Exploring effects of stress

from a cellular and molecular perspective

Gender-related dimorphisms

and implications for pharmacotherapy

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Exploring effects of stress from a cellular and molecular perspective: Gender-related dimorphisms and implications for pharmacotherapy

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Alla mia famiglia,
per aver sempre creduto in me
ed avermi sostenuto in tutte le scelte

A Sjoukje,
per avermi accompagnato
in questa indimenticabile avventura

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1

General Overview

"Do not tell me the problem is a difficult one.

If it were not difficult it were not a problem"

Marshal Foch

Investigating neuronal functioning from a molecular perspective

Despite the fact that many research papers have been written about stress and stress-related diseases, a clear and scientifically accepted definition of stress does not yet exist. It is generally accepted however, that disruption of body homeostasis evokes a stress response, after wich adaptive compensatory responses are subsequently activated in order to re-establish a new equilibrium. These processes reflect the activation of specific central neurocircuits, which are genetically programmed and constantly modulated by environmental factors [1]. An essential part of the stress response is activation of the hypothalamic-pituitary-adrenocortical (HPA) axis, which results in the secretion of glucocorticoids from the adrenal cortex. A brief period of stress is usually experienced with general excitement and can be beneficial [2,3]. Acute corticosteroid exposure is known to stimulate learning [4-6], enhance memory [7,8], and modulate fear and anxiety-related behaviors [3]. In contrast, prolonged elevation of glucocorticoid concentrations has been associated with altered neurotransmitter function and reduced neuronal plasticity and survival, especially in the hippocampus and prefrontal cortex [9-11]. In accordance, sustained exposure to adverse events has been linked to cognitive impairment, emotional dysregulation, and enhanced vulnerability to psychopathology [12-16].

Although considerable progress has been made in understanding the neurobiological substrates underlying the acute stress response [17], the cellular and molecular mechanisms involved in chronic stress-induced dysfunctions remain largely obscure. An increasing amount of preclinical evidence has suggested that prolonged exposure to uncontrollable and unavoidable stressors can lead to a number of behavioral and biochemical changes resembling those observed in human psychiatric conditions[12,18,19,20]. In the **first chapter** of this thesis, we shall thus examine the neurochemical changes underlying acute and sustained footshock exposure in adult male rats. As stress has been shown to influence brain structure and activity in both a positive and negative manner [9,21,22], we decided to investigate its biphasic action (acute vs. prolonged) on neuronal functions, at the cellular and the molecular level, using a stressful procedure during which conditioned visual and contextual stimuli (CSs) were paired with uncontrollable and inescapable footshocks (USs). “Short-term” aversive paradigms have been extensively used to investigate associative learning and emotional memory [23,24]. However, by extending the length of the training (up to 21 days) and comparing the immunohistochemical changes induced by prolonged footshock exposure with those obtained in response to acute adverse conditions, this “long-term procedure” might prove potentially useful for detailed investigation of the temporal dynamics of stress-induced neuronal impairments.

It is interesting to note that the majority of studies investigating the cellular and molecular events underlying the modulation of cognitive and emotional responses have

been performed in males, leaving the fear system in female brains poorly explored. In the **second chapter**, we have therefore attempted to characterize the mechanisms underlying short- and long-term footshock exposure in both male and cyclic female rats. This data may contribute to the understanding of the neurobiological substrates underlying gender-related differences in cognitive and emotional processing and their relationship with stress-induced cortical-limbic impairments.

Stressful events do not only contribute to the development and/or maintenance of psychopathology in humans [25-27] but they also seem to affect the 'therapeutic power" of antidepressants [28]. The **third** and final **chapter** thus focuses on the neurochemical alterations induced by prolonged footshock exposure and/or concomitant long-term antidepressant treatment. Three different classes of antidepressants were tested including a serotonin reuptake enhancer (tianeptine), a selective serotonin reuptake inhibitor (citalopram), and a selective norepinephrine reuptake inhibitor (reboxetine). Animal models have provided valuable information regarding the role of stress in the development of psychopathology [18,29] as well as antidepressants' mechanisms of action[20,30]. Important discrepancies however exist between animal models and human psychopathology. This is largely attributable to the fact that most of the preclinical research has been performed in male animals [31], while women are reported to have a higher susceptibility to stress-related psychiatric illnesses [32]. Women also constitute the majority of patients receiving antidepressant treatment. To further pursue the gender aspect, this chapter discusses the relationships between chronic footshock exposure and/or long-term antidepressant treatments in cyclic female rats.

Immunohistochemical markers of cellular activity and neuronal plasticity

The differential response of cortical and subcortical structures to stress and/or concurrent antidepressant treatments were investigated by analyzing the changes in the level of expression of key genes (c-fos) and phosphorylation of specific kinases (phospho-ERK1 and phospho-ERK2) and transcription factors (phospho-CREB). The choice of genes and proteins was made upon reviewing their specific cellular functions. Alterations in their expression or phosphorylation allowed us to monitor selective processes such as stress-induced changes of cellular activity and neuronal plasticity. This molecular approach may provide important insights into the neuronal circuits and molecular substrates underlying the response to acute and prolonged stress as well as antidepressant administration. In turn, this may ultimately contribute to our understanding of the dynamic influences exerted by stress on neuronal processes, its role in the development of neuronal abnormalities, and the ability of different pharmacotherapies to attenuate its deleterious effects.

Neuronal activity

The first issue we had to address during this study was the identification of a reliable marker to investigate the patterns of neuronal activity in response to external stimuli. This matter can be easily solved in human or large animals using neuroimaging techniques, as they represent very elegant methods for the investigation of *in vivo* brain activity changes in response to specific tasks [33-36]. When investigating small animals such as mice or rats however, neuroimaging provides only a partial solution. The limited resolution of imaging scanners and the reduced size of the relevant brain structures limit their application in the analysis of *in vivo* responses in rodents. To overcome this matter we chose to investigate neuronal activity from a molecular and cellular perspective using immunohistochemical methods and gene expression techniques.

The immediate early gene c-fos (FOS-ir) is generally used as a marker for neuronal activity [37-40] and changes in its expression level have provided a useful tool to investigate neuronal circuits underlying aversive conditioning [37,41-46], learning [47-50], memory [51-54], or activated by stress [1,55-63] and pharmacological treatments [64-68]. In addition to the traditional manner of FOS analysis, which measures absolute regional c-fos positive cell density, we also introduced a novel approach that we termed "*relative regional FOS-ir*". This alternative analysis likens the neuroimaging concept and provides additional indications with respect to the regional state of activation. Combined with the traditional FOS analysis, this relative interpretation allows a more detailed understanding of the effects of external stimuli on the activity of a "defined" neuronal network.

Neuronal plasticity

The adult brain appears to possess a high degree of plasticity, which is necessary for functional adaptations to a continuously changing environment [69]. Neurotrophins and hormones play an integrative role in the modulation of this plasticity and their multiple actions involve the regulation of the integrity, survival, and vulnerability of neuronal populations [70,71].

Mitogen-activated protein / extracellular signal-regulated kinase (MAPK or ERK) is a family of serine / threonine protein kinases implicated in the transduction of neurotrophic signals from the cell surface to the nucleus [72]. MAPK cascades play a central role in neurodevelopment, regulation of cell growth, proliferation, and differentiation. Interestingly however, several family members, including ERK1 and ERK2, are also widely expressed by post-mitotic neurons in the mammalian nervous system [73]. This evidence has suggested that MAPKs might contribute to the regulation of important functions in the adult brain such as neuronal plasticity, learning, and memory[74,75]. A crucial step in ERK-mediated activities is represented by their dual phosphorylation, which leads to transient activation and translocation from the

cytoplasm to the nucleus [76]. Only phosphorylated ERKs are able to interact with and activate cytoplasmic and nuclear targets like the cAMP/calcium response element binding protein (CREB) [72]. Similar to ERKs, CREB has also been established as a key component in the intracellular transduction system involved in the modulation of neuronal plasticity and survival [77].

Preclinical studies have confirmed the ability of chronic stress to reduce dendritic growth and branching, leading to neuronal atrophy and, in severe cases, cell death [15,78,79]. An intriguing hypothesis holds that stress may affect brain structural plasticity and neuronal survival through its deleterious effects on neurotrophin function and expression [80]. A link between stress, brain-derived neurotrophic factor (BDNF) and depression has recently been put forth [81,82]. The transduction of BDNF signals requires the coordinated activity and interaction of numerous protein kinases and transcription factors, including ERKs and CREB [83-87]. Phosphorylated CREB (phospho-CREB) in turn modulates the expression of several genes underlying neuronal plasticity, including BDNF [88]. Chronic stress has been reported to disrupt this coordinated regulation [89] and the analysis of phospho-ERK1/2 and phospho-CREB expression in response to acute and long-term footshock exposure may thus provide valuable insights into stress-induced changes of neuronal plasticity [74,77,90-92].

Gene expression profiling

Changes in gene expression patterns are essential for the maintenance of normal brain function. Processes such as neuronal plasticity, regeneration, and even cell death, are likely to depend on altered expression of numerous genes [69,93,94]. As these processes are also critical determinants of chronic stress outcome, identifying stress-induced gene expression changes and determining the patterns of temporal expression of these genes is integral for the understanding of the molecular mechanisms underlying abnormal neuronal functions. Several studies have employed molecular biological methods such as RT-PCR and *in situ hybridization* to examine the expression of individual genes whose products contribute to the recovery or the impairment of cognitive functions [95-98]. Although these studies report an altered expression of several individual genes in cortical-limbic regions following stress, a global analysis of mRNA expression in these structures has not yet been carried out. Moreover, although this approach has been used to assess large-scale changes in gene expression patterns in tumor research, metabolic pathways and responses to environmental stresses in yeast cells, only a few reports have been published on their use in studies concerning molecular perturbances that might occur in neuropathological states affecting the mammalian brain [99-101]. Nevertheless, gene expression patterns acquired with these techniques have been successfully applied to identify genes associated with various conditions, including disease states [101-103].

The flood of biological information produced by these experiments has opened new doors into genetic analysis [104]. The use of this approach may help to provide important information regarding processes that reduce survival or cause cell death after exposure to prolonged stress. Although it must be noted that numerous issues related to format, quality, validation, and interpretation of this data remain to be resolved before microarray profiling can become a diagnostic tool of clinical relevance, for those engaged in drug development, this means that therapeutic drug discovery will no longer be hampered by a shortage of targets, but rather, hindered by an excess of targets.

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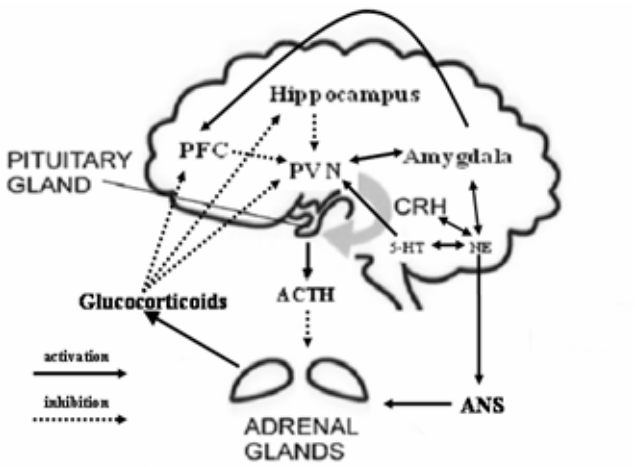
2

Acute vs. prolonged footshock exposure: exploring the role of stress from a cellular and molecular perspective

"The important thing in science
is not so much to obtain new facts
as to discover new ways of thinking about them"
William Bragg

Neuroanatomy of the stress response

Exposure to adverse conditions initiates a series of adaptive responses organized to defend the stability of the internal environment and enhance an organism's survival. This orchestrated process, usually referred to as "stress response", involves various mechanisms, which allow the body to make the necessary physiological and metabolic adjustments required to cope with the demands of a homeostatic challenge. Such changes may occur on the psychological (emotional and cognitive), behavioral (fight and flight), and biological level (altered autonomic and neuro-endocrine function). The unfavorable events that trigger these complex reactions are often termed "stressors" and may be divided into three categories: 1) external changes resulting in pain or discomfort; 2) internal homeostatic disturbances; 3) learned or associative responses to the perception of impending endangerment, pain, or discomfort ("psychological stress") [1].



Stress response system

The primary hormonal mediators of the stress response, glucocorticoids and catecholamines, are often referred to as "stress hormones" and their release is carefully regulated by neural circuits impinging on hypothalamic neurons. Stress hormones have both protective and damaging effects on the body. Whereas in the short run, they are essential for adaptation, homeostatic maintenance, and survival (allostasis), when extended over longer time intervals, they exact a cost (allostatic load) that can accelerate disease processes [2].

Initiation of the stress response

Over the past few years, our understanding of neuroendocrine circuits and neurotransmitter systems involved in the regulation of the stress response has increased substantially. Fundamental aspects of this response involve the perception of the stressor, processing of its specific features, and transduction of this information into neurohormonal, neurobiological, and behavioral responses. The most commonly studied physiologic systems that respond to stress are the HPA axis and the autonomic nervous system (ANS), particularly the response of the adrenal medulla and sympathetic nerves. These systems respond in daily life according to stressful events and to the diurnal cycle

of rest and activity [2]. Modulation of the stress response however, is not limited to these two systems but involves a coordinated interplay of numerous brain structures and neurotransmitters, which interact at various levels to allow a precise activation and/or inhibition of these stress systems. These include the corticotropin releasing factor (CRF)/HPA axis, the CRF/norepinephrine (NE) system, the dopaminergic and serotonergic neurotransmitter systems, the endogenous benzodiazepine, the central glutamate system, γ-aminobutyric acid (GABA), and several other neuropeptides.

The autonomic nervous system

The ANS consists of two fundamental subdivisions, namely the parasympathetic and the sympathetic nervous system, which interact at multiple levels to assure proper functioning. Whereas the former subdivision consists of vagal efferents arising in the medulla oblongata of the brain stem and synapsing in ganglia either embedded in the wall of or close to a wide variety of thoracic and abdominal viscera, the latter includes sympathetic nerves and adrenal medullae. Most organs in the body are innervated by both subdivisions of the ANS, with the exception of the adrenal medulla, sweat glands, and somatic blood vessels, which are regulated exclusively by the sympathetic nervous system [3]. Although sympathetic nervous system activity is regulated by the frontal cortex and the hypothalamus [4,5], the importance of this system is such that its functioning has to be guaranteed even under extreme conditions, including those that impair the connection between higher and lower structures, such as hypothalamic or cortical lesions[6,7]. The relative independence of the sympathetic nervous system from central nervous system (CNS) regulation and the ability of its end-organs to continue functioning under extreme circumstances [3] all contribute to its capacity for autonomous function [8].

The ANS responds rapidly to stressors. Changes of heart rate and blood pressure are some of the primary changes triggered by acute physical and psychological stressors that are mediated by this system. The ANS controls a wider range of activities involved in the maintenance of metabolic homeostasis [9], including cardiovascular [10], respiratory [11], renal [12], endocrine functions [4]. Due to possible paradoxical effects in some instances, the parasympathetic system may assist sympathetic functions by withdrawing or more often by antagonizing sympathetic influences through increased activity [9]. During severe stress, for instance, the vagus nerve mediates some sympathetic-like effects in the gastrointestinal system, such as the suppression of gastric secretion [13]. Epinephrine is released into the circulation from the adrenal medulla and norepinephrine from postganglionic sympathetic nerves innervating the vascular endothelium [3]. A complex hierarchy of central nervous system elements determines neurosympathetic and adrenomedullary activity [4]. A number of discrete neuron populations originating from the nucleus tractus solitarius (NTS), ventrolateral medulla, parabrachial nuclei,

hypothalamus (paraventricular nucleus) and limbic regions (amygdala, prefrontal cortex) project directly and / or indirectly to the sympathetic preganglionic neurons of the intermediolateral column located in the thoracic and lumbar segments of the spinal cord to determine sympathetic output [10]. Viral retrograde transneuronal tracing studies for example have shown significant numbers of infected cells in cortical structures, hypothalamus, amygdala, parabrachial area, nucleus of the solitary tract, ventral medulla oblongata, and intermediolateral cells groups of the thoracic spinal cord after infection of the heart [14] and adrenal gland [15,16]. The hypothalamic paraventricular nucleus (PVN), the main output area of the forebrain limbic system to the pituitary and autonomic system, projects to the dorsal motor nucleus of the vagus nerve as well as the intermediolateral cells groups of the thoracic spinal cord providing the sympathetic innervation [17]. The PVN is thus capable of tuning the delicate balance between the activity of the parasympathetic and sympathetic systems necessary for the maintenance of metabolic homeostasis. Feedback to higher cortico-limbic regions and the hypothalamus from systems signaling changes in the metabolic homeostasis is mediated largely by vagal afferents terminating in the NTS [18]. The NTS, subsequently, relays this metabolic sensory information to the parabrachial nucleus, the locus coeruleus, the amygdala, and the hypothalamus [19,20]. Notably plasma epinephrine levels following aversive stimulation vary depending on stressor intensity [21] and although only a small proportion of norepinephrine release diffuses into the bloodstream, circulating norepinephrine is a useful estimate of neurosympathetic activity [22].

The Hypothalamic-Pituitary-Adrenal (HPA) axis

Prominent amongst the reactions triggered by stress is the release of glucocorticoids by the adrenal glands [23]. A central control station involved in the regulation of this particular response is located in the hypothalamus, namely the paraventricular nucleus. The PVN serves as an integrator of endocrine, autonomic, and behavioral functions under a variety of physiological conditions [17,24-26], as it receives afferent sensory information from several midbrain, cortical, and limbic structures [19,27-29]. This nucleus is divided into several clearly distinguishable subregions [30], including the magnocellular division, which contain neurons that synthesize arginine-vasopressin (AVP) and oxytocin and project to the posterior pituitary, and the parvocellular region, which contain neurons that have efferent projection sites in the median eminence and autonomic centers in the brainstem and spinal cord [17,30-32]. The parvocellular subregion also contains the majority of CRF-synthesizing neurons, which modulate the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary [33]. Arginine-vasopressin neurons in PVN also play a role in ACTH secretion [34-36].

The HPA axis meets the demands of stress primarily through the synthesis and release of 3 key hormones, such as CRF, ACTH, and the species-specific glucocorticoids,

either cortisol (in human and non-human primate) or corticosterone (rodents) [13,23]. Upon stimulation by stress, neurosecretory neurons in the paraventricular nucleus release a cocktail of CRF and AVP into the pituitary portal circulation. CRF and AVP act synergistically augmenting the release of ACTH from the anterior pituitary. Subsequently, ACTH is transported by the systemic circulation to the adrenal glands where it interacts with cortical receptors, causing steroidogenesis and elevation of plasma glucocorticoids [37]. In non-stressful situations, both CRF and AVP are secreted in the portal system in a circadian, pulsatile fashion [38,39]. During acute stress however, the amplitude and synchronization of CRF and AVP pulsations markedly increase, resulting in increases of ACTH and corticosteroid secretory episodes [37]. Glucocorticoids are the final effectors of the HPA axis and participate in the control of body homeostasis and response to stress. They play a key regulatory role in the basal activity of the HPA axis and contribute to the termination of the stress response by acting at hypothalamic and extrahypothalamic levels [40-42]. They also exert powerful inhibitory influences on ACTH secretion [43,44]. The magnitude of the HPA axis response elicited by hypothalamic neurons is thus limited by neuronal and hormonal mechanisms [24,44]. These act synergistically to maintain glucocorticoid levels within tolerable limits by reducing the duration of exposure and minimizing the deleterious effects of these steroid hormones. Hypersecretion as well as prolonged exposure to elevated glucocorticoid levels have been implicated in the etiology of a wide range of neurological and psychiatric illnesses[45]. This illustrates that although adrenal steroids have an important adaptive value, inadequate control of their release may lead to neuronal abnormalities and psychopathology.

The CRF systems

The PVN appears crucial for central regulation of the HPA axis due to its role in both the initiation and inhibition of glucocorticoid secretion. As mentioned above however, modulation of the stress response involves the coordinated activity of multiple systems to allow regulation through interconnections at various levels. The HPA axis also has important functional interactions with the central norepinephrine system. The CRF/norepinephrine system serves as a generalized warning structure to help determine whether, under threat, an individual's attention should turn towards external sensory stimuli or to internal vegetative states [46]. In conjunction with the ANS, the CRF/NE system plays an important role in the maintenance of homeostasis following exposure to stressors.

Besides being closely involved in the modulation of HPA axis activity however, extensive literature indicates that CRF is more than a simple effector of the stress response, as it has also activating properties on behavior. This neuropeptide enhances behavioral responses to stressors, an effect that appears to be independent of the

pituitary and the adrenal axis [47]. There are two different CRF receptors in the brain (CRF_1 and CRF_2) [48,49]. Differential distribution and affinities of these receptors for their specific ligands allow this neuropeptide to exert multiple actions in the brain. The CRF_1 receptor has a higher affinity for its ligand and is most abundant in the neocortex, hypothalamus, amygdala and hippocampus whereas the CRF_2 receptor has a lower affinity and is located in specific subcortical structures such as the amygdala and the hypothalamus [48].

Stimuli that are interpreted by the brain as extreme or threatening elicit an immediate stereotypic response characterized by enhanced cognition, affective immobility, vigilance, and autonomic arousal [13]. The brain's ability to mobilize this particular stress response seems to be mediated by the action of CRF in several subcortical nuclei including the hypothalamus, the amygdala and the locus coeruleus (LC) [13]. This evidence supports the notion of two distinct CRF systems in the brain: one, which is constrained by glucocorticoids (CRF/HPA axis) and another, which is not (CRF/NE system). Although these two CRF systems mutually stimulate each other, responding similarly to messengers, they do differ in their temporal response patterns. Whereas the CRF/NE system is rapidly activated with an earlier response depletion, the CRF/HPA axis response initiates after several minutes yet lasts longer [47].

A fast growing body of clinical and preclinical reports suggests a relationship between alterations in the norepinephrine system and stress [50,51]. Most of the neurobiological evidence supporting this interaction has focused on the locus coeruleus, as this brainstem nucleus contains the majority of noradrenergic cell bodies. Nevertheless, the LC also possesses a dense network of projections that extend throughout multiple cortical and subcortical regions, including the prefrontal cortex, the hippocampus, the amygdala, and the hypothalamus [52]. Numerous studies have supported the notion that stress modulates LC sensitivity to CRF [53,54], which may thus act as an excitatory neurotransmitter during the initiation of stress responses. Stressors increase CRF concentrations in this midbrain region [55] whereas central administration of this neuropeptide has also been shown to activate the LC [56]. Central administration of CRF has also been observed to stimulate the ANS, an effect which appears to be independent and precede the activation of the pituitary-adrenal axis activity [57,58].

It is of interest to note that LC neurons exhibit abundant expression of glucocorticoid receptors [59], which indicates the capacity of this nucleus to respond to fluctuations in circulating corticosteroids. Exposure to adverse experiences has in fact been shown to promote the release of norepinephrine in the PVN [60], whereas neuroanatomical evidence has documented ample norepinephrine-CRF synaptic connections in this nucleus [61,62]. Ascending projections from this midbrain structure may in turn mediate noradrenergic activation of a wide array of cortical and subcortical regions, many of which have been implicated in stress-mediated activation of the PVN [24]. It is conceivable that LC-induced activation of forebrain structures influences the activity

of stress-encoding PVN-projecting pathways, thereby affecting the HPA axis response to stress. LC modulation of PVN activity therefore seems critical for normal neuroendocrine responses to stressors. Abnormal regulation of LC activity may also contribute to the hypothesized increase of neural drive thought to be involved in chronic stress-induced HPA axis hyperactivity, although its role in this response is less crucial and may involve many other stimulatory influences [63].

Another stress-sensitive structure and pivotal component of the CRF system is the central nucleus of the amygdala (CeA). Numerous studies support a central role for the amygdala CRF system in the modulation of behavioral responses to stress [64,65], since acutely stressed rats demonstrate significantly increased CRF levels [66] as well as norepinephrine release in this nucleus [67]. Noradrenergic activation, in turn, further stimulates CRF release [68] suggesting that in the central amygdala, this neuropeptide may itself modulate certain behavioral responses to stress. This nucleus is consistently involved in the organization of processes of passive coping, reflected by immobile behavior and parasympathetic activity [69]. Furthermore, differential regulation of the CeA via its peptidergic neuronal input may underlie distinct behavioural and physiological stress patterns accompanying differing coping styles. The CeA exerts a general, modulatory influence on the neuroendocrine response to acute and unconditioned stressors, whereas during conditioned stress this output seems to be mediated by other amygdalar nuclei [70]. The neuroendocrine state as achieved during acute stress is of importance in learning about the situation and consolidating the experience [71].

Within the amygdala, two other subregions, the lateral (LaA) and the basolateral nucleus (BslA), play a fundamental role in emotional and cognitive processing [72-75]. These nuclei are crucial for accurate modulation of stress response, although, in contrast to the CeA, they constitute input elaboration centers and are involved in the process that attributes the proper valence to specific stressors [73,76]. These subregions also regulate the activity of other structures, such as the hippocampus and the prefrontal cortex, influencing their time-dependent activation, which is requisite to guarantee appropriate adaptive responses to emotional and/or stressful events [77,78]. By affecting neuronal plasticity in the hippocampus for instance, the BslA modulates memory processes, presumably via mediation of stress hormones such as norepinephrine and corticosteroids, in order to establish a discrete memory of an experience [79-81]. Although prolonged exposure to elevated glucocorticoid concentrations may disrupt cognitive responses [81], it is intriguing to speculate that stress hormones, following acute adverse experiences, permissively mediate neuronal plasticity. Like the BslA, considerable evidence indicates the LaA as a site of plasticity and storage of emotional memory [82-84]. This nucleus receives excitatory input from cortical and subcortical processing areas and is believed to be involved in the evaluation of the affective valence of emotional stimuli [5]. Growing evidence further suggests the relevance of the LaA in mediating the association

between CS-US during learning [86] and synaptic plasticity underlying the acquisition of fear-related memories [84]. Taken together, these findings support the view of the amygdala as a heterogeneous structure involved in the coordination of behavioral, neuroendocrine, and autonomic responses to stress, while playing a central role in the processing of cognitive and emotional stimuli.

