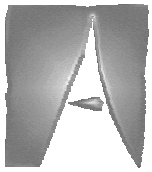

Brain changes underlying the long-term effects of a single previous exposure to emotional or systemic stressors in rats: a view from the hypothalamic-pituitary-adrenal axis

Astrid Vallès Sánchez

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Aquest treball ha estat realitzat sota la direcció del Dr. Antonio Armario García i del Dr. Octavi Martí Sistac, professors del Departament de Biologia Cel.lular, de Fisiologia i d'Immunologia, Unitat de Fisiologia Animal, Facultat de Ciències i Institut de Neurociències, de la Universitat Autònoma de Barcelona

Directors de tesi: Doctoranda:

Dr. Antonio Armario García
Catedràtic d'Universitat

Dr. Octavi Martí Sistac
Professor Associat

Astrid Vallès Sánchez

A la meva família

"Stress is life, and life is stress"

Hans Selye

"Shake the cloud from off your brow,
fate your wishes does allow;
empire growing, pleasures flowing,
fortune smiles and so should you"

Henry Purcell *(Dido & Aeneas)*

"La paciència és un arbre d'arrel amarga,
però de fruits molt dolços"

Proverbi persa

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LIST OF ABBREVIATIONS

6	abducens nucleus
7	facial nucleus
12	hypoglossal nucleus
AA	adjuvant-induced arthritis
AAm	anterior amygdaloid area
AC	adenilate ciclase
Acb	acumbens nucleus
ACo	anterior cortical amygdaloid nucleus
ACTH	adrenocorticotropin hormone
AD	anterodorsal thalamic nucleus
AH	anterior hypothalamic area
ANOVA	analysis of variance
AP	area postrema
AP-1	activator protein-1
Arc	arcuate hypothalamic nucleus
AUC	area under the curve
AV	anteroventral thalamic nucleus
AVP	arginin-vasopressin
AVPO	anteroventral preoptic nucleus
BBB	blood-brain barrier
BLA	basolateral amygdala
BSA	bovine serum albumin
BST	bed nucleus of the stria terminalis
BSTL	bed nucleus of the stria terminalis, lateral division
BSTMa	bed nucleus of the stria terminalis, medial division, anterior
BSTMv	bed nucleus of the stria terminalis, medial division, ventral
C3aR	receptor for the C3 protein of the complement system
cAMP	cyclic adenosin monophosphate
CBG	corticosteroid-binding globulin
CD14	lipopolysaccharide receptor
CeA	central amygdala
CG	central (periaqueductal) gray
Cl	claustrum
CM	central medial thalamic nucleus

CNS	central nervous system
CPu	striatum
CREB	cAMP responsive element binding protein
CRF	corticotropin-releasing factor
Ctx	cerebral cortex
Cu	cuneate nucleus
CV	coefficient of variation
CVOs	circumventricular organs
DC	dorsal cochlear nucleus
DEn	dorsal endopiriform nucleus
DEPC	diethylpyrocarbonate
DLG	lateral geniculate nucleus, dorsal part
DM	dorsomedial hypothalamic nucleus
DMM	dorsomedial medulla
DpMe	deep mesencephalic nucleus
DR	dorsal raphe nucleus
DTT	dithiothreitol
ECIC	external cortex of the inferior colliculus
Ecu	external cuneate nucleus
EDTA	ethylenediaminetetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
EP_4R	prostaglandin receptor, type 4
GABA	γ-aminobutyric acid
Glu	glutamate
GP	globus pallidus
FLI	Fos-like immunoreactivity
GR	glucocorticoid receptor (or glucocorticoid receptor type II)
hnRNA	hereronuclear RNA
HPA	hypothalamic pituitary-adrenal
IEG	immediate-early gene
IκBα	inhibitory factor κBα
IL-1β	interleukin-1β
IL1R	interleukin-1β receptor
IL-6	interleukin-6
IL6R	interleukin-6 receptor
IMD	intermediodorsal thalamic nucleus
IMO	immobilisation in wooden boards
IO	inferior olive

ip	intraperitoneal
KPBS	potassium phosphate buffered saline
LBP	LPS-binding-protein
LC	locus coeruleus
LD	laterodorsal thalamic nucleus
LDTg	laterodorsal tegmental nucleus
LH	lateral hypothalamic area
LHbL	lateral habenular nucleus, lateral division
LHbM	lateral habenular nucleus, medial division
LM	lateral mammillary nucleus
LP	lateral posterior thalamic nucleus
LPB	lateral parabrachial nucleus
LPGi	lateral paragigantocellular nucleus
LPOA	lateral preoptic area
LPS	lipopolysaccharide
LRt	lateral reticular nucleus
LS	lateral septum
LSd	lateral septum, dorsal division
LSi	lateral septum, intermediate division
LSv	lateral septum, ventral division
mCD14	lipopolysaccharide receptor, membrane form
MCN	melanocortin
MD	mediodorsal thalamic nucleus
ME	median eminence
MeA	medial amygdala
MG	medial geniculate nucleus
MnR	median raphe nucleus
MPB	medial parabrachial nucleus
mPFC	medial prefrontal cortex
MPO	medial preoptic nucleus
MPOA	medial preoptic area
mPVN	paraventricular hypothalamic nucleus, magnocellular division
MR	mineralocorticoid receptor (or glucocorticoid receptor type I)
MS	medial septal nucleus
MVe	medial vestibular nucleus
NA	noradrenaline
NSR	normal rabbit serum
NST	nucleus of the solitary tract

NF-κB	nuclear factor-κB
OVLT	organum vasculosum of the lamina terminalis
PAMPs	pathogen-associated molecular patterns
PB	parabrachial nucleus
PBS	phosphate buffered saline
PC	paracentral thalamic nucleus
PCPA	paraclorophenylalanine
pCREB	cAMP responsive element binding protein, phosphorilated
PCRt	parvocellular reticular nucleus
PDTg	posterodorsal tegmental nucleus
PeV	periventricular hypothalamic nucleus
PFA	paraformaldehyde
PFC	prefrontal cortex
PGE_2	prostaglandin, E_2 type
PH	posterior hypothalamic area
Pir	piriform cortex
PKA	protein kinase A
PMD	premammillary nucleus, dorsal part
PMV	premammillary nucleus, ventral part
Pn	pontine nuclei
PnC	pontine reticular nucleus, caudal part
PnO	pontine reticular nucleus, oral part
PnV	pontine reticular nucleus, ventral part
Po	posterior thalamic nuclear group
POMC	proopiomelanocortin
PP	peripeduncular nucleus
pPVN	paraventricular hypothalamic nucleus, parvocellular division
PrH	prepositus hypoglossal nucleus
PS	parastrial nucleus
PT	paratenial thalamic nucleus
PVa	paraventricular thalamic nucleus, anterior division
PVp	paraventricular thalamic nucleus, posterior division
PVN	paraventricular hypothalamic nucleus
RCh	retrochiasmatic area
Re	reuniens thalamic nucleus
Rh	rhomboid thalamic nucleus
RIA	radioimmunoassay
RMg	raphe magnus

RPa	raphe pallidus
Rt	reticular thalamic nucleus
RT	room temperature
SC	superior colliculus
sCD14	lipopolysaccharide receptor, soluble form
SCh	suprachiasmatic nucleus
SE	standard error
SFO	subfornical organ
SHy	septohypothalamic nucleus
SMA	sympathomedulloadrenal
SNK	Student-Newman-Keuls
SO	supraoptic nucleus
Sp5	spinal trigeminal nucleus
SPF	subparafascicular thalamic nucleus
SPO	superior paraolivary nucleus
SSC	saline-sodium citrate
ssDNA	single-stranded DNA
STh	subthalamic nucleus
SuM	supramammillary nucleus
TEA	triethanolamine
TLR4	Toll-like receptor 4
TLRs	Toll-like receptors
TMB	tetramethylbenzidine
TNF-α	tumor necrosis factor-α
TNFR1	tumor necrosis factor-α receptor type 1 (or p55 subtype)
TT	tenia tecta
Tz	nucleus of the trapezoid body
UCN	urocortin
VC	ventral cochlear nucleus
Veh	vehicle
VL	ventrolateral thalamic nucleus
VLG	lateral geniculate nucleus, ventral part
VLM	ventrolateral medulla
VMePOA	ventromedial preoptic area
VMH	ventromedial hypothalamic nucleus
VP	ventral pallidum
VPM	ventral posteromedial thalamic nucleus
VTA	ventral tegmental area

vWF	von Willebrand factor
ZEME	external zone of the median eminence

GENERAL INTRODUCTION

1. STRESS

It is common knowledge nowadays that stressful situations can lead to many physiological and psychological alterations. Maybe the word "stress" is one of the most universal ones under the current way of life in developed countries. Some people might even consider "stress" as "a trendy word" or as one of the inherited "new diseases" from the last part of the twentieth century. Nonetheless, it is not stress "per se" that may lead to disease, but the disruptions in the ability of the organisms to respond to stressful situations.

The concept of stress cannot be understood without first introducing that the organisms have physiological mechanisms to maintain an internal balance. Living organisms are not isolated, but in a constant interaction with a changing, and sometimes challenging, environment. The existence of complex regulatory mechanisms allows the survival and adaptation of the organisms to different external situations. The French physiologist Claude Bernard, back in 1870, already introduced the idea that the organisms have an "internal environment" and control systems to maintain an internal equilibrium:

"*The constancy of the internal environment is the condition that life should be free and independent… So far from the higher animal being indifferent to the external world, it is on the contrary in a precise and informed relation with it, in such a way that its equilibrium results from a continuous and delicate compensation, established as by the most sensitive of balances*".

At the beginning of the twentieth century, this idea was taken further by Walter B. Cannon, who introduced the word "homeostasis", defined as "*the many regulatory processes that maintain the stability of various constituents of extracellular fluids within multicellular organisms*". The word "stress" was initially borrowed from physics (Hooke's equation, 1635-1703) by Cannon and others [127] to picture a state of threatened homeostasis [257, 279]. Cannon also described the "fight or flight" sympathetic adrenal-medullary response as the natural animal reaction to threatening situations. But it was Hans Selye, back in 1936, the one who really popularised the concept of "stress" through his numerous scientific studies leading him to define the "stress syndrome" or "general adaptation syndrome" as "*the non-specific response of the organism to any demand upon it*" [245]. He studied the response of the organisms to various threatening situations such as cold, surgical injury, spinal shock, muscular

exercise and intoxications. This syndrome was generally characterised by adrenal enlargement, gastrointestinal ulceration and thymico-lymphatic involution.

Since Selye's initial definition, there have been many attempts to redefine or broaden the concept of stress [149]. It is generally agreed that the term "stress" comprises a wide range of physiological and/or behavioural changes that have evolved along phylogeny and that take place in the organisms under different challenging situations [289]. This wide range of situations can be either a real, anticipated or symbolic threaten for the integrity of the organism, and cannot be coped by means of normal homeostatic mechanisms [289]. The stress response, regarded as a positive adaptative process, comprises a set of functional and behavioural reactions to cope with these challenging situations. A coordinated and adequate set of responses to stress is crucial for the survival of the organisms in front of these situations. However, exaggerated responses to stress appear to be closely related to a wide range of physiological and psychological dysfunctions such as cardiovascular [29, 256] and sleep disorders [120], infertility [183], anxiety-related disorders such as depression [87] or anorexia [33, 67], and neurodegeneration [228]. It has also been shown that stress increases the susceptibility of the organisms to immune-mediated diseases [186], cancer [255] or addiction to drugs [202]. All these important pathological features of the stress response have triggered an intense study of the different systems activated by stress.

In mammals, very different kinds of stressful situations are able to activate the response of two main physiological systems: the sympathomedulloadrenal (SMA) system and the hypothalamic-pituitary-adrenal (HPA) axis, the latter being considered to play an important role in the pathological consequences of stress.

2. CATEGORISATION OF STRESSFUL STIMULI

Even if the concept of stress implies a non-specific response to any kind of challenging situation, the fact is that there is also a very important component of specificity of the stress response depending on the nature of the stressful stimulus or "stressor". It is generally acknowledged that, depending on the characteristics of each stressor, the brain routes involved in the activation of the stress response and the final pattern of this response will be different. Thus, the "non-specificity" of Selye's stress concept has been subject of discussion and it is still a controversial issue. Nonetheless, most authors agree that each stressful stimulus has its own central

neurochemical and peripheral neuroendocrine "signature" (e.g. [197], see [196, 225] for a review).

Depending on the main characteristics of each stressful stimulus, they have been classified in two main categories: emotional stressors (also described as neurogenic, psychological or processive) and systemic stressors (also labelled as physical, homeostatic or physiological). This categorisation is not always clear or easy to make, since some stressful situations have a mixed emotional/physical component. The general criterion is to classify different stressors depending on the main component that characterizes them. Examples of psychological stressors are social stressors (novelty, social defeat, social isolation), and other mainly emotional stressors with a physical component (noise, footshock, forced swim, restraint, immobilisation (IMO)). Physical stressors would range from immunological (endotoxin and cytokine administration) to metabolic and osmotic challenges (insulin and 2-deoxiglucose administration, hypertonic saline injection), among others (ether exposure, exercise, cold exposure, hypoxia, haemorrhage).

2.1. Central stress pathways

The routes converging to the stress-induced brain activation are very different depending on each stressor. In order to be able to describe this differential activation depending on the category of the stressful stimulus, first we need to illustrate the main routes involved in the stress response. Stressful stimuli are processed by the brain and convey information into the paraventricular hypothalamic nucleus of the hypothalamus (PVN), one of the key elements of the hypothalamic-pituitary-adrenal axis (see below). Based on cytoarchitectural, cytochemical and connectional features, the PVN is divided in two main regions: the magnocellular (mPVN) and parvocellular (pPVN) divisions [259]. The mPVN consists of large neurons projecting to the posterior pituitary and mainly synthesizing arginin-vasopressin (AVP) and oxitocin. The pPVN can be further divided into five subdivisions: periventricular and anterior, medial (divided in dorsal and ventral), dorsal and lateral parvocellular regions. Neurons located in the periventricular and medial dorsal divisions of the pPVN project to the external lamina of the median eminence (ME). Medial dorsal pPVN neurons mainly synthesize corticotropin-releasing factor (CRF), the principal origin of HPA axis activation (see below). In addition, periventricular and medial dorsal pPVN subdivisions synthesize other neuropeptides (somatostatin, growth hormone releasing hormone, thyrotropin-releasing hormone) and also neurotransmitters (dopamine), thus regulating the release of other hormones (e.g. growth hormone, thyrotropin,

prolactin) from the anterior pituitary [260]. The remaining subdivisions of the pPVN project mainly to the spinal chord and/or dorsal vagal complex, and play an important role in the regulation of the autonomic nervous system.

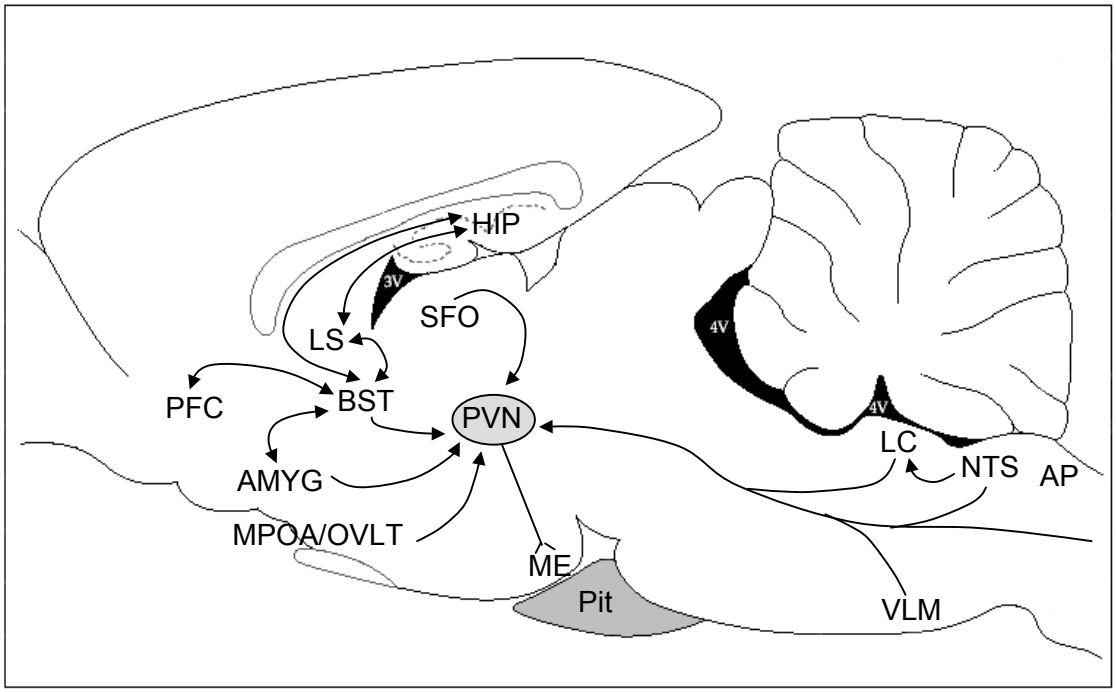

Figure 1. Schematic representation of the main central pathways involved in the activation of the PVN after stress. As explained in the text, the main afferents to the PVN originate from the limbic system, brainstem and circumventricular organs. Abbreviations: AMYG, amygdala; AP, area postrema; BST, bed nucleus of the stria terminalis; HIP, hippocampal formation; LC, locus coeruleus; LS, lateral septum; NTS, nucleus of the solitary tract; ME, median eminence; MPOA, medial preoptic area; Pit, pituitary; PVN, paraventricular hypothalamic nucleus; OVLT, organum vasculosum of the lamina terminalis; PFC, prefrontal cortex; SFO, subfornical organ; VLM, ventrolateral medulla. Modified from [47, 201, 231].

The activity of the PVN is regulated by inputs originating from different areas of the brain such as the brainstem, the limbic system, the hypothalamus and the circumventricular organs (CVOs) (summarised in Figure 1, for reviews see [47, 96]), with different neurotransmitter systems involved in this regulation. The afferents originating from the brainstem involve mainly catecholaminergic pathways from ventrolateral medulla (VLM, A1/C1), nucleus of the solitary tract (NST, A2/C2), locus coeruleus (LC, A6) and dorsomedial medulla (DMM, C3) [49, 50]. The noradrenergic inputs to the PVN are generally acknowledged to be stimulatory [261], and whereas the A1 projection innervates primarily the mPVN, the major projection to the medial dorsal division of the pPVN originates from the A2 and, to a lesser extent, from the A6 cell groups [50]. Although all the adrenergic cell groups cited before have direct projections to the pPVN [49], their exact role on stress-induced PVN activation is still controversial, since mainly stimulatory [262, 298] but also inhibitory [181] effects have

been reported. There is also a modest serotoninergic projection from B7-B9 cell groups [143, 236], although their importance in PVN activation has not been yet clearly determined [65]. Finally, there are also some mesopontine afferents to the PVN and they are thought to be important in the relay of somatosensory information to the PVN, although the main neurotransmitter system involved is still under discussion (reviewed in [47]).

Although it has been widely demonstrated that different components of the limbic system (medial prefrontal cortex (mPFC), hippocampus, lateral septum (LS) and amygdala) have a very important influence on stress-induced PVN activation, the fact is that they lack substantial direct inputs to the PVN. There are only modest afferents to the PVN originating from the central amygdala (CeA) and possibly the medial amygdala (MeA) [84]. Instead, it is generally acknowledged that limbic influence on PVN activity is mediated by neuronal relays, and the main candidate for this role is the bed nucleus of the stria terminalis (BST), as suggested from multiple experiments (e.g. [70, 78, 98]). In fact, there are numerous interconnections between this nucleus and some of the components of the limbic system (hippocampus, LS and amygdala) [296], and the BST sends important projections (mainly GABAergic) to the medial dorsal division of the pPVN [48]. Nonetheless, the exact role of the projections of the BST to the PVN (excitatory or inhibitory) is thought to change, depending on the particular division of this nucleus [96].

In addition to the BST, the hypothalamus itself is also regarded as a candidate to relay limbic information to the PVN (see [101] for a review). There are numerous hypothalamic areas that receive afferents from different regions of the limbic system (ventral subiculum, prefrontal cortex (PFC), MeA, LS, paraventricular thalamic nucleus) and project to the medial dorsal division of the PVN [233, 274]. The main hypothalamic areas involved in this relay are the anterior pPVN, anterior hypothalamic area, perinuclear region of the supraoptic nucleus (SO) and dorsomedial hypothalamus. Apart of functioning as a limbic relay, this local hypothalamic circuit is also thought to convey information from ascending brainstem pathways (see review [101]). Although GABA is the main neurotransmitter involved in this hypothalamic innervation [31], it is thought that a fine interplay between glutamate (Glu) and GABA neurotransmission through multisinaptic connections would be determining the final output (stimulatory/inhibitory) to the PVN, depending on multiple factors such as the nature of the stressor [101]. Finally, other neurotransmitter systems have also been involved in the PVN regulation by other hypothalamic nuclei. For instance, the arcuate nucleus (Arc) sends both putative

inhibitory (β-endorphin) [124, 235] and stimulatory (neuropeptide Y) [20, 291] projections to the PVN. In addition, substance P afferents to the PVN have also been described and are thought to play and inhibitory role [77]. The main source of substance P afferents is the lateral hypothalamic area (LH) and, to a lesser extent, the anterior periventricular (PeV), dorsomedial (DM) and ventromedial hypothalamic nuclei (VMH) [27].

The other group of afferents to the PVN originates from CVOs (organs devoid blood-brain barrier (BBB)) such as the subfornical organ (SFO) and forebrain regions associated with the lamina terminalis (medial preoptic nucleus (MPO), organum vasculosum of the lamina terminalis (OVLT)) [193]. Although these areas principally innervate the mPVN (presumably through GABA and angiotensin II projections), they also seem to influence the activity of the medial dorsal pPVN [79, 133]. These projections are thought to mediate the effects of blood-borne signals on the activity of the PVN, being involved in the control of water balance, cardiovascular regulation and neuro-immune interactions [38, 248].

2.2. Processive versus systemic stress pathways

We have seen that there are many routes converging to the activation of the PVN, suggesting that very different levels of the brain (sensory, autonomic and integrative) are involved in the coordination of the stress response. Nonetheless, as previously advanced, each particular stressor activates the PVN through a particular set of these pathways. These different stress pathways have been generally classified following the same nomenclature as in stressor categorisation: processive and systemic [96]. In general, processive stimuli would first be processed by higher structures of the brain and would activate the PVN through limbic pathways, whereas systemic stressors have been suggested to be "limbic-insensitive" and would follow more direct and rapid PVN-activating pathways, such as the ones originating from the brainstem. This has been observed following different experimental approaches: lesion studies and immediate-early gene (IEG) expression (for a summary, see [96]), the first approach being the more direct in order to elucidate the brain routes involved in PVN activation. Study of the stress responses affected or unaffected after lesioning specific brain areas has shown that lesions of different forebrain nuclei (e.g. amygdala, BST) mainly modify the response to processive stressors (e. g. conditioned fear, restraint, IMO), whereas brainstem lesions principally affect the response to systemic stressors (e.g. ether, haemorrhage, cytokines, hypoglycaemia) [96]. Studies of IEG activation have also shown a differential activation depending on the

category of the stressor: a widespread activation of brain nuclei, including forebrain regions, has been found following processive stressors, whereas a more restricted IEG activation, mainly in areas of the brain related to autonomic regulation, has been described after systemic challenges [231, 246].

However, even if this general classification is very comprehensive, it also tends to simplify the real picture. That is, each specific stressor follows its own and characteristic pathways depending on its many intrinsic characteristics, and there are some differences in the pathways or brain centres activated within the same category of stressor. For example, two psychological stressors such as forced swim and restraint elicit a similar pattern of IEG activation, but also differ in some of the areas activated: a higher number of regions involved in sensorimotor processing were activated after swim, since it involves movement, whereas restraint does not [46]. There are also differences within systemic stressors, since both the sensory systems involved in each particular stressor and the routes for the transduction of the peripheral information to the brain are quite specific for each particular challenge. For example, hypoxia is detected by sensory elements in the carotid body or carotid sinus, relaying the information to the PVN through the NST or the VLM. On the other hand, immunological stimuli such as lipopolysaccharide (LPS) or cytokine administration, although triggering the activation of brainstem nuclei, are believed to activate the brain by means of transduction signals in both the CVOs and endothelial cells of the brain [212, 213]. Overall, the different pathways involved in the response of the organisms to stressful situations suggest that both the mechanisms of activation and the pattern of the stress response are very much dependant on the type of stressor applied.

3. THE HYPOTHALAMIC-PITUITARY-ADRENAL AXIS AND STRESS

One of the main physiological systems involved in the response of the organisms to stressful situations is the HPA axis. This system has been very well characterised from both nervous and hormonal points of view, due to the fundamental role it plays regarding the pathophysiological and pathopsychological consequences triggered by the exposure of the organisms to stress [182].

The general mechanisms of HPA activation in response to stress are shown in Figure 2. As previously described, stressful stimuli are processed by the central nervous system (CNS) and converge information to the hypothalamus, where they are able to stimulate the synthesis of CRF from the parvocellular neurosecretory neurons of the

PVN [47, 51, 116]. CRF is the main molecule driving the HPA axis response to stress [12], but there are also other factors involved in HPA axis control, such as AVP and other hypothalamic neuropeptides, AVP being considered the most important of these cosecretagogues [203].

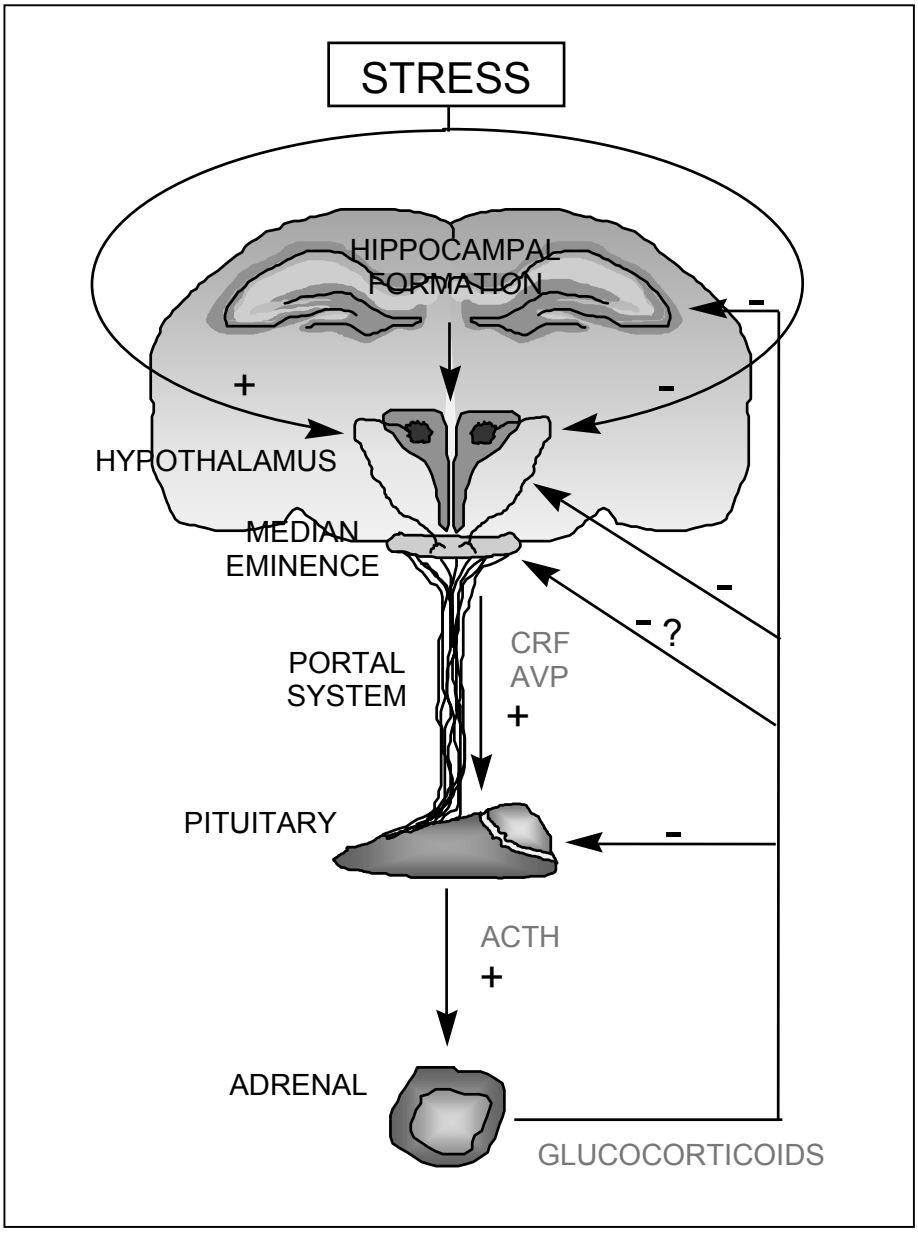

Figure 2. General view of the functioning of the hypothalamic-pituitary-adrenal (HPA) axis, including the negative feedback exerted by glucocorticoids (see text for details). Abbreviations: CRF, corticotropin-releasing factor; AVP, arginin-vasopressin; ACTH, adrenocorticotropic hormone. Adapted from [174].

CRF is released into the hypophyseal portal blood of the ME, reaching the anterior pituitary, where it stimulates both the transcription and cleavage of proopiomelanocortin (POMC), the precursor molecule, among other peptides, of adrenocorticotropin hormone (ACTH). CRF, together with the other hypothalamic

factors released into the ME, is also able to stimulate the release of ACTH into the peripheral circulation. ACTH, in turn, stimulates the synthesis and release of glucocorticoids (cortisol in humans, corticosterone in rat) from the adrenal cortex. Finally, glucocorticoids, apart from exerting multiple metabolic actions on the organism and being the main factor involved in the negative consequences of stress (e.g. infertility, immunosupression, psychopathology), are also able to regulate the activity of the HPA axis by means of retroinhibitory mechanisms acting mainly at the hippocampal, hypothalamic and pituitary levels [61] (see below).

3.1. Corticotropin-releasing factor

The role of CRF in the overall control of the organism response to stress has been extensively studied since its characterisation more than 20 years ago [280], and nowadays the most abundant and relevant data about stress neurochemistry correspond to CRF [276]. As previously described, this 41-aminoacid peptide, synthesised in the medial dorsal pPVN in response to a wide variety of stressors, is considered the key component of the HPA axis by being the principal stimulator of the synthesis and release of ACTH from the anterior pituitary. In addition, CRF is also one of the most widely distributed peptides throughout the CNS [232], being found both in the hypothalamus (e.g. medial preoptic area (MPOA), SO, LH) and other areas of the brain including the limbic system (e.g. BST, CeA, hippocampus) and the brainstem (e.g. Barrington´s nucleus, parabrachial nucleus (PB), inferior olivary complex). Interestingly, the PVN itself receives CRFergic inputs originating from the hypothalamus (dorsal hypothalamic and perifornical areas, DM), limbic system (BST) and brainstem (Barrington´s nucleus, dorsal raphe nucleus (DR)) [43]. However, whereas there is also a wide and abundant distribution of CRF mRNA throughout the brain, the CRF primary transcript, a much more sensitive and reliable index for determining the activity of the CRF gene [99], has only been detected in the PVN [68].

In this regard, the measure of the CRF primary transcript by using probes directed against the intronic sequence of the CRF gene (illustrated in Figure 3) is a newly developed methodology [99] that has been shown to be a more powerful and sensitive tool than the measure of CRF mRNA to study the HPA axis response to stress (see also Chapter 3, Introduction). In the last few years, the number of studies describing the early transcriptional activity of the CRF gene after stress has increased dramatically, showing that a wide range of stressful stimuli are able to induce CRF primary transcript. These include (i) immunological or immunological-related

challenges [137, 139, 145, 146, 214, 281, 282]; (ii) pharmacological manipulations [138, 273], including CRF administration [170]; (iii) glucocorticoid depletion and/or manipulation [99, 158, 162]; (iv) physical stimuli such as hypertonic saline injection [13, 159, 164], hypovolemia [264-266], ethanol administration [147, 192, 218], exposure to ether vapor [129, 131, 132] or exercise [210]; and (v) neurogenic stimuli like restraint [95, 107-110, 160, 161, 163] or footshock [146].

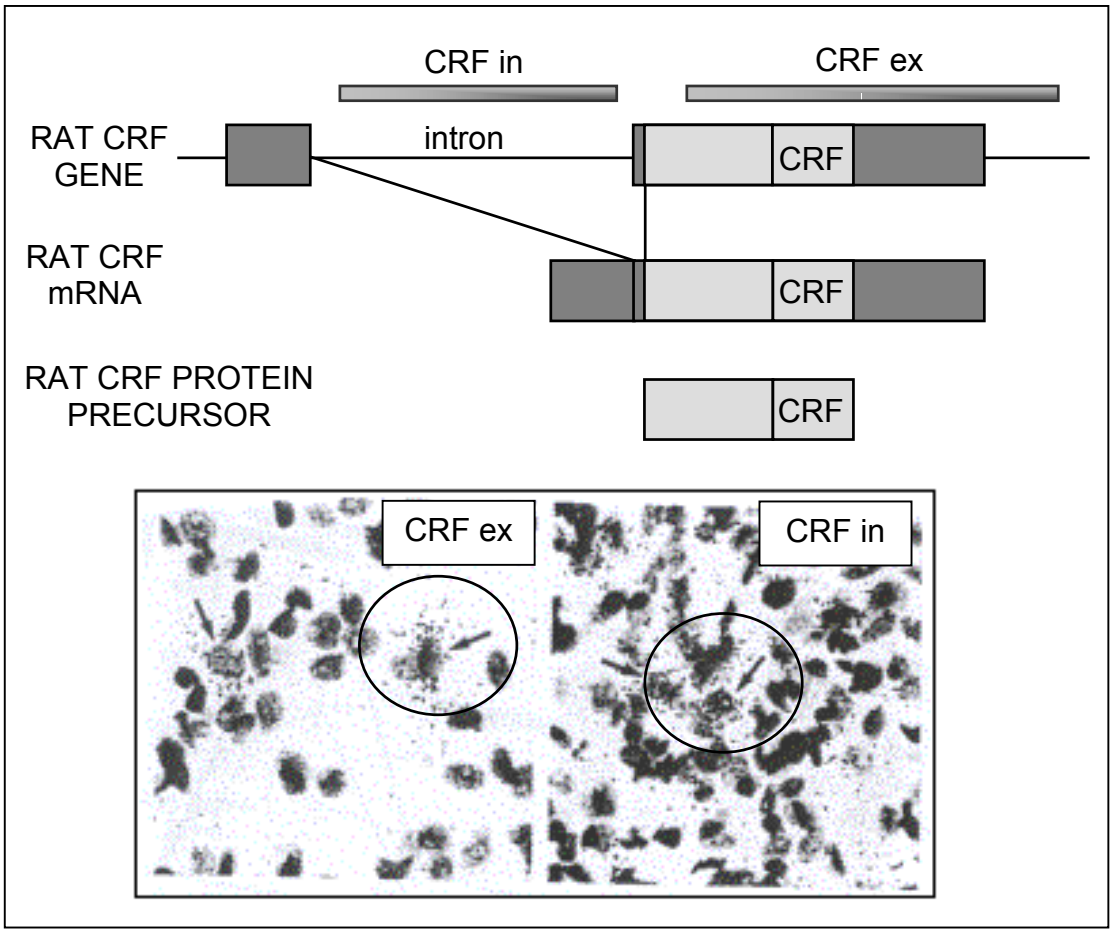

Figure 3. Schematic view of two of the probes directed against the CRF gene. The classical exonic probe (CRF ex) detects principally the mature transcript (CRF mRNA), whereas the intronic probe (CRF in) is directed against the intronic sequence of the rat CRF gene, thus detecting only the primary CRF transcript (before being processed), also named hereronuclear RNA (hnRNA). The pictures below display the classical localisation of the signal in the CRFergic neuron: whereas mRNA is located around the nucleus of the cell (cytoplasm), the signal for hnRNA is overlapping the nucleus, indicative of its immature state. Adapted from [99, 110].

The action of CRF is mediated through two types of receptors: CRF_1 and CRF_2 (with two subtypes: $CRF_{2\alpha}$ and $CRF_{2\beta}$) (see [55] for a review), which are very differently distributed in the CNS [42]. The CRF_1 receptor is widely distributed throughout the brain, including the olfactory bulb, cerebral cortex, limbic system (amygdala, hippocampus), red nucleus, central gray (CG), cerebellum and pituitary [205]. On the

other hand, the $CRF_{2\alpha}$ receptor is generally restricted to limbic structures (LS, VMH, amygdala), whereas the $CRF_{2\beta}$ receptor is found in non-neuronal elements of the brain (choroid plexus, cerebral blood vessels) and in the periphery (cardiac and skeletal muscle, lung, intestine) [155]. CRF has a higher affinity for the CRF_1 than for the CRF_2 receptor, whereas urocortin (UCN), a new mammalian member of the CRF peptide family [288], displays a similar affinity for both receptors. Two more CRF-related peptides have been recently identified (UCN II and III), and both of them bind preferentially the CRF_2 receptor [150, 208]. An interesting study has described the IEG activation after an icv administration of either CRF or UCN reporting that, in general, the areas displaying IEG activation after CRF or UCN are very close to the areas expressing the CRF_1 or CRF_2 receptors, respectively [28] and, more recently, the distribution of UCN III immunoreactive projections has been found to be partially overlapped with the distribution of CRF_2 receptor [151].

A high expression of CRF_1 receptor has been observed throughout the brain and found to be insensitive to stress. In contrast, low to undetectable levels have been observed in the PVN, but different kind of stressful stimuli have been found to trigger the transcriptional activation of the CRF_1 receptor quite exclusively in the PVN and, to a lesser extent, in the SO [157, 168, 215]. In addition, exogenous CRF has been found to trigger the transcription of both CRF [200] and CRF_1 receptor [170], but not CRF_2 receptor [169], in the PVN. On the basis of these data and together with the selective transcription of CRF in the PVN, the existence of an ultrashort positive feedback loop within the PVN has been suggested, by which CRF would modulate both its own biosynthesis and that of its CRF_1 receptor [68, 106]. In contrast, a stress-induced decrease of CRF_1 receptor transcript in the anterior pituitary has also been described, and it has been found to be partially blocked by experimental removal of corticosteroids, suggesting an alternative mechanism of glucocorticoid negative feedback on the HPA axis [168]. Nonetheless, the regulation of the binding and expression of CRF_1 receptor in the pituitary during exposure to chronic (prolonged and/or repeated) stressful situations is highly more complex (for a review, see [1]). In this regard, during repeated stress, an increase of pituitary CRF_1 receptor mRNA, probably through AVP stimulation, together with a decrease of CRF_1 receptor binding has been described. It is suggested that changes in the efficiency of translation and/or desensitisation and internalisation of the receptors would explain this dissociation, and that the elevated CRF_1 receptor mRNA levels would serve as a mechanism to maintain permissive levels of this receptor in the pituitary.

Finally, compatible with the widespread distribution of CRF in the CNS, the function of CRF is not only restricted to its hypophysiotropic action, but it is also involved in the control of a wide range of autonomic and behavioural responses to stressful stimulus, including anxiety-like behaviours, food intake, arousal, learning and memory [45, 69, 195, 243]. In this respect, it has been suggested that CRF_1 receptors would be related to cognitive aspects of behaviour including attention, executive functions, emotions and possibly, learning and memory, whereas CRF_2 receptors would influence survival-related processes such as feeding, reproduction and defence [249]. Recently, it has been found that acute blockade of CRF_1 or $CRF_{2\alpha}$ receptors within the CeA or LS, respectively, decreases stress-induced defensive behaviour (freezing) [21], suggesting that both receptor types would play parallel roles in the regulation of stress-related behaviour, and that this regulation might take place through different brain regions.

CRF_1 receptors are positively coupled to adenilate ciclase (AC), and the stress-induced activation of these receptors in the corticotroph pituitary cells provokes the increase of intracellular cAMP that leads to PKA activation and the subsequent phosphorilation of molecules stimulating the synthesis of ACTH. In addition, stimulation of these receptors also leads to increases in intracellular Ca^{2+}, stimulating the release of ACTH into the circulation (see [119, 123] for reviews).

3.2. Arginin-vasopressin

AVP is a 9-aminoacid peptide that is principally produced by the posterior-pituitary projecting magnocellular neurons of the PVN and SO of the hypothalamus, playing an important role in the regulation of fluid and volume balance. It has been suggested that AVP originating from PVN and SO magnocellular neurons passing through the internal lamina of the ME may play a role in HPA axis modulation [104]. Nonetheless, it is generally assumed that the main source of this peptide in relation to the HPA axis is found in a subset of CRFergic neurons coexpressing AVP in the parvocellular region of the PVN [297]. Both AVP mRNA and its primary transcript in the pPVN have been found to increase in response to different stressful stimuli (e.g. [22, 132, 159, 160, 299]). More importantly, different types of stressors stimulate the release of AVP into the ME and, in turn, AVP targets the anterior pituitary to stimulate the release of ACTH (for a review see [125]). The actions of AVP are mediated through its receptor of V_{1b} subtype, expressed in the pituitary and positively coupled to phospholipase C, stimulating the increase in intracellular Ca^{2+} that leads to AVP-induced ACTH release [123]. It is important to note that AVP does not stimulate ACTH sythesis, but only its release from pituitary corticotrophs. However, although AVP alone

is not a powerful secretagogue of ACTH, it plays an important role in HPA axis activity because of its powerful synergistic effects on CRF activity [81, 221]. The role of AVP in ACTH release has been reported to gain importance under prolonged stress situations, when the ratio of AVP-expressing cells in the PVN has been found to increase substantially [58, 59].

3.3. Peripheral stress hormones: ACTH and corticosterone

As introduced previously, the stress-induced activation of pituitary corticotroph cells results in the synthesis (from its precursor POMC) and release into the peripheral circulation of the 39-aminoacid peptide ACTH, considered to be the primary peripheral stress hormone, which synthesis and secretion is triggered by a wide range of stressful situations. As previously pointed out, CRF, but not AVP, has been shown to stimulate ACTH synthesis [103], whereas both CRF and ACTH are able to stimulate its secretion [222]. In addition, CRF or AVP antagonists [222, 223], CRF or AVP immunoneutralisation [219, 221] and PVN lesions [216] have been found to reduce or abolish stress-induced ACTH release, confirming the main role CRF and AVP as secretagogues of ACTH. Once in the circulation, ACTH acts in the adrenal cortex through its specific cell membrane receptor from the family of melanocortin (MCN) receptors MC2-R, which is positively coupled to AC [267]. The cAMP subsequent increase and PKA activation initiate a cascade of events leading to both synthesis and release of glucocorticoids from the adrenal cortex (corticosterone in rat). The main effects of ACTH in this regard are mediated by driving cholesterol obtaining from the cell and stimulating the limiting step of steroidogenesis (conversion of cholesterol to pregnenolone). In addition to the stimulatory actions on glucocorticoid synthesis and release, ACTH exerts trophic effects on the adrenal gland. In this regard, high doses of ACTH have been shown to provoke adrenal hypertrophy and hyperplasia, whereas ACTH depletion induces the atrophy of the adrenal glands.

As previously described, glucocorticoids exert multiple actions on the organisms. To further understand the role of glucocorticoids on the response to stress, their actions have been classified in two main categories: modulating and preparative [229]. Modulating actions of glucocorticoids are those altering the response of the organisms to a stressor, and are further divided into three more categories: permissive, suppressive and stimulating actions. Permissive actions are manifested during the initial phase of the stress response and prepare the mechanisms of defence of the organism to cope with stress. Suppressive actions take place after one hour or more after the onset of stress, and prevent the organism from the negative consequences

of an excessive response to stress. On the contrary, stimulating actions, also taking place from about one hour or more after the beginning of stress, enhance the effects of the hormones released after stress, thus helping to mediate the stress response. Finally, glucocorticoid preparative actions, which can be either mediating or suppressive, are those modulating the future response of the organisms to stress.

Most of the multiple metabolic effects of glucocorticoids on the periphery appear to provide a redirection of energy (for a review see [186]). These effects include the stimulation of liver gluconeogenesis, inhibition of insulin secretion and stimulation of lipolysis to increase blood glucose levels. Glucocorticoids also induce a wide number of enzymes and proteins including the protein metallothionein, which is believed to play an important role in glucocorticoid-mediated detoxification. In addition, glucocorticoids have an important role in inhibiting inflammatory and immune activity. Finally, glucocorticoids play an important role in regulating the HPA axis activity by means of retroinhibitory mechanisms.

3.4. Glucocorticoids and feedback

The classical regulation of the HPA axis activity by glucocorticoids is mediated by their interaction with specific receptors [51, 61]: type I or mineralocorticoid type (MR) and type II or glucocorticoid type (GR). Glucocorticoid receptors have a similar structure, with both DNA and steroid-binding domains, and non-occupied receptors are located predominantly in the cytoplasm, forming large heterocomplexes with heat shock proteins, which dissociate after hormone binding. The activated hormone-receptor complex can then translocate into the nucleus and target glucocorticoid response elements found in the promoter region of different genes, thus modulating their transcription. Glucocorticoid receptors can also modulate gene transcription by direct binding to transcription factors [83] such as activator protein-1 (AP-1), cAMP responsive element binding protein (CREB) and nuclear factor-κB (NF-κB).

The binding properties and distribution of the two types of glucocorticoid receptors are quite different [60]. MRs have a high affinity for corticosterone, and their brain expression is abundant in the hippocampal formation, layer II of the cortex, lymbic system (LS, MeA, CeA, olfactory nucleus) and brainstem sensory and motor neurons. On the other hand, GRs, with a lower affinity for corticosterone, are widely distributed throughout the CNS and also in the pituitary. The higher levels of GR are found in the limbic system (hippocampal formation, septum), pPVN and SO, and, to a lesser extent, in ascending monoaminergic neurons of the brainstem. Moderate GR levels

are also found in many thalamic nuclei, striatal areas, CeA and throughout the cerebral cortex.

On the basis of the differential binding properties and distribution of glucocorticoid receptors, each subtype has been suggested to play a differential role in HPA axis regulation. Due to their high affinity for glucocorticoids, MRs display more than an 80% of occupancy throughout the diurnal cycle of the HPA axis. On the other hand, GRs are partially occupied during the phase of maximal corticosterone secretion of the circadian rhythm (afternoon/evening in the rat) and, more importantly, when there are elevated concentrations of glucocorticoids provoked by exposure to stressful situations [60, 61]. In general, MRs are thought to mediate the tonic inhibitory control of the HPA axis to control its basal activity throughout the circadian circle, whereas GRs are assumed to be the main contributors to the retroinhibitory effects exerted by glucocorticoids in the pPVN and anterior pituitary to switch-off the HPA axis activity after stress. In addition, the two receptors are thought to mediate coordinately the hippocampal regulation of the HPA axis activity, and it has been suggested that an adequate MR/GR balance is critical for determining the effects of glucocorticoids on cellular homeostasis, behavioural adaptation and susceptibility to disease [60].

The effects of glucocorticoids through these two types of receptors necessarily involve gene transcription, and therefore cannot account for some rapid effects taking place within 15 minutes after the onset of stress. To explain these rapid effects of glucocorticoids, some alternative mechanisms have recently been suggested, such as the existence of putative glucocorticoid membrane receptors and/or the modulation of membrane channels or neurotransmitter receptors through glucocorticoid binding [167].

Depending on their speed of action, feedback mechanisms on the HPA axis have been divided into three categories: fast, intermediate and slow [122]. Fast feedback includes the actions of corticosterone on the control of the release of ACTH secretagogues in the ME, and takes place within 10 minutes after the onset of stress. Intermediate feedback mechanisms, developping within 30-60 minutes after the onset of stress, involve gene-mediated glucocorticoid effects on the coupling of stimulus and secretion, excitability and intracellular signal transduction pathways. Finally, slow feedback also develops within 30-60 minutes after the onset of stress and, in contrast to intermediate feedback, may last for several hours. The effects of slow feedback include the blockade of stress-induced CRF, AVP and POMC gene expression.

4. LONG-TERM EFFECTS OF STRESS ON THE HPA AXIS ACTIVITY

4.1. Effects of chronic stress: overview

There is a wealth of studies regarding the consequences of chronic exposure to stress on the HPA axis activity because of its implications in stress-related pathology. The changes observed after chronic stress depend on multiple factors such as the type of stimulus or the intensity/duration of the stressor, making it difficult to generalize them. Nonetheless, it is generally acknowledged that the main changes include [172, 173]: (i) reduction of food intake and body weight gain; (ii) adrenal hypertrophy and enhanced adrenocortical response to ACTH; (iii) increased basal levels of corticosterone, generally with normal levels of ACTH; (iv) enhanced expression of POMC in the anterior pituitary; (v) down-regulation of CRF receptors and increased response to exogenous CRF; (vi) enhanced expression of CRF and AVP in the PVN; and (vii) down-regulation of type II, but not type I, glucocorticoid receptors in the hippocampal formation.

One of the most characteristic phenomena of chronic stress is the progressive reduction of the stress response after repeated exposure to the same stressor [173]. Depending on the subjacent mechanisms believed to be involved in this phenomenon, it has been termed adaptation or habituation. Whereas the concept of adaptation does not assume any particular process, that of habituation assumes that the process involved in the reduced response follows the rules of habituation as defined by Groves and Thompson [86]. However, it is unclear whether or not adaptation to stress is a habituation process. Regardless of this theoretical discussion, the reduced response to repeated stress may either involve biochemical mechanisms (e.g. receptor down-regulation) or, in contrast, could be more related to the cognitive processing of the stressful situation, involving a reduced emotional activation of the animal in front of the same stimulus. Therefore, it is crucial to determine if the reduced response to stressful stimuli observed after chronic exposure to stress is more likely to be explained by a phenomenon of biochemical adaptation or by some kind of learning mechanism. To this end, the best experimental strategy is the study the stress-specificity of the phenomenon, consisting on the characterisation of the HPA axis response to a different (heterotypic) stressor after the chronic exposure to the same (homotypic) stressor. If the response to the superimposed heterotypic stressor is still reduced after chronic stress, we are presumably dealing with a phenomenon of biochemical adaptation (cross-desensitisation), whereas if

there is a normal response, it would be generally regarded as a learning-like process related to the experience of the animal with stress.

There is also another phenomenon caused by chronic stress exposure different from adaptation/habituation and termed as facilitation [26, 52]. It consists of a normal or even enhanced, instead of reduced, response to a novel (heterotypic) stressor after chronic stress exposure. It is thought that facilitation involves stimulating (facilitatory) mechanisms of stress itself that would balance or even overcome the inhibitory control exerted by glucocorticoids on the HPA axis. Although it is generally agreed that facilitation is an important phenomenon regarding chronic exposure to stress, the exact meaning and adaptative value of this exacerbated response of the HPA axis after chronic stress still remain to be fully determined.

4.2. Long-term effects of stress in young animals: general features

It is relatively well established that exposure of laboratory animals to different kind of manipulations during the neonatal period can induce different alterations in the CNS, which are persistent in the adult stages [180]. Among these changes, the most prominent ones include the modification of anxiety and the alteration of the HPA axis response to stress. The exact direction of these changes (increased/reduced anxiety or HPA axis response) depends on the nature of the manipulations that the animal received during early life [154, 180]. It has been shown that both postnatal handling and maternal care provoke a reduced HPA axis response to stress in adult stages. In contrast, early-life traumatic events such as maternal separation, physical trauma or endotoxin administration enhance the future HPA axis response to stress in adult life.

Independently of the direction of these changes, they persist throughout the life of the animal and are also accompanied by permanent alterations in the HPA axis [180]. Postnatal handling induces a reduction of basal CRF mRNA levels in the hypothalamus and of CRF and AVP levels in the ME, in accordance with the described reduction of the HPA axis response to stress. In contrast, early traumatic events induce a permanent increase of basal hypothalamic CRF and/or AVP mRNA and of CRF and/or AVP in the ME, in line with the enhanced HPA axis response to stress found in these animals. It is thought that the alterations of hypothalamic CRF mRNA levels and of CRF and AVP levels in the ME are partially related with a differential regulation of the expression of glucocorticoid receptors in the hippocampal formation. In this respect, an inverse relationship between type II receptors in the hippocampus and the magnitude of HPA axis response to stress has

been described [180]. All these changes are considered a good example of synaptic plasticity in young animals.

4.3. Long-term effects of stress in adult animals: an emerging field of study

4.3.1. The beginning of the story: initial reports

In contrast to the long-term effects provoked by manipulations in the early-life of the organisms, the degree of CNS plasticity in adult animals has not been so widely studied. The first evidences in this regard are related to the phenomenon known as learned helplessness [165, 166], in which the animals show relatively long-lasting motivational and associative deficits after being exposed to uncontrollable stressful situations (e.g. electric shock). Later on, Antelman and co-workers described a very interesting although complex phenomenon. It consists on the drug-induced modification, days to weeks later, of the behaviour and/or survival of the organisms to a new administration of the same or a different drug [5, 6, 8, 9, 11]. Interestingly, the effects described are thought to be triggered by the stressful component of the different drugs tested rather than by their pharmacological actions, since the drugs they administered (e.g. amphetamine, haloperidol, diazepam, fluphenazine hydrocloride, amitriptyline, cocaine) had very different properties. In addition, they found that a previous drug administration is able to modify the drug-induced HPA axis response in the long-term [3]. They also studied the effects of a single exposure to stress (e.g. IMO, 2-deoxy-D-glucose, ethanol, novelty) on the action of different drugs (e.g. cocaine, amphetamine, haloperidol, diazepam) on the organism [4, 5, 7, 10], showing that stress is able to modulate the multiple effects of drugs in the long-term (weeks to months). Interestingly, both the intensity of the stressor and the time elapsed between the stress session and the administration of the drug have been shown to be of particular importance to determine the detection and direction of the changes observed (sensitisation/desensitisation). For instance, they showed that stressors of low or high intensity, as determined by the plasma corticosterone response, were able to sensitise or desensitise, respectively, the cataleptic response induced by haloperidol injection 2 weeks, but not 1h, after this single stress experience [4].

After these initial studies, there has been a growing interest on the study of the long-term effects of a single stress experience on the organism, because of its potential relevance regarding some human pathologies such as post-traumatic stress disorder [303]. Many of these studies have been published along the elaboration of the experimental work corresponding to this thesis, which is indicative of the importance and implication of these effects.

4.3.2. Long-term stress-induced sensitisation

The first studies, leaded by Tilders and co-workers (for a review see [271]), have been focused two main aspects: (i) the stress-induced changes on the future behavioural response of the animals to either a new stress session or a drug administration [241, 285-287] and (ii) the alteration of the HPA axis activity, both under resting conditions or in response to a new stress session, weeks after a single stressful experience [237-241, 285, 287]. One of their first reports showed that a single exposure to a psychological stressor with a pain component such as electric shock is able to modify the future behavioural and HPA axis responses to a novel emotional stimulus [285]. Briefly, rats receiving a single session of footshock displayed, two weeks later, an increased immobility in a novel environment (noise test), which is regarded as a sign of increased fear/anxiety. These changes at the behavioural level were accompanied by a sensitised initial plasma ACTH response to the new stressor (noise), together with a faster reduction of both ACTH and corticosterone levels in the post-stress period. In addition, there was a tonic increase of the AVP content in the external zone of the ME (ZEME) in previously stressed rats. In the same experiment, CRF stores in the ZEME were not altered by a previous footshock experience, although the same researchers have reported an increase of CRF levels in the ZEME both 7 and 11 days after a single footshock experience [237].

This apparent discrepancy about the effects of footschock might be caused by the slight differences in the application of the stressful paradigm. Whereas in their earliest report the animals received a total of 10 footshocks of 1 mA, which were irregularly distributed over a 15 min period [285], in the second one they received a total of 20 footshocks of 0.5 mA in a regular pattern (4 shocks/min) during 5 min [237] inducing, respectively, either no effect or an increase of resting CRF levels in the ZEME. This suggests that the intensity and/or regularity of the initial stressor might be important in determining the direction of the changes observed in the long-term, as previously pointed out by Antelman and co-workers [4]. Additionally, the first experiment was carried out during the dark period, when the animals have a greater activity, and the second one was performed with lights on. Very recently, other authors have reported that the expression of footshock-induced long-term behavioural sensitisation to mild stressors is more clearly expressed at the beginning of the dark phase [253], indicating that the circadian period is an additional factor that can affect at least the intensity of the changes observed, in this case, at the behavioural level.

Another example of the complexity of this phenomenon is the recent finding regarding the long-term effects of a single drug challenge. For instance, a single dose of amphetamine has been shown to exert long-term sensitisation on the behavioural (locomotor activity) and neuroendocrine (ACTH, corticosterone) responses to a new amphetamine challenge 1 or 3 weeks later [287]. However, this is not accompanied by an increase of AVP levels in the ZEME, but rather by a decrease of CRF levels 22 (but not 11 or 3) days later [240]. Finally, morphine administration for 14 days induces, 3 weeks later, behavioural sensitisation (locomotor activity), but neuroendocrine desensitisation (ACTH, corticosterone) in response to an amphetamine challenge [241], suggesting that behavioural and neuroendocrine sensitisation are not always associated.

In addition to these studies on long-term stress- and drug-induced behavioural and neuroendocrine changes, other researchers have also described autonomic changes provoked by a single stress situation. In this respect, Wiegant and co-workers (for a review see [251]) have found that a single session of footshock is also able to enhance both colonic spike burst frequency [252], a measure of intestinal activity, and blood pressure [36] in response to a novel stress situation (electrified prod in the cage) two weeks later. Interestingly, these autonomic changes are accompanied by a sensitisation of the CRF response to the novel stressor in the PVN, ME and CeA [35]. Furthermore, the same experimental paradigm induced a greater expression of Fos-like immunoreactivity (FLI) in a wide variety of cortical and subcortical areas generally agreed to be involved in fear/anxiety, neuroendocrine and autonomic responses to stress [34]. They have also attempted to elucidate the possible mechanisms involved in this phenomenon, suggesting that metabotropic Glu receptors ($mGlu_{2/3}$) could play at least a partial role in the expression of long-term stress-induced sensitisation at the behavioural level [37]. Finally, they have recently described some of the factors that can affect the intensity and direction of the long-term behavioural changes observed after a single footshock session. As previously introduced, these factors include the intensity of the superimposed novel stressor, the circadian phase when the study is performed and the sex of the animals [253].

After their first studies with a mainly psychological stressor such as footshock, Tilders and co-workers showed that long-term stress-induced sensitisation could be generalised to stressors of different nature. In this regard, a stressful stimulus with a clear immune component such as interleukin-1β (IL-1β) administration, increases, two weeks later, tonic AVP immunoreactivity in the ZEME and enhances ACTH and corticosterone responses to another IL-1β administration or to a completely different

stressful situation such as electric shock [239]. These data suggest that a single exposure to different stressors sensitises the HPA axis thus resulting in an enhanced HPA response after a further exposure to the same (homotypic) or to a novel (heterotypic) stressor. The fact that the effects of a single stress exposure could be generalised to stressors of such a different nature was an important finding and an indication of the importance of this phenomenon. Nonetheless, further studies with other stressors have suggested that long-term effects of stress on the HPA axis appear to depend, at least to some extent, on the nature of the stressors.

Apart from footshock and IL-1β, other stressors such as LPS and brain surgery result in increased AVP content in the ZEME weeks after the stressful situation [237]. In contrast, social-defeat or amphetamine, both eliciting a quantitative HPA response similar to some of the above-mentioned stressors, do not increase AVP content in the ZEME [39, 240]. Similarly, a much more intense stimulus such as IMO, although inducing long-lasting increases in AVP mRNA in the PVN (at least 4 days after the single stress experience), does not increase AVP content in the ZEME in the long-term [19]. All these studies further support the idea that the intensity and/or nature of the stressors are important factors in the determination of the future changes in the HPA axis activity. Additionally, other experimental conditions, such as the housing of the animals (individually or grouped) have also been suggested to be important in determining the intensity of the behavioural and neuroendocrine changes observed in the long-term [227].

Following the initial report on the long-term effects of an immunological stressor such as IL-1β [239], an increasing interest has recently emerged regarding the effects of cytokines and other immune-related factors on the future response of the organisms to a new immune or emotional challenge. In this regard, a single injection of tumor necrosis factor-α (TNF-α) induces a progressive sensitisation of sickness behaviour, corticosterone release and noradrenaline (NA) utilisation in the PVN in response to a new injection of TNF-α. Interestingly, the corticosterone response was desensitised at 1 day after the first TNF-α administration, normal response at 1 week and sensitised at 2 weeks, being maximal 4 weeks after the initial treatment [91]. A progressive increase of AVP and CRF immunoreactivity and their colocalisation in the ZEME was also found both 7 and 14 days after TNF-α injection, returning to normal levels 28 days after the initial stress exposure. Additionally, a single TNF-α experience induces a marked sensitisation of the c-fos mRNA response to a new TNF-α injection in the PVN, SO and CeA 7 to 14 days later [93]. After these studies with TNF-α, the same authors have reported that a previous LPS administration sensitises the sickness behaviour caused

by an injection of TNF-α or IL-1β and the corticosterone response to TNF-α or restraint [92]. Nonetheless, these effects were evident 1, but not 28, day after LPS injection, suggesting that the development of sensitisation induced by LPS and TNF-α involve different time frames.

Very recently, the effects of emotional stressors on the future HPA axis response to a superimposed stressor of immunological nature have also been reported. Prior exposure to inescapable tailshock increased the initial corticosterone, ACTH and cytokine responses to a low dose of LPS one day later [117, 118]. Nonetheless, the duration of these sensitisation effects differed depending on the parameter under study. Plasma IL-1β response to LPS in previously stressed rats was still sensitised 4 days after the tailshock, but vanished thereafter. Additionally, the initial corticosterone response to LPS (1 hour post-injection) was still increased 10, but not 21, days after the initial challenge, whereas the effects on the ACTH response were not studied with these inter-stress intervals.

Finally, it has been suggested that long-term stress-induced sensitisation would be independent of the stimulus used, since peripheral administration of a CRF antibody is able to induce slowly-developing and long-lasting increases in tonic AVP levels in the ZEME [238], similarly to the previously reported results with stress. These authors suggest that long-term sensitisation would be caused by the blockade of the negative feedback exerted by CRF at the level of the ME, facilitating the release of CRF independently from the stimulation of PVN neurons. At the same time, a transient suppression of resting corticosterone levels provoked by the administration of metyrapone, an inhibitor of glucocorticoid synthesis, caused similar sensitising effects on AVP levels in the ZEME [238]. This also suggests that the transient blockade of glucocorticoid-mediated feedback on CRF/AVP neurons can induce long-term sensitisation of the HPA axis, and the selectivity of these effects on AVP is in accordance with the reported greater sensitivity of AVP, in comparison to CRF, to glucocorticoid feedback [131]. Nonetheless, the hypothesis that long-term sensitisation of the HPA axis activity would only be dependent on the activation of CRF neurons is not in accordance to other reports of the same and other groups where they failed to find any sensitising effects on the levels of AVP at the ZEME of other stressors such as insulin, ether or social defeat [39, 237], in spite of the known activation of the synthesis and release of CRF by these stressors.

In sum, a single stress experience with stressors of low intensity is able to sensitise the response of different parameters at neuroendocrine, autonomic and behavioural

levels to a new stress situation. The stressors exerting these effects can be of either emotional or immunological nature, and it seems that these effects are not specific for the stressor used since many cross-sensitisation effects have been reported. In addition, these changes are generally, but not always, accompanied by an increase of the tonic HPA activity, as shown by the increased AVP, and sometimes CRF, immunoreactivity in the ZEME. Nonetheless, the nature and intensity of the stress experience seem to be important in order to detect this long-term sensitisation. The dynamics of the changes appears to depend on the stimulus used and the variables studied, some changes appearing but also disappearing very rapidly, whereas others develop slowly but are extremely long-lasting.

4.3.3. Long-term stress-induced desensitisation

Most of the previously exposed reports have described sensitising effects of a single stress experience on the HPA axis response to a further stress situation. Nonetheless, some studies have also reported desensitisation effects. For instance, a three-day treatment with ethanol was able to reduce the tonic HPA activity 7 days later, as shown by a reduction of CRF and AVP stores in the ZEME [147]. Those changes were accompanied by a desensitisation of the peripheral (ACTH, corticosterone) and central (PVN CRF heteronuclear RNA (hnRNA), CRF_1 receptor mRNA, NGFI-B mRNA and c-fos mRNA) HPA axis responses to a new ethanol injection 7 days later [144, 147]. This phenomenon was stressor-specific since there was a normal response to footshock or LPS in previously alcohol-injected animals [144, 217].

Similarly, our group has also reported that a single exposure to an emotional stressor of high intensity such as IMO accelerates the recovery of ACTH and corticosterone levels after the termination of exposure to the same stressor applied weeks later and also decreases CRF mRNA response in the PVN [175]. Interestingly, the desensitisation effects at the peripheral level of the HPA axis (ACTH, corticosterone) are already detected at least 1 week after the first stressful experience, whereas an incubation period of at least 4 weeks is needed in order to observe any changes at the central (CRF mRNA) level. This phenomenon seems to be abolished in adrenalectomised animals, suggesting that stress-induced release of glucocorticoids might be one of the mechanisms involved in the induction of these effects [54]. In addition, this desensitisation of the HPA axis also appears to be stressor-specific so far as a previous exposure to IMO did not modify the HPA response to a novel stressor (forced swimming) [175]. Forced swimming reduced the levels of hypothermia and struggling after a new exposure to the same stimulus two weeks later [53]. However, these

modifications were not accompanied by major neuroendocrine changes at the level of the HPA axis, except for a slight enhancement the recovery of basal corticosterone, but not ACTH, levels in the post-stress period. Very recently, other researchers have reported that two sessions of social defeat reduce the corticosterone response to a high, but not to a low, dose of LPS one week later [41]. Finally, a single LPS injection, but not footshock, reduces the future susceptibility of the animals to adjuvant-induced arthritis (AA), although the HPA activity in control and previously LPS-injected AA remains unchanged [88].

At present, the studies on long-term stress induced desensitisation are less numerous than those reporting sensitising effects of a previous stress exposure. Nonetheless, the reports from our and other groups are quite consistent and it appears that the intensity, and may be the nature, of the stressor, are important factors to take into account. Furthermore, whereas sensitisation is not a specific phenomenon for the stimulus applied, it is not completely clear if desensitisation is a stressor-specific phenomenon, since there are some contradictory results. Finally, all these studies on the long-term effects of a single stress exposure, involving either sensitising or desensitising effects, suggest the existence of an important degree of synaptic plasticity in adult animals. Due to the complexity and important functional and pathological implications of this phenomenon, the further study of the factors and mechanisms involved are of great conceptual importance.

OBJECTIVES

Previous data from our laboratory have shown that a single exposure an emotional stimulus such as IMO induces a slowly developing and long-lasting desensitisation of the central (CRF mRNA) and peripheral (ACTH, corticosterone) HPA axis response to the same stimulus. The characteristics of the phenomenon do not fit with any current theory about stress and adaptation and, therefore, we suspect that it represents a new learning-like type of synaptic plasticity. The main purposes of the present study have been to define more precisely the long-term changes caused by stress at the level of the PVN and define the brain areas putatively involved in this desensitisation of the HPA axis. More specifically, the objectives of the present study were the following:

1. To demonstrate whether the long-term effects of stress on the HPA axis are or not restricted to emotional stressors. To this end, we studied the long-term effects of a single exposure to a systemic stressor of immune characteristics (LPS administration) on the central and peripheral HPA axis.

2. To demonstrate that long-term effects of stress are specific for the homotypic stressor and therefore, the HPA response to other stressors is essentially maintained intact.

3. To further characterise the long-term effects of stress at the level of the PVN by measuring the transcriptional activity of c-fos and that of the two main hypothalamic secretagogues of ACTH (CRF and AVP).

4. To explore the brain areas sensitive to a single previous experience with the stressors (IMO or LPS) using the induction of c-fos as a marker of neuronal activation.

MATERIAL AND METHODS

1. ANIMALS

Adult (approximately 55-60 day-old) male Sprague-Dawley rats were obtained from the Animal Breeding Centre of the Universitat Autònoma de Barcelona. They were housed one or two per cage, depending on the experiment, under standard conditions of light (lights on from 07:00 to 19:00 h) and temperature (22 ± 1 °C). Food and water were provided *ad libitum*. The animals were allowed to acclimate to the animal rooms in our laboratory for a period of at least one week before the beginning of the experiments. All the experimental procedures described below were previously approved by the Ethical Committee in Animal and Human Research of the Universitat Autònoma de Barcelona.

2. STRESS PROCEDURES

2.1. Immobilisation in wooden boards (IMO)

Animals were immobilised as previously described [134] by taping their four limbs to metal mounts attached to a wooden board; their forelimbs were protected with plastic tubes to prevent the animals from biting themselves. Their head movements were restricted by means of two metal loops around their neck attached to the board. It is accepted that IMO is a severe stressor with a mainly psychological component, being able to activate the HPA axis in a very consistent manner [15-17].

2.2. Lipopolysaccharide administration (LPS)

LPS (*Escherichia coli*, 055:B5, Sigma, Spain) was dissolved in sterile saline and injected intraperitoneally (*ip*) in a volume of 2 ml/kg at a dose of 1 mg/ml. Many studies have shown that LPS is a very powerful HPA axis activator [214, 263, 275], being considered as a mainly physical stressor and an immune activator [277].

3. SAMPLING

3.1. Decapitation

Animals were sacrificed within 30 s from being removed from the animal rooms and trunk blood was collected in 10 ml plastic tubes in an ice-cold bath. Brains were rapidly removed, placed in a recipient containing isopentane (Fluka, Spain), frozen by dipping it in liquid nitrogen, and then stored at – 80 °C until being processed. Serum was obtained by centrifugation (1600 g, 15 min, 4 °C) and aliquots were made and stored at –20 °C until being processed.

3.2. Transcardiac perfusion

Animals were anaesthetised with a mixture of ketamine (Ketolar 50, Parke-Davis, Spain) and xilazine (Rompun 2%, Bayer, Germany) injected *ip* in a volume of 2.3 ml/kg to obtain a dose of 90:10 mg/kg. Once anaesthetised, they were transcardiacly perfused for 1-2 mins with ice-cold 0.9% saline and then for approximately 10 min with an ice-cold fixative containing 4% paraformaldehyde (Electron Microscopy Sciences, USA) and 3.8% borax (Sigma, Spain) (PFA+Borax). Brains were then removed and stored at 4 °C in the same fixative buffer before being processed.

3.3. Tail-nick

Repeated blood samples were taken by tail-nick, by wrapping the animal with a cloth and performing a 2 mm incision at the end of one of the tail arteries, and then massaging the tail of the animal while collecting 300 μl of blood in an EDTA-coated capillary tube (Sarstedst, Germany). After obtaining the blood, plasma was obtained by centrifugation (5000 g, 25 min, 4 °C) and aliquots were made and stored at –20 °C.

4. ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA): TNF-α

Plasma TNF-α levels were determined by immunoassay using a commercial kit (Biotrak, Amersham Pharmacia Biotech, UK). The assay is based on a solid phase ELISA, using a rTNF-α antibody bound to a microtitre plate. rTNF-α standards ranging from 31 to 2500 pg/ml and plasma samples (diluted 1/1, 1/2 or 1/10 depending on the expected concentration) were added into each well. After 1h of incubation at room temperature (RT), the wells were thoroughly washed and a biotinilated antibody against rTNF-α was added and incubated for 2 h at RT. The wells were washed again and a streptavidin-horseradish peroxidase conjugate was added and incubated for 30 min at RT. After washing it, the plate was developed by adding tetramethylbenzidine (TMB) substrate and incubating it for 30 min at RT in the dark. The reaction was then stopped and the optical density was determined at 450 nm using a spectrophotometer (Labsystems Multiskan Bichromatic, Finland). The sensitivity of the assay was <10 pg/ml and the average intra-assay coefficient of variation (CV) was 10%.

5. RADIOIMMUNOASSAY (RIA)

5.1. ACTH RIA

ACTH concentration was determined by a double-antibody RIA in non-equilibrium following the protocol described by Dr. W. C. Engeland (personal communication, University of Minessota, Minn, USA), slightly modified in our laboratory. The RIA buffer contained 50 mM sodium phosphate, 25 mM dissodium EDTA, 0.1% Triton X-100 and 0.25% BSA (Sigma, Spain) diluted in MilliQ water, pH 7.4. All the procedure was carried out at 4 ºC to avoid ACTH degradation. We used rat synthetic ACTH $_{1\text{-}39}$ (Sigma, Spain) as the standard, 3-[^{125}I]*iodotirosyl* ^{23}-ACTH $_{1\text{-}39}$ (specific activity 2000 Ci/mmol, Amersham Pharmacia Biotech, UK) as the tracer and an antibody raised against rat ACTH (Ab Rb 7), generously provided by Dr. W. C. Engeland. The free and bound fractions of the hormone were separated with a second antibody (*donkey anti-rabbit* IgG, Chemicon, USA) diluted in RIA buffer containing 1.2% normal rabbit serum (NSR). The radioactivity of the pellets was measured with a gamma counter (Wallac 1272 Clinigamma, Finland) and calculations to determine ACTH concentration were done using a logit transformation. The average inter- and intra-assay CV were 10%.

5.2. Corticosterone RIA

Serum corticosterone levels were determined by RIA as previously described by Lahmame et al [142]. The RIA buffer was prepared with 0.01 M phosphate buffer, 0.9% NaCl, and 0.1% gelatine (Merck, Germany) in bidistilled water, and adjusted to pH 8.2. Corticosterone (Merck, Germany) was used as the standard and 1, 2, 6, 7-[^{3}H]-corticosterone (specific activity 72-93 Ci/mmol, Amersham Pharmacia Biotech, UK) as the tracer. Samples were incubated at 70 ºC for 30 min to denature the corticosteroid-binding globulin (CBG) and thus avoid interferences between this protein and the antibody. After incubating 18-24 h at 4 ºC, the free and bound fractions of the hormone were separated with charcoal (Merck, Germany) diluted in RIA buffer. Corticosterone concentration was determined by measuring the radioactivity remaining in the supernatant in a beta counter (Wallac 1409 Liquid Scintillation Counter, Finland) and applying logit transformations. The average inter- and intra-assay CV were 10% and 8%, respectively.

6. IN SITU HIBRIDISATION

6.1. Oligoprobes

Frozen brains were allowed to thaw from – 80 to – 20 °C before being cut in a cryostat (Frigocut 2800, Leica, Germany). Coronal brain sections from the medial part of the PVN were sectioned at a thickness of 12 μm. Stereotaxic coordinates were obtained according to the atlas of Paxinos and Watson [201]. The sections were collected onto gelatine-coated slides, which were prepared by submerging pre-cleaned microscope slides twice into 0.25% gelatine (Merk, Germany) dissolved in diethylpyrocarbonate- (DEPC, Sigma, Spain) treated MilliQ water containing 0.025% $CrK(SO_4)_2$ (Sigma, Spain). All the sections obtained were stored at – 80 °C until being further processed.

A synthetic 48-base oligonucleotide complementary to an exonic part of CRF mRNA (courtesy of Dr. M.S. Harbuz, Bristol, UK) was used as the probe, its sequence being as follows:

CRF *5' GCCAGGGCAGAGCAGTTAGCTCAGCAAGCTCACAGCAACAGGAAACTG 3'*

The probe was radioactively labelled using terminal deoxinucleotidyl transferase (Roche, Switzerland) to add a [α-^{35}S]dATP tail (specific activity >1000 Ci/mmol, Amersham Pharmacia Biotech, UK) to the 3'OH end of the probe. The excess of label was removed using a commercial kit (QIAquick nucleotide removal kit, Qiagen, USA).

In situ hybridisation histochemistry using oligoprobes was performed as previously described [244]. All buffers were prepared with DEPC-treated MilliQ water and autoclaved before use in order to avoid RNA degradation. Once defrosted and dried, sections were prefixed with 4% formaldehyde in PBS (pH 7.4) for 5 min. They were rinsed twice in PBS, acetylated (0.25% acetic anhydrous in TEA-HCl 0.1M/0.9% NaCl) for 10 min, dehydrated with ethanol (70% (1 min), 80% (1 min), 95% (2 min) and 100% (1 min)), defatted in chloroform for 5 min and partly re-hydrated (ethanol 100% (1 min) and 95% (1 min)). Slides were air-dried and then 70 μl of hybridisation buffer (50% formamyde, 4 X SSC, ssDNA 500 μg/ml, yeast tRNA 250 μg/ml, 1 X Denhardts, 10% dextrane sulphate and 10 mM DTT) containing the labelled probe (150000 to 200000 cpm/70 μl) were spotted onto each slide and coverslipped with parafilm. Sections were incubated for 16-18 h in a humid chamber at 37 °C. After hybridisation, coverslips were removed in 1 X SSC, and each slide was briefly washed 3 X in 1 X SSC. Then sections were washed in 1 X SSC (3 x 15 min at 55 °C), 0.5 X SSC (1 X 15 min at 55

ºC and 1 X 30 min at RT) and 0.01 X SSC (1 X 30 min at RT), rinsed twice in distilled water and air-dried. Finally, sections were exposed to Hyperfilm-MP autoradiography film (Amersham Pharmacia Biotech, UK) for 3 or 14 days for AVP or CRF mRNA, respectively.

6.2. Riboprobes

Fixed brains were post-fixed for at least 24 h in PFA+Borax at 4 ºC before being cryoprotected by placing them in PFA+Borax containing 10% sucrose (Merk, Germany) for at least 24 h more at 4 ºC. They were frozen in dry ice, mounted onto a cryostat (Frigocut 2800, Leica, Germany) and cut into 30 µm coronal sections. Sections were collected in cryoprotectant solution (0.05 M sodium phosphate buffer, pH 7.3, 30% ethyleneglycol, 20% glycerol) and stored at – 20 ºC.

The protocol used for in situ hybridisation histochemistry using riboprobes was adapted from Simmons et al [247]. All the solutions were pre-treated with DEPC and sterilised before use. One every sixth section of the entire brain (from the olfactory bulb until the end of the medulla) were mounted onto gelatine and poly-L-lysine-coated slides. The slides were prepared by submerging pre-cleaned slides into 10% gelatine (Merk, Germany) containing 1% $CrK(SO_4)_2$ (Sigma, Spain) for 2 min, letting them dry overnight at 37 ºC and then submerging them into 0.1 mg/ml poly-L-lysine (Sigma, Spain) for 30 min and dried overnight at 37 ºC. Mounted sections were desiccated and stored under vacuum before further processing.

The GR probe was obtained by linearisation of a pGem3 plasmid with Bam HI (Amersham Pharmacia Biotech, UK). The c-fos probe was generated from the Eco RI fragment of rat c-fos cDNA (Dr. I. Verma, The Salk Institute), subcloned into pBluescript SK-1 (Statagene, USA) and linearised with Sma I. pGem3 plasmid containing a pure CRF intronic piece was linearised with Hind III (530 bp) to detect specifically CRF hnRNA (Dr. S. Watson, The University of Michigan, Ann Arbor, USA [99]). AVP intronic probe (Dr. T. G. Sherman, University of Pittsburgh, PA, USA) was constructed from a pGem3 vector containing a Sprague-Dawley rat AVP gene fragment of intron I (pGem3-sdAVPgInI), and a 700-bp Pvu II fragment contained within intron I was subcloned into the Hinc II site of pGem3 and linearised with Hind III. The GR plasmid and the c-fos, hnCRF and hnAVP plasmids were generously provided by Dr. E. R. de Kloet (Leiden University, Leiden, The Netherlands) and Dr. S. Rivest (Laval University, Québec, Canada), respectively.

Radioactive antisense cRNA copies were generated using a transcription kit (Promega, USA). Once digested, 250 ng of linearised plasmid were incubated in transcription buffer (40 mM Tris-HCl pH 7.9, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl), 10 mM DTT, 0.2 mM GTP/ATP/CTP, 200 µCi [α-^{35}S]UTP (Specific activity 1300 Ci/mmol, NEN Life Science Products, USA), 40 U RNasin and 20 U of SP6 (GR) or T7 (c-fos, hnCRF and hnAVP) RNA polymerase for 60 min at 37 °C. The DNA template was then digested with RNase-free DNase (Promega, USA; 1 U DNase in 0.25 µg/µl tRNA and 9.4 mM Tris/9.4 mM $MgCl_2$) for 10 min at RT and extracted with phenol-chloroform-isoamilalcohol 25:24:1 (Sigma, Spain). The cRNA was precipitated with 80 µl of 5 M ammonium acetate and 500 µl of 100% ethanol for 20 min at – 80 °C. The pellet was obtained by 15 min centrifugation at 15000 g, dried, ressuspended in 100 µl of 10 mM Tris/1 mM EDTA, pH 8.0, and stored at – 20 °C.

Sections were post-fixed in 4% PFA+Borax for 30 min, rinsed twice in KPBS for 5 min, then digested with proteinase K (Roche, Switzerland; 0.01 mg/ml in 100 mM Tris-HCl pH 8.0 and 50 mM EDTA pH 8.0) for 25 min at 37 °C, rinsed in DEPC-treated water and 0.1 M trietanolamine pH 8.0 (TEA, Sigma, Spain) and acetylated in 0.25% acetic anhydride in 0.01 M TEA. Finally, they were washed for 5 min in 2 X SCC, dehydrated through graded concentrations of ethanol (50, 70, 95 and 2 X 100%, 3 min each) and air-dried. Thereafter, 90 µl of hybridisation buffer (50% formamide, NaCl 0.3 M, Tris-HCl 10 mM pH 8.0, EDTA 1 mM pH 8.0, 1 X Denhardts, 10% dextrane sulphate, yeast tRNA 500 µg/ml and 10 mM DTT) containing the labelled probe (1 X 10^6 cpm/90 µl) were pre-heated at 60-65 °C, spotted onto each slide and sealed with a coverslip. Sections were incubated for 16-18 h in a humid chamber at 60 °C.

After hybridisation, the slides were dipped for 20-30 min in 4 X SSC, and then coverslips were removed before washing the slides 4 X 5 min in 4 X SCC containing 1 mM DTT (Sigma, Spain). Both the unspecifically hybridised and the excess of probe were removed by digestion with RNase A (Amersham Pharmacia Biotech, UK; 0.02 mg/ml in 0.5 M NaCl, 10 mM Tris-HCl pH 8.0 and 1 mM EDTA pH 8.0) for 30 min at 37 °C to reduce non-specific binding. Thereafter, the sections were washed in descending concentrations of SSC containing 1 mM DTT (2 X 5 min 2 X SCC, 5 min 1 X SSC, 10 min 0.5 X SSC, 30 min 0.1 X SSC at 60-65 °C and a short rinse in 0.1 X SSC at RT), dehydrated through a series of ethanol solutions containing 1 mM DTT (50, 70, 95 and 2 X 100%, 3 min each) and air-dried. The slides were then exposed to a XAR-5 Kodak Biomax MR autoradiography film (Kodak, Spain) for 24 h for AVP hnRNA and c-fos mRNA, 3 days for GR mRNA or 3-6 days for CRF hnRNA. After developing the films, the slides were cleaned and defatted with a series of ethanols and xylene (5 min 95%, 3 X 5 min

100%, 10 min xylene, 30 min xylene and 3 X 5 min 100%) and they were dipped into NTB2 nuclear emulsion (Kodak, Spain). After being exposed for 7, 21 or 21-42 days, depending on the probe, they were developed at 14-16 ºC in D19 developer (Kodak, Spain) for 3.5 min, rinsed in MilliQ water, and fixed in rapid fixer (Kodak, Spain) for 5 min. The slides were rinsed under running tap water for 1-2 h and then counterstained with 0.25% thyonin (Sigma, Spain) for 30-45 sec, dehydrated through increasing concentrations of ethanol, cleared in xylene and coverslipped with DPX (Electron Microscopy Sciences, USA).

6.3. Image analysis

Semiquantitative analysis of the hybridisation signal for CRF mRNA was carried out on the developed films. Densitometric analyses for GR and c-fos mRNA and CRF and AVP hnRNA were done on nuclear emulsion-dipped slides using dark-field illumination in an inverted microscope (Olympus IX70, Japan). Magnification used varied depending on the area under analysis. The sections to be analysed were digitalised and quantified using Image software (W. Rasband, NIH, USA; available on the web at http://rsb.info.nih.gov/nih-image or http://www.scioncorp.com) by setting up the best threshold to avoid detecting any background signal and obtaining measures in arbitrary units (pixel area x average sum grey). In general, the measured area was the whole nucleus under analysis or an average of different measures along the area under analysis. In the case of AVP hnRNA, densitometric analysis was performed in the pPVN, the area generally acknowledged as being the most relevant source of AVP for the control of the HPA axis. The pPVN area was identified on an anatomical basis (medial location in the PVN), since parvocellular and magnocellular AVP hnRNA-containing neurons cannot be distinguished on the basis of grain density, differently from AVP mRNA [95]. All samples to be statistically compared were processed in the same assay to avoid inter-assay variability.

7. STATISTICAL ANALYSIS

Depending on the design of the experiment and the groups to be compared, data were analysed using the Student's t-test, one-way or two-way analysis of variance (ANOVA). In the case of one-way ANOVA, when appropriate, data were further analysed with the post-hoc Student-Newman-Keuls (SNK) test at the 0.05 level of significance. Similarly, in the case of two-way ANOVA, post-hoc analyses were performed when necessary. In some cases logarithmic transformation was used in order to obtain homogeneity of variances.

CHAPTER 1

Long-term effects of stress: LPS as well?

INTRODUCTION

A single exposure to a severe stressor such as IMO has been shown to cause desensitisation of the HPA response to the same stressor applied days or weeks later [175]. As evaluated by its physiological consequences, IMO is a severe stressor [14, 16, 18], in which emotional components predominate over physical ones [198]. Because of the particular characteristics of this stressor, it may well be that the previously described effects of a single IMO exposure on the long-term would only be triggered by this specific neurogenic stimulus.

In order to know whether the long-lasting effects of stress on the HPA axis are or not restricted to emotional stressors such as IMO, we decided to study whether a similar long-lasting desensitisation of the HPA axis may develop after exposure of animals to a systemic stressor. Bacterial endotoxins are lipopolysaccharides located in the outer membrane of Gram-negative bacteria, and they have been widely used to mimic the acute response to bacterial infection without actually infecting the host (see [270] for a review). The specific effects of endotoxin administration depend on different factors such as the dose, route of administration, and bacterial strain, although, in general, LPS can induce a wide range of physiological changes that include fever, sickness behaviour and activation of the HPA axis [270]. Endotoxin appears to be an ideal candidate in order to generalise the study of the long-term effects of stress, since it is considered a physical/immunological stressor that causes CNS changes very different from those of mainly psychological stressors such as restraint or IMO and activates the HPA axis following different mechanisms and pathways, as indicated by brain mapping of c-fos mRNA activation [30, 214]. In fact, during these last few years there has been a wealth of studies trying to elucidate how circulating LPS and peripheral cytokines released after endotoxin administration can trigger the activation of the HPA axis (see [212, 213, 277] for reviews). The mechanisms of HPA axis activation by LPS are highly complex and involve many different factors. The key appears to be the transduction of signals through different cells located both outside (around the CVOs) and inside the BBB, where information from both LPS and peripherally-released cytokines would trigger a central immune response and, in turn, the activation of the HPA axis [212, 213, 277].

We will try to make a brief description of these mechanisms after an intraperitoneal (*ip*) injection of LPS (thoroughly reviewed in [212, 213]), a summary of which is depicted in Figure 4. Once in the bloodstream, LPS binds to the LPS-binding-protein (LBP) [242] and this complex is able to activate the production of different cytokines (mainly TNF-α, IL-1β and interleukin-6 (IL-6)) from peripheral monocytes/macrophages expressing the membrane LPS receptor mCD14 [301]. Until very recently, the mechanisms involved in the activation of the proinflammatory transduction pathways after binding of LPS-LBP to CD14 were not

completely understood, since this receptor is not able to discriminate LPS from host lipids and, in addition, does not have a cytoplasmic signalling domain. Studies with mutant mice showing hyporresponsiveness to LPS together with the characterisation of the family of Toll-like receptors (TLRs) has lead to the conclusion that Toll-like receptor 4 (TLR4) is the main receptor involved in both (i) the specific recognition of pathogen-associated molecular patterns (PAMPs) of Gram-negative bacteria such as LPS, and (ii) the consequent activation of proinflammatory signalling pathways [105, 300]. It is thought that LPS-LBP, once bound to mCD14, would reach adjacent TLR4 and this newly formed complex would then be able to activate the signal transduction through the adaptor protein Myd88, leading to the production of cytokines from peripheral myeloid cells [25, 121].

Apart from binding peripheral monocytes/macrophages, it is thought that the complex LPS-LBP could also enter the brain through the CVOs and trigger cytokine production (mainly TNF-α) from the macrophages/microglia located there. Peripherally generated TNF-α would also access these myeloid cells located around the CVOs and trigger its own production there. This centrally produced TNF-α would then be able to access parenchymal cells (microglia) to trigger both its own production and also mCD14 expression in cells located in deeper parts of the brain. It is thought that centrally-produced mCD14 could play a role as an opsonic receptor, enhancing the clearance of LPS that has been able to enter the brain and thus avoiding possible neurotoxic effects of the endotoxin [213].

It has been suggested that centrally-produced TNF-α might trigger HPA axis activation through stimulation of both pPVN CRF-expressing cells and the ME, since the administration of CRF antibody as well as bilateral lesions of the PVN inhibit the TNF-α-induced HPA axis activation [24, 130]. Nonetheless, the effects of centrally produced TNF-α on the PVN do not seem to be direct, since the PVN only depicts very low amounts of the transcript for its specific receptor TNFR1 (also named as p55 subtype) [188]. Instead, it has been suggested that intermediary molecules produced by the endothelium of blood vessels penetrating the PVN would directly mediate the effects of TNF-α in the PVN, taking into account the profound stimulatory effects of TNF-α in these cells [188]. In this regard, it has been shown that brain endothelial cells express constitutively the soluble form of the LPS receptor (sCD14) and also receptors for TNF-α (TNFR1) and IL-1β (IL1R) (see reviews [212, 213]). The brain endothelium could be activated by peripheral LPS-LBP, TNF-α and IL-1β, triggering the production of more IL-1β (thus functioning as a positive feedback) and of prostaglandins, mainly of the E_2 type (PGE_2). PGE_2 would be able to diffuse through the brain parenchyma, having access to different areas of the brain, among them the pPVN, and inducing the expression of the prostaglandin receptor EP_4R in these nuclei. PGE_2 would then be able to activate c-fos and CRF transcription in pPVN neurons and, in turn, the activation of the HPA

axis. Nonetheless, PGE_2 does not seem to be the only mediator of cytokine-induced PVN activation, since administration of indomethacin, an inhibitor of prostaglandin synthesis, only reduces LPS-induced c-fos mRNA activation in selective brain areas after a medium (0.25 mg/kg) but not a high (2.5 mg/kg) dose of LPS [136]. The role of IL-6 in this story is not completely clear yet, but it seems that centrally-produced IL-6 would contribute to the maintenance of CRF stimulation during the late phases of endotoxemia, since the expression of its receptor IL6R is also induced in the pPVN after LPS injection (see review [212]). Finally, the vagus nerve could also play a role in HPA axis activation by peripheral cytokines released after *ip* LPS injection, but only at very low doses of LPS (reviewed in [75]).

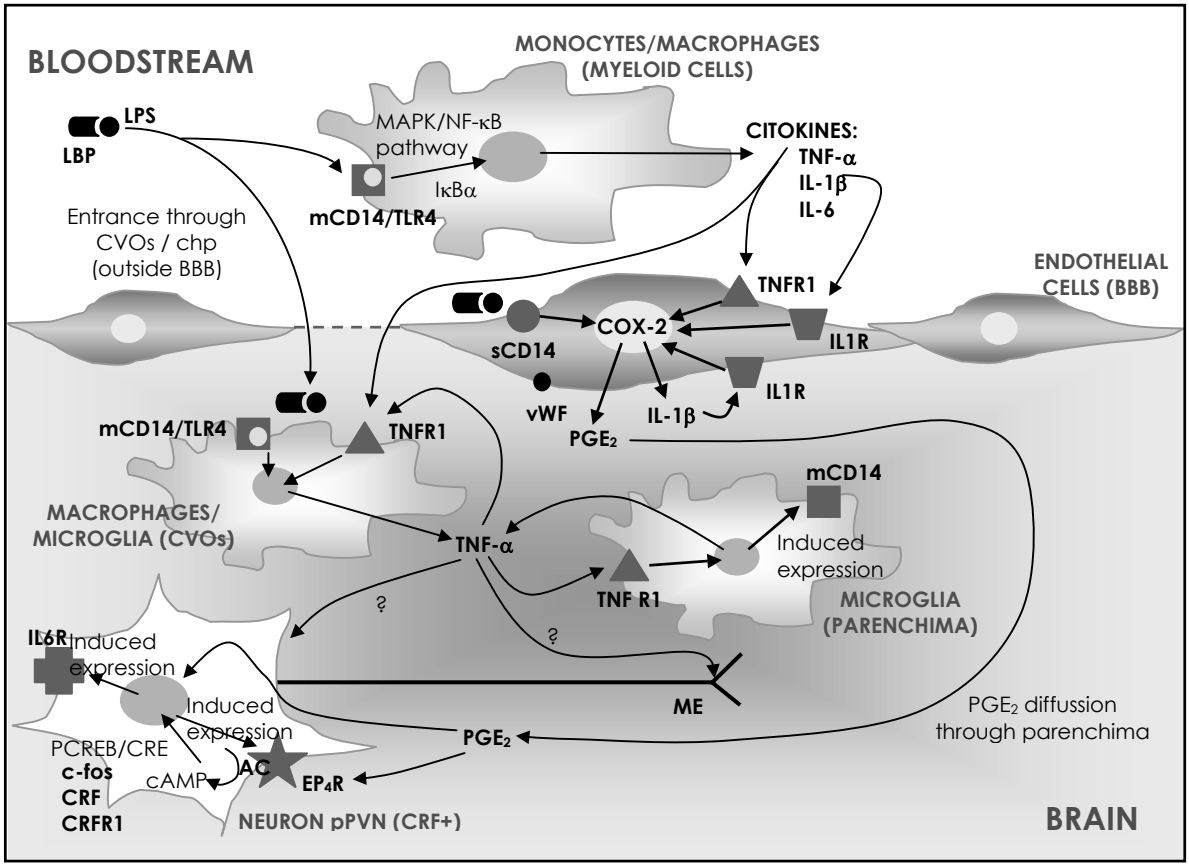

Figure 4. Summary of the mechanisms involved in the activation of the PVN by LPS and cytokines. Briefly, the complex LPS-LPB binds to its specific receptors in the surface of myeloid cells located both in the periphery and in the brain (in the vicinity of the CVOs) triggering the synthesis and release of different cytokines both in plasma and in the brain. This leads to a cascade of events that will ultimately activate CRFergic neurons in the pPVN, leading to the transcription of c-fos, CRF and CRFR1 (see text for details). Adapted from the information reviewed in [212, 213]. Abbreviations: AC, adenilate cyclase; BBB, blood-brain barrier; cAMP, cyclic AMP; chp, choroid plexus; COX-2, ciclooxigenase-2 (PG endoperoxide H synthase isoform 2); CRE, cAMP responsive element; CRF, corticotropin-releasing factor; CVOs, circumventricular organs; EP_4R, PG receptor, type 4; IL-1β, interleukin 1β; IL-6, interleukin 6; IL1R, IL-1β receptor type 1; IL6R, IL-6 receptor; IκBα, inhibitory factor κBα; LBP, LPS-binding protein; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; mCD14, LPS receptor (membrane form); ME, median eminence; NF-κB, nuclear factor κB; PCREB, cAMP responsive element binding protein 1, phosphorilated form; PGE_2, prostaglandin of E_2 type; pPVN, paraventricular hypothalamic nucleus, parvocellular; sCD14, LPS receptor (soluble form); TLR4, toll-like receptor 4; TNF-α, tumor necrosis factor α; TNFR1, TNF-α receptor type 1; vWF, von Willebrand factor (specific marker of endothelial cells).

When speaking about the possible long-term effects of a single LPS injection on the HPA axis, the concept of tolerance to bacterial endotoxin, a well-studied phenomenon of highly clinical relevance, should be taken into account. This term was coined to conceptualise the finding that previous exposure of animals to endotoxin reduces the immune and metabolic impact as well as lethality after a new endotoxin challenge hours or even weeks later [85]. Although tolerance to endotoxin has been extensively studied and has been found to be a more complex phenomenon than previously believed [148, 305], the possible active role of the CNS in the development of tolerance has not been addressed. The fact that tolerance to endotoxin of some CNS-mediated functions is not a passive consequence of changes in the responsiveness of blood cells to endotoxin and could be, at least in part, brain-mediated is of great conceptual importance.

In contrast to the well-established concept of tolerance, it has been reported that previous IL-1β or LPS administration results in enhanced AVP content in the ZEME 7 to 11 days later [237, 239]. Similarly, IL-1β induces long-term sensitisation of the ACTH and corticosterone responses to heterotypic stimuli such as an amphetamine challenge or novelty stress [240]. A single TNF-α injection has also been shown to induce a progressive sensitisation of sickness behaviour, plasma corticosterone, NA utilisation in the PVN and FLI in specific brain nuclei after a new TNF-α administration days to weeks later, together with an increased tonic AVP and CRF immunoreactivity in the ZEME [91, 93]. Very recently, a low dose of LPS has also been shown to sensitise the corticosterone response to LPS or TNF-α administered one, but not 28, days later [92]. Therefore, it is clear that LPS may exert long-term effects on those HPA parameters that have been found to be sensitive to previous stress (IMO) exposure, such as ACTH, corticosterone and CRF mRNA [175]. However, the direction of the changes (sensitisation *vs* desensitisation) is difficult to predict and the protracted effects of a high dose of endotoxin on the HPA axis have not been studied. This is important because we believe that long-term effects of a single exposure to stress are dependent on the intensity (dose) of the stressor used, since sensitisation effects have been detected with stressors of low intensity (e.g. [237, 285]), whereas we and others have described desensitisation effects with a stressor of high intensity such as IMO or alcohol injection [144, 175]. In this study, we wanted to assess the effects of a previous high dose of LPS on the future HPA response to the homotypic stimulus. We consider that these experiments using LPS as a stressor are of great importance in order to extend our previous findings with IMO to systemic stressors.

EXPERIMENTAL DESIGNS AND RESULTS

Experiment 1-1

Since we have previously reported that the effect of a single exposure to IMO on the HPA axis response is enhanced with the time elapsed between the first and the second exposure to the stressor, the purpose of this experiment was to study whether or not a single exposure to LPS would be able to modify the HPA axis response to another exposure to LPS and the time-course of the effect. On their arrival, rats were assigned to two experimental groups: 1/ rats injected with saline (vehicle, Veh) and 2/ rats injected with LPS (LPS). Two or 4 weeks later, rats from all groups were sacrificed 3 h after saline or LPS administration.

T-test showed that acute LPS increased CRF mRNA levels in the PVN of the two groups of rats previously injected with Veh 14 or 28 days before ($p<0.05$ and $p<0.01$, respectively) and also in those previously treated with LPS 14 days before ($p<0.01$). However, the CRF mRNA response was abolished in the rats previously treated with LPS 28 days before (Figures 5 and 6A). One-way ANOVA of groups given LPS the last day further confirmed these results, since CRF mRNA levels after LPS injection were significantly lower in the animals with previous LPS experience 28, but not 14, days before ($p<0.03$, SNK). Acute LPS increased corticosterone levels in all groups (t-test, $p<0.001$ in all cases) and one-way ANOVA revealed no effect of previous LPS treatments 28 or 14 days before (Figure 6B).

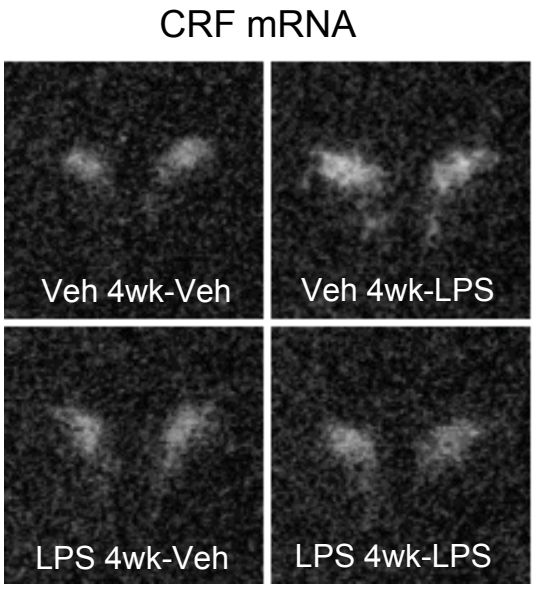

Figure 5. Negative images of some representative autoradiographs of the *in situ* hibridisation for CRF mRNA in the PVN, corresponding to experiment 1-1. Rats received saline (Veh) or 1 mg/kg of lipopolysaccharide (LPS) and were sacrificed, 4 weeks later, 3 h after receiving a new Veh or LPS injection. The legend should be read as "treatment received 4 weeks before-treatment received on the day of sacrifice".