The serotonergic system

Besides norepinephrine, other neurotransmitter systems have been implicated in the modulation of the stress response, including the serotonergic system [47,87,88]. Serotonin is involved in the regulation of a variety of different processes, including fear, anxiety, arousal, aggression, mood, impulsivity, and food-intake regulation [89]. Anatomical as well as functional evidence support a role of this neurotransmitter in the modulation of stress-induced HPA axis activity [90] and serotonin seems to facilitate CRF, ACTH, and glucocorticoid release [91-93]. Animals exposed to a variety of stressors, including footshock, have shown an enhanced serotonin turnover in various limbic regions. The latter include the medial prefrontal cortex, the amygdala, the hypothalamus, and the LC[94-97]. A more widespread serotonergic activation following more severe stress is thought to be related to behavioral changes reflecting augmentation of fear [96]. Chronic electric shock treatment producing "learned helplessness" behavioral deficits has been associated with reduced *in vivo* release of serotonin in the frontal cortex [98], probably reflecting a situation in which synthesis is not able to keep pace with demand. After inescapable stress, 5-HT_{2A} receptor density has been found reduced in the hypothalamus of helpless rats. While no changes have been found in 5-HT_{1A} receptor density in any brain region, a significantly decreased 5-HT_{2A} density has been found in the hippocampus and amygdala in response to stress yet unrelated to helplessness. In the medial prefrontal cortex, a reduction of serotonin transporter density has also been observed in helpless rats [99]. Notably, stressors have been shown to influence serotonin receptor densities in differential ways. An increased 5-HT_{1A} receptor binding was reported in the hippocampal dentate gyrus of socially stressed rats, while a decreased 5-HT_{2A} receptor binding was observed in the parietal cortex [100]. Serotonin antagonists appear to be able to produce behavioral deficits similar to those observed in response to inescapable shock. The latter may prove of relevance since drugs that stimulate serotonergic transmission (imipramine) thus prevent stress-induced decreases in serotonin and 5-HT_{1A} agonists (buspirone) effectively reverse stress-induced behavioral deficits [101,102].

Termination of the response to stress

Since abnormal regulation of the stress response may lead to prolonged exposure to elevated glucocorticoid levels, appropriate modulation of HPA axis activity becomes

fundamental to prevent the development of neuronal dysfunctions. Efficient activation and feedback inhibition of the HPA axis are essential aspects for optimal coping ability and long-term well being. The termination of the stress response, after diminishment of the stressor, is as important as its initiation. Proper regulation of glucocorticoid release however, is a complex process, requiring appropriate mechanisms to inhibit stress-integrative PVN neurons, final mediators of the stress response system.

The paraventricular nucleus of the hypothalamus

The HPA axis is generally considered to function as a closed loop autoregulatory system modulated by glucocorticoid-mediated negative feedback and operating over multiple time domains, at different levels and by several sources [103]. Glucocorticoid-mediated negative feedback acts directly at the PVN level, since the expression of both CRF and AVP is under regulatory control by the adrenal steroids themselves [41,104]. This inhibition is partly achieved by the binding of circulating glucocorticoids to specific cytoplasmic receptors in the hypothalamus, where they inhibit further release of CRF and, consequently, ACTH secretion in the pituitary [25]. Glucocorticoids can modulate the transcription of responsive genes by interacting with two types of intracellular receptors, the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR), which markedly differ in their neuroanatomical distribution and ligand affinity [105]. Glucocorticoid and mineralocorticoid receptors are localized in discrete brain regions, especially in the limbic system, and exert inhibitory control over the HPA axis [106].

Inhibition of stress responsiveness by adrenal steroids appears to operate through three different and partially independent mechanisms: a rate sensitive fast feedback, an intermediate feedback, and a delayed feedback mechanism [103]. The fast feedback mechanism is a very rapid phenomenon (with a time domain of 5-15 minutes), activated by the rate of rise of plasma glucocorticoid levels rather than their absolute concentration. This inhibition is achieved by glucocorticoid binding to specific receptors in selective limbic regions including the hypothalamus and the hippocampus. Intermediate and delayed-feedback operate relatively slowly over the course of hours to days and are activated by the interaction of the glucocorticoid-receptor complex with selective genes in the hypothalamus [103]. The result of this interaction is the suppression of the expression of selective genes, including CRF [107], thereby decreasing the secretory drive in the pituitary. This direct feedback mechanism however cannot account for all aspects of HPA axis inhibition supporting the hypothesis of the existence of several neural inhibitory pathways working in parallel with steroid feedback. Thus, although direct glucocorticoid inhibitory action at the level of CRF-releasing neurons accounts in part for the ability of maintaining the organism in an ideal state of stress responsiveness, neural connections from the hippocampus and prefrontal cortex to the hypothalamus also play a critical role in the regulation of HPA axis response to stress.

The hippocampus

A central player in the modulation of the stress response, but also a major target of glucocorticoid-mediated effects, is the hippocampus [108]. An inhibitory role of this limbic structure on HPA axis regulation is supported by both clinical and preclinical studies, illustrating that hippocampal stimulation results in decreased HPA activity in both rats and humans [109]. In contrast, lesions occurring in different hippocampal areas cause CRF mRNA up-regulation in the PVN, increased ACTH release, and elevation of circulating corticosterone levels [110]. Reduced hippocampal activity leads to increased basal drive of the HPA axis, possibly caused by the reduction of hippocampal-mediated negative feedback, albeit no direct connections between the hippocampus and the PVN have been identified [17].

The medial prefrontal cortex

Although the hippocampus has long been regarded as the principal control center, many other cortical and limbic structures are involved in the modulation of HPA axis activity, either facilitating its activation or providing inhibitory feedback control [111,112]. The need to better understand the role of higher cortical areas in the modulation of stress response system is clear when considering that a wide variety of psychiatric conditions is associated with both dysfunctional HPA axis regulation and cortical-limbic abnormalities [45,113]. The important role of the medial prefrontal cortex (mPFC) as part of the stress response circuitry has been well documented [112]. It has been known for some time that a high density of corticosteroid receptors is present in the rat frontal cortex and frontocortical glucocorticoid receptors are responsive to changes in circulating corticosterone levels [114]. Exposure to stress causes marked increases in mPFC activity, as reflected by FOS expression [115,116]. It is also interesting to note that, in rhesus monkeys, GR immunoreactivity is much greater in the mPFC than in the hippocampus [114]. This would suggest that, in primates, prefrontocortical regions play a relatively greater role in glucocorticoid-mediated feedback than the hippocampus, which mediates corticosteroid actions primarily though MR activation. Lesions in the medial prefrontal cortex, for instance, were found to significantly increase plasma ACTH and corticosterone levels in response to stress, an effect consistent with a reduced negative feedback action [111,117]. It is also of interest to note however, that a number of behavioral and stress-related processes are differentially regulated by different subregions of the mPFC [118-121]. Thus, while dorsal prefrontocortical regions normally act to inhibit HPA axis functions, ventral areas play a facilitating role in activating the HPA axis. An activational role in HPA axis modulation by the ventromedial PFC is consistent with the fact that electrical stimulation of this area increases plasma corticosterone in the rat [122].

The neurobiology of fear conditioning

Another important aspect of the stress response concerns the ability of glucocorticoids to both promote and disrupt cognitive processing. Much of what we know about the molecular mechanisms underlying learning and memory comes from studies of Pavlovian fear conditioning [74,123-126]. In this learning paradigm, an initially neutral stimulus (conditioned stimulus, CS), such as a tone or a light pulse, acquires the ability to elicit fear-related responses after association with a painful stimulus (unconditioned stimulus, US), such as a brief electric shock to the feet. The last decade has witnessed an unprecedented growth of interest in the investigation of the molecular and cellular mechanisms involved in the acquisition of these conditioned responses. The interest for this topic is mainly related to the possibility that abnormalities in the modulation of these processes may represent an important predisposing factor in the development of psychopathology [73,127,128]. As shown for stress response modulation, multiple brain structures also play an essential role in cognitive processing and interestingly, important similarities exist between the neuronal circuits underlying the modulation of stress and cognitive responses.

The amygdala

The amygdala has long been thought to be involved in emotional behavior. Its role in anxiety and conditioned fear has also been highlighted [78,127,129,130]. This limbic structure modulates memory consolidation, mediates the storage of emotionally relevant information, and comprises a site of neuronal plasticity during associative learning [73-75]. Two distinct neural subsystems within the amygdala seem to mediate different types of conditioned fear-related behavior [131,132]. The first subsystem includes the LaA and the BslA and represents the primary sensory interface of the amygdala. Lesions of these two nuclei produce severe deficits in both the acquisition and expression of fear conditioning[133-137]. The second subsystem consists of the CeA and constitutes the amygdala's interface to extra-amygdala fear response systems. Lesions of the CeA also produce profound deficits in both the acquisition and expression of conditioned fear [138-141] as this nucleus is thought to represent the final common pathway for the generation of learned fear responses [74].

At least two temporally and mechanistically distinct forms of memory are conserved across many species: short-term memory, which persists minutes to hours after training, and long-term memory, which persists for days or longer [142,143]. “New” memories are initially labile and sensitive to disruption before being consolidated into stable long-term memories [144]. The formation of fear-related memories in the amygdala is associated with changes of a broad array of transcriptionally regulated genes [145]. These include genes encoding for transcription factors, cytoskeletal proteins, adhesion

molecules, and receptor stabilization molecules. Requisite activity of phosphatidylinositol 3-kinase (PI-3K) and mitogen-activated protein kinase (MAPK) for both consolidation of fear-related memories and neuronal plasticity in the amygdala[82,146], renders these two signaling pathways an interesting candidates in the series of biochemical events underlying cognitive processing. The participation of these intracellular cascades in the biochemical events underlying learning and memory is also supported by numerous reports concerning the involvement of the cAMP response element binding protein (CREB) in the regulation of the synthesis of new proteins necessary for the consolidation of new fear-related memories [147]. CREB, in fact, represents a common target for both PI-3 kinase and the MAPK cascade members[146,148,149] and its phosphorylation in the amygdala may serve as a molecular switch for the formation of long-term memory in fear conditioning [150].

The hippocampus

Several theories have proposed a role for the hippocampus in the acquisition and retrieval of contextual memories [151-153]. In a typical fear conditioning experiment, rats acquire fear of the CS paired with the US, as well as the contextual cues associated with US delivery. Memory is a complex process composed of several different aspects, which are all supported by different brain systems [154,155]. The neuronal pathways involved in processing aversive stimuli before they come into association with shock are quite different and may thus involve additional subcortical structures, such as the hippocampus. Whereas information regarding discrete CSs appears to reach the amygdala via direct projections from primary sensory areas, information concerning contextual CSs is transmitted to the amygdala via other multisensory pathways [156]. Recent work has supported the hypothesis that the hippocampal formation modulates contextual fear conditioning by storing a conjunctive representation of context [157], assembling contextual representations, and transmitting these representations to the amygdala for association with USs [158].

The fundamental role of hippocampus in cognitive processing is attributable to the almost unique ability of this limbic structure, to generate new neurons throughout adulthood [159-161]. Recent studies have indicated that these newly generated cells possess the morphology and physiological properties of more established neurons. Although the biological relevance of these new neurons has been a matter of discussion for many years, recent evidence suggests that neurogenesis may play a critical role in the formation of some types of hippocampal-dependent memories [162]. Furthermore, these newly generated neurons in the adult brain are not only affected by the formation of hippocampal-dependent memory, but also participate in it [163]. These new cells most likely represents the first step of a complex process necessary to guarantee the appropriate processing of stimuli associated with the acquisition of associative learning

and/or the consolidation of fear-related memory. Neurogenesis alone is not enough to support cognition, and other changes have been suggested to take place in the hippocampus, including the formation of new synapses as well as the remodeling of existing ones [164]. Dendritic spines are sources of synaptic contact that can be altered by experience and, as such, may be involved in memory consolidation. In support of this view, recent studies have shown that the formation and expression of associative memories increase the availability of dendritic spines and the potential for synaptic contact [165].

The structural changes underlying learning and memory require new protein synthesis as well as the activation of specific intracellular signaling pathways in order to be stored. Although the biochemical mechanisms involved in these processes have not yet been fully elucidated, growing evidence suggests that activation of specific protein kinases and phosphorylation of their downstream effectors play a major role [148,166,167]. The extracellular signal-regulated kinases (ERKs) and its effector CREB have been shown to play a key role in hippocampal plasticity and memory formation [168-170], as documented by a rapid and transient activation of ERK and CREB in response to aversive experiences[171,172]. Classical conditioning is known to activate ERK cascade in the hippocampus and this pathway appears to be necessary for the consolidation of the resultant learning [173].

The medial prefrontal cortex

The multiple learning system framework provides a simple set of principles, derived from converging biological, psychological and computational constraints, for understanding the contributions of the medial prefrontal cortex to learning and memory[174,175]. The central principle is that the neocortex has a low learning rate and is not crucially involved in the acquisition, expression, and maintenance of fear-conditioned responses [176]. Other subcortical structures, such as the amygdala and the hippocampus, play a more important role in this process. In addition to the understanding of the processes by which fear-related memories are established and expressed however, there is considerable interest in the mechanisms through which fear-related memories are inhibited. Understanding fear reduction has important clinical implications for treating disorders of fear and anxiety, such as posttraumatic stress disorder, panic disorder, and depression [77,177]. Since animal studies have shown that the medial prefrontal cortex has direct connections with limbic structures that are important in the expression of fear, this may support a functional role of this cortical region in mediating cognitive processing and modulating central states of fear and anxiety [178,179].

Conditioned fear responses to a stimulus previously paired with a shock diminish if the tone is repeatedly presented without the shock, a process known as extinction [164]. A growing amount of evidence has implicated the prefrontal cortex in the inhibition or

extinction of conditional fear [180-183]. Although considerable efforts have been made to elucidate the molecular mechanisms underlying memory, a full comprehension of this process requires the investigation of synaptic plasticity changes related to extinction. As opposed to erasing conditioning, extinction has also been hypothesized to form new memories [182]. Conversion of these new memories into a lasting form may involve the gradual refinement and linking together of neural representations stored widely throughout the neocortex [184]. Destruction of the medial prefrontal cortex blocks recall of fear extinction, indicating that this region might store long-term extinction memory. As a result of its modulatory function, abnormal prefrontocortical activity may also lead to impaired regulation of fear-related responses, a condition frequently observed in depression and anxiety.

The role of glucocorticoids in the modulation of fear conditioning

As mention earlier, whereas a brief period of stress can be exciting and beneficial, chronically elevated levels of circulating glucocorticoids are believed to enhance vulnerability to subsequent insults and lead to psychopathology. It is the timing of corticosteroid increase that determines whether and how neuronal activity and behavior will be affected [2].

Exposure to acute stressful experience has been shown to facilitate classical conditioning in male rats [185]. Such learning and memory is essential for every living organism, as these processes are fundamental when coping with environmental demands, enabling rapid adaptations to changes in the conditions of life. Transient exposure to elevated glucocorticoid concentrations exerts a beneficial effect on an organism's survival as it promotes proper behavioral and neurochemical responses to stress [2,186]. Animals, for instance, immediately freeze and remain alert when a predator or other source of danger is detected. This behavioral response reduces the likelihood of detection and attack from a predator and notably it can already be observed before the HPA axis is activated. After a threat has dissipated and the HPA axis is activated, glucocorticoids promote the consolidation of acquired information [187,188]. Such memories are helpful to predict the occurrence and nature of the next encounter, thereby maximizing the likelihood of survival. The extent of fear and the levels of plasma corticosterone are dependent upon the intensity of the stimulus. In fact, literature reports a positive correlation between the magnitude of corticosterone levels and fear-related behaviors [189]. This evidence supports the involvement of corticosterone in the storage of fear-related stimuli and their consolidation as long-term memories [189].

It has been speculated however, that some individuals may become more sensitive to subsequent stressors if the initial stressor is too strong or the extinction period is too short [186]. The activation of stress response systems is meant to be acute or at least of a limited duration. The time-limited nature of this response renders its effects

temporarily beneficial rather than adverse [2]. In contrast, sustained stress exposure is likely to seriously threaten the welfare of both humans and animals. Since stress response systems coordinate behavioral, neuroendocrine, autonomic, and immune adaptations during adverse situations, their prolonged activation could lead to pathogenesis and all manifestations of the "stress syndrome", including psychiatric, neuroendocrine, cardiovascular, metabolic, and immune components [2,186].

Stress-related psychiatric illnesses, such as melancholic depression, have been characterized by persistent HPA axis activation, possibly due to impaired feedback-inhibition [190]. A full understanding of the molecular mechanisms leading to psychopathology however, remains mostly obscure. Not only is the pathologic process very complex (targeting multiple brain systems, such as those involved in the modulation of stress- and fear-related responses as well as various neurotransmitters, neuropeptides, and stress hormones), but there is also a fine line between adaptation and psychopathology. Prolonged exposure to elevated corticosteroid concentrations, for instance, has been shown to down-regulate MRs rather than GRs [191,192]. Downregulation of GRs requires extensive and prolonged exposure to extremely high levels of corticosteroids [193]. Interestingly, MR may inhibit GR biosynthesis in the dorsal hippocampus [194] by binding to glucocorticoid response elements present in the GR promoter region [195,196]. Due to downregulation of MRs after acute stress, GR numbers may thus increase. This initial MR downregulation and GR upregulation however, seems to be functional [197]. It is postulated that a reduction in the population of MRs presents a risk of reduced fear extinction, whereas elevated numbers of GRs presents a risk of increased fear responsiveness, strong consolidation of traumatic memories, and increased fear potentiation [128]. Fear potentiation can be seen as an adjustment in anticipation of changing demands. Such feed-forward regulation (allostasis) however, may be particularly vulnerable to dysfunctions promoting stress-sensitization [198-200]. Therefore, the initial adaptive hormonal stress response may have maladaptive consequences.

Prolonged stress has been shown to down-regulate both central MRs and GRs, resulting in elevated baseline plasma corticosteroid levels (due to decreased MR function) and increased stress-induced corticosteroid levels that remain high longer after stress (due to decreased GR function and thus feedback resistance) [201]. Elevated plasma corticosteroid levels over a prolonged period may stimulate CRF systems [202-204]. Glucocorticoids may also activate the PVN [205] with a descending CRF projection to brainstem NE-containing neurons [206]. Another NE-CRF interaction may occur in the terminal projections of forebrain noradrenergic systems, including the BST and the CeA, where NE stimulates CRF release [207]. Corticosteroids may also increase the firing rate of 5-HT neurons in the raphe nuclei and stimulate synthesis and release of 5-HT in the limbic system [208,209]. Increased serotonergic and noradrenergic neurotransmission in the

limbic system, together with increased CRF activity in the amygdala, cause a higher "anxiety state" that may represent a key predisposing factor to depression [190]. It has been hypothesized that chronic stress-induced GR downregulation may initially lower this "anxiety state" [128]. This action however brings the organism in a vicious circle, since it causes feedback resistance and even a stronger CRF hyperdrive. Furthermore, due to the impaired GR-function, the central nervous system is bombarded with sensory stimuli at the expense of stimulus integration [128]. As a consequence of these conditions, the organism may have difficulties in adequately evaluating cues of danger and safety. The chronic hyperactivity of the stress response system, together with multiple abnormalities of the norepinephrine and serotonin neurotransmitter systems, represent common features of depression and anxiety disorders [45,89]. Most findings support an underactivation of serotonergic function and a complex noradrenergic dysregulation, most consistent with overactivation of this system [210,211]. Remarkably, impaired stress response regulation has been reported by approximately 50% of depressed subjects, leading to chronic activation of the LC / NE system, HPA axis hyperactivity, and relative immunosupression [212-216]. Furthermore, CRF levels in the cerebrospinal fluid (CSF) are also elevated in these subjects [217]. CRF hypersecretion may also participate in the initiation and / or perpetuation of a vicious cycle involved in the pathophysiology of depression. An increased numbers of PVN CRF and AVP neurons [218], marked hippocampal atrophy [219,220], and a small and hypofunctional medial frontal lobe have been reported in depressed patients [221].

Materials and Methods

Animals

To perform this experiment male Wistar rats were used (n°=48: 212-240 gr). The animals were individually housed with food and water available ad libitum and maintained on a 12/12-hr light/dark cycle. All rats were weighed and handled daily for 5-8 min to minimize the non-specific stress response. Twelve rats (6 control and 6 test-rats) were used in both the acute (duration 3 days) and subchronic experiment (duration 10 days), while 24 rats (12 control and 12 test-rats) were used in the chronic experiment (duration 21 days). Measures were taken to minimize pain and discomfort of the animals during the experiments. The experiments were carried out in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC) and with the guidelines of the Animal Bio-ethics Committee of the University of Groningen (FDC: 2509).

Footshock procedure

The rodent test-chamber consists of a box containing an animal space placed on a gridfloor connected to a shock generator and scrambler. A light, placed on the wall, was used for the conditioning. Test-rats received one session of 30 min/day in the footshock chamber during which 5 inescapable footshocks were given (0.8 mA in intensity and 8 sec in duration: unconditioned stimulus; US) with different inter-shock intervals in order to make the procedure as unpredictable as possible. Each footshock was preceded by a pulse of light (10 sec) in order to condition the rats to it (conditioned stimulus; CS). This conditioning procedure was followed for 2 (acute experiment), 9 (subchronic experiment) or 20 days (chronic experiment). On the final day of each experiment (3rd for the acute, 10th for the subchronic and 21st for the chronic) all rats received 5 CSs only without being exposed to any USs. The coupling of CSs to USs was fundamental on the final day of each experiment as it allowed us to investigate the patterns of protein expression and/or phosphorylation induced by neutral stimuli (CS) previously coupled with painful footshocks (USs).

Control rats. Control animals were exposed to the same stimuli as the test-rats (as they were housed in the same room and similarly exposed to the footshock box and CSs). They did not however receive USs throughout the entire duration of the experiment.

Physiological and neuroendocrine measurements

To define the changes induced by prolonged footshock stress, physiological and neuroendocrine parameters were measured. Weight gain was monitored on a daily basis throughout the experiment, and upon termination, adrenal glands were removed and weighed. Graphs were constructed to serve as a reference to verify the severity of stress perceived by the animals. In addition, blood samples were also drawn by transcardial puncture immediately upon termination and stored at -20°C. These samples were used to determine plasma corticosterone and adrenaline concentrations with HPLC.

Extraction and Chromatography

Adrenaline. Adrenaline was extracted from plasma using liquid/liquid extraction with 3,4-dihydroxybenzylamine as internal standard [222,223]. Briefly, plasma adrenaline was bound to diphenylborate-ethanolamine at pH 8.6. The extraction was performed with n-heptane (containing 1% octanol and 25% tetraoctylammoniumbromide). Finally, adrenaline was extracted from the organic phase with diluted acetic acid. Adrenaline (20 µl acetic acid extract) was analyzed using an HPLC/auto-injector (CMA, Sweden) and a Shimadzu LC-10AD pump (Kyoto, Japan) connected to a reversed phase column (Hypersil, C18, 3µm, 150x2.0mm), followed by an electrochemical detector (Antec Leyden, The Netherlands) working at a potential setting of 500mV vs. Ag/AgCl reference. The mobile phase consisted of 50mM acetate buffer, 150mg/l octane sulphonic acid, 150mg/l tetramethylammonium, 15mg/ml Na_2EDTA and 3% methanol, adjusted to pH 4.1. The flow-rate was 0.35ml/min. Temperature was 30°C. The detection limit of the method was 0.1nM.

Corticosterone. For the assay, dexamethasone was used as internal standard. After addition of the internal standard, plasma was extracted with 3 ml of diethylether, vortexed for 5 min and then centrifuged for 5 min at 3000 x *g*. The extraction procedure was repeated twice. The organic phase was evaporated to dryness in a 50°C waterbath. The residue was reconstituted with 200µL of mobile phase and 50µL was injected into the HPLC system. The mobile phase (flow rate 1.0mL/min) for the determination consisted of acetonitrile in ultrapure water (27:73 v/v). The concentration of both corticosterone and the internal standard was determined with UV detection at a wavelength of 254nm. The detection limit of the method was 10nM.

Histological procedure - Molecular biology

Tissue and RNA Preparation

Thirty minutes after the start of the final session, rats used for the molecular biology were anesthetized with halothane and decapitated. The prefrontal cortex was dissected, quick-frozen in liquid nitrogen and stored at -80°C. Total RNA was isolated from the prefrontal cortex of each animal by using Trizol (Life Technology, Gaithersburg, MD, USA) according to the manufacturer's instructions. Integrity of total RNA was confirmed on an agarose gel and final concentrations were assessed spectrophotometrically.

cDNA microarray

RNA extracted from the prefrontal cortex was pooled from all the rats within each group (MS MC FS FC; 2µg/pool) and converted into a ^{32}P-labeled first-strand cDNA, used to hybidize cDNA microarrays (rat atlas cDNA array 1.2; Clontech, Palo Alto, CA, USA). Use of a broad coverage array instead of a stress array was intentionally chosen because of our interest in the role of transcription factors and second messengers in stress-induced neuronal dysfunction which could involve the expression of numerous candidate genes. In this microarray, plasmid and bacteriophage DNAs are included as negative controls, along with several housekeeping cDNAs as positive controls. A complete list of the genes and controls

spotted on the array, as well as array coordinates and GenBank accession numbers, is available at Clontech's web site, (http://www.clontech.com). In order to suppress non-specific background each membrane was prehybridized for 30 min at 68ºC in 5ml of hybridization solution (ExpressHyb, Clontech) with continuous agitation. Hybridization was subsequently carried out by the addition of the denatured, labeled cDNA to the prehybridization solution at 68ºC for overnight incubation to reach a final probe concentration of 2-5 x 10^6cpm/ml. Membranes were stringently washed with continuous agitation at 68ºC in 2 x SSC, 1% SDS (4x30 min) and then in 0.1 SSC, 0.5% SDS (30 min) After a final rinse in 0.1 x SSC (5 min), membranes were mounted on Whatman paper, plastic-wrapped, exposed to x-ray film overnight at -80ºC followed by exposure to a phosphoimager screen for 3 days.