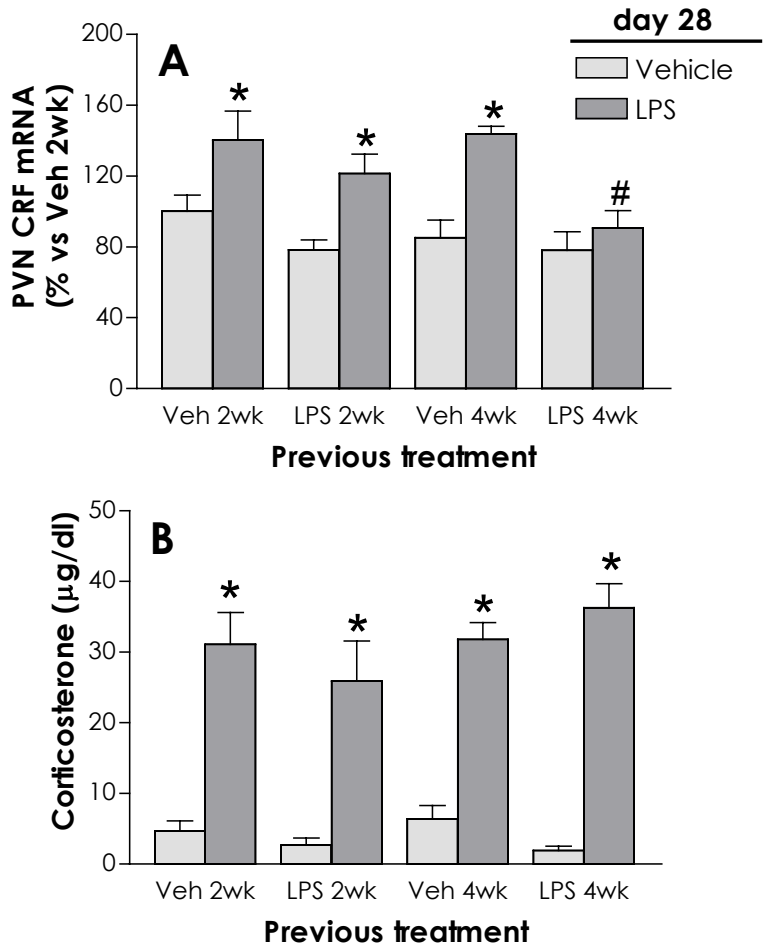

Figure 6. Effect of previous LPS administration on PVN CRF mRNA (A) and corticosterone (B) responses to another LPS administration 2 or 4 weeks later. Stress-naïve animals received saline (Veh) as the previous treatment. Results are expressed as mean ± SE (n = 6-8 per group). * at least $p<0.05$ *vs* respective day 28 Veh (Student's t-test); # $p<0.05$ *vs* the groups receiving the same treatment on day 28 (one-way ANOVA, SNK).

Experiment 1-2

The objective of this experiment was two fold: firstly, we wanted to assess whether a previous LPS administration would be able to alter a physiological response other than the HPA axis, such as rectal temperature changes provoked by LPS injection in our specific experimental conditions. Secondly, we wanted to perform a more detailed analysis of the peripheral HPA axis response to a second LPS administration in previously LPS-injected rats, since the single time-point studied in experiment 1-1 might not be informative enough. Rats were assigned to the following experimental groups: 1/ rats injected with saline on day 1 and with LPS on day 28 (Veh-LPS), 2/ rats injected with LPS on days 1 and 28 (LPS-LPS), and 3/ rats injected with saline on day 28 (∅-Veh). On day 28, rectal temperature was measured 30 min before injection and then for 4 h at 30 min intervals after injection. Repeated blood samples were taken by tail-nick at 4, 6, 8, 10, 12, 18 and 24 h after injection, since it is known that LPS elicits a sustained activation of the hormones of the HPA axis [82].

Figure 7A shows the effect of LPS administration on rectal temperature in previously LPS-injected and stress-naïve rats. Two-way ANOVA for repeated measures of rectal temperature after LPS administration (groups Veh-LPS and LPS-LPS) showed significant effects of sampling time ($p<0.001$), no effects of treatment and a significant interaction between these two factors ($p<0.05$). One-way ANOVAs for each sampling time, now including rats given Veh the last day (∅-Veh), revealed that LPS administration to stress-naive rats caused hypothermia from 1h 30 min to 4h post injection (p at least <0.05, SNK), when compared to the ∅-Veh group. However, this hypothermic response to LPS was abolished in rats administered LPS 4 weeks before, since no differences were found between ∅-Veh and LPS-LPS treated rats at any of the time points studied. In addition, rectal temperature in LPS-LPS animals was significantly higher than in Veh-LPS rats at 1h 30 min and 4h post-injection ($p<0.02$ and $p<0.03$, respectively, SNK). When the area under the curve (AUC) for rectal temperature was calculated from 1h to 4h post-injection (Figure 7B), the comparison of AUC between the different groups displayed significant differences (one-way ANOVA, $p<0.03$), the AUC of Veh-LPS animals being lower than that of the other two groups (SNK).

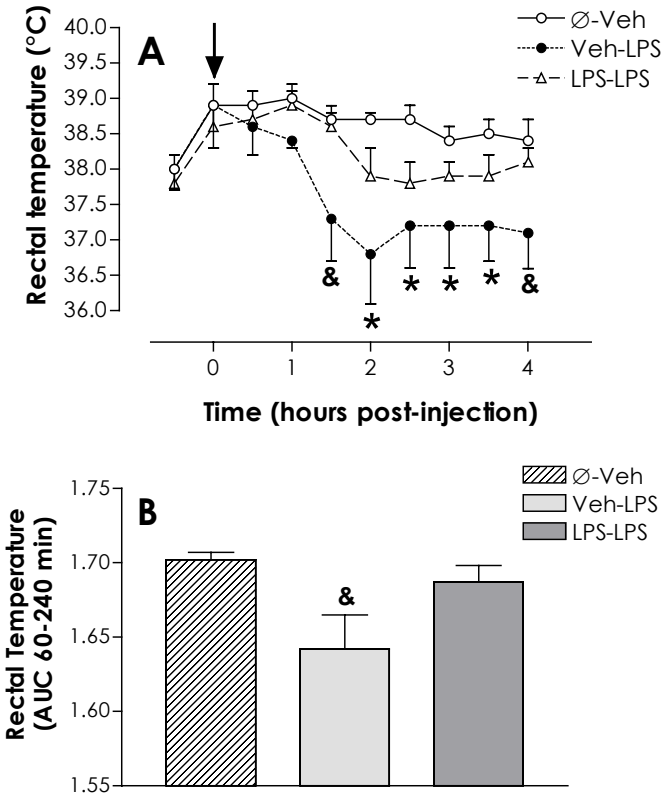

Figure 7. Effects of Veh or LPS administration on the dynamics of rectal temperature **(A)** in stress-naïve and rats previously given LPS 4 weeks before; the arrow indicates the time of injection. The area under the curve (AUC) of rectal temperature levels from 60 to 240 min after injection was also calculated **(B)**. Results are expressed as mean ± SE (n = 3-6 per group). The groups are as follows: 1/ ∅-Veh (animals receiving no treatment on day 1 and injected with Veh on day 28), 2/ Veh-LPS (animals receiving Veh on day 1 and 1 mg/kg of LPS on day 28) and 3/ LPS-LPS (animals receiving 1 mg/kg of LPS on day 1 and the same dose of LPS on day 28). * $p<0.05$ *vs* ∅-Veh group; & $p<0.05$ *vs* ∅-Veh and LPS-LPS groups (one-way ANOVA, SNK).

Figure 8A shows corticosterone levels from 4h to 24h post-injection. Corticosterone levels in ∅-Veh rats were relatively high 4 h after saline injection due to repeated handling and rectal temperature measurements, but showed thereafter a consistent circadian rhythm with a peak at the beginning of the dark period. Two-way ANOVA for repeated measures after LPS administration (groups Veh-LPS and LPS-LPS) displayed significant effects of sampling time ($p<0.001$), no effects of treatment and a marginal interaction between these two factors ($p=0.093$). Although at some sampling times corticosterone levels in LPS-LPS rats were below those in Veh-LPS rats, the differences did not reach statistical significance. AUC for corticosterone levels was also calculated (Figure 8B), including the ∅-Veh group, but no significant effects were detected (one-way ANOVA).

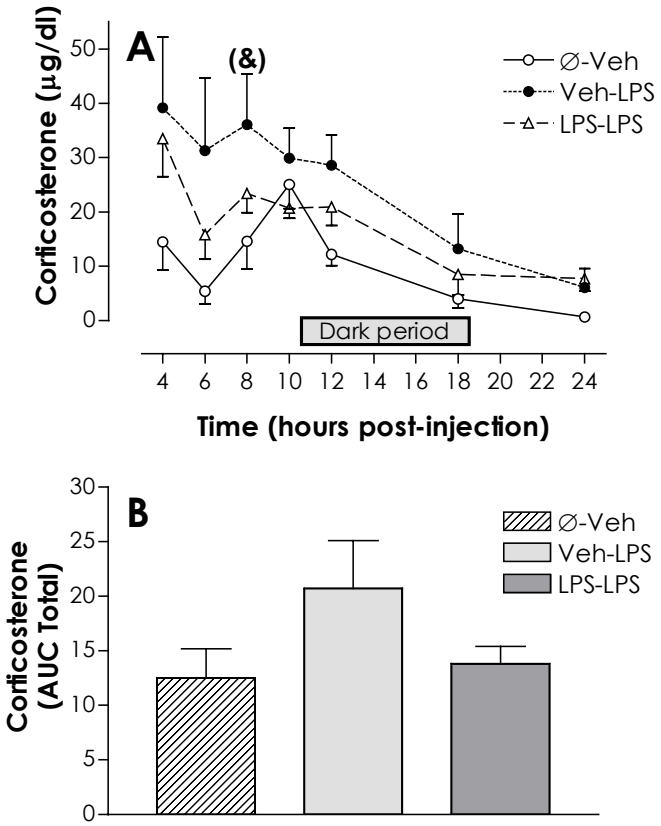

Figure 8. Effects of Veh or LPS administration on corticosterone levels **(A)** in stress-naïve rats previously given LPS 4 weeks before. The area under the curve (AUC) of total corticosterone levels was also calculated **(B)**. Results are expressed as mean ± SE (n = 3-6 per group). The groups are as follows: 1/ ∅-Veh (animals receiving no treatment on day 1 and injected with Veh on day 28), 2/ Veh-LPS (animals receiving Veh on day 1 and 1 mg/kg of LPS on day 28) and 3/ LPS-LPS (animals receiving 1 mg/kg of LPS on day 1 and the same dose of LPS on day 28). (&) denotes marginal significance *vs* ∅-Veh group (one-way ANOVA, $p<0.1$, SNK).

Experiment 1-3

In the previous experiments we were not able to detect any statistical differences in the peripheral HPA axis response to LPS between previously LPS-treated and control rats. In

experiment 1-1 there was only a single and early sampling time point, and in experiment 1-2 the sustained activation of corticosterone partially coincided with the peak of the normal circadian corticosterone rhythm [176], thus greatly masking the response to LPS. In this experiment we decided to inject LPS very early in order to avoid this possible masking effect of the HPA axis circadian rhythm. In addition, we wanted to extend our study on two additional aspects. First, we studied if there was any influence of the time elapsed between the first and second exposure to LPS on the peripheral HPA axis response to this stressful stimulus. In this regard, it should be noted that the time of inter-stress interval seems to be a critical factor, when using IMO, in order to detect a blunted CRF mRNA response at the PVN level [175]. Second, having found an effect of a previous LPS injection on a parameter not directly related to the HPA axis itself (rectal temperature), we wanted to extend our findings by studying circulating levels of TNF-α, one of the primary cytokines released in response to LPS [213, 277] and very sensitive to tolerance [226, 306].

Rats were assigned to the following experimental groups: 1/ rats injected with saline on days 1, 21 and 28 (Veh-Veh), 2/ rats injected with saline on days 1 and 21 and with LPS on day 28 (Veh-LPS), 3/ rats injected with saline on day 1 and with LPS on days 21 and 28 (LPS1wk-LPS) and 4/ rats injected with saline on day 21 and with LPS on days 1 and 28 (LPS4wk-LPS). On day 28 blood samples were taken by tail-nick 0, 2, 5, 8 and 11 h after saline or LPS administration for corticosterone and ACTH measurements. In order to avoid the interference of the peak of the circadian rhythm of ACTH and corticosterone [176] with the response to LPS on day 28, administration of saline or LPS was done at 03:00 AM.

Two-way ANOVA for repeated measures (of the groups receiving LPS on the day 28) for both ACTH (Figure 9A) and corticosterone (Figure 9B) revealed significant effects of sampling time, treatment, and an interaction between the two factors ($p<0.001$ in all cases and for both hormones). For each sampling time, ACTH and corticosterone data from the four experimental groups (Veh-Veh, Veh-LPS, LPS1wk-LPS and LPS4wk-LPS) were analysed with one-way ANOVA and post-hoc comparisons with the SNK test. There were no differences among groups in basal levels of the two hormones. One-way ANOVAs revealed that ACTH differed among groups at 2, 5, 8 and 11 h after the injections ($p<0.001$, $p<0.001$, $p<0.003$ and $p<0.002$, respectively). Similarly, corticosterone differed among groups at all time points after the injections ($p<0.001$ in all cases). Post-hoc comparisons showed that all groups receiving LPS the last day had higher ACTH and corticosterone levels at 2 h after the injection than those receiving Veh and no effect of previous LPS administration 1 or 4 weeks before was observed. ACTH levels were still higher than in Veh-treated rats in all groups 5h after LPS injection, but the two previously LPS-treated groups displayed lower ACTH levels than LPS-naïve rats. Both at 8h and 11h after injection, ACTH levels in the two previously-stressed

groups were comparable to Veh-treated rats, but in those animals having no previous experience with the stressor higher ACTH levels 8h post-injection were observed when compared to the other groups. Corticosterone dynamics was parallel but delayed when compared to ACTH profile: 5 h after injection, corticosterone levels were still higher in all groups receiving LPS as compared to the Veh-Veh group, although the levels in LPS1wk-LPS group were lower than in Veh-LPS and LPS4wk-LPS groups. At 8 h post-injection, a similar pattern was observed, with the LPS1wk-LPS group reaching the values of Veh-Veh rats and the LPS4wk-LPS displaying lower levels than Veh-LPS group. Finally, 11 h after injection, the only group maintaining higher corticosterone levels was the one having no previous experience with LPS (Veh-LPS).

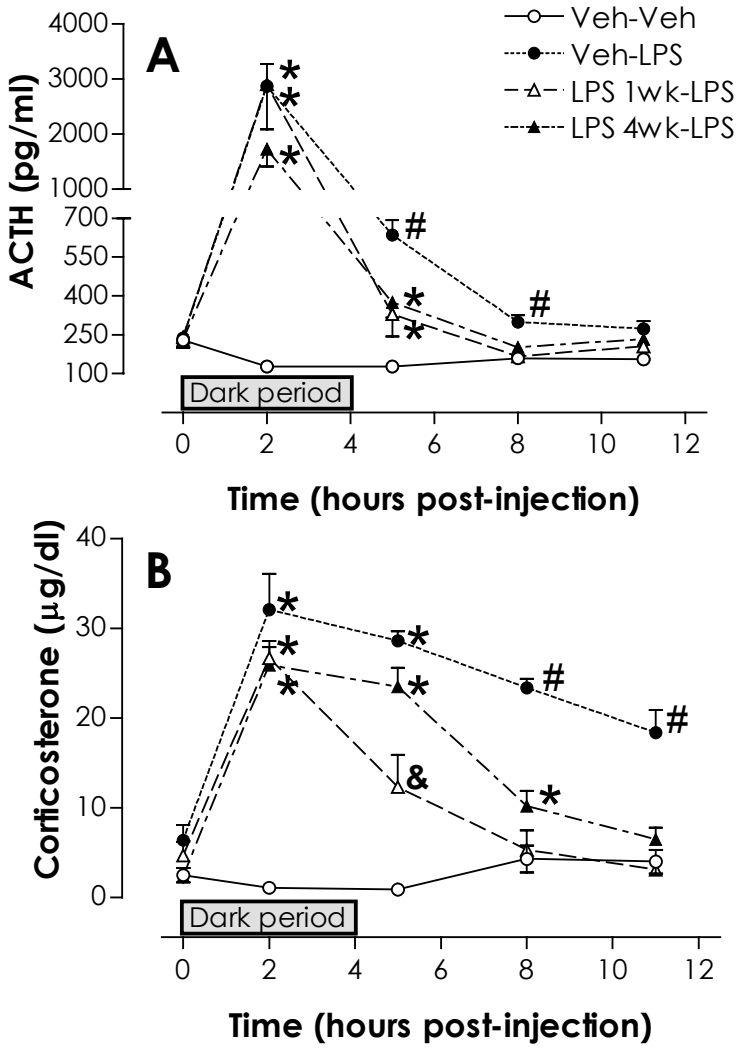

Figure 9. Effect of previous LPS administration on ACTH **(A)** and corticosterone **(B)** responses to another LPS injection 1 or 4 weeks later. Values are the means ± SE (n = 6-8 per group). The groups are as follows: 1/ Veh-Veh (animals receiving Veh on days 1, 21 and 28), 2/ Veh-LPS (animals receiving Veh on days 1 and 21 and 1 mg/kg of LPS on day 28), 3/ LPS 1wk-LPS (animals receiving Veh on day 1 and 1 mg/kg of LPS on days 21 and 28) and 4/ LPS 4wk-LPS (animals receiving Veh on day 21 and 1 mg/kg of LPS on days 1 and 28). * $p<0.05$ *vs* Veh-Veh; # $p<0.05$ *vs* Veh-Veh, LPS 1wk-LPS and LPS 4wk-LPS; & $p<0.05$ *vs* Veh-Veh, Veh-LPS and LPS 4wk-LPS (one-way ANOVA within each particular sampling time, followed by SNK).

One-way ANOVA revealed significant effects of previous LPS treatment on the plasma TNF-α response to the acute dose of LPS ($p<0.003$ at 2 h post-injection and $p<0.001$ at 5 h after LPS) (Figure 10). Further post-hoc comparisons showed a reduction of TNF-α response to LPS in rats given LPS 1 or 4 weeks before, with no effect of the time elapsed between the two injections (SNK test).

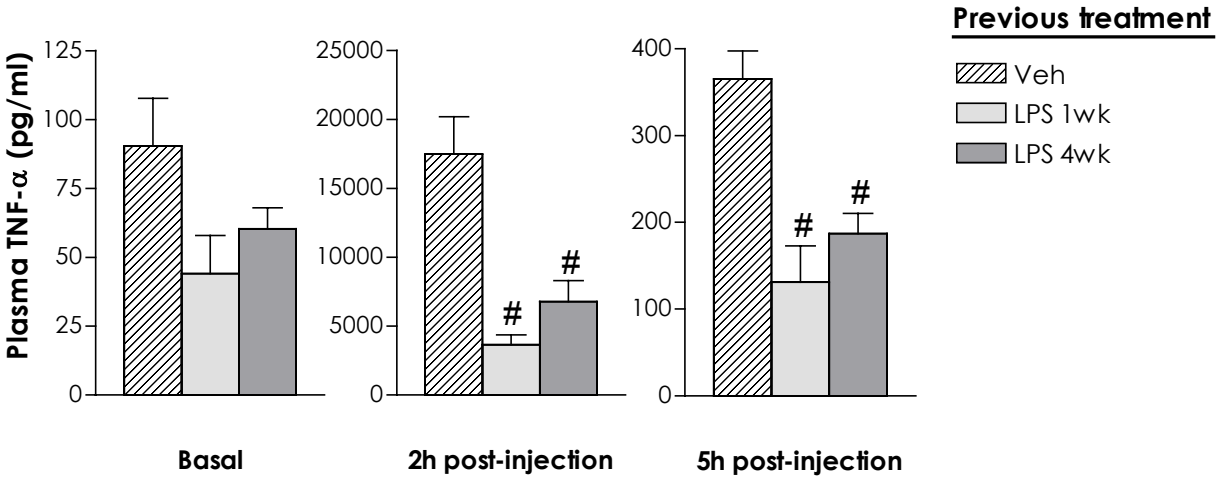

Figure 10. Effect of a final LPS administration on plasma TNF-α in rats given a single LPS injection 1 or 4 weeks before. Stress-naïve animals received saline (Veh) on days 1 and 21. Basal samples were taken before the LPS challenge. Values are the means ± SE (n = 6-8 per group). Note that the scale changes dramatically in function of the sampling time. The changes with respect to basal levels were always significant in all experimental groups at both 2 and 5 h post-injection. Only comparisons within the same sampling time period are indicated. # $p<0.05$ *vs* rats receiving Veh as pre-treatment.

DISCUSSION

We have recently shown that a severe, mainly psychological, stressor such as IMO is able to exert long-term effects on the response of the HPA axis to the same stressor, consisting of a faster termination of the ACTH and corticosterone responses, and an abolition of the CRF mRNA response in the PVN to a second exposure to the same stressor [175]. In the present study, we aimed at studying if such effects could be extended to a physical-immunological stressor such as LPS [277].

Since our previous results with IMO showed that the long-term effects in the PVN were delayed with respect to the peripheral changes [175], in experiment 1 we chose 2 and 4 weeks as the intervals between the first and last exposure to stress to be able to detect changes in both the peripheral and the central levels of the HPA axis. Three hours after LPS injection serum corticosterone levels were significantly higher than in Veh-treated rats, with no differences between control (previously injected with Veh 2 or 4 weeks before) and previously LPS-injected rats. In contrast, the CRF mRNA response in the PVN was abolished in

the group previously injected with LPS 4, but not 2, weeks before. These results demonstrate that LPS is able to exert a long-term desensitisation of the central HPA response to another LPS injection although a period of incubation of more than 2 weeks is needed, in agreement with our previous findings using IMO [175]. The fact that the effects are only apparent 4 weeks after the first exposure to stress would suggest that a minimum amount of time is needed for the development of the long-term effects of stress at the level of the PVN. The present results are also in accordance with those of Lee and Rivier [144] who found that three days of exposure to ethanol did not modify the HPA response to a further ethanol administration on day 4, but did reduce both the c-fos mRNA expression in the PVN and the peripheral HPA response to ethanol 3-7 days after the last administration.

It remains to be explained why previous exposure to IMO reduced 4 weeks later both the central and peripheral responses to the same stressor, whereas previous exposure to LPS only affected the central response to LPS. There are some alternative hypotheses. Firstly, IMO is a mainly emotional stressor [198], whereas LPS is a systemic stressor that activates the HPA axis through not yet well-known mechanisms. Briefly, the effects of LPS are long-lasting and depend on the clearance rate of the endotoxin in the organism and also on the secondary immune activation it elicits [214, 277, 278]. It is thought that LPS would activate the CRF neurons in the PVN in different phases; in the early stages, this activation would be independent of cytokines, involving binding of LPS-LBP to its receptor complex (CD14/TLR4) located in the surface of myeloid cells present both in the bloodstream and CVOs, whereas later in time cytokines would contribute to sustain the HPA axis response (see Introduction, [212, 213]). Finally, some of the cytokines released in response to LPS could have direct effects on the pituitary and the adrenal glands [277], thus explaining a normal peripheral HPA axis response despite no central activation of the PVN.

Taking into account the sustained activation of the HPA axis produced by the high dose of LPS used, it could be that we were not able to detect a faster termination of the corticosterone response to LPS in previously LPS-injected rats because the sampling time point chosen (3 hours after injection) was too short. To test this possibility, we designed a second experiment in which blood samples were obtained throughout a 24-hour period after LPS administration in LPS-naïve and LPS-injected rats 4 weeks before. We decided to measure also rectal temperature during the 4 hours following the injection, to test if changes in rectal temperature provoked by LPS were affected by prior LPS injection. LPS administration to stress-naive rats caused hypothermia for at least 4 h, in accordance with previous results using similar experimental conditions [63, 89, 224]. However, this response to LPS was abolished in rats administered LPS 4 weeks before. These results indicate that LPS is able to exert long-term effects on the rectal temperature changes triggered by another LPS

injection, since hypothermia caused by LPS in our experimental conditions was no longer observed in rats pre-challenged with LPS. Similarly, recent data from our group show that prior single exposure to the forced swimming test reduces the degree of hypothermia produced by another exposure to the test 2 weeks later [53]. If rectal temperature response to endotoxemia is detrimental for the organism, then the lower hypothermia in LPS-LPS treated animals would be protective and might have an adaptative value. More importantly, these results indicate that the long-term effects of a single LPS injection are not only restricted to the central HPA axis response to another exposure to the stressor, but can be generalised to other physiological parameters, not directly related to the HPA axis, such as rectal temperature.

In the same experiment we measured corticosterone levels from 4 h to 24 h after injection. However, no significant effects of the final LPS administration or the previous LPS treatment were found, likely for two reasons: (i) the interference of the handling associated to temperature measurements and (ii) the interference of the normal circadian rhythm of corticosterone, peaking at the beginning of the dark phase of the light-dark cycle [176].

To try to avoid the masking effects caused by the light-dark cycle and the circadian rhythm of the HPA axis, we designed another experiment in which LPS administration on the last day started very early (03:00 AM). In this experiment (experiment 1-3), ACTH and corticosterone measurements displayed significant differences in the response to LPS depending on the treatment received 1 or 4 weeks before. All the groups receiving LPS on day 28 showed increased ACTH and corticosterone levels 2 hours after the injection when compared to the Veh-Veh group. Nonetheless, a previous LPS injection increased the rate of recovery of both ACTH and corticosterone levels after a second challenge, the effect being greater in those receiving LPS 1 week before than in those receiving LPS 4 weeks before.

Two main conclusions can be reached from the present data. Firsly, the primary reason for a shortened peripheral HPA response to a second LPS challenge is not a defective CRF mRNA response, since the latter was not observed in rats administered LPS 2 weeks before. Secondly, the possible direct effects of cytokines released after LPS injection on pituitary and adrenal glands are not masking central desensitisation. TNF-α, mainly produced by macrophages in response to LPS, is exquisitely sensitive to tolerance [226, 306]. It is however unlikely that reduced responsiveness of the HPA axis was merely due to a reduced release of TNF-α (and presumably other cytokines) for two main reasons. Firstly, macrophages are the main source of circulating cytokines and the activation of the HPA axis by a high dose of LPS is observed in macrophage-depleted animals [62]. Secondly, desensitisation of the peripheral TNF-α, ACTH and corticosterone responses to LPS were similar both 1 and 4 weeks

after the first LPS challenge, whereas the reduction of the CRF mRNA response was only observed with a 4-week interval between LPS injections.

It is noteworthy that the effects of a single exposure to LPS (present study) and IMO [175] on the peripheral HPA response to the same stressor were already observed 1 week after the first stress session, whereas the central response was affected only after 4 weeks. It is therefore clear that there is a dissociation between central and peripheral levels of the HPA axis as a consequence of previous exposure to a stressor. There are at least two alternative hypotheses to explain these apparent discrepancies. Firstly, peripheral changes in the HPA axis might be related to ACTH secretagogues other than CRF, such as AVP [12, 119]. However, preliminary results from our laboratory suggest that long-term stress-induced desensitisation of the HPA axis primarily involves changes in the mRNA for CRF, but not for AVP (data not shown). Secondly, it should be taken into account that, in terms of underlying intracellular mechanisms, release of hypothalamic corticotropin-releasing factors into the ME (CRF, AVP) and the subsequent release of ACTH is a parallel but distinct phenomena than the enhanced expression of CRF mRNA after stress. In fact, release of ACTH and corticosterone is more sensitive to stressors than changes in CRF hnRNA or CRF and c-fos mRNA [264]. From the above considerations, it appears that long-term changes caused by stress on the activation of PVN neurons (leading to release of stimulatory factors to the ME) develop faster in time and appear to be, at least, partially independent of changes in gene expression. In fact, the latter changes might be an anticipatory and adaptative response for the possibility of a prolonged exposure to the present stressor or future exposures stressors rather than the determinant of the initial response to the stressor.

Our present data with LPS and our previous observations with IMO [175] indicate that these two greatly differing stressful stimuli are able to induce long-term effects on the response of the HPA axis to the same stressor. In apparent contrast with the above results, it has been reported that a single exposure to some stressors such as footshock causes long-term sensitisation of the HPA response to further exposure to a different stressor such as noise or an amphetamine challenge [241, 285]. Similarly, a single IL-1β injection sensitises the HPA response to a new IL-1β administration or to a different stressor such as footshock [239], and a single TNF-α administration sensitises the future HPA response to a new TNF-α injection [91]. A single administration of amphetamine also exerts long-term sensitisation of the HPA response to a new injection of the same drug, although it desensitises the HPA response to an injection of a different drug such as morphine [241]. The exact reason for the discrepancies between our results and those cited above is unclear. One possibility is that the direction of the long-term effects might depend on the nature of the stressor. However, this appears to be unlikely since footshock, like IMO, is mainly an emotional stressor and IL-1β, like LPS, is a systemic

stressor. Another possibility is that the genetic background of the animals or some environmental rearing conditions might be important to explain these discrepancies, although preliminary studies from our laboratory do not seem to indicate this (unpublished observations). Nonetheless, it may well be that the intensity and/or duration (or the dose) of the first exposure to the stressor would be the key factor in determining the exact direction of the changes caused by a single exposure to stress. A recent study has described sensitising effects of a single low dose of LPS on the behavioural and neuroendocrine responses to a new stressor [92], whereas clear desensitising effects have been observed with a high dose of LPS (present results) and with IMO [175], a severe stressor [171]. In addition, the intensity of the last stressor might be determinant for the detection of the long-term stress-induced changes, since two sessions of social defeat exert long-term desensitisation of the corticosterone response to a high dose of LPS, whereas no significant changes were detected after a lower dose [41].

In summary, it appears that exposure to a high dose of LPS allows the development and progressive consolidation of some kind of memory about the previously encountered stressor, thus leading to a reduced HPA activation after a second exposure to LPS. That this is not restricted to the HPA axis and might have an adaptive value for the animals is reflected by the finding that LPS-induced hypothermia was blunted and TNF-α response was reduced in animals having a previous experience with the stressor. The phenomenon observed with both LPS (present results) and IMO [175] represents a good model of synaptic plasticity in adult animals characterised by some kind of maturational process that affects the peripheral HPA response before the central one. The full adaptive consequences of this phenomenon and its implications in understanding adaptation to repeated stress remain to be established.

CHAPTER 2

Are long-term effects of stress stressor-specific?
A study using IMO and LPS as stressors

INTRODUCTION

A myriad of classical studies using chronic (daily repeated) stress exposure using the same (homotypic) stimulus have brought a wealth of data generally reporting adaptation of many different neurochemical and hormonal responses to stress (see [173] for a review). Nevertheless, the mechanisms accounting for this adaptation to stress may relay at least on two very different factors: 1/ a reduced emotional activation of the animals by getting used to the same daily stress situation or 2/ a pure biochemical adaptation such as down-regulation of receptors or other neurochemical counter-regulatory mechanisms. A single challenge using a different (heterotypic) stressor after chronic stress exposure has been found to be useful in order to elucidate which is the main of the above-mentioned mechanisms taking part in the adaptation to chronic stress exposure. If the response to the heterotypic stressor is also adapted in chronically stressed rats, a general biochemical adaptation may be taking part, whereas if this response is found to be maintained intact or even enhanced, emotional and not purely biochemical mechanisms are probably involved. In this vast amount of studies, all these possibilities have been reported in relation to the HPA axis, depending on many different factors such as the nature or duration of the stressor/s, although adaptation appears to be generally restricted to the homotypic stressor [173].

We have already introduced that there are different categories of stressful stimuli depending on several parameters, and that the routes involved in the activation and the final outcome of the stress responsive systems are greatly depending on the nature of the stressor (see General Introduction). In the last few years, a growing interest has emerged in order to elucidate, using different experimental approaches, the routes and signals involved in HPA axis activation depending on the nature of the stressor (for reviews, see [96, 231, 246]). One of the most extensively used tools has been the description of IEG activation in different brain areas in function of the category of stressor used (e.g. [76, 269, 304]). More direct approaches, such as selective lesions of brain nuclei or transections of projection fibres, have also been used before studying the HPA and/or IEG response to stress in these animals. One of these studies used unilateral transection of the ascending/descending projections from the medulla to rostral brain and showed that Fos activation in the PVN after systemic IL-1β injection was abolished in the side ipsilateral to the medullary transection, whereas the IL-1β-induced activation of this IEG remained intact in the medullary nuclei studied (VLM and NTS) [152]. In contrast, the Fos response to footshock in the PVN was not affected by unilateral transection, whereas there was

an abolition of the Fos activation after the same stimulus in the VLM and NTS. This elegant report clearly demonstrated that the activation of the PVN after an immunological challenge such as IL-1β depends on brainstem activation, whereas after a prototypic emotional stimulus such as footshock does not. Overall, these studies have confirmed that the routes leading to PVN activation after stress may be different depending on the nature of the stressor (see [231, 246] for reviews).

We have so far described that two greatly differing stressful paradigms such as IMO [175] and LPS (Chapter 1) are able to modify the HPA axis response to the same (homotypic) stimulus days to weeks after the first stressful experience. However, we do not know if these long-term effects of stress are specific for the stimulus previously applied. In fact, cross-sensitisation of the HPA response has been observed with stressors greatly differing in nature (i.e. IL-1β and footshock) [239]. Very recently, a long-lasting enhancement of ACTH and corticosterone responses to either emotional (pedestal exposure) or systemic (LPS) stressors has been observed in rats previously exposed to footshock [117]. Therefore, we decided to study the possible cross-desensitisation between IMO and LPS not only for theoretical reasons, but because the degree of specificity of the protracted changes caused by exposure to stress may give us preliminary information about the places or mechanisms more likely to be involved in the changes, as it has been shown with chronic stress [173]. In this regard, if the main loci of the long-term changes caused by a single stress exposure would be within the final common pathways of the HPA axis (e.g. PVN, anterior pituitary) or within the areas exerting an overall control of HPA responsiveness (e.g. hippocampal formation), then the long-term desensitisation of the HPA axis response should be observed in response to both homotypic and heterotypic stressors. In contrast, if the mechanisms responsible of the long-term stress-induced changes would be laying in some of the brain areas involved in the primary processing of the stressor, then the response to a heterotypic stressor widely differing in nature should be maintained essentially intact.

EXPERIMENTAL DESIGNS AND RESULTS

Experiment 2-1

The purpose of this experiment was to demonstrate the specificity of the long-term effects of a single exposure to stress on HPA responsiveness. To this end, rats were treated with LPS or exposed to IMO and 28 days later they were exposed to the homotypic or the heterotypic stressor. We chose a 4 weeks interval between the first and last stress session, since we have previously observed that this is the minimum time

lag required for being able to detect changes at the PVN level. On the first day of the experiment, three groups of animals were defined depending on the treatment received: 1/ saline (Veh), 2/ LPS or 3/ 2h of IMO. On day 28, rats from the three groups were subjected to one of the following treatments: 1/ Veh, 2/ LPS or 3/ 1h of IMO. All animals were sacrificed 4 hours after the onset of the treatments. Rectal temperature was also measured just prior to sacrifice only in the groups receiving LPS on day 28, as this time was appropriate to detect tolerance according with the results obtained previously (see Chapter 1).

One-way ANOVA revealed differences between groups in rectal temperature 4 h after LPS ($p<0.001$). As shown in Figure 11, rectal temperature was higher in those animals previously treated with LPS compared to the other two groups (SNK), suggesting protection from LPS-induced hypothermia.

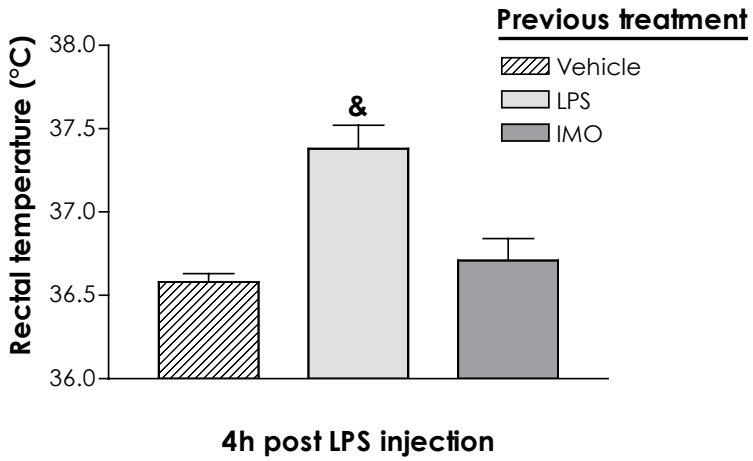

Figure 11. Effect of previous stress exposure (LPS or IMO) on rectal temperature changes 4 h after LPS injection 28 days later. Stress-naïve animals received saline (Vehicle) on day 1. Results are expressed as mean ± SE (n = 8 per group). & $p<0.05$ *vs* the other two groups (one-way ANOVA, SNK).

Figure 12 (A-C) displays CRF mRNA, ACTH and corticosterone responses to the acute stressors (LPS and IMO). Two-way ANOVA indicated significant effects of acute treatment ($p<0.001$ for all variables). There were also significant effects of previous treatment for CRF mRNA and ACTH (p at least <0.01), and marginally significant effects for corticosterone ($p=0.068$). Finally, there was a significant interaction between the two factors for CRF mRNA ($p<0.001$), a marginal interaction for ACTH ($p=0.064$), and a significant interaction for corticosterone ($p<0.01$). Appropriate post-hoc comparisons showed that previous stress exposure did not modify basal levels of the variables studied, except for a small but significant decrease of ACTH basal levels

in previously immobilised animals ($p<0.05$). More importantly, post-hoc analysis revealed that previous experience with LPS only reduced the CRF mRNA response to the homotypic stressor ($p<0.001$), whereas previous experience with IMO reduced CRF mRNA, ACTH and corticosterone responses to the same stressor (p at least <0.01). The fact that no differences were found among ACTH and corticosterone levels 4 h after LPS injection between previously LPS-treated and control rats is probably due to the sampling time point, since we found previously that later in time (from 5 h post-injection) both hormones display significantly lower levels when compared to those of stress-naïve rats (see Chapter 1).

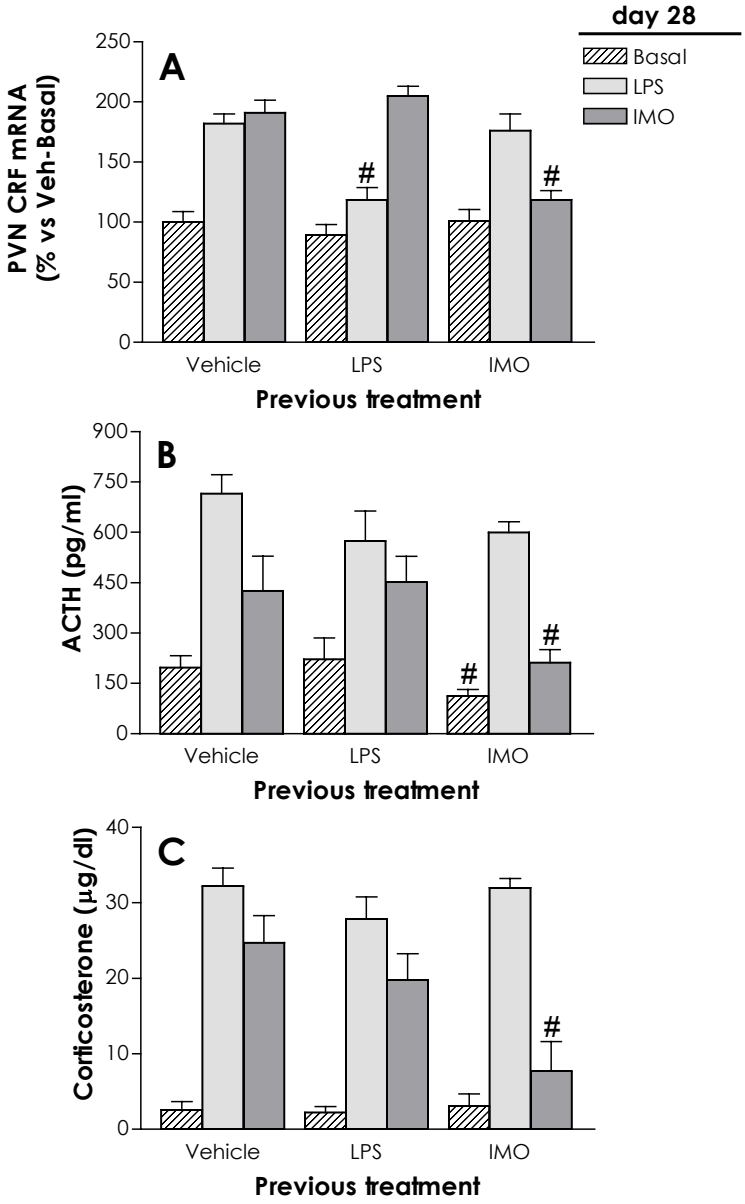

Figure 12. Effect of previous stress exposure (LPS or IMO) on PVN CRF mRNA **(A)** ACTH **(B)** and corticosterone **(C)** responses to the homotypic or the heterotypic stressor 28 days later. Stress-naïve animals received saline (Vehicle) on day 1. Values are the means ± SE (n = 6-8 per group). # $p<0.05$ *vs* groups receiving the same treatment on day 28 (one-way ANOVA, SNK).

Experiment 2-2

The previous experiment showed no cross-desensitisation between LPS and IMO in the long-term at the different HPA axis levels studied. However, we cannot be sure that previous IMO exposure did not affect plasma ACTH and corticosterone responses to LPS, as we have seen that the sampling time point studied (4 h post-injection) is not optimal for detecting a faster shortening of the peripheral HPA response to LPS in previously LPS-treated animals (see Chapter 1). For this reason, animals were subjected to 2h of IMO or injected with Veh on day 1, and 4 weeks later each of these groups received either Veh or LPS. Blood samples were taken by tail-nick 0, 2, 5, 8 and 11 h after Veh or LPS administration for corticosterone and ACTH measurements. On the basis of our previous results (see Chapter 1), on day 28, administration of saline or LPS was done at 03:00 AM.

ACTH and corticosterone levels after Veh or LPS administration on day 28 are shown in Figure 13 (A-B). Two-way ANOVA for repeated measures (without including the groups receiving Veh on day 28 to avoid uninformative interactions) revealed significant effects of sampling time ($p<0.001$ for both hormones), but not of previous IMO or the interaction between these two factors. To know the statistical significance of the effects of LPS administration on ACTH and B we used one-way ANOVA (followed by SNK test) for each sampling time comparing rats given Veh the last day (Average Veh) to those receiving LPS (Veh-LPS, IMO-LPS). There were no differences among groups in the basal levels of both hormones, but both ACTH and corticosterone differed among groups at 2, 5, 8 and 11 h after the injections ($p<0.001$ in all cases). Post-hoc comparisons showed that acute LPS injection increased both ACTH and corticosterone levels irrespective of previous IMO treatment and that their levels, when compared with Veh-treated animals, remained still above control levels 11 h after LPS injection.

Experiment 2-3

The HPA axis activity is regulated by means of negative feedback mechanisms exerted by glucocorticoids at different levels, mainly in the pituitary, PVN and hippocampal formation [60]. These retroinhibitory mechanisms are mediated by the interaction of glucocorticoids with specific receptors, the classical ones being MR (or type I) and GR (or type II). Whereas MR plays a more significant role in the regulation of the circadian rhythm of the HPA axis, it is generally agreed that GR is mainly involved in the negative feedback mechanisms taking place after stress exposure. To further extend the previous results on the specificity of the long-term effects of a single

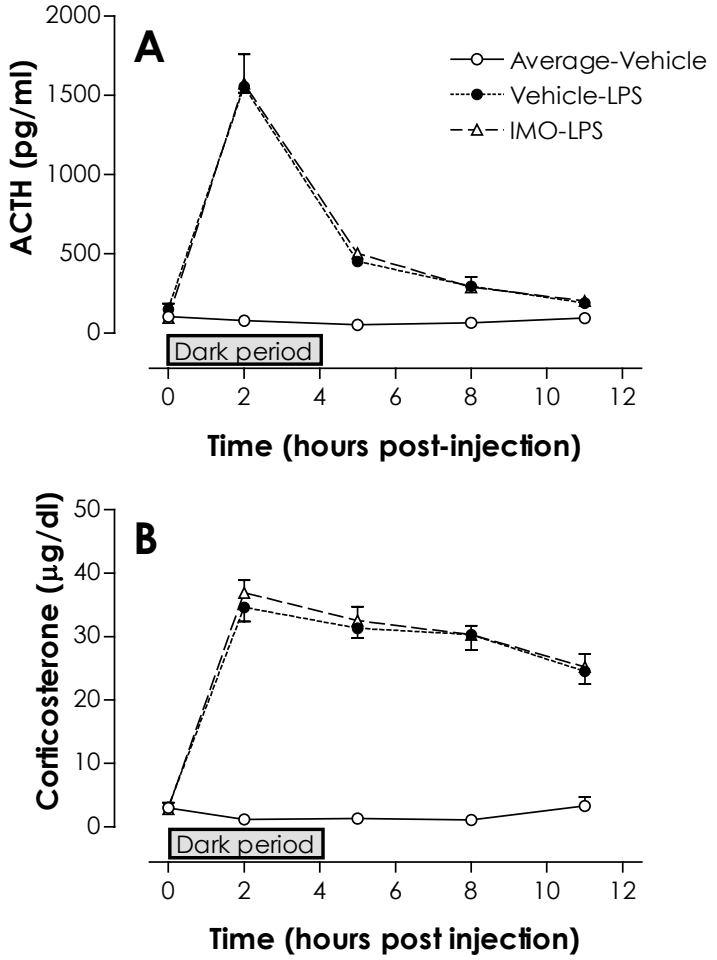

Figure 13. Effect of previous IMO exposure on ACTH **(A)** and corticosterone **(B)** responses to an LPS injection 4 weeks later. The group Average-Vehicle corresponds to animals that received Veh on day 28, having received either Veh or IMO 4 weeks before. Results are expressed as mean ± SE (n = 7-8 per group). Although symbols are not displayed in the figure, ACTH and corticosterone levels after LPS injection were above Average-Vehicle group in all the time points studied (one-way ANOVA, SNK).

stress exposure, we wanted to study whether LPS or IMO exerted long-term effects on the expression of GR in two areas critically involved in the regulation of the HPA axis: the PVN and the hippocampal formation. Changes in GR in these areas might be important in the negative feedback of glucocorticoids on the HPA axis [60, 100, 131] and there is some evidence for long-term changes in MR/GR after a single exposure to stress [39, 153, 285].

To this end, animals received either Veh, LPS or 2h IMO+Veh on day 1, and 4 weeks later they were perfused under basal conditions. Basal GR mRNA levels on day 28 in both the PVN and hippocampal formation are shown in Figure 14 (A-B). All groups displayed similar levels of this transcript in the PVN. A more robust signal was obtained in the hippocampus, showing different degrees of GR mRNA expression depending on the subfields, as previously described [250]. No differences were found depending

on the previous treatment in the CA1-2 and CA3-4 hippocampal subfields, but the animals with previous IMO experience displayed significantly higher GR mRNA levels in the DG subfield when compared to both control and previously LPS-injected groups (one-way ANOVA, $p<0.05$, SNK).

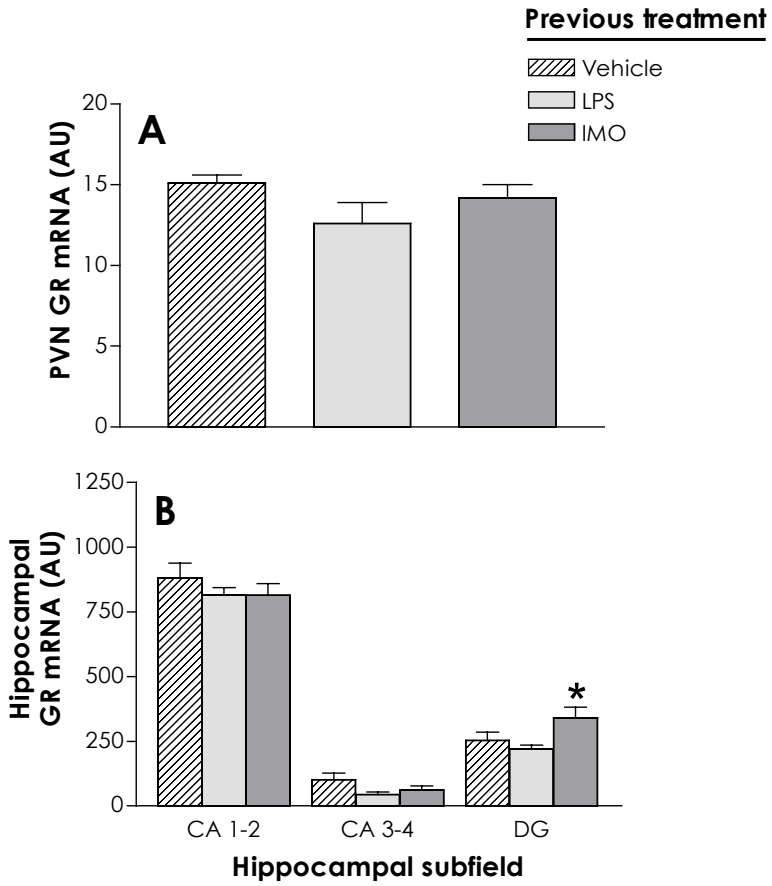

Figure 14. Basal GR mRNA levels in **(A)** the PVN and **(B)** the hippocampal formation of rats previously exposed to LPS or IMO 4 weeks before. Stress-naïve animals received saline (Vehicle) on day 1. Results are expressed as mean ± SE (n = 8 per group). AU: arbitrary units (pixel area x sum grey). * $p<0.05$ *vs* Control group within the same hippocampal subfield (one-way ANOVA, SNK).

DISCUSSION

Our previous observations with IMO [175] and the present data using LPS (Chapter 1) indicate that these two greatly differing stressful stimuli are able to induce long-term effects on the response of the HPA axis to the same stressor. Since desensitisation of the stress response caused by a previous single stress exposure is a novel and challenging finding from an adaptative point of view, it is also crucial to determine whether or not this is a stressor-specific phenomenon. To answer this question we performed an experiment using the above-mentioned stimuli. The animals received saline, 2h IMO or LPS the first day of the experiment, and 28 days later they were

sacrificed under basal conditions or 4 hours after the onset of another exposure to the homotypic or the heterotypic stressor. In the animals receiving LPS on day 28, rectal temperature was measured just before sacrifice.

Acute exposure to both LPS and IMO elicited a robust central and peripheral HPA response in stress-naive rats, as evaluated 4 h after the beginning of the stress. Whereas the HPA response to the heterotypic stressor was not modified by previous exposure to stress 4 weeks before, the CRF mRNA response in the PVN was reduced (for LPS) or even abolished (for IMO), in the animals receiving the homotypic stressor 4 weeks earlier. At the peripheral level, previous exposure to LPS did not modify either ACTH or corticosterone response to the same stressor at the time point studied, whereas previous exposure to IMO resulted in a greatly attenuated (for ACTH) or even absent (for corticosterone) response in those animals that had received IMO on day 1. All these data confirm the long-term effects of both a single LPS administration (Chapter 1) and an exposure to IMO [175] on the HPA response to the same stressor. In this latter work, the peripheral HPA response to a novel stressor (forced swimming) was also normal in animals previously exposed to IMO [175]. The fact that the endocrine response to the heterotypic stressor on day 28 remains intact argues against a cross-desensitisation between stressors and also indicates that a single exposure to a stressor does not impair the capability of the HPA axis to respond to heterotypic stressors despite possible underlying changes in the HPA axis. A similar conclusion is usually achieved after exposure to chronic stress [52, 173].

It is generally assumed that activity of the HPA axis is the result of the balance between the stimulatory inputs to the PVN and the negative feedback exerted by corticosteroids acting through the concerted action of type I and type II glucocorticoid receptors (MR and GR, respectively) [60]. Since the long-term effects of LPS and IMO are characterised by a faster termination of the HPA axis response to a second exposure to the same stressor, it would be at first glance reasonable to propose the existence of increased efficacy of the feedback mechanisms. In fact, an increase of GR and MR binding in the hippocampal formation 14 days after a single footshock exposure has been described [285]. Similarly, it has also been shown that a single prolonged stress session (a single session of combined exposures to restraint, forced swim and ether anaesthesia) is able to increase GR mRNA levels in the hippocampal formation 7 days later [153]. However, the results are not consistent with the fact that a single defeat experience resulted in a decrease in GR binding in the hippocampus and hypothalamus 7 days later [39]. Taking into account this variety of

results in literature, we wanted to directly assess this item by measuring the tonic status of GR mRNA 4 weeks after a single stress (IMO or LPS) experience.

We were not able to find any important change in the levels of this transcript in previously LPS-challenged animals, except for a slight increase in GR mRNA levels detected in the DG of rats exposed to IMO 4 weeks before. Nonetheless, it is unlikely that changes in GR negative feedback mechanisms are relevant to explain the present findings for several reasons. Firstly, the HPA response to a heterotypic stressor is maintained intact (present results, [175]). Secondly, we have not been able to find any changes in the efficacy of dexametasone in inhibiting ACTH and corticosterone responses to IMO in rats exposed to the same stressor 1 week earlier (data not shown). Thirdly, the speed of post-stress recovery of the HPA axis, the aspect most clearly affected by previous experience with the stressor, appears to be regulated by factors at least partially independent of negative corticosteroid feedback [80].

Our results could more likely be explained assuming that previous experience with a stressor results in lower stimulatory inputs to the PVN or enhanced inhibitory inputs when confronted again with the same stressor. The exact nature and the origin of these changes remains to be determined, although the inhibitory inputs may be glucocorticoid-independent (e.g. GABA-A, substance P) [96, 114] or related to proposed membrane glucocorticoid receptors [167]. Nonetheless, this last possibility seems less likely since the non-genomic effects of glucocorticoids take place very rapidly (within 5-20 minutes), and we do not detect changes in the response of the HPA axis in previously stressed animals until much prolonged post-stress times. Finally, the areas most likely to be involved would be those activated by stress and sending (directly or indirectly) inhibitory signals to the PVN: hippocampal formation, septum and amygdala-BST, among others [96]. Although IMO and LPS, two very different stressors, induce similar changes on the HPA axis activity in the long-term, it is unlikely that the brain areas sensitive to a previous experience with the stressor would be the same for the two stimuli, taking into account the different pattern of brain activation they elicit (reviewed in [231, 246]). Since it is now accepted that physiological mechanisms underlying memory about past experiences can reside in different brain areas, depending on the particular characteristics of the learning task [268], we suggest that the brain areas sensitive to memory about past stressors could be stressor-dependent.

Despite apparent a similarity in the time course, the present phenomenon is different from that described by other researchers since they found a non-specific

enhancement of HPA responsiveness to different stressors (e.g. [91, 92, 117, 239, 285]). In contrast, our desensitisation process exhibits a marked specificity, in accordance with previous studies reporting chronic stress-induced desensitisation [144, 217]. The sensitisation observed by other authors is not restricted to the homotypic stressor and therefore is probably reflecting changes in the HPA axis at levels proximal to the PVN or downstream.

Altogether, the present results indicate that long-term stress-induced desensitisation of the HPA axis is stress-specific, since there is no cross-desensitisation between two different stimuli such as IMO and LPS. This lack of cross-sensitisation would go against an unspecific upregulation of glucocorticoid receptors as the mechanism implied in the long-term effects of stress as we have previously discussed, but would suggest that other, learning-like, mechanisms would be involved.

CHAPTER 3

Study of the influence of a previous single experience with IMO or LPS on the transcriptional activation at the PVN level

INTRODUCTION

We have described so far that a single stressful experience (either psychological or immunological in nature) can induce long-term effects on the HPA axis response to the same stimulus, characterised by a faster termination of the peripheral HPA axis response to the same stimulus and also by a reduced or even abolished CRF mRNA response to the homotypic stressor at the PVN level. However, whereas the effects at the peripheral level (ACTH, corticosterone) are already noticeable one week after the first experience with the stressor, an incubation time of at least four weeks is needed for observing any effects at the PVN level (CRF mRNA).

At first sight, those results might seem contradictory and we should consider different hypotheses to explain these apparent discrepancies. Firstly, peripheral changes in the HPA axis might be related to ACTH co-secretagogues such as AVP [12, 119], being recognised as dominant among several ones in promoting CRF actions on anterior pituitary corticotropes. However, preliminary results from our laboratory suggest that long-term stress-induced desensitisation of the HPA axis primarily involves changes in mRNA for CRF, but not for AVP (data not shown). Secondly, it should be taken into account that whereas the peripheral HPA response takes place immediately after the onset of the stressful stimulus, the response of the central parameter we measured (CRF mRNA) is not detectable until 3 or 4 hours after the beginning of stress; therefore, these parameters (peripheral *vs* central) can not be directly related. In fact, activation of PVN neurons leading to the secretion of CRF/AVP into the ME and therefore to the release of ACTH is not necessarily linked to the intracellular biochemical processes leading to mRNA induction. For instance, it has been shown, using hypovolemia as a stressor [264], that central (CRF hnRNA, CRF and c-fos mRNA) and peripheral (ACTH, corticosterone) HPA axis responses have different thresholds of activation, the peripheral response being more sensitive than the central one. Furthermore, we should take into account that the measure of CRF mRNA is a reflex of both the newly synthesised transcript after stress but, more importantly, of the amount previously stored under basal conditions [68, 99], thus resulting, in some cases, in non-significant increases after stress because of the already large quantity of CRF mRNA in storage. The measure of CRF mRNA is more likely to indicate the capability of the HPA axis to respond to further stressful situations rather than being related to the immediate and dynamic response during an acute stressful situation.

To assess the latter point, a new in situ hybridisation technique has recently been developed, using probes directed against the intronic sequence of the newly

synthesised transcript [99]. This methodology is thought to be a more reliable tool to study the early central HPA response to stress, as shown by an increasing number of studies in the last few years measuring its response to different stimuli (see General Introduction). All these studies have shown that measurement of CRF and AVP primary transcripts, namely heteronuclear RNA (hnRNA), is a very consistent tool to investigate the early PVN response during stress; in fact, it is a more direct index of the transcriptional activity in this nucleus. Therefore, we decided to measure these parameters using the stress models already described in the previous chapters (IMO, LPS).

Apart from studying the CRF and AVP hnRNA responses in the PVN using our stress paradigm, we also wanted to measure a parameter reflecting neuronal activation in this hypothalamic nucleus. We chose the IEG c-fos, since it has been widely used as a marker of neuronal activation (see [102, 185] for reviews). Even if its functional role is complex and not yet completely understood [128], it is still regarded as a useful tool to study the rapid activation of neurons (its transcription does not depend on protein synthesis) [230]. Furthermore, since c-fos transcript is not usually expressed under basal conditions [185], its measurement is a very sensitive tool to detect stress-triggered neuronal activation. Finally, but not less important, it has been shown that the response of this transcript in the PVN is able to adapt after repeated exposure to both emotional [126, 278, 294] and immunogical [263] stressors, and therefore it would be interesting to evaluate if a single stress session might or not be able to exert long-term effects on the c-fos mRNA response to the same stressor.

Altogether, these early-activation studies will most probably give us a clearer view of what is actually happening at the PVN level in previously stressed rats and might clarify the apparent contradictions in the time lag required for observing long-term effects peripherally and centrally in the HPA axis.

EXPERIMENTAL DESIGNS AND RESULTS

Experiment 3-1

To study the early PVN response to IMO, rats were assigned to the following groups: 1/ control (no stress), 2/ 2h of IMO on day 1 (IMO 4wk) and 3/ 2h of IMO on day 21 (IMO 1wk). On day 28, rats were anaesthetised and perfused either 1/ under basal conditions, 2/ just after 1h of IMO or 3/ 1h after the end of IMO for 1h. This temporal dynamics was chosen on the basis of the early response of the parameters we

wanted to analyse (c-fos mRNA, CRF and AVP hnRNA), but bearing in mind that IMO is a very powerful stressor and thus it might trigger a more sustained PVN activation when compared to the early PVN response that has been described using other neurogenic stressful stimuli like restraint [107-110, 160, 161, 163].

The peripheral HPA axis response could not be studied using these sampling time points since we have previously observed that the long-term effects of IMO on ACTH and corticosterone are not detected until post-stress periods longer than 1h [175]. Thus, we included a small group of animals receiving the same treatments as above but submitted instead to repeated blood sampling at the following time-points: basal, just after 1h of IMO and 1 and 2 h after stress.

c-fos mRNA in the PVN (Figures 15 and 18A) was not detectable under basal conditions, therefore density measurements were not performed in this group. In contrast, a strong signal was obtained just after 1h of IMO, independently of the previous treatment received, and a still convincing signal was detected 1h after the end of IMO. Two-way ANOVA indicated significant effects of previous treatment and time point ($p<0.001$ in both cases) and a significant interaction between the two factors ($p<0.005$). Post-hoc analysis indicated no effect of previous treatment just after 1h of IMO, but an effect at 1h post-IMO: c-fos mRNA levels were greater in the control group than in the two previously-stressed groups, with IMO 4wk group displaying higher levels than IMO 1wk group (p at least <0.02).

Analysis of the transcriptional activity at the PVN level by measuring CRF hnRNA (Figures 16 and 18B) showed barely detectable levels of this transcript under basal conditions, as previously described [99]. A more convincing signal was obtained just after 1h of IMO. At 1h post-IMO a still detectable but weaker signal was observed. Two-way ANOVA revealed significant effects of previous treatment ($p<0.005$), time point ($p<0.001$) and the interaction between these two factors ($p<0.02$). Post-hoc analysis showed no differences in CRF hnRNA levels under basal conditions in function of previous treatment, but significant differences after 1h of IMO and at 1h post-IMO, the animals receiving IMO 28 days before showing lower CRF hnRNA levels when compared to the other two groups (p at least <0.025).

Two-way ANOVA of the AVP hnRNA levels in the pPVN (Figures 17 and 18C) revealed a significant effect of time point ($p<0.001$) but not of previous treatment or the interaction between these two factors.

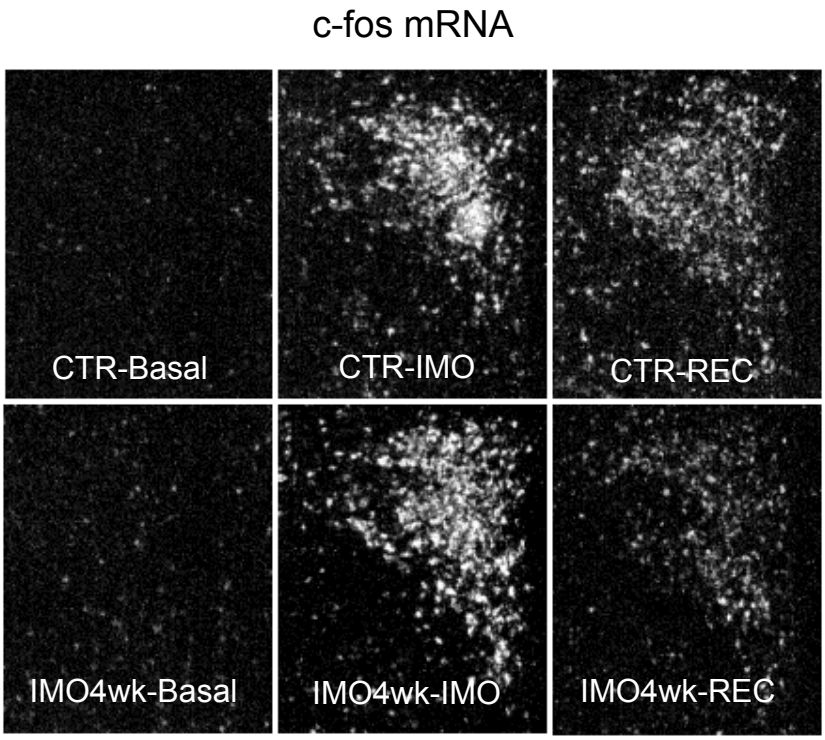

Figure 15. Representative dark-field photomicrographs displaying the effects of a previous single exposure to IMO 4 weeks before on PVN c-fos mRNA expression immediately after 1h of IMO (IMO) and at 1h post-IMO (REC). Control (CTR, stress-naïve) animals did not receive any treatment on day 1. The legend should be read as "previous treatment-day 28 treatment", e.g. CTR-Basal are animals that did not receive any treatment on day 1 and were sacrificed under basal conditions on day 28. Magnification: 60X.

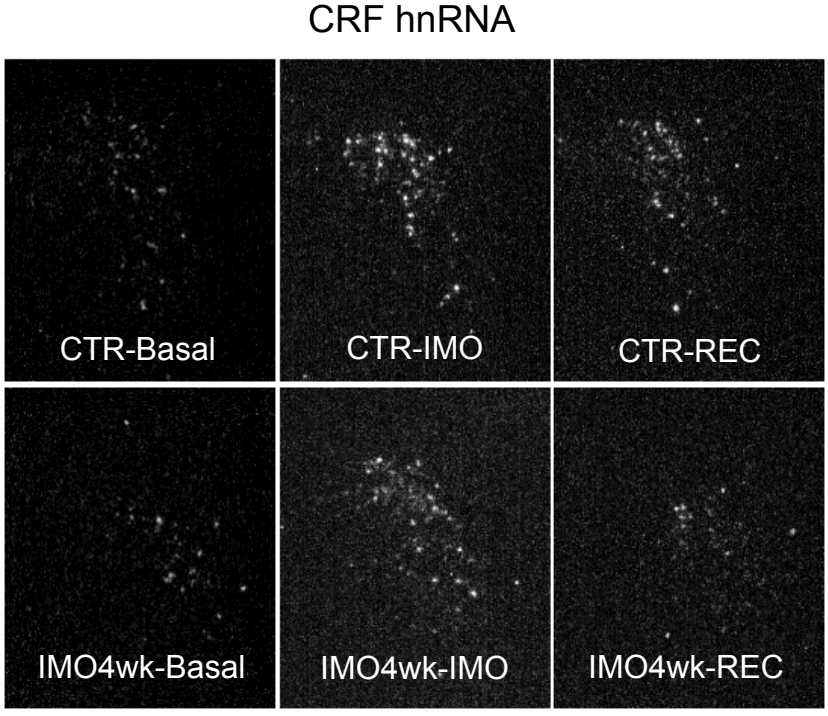

Figure 16. Representative dark-field photomicrographs displaying the effects of a previous single exposure to IMO 4 weeks before on PVN CRF hnRNA expression immediately after 1h of IMO (IMO) and at 1h post-IMO (REC). Control (CTR, stress-naïve) animals did not receive any treatment on day 1. The legend should be read as "previous treatment-day 28 treatment", e.g. CTR-Basal are animals that did not receive any treatment on day 1 and were sacrificed under basal conditions on day 28. Magnification: 60X.

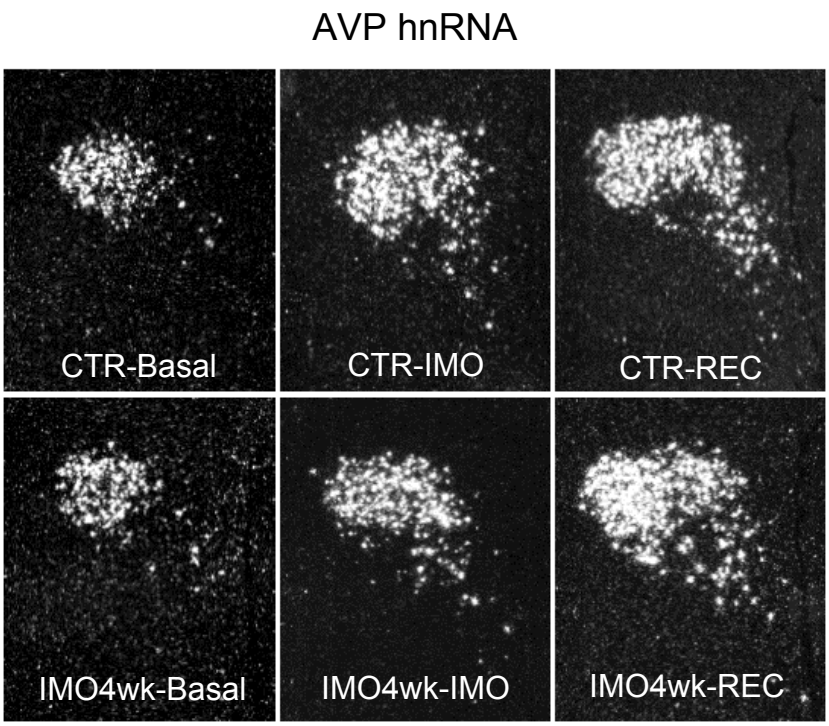

Figure 17. Representative dark-field photomicrographs displaying the effects of a previoussingle exposure to IMO 4 weeks before on parvocellular PVN AVP hnRNA expression immediately after 1h of IMO (IMO) and at 1h post-IMO (REC). Control (CTR, stress-naïve) animals did not receive any treatment on day 1. The legend should be read as "previous treatment-day 28 treatment", e.g. CTR-Basal are animals that did not receive any treatment on day 1 and were sacrificed under basal conditions on day 28. Magnification: 60X.

Peripheral HPA activity (Figure 19) was modified by previous stress exposure similarly to what has previously been described [175]. Two-way ANOVA for repeated measures of plasma ACTH levels revealed significant effects of sampling time ($p<0.001$), previous treatment ($p<0.05$) and the interaction between the two factors ($p<0.01$). Comparisons of previous treatment groups within each sampling time showed that IMO 1wk group displayed significantly lower ACTH levels at 1h post-IMO respect control animals (one-way ANOVA, $p<0.05$, SNK). At 2h post-IMO, ACTH levels were still different between control and IMO 1wk groups (one-way ANOVA, $p<0.02$, SNK). Two-way ANOVA for repeated measures of plasma corticosterone levels revealed a significant effect of sampling time ($p<0.001$), a marginally significant effect of previous treatment ($p=0.071$) and a significant interaction between the two factors ($p<0.002$). Comparisons of previous treatment groups within each sampling time showed that corticosterone levels were elevated to the same extent in all groups except for the time 2h post-IMO, when both previously-stressed groups displayed substantially lower levels of this hormone than control animals (one-way ANOVA, $p<0.001$, SNK).

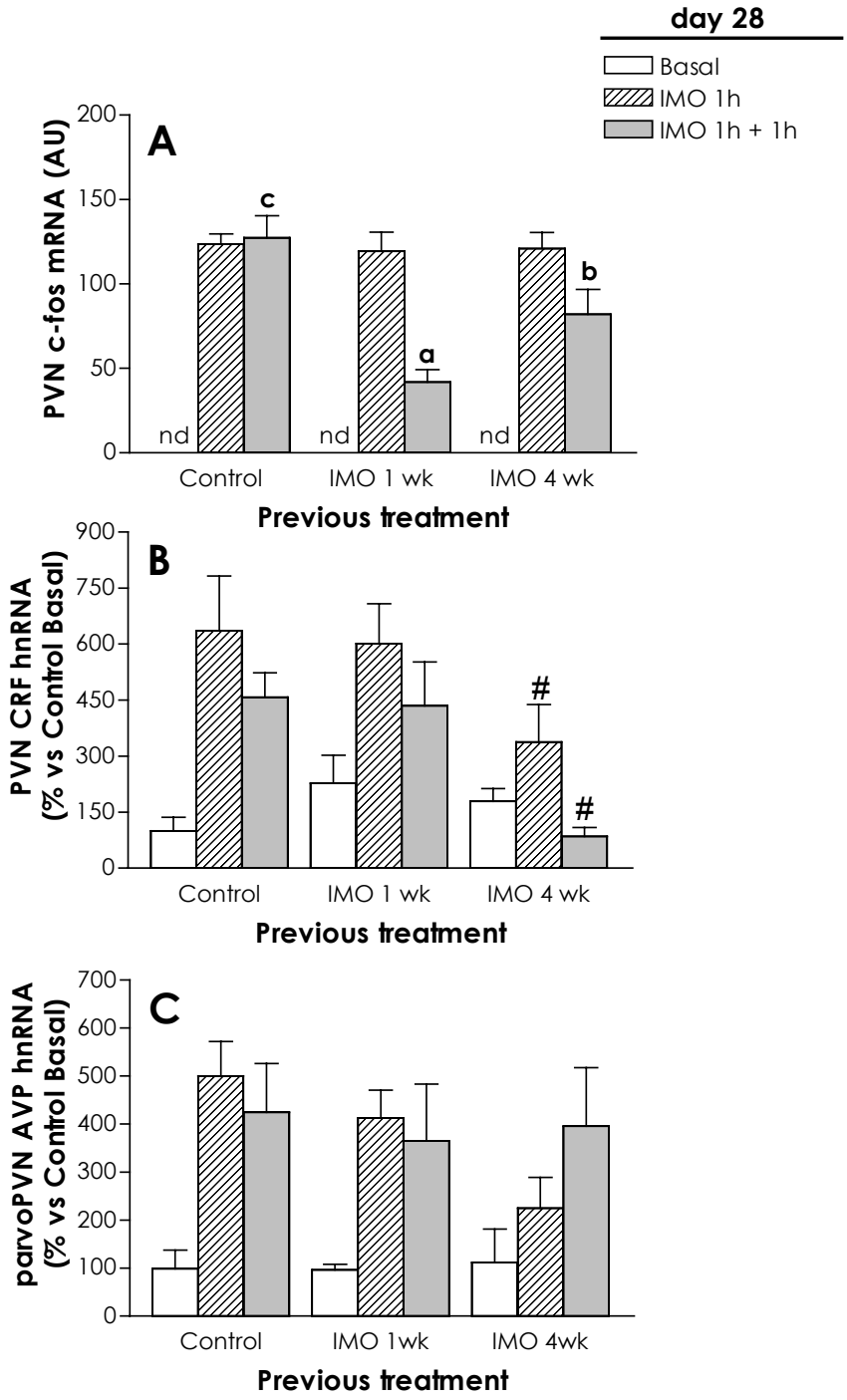

Figure 18. Effects of a single previous IMO experience on the PVN c-fos mRNA **(A)** PVN CRF hnRNA **(B)** and parvocellular PVN AVP hnRNA **(C)** responses to IMO 1 or 4 weeks later. Basal c-fos mRNA levels were not determined due to the almost undetectable signal. Results are expressed as mean ± SE (n = 4-7 per group). AU: arbitrary units (pixel area x sum grey). The different letters (a,b,c) indicate differences between the groups within the same time point on day 28; # $p<0.05$ *vs* the same time point in control and IMO 1wk groups (two-way ANOVA, post-hoc analysis).

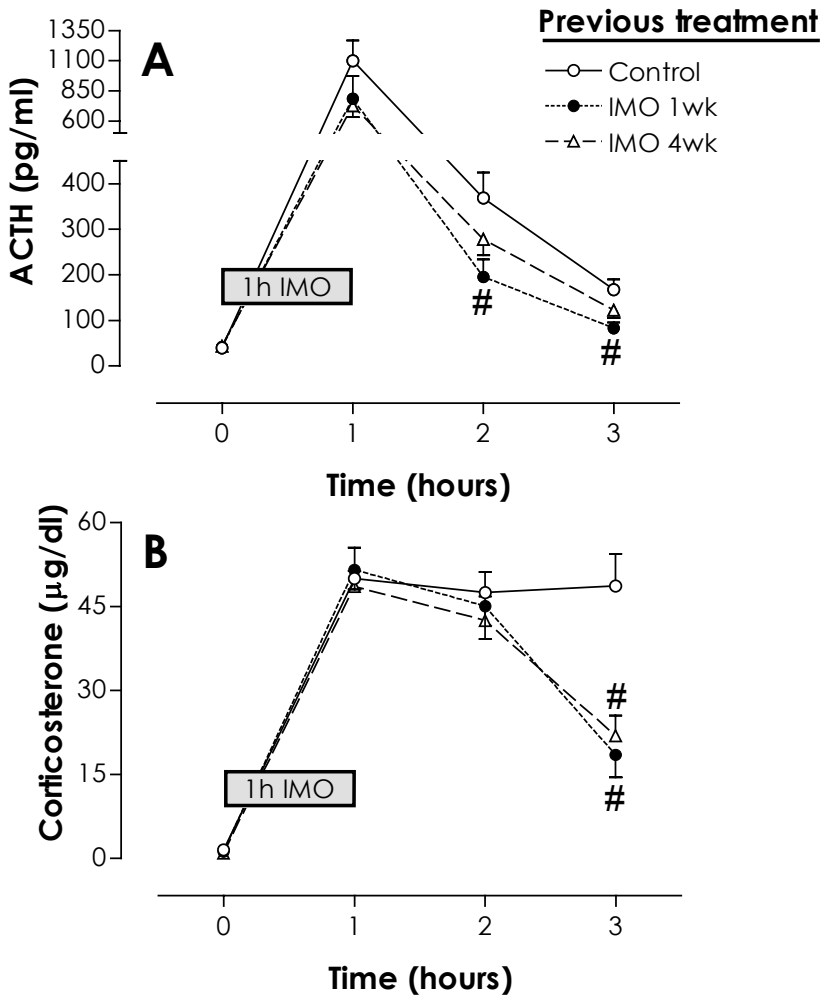

Figure 19. ACTH **(A)** and corticosterone **(B)** responses to acute IMO in stress-naïve rats (control) and rats previously immobilised 1 or 4 weeks before. Results are expressed as mean ± SE (n = 6 per group). # $p<0.05$ *vs* control group within the same sampling time (one-way ANOVA, SNK).

Experiment 3-2

The purpose of this experiment was two fold: (i) to study the PVN response (c-fos mRNA, CRF and AVP hnRNA) to LPS and (ii) to further assess if there was any sign of cross-desensitisation between IMO and LPS measuring these early-response markers. On day 1, animals received 1/ saline (Veh), 2/ LPS or 3/ 2h of IMO+Veh (note that animals receiving IMO were also administered with Veh, thus the Veh group served as a control for both LPS and IMO groups). After 28 days, rats were anaesthetised and perfused after one of the following treatments: 1/ under resting conditions (Basal), 2/ 2h after LPS injection, 3/ 4h after LPS injection, 4/ just after 20 min of IMO or 5/ 30 min after the termination of 20 min of IMO (30 min post-IMO). The group of animals that had previously received 2h IMO+Veh did not receive any of the IMO treatments on

day 28, since the long-term effects of IMO on early response at the PVN level using the homotypic stressor was already studied in experiment 3-1.

On day 28, we chose shorter sampling times for IMO than in experiment 3-1, since it has previously been shown that a shorter exposure to another stressor such as restraint is already able to trigger CRF transcriptional activity [108-110] and therefore it was not necessary to apply longer stress exposures. Furthermore, in a pilot experiment we were able to observe a better signal for the CRF primary transcript using shorter rather than longer times of stress exposure.

When doing the statistical analysis, the groups were separated depending on the treatment received on day 28 (LPS or IMO); thus, animals receiving LPS or IMO on day 28 were not directly compared, since the design did not allow a biologically significant comparison.

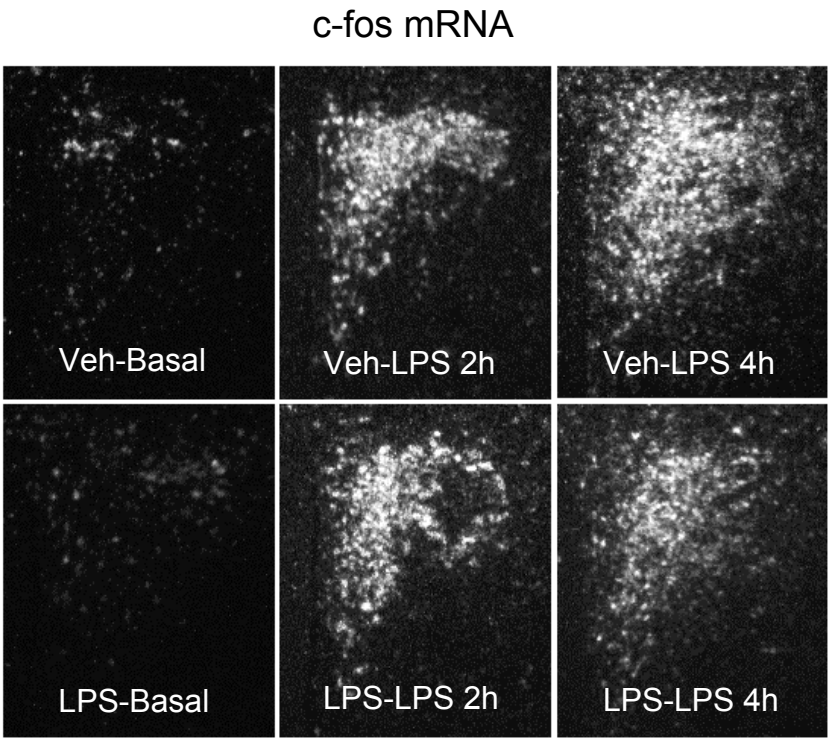

Figure 20. Representative dark-field photomicrographs of PVN c-fos mRNA levels after an LPS challenge in stress-naïve and previously LPS-injected animals. The legend should be read as "previous treatment-day 28 treatment", e.g. Veh-Basal are animals that received a Veh injection on day 1 and were sacrificed under basal conditions on day 28. The hours (LPS2h, LPS4h) indicate the time of sacrifice after LPS injection on day 28. Magnification: 60X.

When measuring the PVN c-fos mRNA response to the acute LPS challenge (Figures 20 and 23A), a long-lasting activation was observed both 2 and 4 h after the

injection, with no detectable levels under basal conditions. Two-way ANOVA of c-fos mRNA levels indicated a significant effect of previous treatment ($p<0.005$) and time point ($p<0.001$), but not of the interaction between these two factors. Post-hoc analysis indicated that c-fos mRNA levels in stress-naïve animals were significantly different from the other two groups (receiving LPS or IMO 28 days before; p at least <0.01). Two-way ANOVA of CRF hnRNA levels after LPS injection (Figures 21 and 23B) displayed significant effects of time point ($p<0.001$), but not of previous treatment nor of the interaction between the two factors. Similarly, two-way ANOVA of AVP hnRNA levels (Figures 22 and 23C) in the pPVN indicated significant effects of time point ($p<0.01$) but not of previous treatment or the interaction between the two factors.

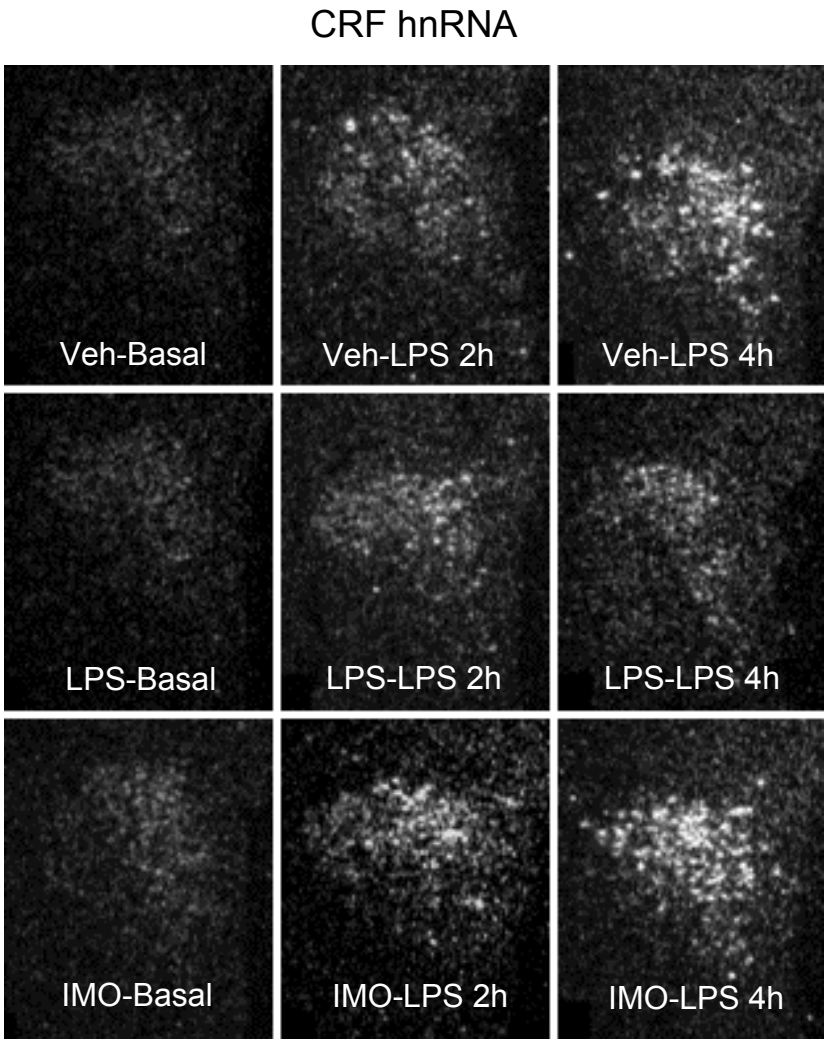

Figure 21. Representative dark-field photomicrographs of PVN CRF hnRNA levels after an LPS challenge in stress-naïve and previously stressed (LPS or IMO 4 weeks before) animals. The legend should be read as "previous treatment-day 28 treatment", e.g. LPS-Basal are animals that received an LPS injection on day 1 and were sacrificed under basal conditions on day 28. The hours (LPS2h, LPS4h) indicate the time of sacrifice after LPS injection on day 28. Magnification: 60X.

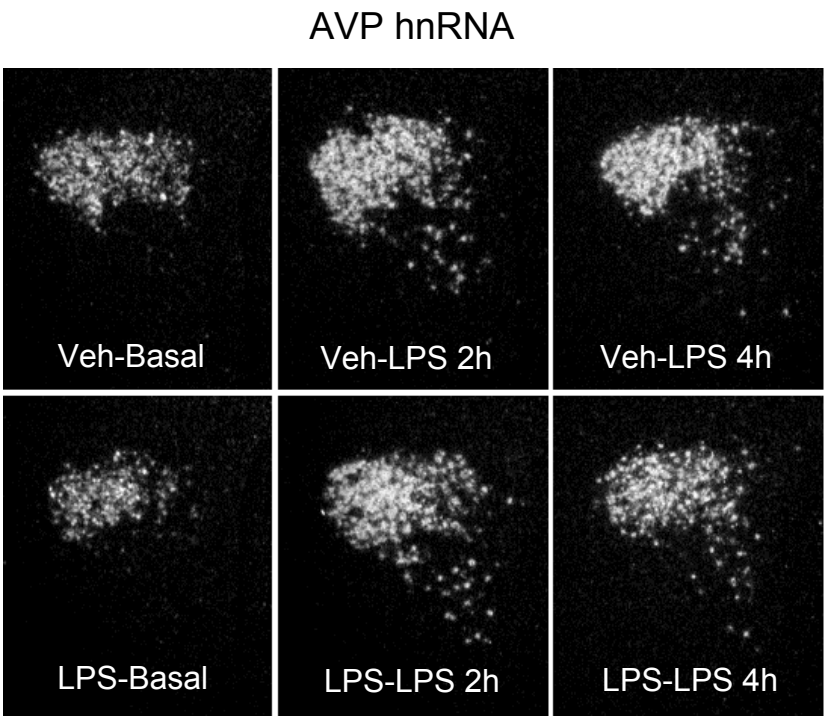

Figure 22. Representative dark-field photomicrographs of parvoPVN AVP hnRNA levels after an LPS challenge in stress-naïve and animals previously injected with LPS 4 weeks before. The legend should be read as "previous treatment-day 28 treatment", e.g. Veh-Basal are animals that received a Veh injection on day 1 and were sacrificed under basal conditions on day 28. The hours (LPS2h, LPS4h) indicate the time of sacrifice after LPS injection on day 28. Magnification: 60 X.

As to the c-fos mRNA response to IMO on day 28 (Figure 24A), two-way ANOVA revealed a significant effect of time point ($p<0.001$), but not of previous treatment or the interaction between the two factors. Two-way ANOVA of CRF hnRNA levels after an acute IMO exposure (Figure 24B) displayed significant effects of time point ($p<0.001$), but not of previous treatment or the interaction between the two factors. Finally, acute IMO did not provoke any changes in AVP hnRNA levels at the time points studied (Figure 24C), although two-way ANOVA indicated marginally significant effects of time point ($p=0.06$).

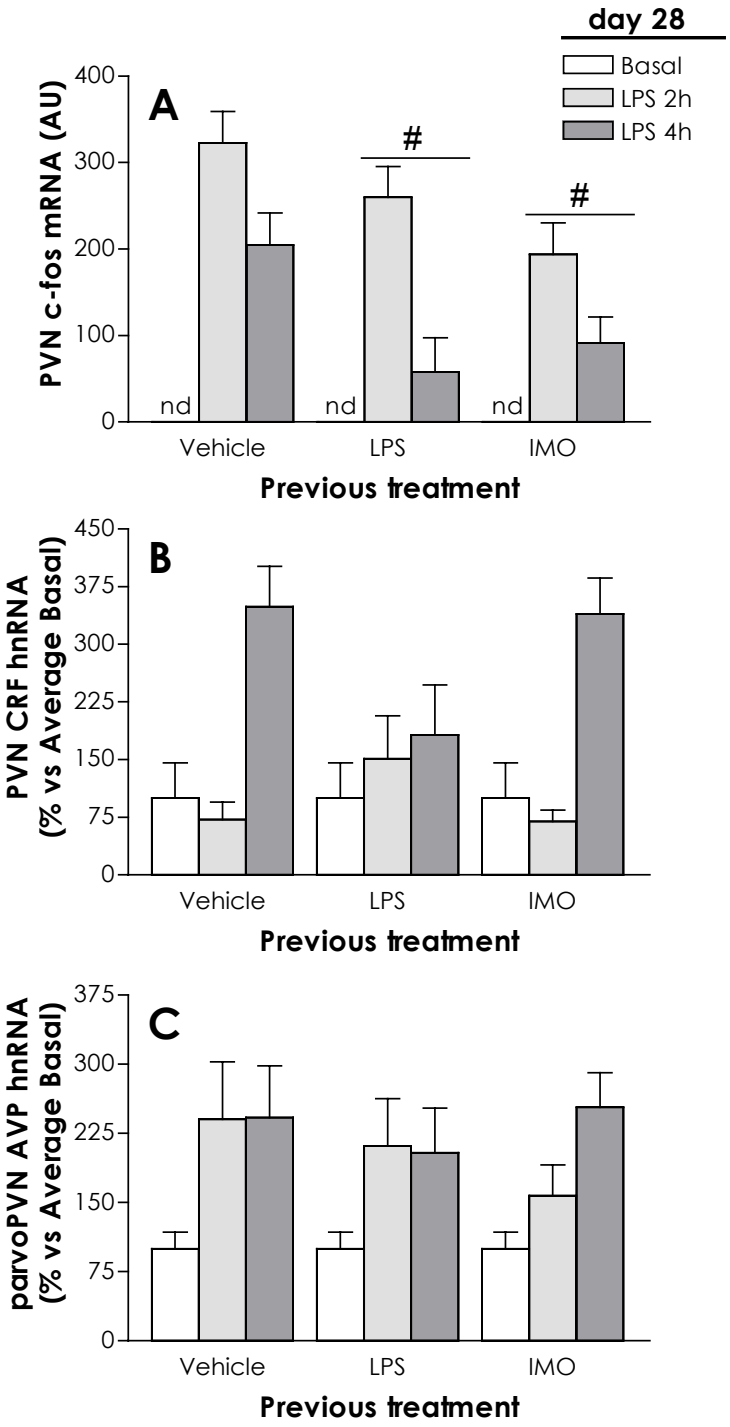

Figure 23. Effects of previous LPS or IMO experience on the PVN c-fos mRNA **(A)** PVN CRF hnRNA **(B)** and parvocellular PVN AVP hnRNA **(C)** responses to LPS 4 weeks later. Stress-naïve (control) animals received saline (Veh) on day 1. On day 28, animals were sacrificed under basal conditions and 2 or 4 hours after administration of 1 mg/kg of LPS (LPS 2h and LPS 4h, respectively). Basal c-fos mRNA levels were not determined due to the almost undetectable signal. Results are expressed as mean ± SE (n = 4-7 per group). AU: arbitrary units (pixel area x sum grey). # $p<0.05$ *vs* control group (two-way ANOVA and post-hoc analysis).

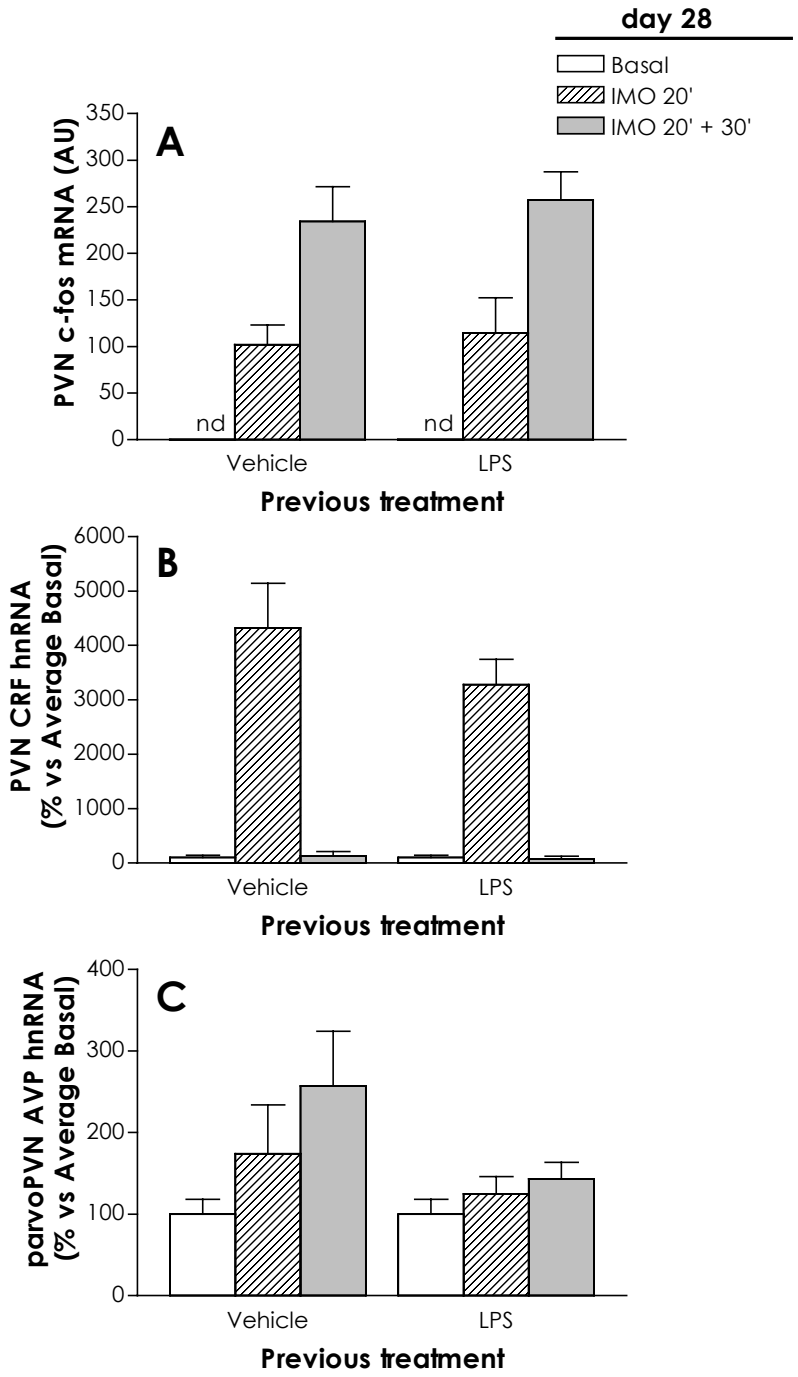

Figure 24. Effects of previous LPS experience on the PVN c-fos mRNA **(A)** PVN CRF hnRNA **(B)** and parvocellular PVN AVP hnRNA **(C)** responses to IMO 4 weeks later. Stress-naïve (control) animals received saline (Veh) on day 1. On day 28, animals were sacrificed under basal conditions, just after 20 mins of IMO (IMO 20') or 30 mins after finished 20 mins of IMO (IMO 20' + 30'). Basal c-fos mRNA levels were not determined due to the almost undetectable signal. Results are expressed as mean ± SE (n = 4-7 per group). AU: arbitrary units (pixel area x sum grey). No differences in function of the previous LPS treatment were observed.

DISCUSSION

The effects of a single stressful experience of both emotional and immunological nature have been shown to develop with time, triggering a faster termination of the peripheral HPA response and an abolition of the central HPA response to the same stressful stimulus. Nevertheless, even if the peripheral response is already modified at least 1 week after the first stressful experience, the central parameter we measured in the previous chapters (CRF mRNA) was not found to be altered until an inter-stress interval of at least 4 weeks. In order to further study this apparent discrepancy, in this chapter we wanted to study a closer approach to the transcriptional activity in the PVN by measuring only the newly synthesised transcript. We used in situ hybridisation with intron-directed probes, a new methodology that has been proved to be a reliable tool for this purpose [68, 99]. To complete this early activation study, we also measured the AVP hnRNA response in the pPVN as the second main secretagogue of ACTH [12, 119] and the c-fos mRNA response, a well-accepted marker of neuronal activation [102, 128, 185].

In the first experiment we used IMO as a stressor of emotional characteristics. Measurement of CRF hnRNA levels in the PVN showed an increased transcriptional activity of this gene in the control group just after 1h of IMO, with lower, but still measurable, CRF hnRNA levels at 1h post-IMO. A similar pattern was observed in the group of animals receiving the same stressful stimulus 1 week before. In contrast, the group previously exposed to IMO 4 weeks before displayed significantly lower levels of this primary transcript both immediately after 1h of IMO and at 1h post-IMO. Those findings are in accordance with the previous studies measuring CRF mRNA [175]. When measuring AVP hnRNA levels in the pPVN after stress, we were not able to detect significant differences depending on the previous stress experience of the animals. This is in accordance with preliminary data indicating that a previous LPS injection does not compromise the AVP mRNA response to another LPS injection 4 weeks later (data not shown). Therefore, transcriptional activity of the AVP gene does not appear to be sensitive to a previous experience with a stressor, similarly to what has been found with chronic stress. In this regard, chronic (daily repeated) exposure to another emotional stimulus such as restraint has been shown to abolish the response of CRF mRNA and hnRNA to acute restraint, whereas the response of AVP mRNA and hnRNA was maintained intact [160].

The study of neuronal activation in this hypothalamic nucleus by measuring mRNA levels of the IEG c-fos brought rather surprising results. Whereas in control animals IMO

provoked a powerful and sustained activation of this transcript, with c-fos mRNA levels 1h post-stress being as high as just after 1h of IMO, a different pattern was observed in the two previously-stressed groups: c-fos mRNA levels were comparable to the control group just after stress, but significantly lower levels of this transcript were detected 1h post-IMO in the two groups with previous experience with IMO, those levels being even lower in IMO 1wk group than in IMO 4wk animals. This finding is of interest since it shows a pattern of c-fos mRNA response similar to the peripheral HPA data previously described [175] and therefore adds more information about the process underlying the consequences of a previous experience with the stressor.

The fact that c-fos mRNA showed a pattern close to the peripheral data but clearly distinct from CRF mRNA and hnRNA suggests a dissociation between neuronal activation as evaluated by c-fos mRNA and transcriptional activity of the CRF gene. This dissociation has been nicely shown before. Briefly, acute ether stress provoked a rapid activation of CRF hnRNA in the PVN, which overlapped with the active (phosphorilated) form of CREB (pCREB), whereas the response of c-fos mRNA was delayed, with an increased FLI detected even later in time, in parallel with an activation of the AVP transcriptional activity (AVP hnRNA) [132]. In addition, using cycloheximide to block protein synthesis, it has been reported that the drug did not modify stress-induced CRF hnRNA but, in contrast, abolished and reduced, respectively, Fos and AVP hnRNA responses after stress [129]. The fact that the AVP hnRNA response was not affected by a previous stress experience even if the c-fos mRNA response was reduced in previously stressed rats is not surprising taking into account that complete blockade of Fos activation only reduces, but not abolishes, the AVP hnRNA response to stress [129]. In fact, the AVP gene is thought to be regulated by transcriptional factors other than Fos [44, 184, 199, 280].

In the same experiment we included an extra group of animals to study the peripheral HPA axis response to IMO both 1 and 4 weeks after a previous exposure to the same stressor. This was done to further confirm that previous IMO exposure was able to affect the peripheral HPA axis response to a new IMO session, as previously described [175]. The present results partly confirmed these previous findings, showing an accelerated rate of recovery of corticosterone levels after stress to the same extent in the two previously stressed groups. This was not exactly the case of ACTH, since the increased speed of recovery was only significant in animals receiving a previous IMO session 1, but not 4, weeks before. In fact, corticosterone response has been shown to be more sensitive than the ACTH response to a previous single stressful experience when using a less intense stressor such as restraint [175]. In addition, a

partial dissociation between ACTH and corticosterone cannot be ruled out. This phenomenon has been observed after prolonged acute stressors [64, 90, 111, 220], after chronic stress [15, 177, 191] and during the recovery period once finished the stress exposure [53, 54]. This dissociation might be explained by the existence of humoral or neural factors that regulate the sensitivity of the adrenal cortex to circulating ACTH [113, 194, 290], and the degree of sensitivity could have been modified by previous stress experience, thus explaining this dissociation between ACTH and corticosterone. It also appears that the long-term effects of a single exposure to IMO on ACTH may evanish before than those on corticosterone.

Previous results (Chapter 2) did not show any cross-desensitisation of the HPA axis response to stress, neither at the central (CRF mRNA) nor at the peripheral (ACTH, corticosterone) levels. However, in a second experiment we wanted to extend our studies on the early PVN transcriptional activation using LPS and also confirm whether or not there was any cross-desensitisation between IMO and LPS with the parameters measured in this chapter (hnRNA, c-fos mRNA). Since we already observed that the minimum time lag required for detecting any effects on the CRF transcriptional activity in the PVN was of 4 weeks (experiment 3-1), this was the inter-stress period chosen for the present experiment.

Similarly to what has been previously described [214], CRF hnRNA expression was triggered in the PVN of control (stress-naïve) rats 4h, but not 2h, after an acute endotoxin challenge. In contrast with the results obtained with CRF mRNA (Chapter 1), the group with previous LPS experience did not show a significant desensitisation of the response to a new LPS injection 4 weeks later. In this case, LPS was not able to modify the primary transcription of the CRF gene in response to a further LPS injection. These results suggest that the reduction of LPS-induced CRF mRNA response in previously LPS-injected animals might be dissociated from the primary transcriptional activity of the CRF gene. This dissociation could be explained by additional regulations at the post-transcriptional level, and further studies should be performed in this regard. Similarly to the results obtained with IMO in experiment 3-1, AVP hnRNA levels after an acute LPS injection were not modified by a previous LPS injection. In fact, acute LPS injection did not cause a very marked AVP hnRNA response, although a general analysis indicated a significant increase of this transcript after stress independently of the previous treatment. We also determined c-fos mRNA levels, observing a very strong response of this IEG after LPS injection in control (stress-naïve) animals, with a significant reduction of this response in previously LPS-injected animals.

In this case, previous LPS experience affected the neuronal activation of the PVN, similarly to our previous results with CRF mRNA (Chapter 1).