PhosphoImaging analysis

Membranes were scanned using a Molecular Dynamics STORM PhosphoImager (Molecular Dynamics, Inc., Sunnyvale, CA, USA), and images were analyzed by ImageQuant (Molecular Dynamics, Inc., Sunnyvale, CA, USA). The expression level for each gene was quantified after background correction. Local background for each membrane was calculated on the basis of the negative controls on the array, positions with no DNA spotted. Signals were normalized using the average intensities of a set of 3 least variable housekeeping genes present on each array. For each comparison and for each cDNA represented in the array, an absolute difference (of intensities) was calculated as well as a ratio by dividing normalized intensities of spots on one array by normalized intensities of spots on a second array. Based on a pilot study, a difference was considered eligible for further confirmation with RT-PCR when the hybridization signal of a gene extended to two times the background signal with an absolute difference >10 (as measured by the phosphoimager in arbitrary signal intensity units). According to the Array manufacturer, Clontech, the radioactive cDNA signal is linear for RNAs present at levels of 0.01-3% of the total RNA population. An admonition of this quantitative analysis however, is that the accuracy for the extremely low abundant genes may not be reliable due to the detection limitation.

Quantitative RT-PCR

Total RNA from the prefrontal cortex of the same animals used for the cDNA microarray was used for further confirmation with quantitative PCR. To convert each sample to cDNA, reverse transcription was performed. All reagents were purchased from Roche Molecular Biochemicals. Per sample 2µg of total RNA was diluted with water to a total volume of 34µl, heated to 65°C for 10 min, and then placed on ice. To this was added 10 µl 5x incubation buffer, 2µl M-MuLV (40U), 2µl dNTP mix (2mM), 2µl pT18 (15µM), 0.5µl RNAse inhibitor (20U) and 0.5µl DTT (1M), to a total volume of 51µl. The reaction mixture was incubated at 37°C for 90 min. Real-time, one-step, no-nested PCR for ERK2 mRNA (262 bp) was performed using the LightCycler thermal cycler system (Roche Diagnostics) according to the manufacterer's instructions. The location of the gene was Accession number M64300; position 189 U 19 and 450 L 24. The primers for ERK2 were sense: 5'-GGC GGG CCC GGA

GAT GGT-3' and antisense: 5'-AAT GGT TGG TGC CCG GAT GAT GTC-3' (Biolegio, Malden, the Netherlands). Use of the β-actin gene (Accession number V01217) was included as housekeeping gene and the primers used were sense: 5'-ACC CAC ACT GTG CCC ATC TA-3' and antisense: 5'-GCC ACA GGA TTC CAT ACC CA-3' (TIB Molbiol, Berlin, Germany). PCR reagents excluding the primers were part of the LightCycler DNA Master SYBR Green kit (Roche Molecular Biochemicals, Mannheim, Germany). In brief, 2µl of cDNA was used per reaction and the following program was applied. After an initial denaturation at 95°C, the samples were run for 45 cycles at 95°C, 55°C (15s), and 72°C (20s). At the end of each cycle, the fluorescence was measured in a single step in Channel F1. After the 45th cycle, the PCR products were subjected to a melting curve analysis to confirm amplification specificity and fluorescence was measured continuously (channel F1). The melting curve analysis started at 45°C for both primer pairs and was raised to 95°C in steps of 0.2°C/sec. The melting temperatures for ERK2 and β-actin were 90°C and 89°C respectively. After completion, quantification of signals were analyzed with the Light Cycler analysis software which calculates the relative copy number of target molecules by plotting logarithm of fluorescence versus cycle number and setting a baseline x-axis. The baseline identifies the cycle in which the log-linear signal can be distinguished from the background for each sample. In order to avoid misinterpretation of the expression profiles due to variation in the amount of starting material between the samples, the crossing points of ERK2 were compared with the noise band crossing point cycle number of β-actin from each sample.

Histological procedure

Two hours after CS exposure, the rats were terminated with an overdose of halothane which preceded a transcardial perfusion with 4% paraformaldehyde solution in 0.1M sodium phosphate buffer (pH 7.4). The brains were carefully removed and post-fixed in the same solution overnight at 4°C, before being transferred to a potassium phosphate buffer (KPBS 0.02 M, pH 7.4) and stored at 4°C. Following cryoprotection of the brains by overnight immersion in a 30% glucose solution, coronal serial sections of 40µm were prepared on a cryostat microtome. Sections were collected in KPBS with sodiumazide and stored at 4°C.

Immunohistochemistry

All stainings were performed on free-floating sections under continuous agitation. The sections were pre-incubated in 0.3% H_2O_2 for 15 min to reduce endogenous peroxidase activity, before being incubated with the respective primary antibody solutions:

- polyclonal rabbit anti-c-fos antibody (commercialized by Oncogene Research Products, brands of CN Biosciences, Inc, an affiliate of Merck KGaA, Darmstadt, Germany) 1:10000 dilution in KPBS 0.02 M, pH 7.4, for 60-72 hr at 4°C;
- polyclonal rabbit anti-phospho-CREB (commercialized by Upstate Biotechnology, Charlottesville, VA, USA: www.upstatebiotech.com) 1:1000 dilution in KPBS 0.02 M, pH 7.4, for 60-72 hr at 4°C;

- monoclonal mouse anti-phospho-ERK1/2 antibody (commercialized by New England Biolabs, Inc., Beverly, MA, USA; www.neb.com) 1:5000 dilution in KPBS 0.02 M, pH 7.4, overnight at room temperature.

Subsequently, sections were washed with KPBS and incubated at room temperature with secondary biotinylated goat anti-rabbit (for the anti-c-fos and anti-phospho-CREB antibodies) or goat anti-mouse (for the anti-phospho-ERK1/2 antibody) IgG antibodies (Vector Laboratories, Inc., Burlingame, CA, USA; 1:1000 dilution in KPBS 0.02 M, pH 7.4) followed by ABC complex (Vector ABC kit, Vector Laboratories, Burlingame, CA, USA). After another wash, the reaction product was visualized by adding diaminobenzidine as chromogen and 1% H_2O_2 for 15 min. Finally, the sections were washed, mounted on slides, dehydrated and coverslipped with DePex.

Antibody specificity testing. To control for cross-reactivity due to aspecific binding, immunostainings were performed by incubating the sections without the presence of one of the antibodies needed for the reaction (primary, secondary or tertiary antibody). All these reactions were negative thereby confirming the specificity of the antibodies.

Quantification and data analysis

FOS, phospho-CREB and phospho-ERK1/2-labeled cells were quantified using a computerized image analysis system by an observer who was blind to group assignment. Selected areas from regions of interest (ROIs) were digitized by using a Sony (SONY Corporation, Tokyo, Japan) charge-coupled device digital camera mounted on a LEICA Leitz DMRB microscope (LEICA, Wetzlar, Germany). Quantification was carried out at x100 (FOS and phospho-CREB positive nuclei) or x200 magnifications (phospho-ERK1/2 positive dendrites) using at least 5 coronal serial sections (the rostro-caudal distance between consecutive sections was 0.4mm) for each area or nucleus of interest. ROIs were outlined with a digital pen. Each digitized image was individually set at a threshold to subtract the background optical density. The numbers of cell nuclei or dendrites above the background were counted by use of the computer-based image analysis system LEICA (LEICA Imaging System Ltd., Cambridge, England). Only cell nuclei and dendrites that exceeded a defined threshold were detected by the image analysis system and subsequent counts were reported as number of positive cells/0.1mm^2 (FOS, phospho-ERK1/2, and phospho-CREB immunoreactivity) or number of H+V intersections/0.1mm^2 (phospho-ERK1/2 immunoreactivity). FOS, phospho-ERK1/2, and phospho-CREB-labeled cells and dendrites with gray levels below the defined thresholds were thus classified as “negative”. This is of relevance for proper understanding of our results, since this method does not allow discrimination between negative nuclei with no immunoreactivity and nuclei with (too) low immunoreactivity. This method is thus not suitable for determining absolute protein levels. All areas were measured bilaterally (no left-right asymmetry was found) and therefore the absolute regional FOS (table 1), phospho-CREB, and phospho-ERK1/2 immunoreactivity for each region was reported (mean±standard error (SEM)).

FOS-ir. ROIs included the prefrontal (prelimbic and infralimbic areas; mPFC: Bregma +3.60 to +1.70) and the cingulate cortex (AC: Bregma +3.20 to +0.95); the hippocampal CA1 area (CA1: Bregma –2.45 to –4.60) and the dentate gyrus (DG: Bregma -2.00 to –3.90); the central (CeA: Bregma –1.53 to –2.85), the lateral (LaA: Bregma –2.00 to -3.70), the basolateral (BslA: Bregma –1.78 to –3.25), and the medial nucleus of the amygdala (MeA: Bregma –1.78 to –3.25); the paraventricular (PVT: Bregma –1.33 to –3.90), the dorsomedial (DMT: Bregma –2.00 to –3.90), and the centromedial thalamic nucleus (CMT: Bregma –1.53 to –3.90); the paraventricular (PVN: Bregma –1.08 to –2.00) and the dorsomedial hypothalamic nucleus (DMH: Bregma –2.45 to –3.70); the dorsal raphe (DR: Bregma –7.10 to –9.25), the medial raphe (MR: Bregma –9.25 to –10.35), and the periaqueductal grey (PAG: Bregma –6.53 to –8.30) (table 1) [224].

Phospho-CREB expression. ROIs included the medial prefrontal cortex, the cingulate cortex, the somatosensory cortex (Bregma 2.80 to –0.11), the perirhinal cortex (Bregma +2.80 to 0.00), the hippocampal dentate gyrus, the lateral and the basolateral nucleus of the amygdala [224].

Phospho-ERK1/2 immunoreactivity. ROIs included the medial prefrontal cortex, the cingulate cortex, the somatosensory cortex, the perirhinal cortex. After image acquisition, the number of phospho-ERK1/2-stained dendrites were quantified as the number of horizontal (H) and vertical (V) intersections (H+V contacts) between positive dendrites and an imaginary detection grid (composed by 514 horizontal x 698 vertical lines) present in the quantification field [225].

Relative regional FOS-ir

The prefrontal cortex, the cingulate cortex, the amygdala, the hippocampus, the thalamus, and the hypothalamus are considered to be part of a complex neural network, the cortical-limbic system. A specific exchange of information between its components, most likely, determines whether this system can properly carry out its functions, such as modulating mood and emotions and regulating stress response. With this in mind, we decided to investigate the response of individual brain regions relative to the network as a whole rather than limiting the analysis to their absolute levels of activation (c-fos positive cell densities)[226]. This manner allows one to consider individual cortical-limbic structures as parts of a larger, more complex system. In order to perform this calculation, we determined the average regional surface (ARS) of all the regions of interest (table 1). The ARS of each cortical-limbic component was calculated by determining the mean surface area of each region across all the animals. The c-fos positive cell densities of each region was then multiplied by the average regional surface for all animals (regional cell density $_{rat\ n}$ * ARS). This was done in order to correct for eventual differences in quantified areas between different rats, thereby providing c-fos positive cell numbers across a similar cortical-limbic quantified surface area in all rats, suitable for comparison. By adding the number of c-fos positive cells of every region (regional cell density $_{rat\ n}$ * ARS) together for each animal we obtained the total number of cortical-limbic c-fos positive cells (TOT $_{rat\ n}$). To acquire the relative regional activation values of each animal (% regional activation $_{rat\ n}$), it sufficed to divide the “regional cell density $_{rat\ n}$ *

ARS" by the $TOT_{rat\ n}$. The example below illustrates the formulas and their calculations.

BRAIN AREAS (ARS)	No stress		Acute stress		Chronic stress	
	Absolute	*Relative* (%)	*Absolute*	*Relative* (%)	*Absolute*	*Relative* (%)
FRONTAL CORTEX						
Prefrontal cortex (6.5mm^2)	54±6	61.8±1.9	66±9	45.8±1***	118±9***	68.6±1.9*
Anterior cingulate (2.9mm^2)	15±1	13.1±0.9	41±6**	20.5±1.3**	20±3	8.5±0.8**
HIPPOCAMPUS						
Dentate gyrus (1.25mm^2)	12±2	2.08±0.31	10±1	1.05±0.15*	6±1*	0.58±0.07**
CA1 (1.18mm^2)	2±1	0.2±0.07	11±4*	0.67±0.16*	4±1	0.24±0.05
AMYGDALA						
Central (1.0mm^2)	7±3	0.88±0.31	39±7**	3.09±0.48**	16±3*	1.34±0.25
Lateral (1.4mm^2)	7±2	1.24±0.24	17±2**	2.08±0.22*	11±1	1.02±0.04
Basolateral (1.6mm^2)	6±1	0.96±0.1	17±4*	1.80±0.15**	19±3**	1.54±0.17*
Medial (2.0mm^2)	9±1	3.12±0.34	17±3*	3.72±0.47	16±2**	2.74±0.11
THALAMUS						
Centromedial (0.8mm^2)	11±3	0.76±0.29	40±8*	1.38±0.19	7±2	0.2±0.06
Dorsomedial (1.6mm^2)	2±1	0.28±0.08	4±2	0.22±0.06	2±1	0.12±0.02
Paraventricular (1.8mm^2)	39±6	4.54±0.67	69±6*	4.90±0.37	40±8	2.28±0.37*
HYPOTHALAMUS						
Paraventricular (1.5mm^2)	27±2	4.66±0.79	115±31*	10.42±0.65*	89±10***	7.44±0.71*
Dorsomedial (2.0mm^2)	44±6	6.4±1.01	52±5	4.48±0.42	80±10**	5.58±0.84
Cortical-limbic system		**100%**		**100%**		**100%**
MIDBRAIN						
Medial raphe (MR)	6±1	--	19±5*	--	23±6*	
Dorsal raphe (DR)	10±2	--	18±3*	--	34±5**	

Table 1. ***** = p<0.05; ****** = p<0.01; ******* = p<0.001; non-stressed vs. stressed rats.

Example: **Rat 1**

Total cortical-limbic activation $_{rat\ 1}$: Σ (cell density $_{rat\ 1}$ * ARS) $_{mPFC}$ + (cell density $_{rat\ 1}$ * ARS) $_{AC}$ + …….. + (cell density $_{rat\ 1}$ * ARS) $_{PVN}$ = $TOT_{rat\ 1}$

Relative regional activation: % $mPFC_{rat\ 1}$: (cell density $_{rat\ 1}$ * ARS) $_{mPFC}$ / $TOT_{rat\ 1}$

Mean *relative regional activation* of each animal group:

Σ (% $mPFC_{rat\ 1}$ + …… + % $mPFC_{rat\ n}$) / n

The above equations allow a rapid calculation of the relative FOS-ir using the absolute FOS-ir densities and ARSs listed in table 1. To illustrate this with two examples, we have used 2 fictitious rats (CTR and STR) with absolute FOS densities coinciding with the mean values presented in table 1.

Example 1: Rat $_{CTR}$

Total cortical-limbic activation $_{rat\ CTR}$:

Σ (54 * 65) $_{mPFC}$ + (15 * 29) $_{AC}$ + (12 * 12.5) $_{DG}$ + (2 * 11.8) $_{CA1}$ + (7 * 10) $_{CeA}$ + (7 * 14) $_{LaA}$ +

$(6 * 16)_{BslA} + (9 * 20)_{MeA} + (11 * 8)_{CMT} + (2 * 16)_{DMT} + (39 * 18)_{PVT} + (44 * 20)_{DMH} + (27 * 15)_{PVN} = TOT_{rat\ CTR} = 6669.6$

Each ARS has been multiplied by a factor 10 since the regional densities express the number of positive cells / $0.1mm_2$ (mPFC: ARS * 10 = $6.5mm_2$ * 10). The regional relative FOS-ir can be thus calculated:

Relative regional activation:

$\%\ mPFC_{rat\ CTR}$: $(cell\ density_{rat\ CTR} * ARS)_{mPFC} / TOT_{rat\ CTR} = (54 * 65)_{mPFC} / 6669.6 =$

$= 3510 / 6669.6 = 52.6\%$

$\%\ AC_{rat\ CTR}$: $(cell\ density_{rat\ CTR} * ARS)_{AC} / TOT_{rat\ CTR} = (15 * 29)_{AC} / 6669.6 = 435 / 6669.6 =$

$= 6.52\%$

Example 2: Rat $_{STR}$

Total cortical-limbic activation $_{rat\ STR}$:

$\Sigma\ (118 * 65)_{mPFC} + (20 * 29)_{AC} + (6 * 12.5)_{DG} + (4 * 11.8)_{CA1} + (16 * 10)_{CeA} + (11 * 14)_{LaA} +$

$(19 * 16)_{BslA} + (16 * 20)_{MeA} + (7 * 8)_{CMT} + (2 * 16)_{DMT} + (40 * 18)_{PVT} + (80 * 20)_{DMH} +$

$+ (89 * 15)_{PVN} = TOT_{rat\ STR}\ 13053.2$

Relative regional activation:

$\%\ mPFC_{rat\ STR}$: $(cell\ density_{rat\ STR} * ARS)_{mPFC} / TOT_{rat\ STR} = (118 * 65)_{mPFC} / 13053.2$

$= 7670 / 13053.2 = 58.76\%$

$\%\ AC_{rat\ STR}$: $(cell\ density_{rat\ STR} * ARS)_{AC} / TOT_{rat\ STR} = (20 * 29)_{AC} / 13053.2$

$= 580 / 13053.2 = =4.44\%$

The relative regional c-fos expression (mean±standard error (SEM)) for each region was reported (table 1).

Statistics

The mean±standard error (SEM) for each region was reported. One-Way-Anova and *F* tests of variance were run on numbers of immuno-positive dendrites and cell nuclei from individual brain regions of interest from experimental and control conditions. That value determined whether *post-hoc t tests* for equal or unequal variance were performed to compare the cell counts from individual brain regions of control and experimental conditions. $P<0.05$ was defined as the level of significance between groups.

Results

To define the changes induced by prolonged footshock exposure, various physiological and neuroendocrine parameters were measured. These include body weight gain during the experiment, plasma corticosterone and adrenaline levels as well as adrenal weights on the final day.

Body weight gain

Body weights of control and chronically stressed rats were measured daily during the acclimatization period and following the footshock procedure (fig. 1). During the acclimatization period both groups showed an identical weight gain. Immediately after initiation of the footshock procedure however, a consistent reduction in body weight gain was observed in chronically stressed rats, while control animals grew as expected. The difference in weight gain between controls and stressed rats increased progressively reaching a significant value on day 6 of the procedure ($F=6.13$, $p<0.033$) and continued increasing until the final day ($F=18.09$, $p<0.0019$) (fig. 1).

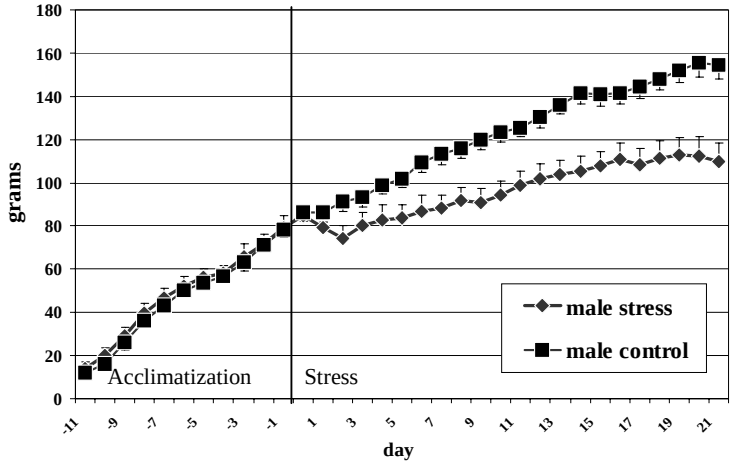


Figure 1. Body weight gain following prolonged stress exposure.

Plasma corticosterone and adrenaline concentrations – Adrenal weights

Blood samples were taken on the final day after exposure to 5 CSs. Corticosterone and adrenaline concentrations were measured by HPLC. Interestingly, although only exposed to the CSs, chronically stressed rats demonstrated significantly higher plasma corticosterone concentrations ($F=8.14$, $p<0.021$), indicating that no habituation of the HPA axis response occurred following repeated footshock exposure. Adrenaline concentrations, although higher in chronically conditioned rats, did not reach a statistical difference compared to non-stressed animals ($F=2.87$, $p<0.12$) (fig. 2a). Chronically stressed rats also showed significant adrenal hypertrophy ($F=24.20$, $p<<0.001$) which, combined with higher corticosterone levels, may suggest a prolonged HPA axis hyperactivity (fig. 2b).

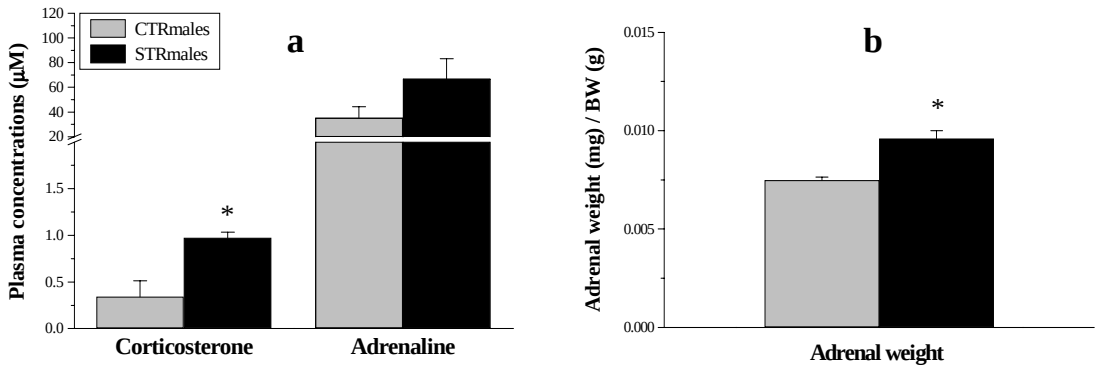


Figure 2. Plasma corticosterone and adrenaline levels measured on the final day of the experiment (a). Chronically stressed rats also reported a significant adrenal hypertrophy in response to repeated footshock exposure (b).

Immunohistochemistry

FOS-ir

As aforementioned, FOS-ir was examined throughout several cortical and subcortical regions (fig. 3) including the prefrontal cortex, the hippocampus, the amygdala, the thalamus, the hypothalamus (fig. 4), and the midbrain (table 1) in rats exposed to acute (two days) and long-term footshock procedure (20 days).

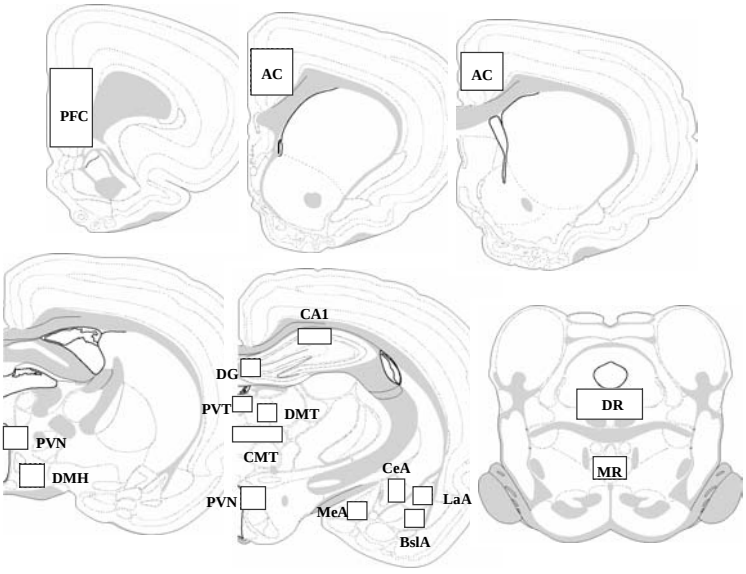


Figure 3. Schematic diagram illustrating the location of cortical-limbic regions used in the quantification of FOS-ir.

Acutely stressed rats showed a significantly increased absolute cortical-limbic FOS-ir compared to non-stressed animals ($F^{absolute(ab)}$=6.3, $p^{absolute(ab)}$<0.03) (fig. 5a). Absolute FOS-ir was found significantly increased in the AC (F^{ab}=14.35, p^{ab}<0.0043) (fig. 5b), the CA1 (F^{ab}=5.39, p^{ab}<0.045) (fig. 5c), the CeA (F^{ab}=13.45, p^{ab}<0.0063), the LaA (F^{ab}=16.39, p^{ab}<0.0037), the BslA (F^{ab}=8.93, p^{ab}<0.017) and the MeA (F^{ab}=6.12, p^{ab}<0.038) (fig. 5d), the CMT (F^{ab}=9.54, p^{ab}<0.013) and the PVT (F^{ab}=11.42, p^{ab}<0.008) (fig. 5e), the PVN (F^{ab}=6.45, p^{ab}<0.032) (fig. 5f), the MR (F^{ab}=6.24, p^{ab}<0.034) and the DR (F^{ab}=8.46, p^{ab}<0.02) (fig. 5g).