When studying the specificity of the phenomenon, we found that previous IMO exposure did not modify LPS-induced response of CRF and AVP primary transcripts, similarly to the previous results with CRF mRNA (Chapter 2). However, animals previously immobilised 4 weeks before showed a reduced c-fos mRNA response to LPS when compared to control (stress-naïve) animals. These results with c-fos mRNA were at first quite surprising, since we found no cross-desensitisation between IMO and LPS when measuring CRF mRNA and HPA hormones (Chapter 2). These non-specific effects of IMO could be explained in several ways. First, although IMO is mainly an emotional stressor, it provokes a weak immune/inflammatory reaction, since the limbs of the animals display some inflammation after exposure to this stressor, and this could account for the observed cross-desensitisation with an immune stimulus such as LPS. Second, in a pilot study in mouse using c-fos mRNA mapping to compare the areas activated after an LPS challenge or an exposure to restraint we observed a very different profile of expression of this IEG after these very different stress paradigms (data not shown), but there are some common areas activated after both stimuli such as LS, BST, PVN, CG and LC, and activation of these areas could play a role in the non-specific effects previously described, if some of these common areas would be the place of synaptic plasticity involved in the long-term effects of stress. Nonetheless, these non-specific effects of IMO on the LPS-induced c-fos mRNA response do not seem to affect the HPA responsiveness since no effects have been found in the other parameters studied.

The effect of previous LPS challenge on the HPA response to IMO at this level was also assessed in this experiment. Both control (stress-naïve) and previously LPS-injected animals displayed a rapid but transient CRF hnRNA activation just after 20 min of IMO, since very low levels of this transcript were detected at 30 min post-IMO. Giving further support to our previous results (Chapter 2), no cross-desensitisation was detected between IMO and LPS when measuring, in this case, CRF primary transcript. Similarly, AVP hnRNA and c-fos mRNA levels after an acute LPS injection were not modified by previous IMO exposure.

When comparing the response of CRF hnRNA to acute IMO in stress-naïve groups of experiments 3-2 and 3-1, a substantially different dynamics can be observed. Whereas in experiment 3-1 CRF hnRNA levels were increased immediately after 1h of IMO and there were still measurable levels of this transcript at 1h post-IMO, in

experiment 3-2 20 min of IMO triggered a very powerful CRF hnRNA response but 30 min after this shorter stress exposure no levels of this transcript were detected. This might be at least partially explained by technical reasons, since the threshold we used for determining optical density needed to be different for each experiment. In fact, in experiment 3-2 the signal for CRF hnRNA 20 min after the onset of IMO was very strong when compared to the signal observed at 30 min post-IMO. It might be that the optimal threshold for measuring grey levels just after IMO was not so good for the other time point, and therefore we were not able to perform accurate enough measurements at the post-stress time point. Ideally, a different threshold should have been set up for each intensity of signal, but this would not let us compare different groups. In experiment 3-1 the signal obtained was lower both 1 and 2h after the onset of 1h IMO, probably because there was enough time for the CRF hnRNA to start being processed into mRNA, and the threshold chosen in this experiment was ideal for both sampling time points.

Overall, these early activational studies indicate that previous IMO or LPS exposure is able to compromise the future PVN response (CRF hnRNA and c-fos mRNA for IMO and c-fos mRNA for LPS) to the same stressful stimulus, although this is not the case of AVP hnRNA response. When an effect on CRF hnRNA was observed, an inter-stress interval of at least 4 weeks was needed, similarly to CRF mRNA. Nonetheless, c-fos mRNA response in the PVN was already affected 1 week after the first stressful experience, similarly to that observed with ACTH and corticosterone. Therefore, it appears that c-fos mRNA induction roughly reflects the changes in firing activity (electrophysiological stimulation) of PVN neurons, but not the intracellular changes controlling CRF gene expression. These differences may result from subtle changes in the type of stimulatory inputs reaching the PVN as a consequence of the previous experience with the stressor. The knowledge of these kind of signals may be of great importance, not only to explain the long-term effects of stress, but also to a better characterisation of the relationship between the electrophysiological activation of PVN neurons and the biochemical processes leading to an altered transcriptional activity.

CHAPTER 4

Effects of a previous stressful experience on the future stress-induced c-fos mRNA activation across the brain

INTRODUCTION

We have previously described the long-term effects of a single stress exposure on the future HPA response to stress ([175], previous chapters). These effects are characterised by a reduced or even blunted central HPA axis response and an increased rate of return to basal levels of the peripheral HPA hormones. The effect develops at least with two stressful stimuli (IMO, LPS administration), and appears to be stressor-specific since, in general, no cross-desensitisation was found between IMO and LPS. This is not so surprising taking into account the differential mechanisms of HPA axis activation by emotional stressors such as IMO and immunological stressors such as LPS [96, 231, 246].

There is an important common feature of these long-term effects of stress: the peripheral HPA response (ACTH, corticosterone) is already affected one week after the initial exposure to stress, whereas the central HPA axis response is not modified at least until 4 weeks after the first experience with the stressor. We have previously suggested that these effects might represent a new form of learning linked to aversive stimuli that progressively consolidate with time [175], in accordance with the progressive process of memory consolidation [178]. The fact that both IMO and LPS are able to induce long-term effects on the future HPA activation induced by the same stimulus suggests that this phenomenon is not a simple biochemical adaptation, but that some kind of memory-like synaptic plasticity is involved.

C-fos mRNA activation throughout the whole brain has been used to characterise the areas involved in the HPA axis response to stress [175]. Although some controversies have emerged regarding the exact functional meaning of the activation of this IEG, it is still a powerful tool to describe the overall activation of the brain after stress [128]. As a first approach to elucidate some of the possible brain areas involved in the long-term effects of stress on the HPA axis, we mapped the c-fos mRNA response to acute stress in previously-stressed rats, using the two previously characterised stressors (IMO and LPS).

EXPERIMENTAL DESIGNS AND RESULTS

Experiment 4-1

The animals used correspond to Experiment 3-1. Briefly, the animals were assigned to the following groups: 1/ no stress (stress-naïve), 2/ 2h of IMO on day 1 (IMO 4wk) and 3/ 2h of IMO on day 21 (IMO 1wk). On day 28, all the animals were anaesthetised and

perfused under basal conditions (Basal), just after 1h of IMO (IMO 1h) or 1h after the end of IMO for 1h (IMO 1h+1h).

Description of c-fos mRNA activation in the brain after an acute IMO exposure

Figure 25 and Table 1 summarise the levels of c-fos mRNA across the brain under basal conditions, just after 1h of IMO and at 1h post-IMO. In line with previous studies, c-fos mRNA was not detectable under basal conditions except for a moderate signal in the piriform cortex (Pir) and pontine nuclei (Pn) and a low to very low signal in the dorsal endopiriform nucleus (DEn), cerebral cortex (Ctx) and ventral and dorsal cochlear nucleus (VC, DC).

Telencephalon – IMO caused a wide and robust activation of c-fos mRNA in different telecephalic nuclei. Just after 1h of IMO, there was a strong to very strong c-fos mRNA signal in the DEn, Ctx and Pir. A moderate to strong signal was detected in the intermediate, ventral and dorsal divisions of the LS (LSi, LSv, LSd), ventral division of the medial BST (BSTMv), SFO and MeA. A low to moderate signal was displayed in the tenia tecta (TT) and septohypothalamic nucleus (SHy). There was a very low to undetectable signal in the acumbens nucleus (Acb), medial septal nucleus (MS), striatum (CPu), globus pallidus (GP), ventral pallidum (VP), anterior division of the medial BST (BSTMa), parastrial nucleus (PS), anterior amygdaloid area (AAm), anterior cortical amygdaloid nucleus (ACo) and the CA1, CA2 and CA3 hippocampal divisions.

One hour after the end of IMO, c-fos mRNA remained elevated in the DEn, Ctx and Pir. The signal in LSi, LSv, LSd, BSTMv and MeA was diminished but still within low to moderate levels. Interestingly, there was a moderate increase of c-fos mRNA levels in the CA1, CA2 and CA3 hippocampal divisions. Finally, the rest of the areas described before showed very low to undetectable c-fos mRNA levels at that time point.

Diencephalon –Different thalamic nuclei displayed a positive c-fos mRNA signal just after 1h of IMO. Nonetheless, a moderate to strong signal was only detected in the posterior division of the paraventricular thalamic nucleus (PVp) and medial division of the lateral habenular nucleus (LHbM). Many more areas displayed a moderate c-fos mRNA signal at that time point, such as the anterodorsal thalamic nucleus (AD), anterior division of the paraventricular thalamic nucleus (PVa), central medial thalamic nucleus (CM), paracentral thalamic nucleus (PC), rhomboid thalamic nucleus (Rh), ventrolateral thalamic nucleus (VL) and medial geniculate nucleus

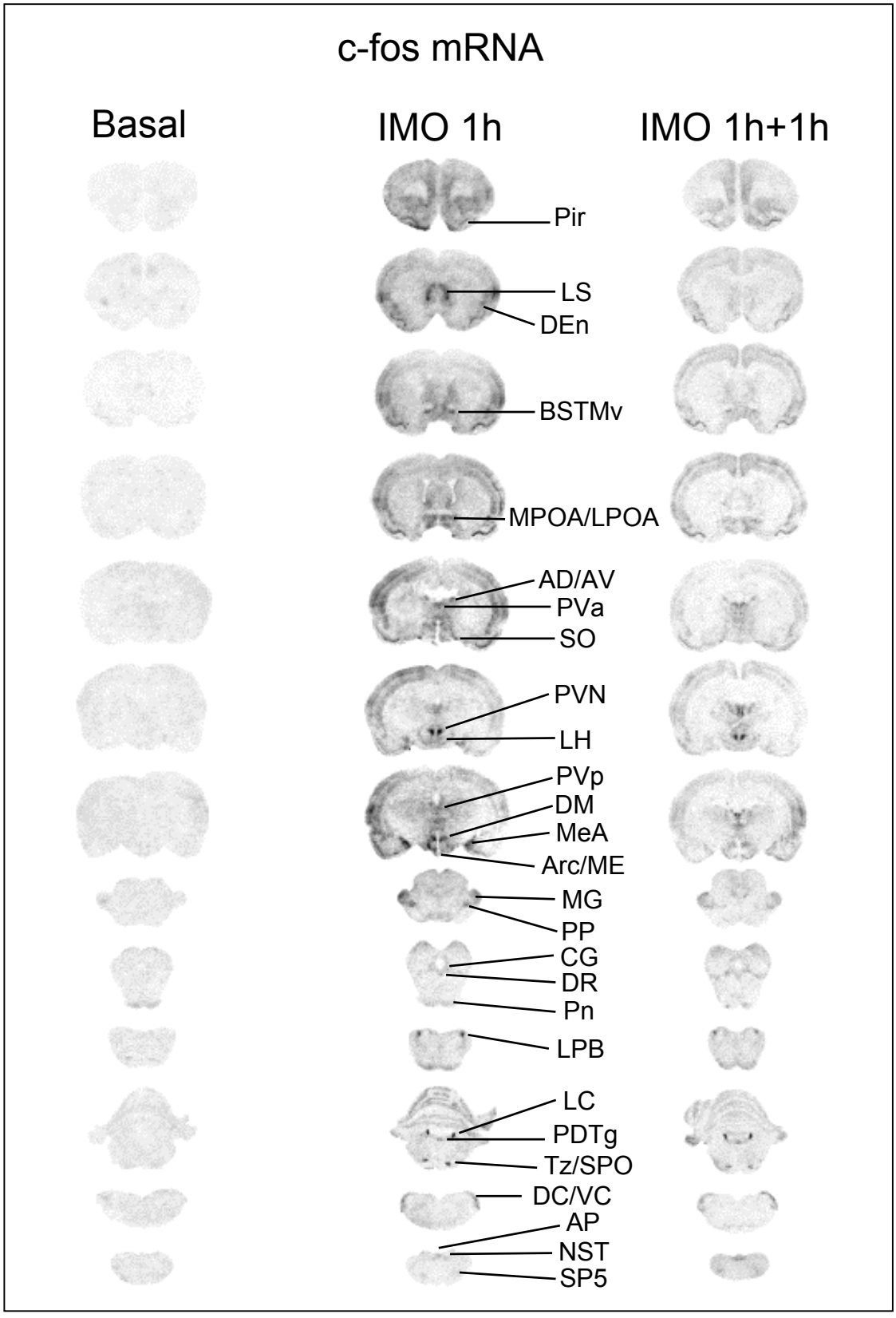

Figure 25. Representative images of the distribution of c-fos mRNA throughout the brain of rats immediately after 1h of IMO and 1h after the termination of IMO (post-IMO). Abbreviations: AD, anterodorsal thalamic nucleus; AP, area postrema; Arc, arcuate nucleus; AV, anteroventral thalamic nucleus; BSTMv, medial bed nucleus of the stria terminals, ventral subdivision; CG, central gray; DC, dorsal cochlear nucleus; DEn, dorsal endopirifom nucleus; DM, dorsomedial (continues in next page)

(MG). A low to moderate signal was displayed in the intermediodorsal thalamic nucleus (IMD), reuniens thalamic nucleus (Re), reticular thalamic nucleus (Rt), mediodorsal thalamic nucleus (MD), laterodorsal thalamic nucleus (LD), ventral posteromedial thalamic nucleus (VPM), lateral posterior thalamic nucleus (LP), dorsal and ventral parts of the lateral geniculate nucleus (DLG/VLG) and subparafascicular thalamic nucleus (SPF). There was a very low to undetectable signal in the anteroventral thalamic nucleus (AV), paratenial thalamic nucleus (PT), posterior thalamic nuclear group (Po), lateral division of the lateral habenular nucleus (LHbL) and subthalamic nucleus (STh).

One hour after finishing IMO, there was still a moderate to strong c-fos mRNA signal in the PVa and PVp. A moderate to low signal was detected in the AD, CM, PC, LHbM, DLG and MG. The signal was low to undetectable in the AV, IMD, PT, Rh, Re, Rt, MD, VL, LD, VPM, Po, LP, LHbL, SPF and STh.

Just after 1h of IMO, there was a strong to very strong c-fos mRNA activation in the PVN. At the same time point, the SO displayed moderate to strong c-fos mRNA levels. A moderate c-fos mRNA activation was observed in other hypothalamic areas, such as the anteroventral preoptic nucleus (AVPO) and supramammillary nucleus (SuM). A low to moderate signal was detected in the MPOA and lateral preoptic area (LPOA), anterior, lateral and posterior hypothalamic areas (AH, LH, PH), DM, dorsal and ventral parts of the premammillary nucleus (PMD/PMV) and the lateral mammillary nucleus (LM). There was a very low to undetectable signal in the retrochiasmatic area (RCh) and Arc.

At 1h post-IMO, the signal in the PVN remained strong. The PMV and PMD displayed moderate to strong c-fos mRNA levels. Low to moderate levels were detected in the AVPO, MPOA, LPOA, SO, DM, SuM and LM. The signal in the AH, LH, RCh, Arc and PH was low to undetectable.

Figure 25 (continues from previous page). hypothalamic nucleus; DR, dorsal raphe nucleus; LC, locus coeruleus; LH, lateral hypothalamic area; LPB, lateral parabrachial nucleus; LPO, lateral preoptic area; LS, lateral semptum; ME, median eminence; MeA, medial amygdaloid nucleus; MG, medial geniculate nucleus; MPOA, medial preoptic area; NST, nucleus of the solitary tract; PDTg, posterodorsal tegmental nucleus; Pir, piriform cortex; Pn, pontine nuclei; PP, peripeduncular nucleus; PVa, paraventricular thalamic nucleus, anterior division; PVN, paraventricular hypothalamic nucleus; PVp, paraventricular thalamic nucleus, posterior division; SO, supraoptic nucleus; SP5, spinal trigeminal nucleus; SPO, superior paraolivary nucleus; Tz, nucleus of the trapezoid body; VC, ventral cochlear nucleus.

Brain Stem – There were some mesencephalic nuclei activated after 1h of IMO. The intensity of the signal was low to moderate in the CG and external cortex of the inferior colliculus (ECIC) and very low to undetectable in the ventral tegmental area (VTA), peripeduncular nucleus (PP), deep mesencephalic nucleus (DpMe) and superior colliculus (SC). One hour after IMO, c-fos mRNA levels remained low to moderate in the CG and diminished to low in the ECIC. They stayed very low in the rest of the areas, except for the SC where c-fos mRNA was undetectable.

In the pons, there were several areas displaying a positive c-fos mRNA signal just after 1h of IMO. A moderate to strong level was found in the nucleus of the trapezoid body (Tz), lateral and medial PB (LPB, MPB) and LC. Low to moderate levels were detected in the Pn, DR and posterodorsal tegmental nucleus (PDTg). Very low c-fos mRNA levels were found in the median raphe nucleus (MnR), oral part of the pontine reticular nucleus (PnO), A5, laterodorsal tegmental nucleus (LDTg), superior paraolivary nucleus (SPO), ventral and caudal parts of the pontine reticular nucleus (PnV, PnC), abducens nucleus (6), facial nucleus (7) and area postrema (AP). At 1h post-IMO, there was a strong to very strong signal in the LC, and the DR and Tz displayed a moderate to strong signal. A low to moderate signal was found in the Pn, LPB, MPB and PDTg. All the other areas displayed very low to undetectable c-fos mRNA levels.

Some medullary areas were also activated just after 1h of IMO. Moderate to strong levels were detected in the VC and DC. Low to moderate levels were found in the NST, external cuneate and cuneate nucleus (Ecu, Cu) and lateral reticular nucleus (LRt). Only very low levels were detected in the raphe magnus and raphe pallidus nuclei (RMg, RPa), medial vestibular nucleus (MVe), lateral paragigantocellular nucleus (LPGi), spinal trigeminal nucleus (Sp5), prepositus hypoglossal nucleus (PrH), VLM, parvocellular reticular nucleus (PCRt), inferior olive (IO) and hypoglossal nucleus (12). At 1h post-IMO, moderate to strong levels were still found in VC and DC, whereas moderate levels were seen in the NST. Ecu, Cu and LRt displayed low to moderate c-fos mRNA levels, and in the PrH and VLM the levels were low. In the rest of the areas, c-fos mRNA levels were almost undetectable.

TABLE 1. c-fos mRNA activation in the brain after IMO exposure: effects of previous IMO experience

AREA	CONTROL			IMO 1wk			IMO 4wk		
	Basal	IMO 1h	REC 1h	Basal	IMO 1h	REC 1h	Basal	IMO 1h	REC 1h
TELENCEPHALON									
Tenia tecta (TT)	-	+/++	+	-	+/++	+/-	-	+/++	+
Dorsal endopiriform nucleus (DEn)	+	+++	++/+++	+	++/+++	++/+++	+	++/+++	++/+++
Neocortex (Ctx)	+/-	+++	++/+++	+/-	++/+++	++/+++	+/-	++/+++	++/+++
Piriform cortex (Pir)	+/++	++++	++++	+/++	++++	++++	+/++	+++/++++	++++
Accumbens nucleus (Acb)	-	+/-	+/-	-	+/-	+/-	-	+/-	+/-
Lateral septal nucleus, intermediate part (LSi)	-	++	+	-	++	+	-	++	+
Lateral septal nucleus, ventral part (LSv)	-	++/+++	+/++	-	++	+	-	++/+++	+
Lateral septal nucleus, dorsal part (LSd)	-	++/+++	+/++	-	++	+	-	++/+++	+
Medial septal nucleus (MS)	-	+/-	-	-	+/-	-	-	+/-	-
Striatum (CPu)	-	+/-	+/-	-	+/-	+/-	-	+/-	+/-
Globus pallidus (GP)	-	+/-	+/-	-	+/-	+/-	-	+/-	+/-
Ventral pallidum (VP)	-	+/-	+/-	-	+/-	+/-	-	+/-	+/-
Bed nucleus stria terminalis, med div, ant (BSTMa)	-	+	+	-	+/++	+	-	+	+/-
Bed nucleus stria terminalis, med div, ventr (BSTMv)	-	++/+++	++	-	++/+++	++/+++	-	++	++
Parastrial nucleus (PS)	-	+/-	+/-	-	+/-	+/-	-	+/-	+/-
Septohypothalamic nucleus (SHy)	-	+/++	+	-	+/++	+	-	+/++	+
Subfornical organ (SFO)	-	++/+++	+	-	++/+++	+/-	-	++	+/-
Anterior amygdaloid area (AA)	-	+/-	+/-	-	+/-	+/-	-	+/-	+/-
Medial amygdala (MeA)	-	++/+++	++	-	++/+++	+/++	-	++/+++	+/++
Anterior cortical amygdaloid nucleus (ACo)	-	+/-	+/-	-	+/-	+/-	-	+/-	+/-
Hippocampal formation									
CA1	-	+/-	+/++	-	+/-	+/++	-	+/-	+/++
CA2	-	+/-	+/++	-	+/-	+/++	-	+/-	+/++
CA3	-	+/-	+/++	-	+/-	+/++	-	+/-	+/++
Dentate gyrus (DG)	-	-	-	-	-	-	-	-	-
DIENCEPHALON									
Thalamus									
Anterodorsal thalamic nucleus (AD)	-	++	++	-	++	++/+++	-	++	++/+++

(-) undetectable; (+) low; (++) moderate; (+++) strong; (++++) very strong

TABLE 1 (continued). c-fos mRNA activation in the brain after IMO exposure: effects of previous IMO experience

AREA	CONTROL			IMO 1wk			IMO 4wk		
	Basal	IMO 1h	REC 1h	Basal	IMO 1h	REC 1h	Basal	IMO 1h	REC 1h
Anteroventral thalamic nucleus (AV)	-	+/-	-	-	+/-	-	-	+/-	-
Central medial thalamic nucleus (CM)	-	++	++	-	++	++	-	+/++	++
Paraventricular thalamic nucleus, anterior (PVa)	-	++	++/+++	-	++	++	-	++	++
Paraventricular thalamic nucleus, posterior (PVp)	-	++/+++	++/+++	-	++/+++	++/+++	-	++	++/+++
Paracentral thalamic nucleus (PC)	-	++	++	-	++	++	-	+/++	++
Intermediodorsal thalamic nucleus (IMD)	-	+	+/-	-	+	+/-	-	+	+/-
Paratenial thalamic nucleus (PT)	-	+/-	+/-	-	+/-	+/-	-	+/-	+/-
Rhomboid thalamic nucleus (Rh)	-	++	+	-	++	+	-	++	+
Reuniens thalamic nucleus (Re)	-	+	+	-	+	+	-	+	+
Reticular thalamic nucleus (Rt)	-	+	+/-	-	+	+/-	-	+	+/-
Mediodorsal thalamic nucleus (MD)	-	+	+	-	+	+	-	+	+
Ventrolateral thalamic nucleus (VL)	-	++	+	-	++	+	-	++	+
Laterodorsal thalamic nucleus (LD)	-	+	+	-	+	+	-	+	+
Ventral posteromedial thalamic nucleus (VPM)	-	+/++	+	-	+/++	+	-	+/++	+
Posterior thalamic nuclear grup (Po)	-	+/-	+/-	-	+/-	+/-	-	+/-	+/-
Lateral posterior thalamic nucleus (LP)	-	+	+	-	+	+	-	+	+
Lateral habenular nucleus, medial (LHbM)	-	++/+++	++	-	++/+++	++	-	++/+++	++
Lateral habenular nucleus, lateral (LHbL)	-	+/-	+/-	-	+/-	+/-	-	+/-	+/-
Lateral geniculate nucleus, dorsal and ventral (DLG, VLG)	-	+/++	+/++	-	++/+++	++	-	++	++
Subparafascicular thalamic nucleus (SPF)	-	+	+	-	+	+	-	+	+
Medial geniculate nucleus (MG)	-	++	+/++	-	++/+++	++	-	++	++
Hypothalamus									
Anteroventral preoptic nucleus (AVPO)	-	++	+/++	-	++	+/++	-	++	+/++
Medial preoptic area (MPOA)	-	+/++	+/++	-	++/+++	++	-	++	+/++
Lateral preoptic area (LPOA)	-	+/++	++	-	++/+++	++	-	++	+/++
Supraoptic nucleus (SO)	-	++/+++	++	-	++	+/++	-	++	+/++
Anterior hypothalamic area (AH)	-	+/++	+	-	+/++	+	-	+/++	+
Lateral hypothalamic area (LH)	-	+/++	+	-	+/++	+	-	+/++	+
Retrochiasmatic area (RCh)	-	+/-	+/-	-	+/-	+/-	-	+/-	+/-

(-) undetectable; (+) low; (++) moderate; (+++) strong; (++++) very strong

TABLE 1 (continued). c-fos mRNA activation in the brain after IMO exposure: effects of previous IMO experience

AREA	CONTROL			IMO 1wk			IMO 4wk		
	Basal	**IMO 1h**	**REC 1h**	**Basal**	**IMO 1h**	**REC 1h**	**Basal**	**IMO 1h**	**REC 1h**
Paraventricular hypothalamic nucleus (PVN)	-	+++/++++	+++/++++	-	+++/++++	+/++	-	+++/++++	++/+++
Arcuate hypothalamic nucleus (Arc)	-	+	+	-	+	+/-	-	+	+/-
Dorsomedial hypothalamic nucleus (DM)	-	+/++	+/++	-	++	+	-	++	+/++
Posterior hypothalamic area (PH)	-	+/++	+	-	+/++	+	-	+/++	+
Premammillary nucleus, ventral part (PMV)	-	+/++	++/+++	-	++/+++	++/+++	-	++/+++	+++
Premammillary nucleus, dorsal part (PMD)	-	+/++	++/+++	-	++/+++	++/+++	-	++/+++	++/+++
Suparamammillary nucleus (SuM)	-	++	++	-	++/+++	+/++	-	++/+++	++
Lateral mammillary nucleus (LM)	-	+/++	+/++	-	++	++	-	++	+/++
Subthalamic nucleus (STh)	-	+	+	-	+	+	-	+	+
MESENCEPHALON									
Ventral tegmental area (VTA)	-	+/-	+/-	-	+/-	+/-	-	+/-	+/-
Peripeduncular nucleus (PP)	-	+/-	+/-	-	+/-	+/-	-	+/-	+/-
Deep mesencephalic nucleus (DpMe)	-	+/-	+/-	-	+/-	+/-	-	+/-	+/-
Superior colliculus (SC)	-	+/-	-	-	+/-	-	-	+/-	-
Central (periaqueductal) grey (CG)	-	+/++	+/++	-	++	+/++	-	+/++	+/++
External cortex of the inferior colliculus (ECIC)	-	+/++	+	-	+/++	+	-	+/++	+/++
PONS									
Pontine nuclei (Pn)	+/++	++	+/++	+/++	++	++	+/++	++	++/+++
Dorsal raphe nucleus (DR)	-	+/++	++/+++	-	++	++/+++	-	++	++/+++
Median raphe nucleus (MnR)	-	+/-	+/-	-	+/-	+/-	-	+/-	+/-
Pontine reticular nucleus, oral part (PnO)	-	+/-	+/-	-	+/-	+/-	-	+/-	+/-
A5	-	+	+	-	+	+	-	+	+
Nucleus of the trapezoid body (Tz)	-	++/+++	++/+++	-	++/+++	++	-	++	++/+++
Laterodorsal tegmental nucleus (LDTg)	-	+/-	+/-	-	+/-	+/-	-	+/-	+/-
Lateral parabrachial nucleus (LPB)	-	++/+++	+/++	-	++/+++	+/++	-	++/+++	+/++
Medial parabrachial nucleus (MPB)	-	++/+++	+/++	-	++/+++	+/++	-	++/+++	+/++
Superior paraolivary nucleus (SPO)	-	+/-	+/-	-	+/-	+/-	-	+/-	+/-

(-) undetectable; (+) low; (++) moderate; (+++) strong; (++++) very strong

TABLE 1 (continued). c-fos mRNA activation in the brain after IMO exposure: effects of previous IMO experience

AREA	CONTROL			IMO 1wk			IMO 4wk		
	Basal	IMO 1h	REC 1h	Basal	IMO 1h	REC 1h	Basal	IMO 1h	REC 1h
Pontine reticular nucleus, ventral part (PnV)	-	+/-	+/-	-	+/-	+/-	-	+/-	+/-
Pontine reticular nucleus, caudal part (PnC)	-	+/-	+/-	-	+/-	+/-	-	+/-	+/-
Locus coeruleus (LC)	-	+++	+++/++++	-	+++	+/++	-	+++	++/+++
Posterodorsal tegmental nucleus (PDTg)	-	++	++	-	++	++	-	+/++	++
Abducens nucleus (6)	-	+/-	+/-	-	+/-	+/-	-	+/-	+/-
Facial nucleus (7)	-	+/-	+/-	-	+/-	+/-	-	+/-	+/-
Area postrema (AP)	-	+/-	-	-	-	-	-	-	-
MEDULLA									
Ventral cochlear nucleus (VC)	+	++/+++	++/+++	+	+++	+++	+	+++	+++
Dorsal cochlear nucleus (DC)	+	++/+++	++/+++	+	+++	+++	+	+++	+++
Raphe magnus nucleus (RMg)	-	+	+/-	-	+	+/-	-	+	+/-
Raphe pallidus nucleus (RPa)	-	+	+/-	-	+	+/-	-	+	+/-
Spinal trigeminal nucleus (Sp5)	-	+/-	+/-	-	+/-	+/-	-	+/-	+/-
Medial vestibular nucleus (MVe)	-	+	+/-	-	+	+/-	-	+	+/-
Lateral paragigantocellular nucleus (LPGi)	-	+/-	+/-	-	+/-	+/-	-	+/-	+/-
Nucleus of the solitary tract (NST)	-	+/++	++	-	+/++	+/++	-	+	++
Prepositus hypoglossal nucleus (PrH)	-	+	+	-	+	+	-	+	+
Ventrolateral medulla (VLM)	-	+	+	-	+	+	-	+	+
Parvocellular reticular nucleus (PCRt)	-	+/-	+/-	-	+/-	+/-	-	+/-	+/-
Inferior olive (IO)	-	+/-	+/-	-	+/-	+/-	-	+/-	+/-
External cuneate nucleus (Ecu)	-	+/++	+/++	-	+/++	+	-	+/++	+/++
Cuneate nucleus (Cu)	-	+/++	+/++	-	+/++	+	-	+/++	+/++
Hypoglossal nucleus (12)	-	+/-	+/-	-	+/-	+/-	-	+/-	+/-
Lateral reticular nucleus (LRt)	-	+/++	+/++	-	+/++	+/++	-	+/++	+/++

(-) undetectable; (+) low; (++) moderate; (+++) strong; (++++) very strong

Semiquantitative analysis of c-fos mRNA levels in different brain areas after IMO exposure: effects of a previous IMO experience

Semiquantification of c-fos mRNA levels using dark field illumination was performed in some of the areas described above. The selection of the areas submitted to this analysis was done on the basis of a previous qualitative analysis of c-fos mRNA levels in control and previously-stressed animals. Those areas potentially interesting or found to display a differential c-fos mRNA response depending on the previous stress exposure were selected for quantification. Nonetheless, quantification was not performed in the areas were c-fos mRNA signal was difficult to measure either because it was very low or scattered.

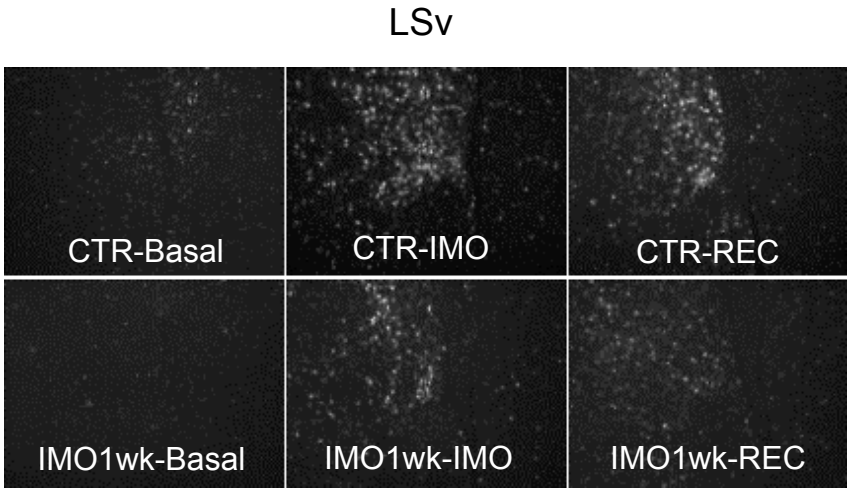

Figure 26. Representative dark-field photomicrographs displaying the effects of a single previous IMO session 1 week before on c-fos mRNA expression in the ventral division of the lateral septal nucleus (LSv) immediately after 1h of IMO (IMO) and 1h after the end of exposure to IMO (REC). Control (CTR, stress-naïve) animals did not receive any treatment on day 1. The legend should be read as "previous treatment-day 28 treatment", e.g. CTR-Basal are animals that did not receive any treatment on day 1 and were sacrificed under basal conditions on day 28. Magnification: 60X.

Figure 27 displays c-fos mRNA levels in the different subdivisions of the LS (LSi, LSv, LSd) in control and rats previously immobilised 1 or 4 weeks before. In the LSi, two-way ANOVA showed significant effects of time point ($p<0.001$) but not of previous treatment or their interaction. Two-way ANOVA of LSv c-fos mRNA levels (Figure 26) indicated significant effects of previous treatment and time point (day 28) ($p<0.001$ for both factors) and a marginally significant interaction ($p=0.051$). Post-hoc analysis revealed that just after 1h of IMO, c-fos mRNA levels were lower in IMO 1wk animals when compared to control and IMO 4wk groups. At 1h post-IMO, both previously-immobilised groups 1 or 4 weeks before showed lower c-fos mRNA levels when compared to stress-naïve animals. Two-way ANOVA of LSd c-fos mRNA levels only

showed significant effects of time point ($p<0.001$) but not of previous treatment or their interaction.

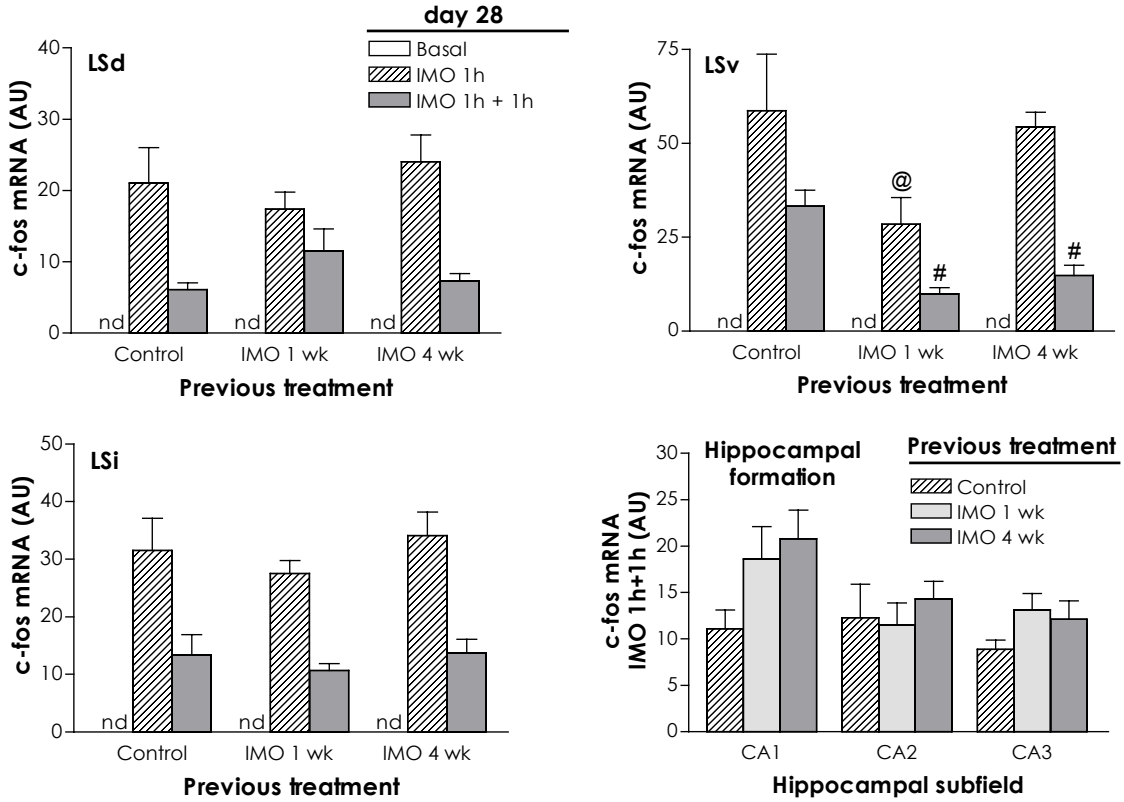

Figure 27. Effects of a single previous IMO experience on the c-fos mRNA responses to IMO, 1 or 4 weeks later, in the dorsal, intermedial and ventral divisions of the lateral septal nucleus (LSd, Lsi, LSv). C-fos mRNA levels at 1h post-IMO in the different divisions of the hippocampal formation are also displayed (bottom, right); note that, in this case, the legend depicted is different from the other three graphs. Basal c-fos mRNA levels were not determined due to the almost undetectable signal. Results are expressed as mean ± SE (n = 4-7 per group). AU: arbitrary units (pixel area x sum grey). @ p at least <0.03 *vs* the same time point in stress-naïve (control) and IMO 4wk groups; p at least <0.004 *vs* the same time point in the control group (two-way ANOVA, post-hoc analysis).

C-fos mRNA levels were analysed in the CA1, CA2 and CA3 divisions of the hippocampal formation at 1h post-IMO (Figure 27), since no detectable levels of the transcript were found just after 1h of IMO. The DG division did not display a positive c-fos mRNA signal in any of the time points studied. One-way ANOVA of c-fos mRNA levels showed marginally significant differences in the CA1 division ($p=0.056$), but not in the CA2 or CA3 divisions.

C-fos mRNA levels were also quantified in both the LPOA and MPOA (Figure 28). Two-way ANOVA did not display any significant effects of previous treatment, time point or their interaction in any of the two areas. C-fos mRNA levels in the SFO after IMO are

displayed in Figure 28. Two-way ANOVA only showed significant effects of time point ($p<0.01$) but not of previous treatment or their interaction.

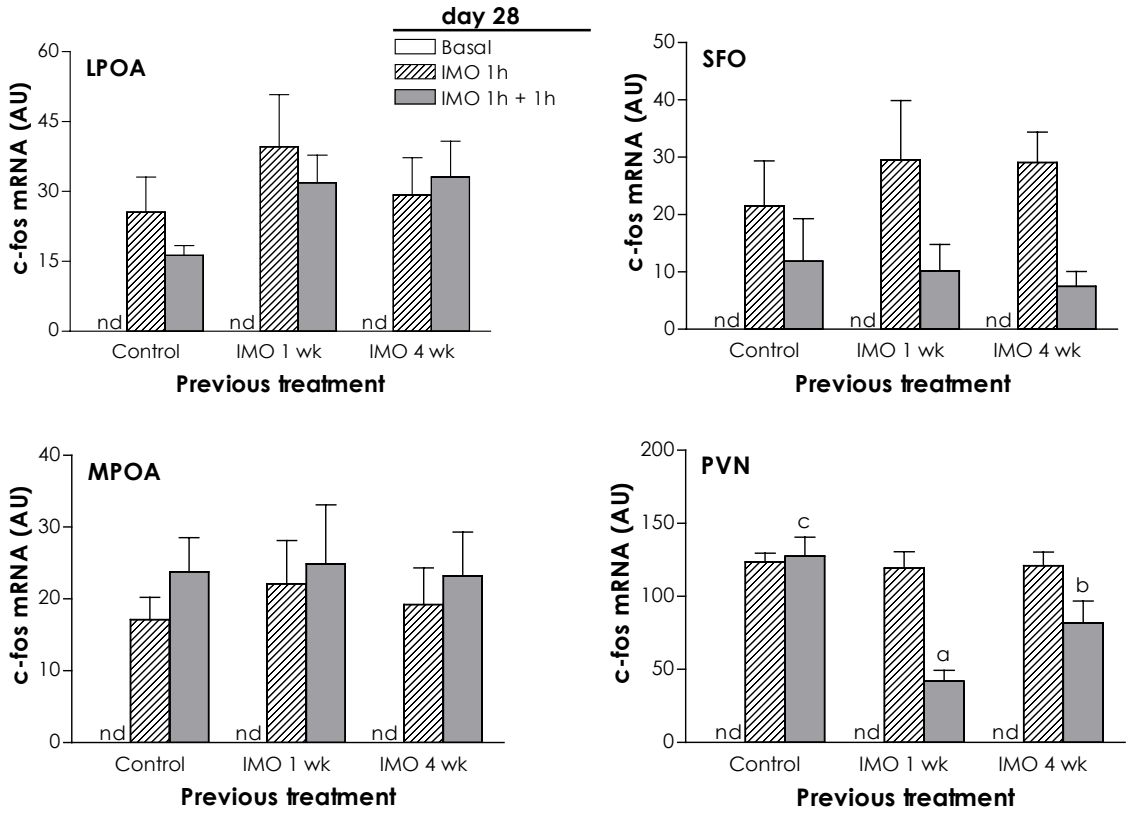

Figure 28. Effects of a single previous IMO experience on the c-fos mRNA responses to IMO, 1 or 4 weeks later, in the lateral preoptic area (LPOA), medial preoptic area (MPOA), subfornical organ (SFO) and paraventricular hypothalamic nucleus (PVN). Basal c-fos mRNA levels were not determined due to the almost undetectable signal. Results are expressed as mean ± SE (n = 4-7 per group). AU: arbitrary units (pixel area x sum grey). The different letters (a,b,c) indicate differences between the groups receiving the same treatment on day 28; # $p<0.05$ vs the same time point in stress-naïve (control) and IMO 1wk groups (two-way ANOVA, post-hoc analysis).

Figure 28 shows c-fos mRNA levels in the pPVN. Two-way ANOVA revealed significant effects of previous treatment and time point ($p<0.001$ in both cases) and a significant interaction between the two factors ($p<0.005$). Post-hoc analysis indicated no effect of previous treatment just after 1h of IMO, but a significant effect at 1h post-IMO: c-fos mRNA levels were greater in the control group than in the two previously-stressed groups, with IMO 4wk group displaying higher levels than IMO 1wk group (p at least <0.02).

Figure 31 displays the c-fos mRNA activation after IMO in the PVa and PVp. In both divisions, two-way ANOVA showed significant effects of time point ($p<0.05$ and $p<0.005$ for each respective division) but not of previous treatment or their interaction.

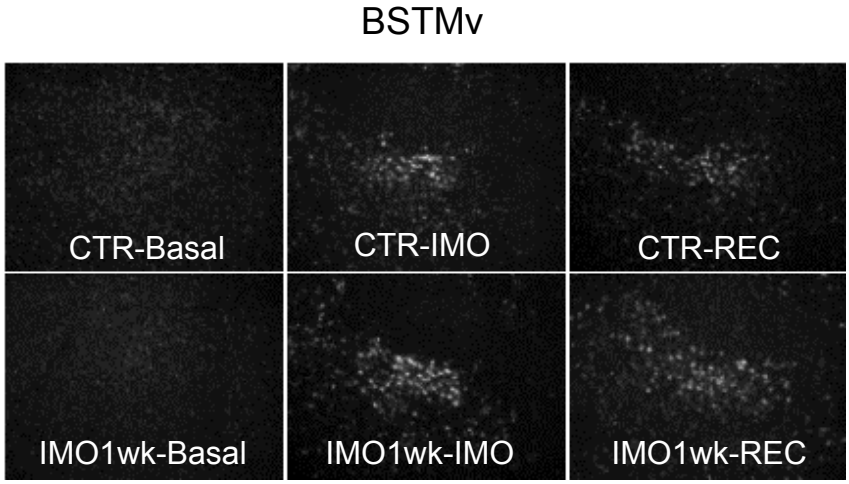

Figure 29. Representative dark-field photomicrographs displaying the effects of a single previous IMO session 1 week before on c-fos mRNA expression in the ventral subdivision of medial division of the bed nucleus of the stria terminalis (BSTMv) immediately after 1h of IMO (IMO) and 1h after the end of exposure to IMO (REC). Control (CTR, stress-naïve) animals did not receive any treatment on day 1. The legend should be read as "previous treatment-day 28 treatment", e.g. CTR-Basal are animals that did not receive any treatment on day 1 and were sacrificed under basal conditions on day 28. Magnification: 60X.

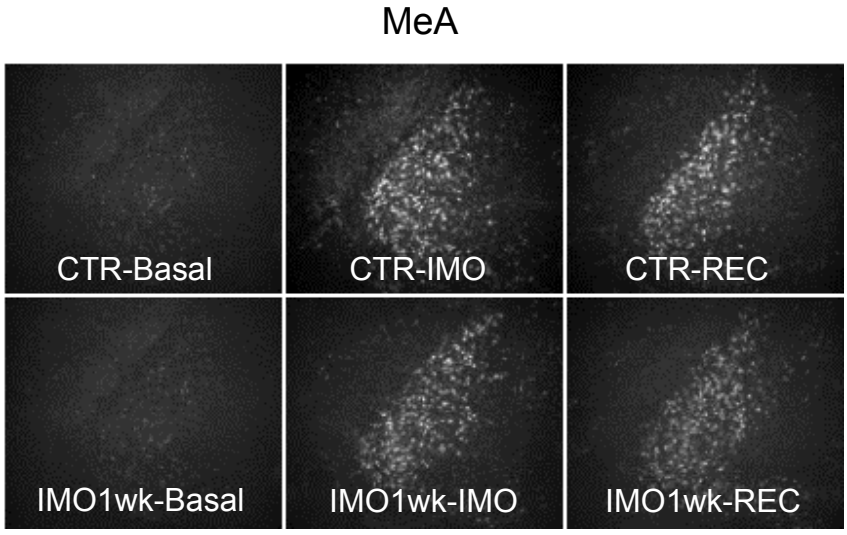

Figure 30. Representative dark-field photomicrographs displaying the effects of a single previous IMO session 1 week before on c-fos mRNA expression in the medial amygdala (MeA) immediately after 1h of IMO (IMO) and 1h after the end of exposure to IMO (REC). Control (CTR, stress-naïve) animals did not receive any treatment on day 1. The legend should be read as "previous treatment-day 28 treatment", e.g. CTR-Basal are animals that did not receive any treatment on day 1 and were sacrificed under basal conditions on day 28. Magnification: 40X.

Figures 29 and 31 show the c-fos mRNA response after IMO in the BSTMv. Two-way ANOVA indicated marginally significant effects of previous treatment ($p=0.054$), no effects of time point and a significant interaction ($p<0.04$). Post-hoc analysis showed that IMO 1wk group displayed higher c-fos mRNA levels just after 1h of IMO when

compared to the other two groups (p at least <0.02). At 1h post-IMO, IMO 4 wk group showed a marginally significant increase of c-fos mRNA levels when compared to control (stress-naïve) animals (p=0.075).

Activation of c-fos mRNA after IMO in the MeA is shown in Figures 30 and 31. Two-way ANOVA revealed significant effects of previous treatment (p<0.05) and time point (p<0.001) but not of their interaction. Post-hoc comparisons revealed that, irrespective of the time point, c-fos mRNA levels were significantly lower in IMO 1wk group with respect to control animals (p<0.02). There was also a marginally significant decrease of c-fos mRNA levels in IMO 4wk group with respect to control animals (p=0.066).

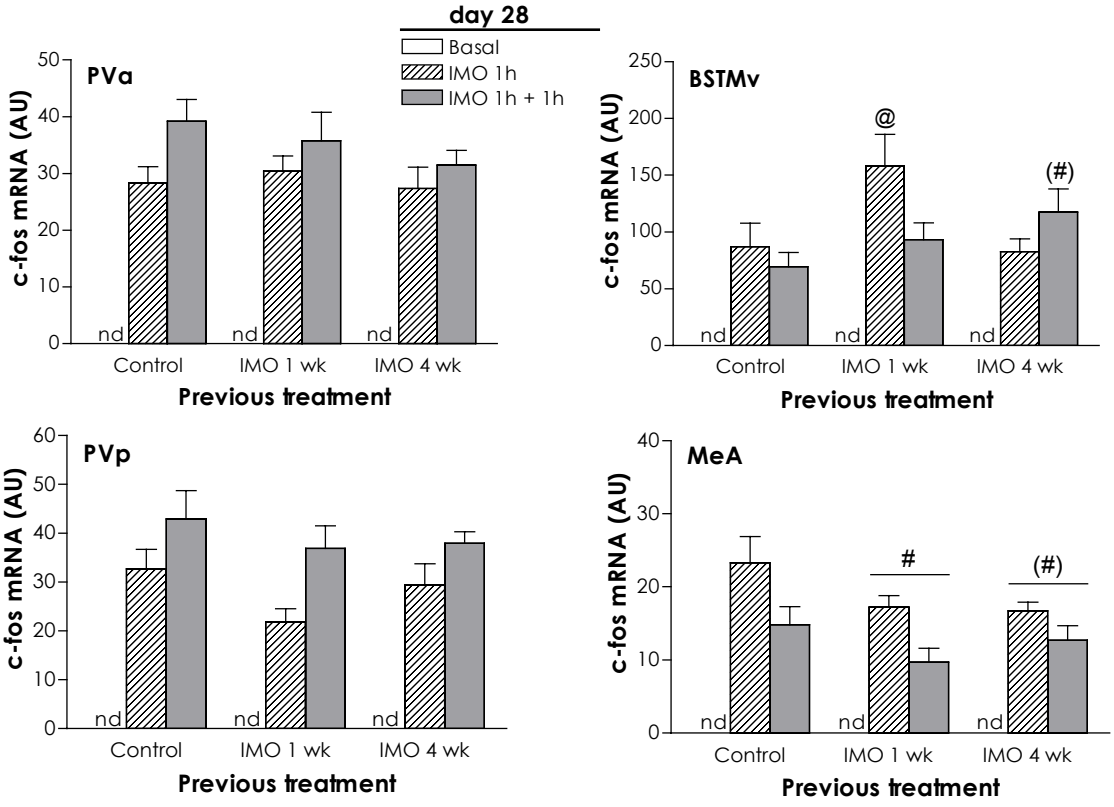

Figure 31. Effects of previous IMO experience on the c-fos mRNA responses to IMO, 1 or 4 weeks later, in the anterior and posterior divisions of paraventricular thalamic nucleus (PVa, PVp), bed nucleus of the stria terminalis medial ventral (BSTMv) and medial amygdala (MeA). Basal c-fos mRNA levels were not determined due to the almost undetectable signal. Results are expressed as mean ± SE (n = 4-7 per group). AU: arbitrary units (pixel area x sum grey). @ p at least <0.015 *vs* the same time point in stress-naïve (control) and IMO 4wk groups; # p<0.02 *vs* the same time point in control and group; (#) indicates marginally significant effects (0.05< p <0.1) *vs* the same time point in control group (two-way ANOVA, post-hoc analysis).

Two-way ANOVA of c-fos mRNA levels in the LC (Figures 32 and 33) revealed significant effects of previous treatment, time point ($p<0.01$ and $p<0.03$, respectively) and their interaction ($p<0.015$). Post-hoc comparisons revealed no differences just after 1h of IMO. In contrast, c-fos mRNA levels were significantly reduced at 1h post-IMO in IMO 1 wk group when compared to the other two groups. At the same time point, IMO 4 wk group displayed a marginally significant reduction of c-fos mRNA levels when compared to the control group ($p=0.064$).

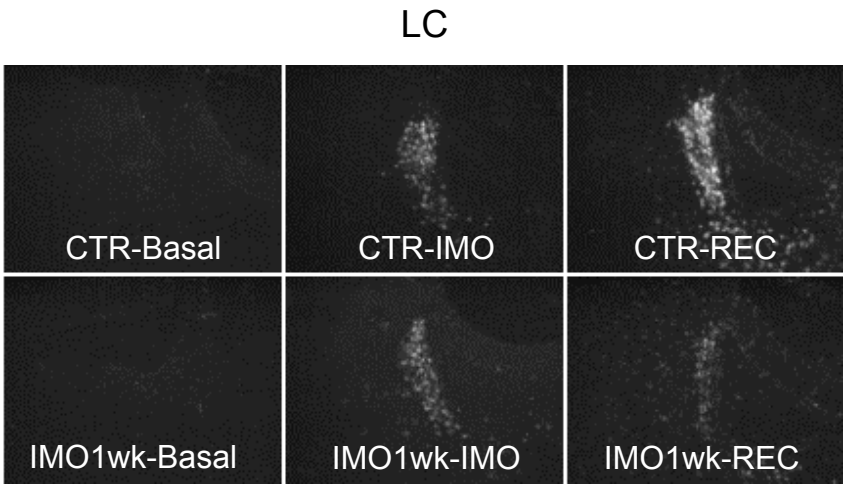

Figure 32. Representative dark-field photomicrographs displaying the effects of a previous IMO session 1 week before on c-fos mRNA expression in the locus coeruleus (LC) immediately after 1h of IMO (IMO) and 1h after the end of exposure to IMO (REC). Control (CTR, stress-naïve) animals did not receive any treatment on day 1. The legend should be read as "previous treatment-day 28 treatment", e.g. CTR-Basal are animals that did not receive any treatment on day 1 and were sacrificed under basal conditions on day 28. Magnification: 60X.

Finally, c-fos mRNA levels were quantified in the NST (Figure 33). Two-way ANOVA revealed marginally significant effects of previous treatment ($p=0.053$), with no effects of time point or the interaction previous treatment by time point. Post-hoc comparisons showed that IMO 1wk animals had lower c-fos mRNA levels, irrespective of the time point studied, when compared to the control group ($p<0.025$). Similarly, there was a marginally significant reduction of c-fos mRNA levels in IMO 4wk animals with respect to control animals ($p=0.078$).

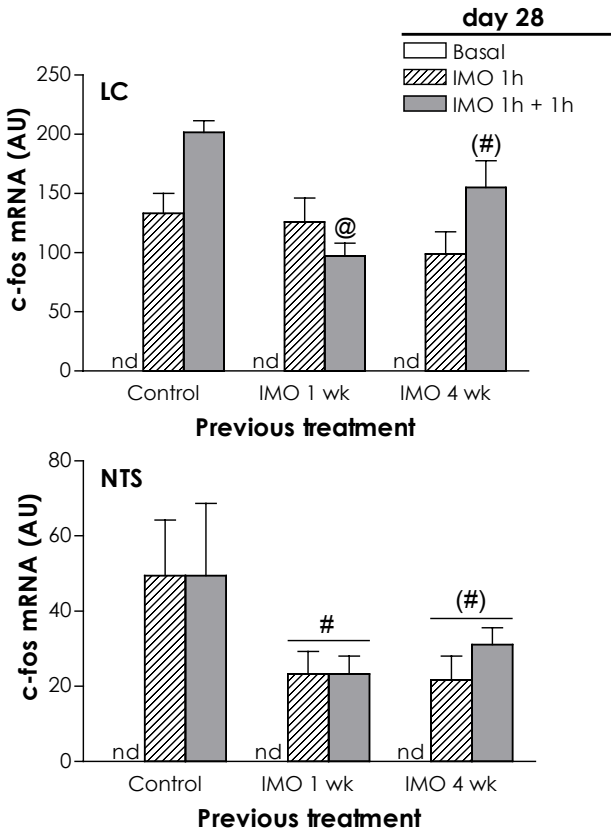

Figure 33. Effects of previous IMO experience on the c-fos mRNA responses to IMO, 1 or 4 weeks later, in the locus coeruleus (LC) and nucleus of the solitary tract (NTS). Basal c-fos mRNA levels were not determined due to the almost undetectable signal. Results are expressed as mean ± SE (n = 4-7 per group). AU: arbitrary units (pixel area x sum grey). @ $p<0.025$ *vs* the same time point in stress-naïve (control) and IMO 4wk groups; # $p<0.02$ *vs* the same time point in control group; (#) indicates marginally significant effects ($p<0.1$) *vs* the same time point in control group (two-way ANOVA, post-hoc analysis).

Experiment 4-2

The animals used correspond to Experiment 3-2. Nonetheless, c-fos mRNA brain mapping was performed only in some of the groups previously described. In brief, animals received either 1/ saline (Veh) or 2/ 1 mg/kg of LPS on day 1. After 28 days, rats were anaesthetised and perfused after one of the following conditions: resting conditions (Basal), 2h after LPS injection (LPS 2h) or 4h after LPS injection (LPS 4h).

Description of c-fos mRNA activation in the brain after an acute LPS injection

Figure 34 and Table 2 summarise the c-fos mRNA activation in the brain after a LPS injection. In accordance with previous studies [214], c-fos mRNA was not detectable in Veh-treated animals in most of the areas studied, except for the Pir and, to a lesser extent, the claustrum (Cl), Ctx and Pn.

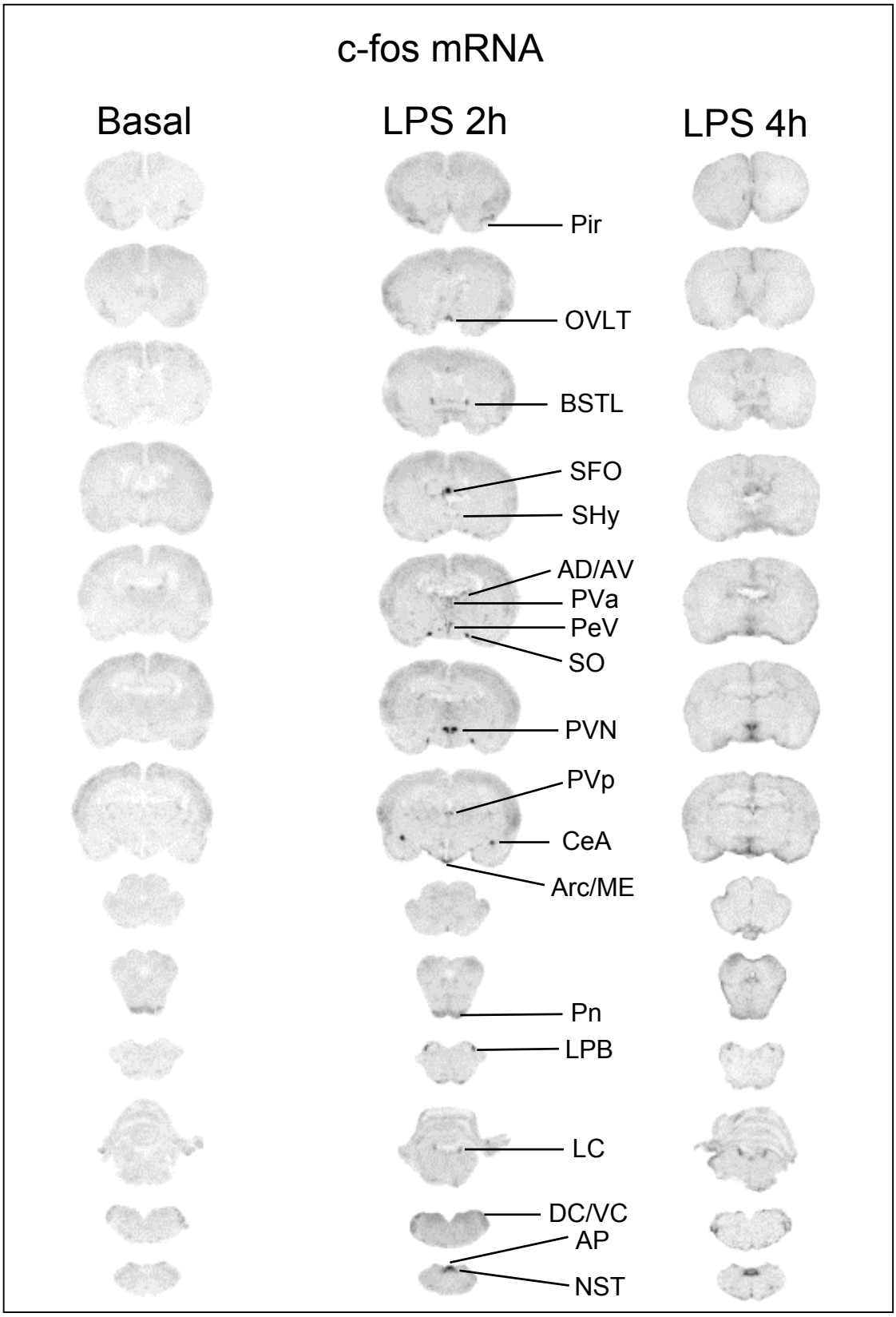

Figure 34. Representative images of the distribution of c-fos mRNA throughout the brain of rats treated with Veh or LPS 1 mg/kg. Abbreviations: AD, anterodorsal thalamic nucleus; AP, area postrema; Arc, arcuate nucleus; AV, anteroventral thalamic nucleus; BSTL, bed nucleus of the stria terminals, lateral division; DC, dorsal cochlear nucleus; LC, locus coeruleus; LPB, lateral (continues in next page)

Circumventricular organs and non-neural components – Two hours after an acute LPS injection there was a profound c-fos mRNA activation in the CVOs, namely OVLT, SFO, ME and AP. At 4h post injection, the signal in these organs almost vanished and seemed to spread in a migratory-like pattern to the surrounding areas, such as the MPOA, fimbria, Arc and NST, respectively. At the same time point, there was a c-fos mRNA activation in the leptomeninges, choroid plexus, ependymal cells of the ventricles and subependymal zone.

Telencephalon – LPS also caused a discrete but robust activation of c-fos mRNA in different brain nuclei. At 2h post-injection there was a positive signal in some telencephalic areas, such as the lateral division of the BST (BSTL). There was also a strong positive signal in the CeA. Four hours after injection, the signal for c-fos mRNA was much lower in these areas. No signal was observed in the hippocampal formation at any time.

Diencephalon – There were different thalamic nuclei displaying a positive c-fos mRNA signal 2h after LPS injection: AD/AV, PVa/PVp and LHbM. To a lesser extent, the CM, DLG/VLG and MG were also activated. The signal at 4h post-injection was greatly diminished in these nuclei, except for the PVp where c-fos mRNA levels were comparable to the levels at 2h post-injection.

There was a strong c-fos mRNA activation in some hypothalamic areas such as the SO and PVN 2h after LPS injection. At 4h post-injection, the signal for c-fos mRNA was considerably lower in the SO but remained elevated in the PVN. At 4h after LPS injection, there was a moderate activation of c-fos mRNA in the MPOA/LPOA, with the positive signal located mainly in what Elmquist et al [72-74] have designed as the ventromedial preoptic area (VMePOA). Low signal was observed in other hypothalamic nuclei such as PeV, suprachiasmatic (SCh), AH, Arc and DM at 2 h post-injection. The c-fos mRNA signal in these areas almost vanished at 4h post-injection, except in the Arc where c-fos mRNA levels were increased, as explained above (see CVOs section). Similarly, there was a migratory-like pattern around the DM at 4h post-injection.

Figure 34 (continues from previous page). parabrachial nucleus; ME, median eminence; OVLT, organum vasculosum of the lamina terminalis; PeV, periventricular hypothalamic nucleus; NST, nucleus of the solitary tract; Pir, piriform cortex; Pn, pontine nuclei; PVa, paraventricular thalamic nucleus, anterior division; PVN, paraventricular hypothalamic nucleus; PVp, paraventricular thalamic nucleus, posterior division; SFO, subfornical organ; SHy, septohypothalamic nucleus; SO, supraoptic nucleus; VC, ventral cochlear nucleus.

TABLE 2. LPS-induced c-fos mRNA activation in the brain: effects of previous LPS experience

AREA	CONTROL			LPS 4wk		
	Veh	LPS 2h	LPS 4h	Veh	LPS 2h	LPS 4h
TELENCEPHALON						
Claustrum (Cl)	+	+/-	+/-	+	+	+/-
Neocortex (Ctx)	+	+	-	+	+	-
Piriform cortex (Pir)	++	++/+++	+/++	++	++/+++	+/++
Lateral septal nucleus (LS)	-	+/-	+/-	-	+/-	+/-
Organum vasculosum of the lamina terminalis (OVLT)	-	++/+++	M	-	++	M
Bed nucleus stria terminalis, lateral div (BSTL)	-	++/+++	+	-	++/+++	+/-
Septohypothalamic nucleus (SHy)	-	++	+/-	-	+/++	+/-
Sufornical organ (SFO)	-	++++	M	-	+++/++++	M
Central amygdaloid nucleus (CeA)	-	+++	+	-	++/+++	+/-
Hippocampal formation						
CA1	+/-	+/-	-	+/-	+/-	-
CA2	+/-	+	-	+/-	+	-
CA3	+/-	+	-	+/-	+	-
Dentate gyrus (DG)	-	-	-	-	-	-
DIENCEPHALON						
Thalamus						
Anterodorsal thalamic nucleus (AD)	+/-	+/++	+	+/-	+/++	+/-
Anteroventral thalamic nucleus (AV)	+/-	+/++	+	+/-	+/++	+/-
Central medial thalamic nucleus (CM)	-	+/-	-	-	+/-	+
Paraventricular thalamic nucleus, anterior (PVa)	-	+/++	+	-	+/++	+
Paraventricular thalamic nucleus, posterior (PVp)	-	++/+++	++/+++	-	++/+++	++
Lateral habenular nucleus, medial (LHbM)	-	+/++	+	-	+	+/-
Lateral geniculate nucleus, dorsal and ventral (DLG	+/-	+	-	+/-	+/-	-
Medial geniculate nucleus (MG)	+/-	+	-	+/-	+/-	-

(-) undetectable; (+) low; (++) moderate; (+++) strong; (++++) very strong
(M) indicates a migratory-like pattern (diffuse signal)

TABLE 2 (continued). LPS-induced c-fos mRNA activation in the brain: effects of previous LPS experience

AREA	CONTROL			LPS 4wk		
	Veh	LPS 2h	LPS 4h	Veh	LPS 2h	LPS 4h
Hypothalamus						
Medial preoptic area (MPOA)	-	+	+/-	-	+/-	+/-
Lateral preoptic area (LPOA)	-	+	+/-	-	+/-	+/-
Periventricular hypothalamic nucleus (PeV)	-	+/-	-	-	+/-	-
Supraoptic nucleus (SO)	-	+++	+	-	++/+++	+
Suprachiasmatic nucleus (SCh)	+/-	+/-	-	+/-	+/-	-
Anterior hypothalamic area (AH)	-	+/-	+/-	-	+/-	+/-
Paraventricular hypothalamic nucleus (PVN)	-	++++	++/+++ M	-	++++	++ M
Median eminence (ME)	-	+++/++++	M	-	+++	M
Arcuate hypothalamic nucleus (Arc)	-	+/-	++/+++ M	-	+/-	++ M
Dorsomedial hypothalamic nucleus (DM)	-	+	+ M	-	+	+ M
Subthalamic nucleus (STh)	-	+/-	-	-	+/-	-
MESENCEPHALON						
Central (periaqueductal) grey (CG)	-	+/-	-	-	+/-	-
PONS						
Pontine nuclei (Pn)	+	+++	+/++	+	+++	+/++
Dorsal raphe nucleus (DR)	-	+/-	+/-	-	+/-	+/-
Laterodorsal tegmental nucleus (LDTg)	-	+	-	-	+	-
Lateral parabrachial nucleus (LPB)	-	+++/++++	+/++	-	+++/++++	+/++
Superior paraolivary nucleus (SPO)	-	+/-	-	-	+/-	-
Locus coeruleus (LC)	-	+/++	++/+++	-	+/++	+/++
Facial nucleus (7)	-	+	-	-	+	-
Area postrema (AP)	-	++++	M	-	+++/++++	M

(-) undetectable; (+) low; (++) moderate; (+++) strong; (++++) very strong
(M) indicates a migratory-like pattern (diffuse signal)

TABLE 2 (continued). LPS-induced c-fos mRNA activation in the brain: effects of previous LPS experience

AREA	CONTROL			LPS 4wk		
	Veh	LPS 2h	LPS 4h	Veh	LPS 2h	LPS 4h
MEDULLA						
Ventral cochlear nucleus (VC)	-	+/++	+	-	+/++	+
Dorsal cochlear nucleus (DC)	-	+/++	+	-	+/++	+
Raphe pallidus nucleus (RPa)	-	-	+	-	-	+/-
Spinal trigeminal nucleus (Sp5)	-	+	-	-	+	-
Medial vestibular nucleus (MVe)	-	++	+/++ M	-	++	+/++ M
Nucleus of the solitary tract (NST)	-	+++/++++	+++/++++ M	-	+++	+++ M
Ventrolateral medulla (VLM)	-	+	+/++	-	+	+
External cuneate nucleus (Ecu)	+/-	+	+	+/-	+	+
Cuneate nucleus (Cu)	+/-	+	+	+/-	+	+
NON-NEURAL COMPONENTS						
Meninges (lepto)	-	-	++/+++	-	-	++
Choroid plexus	-	-	+/++	-	-	+
Ependymal cells of ventricles	-	-	++/+++	-	-	++
Subependymal zone	-	-	+/++	-	-	+

(-) undetectable; (+) low; (++) moderate; (+++) strong; (++++) very strong
(M) indicates a migratory-like pattern (diffuse signal)

Brain Stem – There was no activation of c-fos mRNA in any mesencephalic area, except for a very low activation in CG at 2h post-injection. In contrast, several pontine areas displayed c-fos mRNA activation after LPS. At 2h post-injection, there was a strong signal for c-fos mRNA in the Pn, LPB and, as previously described, the AP (see CVOs section). A moderate positive signal was detected in the LC. There were other areas displaying low to very low c-fos mRNA levels, such as the DR, LDTg, SPO and 7. At 4h post injection, the amount of c-fos mRNA signal was considerably reduced in Pn and LPB and was back to basal levels in LDTg, SPO and 7. There were still detectable but very low levels of c-fos mRNA in the DR. As explained above, the signal from AP was now vanished and there was a positive signal around this area, in a migratory-like pattern. Surprisingly, c-fos mRNA levels in LC were increased at 4h as compared to 2h post-injection.

Some medullary nuclei were also activated 2h after LPS injection. Nonetheless, only the NST displayed a very strong activation. The VLM, MVe and DC/VC displayed a moderate signal. The other areas activated, such as the Sp5, ECu and Cu, displayed a very low positive signal. At 4h post injection, there was still a high c-fos mRNA signal in the NST, although this time it seemed to migrate from the AP (see CVOs section). The same happened in the MVe, where there was a medium to low signal in a migratory-like pattern. There was still a positive signal in DC/VC, VLM, Ecu and Cu. The signal in Sp5 was not detectable any more. In contrast, a very low but positive signal was detected in the RPa.

Semiquantitative analysis of c-fos mRNA levels in different brain areas after LPS administration: effects of a previous LPS experience

In order to elucidate whether or not LPS-induced c-fos mRNA activation in the brain was influenced by a previous LPS injection, some of the nuclei described above were selected for semiquantification of c-fos mRNA levels under dark field illumination. This selection was done after qualitative analysis of c-fos mRNA activation in all the brain areas described above (analysis *de visu*, Table 2). The areas either appearing to be greatly influenced by a previous LPS experience or expected to play an important role in LPS-induced HPA axis activation were the ones chosen for semiquantification. Nonetheless, some of these areas were not subjected to quantification because (i) the amount of signal was already very low in the control group (LHbM, DLG/VLG, MG), (ii) there was a diffuse pattern of activation (Arc) or (iii) it was difficult to chose a comparable area of activation among the different animals (leptomeninges, choroid plexus, ependymal cells, subependymal zone). In general, these areas tended to

display a lesser c-fos mRNA activation in previously LPS-injected animals when compared to the control group. This tendency was found either at 2h post-injection (DLG/VLG, MG), 4h post-injection (Arc, VLM, leptomeninges, choroid plexus, ependymal cells, subependymal zone) or at both time points (LHbM).

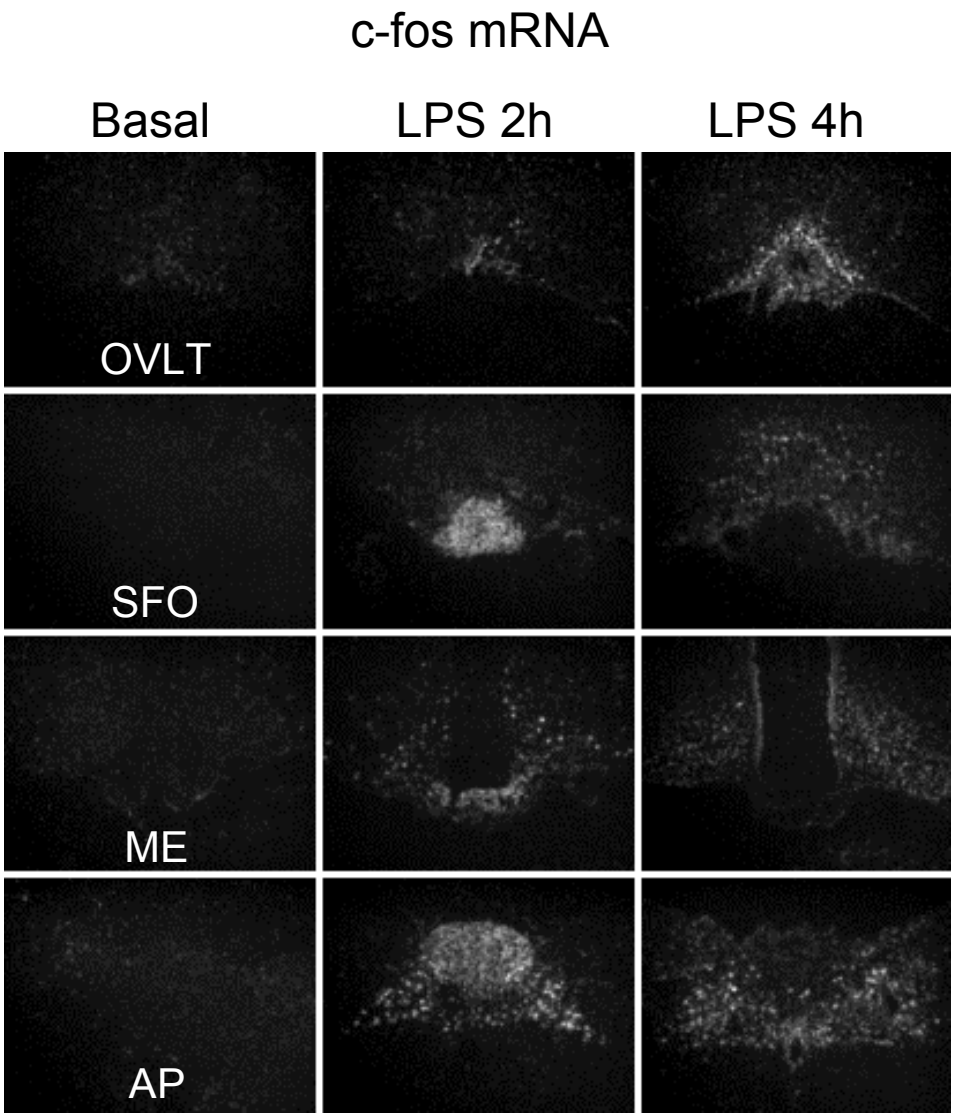

Figure 35. Representative dark-field photomicrographs of the c-fos mRNA activation in the circumventricular organs (CVOs) after an acute LPS injection. As explained in the text, there was a migratory-like pattern of the signal for c-fos mRNA at 4h post-injection, likely as a reflex of the activation of cells neighbouring the CVOs at that time point. Abbreviations: AP, area postrema; Me, median emienence; OVLT, organum vasculosum of the lamina terminalis; SFO, subfornical organ. Magnification: 60X.

Two-way ANOVAs of c-fos mRNA levels in the four CVOs (Figures 35 and 36) did not show significant effects of previous treatment, but revealed significant effects of time point ($p<0.001$ in all CVOs), without a significant interaction between the two main factors. Signal quantification of c-fos mRNA levels in the BSTL (Figures 37 and 39)

revealed significant effects of previous treatment and time point (two-way ANOVA, $p<0.03$ and $p<0.001$, respectively) but not of their interaction, the previous experience with LPS reducing the c-fos mRNA response to the last LPS administration. Two-way ANOVA of c-fos mRNA levels in the CeA (Figures 38 and 39) revealed significant effects of previous treatment and time point (p at least <0.005) but not of their interaction, with a reduced c-fos mRNA response to LPS in previously LPS-injected animals 4 weeks before. Quantification of c-fos mRNA levels in the PVa (Figure 37) and PVp revealed no significant effects of previous treatment, time point or their interaction (two-way ANOVA).

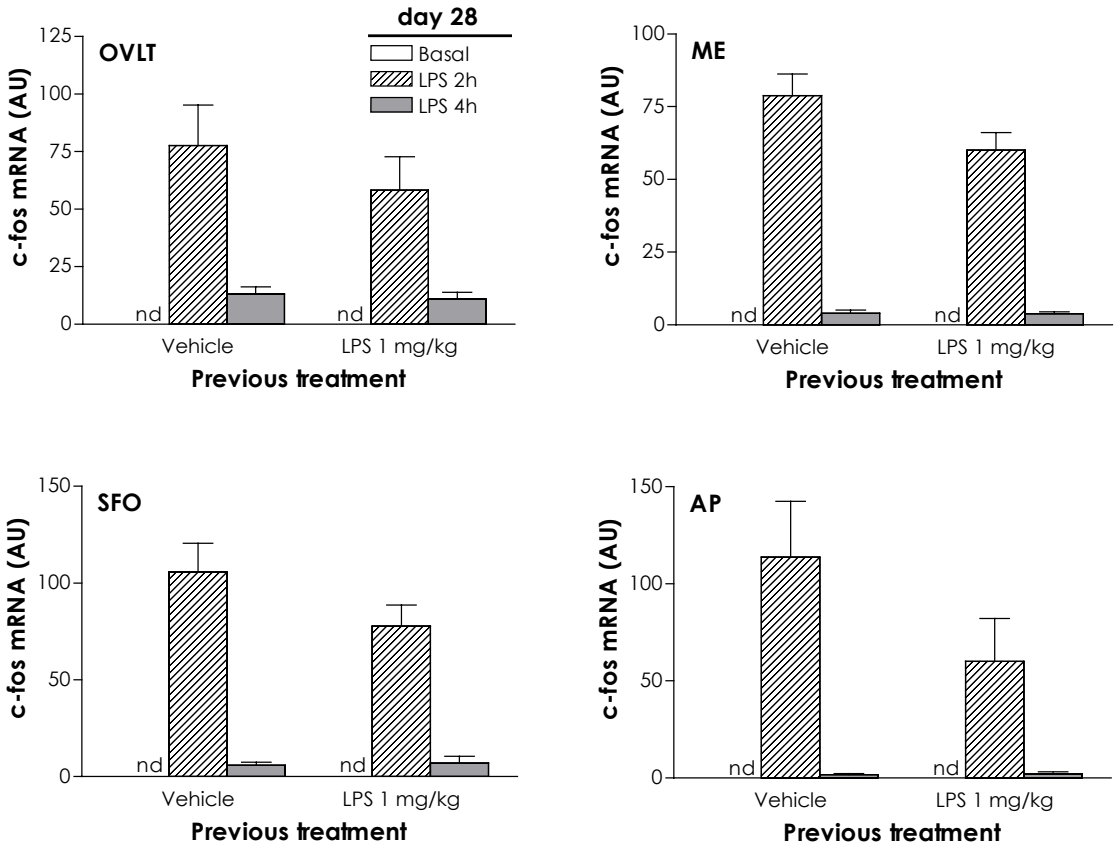

Figure 36. Effects of a previous LPS injection on the LPS-induced c-fos mRNA activation in the circumventricular organs (CVOs) 4 weeks later. Basal c-fos mRNA levels were not determined due to the almost undetectable signal. Results are expressed as mean ± SE (n = 4-7 per group). AU: arbitrary units (pixel area x sum grey). Abbreviations: AP, area postrema; ME, median emienence; OVLT, organum vasculosum of the lamina terminalis; SFO, subfornical organ.

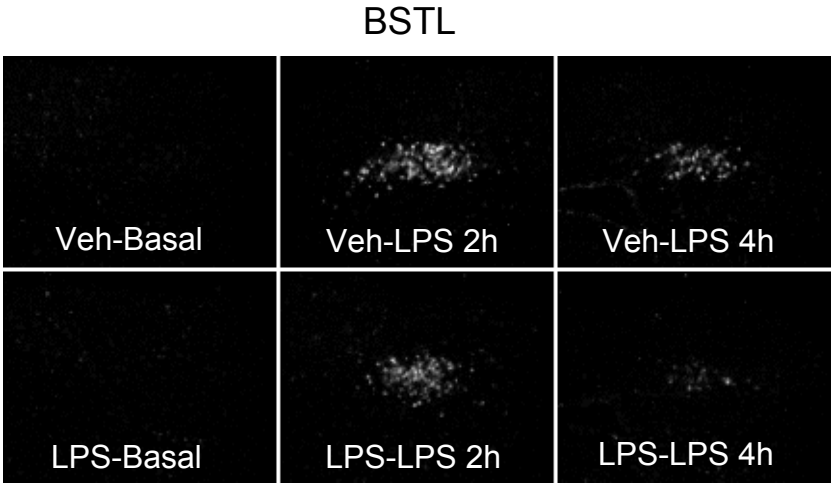

Figure 37. Representative dark-field photomicrographs displaying the effects of a previous LPS injection 4 weeks before on c-fos mRNA expression in the lateral division of the bed nucleus of the stria terminalis (BSTL) 2h or 4h after a new LPS injection. Control (CTR, stress-naïve) animals received Veh on day 1. The legend should be read as "previous treatment-day 28 treatment", e.g. Veh-Basal are animals that received Veh on day 1 and were sacrificed under basal conditions on day 28. Magnification: 60X.

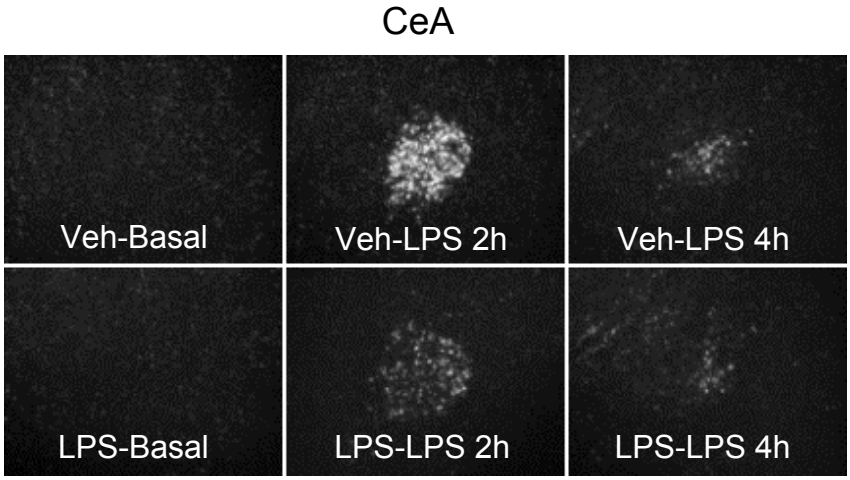

Figure 38. Representative dark-field photomicrographs displaying the effects of a previous LPS injection 4 weeks before on c-fos mRNA expression in the central amygdala (CeA) 2h or 4h after a new LPS injection. Control (CTR, stress-naïve) animals received Veh on day 1. The legend should be read as "previous treatment-day 28 treatment", e.g. Veh-Basal are animals that received Veh on day 1 and were sacrificed under basal conditions on day 28. Magnification: 60X.

Figure 39 displays c-fos mRNA levels in the SO. Two-way ANOVA showed no significant effect of previous treatment, but revealed significant effects of time point ($p<0.001$) and their interaction ($p<0.03$). No differences among SO c-fos mRNA levels were found between control and previously-stressed rats, despite a marginal tendency of previously LPS-injected animals to display lower levels at 2h post-injection when compared to stress-naïve animals (t-test, $p=0.078$).

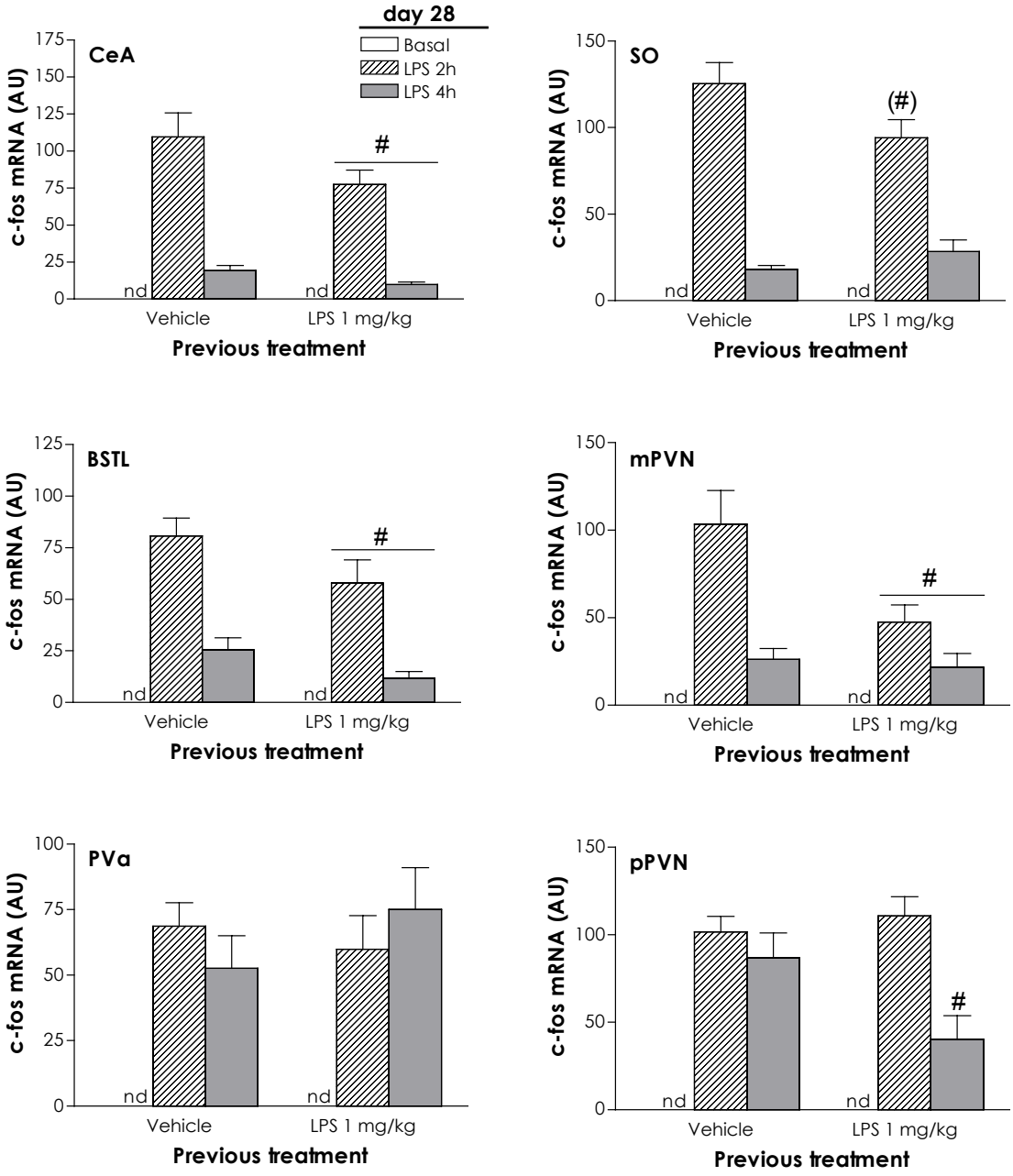

Figure 39. Effects of a previous LPS injection on the LPS-induced c-fos mRNA activation in different thalamic, hypothalamic and limbic structures 4 weeks later. Basal c-fos mRNA levels were not determined due to the almost undetectable signal. Results are expressed as mean ± SE (n = 4-7 per group). AU: arbitrary units (pixel area x sum grey). Abbreviations: BSTL, bed nucleus of the stria terminalais, lateral division; CeA, central amygdala; mPVN, magnocellular paraventricular hypothalamic nucleus; pPVN, parvocellular paraventricular hypothalamic nucleus; PVa, paraventricular thalamic nucleus, anterior division; SO, supraoptic nucleus. # $p<0.02$ *vs* control group; (#) indicates marginally significant effects ($0.05< p <0.1$) *vs* control animals (two-way ANOVA, post-hoc analysis with t-test).

The analysis of the effects of previous stress exposure on the c-fos mRNA levels in the whole PVN after LPS injection has been already described in Chapter 3. Of interest is the difference in the c-fos mRNA signal in this nucleus between 2 and 4 h post-injection, since at the latter time point a migratory-like pattern was detected, the

signal being less compact and more diffuse than 2h before. We wanted to make a more detailed analysis of c-fos mRNA activation in this nucleus by measuring separately the parvo and magnocellular subdivisions (Figure 39). In the mPVN, two-way ANOVA revealed significant effects of previous treatment and time point ($p<0.03$ and $p<0.001$, respectively) but not of their interaction, with a reduced c-fos mRNA response to LPS in animals previously given LPS 4 weeks before. Two-way ANOVA of c-fos mRNA levels in the pPVN did not show a significant effect of previous treatment, but there were significant effects of time point and their interaction ($p<0.001$ and $p<0.03$, respectively). Further comparisons within the same time point showed no differences at 2h post-injection, but a significant reduction of c-fos mRNA levels in previously LPS-injected animals at 4h post-injection when compared to the stress-naïve animals (t-test, $p<0.04$).

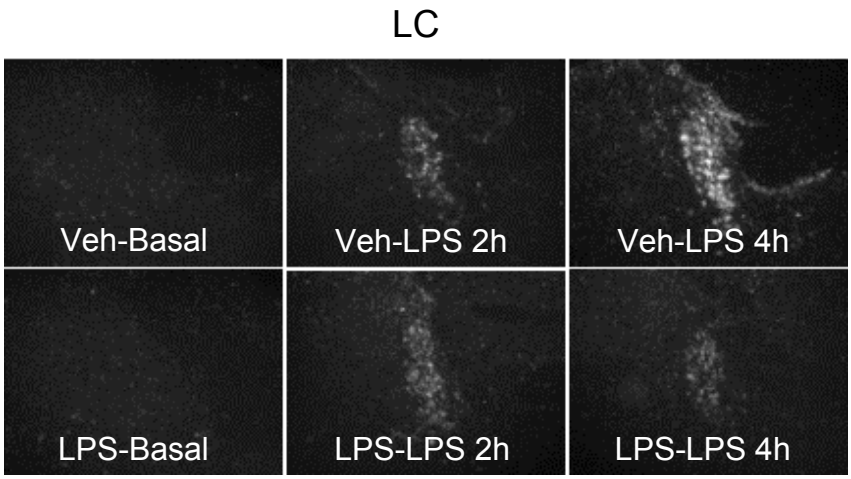

Figure 40. Representative dark-field photomicrographs displaying the effects of a previous LPS injection 4 weeks before on c-fos mRNA expression in the locus coeruleus (LC) 2h or 4h after a new LPS injection. Control (CTR, stress-naïve) animals received Veh on day 1. The legend should be read as "previous treatment-day 28 treatment", e.g. Veh-Basal are animals that received Veh on day 1 and were sacrificed under basal conditions on day 28. Magnification: 60X.

Figures 40 and 41 show c-fos mRNA levels in the LC. Two-way ANOVA revealed a significant effect of previous treatment ($p<0.005$) and marginally significant effects of time point and their interaction ($p=0.090$ and $p=0.052$, respectively). Comparisons within the same time point revealed no differences between stress-naïve and previously LPS-injected animals at 2h post-injection. Nonetheless, at 4h post-injection there was a significant reduction of c-fos mRNA levels in the animals with a previous LPS experience when compared to stress-naïve animals (t-test, $p<0.01$).

C-fos mRNA levels were also quantified in the NST (Figure 41). Two-way ANOVA revealed no significant effects of previous treatment, time point or their interaction.

Finally, c-fos mRNA levels in the RPa were also measured at 4h post-injection (Figure 41), and no differences were found between control and previously LPS-injected rats (t-test).

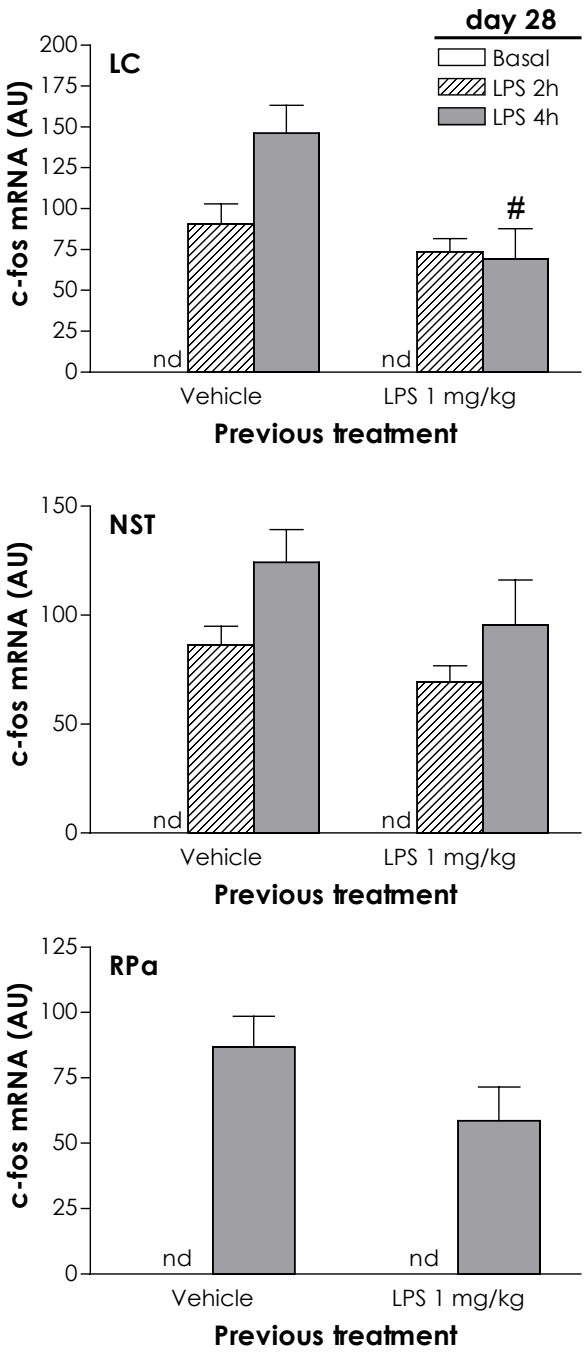

Figure 41. Effects of a previous LPS injection on the LPS-induced c-fos mRNA activation in the locus coeruleus (LC), nucleus of the solitary tract (NST) and raphe pallidus (Rpa) 4 weeks later. Basal c-fos mRNA levels were not determined due to the almost undetectable signal. Results are expressed as mean ± SE (n = 4-7 per group). AU: arbitrary units (pixel area x sum grey). # $p<0.01$ *vs* control group (two-way ANOVA, post-hoc analysis with t-test).

DISCUSSION

We previously described that a single exposure to both IMO and LPS is able to modify the future response of the HPA axis to the same stimulus. It has already been mentioned that these two stressors are greatly differing in nature, activating the HPA axis through different routes, IMO being categorised as an emotional stimulus and LPS as a systemic stimulus [231]. Of interest is the fact that both stressors can affect the HPA response in the long-term, but the mechanisms through which each of these stressors can reduce this response are not known. In order to elucidate if the brain nuclei involved in this phenomenon are or not the same depending on the stimulus applied, we have analysed, as a first approach, the stress-induced c-fos mRNA activation throughout the whole brain in both stress-naïve and previously-stressed rats.

Under basal conditions, there was an almost undetectable signal for c-fos mRNA, whereas the stress-induced c-fos mRNA activation in the brain to an acute stress exposure (control groups), either to IMO or LPS, was in accordance to previous studies using similar stressors (for reviews, see [231, 246]). There was a very different pattern of stress-induced c-fos mRNA activation depending on the stimulus used.

Activation of c-fos mRNA in the brain after acute IMO or LPS exposure

Similarly to restraint [46], 1h of IMO in control animals induced a wide c-fos mRNA expression in the brain: a prominent activation was found in the cerebral cortex, subcortical limbic structures (LS, BSTMv, MeA), thalamic nuclei (AD, PVa/PVp, CM, LHbM, among others), hypothalamic areas (e.g. preoptic and posterior hypothalamic areas and SO, PVN, DM and premamillary nuclei) and brain stem nuclei (CG, DR, LPB/MPB, LC and DC/VC). At 1h post-stress, c-fos mRNA levels remained equal or were slightly reduced in most of these areas, although in some nuclei there was a greater reduction of c-fos mRNA levels (LS, some thalamic nuclei, LPB/MPB). Nonetheless, some areas displayed higher c-fos mRNA levels at this time point, such as the PVa, LC, PMV/PMD and DR. Interestingly, c-fos mRNA levels in some divisions of the hippocampal formation were only detectable in the post-stress period, in accordance with other studies [46].

This widespread activation of brain areas by IMO is in accordance to the proposed central stress circuits of emotional stressors such as restraint, footshock, fear conditioning and novel enviroment [96, 231, 246]. These stressors would activate cortical and limbic areas that in turn would process and convey this activation to the

PVN. In fact, lesions of PFC, hippocampus and amygdala have been shown to alter the HPA axis response to different neurogenic stressors (reviewed in [96, 246]).

The observed c-fos mRNA activation induced by LPS was in accordance with previous studies with LPS or IL-1β [74, 94, 214], and very different from that observed after IMO. At 2h after LPS injection, there was a strong activation of all the CVOs, together with a very slight activation of the cerebral cortex and a strong c-fos mRNA expression in the BSTL and the CeA. There was a discrete activation of some thalamic (AD/AV, PVa/PVp, LHbM) and hypothalamic nuclei (SO, PVN). In the brainstem, strong c-fos mRNA levels were found in the LPB and NST and, to a lesser extent, in the LC. At 4h post-injection, the pattern of c-fos mRNA activation changed quite dramatically. Most of the telecephalic and diencephalic nuclei activated before displayed very low to undetectable levels of this transcript, except for both the thalamic and hypothalamic paraventricular nuclei, were c-fos mRNA levels remained high. In some brainstem nuclei, c-fos mRNA was reduced but still detectable (Pn, LPB), whereas it was increased in the LC and NST. Interestingly, the signal in the CVOs (OVLT, SFO, ME, AP) disappeared and seemed to have migrated to the areas surrounding each of these organs (MPOA, fimbria, Arc and NST, respectively). In addition, there was a positive and strong activation of the leptomeninges, choroid plexus, ependymal cells of ventricles and subependymal zone.

Most of the biological effects of LPS appear to be mediated through the recently characterised TLR4 [204, 207], which is constitutively expressed in the brain, mainly in leptomeninges, choroid plexus and CVOs [141]. LPS-induced c-fos mRNA activation in leptomeninges, ependymal cells and choroid plexus was only found at 4 h post-injection. This delayed c-fos mRNA response is surprising considering that they show constitutive expression of TLR4 [141] and LPS induces the transcription of TNF-α, IL-1β, IL6, CD14 and IκBα in these structures [135, 140, 189, 206, 283]. Nevertheless, two distinct waves of c-fos mRNA activation have been observed in the above-mentioned structures after IL-1β administration, with an intermediate silent period [94]. If this time course of activation is similar to the pattern of c-fos mRNA activation after LPS injection, we possibly might have lost the initial activation of c-fos mRNA caused by LPS in these structures. In fact a weak or null activation of c-fos mRNA in these structures has been reported 1 h after LPS administration in mice, with a greater activation at 4 h post-injection [282]. Whether or not there was an actual prompt activation of these structures in the present work is not known and therefore their role in the initial HPA response to LPS is uncertain.

The initial LPS-induced peripheral HPA axis activation is likely to be mediated by an action on the PVN taking into account the prompt activation of this nucleus [293] and the attenuated initial ACTH response to LPS in PVN-lesioned rats [71]. Combination of c-fos mRNA induction and retrograde labelling has been used to study the PVN-projecting neurons that are activated after LPS injection [73]. It is suggested that there are numerous nuclei directly contributing to the activation of the PVN such as the BST, OVLT and the surrounding MPOA, several intrahypothalamic nuclei and some brainstem areas, mainly the NST, LPB and VLM. Since we have observed a strong activation of the CeA and BSTL, an indirect contribution of the CeA through the BSTL to the activation of the PVN should not be disregarded [2]. However, the primary site of brain LPS-induced activation is unclear. Taking into account the presence of TLR4 and the strong c-fos mRNA induction after LPS injection in the CVOs [141], the primary target of LPS-induced brain activation may be these organs devoid BBB. The activation of the CVOs may be translated to other nuclei such as the NST, neighbouring the AP, or the PVN, which receives projections from the OVLT and SFO [193]. There are no direct projections from the CVOs to the CeA, suggesting that activation of CeA by LPS might arise through signals arriving from brainstem nuclei closely related to AP such as the NST and LPB [115, 209, 272].

Differential pattern of c-fos mRNA activation in the brain after IMO or LPS exposure

This pattern of c-fos mRNA activation caused by IMO or LPS is an indication of the routes involved in HPA axis activation for each of these stimuli, as it has previously been discussed [96, 231, 246]. Of interest is the differential activation of limbic nuclei such as the amygdala and BST depending on the stressor applied: whereas IMO induces c-fos mRNA expression in the MeA and BSTM, LPS induces it in the CeA and BSTL. This is in accordance with the structural organization of these nuclei: briefly, the major output from the MeA innervates the posterior division of the BST, but there are also projections from the MeA to the BSTM [66]. In turn, the CeA innervates predominantly the BSTL [66]. In addition, the BSTM and BSTL are considered an extension of the MeA and CeA, respectively [66]. The MeA is considered as part of the accessory olfactory system component of the amigdalar complex, involved in the control of innate reproductive, defensive and ingestive behaviours [258]. On the other hand, the CeA is considered the autonomic system component of the amigdalar complex, being a specialized region of the striatum that modulates autonomic motor outflow [258]. These neuroanatomic characteristics also correlate with functional studies showing that emotional stressors activate predominantly the MeA, whereas physical stressors activate mainly the CeA [56]. Additionally, MeA

lesions are able to reduce the PVN activation to a neurogenic stressor as restraint, whereas CeA lesions do not affect the PVN response to the same stressor [57] but are able to reduce the central HPA axis response to an immunological stressor such as IL-1β administration [302]. Finally, the BSTM projects mainly to hypothalamic regions associated with the neuroendocrine system, whereas the efferents from the BSTL are autonomic-related regions of the hypothalamus and lower brainstem [66].