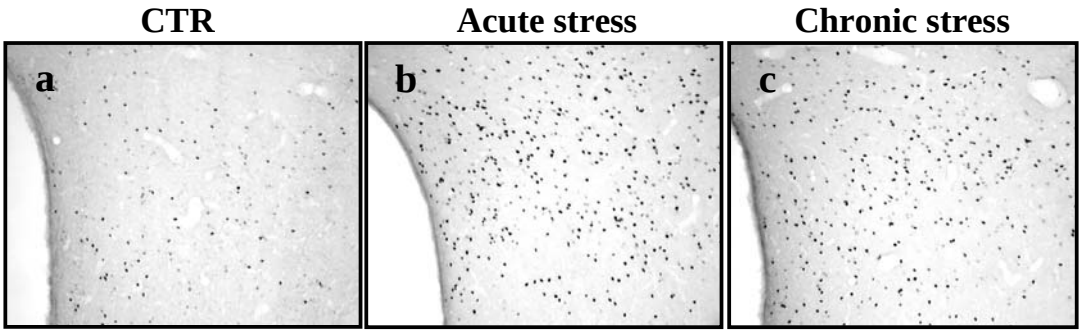


Figure 4. Microphotographs illustrating FOS-ir in the paraventricular hypothalamic nucleus in control (a), acutely (b) and chronically stressed rats (c).

Areas showing a significantly increased *relative FOS-ir* included the anterior cingulate cortex ($F^{relative(re)}$=19.71, $p^{relative(re)}$<0.0016) (fig. 6a), the hippocampal CA1 area (F^{re}=5.93, p^{re}<0.038) (fig. 6b), the central (F^{re}=13.43, p^{re}<0.005), the lateral (F^{re}=6.68, p^{re}<0.029) and the basolateral nucleus of the amygdala (F^{re}=19.41, p^{re}<0.0017) (fig. 6c), and the paraventricular hypothalamic nucleus (F^{re}=8.64, p^{re}<0.016) (fig. 6e). In contrast, the mPFC (F^{re}=31.92, p^{re}<<0.001) (fig. 6a) and the hippocampal DG (F^{re}=9.97, p^{re}<0.012) (fig. 6b) showed a significantly decreased *relative activation*.

Chronically stressed rats showed a significant increase of cortical-limbic activation compared to non-stressed animals, as measured by absolute FOS-ir (F^{ab}=32.26, p^{ab}<<0.001) (fig. 5a). A general increased absolute FOS-ir was observed in the mPFC (F^{ab}=36.90, p^{ab}<<0.001) (fig. 5b), the CeA (F^{ab}=6.52, p^{ab}<0.034), the BslA (F^{ab}=14.72, p^{ab}<0.005) and the MeA (F^{ab}=12.25, p^{ab}<0.0081) (fig. 5d), the DMH (F^{ab}=10.05, p^{ab}<0.013) and the PVN (F^{ab}=35.96, p^{ab}<<0.001) (fig. 5f), the MR (F^{ab}=9.95, p^{ab}<0.016) and the DR (F^{ab}=28.65, p^{ab}<0.0011) (fig. 5g). Only the DG showed an opposite effect, such as a significant decrease of absolute FOS-ir following chronic challenge (F^{ab}=6.63, p^{ab}<0.033) (fig. 5c).

Cortical-limbic structures showing a significantly increased *relative FOS-ir* included the mPFC (F^{re}=6.79, p^{re}<0.031) (fig 6a), the BslA (F^{re}=8.58, p^{re}<0.019) (fig 6c), and the PVN (F^{re}=6.88, p^{re}<0.031) (fig. 6e). A significantly decreased *relative regional activity*, instead, was detected in the AC (F^{re}=14.43, p^{re}<0.0052) (fig 6a), the hippocampal DG (F^{re}=22.10, p^{re}<0.0015) (fig 6b), and the PVT (F^{re}=8.61, p^{re}<0.019) (fig 6d).

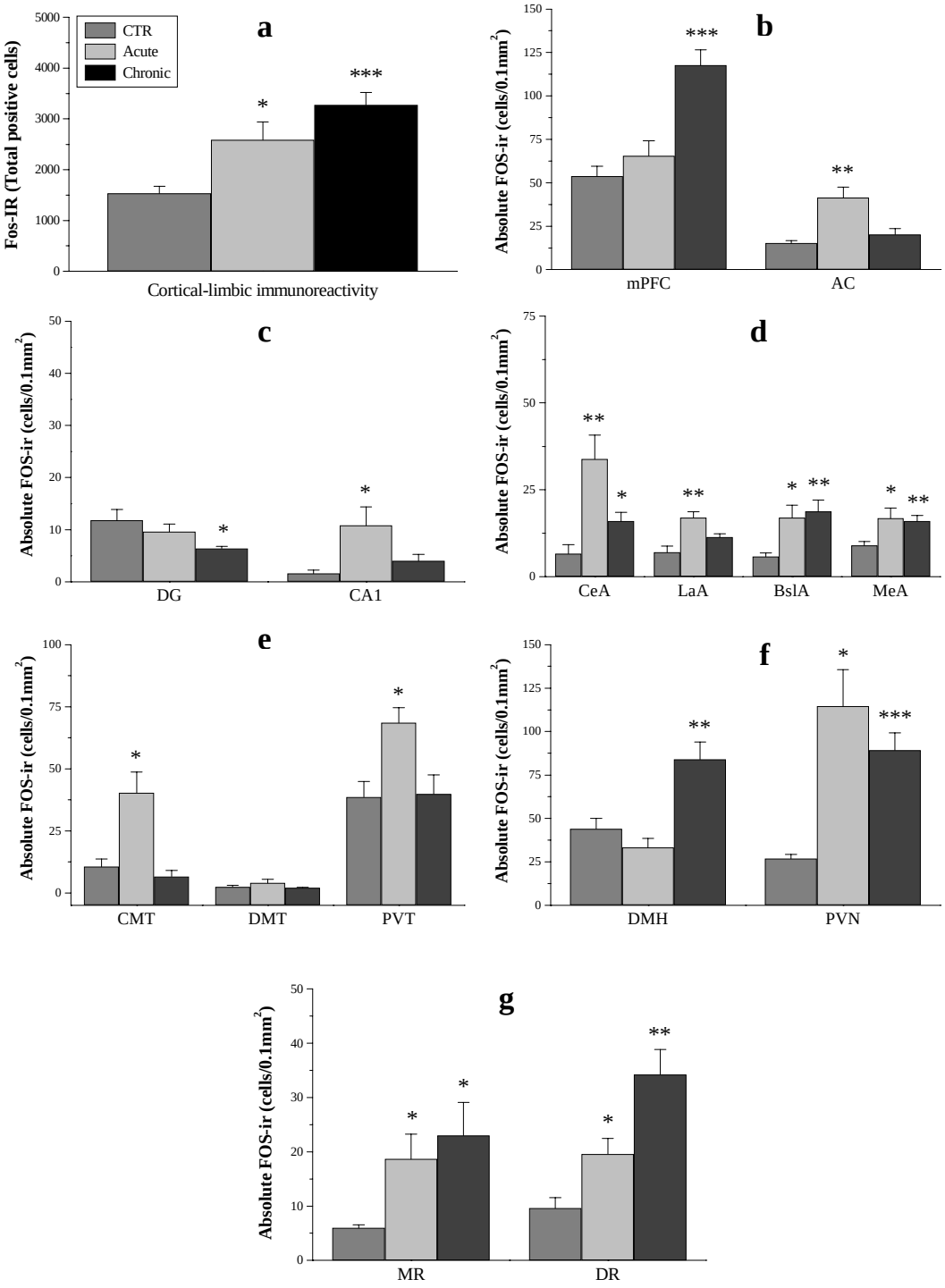


Figure 5. Effect of acute and chronic stress on absolute FOS-ir in: a) cortical-limbic system (total positive cells); b) prefrontal cortex; c) hippocampus; d) amygdala; e) thalamus; f) hypothalamus; g) raphe nuclei. The symbol * expresses the comparison of absolute FOS-ir between stressed rats, both acutely and chronically, and non-stressed animals (*=$p<0.05$; **=$p<0.01$; ***=$p<0.001$).

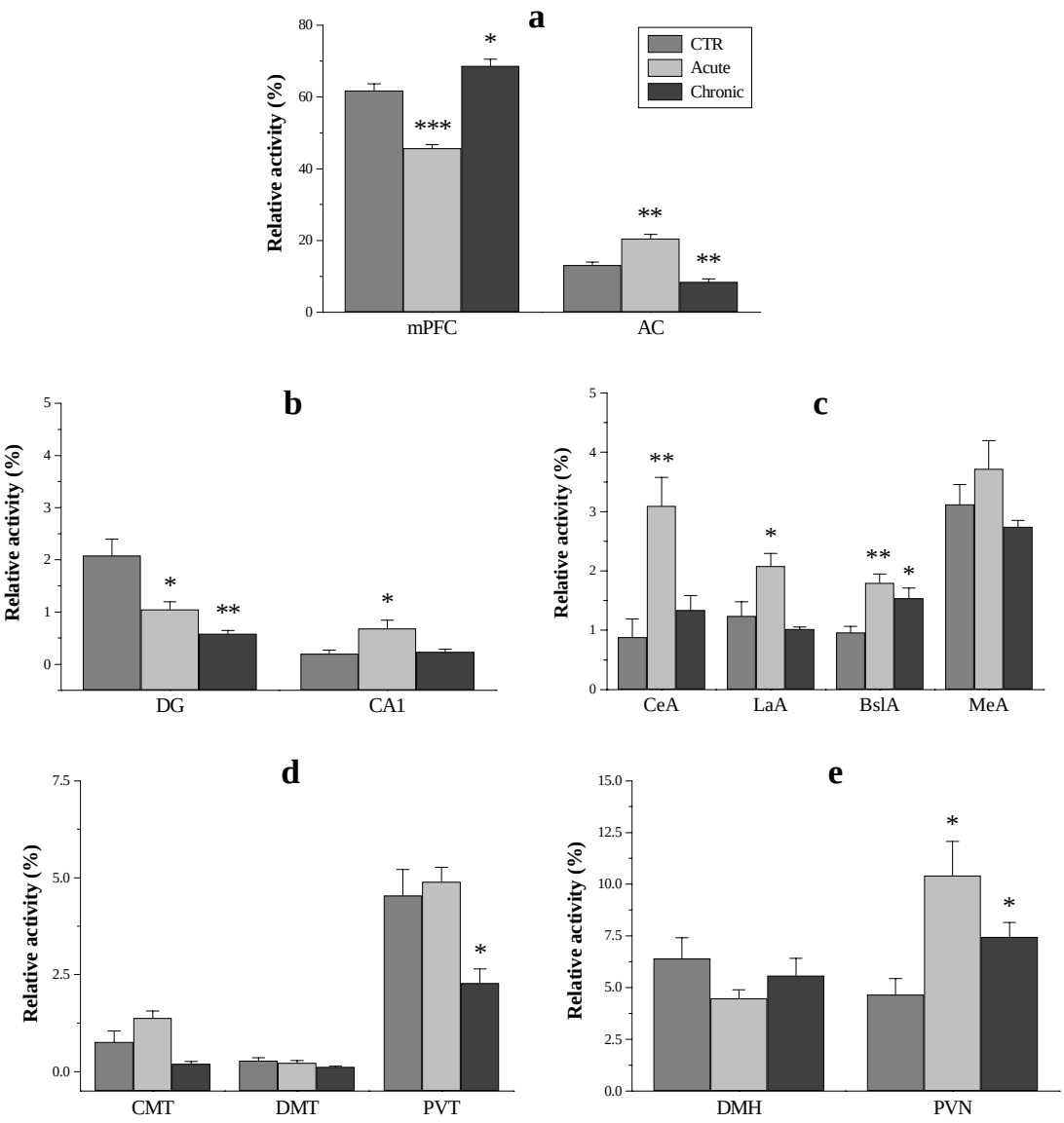


Figure 6. Effect of acute and chronic footshock challenge on relative FOS-ir in: a) prefrontal cortex; b) hippocampus; c) amygdala; d) thalamus; e) hypothalamus. The symbol * expresses the comparison of relative FOS-ir between stressed rats, both acutely and chronically, and non-stressed animals (*=p<0.05; **=p<0.01; ***=p<0.001).

Phospho-ERK1/2-ir

In order to evaluate the changes in the pattern of ERK phosphorylation following footshock stress, three different experiments were performed; an acute (3 day-experiment), a subchronic (10 day-experiment) and a chronic challenge (21 day-experiment). After each experiment, phospho-ERK1/2 immunoreactivity was quantified throughout several cortical regions including the medial prefrontal cortex, the anterior cingulate cortex, the somatosensory cortex, and the perirhinal cortex.

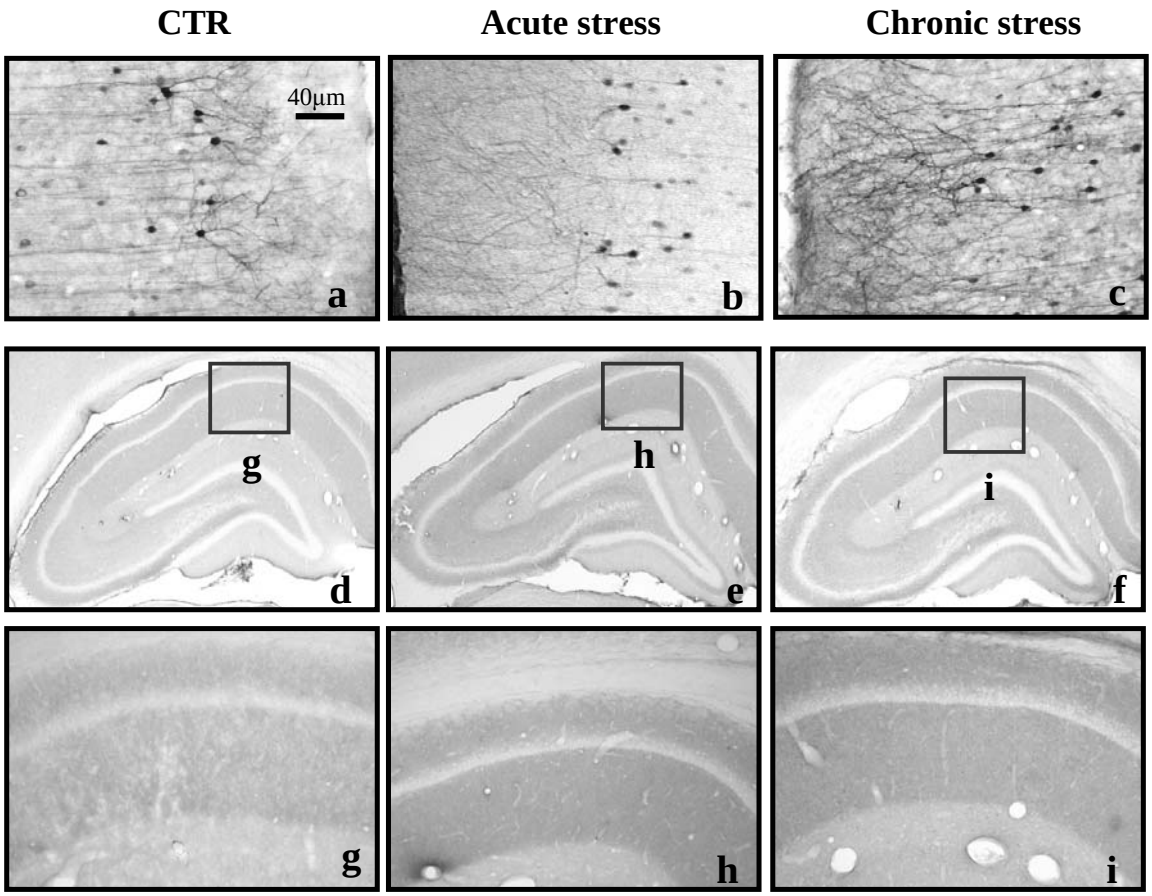


Figure 7. Microphotographs illustrating phospho-ERK1/2-labeled prefrontocortical dendrites in non-stressed (a), acutely (b) and chronically stressed rats (c). Phospho-ERK1/2 immunoreactivity in the hippocampal CA1 area of non-stressed (d,g), acutely (e,h) and chronically stressed rats (f,i).

Following prolonged footshock exposure, a pattern of immunoreactivity indicative of a selective and prolonged ERK1/2 hyperactivation in dendrites of higher medial prefrontocortical layers (II and III) was observed (fig. 7c). Although phospho-ERK1/2 immunoreactivity was analyzed in four different cortical regions, the increased number of positive dendrites observed following chronic footshocks was limited to the mPFC ($F=9.35$, $p<0.005$) (fig. 8a). The increased phospho-ERK1/2 immunoreactivity was specific for the chronic challenge. A lower level of phosphorylated ERK1/2 was observed in rats acutely or subchronically exposed to conditioned stress (fig 8b). Surprisingly, no changes were

observed in the number of prefrontocortical phospho-ERK1/2-labeled cells (fig. 8c). Moreover, while phospho-ERK1/2 expression was homogeneously distributed in the nucleus and proximal dendrites in non-stressed animals, chronically stressed rats showed intense immunoreactivity in the most distal parts of the dendrites (fig. 7a,c).

Phospho-ERK1/2 immunoreactivity was not detected in the hippocampus in any of the experimental groups (fig. 7d-i). This lack of phospho-ERK1/2 immunoreactivity could be the result of a different kinetic of activation of this cascade in the hippocampus, as ERK1/2 phosphorylation begins immediately after stimulus exposure, reaching the maximal peak between 1 and 10 minutes, and returning to baseline 20 minutes following the initial stimulus [227].

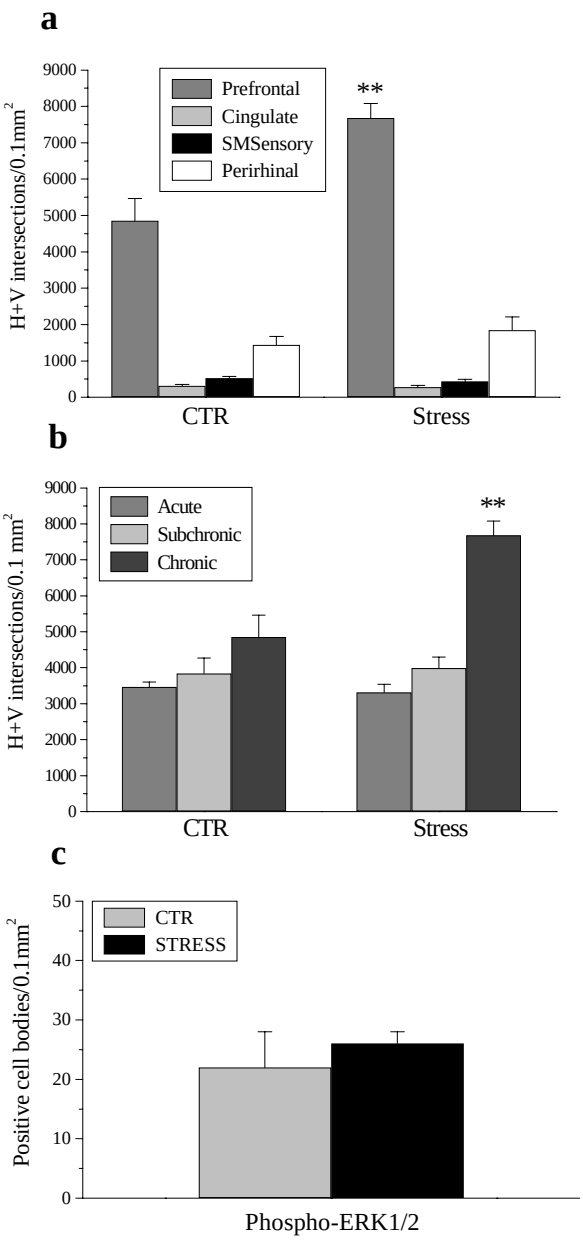


Figure 8. Phospho-ERK1/2-positive dendrites were counted in four cortical regions including prefrontal, cingulate, somatosensory and perirhinal cortex (a). Phospho-ERK1/2-positive dendrites in male rats exposed to acute (3 days), subchronic (10 days) and chronic stress (21 days) (b). No changes were found in the number of prefrontocortical phospho-ERK1/2-labeled neurons (c).

Phospho-CREB-ir

Likewise phospho-ERK1/2 expression, phospho-CREB (ser133) immuno-reactivity was also investigated throughout various cortical and subcortical regions, including the mPFC (fig. 9a,b,c), the AC, the SMS, the PRH, the DG (fig. 9d-i), the LaA, and the BslA (fig. 3). Acute fear conditioning was associated with a significant increase of phospho-CREB expression in the hippocampal dentate gyrus ($F=19.71$, $p<0.002$), the lateral ($F=6.25$, $p<0.037$) and the basolateral nucleus of the amygdala ($F=5.42$, $p<0.048$) (fig 10a). Chronic stress exposure, in contrast, was found to significantly downregulate phospho-CREB expression in these subcortical structures compared to both acutely stressed rats (DG: $F=64.5$, $p<<0.001$; LaA: $F=11.26$,

p<0.01; BslA: F=25.20, p<0.001) and controls (DG: F=5.54, p<0.046; BslA: F=5.74, p<0.043) (fig. 10a). A significant chronic stress-induced reduction of CREB phosphorylation was also observed in the mPFC (F=107, p<<0.001), the AC (F=11.67, p<0.002), the SMS (F=3.52, p<0.068), and the PRH (F=9.35, p<0.005) (Fig. 10b).

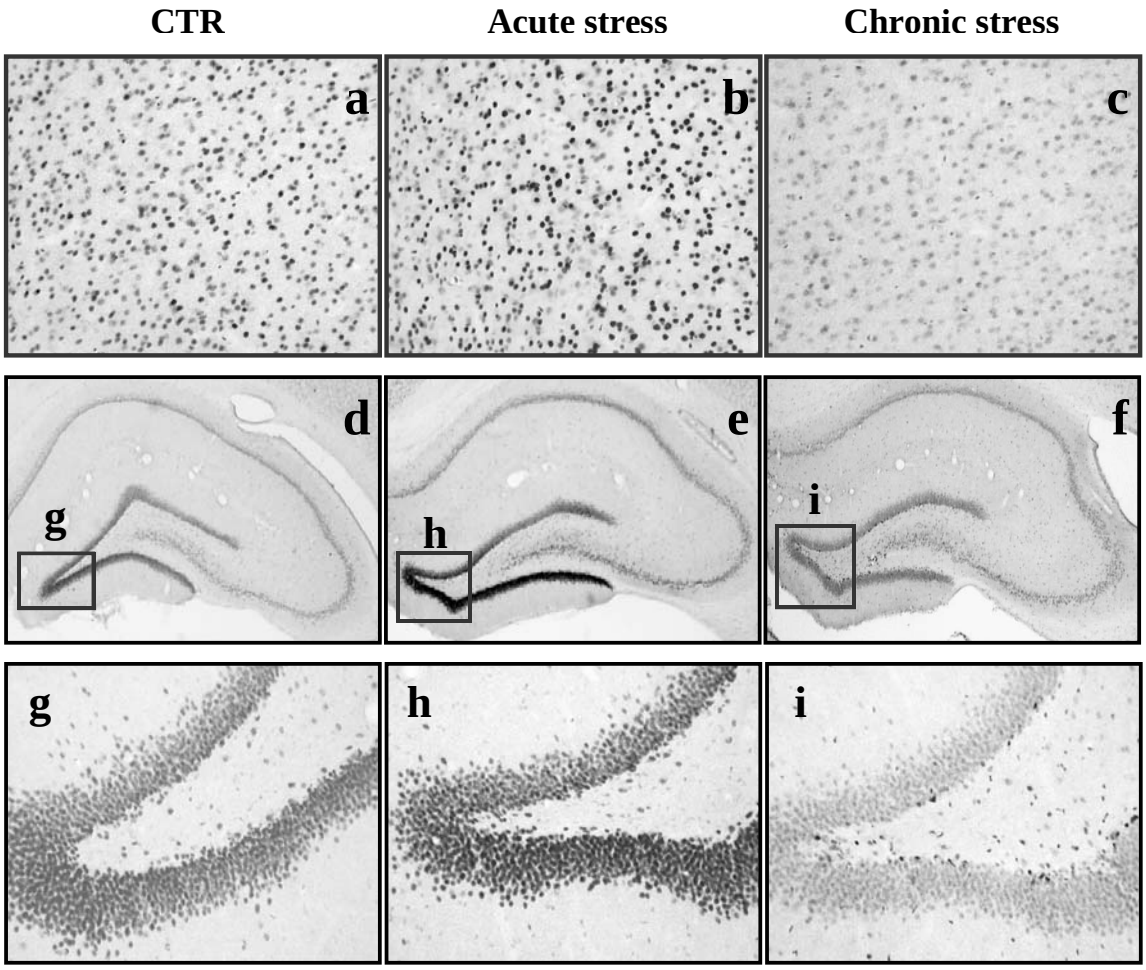


Figure 9. Microphotographs illustrating phospho-CREB immunoreactivity in the mPFC in control (a), acutely (b) and chronically stressed rats (c). Phospho-CREB expression in the hippocampal DG in control (d,g), acutely (e,h) and chronically stressed rats (f,i).