Another important difference between LPS and IMO is the activation of c-fos mRNA in the CVOs and non-neural components by LPS, but not by IMO (except for the SFO, simulated by the two stressors). This suggests that these structures devoid BBB play an important role in the brain transduction of blood-borne signalling induced by LPS and cytokines, as previously discussed [38, 213], but not by IMO. The migratory-like pattern of c-fos mRNA signal after LPS injection is in accordance with a previous study using IL-1β administration [94] and, interestingly, matches similar findings reported for other mRNAs such as the ones coding for TNF-α, CD14 and the receptor for the C3 protein of the complement system (C3aR) [135, 141, 187, 189, 190]. It has been suggested that this migratory-like pattern would reflect the spreading activation to cells of presumably myeloid origin neighbouring the CVOs [135]. Of interest is that c-fos mRNA, considered as a marker of neuronal activation, displays a similar pattern, at least with a high dose of LPS. Although we have not performed dual labelling studies to determine the nature of these cells and we cannot rule out the possibility of them being neurons, the signal was characterised by being very diffuse and not as strong and compact as in other areas of the brain with a high density of neurons. In addition, other studies have described the presence of c-fos mRNA in other cell types such as glia cells [23, 156], thus explaining the present results.

Long-term effects of IMO on the brain c-fos mRNA response to the homotypic stressor

Appart from the above considerations regarding the differential activation of c-fos mRNA by these two different stimuli in control animals, our main objective was to obtain a preliminary clue of the brain areas involved in the long-term effects of a single stress exposure. Considering the greatly differing pattern of neuronal activation depending on the stressor, and the fact that long-term effects of stress appear to be stressor-specific (see Chapter 2), the sites of neuronal plasticity accountable for the blunted CRF and c-fos mRNA response in the PVN may be different, depending on the stressor. Nonetheless, we cannot rule out the possibility that these sites could be common, since there are indeed common areas activated by both stimuli, like the PVN itself and other nuclei such as the SHy, SFO, AD/AV, PVa/PVp, LHbM, SO, Pn, LPB,

DC/VC and LC. Semiquantification of c-fos mRNA levels was performed in the areas thought to play an important role in HPA axis activation and/or found to be differentially activated in previously-stressed rats in a preliminary qualitative analysis.

The c-fos mRNA response in previously immobilised rats both one or four weeks before (IMO 1wk and IMO 4wk groups, respectively) was not altered in most of the areas studied just after 1h of IMO, except for an increased activation in the BSTMv and a lesser activation in the LSv in IMO 1wk group. At 1h post-IMO there was a faster reduction of c-fos mRNA levels in the LSv to the same extent in both IMO 1 and 4wk groups, and also in the PVN and LC. In the PVN, the faster reduction was more accentuated in IMO 1wk than in IMO 4 wk group. A tendency to display greater c-fos mRNA levels in previously-stressed animals was also found in the CA1 subfield of the hippocampal formation. Finally, irrespective of the time point, there was a reduced c-fos mRNA response in the MeA and NST in IMO 1wk group.

The marginally enhanced c-fos mRNA activation in the CA1 division of the hippocampal formation suggests a partial role of this structure in the reduced HPA axis activation of previously-immobilised animals. It is known that the hippocampal formation exerts an inhibitory effect on the HPA axis activation, limiting the duration of the HPA axis response to stress [112], and these effects seem to be driven by the ventral subiculum [97]. Nonetheless, there are no anatomical data showing a direct connection between the ventral subiculum and the PVN. Instead, the ventral subiculum projects to different areas that in turn innervate the PVN, such as the BST, MPOA, DM and AH [48]. Whereas most of these PVN-projecting areas seem to be inhibitory, containing GABAergic neurons [48], projections from the ventral subiculum seem to be essencially excitatory [292]. Thus, the inhibitory effects of this area on the HPA axis activity seem to be mediated through the GABAergic activity of the areas that in turn innervate the PVN [32]. This structural and functional organisation would be consistent with the fact the c-fos mRNA response after IMO is enhanced in the BSTMv of previously-immobilised rats. The ventral division of the BSTM could then be one of the places of neuronal plasticity that would provoke a more rapid switch-off of the central HPA axis response through and increase of the inhibitory pathways to the PVN. In fact, the BST is considered as a source of local inhibitory inputs to PVN neurons [31].

Another area displaying differential c-fos mRNA activation in previously-immobilised animals is the LSv. This division of the lateral septal complex receives inputs from the ventral parts of the CA1 and subiculum, and has dense bi-directional connections

with the periventricular zone of the hypothalamus, including sparse connections with the medial pPVN [211]. It has been suggested that the lateral septal complex influences neuroendocrine responses through both indirect and direct routes projecting to the hypothalamus, since it has been shown to project to the AV, PeV, MPOA, DM and PMV [211]. The LSv has been related to visceromotor and endocrine responses and in modulation of ingestive behaviour [211]. In addition, a decrease in FLI in the LS has been found in learned helplessness, suggesting the importance of this area in mediating behavioural responses to inescapable stress [254].

Interestingly, the MeA, which is known to project to BSTMv [66] and it is also bi-directionally connected with the LSv [211], was also affected by previous IMO exposure. Therefore, this area, generally agreed to be an important mediator of the HPA axis response to an emotional stressor [57], could be the main place of neuronal plasticity involved in the long-term effects of IMO. Furthermore, it has been suggested that the MeA would send projections to the BST through GABAergic neurons, thus limiting the inhibition of the PVN exerted by the BST [96]. This could explain the results described before, since a lesser activation of the MeA could explain the increased neuronal activity observed in the BSTMv. Finally, the decreased c-fos mRNA activation in the LC and NST appears to be secondary to the reduced activation of higher structures such as the MeA and the PVN, when taking into account the mechanisms of activation of mainly emotional stressors such as footshock [152].

Long-term effects of LPS on the brain c-fos mRNA response to the homotypic stressor

At 2h post-injection, there was only a marginal reduction of the c-fos mRNA response to LPS in the SO in animals previously given LPS 4 weeks before. There were more areas affected by previous LPS injection at 4h post-injection: a greater decrease of c-fos mRNA levels was found in the CeA, BSTL, pPVN and LC. C-fos mRNA levels in the mPVN were reduced independently of the time point studied. Interestingly, qualitative analysis of the c-fos mRNA induction in leptomeninges, ependymal cells and choroid plexus suggested a slight reduction in rats having a previous experience with LPS.

Although it has been shown that LPS-induced c-fos mRNA activation originates from neurons of the NST and LPB directly projecting to the CeA [272], a contribution of both areas to the long-term effects of LPS is unlikely since c-fos mRNA activation in these nuclei was not influenced by previous LPS injection. However, we suggest that these areas may be important for the processing of LPS signals, but the place of synaptic

plasticity, leading to a reduced response to the last LPS dose, may be the CeA (see below). The contribution of the VLM to the processing of LPS appears to be less important because a lower activation than that in LPB and NST was observed, in accordance with other reports (e.g. [89]), and there are few VLM neurons activated by LPS that project to CeA [272]. LPS induction of brain c-fos mRNA has been reported to be reduced by depletion of brain serotonin biosynthesis with paraclorophenylalanine (PCPA), an irreversible inhibitor of tryptophan hydroxylase [139]. However, we only observed a weak activation of some raphe nuclei (e.g. RPa) by LPS administration. In addition, c-fos mRNA in the RPa was only detected at 4 h post-injection and was not sensitive to a previous experience with the endotoxin. Therefore, a major role of the serotoninergic system either in the activation of the HPA axis caused by LPS or in the long-term effects of LPS appears to be unlikely, although a permissive role should not be disregarded.

The only brainstem nucleus showing a differential activation depending on previous LPS experience was the LC: there was a reduced c-fos mRNA expression in this nucleus in previously LPS-injected animals. Nonetheless, the primary site of long-term stress-induced plasticity does not appear to be the LC since the main direct stimulatory inputs to the LC originate from the LPGi [284], which was not activated by LPS under our conditions, in accordance with a previous report [295]. Secondly, there are only limited connections between the LC and PVN [179, 234].

We favour the hypothesis that the sensitivity of the LC to previous experience with LPS may be due to descending stimulatory inputs from proencephalic regions. The present results suggest a close relationship between CeA, BSTL and pPVN. The CeA may be the place of synaptic plasticity leading to a reduced response to LPS induced by a previous LPS experience. This area would in turn convey information to the PVN through BSTL [66, 233], thus resulting in a reduced HPA activation caused by another injection of LPS. In addition, the CeA, BSTL and PVN may well contribute to the activation of the LC since all of them send projections to the peri-LC region and dendrites of the LC neurons contact with peri-LC neurons [284], thus explaining the reduction of the c-fos mRNA response to LPS in previously LPS-injected animals.

Conclusions

From these results we observe that the only common areas affected by a previous exposure to the homotypic stimulus are the PVN and the LC. Nonetheless, taking into account the different mechanisms of brain activation by processive or systemic

stressors [96], we can hypothesise that the source the changes in the activation of these two nuclei might be different depending on the stressor, making it difficult to think that the changes in the PVN and LC would have a direct causal relationship. We hypothesise that in the case of IMO, the main site of synaptic plasticity would be the MeA, together with the LSv, and in the case of LPS, it would be the CeA. The changes in the activation in the MeA-LSv and CeA would account, respectively, for the changes observed in the BSTMv and BSTL, the area generally agreed to function as a relay of limbic information to the PVN. The mechanisms involved in the long-term effects of stress on HPA axis activation seem to relay in different brain areas, in accordance with the differential mechanisms of HPA axis activation by stressors of different nature [96]. These results support our previous findings (Chapter 2) suggesting that the long-term effects of stress are stressor-specific. However, it is clear that the limbic system plays a dominant role in the long-term effects of stress, supporting the idea that these effects could be a form of memory linked to stressful (or even traumatic) events. Clearly, more studies will be necessary in order to fully elucidate the mechanisms involved in this phenomenon, although the present results have allowed defining some of the possibly involved areas.

GENERAL DISCUSSION

A previous report from our laboratory has demonstrated that a single exposure to an emotional stressor of high intensity such as IMO is able to modify the forthcoming HPA axis response to the same stressor days to weeks later [175]. In the present work we have extended our studies on this phenomenon and established that a single high dose of a systemic stressor with immune characteristics such as LPS exerts similar effects on the response of the HPA axis to a new LPS injection. Furthermore, we have also performed a more detailed description of the effects of a single IMO or LPS exposure on the transcriptional activation in the PVN after a new exposure to the homotypic stressor. At the peripheral HPA level, these effects are characterised by a faster recovery of basal ACTH and corticosterone levels after the second exposure to the stressor. Whereas the peripheral effects take place in a few days after the first stress experience, an incubation period of at least 4 weeks is needed to observe the reduced response at the central level of the HPA axis, as determined by the measure of CRF mRNA and CRF hnRNA in the PVN, the main regulatory output site of HPA axis activity. Interestingly, the neuronal activity of this hypothalamic nucleus is already reduced 1 week after the first stressful experience, as shown by the determination of the mRNA for the IEG c-fos. In addition, LPS not only affects the HPA axis response, but also modifies other physiological parameters: previous LPS injection reduces the degree hypothermia and the peripheral TNF-α response to a new LPS administration. Generally speaking, these long-term effects of stress appear to be stressor-specific, since no cross-desensitisation has been found between IMO and LPS.

The fact that we were able to generalise our previous findings with IMO to a different stressor such as LPS is of great importance since it shows that long-term stress-induced desensitisation is not restricted to a single particular stressor, indicating that this phenomenon is an important one regarding the long-term consequences of stress. In fact, Rivier and co-workers have shown that a different stimulus such as intragastric alcohol administration, which also stimulates *per se* the HPA axis, is also able to desensitise the central and peripheral HPA axis response to a new exposure to the same compound 1 week later [144, 147]. There are many common features between these studies with ethanol administration and our studies with IMO and LPS. At the peripheral level, there is a faster recovery of ACTH and corticosterone basal levels after the new exposure to the homotypic stimulus ([144, 147, 175], present results). At the central level, there is a desensitisation of the CRF hnRNA and CRF and c-fos mRNA response in the PVN of previously-stressed animals. Nonetheless, whereas the reduction of the CRF mRNA and c-fos mRNA response in this nucleus is observed with both IMO and LPS, we failed to detect any significant changes in the levels of CRF hnRNA after LPS in previously LPS-injected animals, in contrast to what has been

observed with IMO and ethanol administration. We fail to understand the reason of these apparently contradictory results, since there was indeed a blunted response of PVN CRF mRNA to LPS in previously LPS-injected animals. It might be that the different mechanisms of HPA axis activation depending on the category of the stressor could be the reason to explain this differences. Additionally, it could be that the blunted CRF mRNA response after LPS in previously LPS-injected animals would not be explained by a reduction of the transcriptional activity of this gene, but rather by a alternative mechanisms at the post-transcriptional level. It is clear that more studies will be necessary to confirm these results and rule out possible technical problems that might have brought these rather surprising results.

Interestingly, both the studies with ethanol [147] and the present results with IMO and LPS did not show any long-term effects on the AVP hnRNA response in the PVN. However, ethanol pre-administration reduces the AVP, and also CRF, protein levels in both the external and internal zones of the ME [147]. It would be indeed very interesting to extend our studies by measuring CRF and AVP protein levels in the ME with IMO and LPS in order to establish the possible similarities or discrepancies between these stress models in the long-term consequences of stress on HPA axis activity. However, a modified transcriptional activation in the PVN does not necessarily have to be linked to changes in the release of PVN neuropeptides at the level of the ME, since mechanisms regulating neuropeptide synthesis are different from those regulating their release.

The role of the receptors for these neuropeptides in the long-term effects of stress should not be disregarded either. In particular, measurement of CRF_1 receptor levels in the PVN and pituitary would be of great potential interest because of its suggested role in the regulation of HPA axis activity by means of positive (in the PVN) and negative (in the pituitary) feedback mechanisms [68, 106, 168]. In fact, ethanol pre-administration has been shown to reduce the ethanol-induced CRF_1 receptor mRNA activation in the PVN [147]. Nonetheless, preliminary studies from our laboratory failed to detect any differences in the degree of LPS-induced PVN CRF_1 receptor mRNA activation in previously LPS-injected animals (data not shown).

A very important feature of the long-term effects of ethanol, IMO and LPS, is that it appears to be a phenomenon of selective neuroendocrine tolerance. That is, the three stimuli have failed to modify the HPA response to a superimposed heterotypic stimulus in the long-term. Previous ethanol injection did not affect the ACTH response to an emotional stimulus such as electric shock [144] or an immunological one, such

as IL-1β or LPS administration [217]. Similarly, our previous results showed that previous IMO is not able to modify the peripheral HPA axis response to forced swimming [175]. In the present work, either previous IMO or LPS exposure also failed to affect both the central and peripheral HPA axis responses to the respective heterotypic stressor. Nonetheless, some non-specific features have also been found with ethanol and IMO. A small sensitisation of LPS-induced plasmatic IL-6 levels and an increase of the IgG response to an antigenic challenge have been found one week after ethanol administration [217]. In the present work we also found a non-specific desensitisation of the PVN c-fos mRNA after LPS injection in previously immobilized animals. In addition, whereas a previous LPS exposure failed to alter the tonic GR mRNA levels in the hippocampal formation, a previous IMO session induced, 4 weeks later, a small but detectable increase of this transcript in the DG. The exact meaning of these minor non-specific effects is, at present, unknown, and more studies would be necessary in this regard.

The consistence of all these results regarding the long-term desensitisation of the HPA activity by previous stress exposure clearly suggests that this is an important phenomenon. Nonetheless, we cannot overlook that other researchers have described a long-term sensitising effect of a previous stress experience on the HPA axis activity. For instance, two weeks after a single short session of footshock there was a tonic increase in the AVP, but not CRF, immunoreactivity in the ZEME and a sensitised initial (6 minutes after stress) ACTH, but not corticosterone, response to a new stressor such as the noise test [285]. In a separate report they confirmed their results regarding AVP immunoreactivity in the ZEME, although, differently from what they previously described, footshock was also able to increase CRF stores in the ZEME both 7 and 11 days after the stressful experience [237]. These results suggest that the detection of the changes observed in the long-term might depend on subtle differences that seem to be difficult to control. These authors have also indicated that not all stressful stimuli are able to modify the AVP levels in the ZEME in the long term [237], indicating that the nature of the stressor might be an important factor to consider in this phenomenon.

Interestingly, some of the results obtained by Tilders and co-workers share some characteristics with our present results. For instance, both ACTH and corticosterone levels were significantly reduced 1h hour after noise in previously-shocked animals when compared to stress-naïve animals [285]. Although this finding is not specific for the stressor used, differently from the present results, the pattern of peripheral HPA axis activity is similar to what we have described with IMO and LPS, since our results show

no ACTH and corticosterone desensitisation at the initial period after stress but at the post-stress period ([175], present results). In addition, the binding capacity of both GR and MR in the hippocampus was increased by previous shock exposure 2 weeks before [285], suggesting the possibility for an increased efficacy of negative feedback.

This wide range of possibilities regarding the direction (sensitisation/desensitisation) of the changes produced by a single stress exposure in the long-term is indeed intriguing. We suggest that one important factor involved in the direction of the changes, apart from the nature of the stressor already suggested, might be the intensity of the stressor applied. Regarding the type of stressor, we have found desensitising effects of IMO or LPS on the peripheral HPA axis to the same stressor. In contrast, a metabolic challenge such as insulin injection in fasted rats does not affect the future peripheral HPA axis response to the same stressor (data not shown). Regarding the intensity of the stressor, whereas those reports describing sensitising effects of stress use rather mild stressors (short stress exposure or low dose of LPS/cytokine), we have used very intense stressors. The possibility remains that the intensity of the first stress exposure would be crucial in determining the direction of the future induced changes. Additionally, the intensity of final stressor could also be important, since a highly intense stressor might produce a maximal hormonal response, thus masking the possible sensitising effects of previous stress. In a pilot experiment, we addressed these two issues by administering either a low (0.01 mg/kg) or a high (1 mg/kg) dose of LPS on day 1 and, one week later, we evaluated the ACTH response to the low dose of LPS. Animals that had received a previous high dose of LPS showed long-term desensitisation to a low dose of the same stimulus. However, the group receiving a low LPS dose on day 1 showed a non-significant tendency to display a sensitised ACTH response to another low those of LPS. These results have similarities with the long-term sensitising effects of low doses of TNF-α or LPS (0.005 mg/kg) on the HPA axis response to TNF-α and/or LPS [91-93], although, in this case, the effects of LPS (in contrast to the effects of TNF-α) have not shown to be extremely long-lasting [92]. Clearly, more studies will be necessary to fully elucidate the importance of stress intensity on the direction of the long-term observed changes. Nonetheless, at least one previous study supports our view, since it has been shown that a single exposure to stressors of low or high intensity is able to increase or decrease, respectively, the future response of the animals to haloperidol [4].

In spite of the differences that we have been previously discussing, there is an underlying and very important common feature in the long-term effects of a single

stress exposure: independently of the direction (sensitisation/desensitisation) of the changes, the effects develop slowly (days to weeks) and are extremely long-lasting (weeks). This indicates that this phenomenon is not a pure biochemical adaptation but, instead, some kind of maturational process takes place, similar to the process of consolidation of long-term memory [178]. Intriguingly, our results show a clear temporal dissociation between the central and peripheral levels of the HPA axis when taking into account the development of this long-term desensitisation. Whereas a more rapid recovery of basal ACTH and corticosterone levels after IMO or LPS is already evident 1 week after the initial exposure to the homotypic stimulus, or even sooner [175], an incubation period of at least 4 weeks is necessary to detect any changes in the levels of CRF mRNA in the PVN.

We also measured the transcriptional activity of the CRF gene by means of the recently developed intronic technology, and failed to detect any signs of desensitisation of the CRF hnRNA response to IMO in previously immobilised animals 1 week before, in contrast to the group stressed 4 weeks before, which showed reduced CRF hnRNA levels after IMO when compared to the animals without any previous experience with stress. Interestingly, the IMO-induced response of c-fos mRNA in the PVN showed clear signs of desensitisation at 1 week after the first IMO exposure, in parallel with the dynamics of the phenomenon at the peripheral HPA level. The effect of previous IMO on c-fos mRNA induction in the PVN partially evanished 4 weeks after the first stress experience, similarly to plasma ACTH, whereas plasma corticosterone showed the same pattern both 1 and 4 weeks after the first IMO session. These data suggest that c-fos mRNA might be an index of ACTH, but not corticosterone release, and that there is a partial dissociation between both hormones. The dissociation between ACTH and corticosterone is not a new phenomenon in the field of the HPA axis [290] although its true physiological meaning in specific conditions has not been properly explored.

Assuming that c-fos mRNA induction is a marker of the degree of neuronal activation caused by stimulatory inputs arriving at the PVN, it is clear that previous exposure to IMO induced a dissociation between the activation of PVN neurons leading to the release of ACTH secretagogues into the pituitary portal blood and the expression of c-fos mRNA, and particularly CRF mRNA and hRNA. From a mechanistic point of view, such dissociation is not surprising. Some reports have shown that some stressors are able to activate the release of HPA hormones with no effect on c-fos mRNA induction in the PVN (e.g. [40]), suggesting that some stimulatory signals to the PVN are of enough magnitude to activate the release of HPA hormones but not to induce a

significant increase in c-fos mRNA expression. Similarly, it has been demonstrated, using hypovolemia as a stressor, that the threshold for the release of ACTH is lower than that for the induction of CRF gene expresion in the PVN [264].

From the present results, it appears that a single stress experience results in a less sustained stimulation of PVN neurons when the animals were confronted again with the same stressor. Since both the peripheral and central HPA axis response to a novel stressor is normal in previously stressed rats (present results, [175]), it is unlikely the main locus of the long-term changes caused by the first exposure to stress would be the PVN itself. More likely, the differential response observed in the PVN of previously stressed rats could be the result of changes in the inputs reaching the PVN from other brain areas involved in the processing particular stimuli [96]. In order to elucidate the areas involved in this phenomenon, we studied the IMO and LPS-induced degree of neuronal activation by measuring c-fos mRNA levels throughout the whole brain in rats previously subjected to the homotypic stimulus.

Due to the greatly different nature of these two stressors and their mechanisms regarding HPA axis activation, IMO and LPS induced a very different pattern of c-fos mRNA activation, in accordance with previous studies (e.g. [231, 246]). IMO induced a wide expression of this IEG in the whole brain, including the cortex, limbic system (LS, BSTMv, MeA, hippocampus), many thalamic and hypothalamic nuclei and brainstem nuclei. In contrast, the pattern of c-fos mRNA expression after LPS was limited to a few limbic areas (CeA, BSTL), a lesser number of thalamic and hypothalamic nuclei and also to the CVOs (areas of the brain devoid BBB), and meninges.

As hypothesised, a previous experience with the homotypic stimulus induced a differential activation of some of these brain nuclei. In previously immobilised animals, a reduction of the c-fos mRNA response was found in the LSv, MeA, PVN, LC and NST, together with an increased expression of this IEG in the BSTMv and a tendency towards it in the CA1 subfield of the hippocampal formation. In contrast, the areas displaying differential LPS-induced c-fos mRNA activation in previously LPS-injected animals included the CeA, BSTL, PVN and LC. As previously discussed (see Chapter 4), we suggest that the sites of synaptic plasticity would be different depending on the stimulus applied. In previously immobilised animals the areas involved would be the LSv, MeA and BSTMv, whereas the CeA, together with the BSTL, would be the ones in previously LPS-injected rats. The changes observed in brainstem nuclei (LC, NST) would be regarded as a secondary consequence of the reduced activation of

higher brain structures such as the PVN, rather than being the primary site of synaptic plasticity involved in the long-term effects of stress.

These results are roughly in accordance with a previous study regarding the differential FLI found after a novel stress in animals previously exposed to footshock [34]. As previously described, footshock is known to induce neuroendocrine, behavioural and autonomic sensitisation in the long-term [35, 36, 237, 241, 252, 253, 285]. In accordance with these sensitisation effects, a previous experience with this stressor induced a greater FLI in different brain areas, including the BST, basolateral amygdala (BLA), CA1 hippocampal subfield, PVN and LC, among others. These findings, together with the present results, suggest that the main sites of synaptic plasticity involved in the long-term changes produced by a single stress experience would mainly involve integrative and effector areas related to the limbic system. We suggest that the nature of the stressor is a determining factor regarding the exact sites of plasticity, but the fact that limbic-related areas seem to play a main role in this phenomenon is very interesting and suggests that we are dealing with some form of memory related to particularly severe stressors. Now that some of the presumably important areas regarding long-term consequences of stress have been described, the next step would be to lesion some of these areas and study if the phenomenon can be abolished. It would also be important to determine the neurotransmitter systems involved, using selective antagonists locally injected in the area/s of interest in order to find pharmacological tools to block the phenomenon.

In sum, we have reported that an intense single stress experience of either emotional or systemic nature can induce alterations on the future neuroendocrine stress response of the animals to the same, but not to a novel stimulus. These changes are characterised by a slowly developing and long-lasting HPA desensitisation that can be extended to other physiological variables. Study of c-fos mRNA activation throughout the brain suggests that limbic-related areas might be the sites of synaptic plasticity involved in this phenomenon, the specific areas depending on the nature of stressor. These results are of great importance regarding the effects of stress, and we suggest that the observed desensitisation of the HPA axis response to the homotypic stimulus is protective for the organism, when taking into account the negative consequences of an exaggerated stress response on the organisms. Considering the specificity of the phenomenon, this form of synaptic plasticity could be regarded as a new type of learning-like memory related to severe stressors. The implications of the long-term effects of a mainly emotional stimulus such as IMO on some stress-related human pathologies such as posttraumatic stress disorder cannot be disregarded. The

further study of this phenomenon and the elucidation of the exact mechanisms involved will be crucial for better understanding the long-term effects of stress in adult animals.

CONCLUSIONS

1. Long-term effects of a single stress exposure on the HPA axis and other physiological variables can be observed not only in response to IMO, a mainly emotional stressor, but also to a stressor of different nature such as LPS, regarded as a systemic stressor with immune characteristics.

2. A single LPS injection increases the rate of recovery of ACTH and corticosterone after a new LPS injection both 1 and 4 weeks later. The effects on ACTH are comparable with inter-stress intervals of 1 or 4 weeks, whereas enhanced post-stress corticosterone recovery is more marked 1 week after the first LPS injection.

3. At the central level of the HPA axis, the long-term effects of LPS are characterised by a reduction of the LPS-induced CRF mRNA response in the PVN 4, but not 2, weeks later, together with a reduced c-fos mRNA response. The results obtained with CRF hnRNA are inconclusive, whereas AVP hnRNA was not modified by a previous LPS experience.

4. A single LPS exposure does not exclusively affect the future HPA axis response to the same stressor but other physiological parameters as well. We found a reduction of the LPS-induced hypothermia 4 weeks after a single LPS injection and a lower plasma TNF-α response to LPS 1 and 4 weeks after the first LPS experience.

5. A previous IMO session increases, 1 week later, the speed of recovery of ACTH and corticosterone after a new IMO session. These effects are more long-lasting for corticosterone and evanish sooner for ACTH, suggesting a dissociation between ACTH and corticosterone.

6. After a previous single experience with IMO, the rapid transcriptional activation in the PVN is reduced weeks after a new exposure to the same stressor. These effects at the PVN level are characterised by an increased recovery of basal c-fos mRNA and lower CRF hnRNA levels after a new IMO session, although the time between the two exposures needed to reach a maximal effect depended on the variable studied. We failed to detect any effects of a previous IMO session in the degree of AVP hnRNA activation in the PVN.

7. Long-term effects of stress on the HPA axis appear to be stressor-specific, as we found no signs of cross-desensitisation between IMO and LPS at both peripheral and central HPA levels.

8. Whereas previous LPS injection did not modify the PVN c-fos mRNA response to IMO 4 weeks later, previous IMO reduced the c-fos mRNA response to a final LPS administration 4 weeks later. This suggests that there is some cross-desensitisation of c-fos mRNA induction, whose exact meaning remains to be determined.

9. A previous exposure to IMO or LPS does not alter the tonic GR mRNA levels in the PVN 4 weeks later, or the levels of this transcript in the hippocampal formation, except for a small but significant increase in the DG of previously immobilised animals. It is unlikely that such changes are important to explain the long-term effects of stress on the HPA axis.

10. A single stress exposure to IMO or LPS modifies the degree of neuronal activation in specific brain areas, as revealed by c-fos mRNA response to the homotypic stimulus 4 weeks after the first stressful experience. The areas sensitive to previous experience with the stressor included some extended amygdala regions, the PVN and some brainstem nuclei.

11. Considering the projections and neurochemical characteristics of the areas displaying a modified c-fos mRNA activation by a previous stress experience, we suggest that the main sites of neuronal plasticity leading to a reduced response to the homotypic stressor would be the CeA, in the case of LPS, and the LSv and MeA in the case of IMO. Changes in the PVN and brainstem nuclei will be secondary to changes in the above-mentioned areas.

12. The finding that the effects of a single exposure to stress on the HPA axis appears to accentuate with time, suggests that it may be a model to study the process involved in long-term memory consolidation. In addition, the possibility also remains that memory of traumatic events may also be blunted by pharmacological treatments for a period of time after exposure to the situation.

REFERENCES

1. Aguilera, G., C. Rabadan-Diehl, and M. Nikodemova. Regulation of pituitary corticotropin releasing hormone receptors. *Peptides* 22: 769-774, 2001.

2. Alheid, G., J. S. de Omos, and C. A. Beltramino. Amygdala and extended amygdala. In: *The Rat Nervous System* (2nd ed.), edited by G. Paxinos. Sydney: Academic Press, 1995, p. 495-578.

3. Antelman, S. M., A. R. Caggiula, S. Knopf, D. J. Kocan, and D. J. Edwards. Amphetamine or haloperidol 2 weeks earlier antagonized the plasma corticosterone response to amphetamine; evidence for the stressful/foreign nature of drugs. *Psychopharmacology* 107: 331-336, 1992.

4. Antelman, S. M., A. R. Caggiula, D. Kocan, S. Knopf, D. Meyer, D. J. Edwards, and H. Barry III. One experience with 'lower' or 'higher' intensity stressors, respectively enhances or diminishes responsiveness to haloperidol weeks later: implications for understanding drug variability. *Brain Res.* 566: 276-283, 1991.

5. Antelman, S. M., L. A. DeGiovanni, and D. Kocan. A single exposure to cocaine or immobilization stress provides extremely long-lasting, selective protection against sudden cardiac death from tetracaine. *Life Sci.* 44: 201-207, 1989.

6. Antelman, S. M., L. A. DeGiovanni, D. Kocan, J. M. Perel, and L. A. Chiodo. Amitriptyline sensitization of a serotonin-mediated behavior depends on the passage of time and not repeated treatment. *Life Sci.* 33: 1727-1730, 1983.

7. Antelman, S. M., S. Knopf, D. Kocan, D. J. Edwards, J. C. Ritchie, and C. B. Nemeroff. One stressful event blocks multiple actions of diazepam for up to at least a month. *Brain Res.* 445: 380-385, 1988.

8. Antelman, S. M., D. Kocan, D. J. Edwards, and S. Knopf. A single injection of diazepam induces long-lasting sensitization. *Psychopharm. Bull.* 23: 430-434, 1987.

9. Antelman, S. M., D. Kocan, D. J. Edwards, S. Knopf, J. M. Perel, and R. Stiller. Behavioral effects of a single neuroleptic treatment grow with the passage of time. *Brain Res.* 358: 58-67, 1986.

10. Antelman, S. M., D. Kocan, S. Knopf, D. J. Edwards, and A. R. Caggiula. One brief exposure to a psychological stressor induces long-lasting, time-dependent sensitization of both the cataleptic and neurochemical responses to haloperidol. *Life Sci.* 51: 261-266, 1992.

11. Antelman, S. M., D. Kocan, N. Rowland, L. de Giovanni, and L. A. Chiodo. Amitriptiline provides long-lasting immunization against sudden cardiac death from cocaine. *Eur. J. Pharmacol.* 69: 119-120, 1981.

12. Antoni, F. A. Hypothalamic control of adrenocorticotropin secretion: advances since the discovery of 41-residue corticotropin-releasing factor. *Endocrine Rev.* 7: 351-378, 1986.

13. Arima, H., K. Kondo, S. Kakiya, H. Nagasaki, H. Yokoi, T. Murase, Y. Iwasaki, and Y. Oiso. Rapid and sensitive vasopressin heteronuclear RNA responses to changes in plasma osmolarity. *J. Neuroendocrinol.* 11: 337-341, 1999.

14. Armario, A., M. Gil, J. Martí, O. Pol, and J. Balasch. Influence of various acute stressors on the activity of adult male rats in a holeboard and in the forced swim test. *Pharmacol. Biochem. Behav*. 39: 373-377, 1991.

15. Armario, A., J. Hidalgo, and M. Giralt. Evidence that the pituitary adrenal axis does not cross-adapt to stressors: comparison to other physiological variables. *Neuroendocrinology* 47: 263-267, 1988.

16. Armario, A., and T. Jolín. Influence of intensity and duration of exposure to various stressors on serum TSH and GH levels in adult male rats. *Life Sci.* 44: 215-221, 1989.

17. Armario, A., A. López-Calderón, T. Jolín, and J. M. Castellanos. Sensitivity of anterior pituitary hormones to graded levels of psychological stress. *Life Sci.* 39: 471-475, 1986.

18. Armario, A., J. Martí, and M. Gil. The serum glucose response to acute stress is sensitive to the intensity of the stressor and to habituation. *Psychoneuroendocrinology* 15: 341-347, 1990.

19. Aubry, J. M., V. Bartanusz, D. Jezova, D. Belin, and J. Z. Kiss. Single stress induces long-lasting elevations in vasopressin mRNA levels in CRF hypophysiotrophic neurones, but repeated stress is required to modify AVP immunoreactivity. *J. Neuroendocrinol.* 11: 377-384, 1999.

20. Bai, F. L., M. Yamano, Y. Shiotani, P. C. Emson, A. D. Smith, J. F. Powell, and M. Tohyama. An arcuato-paraventricular and -dorsomedial hypothalamic neuropeptide Y-containing system which lacks noradrenaline in the rat. *Brain Res.* 331: 172-175, 1985.

21. Bakshi, V. P., S. Smith-Roe, S. M. Newman, D. E. Grigoriadis, and N. H. Kalin. Reduction of stress-induced behavior by antagonism of corticotropin-releasing hormone 2 (CRH_2) receptors in lateral septum or CRH_1 receptors in amygdala. *J. Neurosci.* 22: 2926-2935, 2002.

22. Bartanusz, V., J.-M. Aubry, T. Steimer, J. Baffi, and J. Z. Kiss. Stressor-specific increase of vasopressin mRNA in paraventricular hypophysiotropic neurons. *Neurosci. Lett.* 170: 35-38, 1994.

23. Bennett, M. R., and W. J. Schwartz. Are glia among the cells that express immunoreactive c-Fos in the suprachiasmatic nucleus? *NeuroReport* 5: 1737-1740, 1994.

24. Bernardini, R., T. C. Kamilaris, A. E. Calogero, E. O. Johnson, M. T. Gomez, P. W. Gold, and G. P. Chrousos. Interactions between tumor necrosis factor-alpha, hypothalamic corticotropin-releasing hormone, and adrenocorticotropin secretion in the rat. *Endocrinology* 126: 2876-2881, 1990.

25. Beutler, B. Tlr4: central component of the sole mammalian LPS sensor. *Curr. Opin. Immunol.* 12: 20-26, 2000.

26. Bhatnagar, S., and M. Dallman. Neuroanatomical basis for facilitation of hypothalamic-pituitary-adrenal responses to a novel stressor after chronic stress. *Neuroscience* 84: 1025-1039, 1998.

27. Bittencourt, J. C., R. Benoit, and P. E. Sawchenko. Distribution and origins of substance P-immunoreactive projections to the paraventricular and supraoptic nuclei: partial overlap with ascending catecholaminergic projections. *J. Chem. Neuroanat.* 4: 63-78, 1991.

28. Bittencourt, J. C., and P. E. Sawchenko. Do centrally administered neuropeptides access cognate receptors? An analysis in the central corticotropin-releasing factor system. *J. Neurosci.* 20: 1142-1156, 2000.

29. Bohus, B., and J. M. Koolhaas. Stress and the cardiovascular system: central and peripheral physiological mechanisms. In: *Stress: from synapse to syndrome*, edited by S. C. Stanford and P. Salmon. San Diego: Academic Press, 1993, p. 75-117.

30. Bonaz, B., and S. Rivest. Effect of a chronic stress on CRF neuronal activity and expression of its type 1 receptor in the rat brain. *Am. J. Physiol.* 275: R1438-R1449, 1998.

31. Boudaba, C., K. Szabó, and J. G. Tasker. Physiological mapping of local inhibitory inputs to the hypothalamic paraventricular nucleus. *J. Neurosci.* 16: 7151-7160, 1996.

32. Bowers, G., W. E. Cullinan, and J. P. Herman. Region-specific regulation of glutamic acid decarboxilase (GAD) mRNA expression in central stress circuits. *J. Neurosci.* 18: 5938-5947, 1998.

33. Brambilla, F. Social stress in anorexia nervosa: a review of immuno-endocrine relationships. *Physiol. Behav.* 73: 365-369, 2001.

34. Bruijnzeel, A. W., R. Stam, J. C. Compaan, G. Croiset, L. M. Akkermans, B. Olivier, and V. M. Wiegant. Long-term sensitization of Fos-responsivity in the rat central nervous system after a single stressful experience. *Brain Res.* 819: 15-22, 1999.

35. Bruijnzeel, A. W., R. Stam, J. C. Compaan, and V. M. Wiegant. Stress-induced sensitization of CRH-ir but not P-CREB-ir responsivity in the rat central nervous system. *Brain Res.* 908: 187-196, 2001.

36. Bruijnzeel, A. W., R. Stam, G. Croiset, and V. M. Wiegant. Long-term sensitization of cardiovascular stress responses after a single stressful experience. *Physiol. Behav.* 73: 81-86, 2001.

37. Bruijnzeel, A. W., R. Stam, and V. M. Wiegant. LY354740 attenuates the expression of long-term behavioral sensitization induced by a single session of foot shocks. *Eur. J. Pharmacol.* 426: 77-80, 2001.

38. Buller, K. M. Role of circumventricular organs in pro-inflamatory cytokine-induced activation of the hypothalamic-pituitary-adrenal axis. *Clin. Exp. Pharmacol. Physiol.* 28: 581-589, 2001.

39. Buwalda, B., S. F. de Boer, E. D. Schmidt, K. Felszeghy, C. Nyakas, A. Sgoifo, B. J. Van der Vegt, F. J. H. Tilders, B. Bohus, and J. M. Koolhaas. Long-lasting deficient dexametasone suppression of hypothalamic-pituitary-adrenocortical activation following peripheral CRF challenge in socially defeated rats. *J. Neuroendocrinol.* 11: 513-520, 1999.

40. Campeau, S., W. A. Falls, W. E. Cullinan, D. L. Helmreich, M. Davis, and S. J. Watson. Elicitation and reduction of fear: behavioural and neuroendocrine indices and brain induction of the immediate-early gene c-fos. *Neuroscience* 78: 1087-1104, 1997.

41. Carobrez, S. G., O. C. Gasparotto, B. Buwalda, and B. Bohus. Long-term consequences of social stress on corticosterone and IL-1b levels in endotoxin-challenged rats. *Physiol. Behav* 76: 99-105, 2002.

42. Chalmers, D. T., T. W. Lovemberg, and E. B. De Souza. Localization of novel corticotropin-releasing factor receptor (CRF_2) mRNA expression to specific subcortical nuclei in the rat brain: comparison with CRH_1 receptor mRNA expression. *J. Neurosci.* 15: 6340-6350, 1995.

43. Champagne, D., J. Beaulieu, and G. Drolet. CRFergic innervation of the paraventricular nucleus of the rat hypothalamus: a tract-tracing study. *J. Neuroendocrinol.* 10: 119-131, 1998.

44. Chan, R. K. W., E. R. Brown, A. Ericsson, K. J. Kovács, and P. E. Sawchenko. A comparison of two immediate-early genes, *c-fos* and NGFI-B, as markers for functional activation in stress-related neuroendocrine circuitry. *J. Neurosci.* 13: 5126-5138, 1993.

45. Croiset, G., M. J. M. A. Nijsen, and P. J. G. H. Kamphuis. Role of corticotropin-releasing factor, vasopressin and the autonomic nervous system in learning and memory. *Eur. J. Pharmacol.* 405: 225-234, 2000.

46. Cullinan, W. E., J. P. Herman, D. F. Battaglia, H. Akil, and S. J. Watson. Pattern and time course of immediate early gene expression in rat brain following acute stress. *Neuroscience* 64: 477-505, 1995.

47. Cullinan, W. E., J. P. Herman, D. L. Helmreich, and S. J. Watson Jr. A neuroanatomy of stress. In: *Neurobiological and clinical consequences of stress. From normal adaptation to post-traumatic stress disorder*, edited by M. J. Friedman, D. S. Charney and A. Y. Deutch. Philadelphia: Lippincot-Raven Publishers, 1995, p. 3-26.

48. Cullinan, W. E., J. P. Herman, and S. J. Watson. Ventral subicular interaction with the hypothalamic paraventriclar nucleus: evidence for a relay in the bed nucleus of the stria terminalis. *J. Comp. Neurol.* 332: 1-20, 1993.

49. Cunningham, E. T. J., M. C. Bohn, and P. E. Sawchenko. The organization of adrenergic projections to the paraventricular and supraoptic nuclei of the hypothalamus in the rat. *J. Comp. Neurol.* 292: 651-667, 1990.

50. Cunningham, E. T. J., and P. E. Sawchenko. Anatomical specificity of noradrenergic inputs to the paraventricular and supraoptic nuclei of the rat hypothalamus. *J. Comp. Neurol.* 274: 60-76, 1988.

51. Dallman, M. F. Stress update. Adaptation of the hypothalamic-pituitary-adrenal axis to chronic stress. *Trends Endocrinol. Metab.* 4: 62-69, 1993.

52. Dallman, M. F., S. F. Akana, K. A. Scribner, M. J. Bradbury, C.-D. Walker, A. M. Strack, and C. S. Cascio. Stress, feedback and facilitation in the hypothalamo-pituitary-adrenal axis. *J. Neuroendocrinol.* 4: 517-526, 1992.

53. Dal-Zotto, S., O. Martí, and A. Armario. Influence of single or repeated experience of rats with forced swimming on behavioural and physiological responses to the stressor. *Behav. Brain Res.* 114: 175-181, 2000.

54. Dal-Zotto, S., O. Martí, and A. Armario. Is repeated exposure to immobilization needed to induce adaptation of the hypothalamic-pituitary-adrenal axis? Influence of adrenal factors. *Behav. Brain Res.* 129: 187-195, 2002.

55. Dautzenberg, F. M., G. J. Kilpatrick, R. L. Hauger, and J.-L. Moreau. Molecular biology of the CRH receptors - in the mood. *Peptides* 22: 753-760, 2001.

56. Dayas, C. V., K. M. Buller, J. W. Crane, Y. Xu, and D. A. Day. Stressor categorization: acute physical and psychological stressors elicit distinctive recruitment patterns in the amygdala and in medullary noradrenergic cell groups. *Eur. J. Neurosci.* 14: 1143-1152, 2001.

57. Dayas, C. V., K. M. Buller, and T. A. Day. Neuroendocrine responses to an emotional stressor: evidence for involvement of the medial but not the central amygdala. *Eur. J. Neurosci.* 11: 2312-2322, 1999.

58. De Goeij, D. C. E., D. Jezova, and F. J. H. Tilders. Repeated stress enhances vasopressin synthesis in corticotropin releasing factor neurons in the paraventricular nucleus. *Brain Res.* 577: 165-168, 1992.

59. De Goeij, D. C. E., R. Kvetnansky , M. H. Whitnall, D. Jezova , F. Berkenbosch, and F. J. H. Tilders. Repeated stress-induced activation of corticotropin-releasing factor neurons enhances vasopressin stores and colocatization with corticotropin-relasing factor in the median eminence of rats. *Neuroendocrinology* 53: 150-159, 1991.

60. De Kloet, E. R. Brain corticosteroid receptor balance and homeostatic control. *Front. Neuroendocrinol.* 12: 95-164, 1991.

61. De Kloet, E. R., M. S. Oitzl, and M. Joëls. Functional implications of brain corticosteroid receptor diversity. *Cell. Mol. Neurobiol.* 13: 433-455, 1993.

62. Derijk, R., N. Van Rooijen, F. J. H. Tilders, H. O. Besedovsky, A. Del Rey, and F. Berkenbosch. Selective depletion of macrophages prevents pituitary-adrenal activation in response to subpyrogenic, but not to pyrogenic, doses of bacterial endotoxin in rats. *Endocrinology* 128: 330-338, 1991.

63. Derijk, R. H., M. van Kampen, N. van Rooijen, and F. Berkenbosch. Hypothermia to endotoxin involves reduced thermogenesis, macrophague-dependent mechanisms, and prostaglandins. *Am. J. Physiol.* 266: R1-R8, 1994.

64. Dhabhar, F. S., B. S. Mc Ewen, and R. L. Spencer. Adaptation to prolongued or repeated stress-comparison between rat strains showing intrinsic differences in reactivity to acute stress. *Neuroendocrinology* 65: 360-368, 1997.

65. Dinan, T. G. Serotonin and the regulation of the hypothalamic-pituitary-adrenal axis function. *Life Sci.* 58: 1683-1694, 1996.

66. Dong, H.-W., G. D. Petrovich, and L. W. Swanson. Topography of projections from amygdala to bed nuclei of the stria terminalis. *Brain Res. Rev.* 38: 192-246, 2001.

67. Donohoe, T. P. Stress-induced anorexia: implications for anorexia nervosa. *Life Sci.* 34: 203-218, 1984.

68. Drolet, G., and S. Rivest. Corticotropin-releasing hormone and its receptors; an evaluation at the transcription level in vivo. *Peptides* 22: 761-767, 2001.

69. Dunn, A. J., and C. W. Berridge. Physiological and behavioral responses to corticotropin-releasing factor administration: is CRF a mediator of anxiety or stress responses? *Brain Res. Rev.* 15: 71-100, 1990.

70. Dunn, J. D. Plasma corticosterone responses to electrical stimulation of the bed nucleus of the stria terminalis. *Brain Res.* 407: 327-331, 1987.

71. Elenkov, I. J., J. Kiss, E. Stark, and B. L. CRF-dependent and CRF-independent mechanisms involved in hypophyseal-adrenal system activation by bacterial endotoxin. *Acta Physiol. Hung.* 79: 355-363, 1992.

72. Elmquist, J. K., M. R. Ackermann, K. B. Register, R. B. Rimler, L. R. Ross, and C. D. Jacobson. Induction of Fos-like immunoreactivity in the rat brain following *Pasteurella multocida* endotoxin administration. *Endocrinology* 133, 1993.

73. Elmquist, J. K., and C. B. Saper. Activation of neurons projecting to the paraventricular hypothalamic nucleus by intravenous lipopolysaccharide. *J. Comp. Neurol.* 374: 315-331, 1996.

74. Elmquist, J. K., T. E. Scammell, C. D. Jacobson, and C. B. Saper. Distribution of Fos-like immunoreactivity in the rat brain following intravenous lipopolysaccharide administration. *J. Comp. Neurol.* 371: 85-103, 1996.

75. Elmquist, J. K., T. E. Scammell, and C. B. Saper. Mechanisms of CNS response to systemic immune challenge: the febrile response. *Trends Neurosci.* 20: 565-570, 1997.

76. Emmert, M. H., and J. P. Herman. Differential forebrain c-fos mRNA induction by ether inhalation and novelty: evidence for distinctive stress pathways. *Brain Res.* 845: 60-67, 1999.

77. Faria, M., P. Navarra, S. Tsagarakis, G. M. Besser, and A. B. Grossman. Inhibition of CRH-41 release by substance P, but not substance K, from the rat hypothalamus in vitro. *Brain Res.* 538: 76-78, 1991.

78. Feldman, S., N. Conforti, and D. Saphier. The preoptic area and bed nucleus of the stria terminalis are involved in the effects of the amygdala on adrenocortical secretion. *Neuroscience* 37: 775-779, 1990.

79. Ferguson, A. V., T. A. Day, and L. P. Reanud. Subfornical organ efferents influence the excitability of neurohypophyseal and tuberoinfundibular paraventricular nucleus neurons in the rat. *Neuroendocrinology* 39: 423-428, 1984.

80. García, A., O. Martí, A. Vallès, S. Dal-Zotto, and A. Armario. Recovery of the hypothalamic-pituitary-adrenal response to stress. *Neuroendocrinology* 72: 114-125, 2000.

81. Gillies, G. E., E. A. Linton, and P. J. Lowry. Corticotropin releasing activity of the new CRF is potentiated several times by vasopressin. *Nature* 299: 355-357, 1982.

82. Givalois, L., J. Dornand, M. Mekaouche, M. D. Solier, A. F. Bristow, G. Ixart, P. Siaud, I. Assenmacher, and G. Barbanel. Temporal cascade of plasma level surges in ACTH, corticosterone, and cytokines in endotoxin-challenged rats. *Am. J. Physiol.* 266: R164-R170, 1994.

83. Göttlicher, M., S. Heck, and P. Herrlich. Transcriptional cross-talk, the second mode of steroid receptor action. *J. Mol. Med.* 76: 480-489, 1998.

84. Gray, T. S., M. E. Carney, and D. J. Magnuson. Direct projections from the central amygdaloid nucleus to the hypothalamic paraventricular nucleus: possible role in stress-induced adrenocorticotropin release. *Neuroendocrinology* 50: 433-446, 1989.

85. Greisman, S. E., and R. B. Hornick. Endotoxin tolerance. In: *The role of immunological factors in infectious, allergic, and autoimmune processes*, edited by R. F. Beers Jr. and E. G. Bassett. New York: Raven Press, 1976, p. 43-50.

86. Groves, P. M., and R. F. Thompson. Habituation: a dual-process theory. *Psychol. Rev.* 77: 419-450, 1970.

87. Halbreich, U. *Hormones and depression*. New York: Raven Press, 1987.

88. Harbuz, M. S., A. Chover-Gonzalez, J. Gilbert-Rahola, and D. S. Jessop. Protective effect of prior acute immune challege, but not footshock, on inflamation in the rat. *Brain Behav. Immun.* 16: 439-449, 2002.

89. Hare, A. S., G. Clarke, and S. Tolchard. Bacterial lipopolysaccharide-induced changes in Fos protein expression in the rat brain: correlation with thermoregulatory changes and plasma corticosterone. *J. Neuroendocrinol.* 7: 791-799, 1995.

90. Hauger, R. L., A. Millan, M. Lorang, J. P. Harwood, and G. Aguilera. Corticotropin-releasing factor receptors and pituitary adrenal responses during immobilization stress. *Endocrinology* 123: 396-405, 1988.

91. Hayley, S., K. Brebner, S. Lacosta, Z. Merali, and H. Anisman. Sensitization to the effects of tumor necrosis factor-a: neuroendocrine, central monoamine, and behavioral variations. *J. Neurosci.* 19: 5654-5665, 1999.

92. Hayley, S., S. Lacosta, Z. Merali, N. van Rooijen, and H. Anisman. Central monoamine and plasma corticosterone changes induced by a bacterial endotoxin: sensitization and cross-sensitization effects. *Eur. J. Neurosci.* 13: 1155-1165, 2001.

93. Hayley, S., W. Staines, Z. Merali, and H. Anisman. Time-dependent sensitization of corticotropin-releasing hormone, arginine vasopressin and *c-fos* immunoreactivity within the mouse brain in response to tumor necrosis factor-α. *Neuroscience* 106: 137-148, 2001.

94. Herkenham, M., H. Y. Lee, and R. A. Baker. Temporal and spatial patterns of *c-fos* mRNA induced by intravenous interleukin-1: a cascade of non-neuronal cellular activation at the blood-brain barrier. *J. Comp. Neurol.* 400: 175-196, 1998.

95. Herman, J. P. In situ hybridization analysis of vasopressin gene transcription in the paraventricular and supraoptic nuclei of the rat: regulation by stress and glucocorticoids. *J. Comp. Neurol.* 363: 15-27, 1995.

96. Herman, J. P., and W. E. Cullinan. Neurocircuitry of stress: central control of the hypothalamo-pitutary-adrenocortical axis. *Trends Neurosci.* 20: 78-84, 1997.

97. Herman, J. P., W. E. Cullinan, M. I. Morano, H. Akil, and S. J. Watson. Contribution of the ventral subiculum to inhibitory regulation of the hypothalamo-pituitary-adrenocortical axis. *J. Neuroendocrinol.* 7: 475-482, 1995.

98. Herman, J. P., W. E. Cullinan, and S. J. Watson. Involvement of the bed nucleus of the stria terminalis in tonic regulation of paraventricular hypothalamic CRH and AVP mRNA expression. *J. Neuroendocrinol.* 6: 433-442, 1994.

99. Herman, J. P., M. K.-H. Schafer, R. C. Thompson, and S. J. Watson. Rapid regulation of corticotropin-releasing-hormone gene transcription in vivo. *Mol. Endocrinol.* 6: 1061-1069, 1992.

100. Herman, J. P., and R. Spencer. Regulation of hipocampal glucocorticoid receptor gene transcription and protein expression *in vivo*. *J. Neurosci.* 18: 7462-7473, 1998.

101. Herman, J. P., J. G. Tasker, D. R. Ziegler, and W. E. Cullinan. Local circuit regulation of paraventricular nucleus stress integration. Glutamate-GABA connections. *Pharmacol. Biochem. Behav*. 71: 457-468, 2002.

102. Herrera, D. G., and H. A. Robertson. Activation of *c-fos* in the brain. *Prog. Neurobiol.* 50: 83-107, 1996.

103. Höllt, V., and I. Haarmann. Corticotropin-releasing factor differentially regulates pro-opiomelanocortin messenger ribonucleic acid levels in anterior as compared to intermediate pituitary lobes of rats. *Biochem. Biophys. Res. Commun.* 124: 407-415, 1984.

104. Holmes, M. C., F. A. Antoni, G. Aguilera, and K. J. Catt. Magnocellular axons in passage through the median eminence release vasopressin. *Nature* 319: 326-329, 1986.

105. Hoshino, K., O. Takeuchi, T. Kawai, H. Sanjo, T. Ogawa, Y. Takeda, K. Takeda, and S. Akira. Cutting edge: toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the *Lps* gene product. *J. Immunol.* 162: 3749-3752, 1999.

106. Imaki, T., M. Naruse, S. Harada, N. Chikada, J. Imaki, H. Onodera, H. Demura, and W. Vale. Corticotropin-releasing factor up-regulates its own receptor mRNA in the paraventricular nucleus of the hypothalamus. *Mol. Brain Res.* 38: 166-170, 1996.

107. Imaki, T., M. Naruse, S. Harada, N. Chikada, K. Nakajima, T. Yosimoto, and H. Demura. Stress-induced changes of gene expression in the paraventricular nucleus are enhanced in spontaneously hypertensive rats. *J. Neuroendocrinol.* 10: 635-643, 1998.

108. Imaki, T., T. Shibasaki, N. Chikada, S. Harada, M. Naruse, and H. Demura. Different expression of immediate-early genes in the rat paraventricular nucleus

induced by stress: relation to corticotropin-releasing factor gene transcription. *Endocr. J.* 43: 629-638, 1996.

109. Imaki, T., X. Q. Wang, T. Shibasaki, S. Harada, N. Chikada, C. Takahashi, M. Naruse, and H. Demura. Chlordiazepine attenuates stress-induced activation of neurons, corticotropin-releasing factor (CRF) gene transcription and CRF biosynthesis in the paraventricular nucleus (PVN). *Mol. Brain Res.* 32: 261-270, 1995.

110. Imaki, T., W. Xiao-Quan, T. Shibasaki, K. Yamada, S. Harada, N. Chicada, M. Naruse, and H. Demura. Stress-induced activation of neuronal activity and corticotropin-releasing factor gene expression in the paraventricular nucleus is modulated by glucocorticoids in rats. *J. Clin. Invest.* 96: 231-238, 1995.

111. Irwin, M. R., and R. L. Hauger. Adaptation to chronic stress. Temporal pattern of immune and neuroendocrine correlates. *Neuropsychopharmacology* 1: 239-242, 1988.

112. Jacobson, L., and R. M. Sapolsky. The role of the hippocampus in feedback regulation of the hypothalamo-pituitary-adrenocortical axis. *Endocr. Rev.* 12: 118-134, 1991.

113. Jasper, M. S., and W. C. Engeland. Splanchnic neural activity modulates ultradian and circadian rhythms in adrenocortical secretion in awake rats. *Neuroendocrinology* 59: 97-109, 1994.

114. Jessop, D. S. Central non-glucocorticoid inhibitors of the hypothalamo-pituitary-adrenal axis. *J. Endocrinol.* 160: 169-180, 1999.

115. Jia, H. G., Z. R. Rao, and J. W. Shi. An indirect projection from the nucleus of the solitary tract to the central nucleus of the amygdala via the parabrachial nucleus in the rat: a light and electron microscopic study. *Brain Res.* 663: 181-190, 1994.

116. Johnson, E. O., T. C. Kamilaris, G. P. Chrousos, and P. W. Gold. Mechanisms of stress: a dynamic overview of hormonal and behavioral homeostasis. *Neurosci. Biobehav. Rev.* 16: 115-130, 1992.

117. Johnson, J. D., K. A. O'Connor, T. Deak, R. Spencer, L. R. Watkins, and S. F. Maier. Prior stressor exposure primes the HPA axis. *Psychoneuroendocrinology* 27: 353-365, 2002.

118. Johnson, J. D., K. A. O'Connor, T. Deak, M. Stark, L. R. Watkins, and S. F. Maier. Prior stressor exposure sensitizes LPS-induced cytokine production. *Brain Behav. Immun.* 16: 461-476, 2002.

119. Jones, M. T., and B. Gillham. Factors involved in the regulation of adrenocorticotropic hormone/b-lipotropic hormone. *Physiol. Rev.* 68: 743-818, 1988.

120. Kant, G. J., R. H. Pastel, R. A. Bauman, G. R. Meininger, K. R. Maughan, T. N. 3rd. Robinson, W. L. Wright, and P. S. Covington. Effects of chronic stress on sleep in rats. *Physiol. Behav.* 57: 359-365, 1995.

121. Kawai, T., O. Adachi, T. Ogawa, K. Takeda, and S. Akira. Unresponsiveness of MyD88-deficient mice to endotoxin. *Immunity* 11: 115-122, 1999.

122. Keller-Wood, M. E., and M. F. Dallman. Corticosteroid inhibition of ACTH secretion. *Endocrine Rev.* 5: 1-24, 1984.

123. King, M. S., and A. J. Baertschi. The role of intracellular messengers in adrenocorticotropin secretion in vitro. *Experientia* 46: 26-40, 1990.

124. Kiss, J. Z., M. D. Cassell, and M. Palkovits. Analysis of the ACTH/b-End/a MSH-immunoreactive afferent input to the hypothalamic paraventricular nucleus of the rat. *Brain Res.* 324: 91-99, 1984.

125. Kjaer, A. Vasopressin as a neuroendocrine regulator of anterior pituitary hormone secretion. *Acta Endocrinol.* 129: 489-496, 1993.

126. Kollack-Walker, S., C. Don, J. W. Watson, and H. Akil. Differential expression of c-*fos* mRNA within neurocircuits of male hamsters exposed to acute or chronic defeat. *J. Neuroendocrinol.* 11: 547-559, 1999.

127. Kopin, I. J. Definitions of stress and sympathetic neuronal responses. *Ann. N. Y. Acad. Sci.* 771: 19-30, 1995.

128. Kovács, K. J. c-Fos as a trancription factor: a stressful (re)view from a functional map. *Neurochem. Int.* 33: 287-297, 1998.

129. Kovács, K. J., C. Arias, and P. E. Sawchenko. Protein syntesis blockade differentially affects the stress-induced transcriptional activation of neuropeptide genes in parvocellular neurosecretory neurons. *Mol. Brain Res.* 54: 85-91, 1998.

130. Kovács, K. J., and I. J. Elenkov. Differential dependence of ACTH secretion induced by various cytokines on the integrity of the paraventricular nucleus. *J. Neuroendocrinol.* 7: 15-23, 1995.

131. Kovács, K. J., A. Földes, and P. E. Sawchenko. Glucocorticoid negative feedback selectively targets vasopressin transcription in parvocellular neurosecretory neurons. *J. Neurosci.* 20: 3843-3852, 2000.

132. Kovács, K. J., and P. E. Sawchenko. Sequence of stress-induced alterations in indices of synaptic and transcriptional activation in parvocellular neurosecretory neurons. *J. Neurosci.* 16: 262-273, 1996.

133. Kovács, K. J., and P. E. Sawchenko. Mediation of osmoregulatory influences on neuroendocrine corticotropin-releasing factor expression in the ventral lamina terminalis. *Proc. Natl. Acad. Sci. USA* 90: 7681-7685, 1993.

134. Kvetnansky, R., and R. Mikulaj. Adrenal and urinary catecholamines in rats during adaptation to repeated immobilization stress. *Endocrinology* 87: 738-743, 1970.

135. Lacroix, S., D. Feinstein, and S. Rivest. The bacterial endotoxin lipopolysaccharide has the ability to target the brain in upregulating its membrane CD14 receptor within specific cellular populations. *Brain Pathol.* 8: 625-640, 1998.

136. Lacroix, S., and S. Rivest. Functional circuitry in the brain of immune-challenged rats: partial involvement of prostaglandins. *J. Comp. Neurol.* 387: 307-324, 1997.

137. Lacroix, S., L. Vallières, and S. Rivest. C-fos mRNA pattern and corticotropin-releasing factor neuronal activity throughout the brain of rats injected centrally with a prostaglandin of E2 type. *J. Neuroimmunol.* 70: 163-179, 1996.

138. Laflamme, N., S. Bovetto, D. Richard, and S. Rivest. Effect of dexfenfluramine on the transcriptional activation of CRF and its type 1 receptor within the paraventricular nucleus of the rat hypothalamus. *Br. J. Pharmacol.* 117: 1021-1034, 1996.

139. Laflamme, N., E. Feuvrier, D. Richard, and S. Rivest. Involvement of serotonergic pathways in mediating the neuronal activity and genetic transcription of neuroendocrine corticotropin-releasing factor in the brain of systemically endotoxin-challenged rats. *Neuroscience* 88: 223-240, 1999.

140. Laflamme, N., and S. Rivest. Effects of systemic immunogenic insuts and circulating proinflamatory cytokines on the transcription of the inhibitory factor kBa within specific cellular populations of the rat brain. *J. Neurochem.* 73: 309-321, 1999.

141. Laflamme, N., and S. Rivest. Toll-like receptor 4: the missing link of the cerebral innate immune response triggered by circulating gram-negative bacterial cell wall components. *FASEB J.* 15: 155-163, 2001.

142. Lahmame, A., F. Gómez, and A. Armario. Fawn-hooded rats show enhanced active behaviour in the forced swimming test, with no evidence for pituitary-adrenal-axis hyperactivity. *Psychopharmacology* 125: 74-78, 1996.

143. Larsen, P. J., A. Hay-Schmidt, N. Vrang, and J. D. Mikkelsen. Origin of projections from the midbrain raphe nuclei to the hypothalamic paraventricular nucleus in the rat: a combined retrograde and anterograde tracing study. *Neuroscience* 70: 963-988, 1996.

144. Lee, S., and C. Rivier. An initial, three-day-long treatment with alcohol induces a long-lasting phenomenon of selective tolerance in the activity of the rat hypothalamic-pituitary-adrenal axis. *J. Neurosci.* 17: 8856-8866, 1997.

145. Lee, S., and C. Rivier. Interaction between corticotropin-releasing factor and nitric oxide in mediating the response of the rat hypothalamus to immune and non-immune stimuli. *Mol. Brain Res.* 57: 54-62, 1998.

146. Lee, S., D. Schmidt, F. Tilders, and C. Rivier. Increased activity of the hypothalamic-pituitary-adrenal axis of rats exposed to alcohol in utero: role of altered pituitary and hypothalamic function. *Mol. Cell. Neurosci.* 16: 515-528, 2000.

147. Lee, S., E. D. Schmidt, F. J. H. Tilders, and C. Rivier. Effect of repeated exposure to alcohol on the response of the hypothalamic-pituitary-adrenal axis of the rat: I. Role of changes in hypothalamic neuronal activity. *Alcohol. Clin. Exp. Res.* 25: 98-105, 2001.

148. Lehner, M., and T. Hartung. Endotoxin tolerance-mechanisms and beneficial effects in bacterial infection. *Rev. Physiol. Biochem. Pharmacol.* 144: 95-141, 2002.

149. Levine, S. A definition of stress? In: *Animal stress*, edited by G. P. Moberg. Baltimore: Americal Physiological Society, 1985, p. 51-69.

150. Lewis, K., C. Li, M. H. Perrin, A. Blount, K. Kunitake, C. Donaldson, J. Vaughan, T. M. Reyes, J. Gulyas, W. Fischer, L. Bilezikjian, J. Rivier, P. E. Sawchenko, and W. W. Vale. Identification of urocortin III, an additional member of the corticotropin-releasing factor (CRF) family with high affinity for the CRF2 receptor. *Proc. Natl. Acad. Sci. USA* 98: 7570-7575, 2001.

151. Li, C., J. Vaughan, P. E. Sawchenko, and W. W. Vale. Urocortin III-immunoreactive projections in rat brain: partial overlap with sites of type 2 corticotropin-releasing factor receptor expression. *J. Neurosci.* 22: 991-1001, 2002.

152. Li, H. Y., A. Ericsson, and P. E. Sawchenko. Distinct mechanisms underlie activation of hypothalamic neurosecretory neurons and their medullary catecholaminergic afferents in categorically different stress paradigms. *Proc. Natl. Acad. Sci. USA* 93: 2359-2364, 1996.

153. Liberzon, I., J. F. López, S. B. Flagel, D. M. Vázquez, and E. A. Young. Differential regulation of hippocampal glucocorticoid receptors mRNA and fast feedback: relevance to post-traumatic stress disorder. *J. Neuroendocrinol.* 11: 11-17, 1999.

154. Liu, D., J. Diorio, B. Tannenbaum, C. Caldji, D. Francis, A. Freedman, S. Sharma, D. Pearson, P. M. Plotsky, and M. J. Meaney. Maternal care, hippocampal glucocorticoid receptors, and hypothalamic-pituitary-adrenal responses to stress. *Science* 277: 1659-1662, 1997.

155. Lovemberg, T. W., D. T. Chalmers, C. Liu, and E. B. De Souza. CRF2a and CRF2b receptor mRNAs are differentially distributed between the rat central nervous system and peripheral tissues. *Endocrinology* 136: 4139-4142, 1995.

156. Ludwig, M., L. E. Johnstone, I. Neumann, R. Landgraf, and J. A. Russell. Direct hipertonic stimulation of the rat supraoptic nucleus increases *c-fos* expression in glial cells rather than magnocellular neurones. *Cell Tissue Res.* 287: 79-90, 1997.

157. Luo, X., A. Kiss, G. B. Makara, S. J. Lolait, and G. Aguilera. Stress specific regulation of corticotropin releasing hormone receptor expression in the paraventricular and supraotic nuclei of the hypothalamus in the rat. *J. Neuroendocrinol.* 6: 689-696, 1994.

158. Ma, X.-M., and G. Aguilera. Differential regulation of corticotropin-releasing hormone and vasopressin transcription by glucocorticoids. *Endocrinology* 140: 5642-5650, 1999.

159. Ma, X.-M., and G. Aguilera. Transcriptional responses of the vasopressin and corticotropin-releasing hormone genes to acute and repeated intraperitoneal hypertonic saline injection in rats. *Mol. Brain Res.* 68: 129-140, 1999.

160. Ma, X.-M., A. Levy, and S. L. Lightman. Emergence of an isolated arginine vasopressin (AVP) response to stress after repeated restraint: A study of both AVP and corticotropin-releasing hormone messenger ribonucleic acid (RNA) and heteronuclear RNA. *Endocrinology* 138: 4351-4357, 1997.

161. Ma, X.-M., A. Levy, and S. L. Lightman. Rapid changes in heteronuclear RNA for corticotrophin-releasing hormone and arginine vasopressin in response to acute stress. *J. Neuroendocrinol.* 152: 81-89, 1997.

162. Ma, X.-M., A. Levy, and S. L. Lightman. Rapid changes of heteronuclear RNA for arginine vasopressin but not for corticotropin releasing hormone in response to acute corticosterone administration. *J. Neuroendocrinol.* 9: 723-728, 1997.

163. Ma, X.-M., and S. L. Lightman. The arginine vasopressin and corticotrophin-releasing hormone gene transcription responses to varied frequencies of repeated stress in rats. *J. Physiol.* 510: 605-614, 1998.

164. Ma, X.-M., S. L. Lightman, and G. Aguilera. Vassopressin and corticotropin-releasing hormone gene responses to novel stress in rats adapted to repeated restraint. *Endocrinology* 140: 3623-3632, 1999.

165. Maier, S. F. Learned helplessness and animal models of depression. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 8: 435-446, 1984.

166. Maier, S. F. Learned helplessness: relationships with fear and anxiety. In: *Stress: from synapse to syndrome*, edited by S. C. Stanford and P. Salmon. San Diego: Academic Press, 1993, p. 207-243.

167. Makara, G. B., and J. Haller. Non-genomic effects of glucocorticoids in the neural system. Evidence, mechanisms and implications. *Prog. Neurobiol.* 65: 367-390, 2001.

168. Makino, S., J. Schulkin, M. A. Smith, K. Pacák, M. Palkovits, and P. W. Gold. Regulation of corticotropin-releasing hormone receptor messenger ribonucleic acid in the rat brain and pituitary by glucocorticoids and stress. *Endocrinology* 136: 4517-4525, 1995.

169. Makino, S., T. Takemura, K. Asaba, M. Nishiyama, T. Takao, and K. Hashimoto. Differential regulation of type-1 and type-2a corticotropin-releasing hormone receptor mRNA in the hypothalamic paraventricular nucleus of the rat. *Mol. Brain Res.* 47: 170-176, 1997.

170. Mansi, J. A., S. Rivest, and G. Drolet. Regulation of corticotropin-releasing factor type 1 (CRF1) receptor messenger ribonucleic acid in the paraventricular nucleus of rat hypothalamus by exogenous CRF. *Endocrinology* 137: 4619-4629, 1996.

171. Márquez, C., X. Belda, and A. Armario. Post-stress recovery of pituitary-adrenal hormones and glucose, but not the response during exposure to the stressor, is a marker of stress intensity in highly stressful situations. *Brain Res.* 926: 181-185, 2002.

172. Martí, O. *Influència de l'estrés crònic sobre l'activitat de l'eix hipotalàmic-pituïtari-adrenal (Thesis)*. Bellaterra: Departament de Biologia Cel.lular i de Fisiologia, Unitat de Fisiologia Animal (Universitat Autònoma de Barcelona), 1994.

173. Martí, O., and A. Armario. Anterior pituitary response to stress: time-related changes and adaptation. *Int. J. Develop. Neurosci.* 16: 241-260, 1998.

174. Martí, O., and A. Armario. Modificacions neuroendocrinològiques amb l'estrès. *Treballs de la SCB* 47: 171-185, 1996.

175. Martí, O., A. García, A. Vallès, M. S. Harbuz, and A. Armario. Evidence that a single exposure to aversive stimuli triggers long-lasting effects in the

hypothalamus-pituitary-adrenal axis that consolidate with time. *Eur. J. Neurosci.* 13: 129-136, 2001.

176. Martí, O., A. Gavaldà, T. Jolín, and A. Armario. Acute stress attenuates but does not abolish circadian rhytmicity of serum thyrotropin and growth hormone in the rat. *Eur. J. Endocrinol.* 135: 703-708, 1996.

177. Martí, O., A. Gavaldà, T. Jolín, and A. Armario. Effect of regularity of exposure to chronic immobilization stress on circadian pattern of pituitary adrenal hormones, growth hormone, and thyroid stimulating hormone in the adult male rat. *Psychoneuroendocrinology* 18: 67-77, 1993.

178. McGaugh, J. L. Memory - a century of consolidation. *Science* 287: 248-251, 2000.

179. McKellar, S., and A. D. Loewy. Organization of some brain stem afferents to the paraventricular nucleus of the hypothalamus in the rat. *Brain Res.* 217: 351-357, 1981.

180. Meaney, M. J., J. Diorio, D. Francis, J. Widdowson, P. LaPlante, C. Caldji, S. Sharma, J. R. Seckl, and P. M. Plotsky. Early enviromental regulation of forebrain glucocorticoid receptor gene expression: implications for adrenocortical responses to stress. *Develop. Neurosci.* 18: 49-72, 1996.

181. Mezey, E., J. Z. Kiss, L. P. Skirboll, M. Goldstein, and J. Axelrod. Increase of corticotropin releasing factor staining in the rat paraventricular nucleus neurones by depletion of hypothalamic adrenaline. *Nature* 310: 140-141, 1984.

182. Michelson, D., J. Licinio, and P. W. Gold. Mediation of the stress response by the hypothalamic-pituitary-adrenal axis. In: *Neurobiological and clinical consequences of stress. From normal adaptation to post-traumatic stress disorder*, edited by M. J. Friedman, D. S. Charney and A. Y. Deutch. Philadelphia: Lippincot-Raven Publishers, 1995, p. 225-238.

183. Moberg, G. P. Influence of stress on reproduction: measure of well-being. In: *Animal stress*, edited by G. P. Moberg. Baltimore: American Physiological Society, 1985, p. 245-267.

184. Mohr, E., and D. Richter. Sequence analysis of the promoter region of the rat vasopressin gene. *FEBS Lett.* 260: 305-308, 1990.

185. Morgan, J. I., and T. Curran. Stimulus-transcription coupling in the nervous system: involvement of the inducible proto-oncogenes *fos* and *jun*. *Annu. Rev. Neurosci.* 14: 421-451, 1991.

186. Munck, A., P. M. Guyre, and N. J. Holbrook. Physiological functions of glucocorticoids in stress and their relation to pharmacological actions. *Endocr. Rev.* 5: 25-44, 1984.

187. Nadeau, S., and S. Rivest. The complement system is an integrated part of the natural innate immune response in the brain. *FASEB J.* 15: 1410-1422, 2001.

188. Nadeau, S., and S. Rivest. Effects of circulating tumor necrosis factor on the neuronal activity and expression of the genes encoding the tumor necrosis factor receptors (p55 and p75) in the rat brain: a view from the blood-brain barrier. *Neuroscience* 93: 1449-1464, 1999.

189. Nadeau, S., and S. Rivest. Regulation of the gene encoding tumor necrosis factor alpha (TNF-alpha) in the rat brain and pituitary in response in different models of systemic immune challenge. *J. Neuropathol. Exp. Neurol.* 58: 61-77, 1999.

190. Nadeau, S., and S. Rivest. Role of microglial-derived tumor necrosis factor in mediating CD14 transcription and nuclear factor kappa B activity in the brain during endotoxemia. *J. Neurosci.* 20: 3456-3468, 2000.

191. Odio, M., and A. Brodish. Age-related adaptation of pituitary-adrenocortical responses to stress. *Neuroendocrinology* 49: 382-388, 1989.

192. Ogilvie, K. M., S. Lee, and C. Rivier. Divergence in the expression of molecular markers of neuronal activation in the parvocellular paraventricular nucleus of the hypothalamus evoked by alcohol administration via different routes. *J. Neurosci.* 18: 4344-4352, 1998.

193. Oldfield, B. J., and M. J. McKinley. The circumventricular organs. In: *The Rat Nervous System* (2nd ed.), edited by G. Paxinos. Sydney: Academic Press, 1995, p. 391-403.

194. Ottenweller, J. E., and A. H. Meier. Adrenal innervation may be an extrapituitary mechanism able to regulate adrenocortical rhythmicity in rats. *Endocrinology* 111: 1334-1338, 1982.

195. Owens, M. J., and B. Nemeroff. Physiology and pharmacology of corticotropin-releasing factor. *Pharmacol. Rev.* 43: 425-473, 1991.

196. Pacák, K., and M. Palkovits. Stressor specificity of central neuroendocrine responses: implications for stress-related disorders. *Endocrine Rev.* 22: 502-548, 2001.

197. Pacák, K., M. Palkovits, G. Yadid, R. Kvetnansky, I. J. Kopin, and D. S. Goldstein. Heterogeneous neurochemical responses to different stressors: a test of Selye's doctrine of nonspecificity. *Am. J. Physiol.* 275: R1247-R1255, 1998.

198. Pan, B., J. M. Castro-Lopez, and A. Coimbra. Chemical sensory deafferentation abolishes hypothalamic pituitary activation induced by noxious stimulation or electroacupunture but only decreases that caused by immobilization stress. A c-fos study. *Neuroscience* 78: 1059-1068, 1997.

199. Pardy, K., R. A. H. Adan, D. A. Carter, V. Seah, J. P. H. Burbach, and D. Murphy. The identification of a *cis*-acting element involved in cyclic 3', 5'-adenosine monophosphate regulation of bovine vasopressin gene expression. *J. Biol. Chem.* 267: 21746-21752, 1992.

200. Parkes, D., S. Rivest, S. Lee, C. Rivier, and W. Vale. Corticotropin-releasing factor activates c-fos, NGFI-B, and corticotropin-releasing factor gene expression within the paraventricular nucleus of the rat hypothalamus. *Mol. Endocrinol.* 7: 1357-1367, 1993.

201. Paxinos, G., and C. Watson. *The rat brain in stereotaxic coordinates*. San Diego: Academic Press, 1998.

202. Piazza, P. V., and M. Le Moal. Glucocorticoids as a biological substrate of reward: physiological and pathophysiological implications. *Brain Res. Rev*. 25: 359-372, 1997.

203. Plotski, P. M. Pathways to the secretion of adrenocorticotropin: a view from the portal. *J. Neuroendocrinol.* 3: 1-9, 1991.

204. Poltorak, A., X. He, I. Smirnova, M. Y. Liu, C. V. Huffel, X. Du, D. Birdwell, E. Alejos, M. Silva, C. Galanos, M. Freudenberg, P. Ricciardi-Castagnoli, B. Layton, and B. Beutler. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* 282: 2085-2088, 1998.

205. Potter, E., S. Sutton, C. Donaldson, R. Chen, M. Perrin, K. Lewis, P. E. Sawchenko, and W. Vale. Distribution of corticotropin-releasing factor receptor mRNA expression in the rat brain and pituitary. *Proc. Natl. Acad. Sci. USA* 91: 8777-8781, 1994.

206. Quan, N., M. Whiteside, and M. Herkenham. Time course and localization patterns if interleukin-1b mRNA expression in the brain and pituitary after peripheral administration of lipopolysaccharide. *Neuroscience* 83: 281-293, 1997.

207. Qureshi, S. T., P. Gros, and D. Malo. Host resistance to infection: genetic control of lipopolysaccharide responsiveness by TOLL-like receptor genes. *Trends Genet*. 15: 291-294, 1999.

208. Reyes, T. M., K. Lewis, M. H. Perrin, K. S. Kunitake, J. Vaughan, C. A. Arias, J. B. Hogenesch, J. Guylas, J. Rivier, W. W. Vale, and P. E. Sawchenko. Urocortin II: A member of the corticotropin-releasing factor (CRF) neuropeptide family that is selectively bound by type 2 CRF receptors. *Proc. Natl. Acad. Sci. USA* 98: 2843-2848, 2001.

209. Ricardo, J. A., and E. T. Koh. Anatomical evidence of direct projections from the nucleus of the solitary tract to the hypothalamus, amygdala, and other forebrain structures in the rat. *Brain Res.* 153: 1-26, 1978.

210. Richard, D., R. Rivest, N. Naimi, E. Timofeeva, and S. Rivest. Expression of corticotropin-releasing factor and its receptors in the brain of lean and obese Zucker rats. *Endocrinology* 137: 4786-4795, 1996.

211. Risold, P. Y., and L. W. Swanson. Connections of the rat lateral septal complex. *Brain Res. Rev*. 24: 115-195, 1997.

212. Rivest, S. How circulating cytokines trigger the neural circuits that control the hypothalamic-pituitary-adrenal axis. *Psychoneuroendocrinology* 26: 761-788, 2001.

213. Rivest, S., S. Lacroix, L. Vallières, S. Nadeau, J. Zhang, and N. Laflamme. How the blood talks to the brain parenchyma and the paraventricular nucleus of the hypothalamus during systemic inflammatory and infectious stimuli. *Proc. Soc. Exp. Biol. Med.* 223: 22-38, 2000.

214. Rivest, S., and N. Laflamme. Neuronal activity and neuropeptide gene transcription in the brains of immune-challenged rats. *J. Neuroendocrinol.* 7: 501-525, 1995.

215. Rivest, S., N. Laflamme, and R. E. Nappi. Immune challenge and immobilization stress induce transcription of the gene encoding CRF receptor in selective nuclei of the rat hypothalamus. *J. Neurosci.* 15: 2680-2695, 1995.

216. Rivest, S., and C. Rivier. Influence of the paraventricular nucleus of the hypothalamus in the alteration of neuroendocrine functions induced by intermitent footshock or interleukin. *Endocrinology* 129: 2049-2057, 1991.

217. Rivier, C. Effect of pretreatment with alcohol on subsequent endocrine and immune responses in the adult male rat. *Alcohol. Clin. Exp. Res.* 21: 1690-1694, 1997.

218. Rivier, C., and S. Lee. Acute alcohol administration stimulates the activity of hypothalamic neurons that express corticotropin-releasing factor and vasopressin. *Brain Res.* 726: 1-10, 1996.

219. Rivier, C., J. Rivier, and W. Vale. Inhibition of adrenocorticotropic hormone secretion in the rat by immunoneutralization of corticotropin-releasing factor. *Science* 218: 377-379, 1982.

220. Rivier, C., and W. Vale. Diminished responsiveness of the hypothalamic-pituitary-adrenal axis of the rat during exposure to prolonged stress: a pituitaty-mediated mechanism. *Endocrinology* 121: 1320-1328, 1987.

221. Rivier, C., and W. Vale. Interaction of corticotropin-releasing factor and arginine vasopressin on adrenocorticotropin secretion *in vivo*. *Endocrinology* 113: 939-942, 1983.

222. Rivier, C., and W. Vale. Modulation of stress-induced ACTH release by corticotropin-releasing factor, catecholamines and vasopressin. *Nature* 305: 325-327, 1983.

223. Rivier, J., C. Rivier, and W. Vale. Synthetic competitive antagosists of corticotropin-releasing factor: effect on ACTH secretion in the rat. *Science* 224: 889-891, 1984.

224. Romanovsky, A. A., C. T. Simons, M. Székely, and V. A. Kulchitsky. The vagus nerve in the thermoregulatory response to systemic inflamation. *Am. J. Physiol.* 273: R407-R413, 1997.

225. Romero, L. M., and R. M. Sapolsky. Patterns of ACTH secretagog secretion in response to psychological stimuli. *J. Neuroendocrinol.* 8: 243-258, 1996.

226. Roth, J., J. L. McClellan, M. J. Kluger, and E. Zeisberger. Attenuation of fever and release to cytokines after repeated injections of lipopolysaccharide in guinea-pigs. *J. Physiol.* 477: 177-185, 1994.

227. Ruis, M. A. W., J. H. A. te Brake, B. Buwalda, S. F. De Boer, P. Meerlo, S. M. Korte, H. J. Blokhuis, and J. M. Koolhaas. Housing familiar male wildtype rats together reduces the long-term adverse behavioural and physiological effects of social defeat. *Psychoneuroendocrinology* 24: 285-300, 1999.

228. Sapolsky, R. M. *Stress, the aging brain and the mechanisms of neuron death*. Cambridge: MIT Press, 1992.

229. Sapolsky, R. M., L. M. Romero, and A. U. Munck. How do glucocorticoids influence stress responses? Integrating permissive, supressive, stimulatory, and preparative actions. *Endocrine Rev.* 21: 55-89, 2000.

230. Sassone-Corsi, P., J. C. Sisson, and I. M. Verma. Transcriptional autoregulation of the proto-oncogene *fos*. *Nature* 334: 314-319, 1988.

231. Sawchenko, P. E., H.-Y. Li, and A. Ericsson. Circuits and mechanisms governing hypothalamic responses to stress: a tale of two paradigms. *Prog. Brain. Res.* 122: 61-78, 2000.

232. Sawchenko, P. E., and L. W. Swanson. Organization of CRF immunoreactive cells and fibers in the rat brain: immunohistochemical studies. In: *Corticotropin-releasing Factor: Basic and Clinical Studies of a Neuropeptide*, edited by E. B. De Souza and C. B. Nemeroff. Boca Raton: CRC Press, 1990, p. 29-52.

233. Sawchenko, P. E., and L. W. Swanson. The organization of forebrain afferents to the paraventricular and supraoptic nuclei of the rat. *J. Comp. Neurol.* 218: 121-144, 1983.

234. Sawchenko, P. E., and L. W. Swanson. The organization of noradrenergic pathways from the brainstem to the paraventricular and supraoptic nuclei in the rat. *Brain Res.* 257: 275-325, 1982.

235. Sawchenko, P. E., L. W. Swanson, and S. A. Joseph. The distribution and cells of origin of ACTH (1-39)-stained varicosities in the paraventricular and supraoptic nuclei. *Brain Res.* 232: 365-374, 1982.

236. Sawchenko, P. E., L. W. Swanson, H. W. M. Steinbusch, and A. A. J. Verhofstad. The distribution and cells of origin of serotonergic inputs to the paraventricular and supraoptic nuclei of the rat. *Brain Res.* 277: 355-360, 1983.

237. Schmidt, E. D., R. Binnekade, A. W. J. W. Janszen, and F. J. H. Tilders. Short stressor induced long-lasting increases of vasopressin stores in hypothalamic corticotropin-releasing hormone (CRH) neurons in adult rats. *J. Neuroendocrinol.* 8: 703-712, 1996.

238. Schmidt, E. D., A. W. J. W. Janszen, R. Binnekade, and F. J. H. Tilders. Transient suppression of resting corticosterone levels induces sustained increase of AVP stores in hypothalamic CRH-neurons of rats. *J. Neuroendocrinol.* 9: 69-77, 1997.

239. Schmidt, E. D., A. W. J. W. Janszen, F. G. Wouterlood, and F. J. H. Tilders. Interleukin-1-induced long-lasting changes in hypothalamic corticotropin-releasing hormone (CRH)-neurons and hyperresponsiveness of the hypothalamus-pituitary-adrenal axis. *J. Neurosci.* 15: 7417-7426, 1995.

240. Schmidt, E. D., A. N. M. Schoffelmeer, T. J. De Vries, G. Wardeh, G. Dogterom, J. G. J. M. Bol, R. Binnekade, and F. J. H. Tilders. A single administration of interleukin-1 or amphetamine induces long-lasting increases in evoked noradrenaline release in the hypothalamus ans sensitization of ACTH and corticosterone responses in rats. *Eur. J. Neurosci.* 13: 1923-1930, 2001.

241. Schmidt, E. D., F. J. H. Tilders, R. Binnekade, A. N. M. Schoffelmeer, and T. J. De Vries. Stressor- or drug-induced sensitization of the corticosterone response is not critically involved in the long-term expression of behavioural sensitization to amphetamine. *Neuroscience* 92: 343-352, 1999.

242. Schumann, R. R., S. R. Leong, G. W. Flaggs, P. W. Gray, S. D. Wright, J. C. Mathison, P. S. Tobias, and R. J. Ulevitch. Structure and function of lipopolysaccharide binding protein. *Science* 249, 1990.

243. Schwartz, M. W., M. F. Dallman, and S. C. Woods. Hypothalamic response to starvation: implications for the study of wasting disorders. *Am. J. Physiol.* 269: R949-R957, 1995.

244. Scott Young III, W. In situ hybridization with oligodeoxyribonucleotide probes. In: *In situ hybridization: a practical approach*, edited by D. G. Wilkinson. Oxford: Oxford University Press, 1992, p. 33-44.

245. Selye, H. A syndrome produced by diverse nocuous agents. *Nature* 138: 32, 1936.

246. Senba, E., and T. Ueyama. Stress-induced expression of immediate early genes in the brain and peripheral organs of the rat. *Neurosci. Res.* 29: 183-207, 1997.

247. Simmons, D. M., J. L. Arriza, and L. W. Swanson. A complete protocol for in situ hybridization of messenger RNAs in brain and other tissues with radio-labeled single-stranded RNA probes. *J. Histotechnol.* 12: 169-181, 1989.

248. Simpson, J. B. The circumventricular organs and the central actions of angiotensin. *Neuroendocrinology* 32: 248-256, 1981.

249. Smagin, G. N., and A. J. Dunn. The role of CRF receptor subtypes in stress-induced behavioural responses. *Eur. J. Pharmacol.* 405: 199-206, 2000.

250. Sousa, R. J., N. H. Tannery, and E. M. Lafer. *In situ* hybridization mapping of glucocorticoid receptor messenger ribonucleic acid in rat brain. *Mol. Endocrinol.* 3: 481-494, 1989.

251. Stam, R., A. W. Bruijnzeel, and V. M. Wiegant. Long-lasting stress sensitisation. *Eur. J. Pharmacol.* 405: 217-224, 2000.

252. Stam, R., G. Croiset, L. M. A. Akkermans, and V. Wiegant. Sensitization of the colonic response to novel stress after previous stressful experience. *Am. J. Physiol.* 271: R1270-R1273, 1996.

253. Stam, R., T. J. van Laar, L. M. Akkermans, and V. M. Wiegant. Variability factors in the expression of stress-induced behavioural sensitisation. *Behav. Brain Res.* 132: 69-76, 2002.

254. Steciuk, M., M. Kram, G. L. Kramer, and F. Petty. Decrease in stress-induced c-Fos-like immunoreactivity in the lateral septal nucleus of learned helpless rats. *Brain Res.* 822: 256-259, 1999.

255. Stefanski, V. Social stress in laboratory rats- Behavior, immune function, and tumor metastasis. *Physiol. Behav.* 73: 385-391, 2001.

256. Steptoe, A. Stress and the cardiovascular system: a psychosocial perspective. In: *Stress: from synapse to syndrome*, edited by S. C. Stanford and P. Salmon. San Diego: Academic Press, 1993, p. 119-141.

257. Stratakis, C. A., and G. P. Chrousos. Neuroendocrinology and pathophysiology of the stress system. *Ann. N. Y. Acad. Sci.* 771: 1-18, 1995.

258. Swanson, L. W., and G. D. Petrovich. What is the amygdala? *Trends Neurosci.* 21: 323-331, 1998.

259. Swanson, L. W., and P. E. Sawchenko. Hypothalamic integration: organization of the paraventricular and supraoptic nuclei. *Ann. Rev. Neurosci.* 6: 275-335, 1983.

260. Swanson, L. W., P. E. Sawchenko, and R. W. Lind. Regulation of multiple peptides in CRF parvicellular neurosecretory neurons: implications for the stress response. *Prog. Brain Res.* 68: 169-190, 1986.

261. Szafarczyk, A., V. Guillaume, B. Conte-Devolx, G. Alonso, F. Malaval, N. Pares-Herbute, C. Oliver, and I. Assenmacher. Central catecholaminergic system stimulates secretion of CRH at different sites. *Am. J. Physiol.* 255: E463-E468, 1988.

262. Szafarczyk, A., F. Malaval, A. Laurent, and R. Girbaud. Further evidence for a central stimulatory action of catecholamines on adrenocortical release in the rat. *Endocrinology* 121: 883-892, 1987.

263. Takemura, T., S. Makino, T. Takao, K. Asaba, S. Suemaru, and K. Hashimoto. Hypotalamic-pituitary-adrenocortical responses to single vs. repeated endotoxin lipopolysaccharide administration in the rat. *Brain Res.* 767: 181-191, 1997.

264. Tanimura, S. M., G. Sanchez-Watts, and A. G. Watts. Peptide gene activation, secretion, and steroid feedback during stimulation of rat neuroendocrine corticotropin-releasin hormone neurons. *Endocrinology* 139: 3822-3829, 1998.

265. Tanimura, S. M., and A. G. Watts. Corticosterone can facilitate as well as inhibit corticotropin-releasing hormone gene expression in the rat hypothalamic paraventricular nucleus. *Endocrinology* 139: 3830-3836, 1998.

266. Tanimura, S. M., and A. G. Watts. Corticosterone modulation of ACTH secretogogue gene expression in the paraventricular nucleus. *Peptides* 22: 775-783, 2001.

267. Tatro, J. B. Receptor biology of the melanocortins, a family of neuroimmunomodulatory peptides. *Neuroimmunomodulation* 3: 259-284, 1996.

268. Thompson, R. F., and D. J. Krupa. Organization of memory traces in the mamalian brain. *Annu. Rev. Neurosci.* 17: 519-549, 1994.

269. Thrivikraman, K. V., C. B. Nemeroff, and P. M. Plotsky. Sensitivity to glucocorticoid-mediated fast-feedback regulation of the hypothalamic-pituitary-adrenal axis is dependent upon stressor specific neurocircuitry. *Brain Res.* 870: 87-101, 2000.

270. Tilders, F. J. H., R. H. DeRijk, A.-M. Van Dam, V. A. M. Vincent, K. Schotanus, and J. H. A. Persoons. Activation of the hypothalamus-pituitary-adrenal axis by bacterial endotoxins: routes and intermediate signals. *Psychoneuroendocrinology* 19: 209-232, 1994.

271. Tilders, F. J. H., E. D. Schmidt, W. J. G. Hoogedijk, and D. F. Swaab. Delayed effects of stress and immune activation. *Bailliere Clin. Endocrinol. Metab.* 13: 523-540, 1999.

272. Tkacs, N. C., and J. Li. Immune stimulation induces Fos expression in brainstem amygdala afferents. *Brain Res. Bull.* 48: 223-231, 1999.

273. Torres, G., J. M. Horowitz, N. Laflamme, and S. Rivest. Fluoxetine induces the transcription of genes encoding *c-fos*, corticotropin-releasing factor and its type 1 receptor in rat brain. *Neuroscience* 87: 463-477, 1998.

274. Tribollet, E., and J. J. Dreifuss. Localization of neurones projecting to the hypothalamic paraventricular nucleus area of the rat: a horseradish peroxidase study. *Neuroscience* 6: 1315-1328, 1981.

275. Turnbull, A. V., S. Lee, and C. Rivier. Mechanisms of hypothalamic-pituitary-adrenal axis stimulation by immune signals in the adult rat. *Ann. N. Y. Acad. Sci.* 840: 434-443, 1998.

276. Turnbull, A. V., and C. Rivier. Corticotropin-releasing factor (CRF) and endocrine responses to stress: CRF receptors, binding protein, and related peptides. *Proc. Soc. Exp. Biol. Med.* 215: 1-10, 1997.

277. Turnbull, A. V., and C. L. Rivier. Regulation of the hypothalamic-pituitary-adrenal axis by cytokines: actions and mechanisms of action. *Physiol. Rev.* 79: 1-71, 1999.

278. Umemoto, S., Y. Kawai, T. Ueyama, and E. Senba. Chronic glucocorticoid administration as well as repeated stress affects the subsequent acute immobilization stress-induced expression of immediate early genes but not that of NGFI-A. *Neuroscience* 80: 763-773, 1997.

279. Ursin, H., and M. Olff. The stress response. In: *Stress: from synapse to syndrome*, edited by S. C. Stanford and P. Salmon. San Diego: Academic Press, 1993, p. 3-22.

280. Vale, W., J. Speiss, C. Rivier, and J. Rivier. Characterization of a 41-residue ovine hypothalamic peptide that stimulates the secretions of corticotropin and b-endorphin. *Science* 213: 1394-1397, 1981.

281. Vallières, L., S. Lacroix, and S. Rivest. Influence of interleukin-6 on neural activity and transcription of the gene encoding corticotrophin-releasing factor in the rat brain: an effect depending upon the route of administration. *Eur. J. Neurosci.* 9: 1461-1472, 1997.

282. Vallières, L., and S. Rivest. Interleukin-6 is a needed proinflamatory cytokine in the prolonged neural activity and trancriptional activation of corticotropin-releasing factor during endotoxemia. *Endocrinology* 140: 3890-3903, 1999.

283. Vallières, L., and S. Rivest. Regulation of the genes encoding interleukin-6, its receptor, and gp130 in the rat brain in response to the immune activator lipopolysaccharide and the proinflamatory cytokine interleukin-1b. *J. Neurochem.* 69: 1668-1683, 1997.

284. van Bockstaele, E. J., D. Bajic, H. Proudfit, and R. J. Valentino. Topographic architecture of stress-related pathways targeting the noradrenergic locus coeruleus. *Physiol. Behav.* 73: 273-283, 2001.

285. van Dijken, H. H., D. C. E. de Goeij, W. Sutanto, J. Mos, E. R. de Kloet, and F. J. H. Tilders. Short inescapable stress produces long-lasting changes in the brain-pituitary-adrenal axis of adult male rats. *Neuroendocrinology* 58: 57-64, 1993.

286. van Dijken, H. H., F. J. Tilders, B. Olivier, and J. Mos. Effects of anxiolytic and antidepressant drugs on long-lasting behavioural deficits resulting from one short stress experience in male rats. *Psychopharmacology* 109: 395-402, 1992.

287. Vanderschuren, L. J. M. J., E. D. Schmidt, T. J. De Vries, C. A. P. Van Moorsel, F. J. H. Tilders, and A. N. M. Schoffelmeer. A single exposure to amphetamine is sufficient to induce long-term behavioural, neuroendocrine, and neurochemical sensitization in rats. *J. Neurosci.* 19: 9579-9586, 1999.

288. Vaughan, J., C. Donaldson, J. Bittencourt, M. H. Perrin, K. Lewis, S. Sutton, R. Chan, A. V. Turnbull, D. Lovejoy, C. Rivier, J. Rivier, P. E. Sawchenko, and W. Vale. Urocortin, a mammalian neuropeptide related to fish urotensin I and to corticotropin-releasing factor. *Nature* 378: 287-292, 1995.

289. Vigas, M. Problems of definition of stress stimulus and specificity of stress response. In: *Stress. Role of catecholamines and other neurotransmitters*, edited by E. R. Usdin, R. Kvetnansky and J. Axelrod. New York: Gordon and Breach Sci. Publ., 1984, p. 27-35.

290. Vinson, G. P., J. P. Hinson, and I. E. Tóth. The neuroendocrinology of the adrenal cortex. *J. Neuroendocrinol.* 6: 235-246, 1994.

291. Wahlestedt, C., G. Skagerberg, R. Ekman, M. Heilig, F. Sundler, and R. Hakanson. Neuropeptide Y (NPY) in the area of the hypothalamic paraventricular nucleus activates the pituitary-adrenocortical axis in the rat. *Brain Res.* 417: 33-38, 1987.

292. Walaas, I., and F. Fonnum. Biochemical evidence for glutamate as a transmitter in hippocampal efferents to the basal forebrain and hypothalamus in the rat brain. *Neuroscience* 5: 1691-1698, 1980.

293. Wan, W., L. Janz, C. Y. Vriend, C. M. Sorensen, A. H. Greenberg, and D. M. Nance. Differential induction of c-Fos immunoreactivity in hypothalamus and brain stem nuclei following central and peripheral administration of endotoxin. *Brain Res. Bull.* 32: 581-587, 1993.

294. Watanabe, Y., E. Stone, and B. S. McEwen. Induction and habituation of c-fos and zif/268 by acute and repeated stressors. *NeuroReport* 5: 1321-1324, 1994.

295. Watkins, L. R., E. P. Wiertelak, L. E. Goehler, K. Mooney-Heiberger, J. Martinez, L. Furness, K. P. Smith, and S. F. Maier. Neurocircuitry of illness-induced hyperalgesia. *Brain Res.* 639: 283-299, 1994.

296. Weller, K. L., and D. A. Smith. Afferent connections to the bed nucleus of the stria terminalis. *Brain Res.* 232: 225-254, 1982.

297. Whitnall, M. H., S. Key, and H. Gainer. Vasopressin-containing and vasopressin-deficient subpopulations of corticotropin-releasing factor axons are differentially affected by adrenalectomy. *Endocrinology* 120: 2180-2182, 1987.

298. Widmaier, E. P., A. T. Lim, and W. Vale. Secretion of corticotropin-releasing factor from cultured rat hypothalamic cells: effects of catecholamines. *Endocrinology* 124: 583-590, 1989.

299. Wotjak, C. T., M. Kubota, G. Liebsch, A. Montokwski, F. Holsboer, I. Neumann, and R. Landgraf. Release of vasopressin within the rat paraventricular nucleus in

response to emotional stress: a novel mechanism of regulating adrenocorticotropic hormone secretion? *J. Neurosci.* 16: 7725-7732, 1996.

300. Wright, S. D. Toll, a new piece in the puzzle of innate immunity. *J. Exp. Med.* 189: 605-609, 1999.

301. Wright, S. D., R. A. Ramos, P. S. Tobias, R. J. Ulevich, and J. C. Mathison. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science* 249: 1431-1433, 1990.

302. Xu, Y., T. A. Day, and K. M. Buller. The central amygdala modulates hypothalamic-pituitary-adrenal axis responses to systemic interleukin-1b administration. *Neuroscience* 94: 175-183, 1999.

303. Yehuda, R., and S. M. Antelman. Criteria for rationally evaluating animal models of posttraumatic stress disorder. *Biol. Psych.* 33: 479-486, 1993.

304. Yokoyama, C., and K. Sasaki. Regional expressions of Fos-like immunoreactivity in rat cerebral cortex after stress; restraint and intraperitoneal lipopolysaccharide. *Brain Res.* 816: 267-275, 1999.

305. Ziegler-Heitbrock, H. W. L. Molecular mechanism in tolerance to lipopolysaccharide. *J. Inflamm.* 45: 13-26, 1995.

306. Ziegler-Heitbrock, H. W. L., A. Wedel, W. Schraut, M. Ströbel, P. Wendelgass, T. Sternsdorf, P. A. Bäuerle, J. G. Haas, and G. Riethmüller. Tolerance to lipopolysaccharide involves mobilization of nuclear factor kappa B with predominance of p50 homodimers. *J. Biol. Chem.* 269: 17001-17004, 1994.

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