Molecular biology

Gene expression patterns

Twelve animals (6 CTR and 6 test-rats) were used for the analysis of altered gene expression patterns in the mPFC following chronic stress exposure (fig. 11a,b). Particular attention was focused on the expression of genes belonging to the MAPK/ERK intracellular pathway including p21, raf, ERK1 and ERK2, RSK. Although several members of the MAPK/ERK cascade including p21 (+49% after stress), H-raf (+27%) and ERK2 (+48%) illustrated slightly increased expression, none were significantly different from the controls. Interestingly, expression levels of two members of the MAPK pathway, ERK1 and RSK, were too low to be detected by the array.

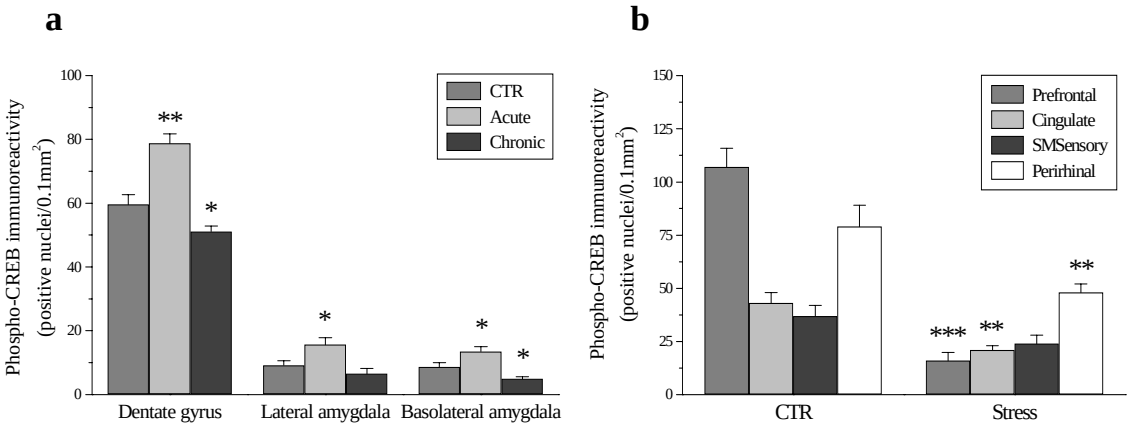


Figure 10. Phospho-CREB expression was quantified in subcortical areas (dentate gyrus, lateral and basolateral nucleus of the amygdala) following acute and prolonged stress (a) and cortical regions (medial prefrontal, cingulate, somatosensory and perirhinal cortex) after long-term footshock challenge (b).

Quantitative RT-PCR

To confirm the results obtained by gene expression analysis, quantitative RT-PCR using the Light Cycler was performed (fig. 11c). Total RNA was extracted from the mPFC of control (n=6) and chronically stressed rats (n=6) and used to quantify levels of ERK2 expression. Although microarrays showed a 48% increase in ERK2 expression in chronically stressed rats, RT-PCR did not confirm this data showing only a minimal and non-significant increased expression ($F=0.028$, $p<0.87$). ERK1 and RSK gene expression were not further quantified as no initial changes were detected by the microarray.

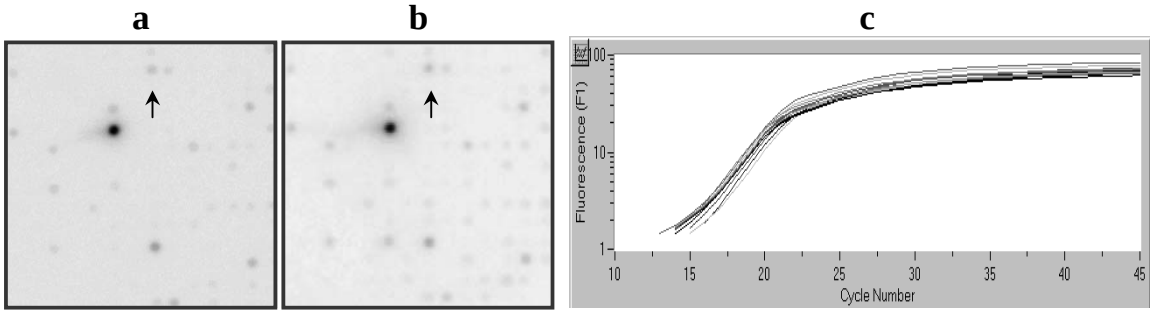


Figure 11. Rat cDNA microarrays revealing prefrontocortical expression of ERK2 in non-stressed (a) and chronically stressed rats (b). The figure only shows a portion of the cDNA microarray. Signal verification using Light Cycler RT-PCR (c). No difference in ERK2 expression was found between non-stressed and chronically stressed animals.

*Selective chronic stress-induced in vivo ERK1/2 hyper-phosphorylation in medial prefrontocortical dendrites: implications for stress-related cortical pathology?**

A. Trentani, S.D. Kuipers, G.J. Ter Horst, J.A. Den Boer

*** Adapted from the manuscript published in Eur. J. Neurosci. (2002), 15, 1681-91.**

In the present chapter, various aspects of the response to acute and repeated stress were examined by evaluating FOS, phospho-ERK1/2, and phospho-CREB expression, in an attempt to define the cellular and molecular mechanisms involved in the biphasic effect of stress (acute vs. chronic) on neuronal functioning. Our working hypothesis proposes the merit of acute aversive conditioning in the investigation of neurobiological substrates involved in the modulation of learning and memory, while long-term footshock exposure, due to the deleterious influences of chronic stress on brain integrity, may prove its utility in the study of the molecular mechanisms underlying stress-induced impairment of neuronal plasticity and cognitive processing.

Transient exposure to adverse experiences is known to promote learning acquisition and memory consolidation [228,229]. Based on the latter, the "acute fear conditioning paradigm" has become a leading model for studying how the brain forms memories about unpleasant events. Nevertheless, although much has been learned concerning the neurobiological substrates underlying Pavlovian conditioning, relatively little is known about the impact of prolonged stressful conditions on the cellular and molecular mechanisms involved in the consolidation of fear-related memories. As short-term adverse experiences may be beneficial for learning and memory, possibly through their positive influence on neuronal plasticity [228-230], sustained stress exposure has been established as a causal factor in the development of emotional and cognitive impairments, both in humans and animals [2,231-233].

Prominent amongst stress-induced reactions is the activation of the HPA axis, culminating in the release of glucocorticoids by the adrenal glands. Abnormal stress-induced HPA axis regulation however, may lead to neuronal dysfunctions and cognitive deficits. Evidence in support of a prolonged activation of this stress response system following repeated footshock stress was provided by altered physiological and neuroendocrine markers, including a marked reduction of body weight gain (fig. 1), higher plasma corticosterone and adrenaline levels (fig. 2a), and more important, a significant adrenal hypertrophy (fig. 2b). Furthermore, consistent with an abnormal HPA axis activation were the immunohistochemical changes observed following sustained

footshock exposure, as they appear to provide indirect support for a stress-induced reduction of neuronal plasticity. Evident were the prolonged and, possibly, uncontrolled prefrontocortical ERK1/2 activation (fig. 7, 8a) and the reduction of CREB phosphorylation in the medial prefrontal and anterior cingulate cortex, the amygdala, and the hippocampus (fig. 9, 10).

Although numerous studies have associated ERK activation with beneficial effects on neuronal activity, sustained ERK phosphorylation has also been linked to excitotoxic degeneration and apoptosis [234]. Abnormal ERK1/2 immunoreactivity, in medial prefrontocortical dendrites, was not detected after acute (3 days) or subchronic footshock stress (10 days) (fig. 8b). It is thus tempting to speculate of a phosphorylated ERK1/2 threshold necessary for inducing neurodegeneration, perhaps via a time dependent stress-mediated dysfunction of MAPK cascade regulation. It is also interesting to note that chronic footshock-induced pattern of ERK1/2 phosphorylation differed from what is known about learning-related ERK1/2 activation, as that begins immediately after CS exposure, reaches a peak between 1 and 10 minutes, and returns to baseline in 20 minute time [227]. Our results, instead, demonstrate an elevated phospho-ERK1/2 immunoreactivity more than two hours following CS exposure (fig. 7c). Furthermore, whereas under physiological conditions phosphorylated ERKs are mainly limited to the nucleus or the proximal part of dendritic trees (fig. 7a,b), chronic stress exposure led to a significant increase of phospho-ERK1/2 immunoreactivity in the distal part of medial prefrontocortical dendrites (fig. 7c). Interestingly, this increased dendritic ERK1/2 phosphorylation was not accompanied by a corresponding increase of prefrontocortical ERK1 and ERK2 mRNAs or phospho-ERK1/2-positive cell nuclei (fig. 7, 8c). In addition to the abnormal ERK1/2 phosphorylation, a significant decrease of phospho-CREB immunoreactivity was observed in both cortical and subcortical structures (fig. 9, 10). Together, these findings thus seem to substantiate our previously-stated hypothesis, suggesting that neuroendocrine and neurochemical changes observed in response to the long-term aversive procedure might be indicative of the harmful influence of the repeated exposure to footshock stress rather than a learning-related process.

Although the negative impact of chronic stress has been demonstrated in various paradigms [235-238], little is known about the intracellular substrates underlying this action. ERK cascade plays a central role in the intracellular response to neurotrophins by stimulating the activity and/or expression of several proteins and transcription factors, including CREB [239-242]. Although numerous studies have associated the activation of this cascade with neuroprotection, MAPK members do not appear to act universally to promote this fundamental function, as recent reports have shown that prolonged ERK activation can contribute to neuronal death [234,243]. MAPK phosphorylated states require careful regulation and perturbed ERK signaling has been related to cytoskeletal

destabilization and neuronal dysfunctions [244]. An important argument supporting the negative effects of sustained footshock exposure on neuronal plasticity as well as a possible abnormal ERK1/2 activation is represented by the prolonged phospho-ERK1/2 hyperphosphorylation coupled with a significant reduction of phospho-CREB immunoreactivity in medial prefrontocortical regions (fig. 7-10). CREB is phosphorylated and activated by ERK1/2 and similarly to these kinases, it plays a central role in neuroprotection and neuroplasticity [245]. It is intriguing to speculate that repeated stress may disrupt neuronal plasticity either directly through the inhibition of BDNF expression [246] or indirectly by down-regulating CREB phosphorylation and, subsequently, reducing BDNF transcription [247,248]. In both cases however, the final target is BDNF and the common result is a reduction of cellular availability of this fundamental neurotrophin. Chronic stress-mediated reduction of CREB phosphorylation was not limited to the medial prefrontal cortex as it also involved other cortical and subcortical structures, such as the anterior cingulate cortex (fig. 10b), the amygdala, and the hippocampus (fig. 10a), regions known to be play a critical role in cognitive and emotional processes [249-251].

Chronic stress has been shown to exacerbate a number of psychiatric disorders, including depression and anxiety, illnesses characterized by prefrontocortical abnormalities and cognitive deficits [252-256]. Numerous reports have documented, in depressed subjects, abnormalities in the neuronal transduction apparatus. In the past few years, increasing attention has focused upon the involvement of MAPK-CREB cascade in the pathophysiology of depression [257,258]. Furthermore, stress has been reported to reduce BDNF expression in the brain [246,259,260], although the mechanisms responsible for this effect remain unclear. An intriguing possibility holds that this may be attributable to the disruption of the coordination of BDNF-ERK-CREB system [225,261]. CREB phosphorylation is crucial for its ability to bind DNA and modulate gene expression, and is therefore essential for the transcription of BDNF [247]. Reduced BDNF availability might result in a reduction of neuronal plasticity, an indispensable feature when facing prolonged stressful conditions. Sustained stressful experiences have indeed been associated with reduced neurotrophin expression [246,259,260], a condition that might disturb the dynamic equilibrium between intracellular signaling cascades. It is plausible that, by indirectly targeting selective transduction pathways, stress may cause defects in more vulnerable neuronal populations. A potential target of this chronic stress-mediated process is the ERK cascade. Footshock-induced inhibition of CREB phosphorylation may lower BDNF synthesis resulting, ultimately, in abnormal ERK activation. However, since ERK1/2 modulates CREB phosphorylation, the abnormal coordination between these kinases and their effector may generate a self-sustaining loop, which acts to augment the reduction of BDNF expression, particularly in medial prefrontocortical territories (fig. 7-10). A dysfunctional ERK modulation that culminates in a persistent activation of these kinases

may also result in the hyperphosphorylation of various cytoskeletal proteins [262]. Abnormal ERK1/2 activity could ultimately weaken dendritic structure, especially in the synaptic terminals where cytoskeletal proteins are particularly abundant [263-265].

As far as we know, the findings presented here represent the first *in vivo* demonstration of a selective chronic stress-induced ERK1/2 hyperphosphorylation in medial prefrontocortical dendrites. Prolonged, and possibly uncontrolled, ERK1/2 phosphorylation combined with marked reduction of phospho-CREB expression might illustrate a stress-induced neuronal impairment. Disrupted ERK1/2-CREB coordination may represent a key mechanism mediating stress-induced damaging effects on selective subpopulations of vulnerable prefrontocortical neurons. Conceivably, the precise kinetics of ERK activation will ultimately dictate whether these activated kinases participate in a cell death-promoting or cell survival pathway. Transient ERK activation leads to increased neuronal plasticity and survival, while permanent and uncontrolled activation might increase neuronal vulnerability to subsequent insults, atrophy and, possibly, cell death. ERK-mediated dendritic abnormalities may thus represent a specific path by which prolonged stress exposure affects medial prefrontocortical functional and structural integrity.

*Amygdala-hippocampal modulation of acute and chronic footshock exposure: exploring the dynamic role of stress on neuronal plasticity**

A. Trentani, S.D. Kuipers, J.A. Den Boer, G.J. Ter Horst

*** Adapted from the manuscript in preparation for Molecular Brain Research**

The experimental evidence presented in the previous section illustrates the negative influence of repeated stress on neuronal plasticity at the molecular level. In the present section, we explored the changes of cellular activity and neuronal plasticity in an integrated network of cortical and subcortical regions, named the cortical-limbic system, in response to acute vs. long-term footshock exposure.

Acute aversive conditioning caused a significant increase of absolute FOS-ir in various forebrain structures, including the anterior cingulate cortex (fig. 5b), the hippocampal CA1 area (fig. 5c), the central, the lateral, the basolateral, and the medial nucleus of the amygdala (fig. 5d), the centromedial and the paraventricular thalamic nuclei (fig. 5e), the paraventricular hypothalamic nucleus (fig. 5f), the medial and the dorsal raphe nuclei (fig. 5g). This induction of FOS-ir was accompanied by a significantly increased CREB phosphorylation in the hippocampus (DG) (fig. 9a,b, 10a) and amygdala (LaA and BslA) (fig. 10a). Numerous studies, in both rats and humans, have "stressed" the importance of these two limbic structures in the modulation of fear-conditioned responses. A growing body of evidence pinpoints the amygdala in particular as a core component of the brain's fear system, essential for the acquisition, storage, and expression of Pavlovian conditioning [73,83,123,124,126,266,267]. Formation and consolidation of fear-related memories however, are dynamic processes and have been related to changes in gene expression [268,269], mRNA transcription [270-272] and protein phosphorylation [171,273,274] not only in the amygdala but also in hippocampal areas. These changes are believed to promote neuronal plasticity and information processing about fear-related stimuli [269]. CREB activity plays a fundamental role in the modulation of these processes. The phosphorylation of this transcription factor is in fact considered a molecular switch for the formation of long-term memories [150,275-277]. Since the amygdala and the hippocampus represent key components of the brain's fear system, the increased cellular activity (absolute FOS-ir) and neuronal plasticity (phospho-CREB expression) observed following acute challenge, may thus provide important immunohistochemical insights into the cellular and molecular mechanisms involved in the acquisition of associative learning and/or formation of emotional memories. In accordance with this view, the

increased absolute FOS-ir in the PVN may substantiate this ongoing process (fig. 5f). The PVN plays a pivotal role in the central regulation of the HPA axis [26,278]. Acute footshock exposure has been reported to strongly activate this hypothalamic center and elevate plasma corticosteroid levels [279,280]. Short-term exposure to high glucocorticoid levels positively influence cognitive abilities, enhancing learning and promoting memory consolidation [229,281,282]. The beneficial effects of adrenal steroid however, is only temporary as sustained exposure to increased glucocorticoid concentrations has been shown to impair cognition, possibly due to their deleterious influence on neuronal plasticity [225,283] and dendritic morphology [284-288]. Our results seem to support this view, as the immunohistochemical changes (increased absolute FOS-ir and enhanced CREB phosphorylation) observed following exposure to acute aversive conditions in key cortical and subcortical structures may validate the concept of an active formation and / or consolidation of fear-related memories.

Repeated footshock stress was associated with significantly increased absolute FOS-ir in the medial prefrontal cortex (fig. 5b), the central, basolateral, and the medial nuclei of the amygdala (fig. 5d), the dorsomedial and the paraventricular hypothalamic nuclei (fig. 5f), the medial and dorsal raphe nuclei (fig. 5g). A significant reduction of absolute FOS expression was instead found in the hippocampal dentate gyrus (fig. 5c). In contrast to acute challenge however, the induction of FOS-ir detected in the amygdala was not accompanied by a similar enhancement of CREB phosphorylation. Chronic stress was associated with a significant reduction of CREB immunoreactivity both in the amygdala and hippocampus (fig. 9d-i, 10a). This data seems thus to support a biphasic action of stress on the molecular events underlying cognitive processing. Acute stress may enhance associative learning and promote the consolidation of new fear-related memories, possibly through the stimulation of CREB phosphorylation, while prolonged footshock exposure could impair cognitive processing through its deleterious cellular (disruption of the neural circuits underlying fear conditioning) and molecular actions (reduction of neuronal plasticity in the medial prefrontal cortex, amygdala, and hippocampus). Interestingly, abnormalities in the neural circuits and molecular substrates underlying cognitive processing and neuronal plasticity are common features of several stress-related neuropsychiatric illnesses, such as post-traumatic stress disorder and anxiety [77,129,289,290]. Although the exact pathways responsible for the opposite effects of acute vs. chronic stress remain largely obscure, a possible candidate involved in this contrasting action is CREB. A growing body of research suggests the participation of this transcription factor in the regulation of neuronal plasticity [291,292], associative learning and memory formation [147,171,273,274,276,277,293], due possibly to the role of phosphorylated CREB in the transcription of several genes such as BDNF [247,248,294,295]. An intriguing possibility is that stress may impair neuronal integration through the inhibition of CREB phosphorylation [225]. New memories are initially labile and sensitive to disruption before

being consolidated into stable long-term memories. Ample evidence indicates that this consolidation involves protein synthesis and phosphorylation. Importantly, CREB phosphorylation in the hippocampus and amygdala has been reported to represent a key molecular event in this process [147,150,277,293,296,297]. Our results illustrate that chronic stress was associated with a significant decrease of phospho-CREB expression in both the amygdala and the hippocampus, suggesting the detrimental influence of prolonged stress exposure on long-term memory consolidation [231,238,287,298]. Prolonged footshock exposure was also associated with reduced absolute FOS-ir in the dentate gyrus (fig. 5c), possibly indicating hippocampal hypoactivity, and increased neuronal activity in the amygdala (CeA, BslA and MeA) (fig. 5d) and hypothalamus (DMH and PVN) (fig. 5f). Sustained footshock stress has been shown to robustly activate the PVN leading to increased glucocorticoid secretion [280], which in turn may promote the development of functional and morphological defects [225,286,288]. The apparent long-term HPA axis hyperactivity observed after chronic challenge was supported by increased absolute FOS-ir in the PVN (fig. 5f), elevated plasma corticosterone levels (fig. 2a), and, more importantly, adrenal hypertrophy (fig. 2b). This neuroendocrine evidence, besides illustrating hyperactivity of the HPA axis, also suggests a lack of habituation of this stress system in response to repeated footshock stress. Whereas acute stress has been related to increased CREB phosphorylation and enhanced BDNF expression [171,292,299], chronic stress has been associated with reduced phospho-CREB immunoreactivity [225] and BDNF expression [259,260,300,301]. The results presented here thus appear to substantiate these findings. It is tempting to speculate that by raising the level of glucocorticoids and inhibiting CREB phosphorylation, chronic stress may limit BDNF availability and reduce neuronal plasticity in the amygdala and hippocampus. The consequent lack of plasticity, particularly essential during persistent stressful conditions, may disrupt sensory integration and impair brain functions.

*Modulation of cortical-limbic activity in response to acute and long-term footshock stress: a perspective on cortical-limbic functioning as revealed by an alternative approach to FOS analysis**

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*** Adapted from the manuscript in preparation for Brain Research**

In the previous section, we compared the cortical-limbic response to acute vs. chronic footshock stress by evaluating the pattern of "absolute expression" of the immediate early gene c-fos. The principal novelty of the present section is the introduction of an alternative means of interpreting FOS-ir data. In addition to the traditional analysis of FOS-ir (absolute density of FOS positive nuclei), we introduced a relative measurement (*relative regional FOS-ir*; see Material and Method section), which in our opinion, could provide an alternative perspective in the evaluation of stimulus-induced changes of neuronal activity and allow a more detailed understanding of the response of "defined" neuronal networks to discrete stimuli. Absolute regional FOS-ir provides only a general idea of the impact of external events on the activity of such "defined networks", since it does not consider the anatomical and functional relationships between the components of a specific system. R*elative regional FOS-ir,* instead, takes into account the fact that individual brain structures do not react to specific stimuli independently, but rather work together in a coordinated manner as part of an integrated network. Moreover, discriminating between changes of regional neuronal activity specifically associated with a discrete stimulus and unspecific response is also necessary to understand the "neuroanatomical substrates" involved in the elaboration of such a stimulus. Absolute regional FOS-ir does not allow this discrimination since it employs an average indication of the regional response to a specific stimulus without considering the individual differences amongst its groups' members. Traditional absolute analysis fails to consider for instance, the regional differences of basal FOS expression among animals and the FOS-ir changes due to unspecific responses. *Relative regional FOS-ir analysis* however, takes into account the individual differences between group members, and more importantly, discriminates between FOS-ir changes related specifically to a discrete stimulus and "background" changes, improving the overall accuracy of the analysis. In the present section, we will use this "*relative FOS-ir analysis*" to investigate the response of a "defined" neuronal network (table 1) to acute and chronic footshock stress [226].

Acute footshock challenge strongly enhanced *relative regional FOS-ir* in a widespread neuronal network that includes the anterior cingulate cortex (fig. 6a), the hippocampal CA1 area (fig. 6b), the central, the lateral and the basolateral amygdala (fig. 6c), and the paraventricular hypothalamic nucleus (fig. 6e). A significant reduction of *relative FOS-ir* was observed in the medial prefrontal cortex (fig. 6a) and the dentate gyrus however (fig. 6b). These regions have been shown to play a key role in the modulation of fear-conditioned responses [73,74,123,302-304]. In accordance with previous observations, these findings also substantiate the merit of "acute footshock challenge" in the investigation of neurobiological substrates and the molecular mechanisms underlying cognitive and emotional processing. Interestingly, most of the structures listed here demonstrated an increased FOS-ir when analyzed both in the traditional (absolute regional FOS-ir) (fig. 5) and the *relative manner* (fig. 6). This supports our suggestion of *relative regional FOS-ir* as a possibly valuable additional tool in the investigation of the mechanisms involved in the response of "defined" neuronal networks to aversive stimuli.

Stimuli that are interpreted by the brain as threatening or extreme provoke a stereotyped pattern of neuronal and endocrine changes known as "stress cascade". This orchestrated process involves different responses that allow the body to make the necessary physiological and metabolic adjustments required to cope with the demands of a homeostatic challenge. Stress-induced release of multiple hormones such as glucocorticoids and catecholamines at different levels of the HPA axis is a prominent part of the stress response [37]. Stimuli that are interpreted by the brain as threatening also trigger a wide variety of additional responses that result ultimately in enhanced cognition, affective processing, and emotional arousal [13], in the attempt to improve the ability of the organism to adjust to the new conditions increasing its chances for survival. Due to their complex nature, the modulation of these processes appears to be channeled through cortical-limbic forebrain circuits [24,44]. As mention earlier, the brain's cortical-limbic system, particularly the prefrontal cortex, the hippocampus, the amygdala, and the hypothalamus, is also intimately involved in the regulation of stress responses. Chronically elevated corticosteroid levels adversely affect brain structure and function, disrupting the integrated information processing between cortical and subcortical regions, leading to both cognitive deficits and emotional impairment [68,305,306]. Appropriate coordination of HPA axis activation thus appears to be fundamental and occurring by multiple interactions between stress-sensitive cortical-limbic circuitry and the neuroendocrine neurons of the PVN [24]. Due to the central role of PVN in the coordination of this stress axis, its state and level of activity is modulated at different levels by several cortical and subcortical structures that act either by stimulating its activation (such as the amygdala) or providing inhibitory feedback control (such as the hippocampus and the medial prefrontal cortex) [26,112,307]. The increased *relative FOS-ir*

observed in the amygdala (fig. 6c), together with the reduction of *relative activity* detected in the medial prefrontal cortex (fig. 6a) and the dentate gyrus (fig. 6b) could thus illustrate adaptive changes in the neuronal network responsible for the modulation of HPA axis activity. The latter may also constitute key events of the physiological response to acute stress leading to PVN activation (fig. 6d), increased HPA axis activity and, ultimately, release of stress hormones.

In recent years, the key role of medial prefrontocortical regions, such as the prelimbic cortex, the infralimbic cortex, and the anterior cingulate cortex in the modulation of affective style, attention, learning, and memory have been clearly established in many species [175,308-310]. A great number of reports have also indicated the medial prefrontal cortex as a functionally dissociable region during cognitive processing in humans [309,311-313]. Prevalent mPFC activation has been associated with maintenance and manipulation of information [314,315], whereas AC activation has been consistently observed in situations requiring divided attention, novel responses, or the overcoming of a prepotent response [308,316]. Increased AC activity has also been associated with evaluative processes during the response to conflicts, which occurs when two incompatible reactions are both compelling [309]. Furthermore, anterior cingulate activation has been specifically correlated with the perception of the affective component of pain, particularly its "unpleasantness" [317,318]. Animal studies have confirmed the involvement of this cortical region in nociceptive and emotional processing [319], although they have failed to report a clear dissociation between AC and mPFC activation. The analysis of absolute regional FOS-ir alone did not clearly document any dissociation between medial prefrontal and anterior cingulate cortex during high-order cognitive tasks (acquisition, expression, and extinction of fear-conditioned responses) (fig. 5b). In contrast, *relative regional FOS-ir*, which allows the investigation of unbiased neuronal activity patterns, provided clear immunohistochemical evidence regarding the differential roles that the medial prefrontal cortex and the anterior cingulate play in the modulation of these responses. This provides support for the existence of a functional antagonism between these two cortical regions in rats as well. While acute challenge was associated with AC *relative hyperactivity* and reduced mPFC *relative regional FOS-ir*, chronic footshock exposure resulted in mPFC *relative hyperactivity* and reduced anterior cingulate *relative regional FOS-ir* (fig. 6a). The opposite engagement of mPFC and AC may support a functional dissociation between these two cortical regions during sensory processing. Considering the enhanced neuronal plasticity observed after acute challenge, the increased anterior cingulate *relative FOS-ir* detected in response to acute stress (fig. 6a), might implicate this region as a key component of a functional circuit activated by emotional learning. The mPFC *relative deactivation*, illustrated by significant reduction of *relative regional FOS-ir*, may also be part of the adaptive and coordinated response to acute aversive conditions. This view was also supported by the increased level of *relative*

regional activity detected in several nuclei of the amygdala following acute challenge (fig. 6c). The amygdala also plays an active role in the modulation of fear-conditioned responses and its activation results in the inhibition of medial prefrontocortical regions[77]. The combination between anterior cingulate and amygdala activation and medial prefrontocortical inhibition may have a crucial adaptive value as it leads to enhanced HPA axis central drive, promoting in turn the release of glucocorticoids by adrenal glands. An increased HPA axis central drive is also supported by the reduced relative FOS-ir observed in the dentate gyrus (fig. 6b), which may suggest a diminished hippocampal feedback-inhibition to the HPA axis. Importantly, these combined changes in the level of activity of key cortical and subcortical regions may result in temporarily elevated glucocorticoid levels that facilitates the consolidation of fear-related memories[145,229,320]. In conclusion, the changes of regional *relative regional FOS-ir* observed following acute footshock challenge may illustrate normal physiological adaptations underlying the response to acute stress and the consolidation of fear-related memories. In addition, our immunohistochemical evidence also suggests that the AC, unlike the mPFC, plays a key role in the modulation of evaluative processes, essential during emotional learning, as it is needed to render affective attributes of an explicit CS and to respond to conflicts. Proper modulation of these processes however also requires the participation of as well as multiple interactions between cortical and limbic structures, such the mPFC, the amygdala, the hippocampus, and the hypothalamus.

It is interesting to note that, although on the final day chronically stressed rats were exposed to the same neutral CSs, their patterns of *relative regional FOS-ir* were significantly different from those observed after acute challenge. An increased r*elative regional FOS-ir* was in fact detected in the mPFC (fig. 6a), the BslA (fig. 6c) and the PVN (fig. 6e), while a marked *relative hypoactivity* was found in the AC (fig. 6a) and the dentate gyrus (fig. 6b). The biological significance of chronic stress-induced changes in *cortical-limbic relative FOS-ir* remains to be elucidated however. Chronic stress has been established as causal factor in the development of cortical-limbic dysfunctions. Experimental evidence has pointed to the hippocampus and the prefrontal cortex as the two main targets of stress-mediated damaging action [108,288,321]. Interestingly, we noted a significant reduction of dentate gyrus and anterior cingulate *relative FOS-ir* following prolonged footshock stress (fig. 6a,b), perhaps indicative of a condition of regional hypofunction. Morphological and functional prefrontocortical and hippocampal defects, including neuronal and glial histopathology [256,288] as well as reduced AC and hippocampal metabolism, have been detected following chronic stress exposure [219,322,323]. The AC plays a critical role in the modulation of high-order processes and is crucial for learning-related and emotional responses. This assumption is supported not only by clinical and preclinical evidence but also by the strong increase of FOS-ir, both absolute and *relative*, observed following acute challenge (fig. 5b, 6a). Although acute stress has

been reported to enhance learning and memory, repeated footshock exposure has been shown to impair neuronal functioning, possibly through its suppressive role on synaptic plasticity. This possibility was supported by our results, as documented by the abnormal neuronal plasticity observed in chronically stressed males (fig. 7-10). These findings were also substantiated by clinical evidence suggesting sustained stress as a critical factor involved in the development of anterior cingulate defects and neuropsychiatric disorders characterized by abnormal modulation of cognitive and emotional responses [256,324]. AC hypoactivity has frequently been reported in depression and the correction of this deficit represents a crucial step in successful clinical recovery [113,115]. The anterior cingulate cortex however, was not the only structure showing abnormal changes in response to chronic stress. A significant reduction of *relative regional FOS-ir* was also detected in the hippocampal DG while a functional *relative hyperactivity* was found in the mPFC, the BslA, and the PVN. These changes appear to differ from those detected after acute challenge and, more importantly, with those reported by Garcia and colleagues which provide direct evidence for the control by the basolateral amygdala of learned fear-induced changes of neuronal activity in medial prefrontal territories [77]. In particular, they found that stimulation of the basolateral amygdala induced predominantly an inhibition in the medial prefrontal cortex, possibly through brainstem putative inhibitory pathways[77]. In our study, the increased relative activity observed in the BslA following repeated stress (fig 6c), was not accompanied by inhibition of the mPFC, a condition that was instead evident after acute stress. Chronic stress exposure was associated with functional mPFC hyperactivity, as illustrated by increased *relative FOS-ir* observed in this region (fig 6a). Since we do not know the direct cause of these *relative FOS-ir changes* or their consequences, we can only hypothesize that the loss of coordination between the amygdala and the medial prefrontal cortex may illustrate a central aspect of chronic stress-induced cortical-limbic impairments.

In conclusion, the evidence presented here seems to suggest that moderate and transient adverse conditions, promote cognitive and emotional processing possibly by facilitating action of acute exposure to elevated glucocorticoid concentrations, thereby enhancing organisms' survival. Prolonged stress exposure, on the contrary, results in both structural and functional abnormalities in cortical (relative mPFC hyperactivity and AC hypofunction) and subcortical regions (relative BslA and PVN hyperactivity; DG hypofunction). This could be due to the long-term overstimulation of the HPA axis that results in sustained exposure to evelated corticosteroids. Stress-induced inhibition of neuronal plasticity and disruption of coordinated input integration between cortical and limbic structures may ultimately account for the development of cognitive and emotional impairments.

Conclusions

In the present chapter, we have provided immunohistochemical evidence to support the notion that acute stress has beneficial effects on sensory integration, and suggesting a detrimental influence of chronic footshock stress on cellular activity and neuronal plasticity, at the molecular, cellular, and systemic level. Acute stress was found to reduce phospho-ERK1/2 immunoreactivity in medial prefrontocortical dendrites (possibly due to an increased translocation of these kinases from the cytoplasm to the nucleus) and enhance CREB phosphorylation in the amygdala and the hippocampus. Prolonged footshock stress, in contrast, was associated with a significant dendritic ERK1/2 hyperphosphorylation and reduced phospho-CREB immunoreactivity. Our data thus seems to point to a disruption of ERK1/2-CREB coordination as one of the decisive aspects underlying chronic stress-induced neuronal defects. It is tempting to speculate that disruption of MAPK-CREB coordination may inhibit neuronal plasticity and this reduced plasticity in the brain's fear system may ultimately result in abnormal cognitive and emotional responses, facilitating the development of stress-related disorders.

A key player involved in stress-induced disruption of brain functions is represented by the HPA axis. Abnormal HPA axis regulation (cause) may constitute a critical mechanism underlying the impairment of the coordinated activity between cortical and subcortical structures (consequence). However, it is also possible that the loss of coordination in the integration of sensory inputs at the cortical-limbic level (cause) may lead to abnormal HPA axis regulation (consequence). Importantly, chronic stressed-induced HPA axis dysregulation may result in prolonged and uncontrolled release of glucocorticoids by adrenal glands. These hormones are known to stimulate neuronal plasticity under acute stressful conditions yet impair this process following long-term exposure. Prolonged stressful conditions may thus generate an auto-sustaining positive feedback loop in which glucocorticoids stimulate their own release and lead, progressively, to reduced neuronal plasticity as well as more severe functional and morphological abnormalities involving both cortical (medial prefrontal and anterior cingulate cortex) and subcortical structures (hippocampus and amygdala). Chronic stress-induced defects may ultimately impair the coordinated activity of the cortical-limbic system disturbing critical processes such as cognitive and emotional responses.

When considering the data and more importantly plausible explanations, one must take into account the restrictions of the analysis within the experimental design, particularly with regard to the interpretation of *relative FOS-ir data*. In this study our interpretation is constrained mainly by our limited knowledge of the neuroanatomy related to mood and cognition and the reciprocal interactions existent between cortical and subcortical structures. For our data acquisition we assimilated a theoretical network of 14 cortical and limbic areas known to be involved in the regulation of the stress

response and the modulation of higher brain functions such as cognition and affective style. The regions selected to constitute this network comprise the classic limbic system as originally proposed by Papez [325]. Depending on one's areas of interest regarding a hypothesis and/or coinciding brain regions however, one could choose to redefine a neural network and discover a wide range of new findings. A possibility for further research could be a reanalysis of the same data by in- or excluding selective regions, thereby gaining additional insight into the specific roles of the regions relative to its predefined network and their significance to stress physiology. Aside from our findings based on the classic limbic system and independent of region specificity, these results suffice to support the argument that data interpretation of a fear/stress-related paradigm would benefit greatly from inclusion of such an additional evaluation of regional network functionality. We have demonstrated the applicability of this approach in investigating the immunohistochemical changes induced by specific aversive stimuli. By evaluating the *relative FOS-ir* in addition to the analysis of absolute regional activation one respects the most basic attribute of the brain, namely its intrinsic nature to function as a network of interconnected regions.

The experimental data presented here represents only the first step of a long-term project. To confirm the present findings, it is necessary to further investigate other aspects of the response to chronic stress and, possibly, the ability of antidepressants to prevent and/or reverse these stress-related structural and functional changes. Future research is thus needed to define the neuroanatomical relationships between cortical-limbic regions, but also to confirm the hypotheses formulated here and further clarify the mechanisms mediating chronic stress-induced cortical-limbic dysfunctions.

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3

Gender-related dimorphisms in the patterns of cellular activity and neuronal plasticity in response to repeated stress

"Experience has shown that science frequently develops most fruitfully once we learn to examine the things that seem the simplest, instead of those that seem the most mysterious"

Marvin Minsky

Stress and psychopathology: is gender an issue?

Ovarian steroids produce a variety of neurochemical effects which affect multiple processes such as cognition, emotional regulation, affective style, pain sensitivity and psychopathology [1-3]. Gender-related differences in the brain however, account for more than merely sex disparities in the effects of sex hormones. Reproductive as well as nonreproductive dimorphisms exist between men and women and it is intriguing to assume that non-reproductive, gender-related cognitive and emotional dissimilarities might reflect functional and/or structural differences in higher-order cortical and subcortical structures.

Gender-related differences in HPA axis regulation

Since the HPA axis represents the final effector in the modulation of the stress response, a large number of clinical and preclinical studies have attempted to define a direct link between sex-related differences in key elements of this system and the higher female susceptibility to stress and stress-related psychopathology. The HPA axis and the female reproductive system are integratively intertwined and exhibit complex relationships [4]. Glucocorticoids, for instance, inhibit pituitary luteinizing hormone (LH), estrogen, and progesterone secretion [5]. Although most of the studies investigating gonadal and adrenal interactions have generally focused on stress-induced disruption of reproductive functions, the relationships between these two endocrine systems are more complex and by no means unidirectional. A partial estrogen response element has been found on in the promoter of the CRF gene [6]. Estrogen-induced increased CRF transcription may thus represent a potential mechanism by which estradiol may enhance stress responsiveness and lead to the higher glucocorticoid levels observed in females [6]. This finding implicates the CRF gene and, therefore, the HPA axis, as an important target of gonadal steroids and a potential element involved in the gender-related differences observed in the modulation of stress response [5].

Differences in the regulation of HPA axis activity may account for the differential response to stress observed between male and female rats, with the latter demonstrating a greater overall reaction, a more rapid onset of glucocorticoid secretion, and a faster elevation of plasma level of adrenal steroids [4]. A steeper rise of circulating stress hormones seems to be necessary to elicit the faster glucocorticoid-mediated feedback inhibition needed in females [7]. In addition, estrogen has also been shown to delay ACTH and glucocorticoid shutoff [8,9], condition that may account for the greater overall reaction to stress observed in females. Progesterone also appears to be involved in the differential modulation of stress response. It is interesting to note that progesterone shows a faster binding time than cortisol on the GR, although it binds the receptor at a different site than glucocorticoids [10,11]. Female rats have a greater number of GRs in the hippocampus

than males [12] and this number is also modulated by progesterone [13]. Although the majority of progesterone-induced effects on the HPA axis are mediated by GR binding, several studies have also demonstrated an affinity of this hormone for the MR, in a range similar to that of dexamethasone [14,15].

The finding of an "exaggerated" response to stress in females, even in ovariectomized animals, has very important implications for our understanding of stress responsiveness. It is now clear that a number of "stress-related" psychiatric disorders are more common in females. These include depression, post-traumatic stress disorder as well as other anxiety disorders [16]. Ovarian hormones have been proposed as key factors in determining this higher female liability to psychopathology [17]. If females indeed demonstrate an increased responsiveness to stress, this finding may explain, at least in part, the greater sensitivity of women to depression and anxiety. An additional aspect that should not be underestimated when considering the increased incidence of psychopathology in women is the greater resistance of female HPA axis response to glucocorticoid-mediated feedback inhibition [18]. If we believe Munck's hypothesis, one of the purposes of glucocorticoids is to terminate not just the HPA axis response to stress but, more in general, the entire stress "cascade" [19]. Recent studies, documenting a role for adrenal steroids in terminating stress-induced activation of the autonomic stress system, appear to substantiate this hypothesis [20,21]. In consequence of this important modulatory role of stress hormones, the higher resistance observed in women to glucocorticoid-mediated feedback inhibition would further exaggerate stress responsiveness [19]. Therefore, whereas estrogen and progesterone exert a protective effect against the negative sequelae of hypercortisolemia, they also antagonize glucocorticoid-mediated terminating action, delaying the recovery from the deleterious consequences of stress [9].

Gender-related differences in cognitive processing

Gender-related differences in structural and functional brain organization may result in sex-related dimorphisms in critical functions, such as cognitive processing and emotional regulation. The existence of sex differences on a cognitive level has become increasingly clear. Males for instance reliably outperform females on tasks that require spatial ability skills. Women, on the other hand, demonstrate superior verbal and object location memory [22], and rely to a greater degree on emotional content during information processing [23]. Despite extensive research however, relatively little is known about the cellular and molecular mechanisms underlying these differences.

The fear conditioning paradigm represents a powerful model to investigate the neurobiological substrates underlying cognition and emotional regulation. Several studies have revealed that male rats exhibit greater contextual fear conditioning than females [24]. Female rats however, acquire fear-conditioned responses much faster than

males [25-27]. These differences were even more pronounced when taking into account the stage of estrous [28]. Recent studies in humans and rodents have shown that the status of both gonadal and adrenal axes strongly influences learning and memory [26]. In rats, for instance, the estrous cycle is approximately 5 days and is separated into four stages: proestrus, estrus, diestrus 1 and diestrus 2. The level of the primary female sex hormone, estrogen, is highest just prior to ovulation, the stage corresponding to proestrus. During this stage, female rats tend to be more active, eat and drink less, and are facilitated in learning fear-conditioned tasks [28]. The mechanism involved in this enhanced acquisition of classically conditioned responses is unknown. Importantly, changes in synaptic efficacy are considered necessary for learning and memory [29,30] and estrogen has been shown to increase the density of dendritic spines and synapses in the hippocampus [31,32] as well as the number of spine synapses formed with multiple synapse boutons [32]. Thus, it has been recently suggested that estrogen may promote cognitive activities by controlling synaptic functions in the central nervous system and enhancing neuronal plasticity, through genomic and non-genomic actions [33]. The latter effects seem to involve the ability of ovarian hormones to modulate the activity of ERK intracellular transduction cascade [34].

The influence of stress on cognitive processing

Previous exposure to acute stressful events has been shown to enhance the acquisition of several types of learning. Adrenal hormones appear to facilitate a wide variety of brain functions [35,36], an effect that is rapidly induced (within 30 minutes) and persist for at least 24 hours [37]. Under acute physiological conditions, the elevation of glucocorticoid levels, during the post-training period, has also been proposed to determine the strength of information storage [38].

Animal and human studies have shown that important sex differences exist in specific cognitive abilities, particularly under stressful conditions. While adverse experiences promote associative learning and memory consolidation in males, female cognitive functions are severely impaired by exposure to the very same events [25,39]. Simply re-exposing the animal to the stressful context days after stressor cessation can reinstate its deleterious influence on learning, suggesting that a psychological manipulation is sufficiently stressful to reinstate the effect of the stressor, at least in females [40]. A growing number of studies support the view that this differential action of stress on memory formation in males and females are determined by sex-related structural differences in the brain [26]. For instance, the induction of learning, in males, is dependent upon the N-methyl-D-aspartate (NMDA) receptor [41]. In females, in contrast, stress-mediated effect on learning does not appear to depend on NMDA receptor activation. An additional structural difference between males and females involved the rate of neurogenesis observed in the adult brain. Neurogenesis plays a critical role in

cognitive processing and new neurons are crucially involved in hippocampal-dependent learning [42,43]. Females produce more immature granule neurons in adulthood than males[44] and some have reported that females learn hippocampal-dependent tasks better than males [45]. Several studies have also indicated that stress inhibits the production of granule cells by suppressing the proliferation of granule cell precursors [46,47]. The relationship between stress and neurogenesis is seemingly complicated when viewed in relation to learning, as exposure to stressful experiences may reduce cell proliferation in the dentate gyrus without affection and, sometimes, even enhancing hippocampal-dependent learning, at least in males [24,48]. In female rats however, stress exposure immediately and persistently impairs associative learning [25,26], possibly through the inhibition of cell proliferation. These results suggest sex-related differences in the structural organization of the brain as a critical factor in both the facilitation and the impairment of cognitive processing in response to stress.

Another factor which may prove of relevance in this dual action of stress on cognitive performance is the role played by ovarian hormones. Estrogen enhances neuronal plasticity, a critical property underlying learning and memory [3,34]. Importantly, the exposure to adverse events has been reported to alter estrogen and progesterone release [39]. The crucial interaction between sex steroids and adverse experiences is supported by the observation that, contrary to cyclic females, ovariectomized animals were not impaired by stress [25]. Furthermore, stress-induced impairment of classical eyeblink conditioning was prevented by estrogen antagonist treatment [26]. Additional evidence to further support the involvement of estrogen in the modulation of the detrimental effects of stress comes from analysis of the consequences of adverse events in different stages of the estrous cycle. The negative impact of stress was most pronounced when females were stressed during the transition into proestrus, consistent with a rise in estrogen levels [28]. The exact mechanisms underlying estrogen-mediated disruption of cognitive processing is unknown, although recent data suggests stress-induced supraelevation of ovarian hormone levels as a critical role in the process [39]. Initially the latter may be perceived as inconsistent since ovarian steroids are known to stimulate neuronal plasticity and promote learning and memory. However, rising levels of estrogen, as well as impaired conditioning have been observed to be rapidly induced (within minutes) and persist for at least 24 h after stressor cessation [39]. Since impaired learning was most evident when rats were stressed during the transition into proestrus, this suggests that a further stress-induced enhancement of estrogen release coupled with already elevated estrogen levels during proestrus and high glucocorticoid concentrations might be detrimental for the formation of new memories.

Sex-related dimorphisms during emotional processing

The functional dimorphisms between male and female brains are not limited to cognitive processing, but they also embrace other higher-order functions such as affective style and emotional regulation. The amygdala, due to its primary involvement in stimulus integration, has for many years been the main focus of clinical and preclinical research[49,50,51]. This structure fulfills an integrative role in behavioural, vegetative, and endocrine activities of animals in relation with their environment and is involved the modulation of mood and affective behaviors in humans [52,53]. Remarkably, marked sex differences have also been observed in the activity of this limbic structure during emotional processing [54-56]. Amygdala activation, for instance, differs in men and women depending upon the valence of emotion. Overall, males express more lateralized amygdala activity than females [57]. As a result, positive emotions produce greater right than left amygdala activation for males compared to females, although both sexes showed greater left amygdala activation during fearful conditions [58]. Gender-related differences observed during perception, experience, and expression of emotional states, may thus be related to the differential use of this structure by men and women [56]. Notably, negative emotions, such as sadness, have been reported to activate a significantly wider portion of the limbic system in women, while men appear to rely more on cortical structures [59]. Remarkably, depressed patients showed increased amygdala volumes compared to healthy subjects. Moreover, this enlargement in patients with first episodes of major depression is more strongly related to enhanced blood flow in the amygdala than to a particular neurodevelopmental structural predisposition [55]. At least one study, investigating the relationship between amygdala volume and depression, found that female patients had significantly larger amygdala than males [60]. This evidence points to differential cortical and subcortical correlates of emotional experience in males and females but also underlines the importance of the amygdala in gender-related differential processing with respect to emotions and mood. Sex-related structural and, more importantly, functional differences may thus be implicated in the mechanisms underlying the differential sensitivity to stress and psychopathology observed between males and females.

Immunohistochemical changes induced by repeated footshock stress: revelation of gender-based differences

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"Neurobiology of Disease", in press

Introduction

Recent advances have been made in understanding the changes of neuronal plasticity in response to stress. Acute stressful experiences, for instance, facilitate the consolidation of new memories and promote cognitive processes [48,61]. In contrast, as a growing literature has proven, adverse experiences, particularly when severe and persistent, may contribute to the development of neuronal dysfunctions and psychopathology [62]. At the cellular level, evidence has emerged indicating dendritic atrophy and neuronal loss in response to chronic stress [63-66]. At the molecular level, it has been suggested that these abnormalities, mostly detected in the hippocampus and prefrontal cortex, result from a decrease of neuronal plasticity associated with persistent exposure to elevated glucocorticoid levels [67-69]. While in the short run adrenal steroids thus play a critical role in the acquisition of fear-conditioned responses [38,70], prolonged elevation of glucocorticoid levels results in functional and structural abnormalities and cognitive impairment [63,66,71,72]. Remarkably however, although considerable progress has been made in elucidating the neurobiological substrates underlying the acute stress response, chronic stress-induced neurochemical changes remain poorly understood. Furthermore, although gender represents a critical aspect in both sensitivity to stress and psychopathology [16,73], most of the clinical and preclinical research concerning stress-related neuronal abnormalities have been conducted in males [74].

With this in mind, the cellular and molecular changes associated with short-term (2 days) and prolonged footshock stress (20 days) were investigated in male and cyclic female rats, in an attempt to gain new insights into the neuronal circuits modulating the response to acute and chronic footshock exposure as well as the mechanisms underlying the biphasic effects of stress on cognitive and emotional processes. By using identical settings (5 footshocks delivered randomly during 30-minute sessions) but extending the length of the exposure from 2 to 20 days, these footshock procedures might prove a potentially useful model for investigating the dynamic of stress-induced disruption of cognitive processing in both a gender comparative setting. Footshock-induced neurochemical changes were examined using molecular and immunohistochemical techniques, including FOS-ir, phospho-ERK1/2 expression, and gene expression microarrays, as markers of cellular activity [75-77] and neuronal plasticity [78-80]. This data may thus contribute to the understanding of the mechanisms underlying gender-related differences in emotional processing and their relationship with the development of stress-induced cortical-limbic dysfunctions.

Materials and Methods

Animals

The experiments were performed using adult male (n=28: 212-240 g) and cyclic female Wistar rats (n=25: 195-212 g). The animals were housed individually (cages 45 x 28 x 20 cm) with food and water available ad libitum, and maintained on a 12/12-hr light/dark cycle. All rats were weighed and handled daily for 5-8 min to minimize the non-specific stress response. The experiments were carried out in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC), and with the guidelines of the Animal Bio-Ethics Committee of the University of Groningen (FDC: 2509).

Footshock procedure

The rodent footshock-chamber consists of a box containing an animal space placed on a grid floor connected to a shock generator and scrambler. Test-rats received one session of 30 min/day in the footshock box during which 5 inescapable footshocks were given (0.8 mA in intensity and 8 sec in duration: unconditioned stimulus; US) with different inter-shock intervals in order to make the procedure as unpredictable as possible. Each footshock was preceded by 10 sec of light (conditioned stimulus; CS). Control rats followed the same schedule in an identical setup but were exposed to CSs only, without receiving any shocks. This conditioning procedure was followed for 2 (acute challenge) or 20 days (chronic challenge). The final day of each experiment (3rd for the acute and 21st for the chronic) all rats were placed in the footshock box and exposed to 5 CSs only, without receiving any painful footshock. This allowed investigation of the pattern of protein expression (FOS-ir) and phosphorylation (phospho-ERK1/2) induced by identical and painless stimuli hereby avoiding exposure of animals to physical stress.

Acute experiment. Six male and six cyclic female rats were used in the acute experiment. Test-rats were conditioned for two consecutive days. The third and last day all rats were subjected to 5 CSs.

Chronic experiment. Eleven males and ten females were used in this experiment. Test-rats were conditioned for 20 consecutive days. On the final (21st) day all rats received 5 CSs without consequent footshocks.

Control rats. Eleven males and nine females were used as control animals. These animals were exposed to the same stimuli as the stressed rats (as they were housed in the same room and similarly exposed to the footshock chamber and CSs). They were never subjected to USs during the entire duration of the experiment.

Physiological and neuroendocrine changes following repeated footshock exposure

To define the dynamics of the response to repeated footshock stress, various physiological and neuroendocrine parameters were measured. Weight gain was monitored on a daily basis throughout the experiment, and upon termination, plasma adrenaline and corticosterone

concentrations were measured and adrenal glands were removed and weighed. Blood samples were drawn by transcardial puncture immediately upon termination and stored at -20°C. These samples were then used to determine plasma corticosterone and adrenaline levels with HPLC. Graphs were constructed to serve as a reference to verify the severity of stress perceived by the animals.

Extraction and Chromatography

Adrenaline. Adrenaline was extracted from plasma using liquid/liquid extraction with 3,4-dihydroxybenzylamine as internal standard [81,82]. Briefly, plasma adrenaline was bound to diphenylborate-ethanolamine at pH 8.6. The extraction was performed with n-heptane (containing 1% octanol and 25% tetraoctylammoniumbromide). Finally, adrenaline was extracted from the organic phase with diluted acetic acid. Adrenaline (20 µl acetic acid extract) was analysed using an HPLC/auto-injector (CMA, Sweden) and a Shimadzu LC-10AD pump (Kyoto, Japan) connected to a reversed phase column (Hypersil, C18, 3µm, 150x2.0mm), followed by an electrochemical detector (Antec Leyden, The Netherlands) working at a potential setting of 500mV vs. Ag/AgCl reference. The mobile phase consisted of 50mM acetate buffer, 150mg/l octane sulphonic acid, 150mg/l tetramethylammonium, 15mg/ml Na_2EDTA and 3% methanol, adjusted to pH 4.1. The flow-rate was 0.35ml/min. Temperature was 30°C. The detection limit of the method was 0.1nM.

Corticosterone. For the assay, dexamethason was used as internal standard. After addition of the internal standard, plasma was extracted with 3ml of diethylether, vortexed for 5 min and then centrifuged for 5 min at 3000 x *g*. The extraction procedure was repeated twice. The organic phase was evaporated to dryness in a 50°C waterbath. The residue was reconstituted with 200µL of mobile phase and 50µL was injected into the HPLC system. The mobile phase (flow rate 1.0mL/min) for the determination consisted of acetonitrile in ultrapure water (27:73 v/v). The concentration of both corticosterone and the internal standard was determined with UV detection at a wavelength of 254nm. The detection limit of the method was 10nM.

Histological procedure - Immunohistochemistry

Two hours after the beginning of the final session, the rats were terminated with an overdose of halothane which preceded a transcardial perfusion with 4% paraformaldehyde solution in 0.1M sodium phosphate buffer (pH 7.4). The brains were carefully removed and post-fixed in the same solution overnight at 4°C, before being transferred to a potassium phosphate buffer (KPBS 0.02 M, pH 7.4) and stored at 4°C. Following cryoprotection of the brains by overnight immersion in a 30% glucose solution, coronal serial sections of 40 µm were prepared on a cryostat microtome. Sections were collected in KPBS with sodiumazide and stored at 4°C.

c-fos and phospho-ERK1/2 immunoreactivity

The stainings were performed on free-floating sections under continuous agitation. The sections were preincubated in 0.3% H_2O_2 for 15 min to reduce endogenous peroxidase activity, before being incubated in primary monoclonal mouse anti-phospho-ERK1/2 (New

England Biolabs, Inc., Beverly, MA, USA; www.neb.com; 1:5000 dilution in KPBS 0.02 M, pH 7.4, overnight at room temperature) or polyclonal rabbit anti-FOS antibody (Oncogene Research Products, brands of CN Biosciences, Inc, an affiliate of Merck KGaA, Darmstadt, Germany; 1:10000 dilution in KPBS 0.02 M, pH 7.4, 60-72 hours at 4˚C) depending on the primary antibody host. Subsequently, sections were washed with KPBS and incubated at room temperature with biotinylated goat anti-mouse or goat anti-rabbit IgG (Vector Laboratories, Inc., Burlingame, CA, USA; 1:1000 dilution) followed by ABC complex (Vector ABC kit, Vector Laboratories, Burlingame, CA, USA). After another wash, the reaction product was visualized by adding diaminobenzidine as chromogen and 1% H_2O_2 for 15 min. Thereafter, sections were washed, mounted on slides, dehydrated and coverslipped with DePex.

Antibody specificity testing. To control for cross-reactivity due to aspecific binding, immunostainings were performed by incubating several sections without the presence of one of the antibodies needed for the reaction (primary, secondary or tertiary antibody). All these reactions were negative thereby confirming the specificity of all antibodies used.

Image analysis and counting procedure (semi-quantitative analysis)

FOS immunoreactive cells were quantified in 14 different brain regions or subregions with reference to the rat Swanson's brain atlas [83] while the quantification of phospho-ERK1/2 immunoreactivity was limited to the medial prefrontal cortex (prelimbic and infralimbic areas) where an abnormal phosphorylation of these kinases was found in chronically stressed male rats [69]. The quantification of FOS and phospho-ERK1/2 immunoreactivity was performed by an observer who was blind to group assignment. For counting of the immunoreactive cell nuclei, at least 4-5 sections per each brain area were analyzed. ROIs included prefrontal (prelimbic and infralimbic area; mPFC: Bregma +3.60 to +1.70) and cingulate cortices (AC: Bregma +3.20 to +0.95); hippocampal CA1 (CA1: Bregma –2.45 to –4.60) and dentate gyrus (DG: Bregma -2.00 to –3.90); central (CeA: Bregma –1.53 to –2.85), lateral (LaA: Bregma –2.00 to -3.70), basolateral (BslA: Bregma –1.78 to –3.25) and medial (MeA: Bregma –1.78 to –3.25) nuclei of the amygala; paraventricular (PVT: Bregma –1.33 to –3.90), dorsomedial (DMT: Bregma –2.00 to –3.90) and centromedial (CMT: Bregma –1.53 to –3.90) thalamic nuclei; paraventricular (PVN: Bregma –1.08 to –2.00) and dorsomedial (DMH: Bregma –2.45 to –3.70) hypothalamic nuclei; dorsal (DR: Bregma –7.10 to –9.25) and medial (MR: Bregma –9.25 to –10.35) raphe nuclei [83]. The area from structures of interest (ROI) were digitized by using a Sony (SONY Corporation, Tokyo, Japan) charge-coupled device digital camera mounted on a LEICA Leitz DMRB microscope (LEICA, Wetzlar, Germany). Each digitized image was individually set at a threshold to subtract the background optical density, and the numbers of cell nuclei above the background were counted by using the computer-based image analysis system LEICA (LEICA Imaging System Ltd., Cambridge, England). After image acquisition, FOS positive nuclei and phospho-ERK1/2-labeled dendrites were quantified. All areas were measured bilaterally (no left-right asymmetry for FOS or phospho-ERKs immunoreactivity was found) and therefore the resulting data was

reported as number of positive cells/0.1mm^2 (FOS-ir) or number of horizontal (H) and vertical (V) intersections (H+V contacts) between positive dendrites and an imaginary detection grid (composed by 514 horizontal x 698 vertical lines) present in the quantification field (H+V intersections/0.1mm^2) [69]. Absolute regional FOS-ir (mean±standard error (SEM)) for each region is reported in Table 1.

Area (ARS)	No stress Males	Acute Males	Chronic Males	No stress Females	Acute Females	Chronic Females
FRONTAL CORTEX						
prefrontal cortex (mPFC)	54±6	66±9	118±9***	214±14^{ooo}	80±7	141±20*
anterior cingulate (AC)	15±1	41±6	20±3	73±9^{ooo}	52±5	49±6
HIPPOCAMPUS						
CA1	2±1	11±4	4±1	28±4^{ooo}	2±1	15±2*
dentate gyrus (DG)	12±2	10±1	6±1	28±2^{oo}	15±2	18±3*
AMYGDALA						
central (CaA)	7±3	39±7	16±3*	30±8^{o}	28±4	16±2
lateral (LaA)	7±2	17±2	11±1	29±2^{ooo}	16±1	16±1***
basolateral (BslA)	6±1	17±4	19±3**	37±6^{ooo}	21±2	22±4
medial (MeA)	9±1	17±3	16±2**	41±6^{ooo}	23±2	27±6
THALAMUS						
centromedial (CMT)	11±3	40±8	7±2	73±6^{ooo}	63±4	37±13*
dorsomedial (DMT)	2±1	4±2	2±1	62±8^{ooo}	2±1	6±2***
paraventricular (PVT)	39±6	69±6	40±8	115±9^{ooo}	33±4	72±18
HYPOTHALAMUS						
paraventricular (PVN)	27±2	115±31	89±10**	55±2^{ooo}	129±19	119±22*
dorsomedial (DMT)	44±6	52±5	80±10*	51±4	33±5	57±3
MIDBRAIN						
medial raphe (MR)	6±1	19±5	23±6*	28±9^{o}	9±1	29±4
dorsal raphe (DR)	10±2	18±3	34±5**	29±6^{o}	22±3	35±5

Table 1. Absolute FOR-ir following acute and chronic stress in male and female rats

* = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$; non-stressed vs. stressed-rats;
^{o} = $p<0.05$; ^{oo} = $p<0.01$; ^{ooo} = $p<0.001$ non-stressed males vs. non-stressed females.

Statistics

One-Way-Anova and *F* test of variance were run on numbers of FOS and phospho-CREB immunoreactive cell nuclei from individual brain ROIs from experimental and control conditions. To compare cell counts from individual ROIs, *t* tests for equal or unequal variance were performed. $P<0.05$ was defined as the level of significance between groups.

Histological procedure - Molecular biology

Tissue and RNA Preparation

Thirty minutes after the beginning of the final session, rats used for the molecular biology were anesthetized with halothane and decapitated. The prefrontal cortex was dissected, quick-frozen in liquid nitrogen and stored at -80°C. Total RNA was isolated from the prefrontal cortex of each animal by using Trizol (Life Technology, Gaithersburg, MD, USA) according to the manufacturer's instructions. Integrity of total RNA was confirmed on an agarose gel and final concentrations were assessed spectrophotometrically.

cDNA microarray

RNA was extracted from the prefrontal cortex of 4 or 5 rats within the groups participating in the chronic experiment and their controls. RNA (2-5µg / rat), subsequently converted into a 32P-labeled first-strand cDNA, was used to hybridize cDNA microarrays (rat atlas cDNA array 1.2; Clontech, Palo Alto, CA, USA). Use of a broad coverage array instead of a stress array was intentionally chosen because of our interest in the role of transcription factors and second messengers in stress-induced neuronal dysfunction, which could involve the expression of numerous candidate genes. In this microarray, plasmid and bacteriophage DNAs are included as negative controls, along with several housekeeping cDNAs as positive controls. A complete list of the genes and controls spotted on the array, as well as array coordinates and GenBank accession numbers, is available at Clontech's web site, (http://www.clontech.com). In order to suppress non-specific background each membrane was prehybridized for 30 min at 68ºC in 5ml of hybridization solution (ExpressHyb, Clontech) with continuous agitation. Hybridization was subsequently carried out by the addition of the denatured, labeled cDNA to the prehybridization solution at 68ºC for overnight incubation to reach a final probe concentration of 2-5 x 10^6cpm / ml. Membranes were stringently washed with continuous agitation at 68ºC in 2 x SSC, 1% SDS (4x30 min) and then in 0.1 SSC, 0.5% SDS (30 min) After a final rinse in 0.1 x SSC (5 min), membranes were mounted on Whatman paper, plastic-wrapped, exposed to x-ray film overnight at -80ºC followed by exposure to a phosphoimager screen for 3 days.

PhosphoImaging analysis

Membranes were scanned using a Molecular Dynamics STORM PhosphoImager (Molecular Dynamics, Inc., Sunnyvale, CA, USA), and images were analyzed by ImageQuant (Molecular Dynamics, Inc., Sunnyvale, CA, USA). According to the Array manufacturer, Clontech, the radioactive cDNA signal is linear for RNAs present at levels of 0.01-3% of the total RNA population. An admonition of this quantitative analysis however, is that the accuracy for the extremely low abundant genes may not be reliable due to the detection limitation of this technique. The expression level for each gene was measured by the phosphoimager in arbitrary signal intensity units. This original raw output was subsequently used to perform the statistical analysis.

Statistics

To perform the following analysis, use was made of Delphi 5.0 and SPSS 11.0 software. Data from the 19 hybridizations was analyzed by log transforming the data points and converting to sequential format. Using analysis of variance the data was checked for systemic position effects. A significant effect was found in the y coordinate of the genes, but since this only explained 1% of the variance, a correction step for this effect would have minimal influence on data quality, and was thus omitted. After transformation to a parallel format, principal component analysis was performed to assess the first principal component. This common component was then removed to yield a better representation of true differences and outliners in a means plot using analysis of variance. To further analyze the data, a reference set of genes was chosen with which to test the hypothesis against a smaller, analysis set. Genes selected for inclusion in the analysis set were those which displayed the greatest variation compared to other genes. To verify the significance of the results, the criterion for inclusion of genes was expression over a range of standard deviations (>1.5, >1.8, >1.9 >2.5). In order to identify the strongest alterations of individual genes a plot was made of the regression factor scores. On the horizontal axis de common variation or expression of the genes was set out against the inter-array differences or variation (second principal component).

Results

Physiological and neuroendocrine changes following repeated footshock exposure

To define the dynamics of the response to prolonged footshock stress, various physiological and neuroendocrine parameters were measured, including body weight gain throughout the experiment, plasma adrenaline and corticosterone concentrations as well as adrenal weights upon termination.

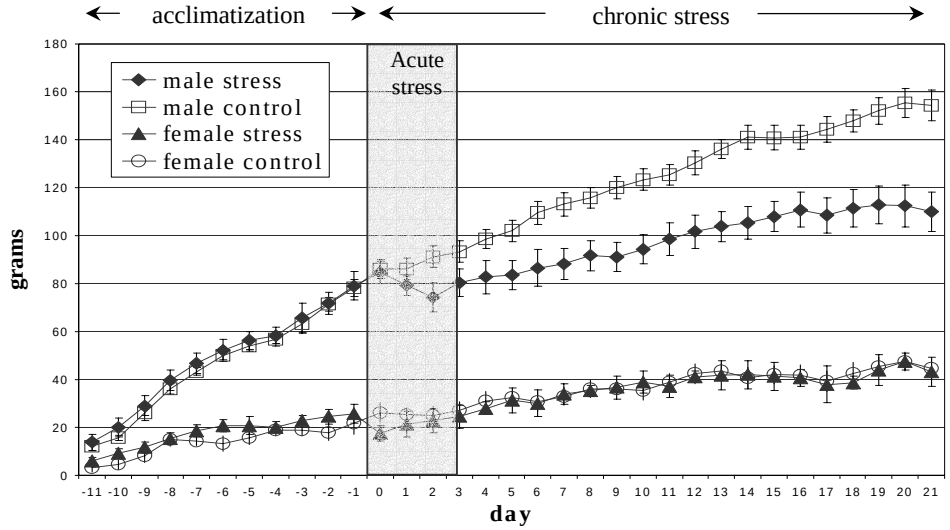


Figure 1a. Body weight gain in response to prolonged footshock stress.

Body weight gain

Body weights were measured daily during the acclimatization period and the chronic stress procedure in control and stressed rats (fig. 1a). During the acclimatization period both groups showed an identical weight gain. Immediately after initiation of stress exposure however, a consistent reduction in body weight gain was observed in stressed males, while weight gain in non-stressed animals continued constantly as expected. The difference in weight gain between non-stressed and stressed males continued to increase progressively until the final day ($F=18.09$, $p<0.0019$). No differences were found between non-stressed and chronically stressed females. This finding was in accordance with previous preclinical data showing that stress exposure, in female rats, does not affect body weight gain as much as it does in males [84].

Plasma corticosterone levels

Corticosterone and adrenaline concentrations were measured by HPLC. Although chronically stressed rats, on the final day, were only exposed to psychological (CSs and exposure to the box) and not to physical stress (USs), they showed significantly elevated plasma corticosterone concentrations ($F=8.14$, $p<0.021$, non-stressed vs. chronically stressed males; $F=9.81$, $p<0.014$, non-stressed vs. chronically stressed females). Adrenaline

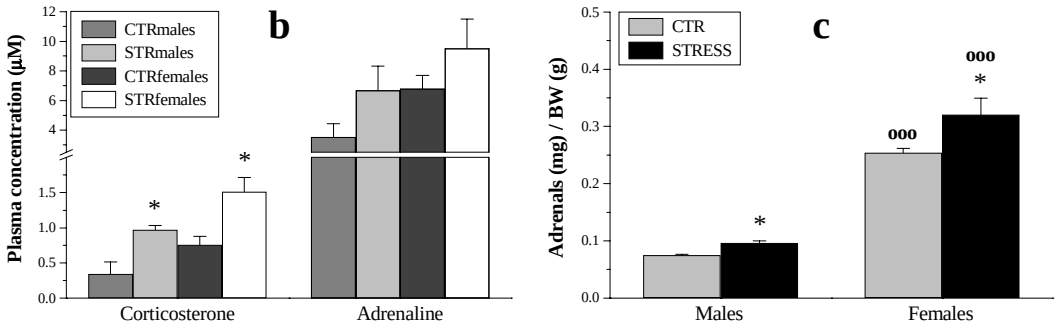


Figure 1b, c. Plasma corticosterone and adrenaline levels following chronic stress exposure (b). Adrenal hypertrophy was detected in both chronically stressed male and female rats (c). The symbol * expresses the comparison between CTR vs. STR rats whereas the symbol ^{o} illustrates the comparison between male and female rats.

concentrations, although higher in chronically stressed rats, did not reach a statistical difference compared to non-stressed animals ($F=2.87$, $p<0.12$, males; $F=1.75$, $p<0.22$, females) (fig. 1b). This evidence seems to suggest the lack of habituation in the neuroendocrine response to repeated footshock stress, both in male and female rats. It is interesting to note that females, both under stressed and non-stress conditions, reported higher plasma corticosterone levels than males ($F=3.86$, $p<0.085$, non-stressed males vs. females; $F=6.26$, $p<0.037$, chronically stressed males vs. females) (fig. 1b).

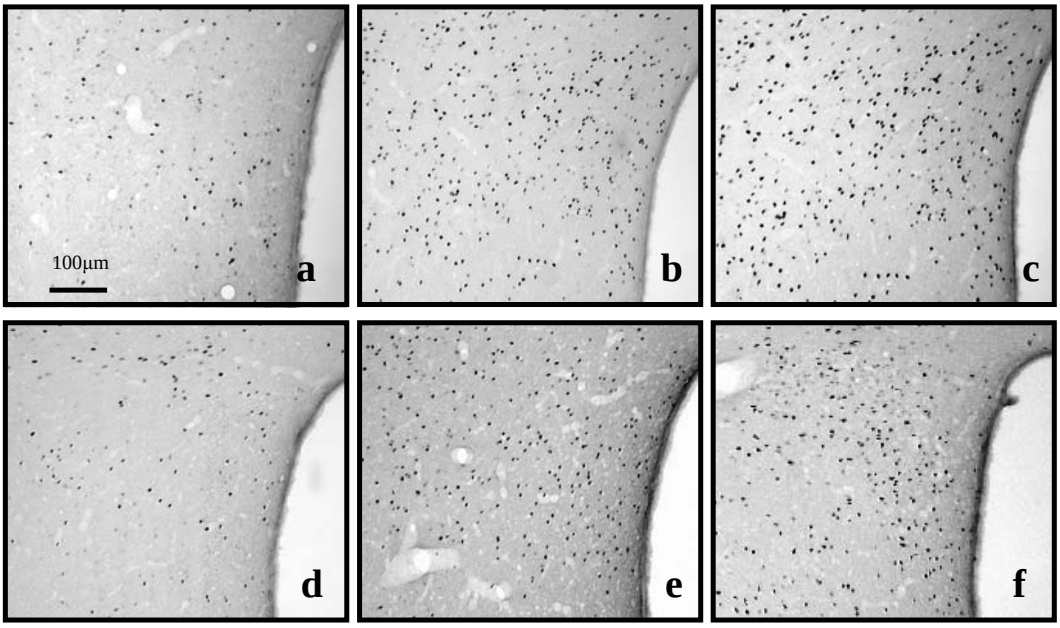


Figure 2. FOS-ir in the paraventricular hypothalamic nucleus.

Adrenal weights

Both male and female rats repeatedly subjected to footshock stress showed a significant adrenal hypertrophy ($F_{males}=24.20$, $p_{males}<0.001$: $F_{females}=6.05$, $p_{females}<0.039$) that appears to suggest a prolonged HPA axis activation (fig. 1c). Additionally, female rats showed higher

adrenal weights compared to males, both under non-stressed and stressed conditions (fig. 1c).

Immunohistochemistry

In the present study, two experiments were performed: a short-term training, consisting of two daily sessions of footshock stress during which 5 electric shocks were delivered, and a chronic experiment, consisting of 20 consecutive daily sessions of footshock stress.

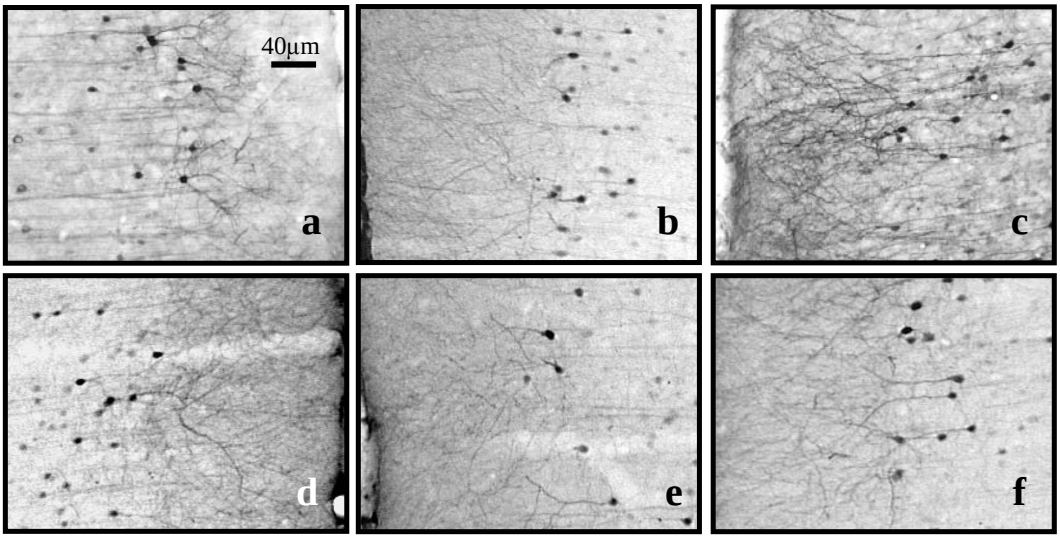


Figure 3. Phospho-ERK1/2 immunoreactivity in medial prefrontocortical dendrites.

After each experiment, FOS-ir (fig. 2) and phospho-ERK1/2 expression (fig. 3) were quantified throughout several cortical and limbic regions involved in the modulation of emotional and stress responses, including the frontal cortex, the hippocampus, the amygdala, the thalamus, the hypothalamus, and the midbrain.

Acute footshock challenge. Gender-related differences in the patterns of cortical-limbic activity were found in response to acute challenge. Male rats showed a significantly increased FOS-ir in the AC (F=14.35, p<0.0043) (fig. 4a), the CA1 (F=5.39, p<0.045) (fig. 4b), the CeA (F=13.45, p<0.0063), the LaA (F=16.39, p<0.0037), the BslA (F=8.93, p<0.017), the MeA (F=6.12, p<0.038) (fig. 4c), the CMT (F=9.54, p<0.013), the PVT (F=11.42, p<0.008) (fig. 4d), the PVN (F=6.45, p<0.032) (fig. 4e), the MR (F=6.24, p<0.034), and the DR (F=8.46, p<0.02) (fig. 4f). In contrast, female rats, acutely exposed to footshock stress, showed a reduction of FOS-ir in the mPFC (F=88.35, p<<0.001), the AC (F=5.81, p<0.039) (fig. 4a), the CA1 (F=94.88, p<<0.001), the DG (F=20.96, p<0.0013) (fig. 4b), the LaA (F=30.52, p<<0.001), the BslA (F=9.49, p<0.013), the MeA (F=11.43, p<0.008) (fig. 4c), the CMT (F=28.15, p<<0.001), the DMT (F=26.15, p<<0.001), the PVT (F=28.89, p<<0.001) (fig. 4d), the DMH (F=5.24, p<0.048) (fig. 4e), and the MR (F=7.18, p<0.028) (fig. 4f). An opposite effect was found in the PVN, where a significantly increased FOS-ir was observed (F=6.61, p<0.033) (fig. 4e).

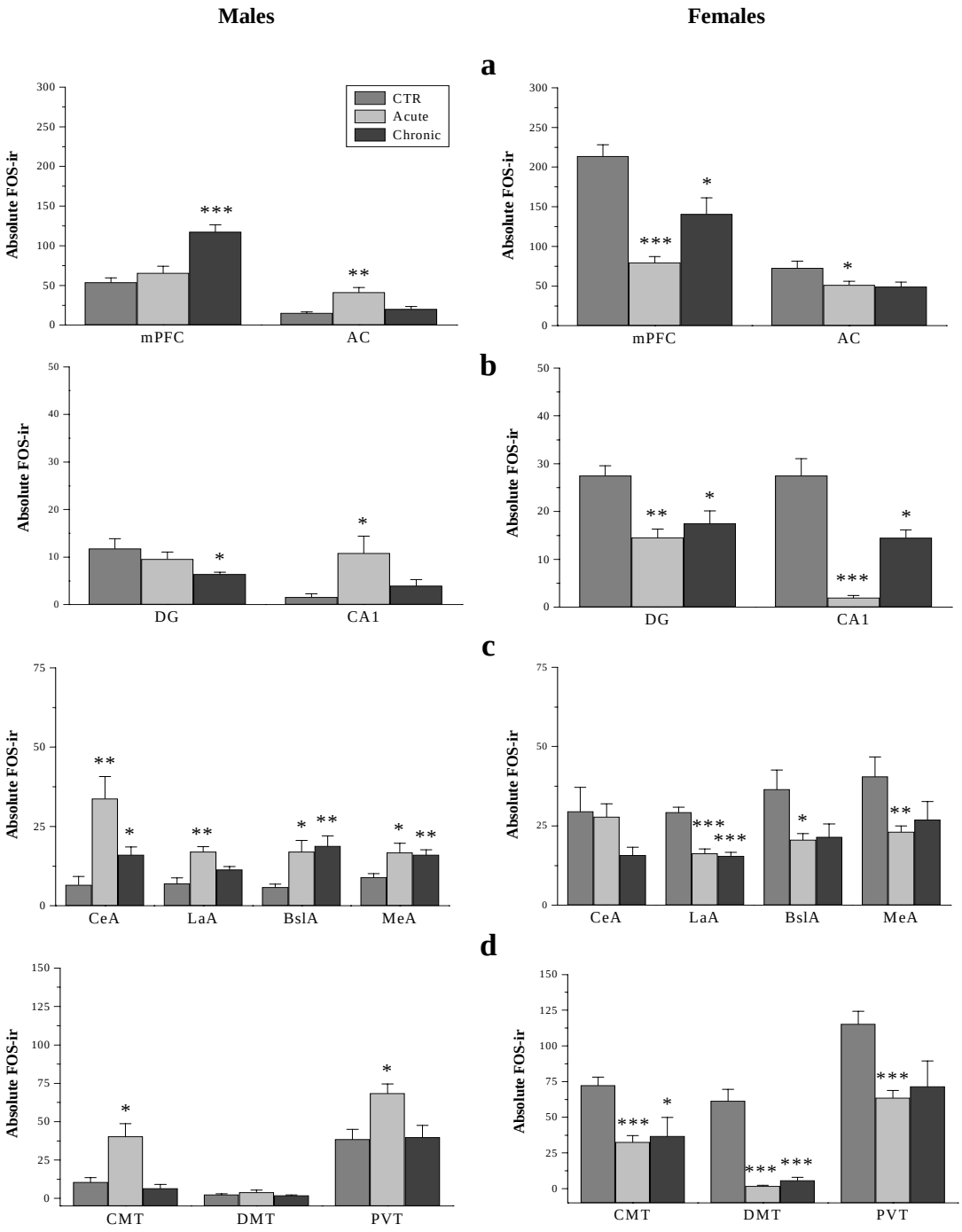
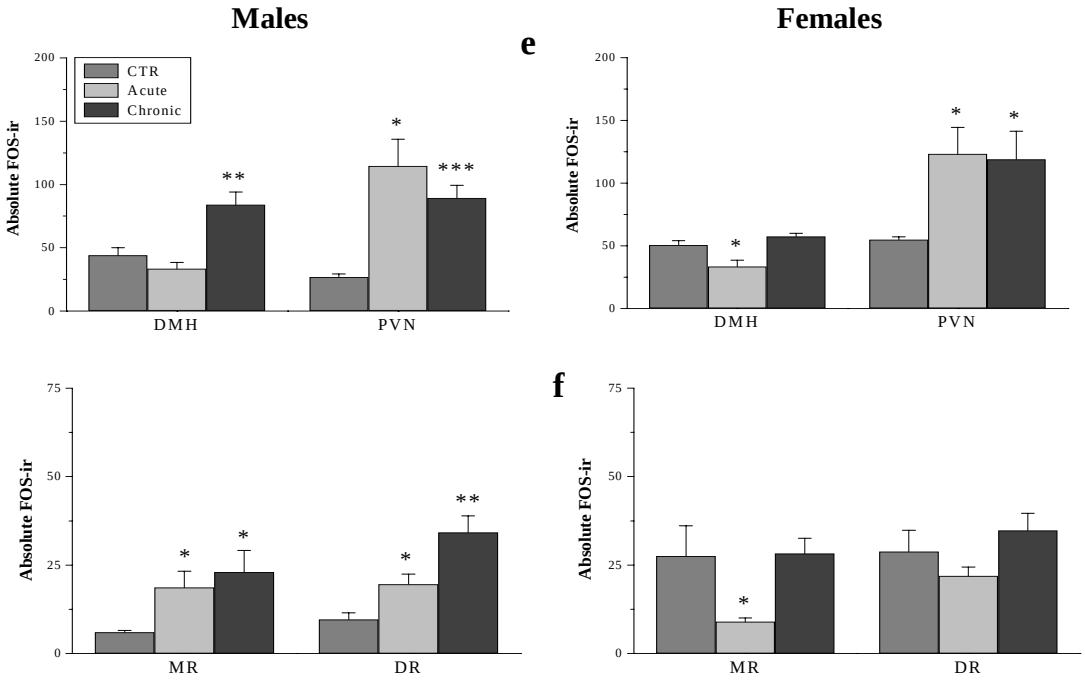


Figure 4. Effect of acute and chronic stress on absolute FOS-ir in: a) medial prefrontal cortex; b) hippocampus; c) amygdala; d) thalamus; e) hypothalamus; f) raphe nuclei. The symbol * expresses the comparison of absolute FOS-ir between stressed rats, both acutely or chronically, and non-stressed animals (*=p<0.05; **=p<0.01; ***=p<0.001).



Unexpectedly, acute footshock exposure caused a significant decrease of phospho-ERK1/2 immunoreactivity in medial prefrontocortical dendrites in male rats while no changes were found in cyclic females (fig. 5).

Chronic footshock challenge. Gender specific patterns of neuronal activity were also observed in response to repeated footshock exposure. Male rats showed an increased cortical-limbic FOS-ir in 12 of the 15 regions (80%) (table 1): the increase was significant in 8 of the 15 areas (53%), including the mPFC (F=36.90, p<0.0003) (fig. 4a), the CeA (F=6.52, p<0.034), the BslA (F=14.72, p<0.005), the MeA (F=12.25, p<0.0081) (fig. 4c), the DMH (F=10.05, p<0.013), the PVN (F=14.72, p<0.005) (fig. 4e), the MR (F=9.95, p<0.016), and the DR (F=28.65, p<0.0011) (fig. 4f). Only DG (F=6.63, p<0.033) and CMT showed an opposite effect such as a decreased FOS-ir after prolonged footshock stress (fig. 4b). Chronically stressed females, in contrast, showed a widespread reduction of cortical-limbic FOS-ir (11 out of 15 regions analyzed). The effect was significant in the mPFC (F=8.62, p<0.026) (fig. 4a), the CA1 (F=11.14, p<0.016), the DG (F=8.96, p<0.024) (fig. 4b), the LaA (F=45.60, p<0.001) (fig. 4c), the CMT (F=6.08, p<0.049), and the DMT (F=44.20, p<0.001) (fig. 4d). An opposite effect was found in the PVN where a significantly increased FOS-ir was observed after chronic stress exposure (F=8.04, p<0.03) (fig. 4e). In addition, chronic footshock stress caused a selective and prolonged ERK1/2 hyperactivation in dendrites of the higher medial prefrontocortical layers (II and III) in males but not in cyclic female rats (fig. 5).

Basal level of protein expression and phosphorylation. A significant gender-related dimorphism in the level of basal FOS immunoreactivity (FOS-ir quantified under non-stressed conditions) was found in several cortical and subcortical areas (fig. 4). Non-stressed females showed in fact a significantly higher FOS-ir than male rats in the mPFC (F=131.45, p<0.001), the AC (F=55.54, p<0.001) (fig. 4a), the CA1 (F=65.96, p<0.001), the DG (F=28.23, p<0.0011) (fig. 4b), the CeA (F=9.69, p<0.017), the LaA (F=77.98, p<0.001), the BslA (F=30.81, p<0.001), the MeA (F=31.82, p<0.001) (fig. 4c), the CMT (F=97.25, p<0.001), the DMT (F=68.07, p<0.001), the PVT (F=51.23, p<0.001) (fig. 4d), the PVN (F=64.79, p<0.001) (fig. 4e), the MR (F=8.09, p<0.025), and the DR (F=11.10, p<0.013) (fig. 4f). No differences however were observed in the pattern of medial prefrontocortical phospho-ERK1/2 between male and cyclic female rats (fig. 5).

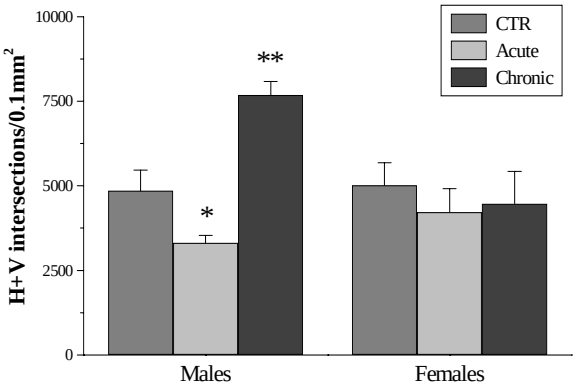


Figure 5. Phospho-ERK1/2-labeled prefrontocortical dendrites.

Molecular biology - Gene expression patterns

Since functional and morphological changes have been reported in medial prefrontocortical regions following chronic stress exposure, nineteen animals where assigned randomly to 4 groups and used for the analysis of gene expression patterns in this cortical region in response to prolonged footshock stimulation. The animals were assigned as follows:

- CTR-males (n=5) and CTR-females (n=5): these rats were exposed to the footshock box and CSs but did not receive any footshocks.
- STR-males (n=4) and STR-females (n=5): These animals were exposed daily to the footshock procedure for 20 consecutive days. On the final day of the experiment they only received CSs without consequent footshocks.

The results illustrate a significant gender difference with regard to gene expression following repeated footshock exposure. The males responded with stronger changes following stress and display an opposite change compared to stressed females. Whereas females illustrated a reduced mRNA transcription (-0.0080 to –0.0252), males demonstrated a strong increase in prefrontocortical gene expression following chronic footshock stress (-0,284 to 0.0594). This interaction effect is quite significant (p=0.006) although it only explains a minimal amount of variation (~0.1 %). If the genes to be compared relative to the others are selected for higher variance, the number of analyzed genes decreases and noise becomes more important. The significance of the results depends on the amount of data included, yet despite the chosen threshold, the trend remains the same. The strongest effect, illustrated below, was obtained with a standard deviation threshold of 1.8 (table 2).

Descriptive Statistics

Dependent Variable: EXPRESS

STRESS	SEX	Mean	Std. Deviation	N
1	1	-.0080	.72685	1395
	2	-.0284	.69032	1395
	Total	-.0182	.70877	2790
2	1	-.0252	.71204	1395
	2	.0594	.59476	1116
	Total	.0124	.66369	2511
Total	1	-.0166	.71941	2790
	2	.0106	.65093	2511
	Total	-.0037	.68789	5301

Tests of Between-Subjects Effects

Dependent Variable: EXPRESS

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Mode	5.960[a]	3	1.987	4.206	.006
Intercept	1.626E-03	1	1.626E-03	.003	.953
STRESS	1.631	1	1.631	3.452	.063
SEX	1.350	1	1.350	2.859	.091
STRESS * SEX	3.621	1	3.621	7.666	.006
Error	2501.967	5297	.472		
Total	2508.001	5301			
Corrected Total	2507.928	5300			

a. R Squared = .002 (Adjusted R Squared = .002)

Table 2

These opposing findings in gene expression seem to confirm FOS-ir data concerning a sex-related dimorphism in the pattern of protein expression and, possibly, neuronal activity in response to repeated footshock exposure. In support of recent studies that documented atrophy of prefrontocortical dendrites in chronically stressed male rats [66], the present investigation reports an abnormal pattern of prefrontocortical ERK1/2 phosphorylation in chronically stressed males (fig. 5). Due to the pivotal role played by ERK1 and 2 in this neuronal function, the expression arrays were further analyzed to identify changes in genes that have been reported to modulate synaptic plasticity in the medial prefrontal areas and might be affected by prolonged footshock exposure. When regression factor score 1 was plotted against factor 2 a skewed distribution was evident (table 3).

In line with the gender effects, the majority of outliners were located on the negative part of the X axis, coinciding with the greatest variation between highly expressive genes in males and low expressive genes in females. It is of interest to note that the expression of several genes involved in the modulation of neuronal plasticity, such as synapsin II, SNAP25, calmodulin, and ERK2, was differentially affected by repeated footshock stimulation in male compared to female rats (table 4).

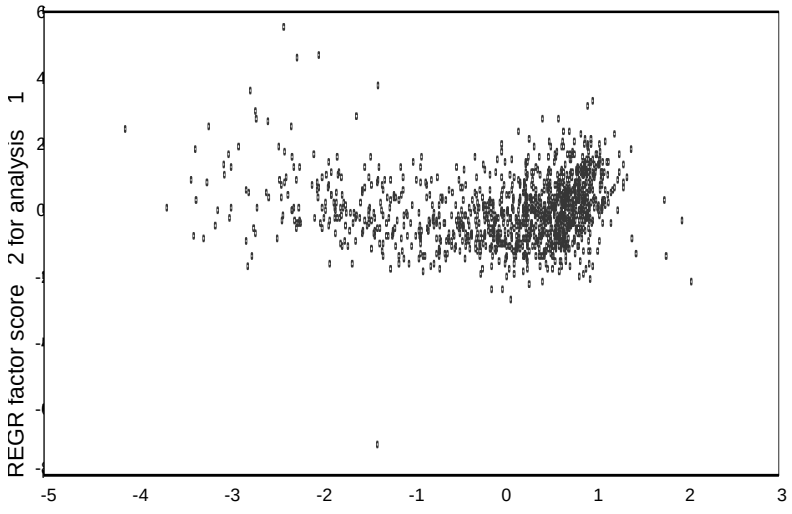


Table 3

GenBank access. No.	Genes
J03754	ATPase isoform 2, Na+K+ transporting, beta polypeptide 2
L08831	Glucose-dependent insulinotropic peptide
X62908	cofilin 1, non-muscle
M84416	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, epsilon polypep
S79304	cytochrome oxidase, subunit I, Sertoli cells
M19007	Protein kinase C beta
M18416	Early growth response 1
K03502	elongation factor 2 (EF2)
D17615	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypep
S45392	heat shock 90-kDa protein beta (HSP90-beta
X67241	Ras-GRF (p140)
D10952	cytochrome c oxidase subunit Vb
D10874	vacuolar ATP synthase 16-kDa proteolipid subunit; ATP6C
M84417	mitogen-activated protein kinase 2 (MAPK2, ERK2)
S68944	sodium/chloride neurotransmitter transporter
S59158	solute carrier family 1, member 3
M25890	Somatostatin
M11185	Proteolipid protein (Pelizaeus-Merzbacher disease, spastic paraplegia 2, uncomplicated)
M35862	Male germ cell-associated kinase
M27925	synapsin II
M25889	Myelin basic protein
AB003991	Synaptosomal associated protein 25; SNAP-25
AF005099	neuronal pentraxin receptor
M95735	Syntaxin 2
X14209	cytochrome c oxidase, subunit IV
D17445	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, eta polypep
M31602	Carboxypeptidase E; carboxipeptidase H
M91808	sodium channel, voltage-gated, type I, beta polypeptide
L12382	ADP-ribosylation factor 3 (ARF3)
M19044	Mitochondrial ATP synthase beta subunit precursor (ATP5B)
M27905	ribosomal protein L21
M80550	adenylyl cyclase 2
U02983	Secretogranin 3 (Sg3)
M26161	voltage-gated potassium channel protein KV1.1
M17526	GTP-binding protein
M76426	Dipeptidylpeptidase 6
M16736	growth accentuating protein 43
U38665	inositol 1,4,5-triphosphate 3-kinase receptor 2 (INSP3R)
U25651	Phosphofructokinase, muscle
D10666	Neural visinin-like protein 1
J02650	ribosomal protein L19
M24105	Vesicle-associated membrane protein (synaptobrevin 2)
M16112	Ca++/calmodulin-dependent protein kinase II, beta subunit
M63901	Secretory granule neuroendocrine, protein 1 (7B2 protein)
L19181	Receptor-linked protein tyrosine phosphatase (PTP-PS)
M61177	mitogen activated protein kinase 3
X16956	Microglobulin; beta-2-microglobulin + prostaglandin receptor F2a
M86870	Endoplasmic reticulum stress protein 72 precursor (ERP72)
M18547	ribosomal protein S12
M28647	ATPase, Na+K+ transporting, alpha 1 polypeptide
J04629	ATPase, Na+K+ transporting, beta polypeptide 2
X13817	Calmodulin III
X62146	ribosomal protein L11
J02942	Ca2+/calmodulin-dependent protein kinase II alpha
M18332	protein kinase C, zeta
J03773	Guanine nucleotide binding protein, alpha
L31622	cholinergic receptor, nicotinic, beta subunit 2
AF019973	neuron-specific enolase (NSE)
D13374	nucleoside diphosphate kinase A (NDKA; NDP kinase A)
M29275	Calcineurin subunit A alpha
J02701	ATPase Na+/K+ transporting beta 1 polypeptide
L31884	tissue inhibitor of metalloproteinase 2
X06942	A-raf

Table 4. Differential gene expression in prefrontal cortex of male and female rats exposed to prolonged footshock stress evaluated by cDNA array.

Discussion

Upon analysis of neuroendocrine and immunohistochemical changes induced by acute and prolonged footshock exposure, important gender-related differences emerged in the patterns of cortical-limbic FOS-ir and prefrontocortical phospho-ERK1/2 expression. The choice of investigating the level of expression and phosphorylation of these specific proteins was made upon reviewing their specific cellular functions. Changes of FOS-ir have been widely used as molecular marker of neuronal activity [75-77]. The analysis of FOS-ir has thus become a molecular tool to investigate complex processes, such as learning [85-91] and memory [92-95] as well as the neurocircuits activated by stress [96-105]. The extracellular signal-regulated kinase (ERK) is a member of a family of serine/threonine protein kinases implicated in the transduction of neurotrophic signals from the cell surface to the nucleus [78]. The ERK cascade plays a central role during neurodevelopment in the regulation of cell growth, proliferation, and differentiation but, interestingly, several family members, including ERK1 and ERK2, are also widely expressed by post-mitotic neurons in the mammalian nervous system [106]. This evidence has suggested that ERKs might contribute to the regulation of important functions in the adult brain, including neuronal plasticity, learning, and memory [107,108]. A critical step in the regulation of ERK-mediated activities is the dual phosphorylation of these kinases that leads to their transient activation and translocation from the cytoplasm to the nucleus [109]. Only phosphorylated ERKs (phospho-ERKs) are able to interact with and activate cytoplasmic and nuclear targets, and modulate such critical neuronal functions [78]. Changes in the levels of ERK1/2 phosphorylation may thus provide important indications concerning the ability of stress to influence neuronal plasticity.

Immunohistochemical changes in response to acute footshock challenge

Acute footshock exposure activated, in male rats, cortical and subcortical structures, including the cingulate cortex (fig. 4a), the hippocampal CA1 (fig. 4b), the central, lateral, basolateral, and medial nucleus of the amygdala (fig. 4c), the centromedial and paraventricular nucleus of the thalamus (fig. 4d), the paraventricular hypothalamic nucleus (fig. 4e), the median and dorsal raphe nucleus (fig. 4f). In contrast, acute footshock stress was associated, in female rats, with a significant reduction of FOS-ir in most of the above-mentioned cortical-limbic regions (fig. 4), with the only exception of the PVN where, similarly to males, a marked increase of neuronal activation was observed (fig. 4e).

Acute emotional experiences have been reported to promote learning and memory [37,110,111]. A growing body of evidence has pinpointed in particular the amygdala and the hippocampus as core components of the brain's fear system [112-118]. Thus, the

increased FOS-ir reported by males in the amygdala (CeA, LaA, BslA, and MeA) (fig. 4c) and hippocampus (CA1) (fig. 4b) may support the participation of these limbic structures in the modulation of acute fear-related responses. Surprisingly, a different pattern of cortical-limbic FOS-ir was observed in female rats following short-term aversive challenge. In contrast to males, acutely stressed females reported a significant reduction of FOS expression in both amygdala (LaA, BslA and MeA) and hippocampus (CA1) compared to non-stressed animals (fig. 4b,c). This differential pattern of neuronal activation was not limited to these two limbic structures but also involved the anterior cingulate cortex (fig. 4a), the thalamus (centromedial and paraventricular nuclei) (fig. 4d), and the midbrain (median raphe nucleus) (fig. 4f). Interestingly, a decreased phospho-ERK1/2 immunoreactivity was observed in medial prefrontocortical dendrites of acutely stressed males while no changes in the level of kinase phosphorylation were found in cyclic females (fig. 5). A critical step in ERK-mediated facilitation of neuronal plasticity involves their dual phosphorylation followed by translocation from the cytoplasm to the nucleus [109]. Reduced phospho-ERK1/2 immunoreactivity in medial prefrontocortical dendrites of male rats, might thus illustrate the translocation of these enzymes from the periphery to the nucleus (fig. 3a,b, 5) and support the molecular changes underlying the consolidation of fear-related memories.

In recent years, a cumulative body of evidence concerning the existence of morphological [56,119-124] and functional differences [49,50,59,125-127] between the male and female brain has emerged. Cognitive processes, such as learning and memory, as well as behavioral responses to stress are influenced by sex [4,22,25,50,125,128-130]. The gender-related patterns of neuronal activity observed in the present study following acute challenge might represent sex-specific coping strategies under aversive conditions. The notion that males and females may differ in their coping strategies has been proposed by Taylor and colleagues [131]. Taylor stated that the male response to stress in humans, along with some animal species, is characterized by a “fight-or-flight” response whereas the female response is more typically characterized by a pattern termed “tend-and befriend” [131]. These gender-related behavioral responses may reflect the involvement of different neural pathways and our results might offer indirect immunohistochemical evidence linking such gender-related coping styles with differential patterns of neuronal activity. A closer look at our data reveals that the divergent response to acute footshock challenge appears to be strongly related to the different level of basal FOS-ir (FOS expression under non-stress conditions) (fig. 4). Non-stressed females in fact, illustrated overall higher neuronal activation than males (up to 5-7 times higher), especially in frontocortical areas (fig. 4a). In line with these findings, neuroimaging investigations have also found gender-related diversities in brain activity patterns in humans, as women illustrated higher values than men [59,132,133]. Furthermore, Esposito and colleagues reported substantial gender-related differences in the frontal lobe rCBF during

performance of a variety of cognitive tasks with women showing a significantly higher activation [125]. Although functional brain imaging studies have illustrated sex differences in global as well as in regional brain activity, reports of differential activation in the frontal lobes have been particularly prevalent [59,134]. The results presented here may thus provide new insights into gender-related differences in the neuronal circuitry engaged in the acute stress response and the molecular mechanisms underlying its modulation.

Gender-related dimorphism following repeated footshock exposure

Brief elevations of glucocorticoid levels plays a critical role in the modulation of fear-related responses [135], promoting learning acquisition and memory consolidation [37,48,136]. This beneficial effect of adrenal steroids however, is only temporary as prolonged exposure to high glucocorticoid concentrations has been shown to impair cognitive processes, possibly through the deleterious effects of stress hormones on neuronal plasticity [137-143]. It is intriguing to speculate that gender-related differences in FOS-ir and ERK1/2 phosphorylation, detected following repeated footshock stress, may illustrate the deleterious effects of prolonged exposure to hostile conditions on functional and structural integrity of the brain. The latter is supported by physiological and neuroendocrine evidence, such as the reduction of body weight gain (fig. 1a, non-stressed vs. chronically stressed males), the significant elevation of corticosterone levels (fig. 1b), the hyperactivity of the PVN (fig. 4e) and, more importantly, the adrenal hypertrophy, observed in both chronically stressed male and female rats (fig. 1c). Footshocks have been reported to strongly activate the PVN, elevating plasma corticosteroid concentrations [144,145]. Given the pivotal role of this hypothalamic nucleus in the regulation of the HPA axis [146,147], these neuroendocrine changes seem to substantiate a lack of habituation and, possibly, an abnormal HPA axis activation in response to repeated stress. An intriguing possibility is that prolonged footshock exposure promotes functional and morphological impairments by persistently elevating corticosteroid concentrations in the brain [66,69,148]. Glucocorticoids have been known to exert a deleterious influence on neuronal plasticity [47,63,149] and cause functional and morphological abnormalities in vulnerable regions, such as the hippocampus [71,150-152] and the prefrontal cortex [66,69]. Functional cortical-limbic alterations included, in chronically stressed males, a reduced neuronal activation in the DG (fig. 4b) and an increased FOS-ir in the mPFC (fig. 4a), amygdala (CeA, BslA and MeA) (fig. 4c), hypothalamus (DMH and PVN) (fig. 4e) and raphe (MR and DR) (fig. 4f). The abnormal ERK1/2 phosphorylation in medial prefrontocortical dendrites (fig. 5) may, instead, document a stress-related structural impairment. In female rats, on the contrary, prolonged aversive stimulation was associated with a general reduction of FOS-ir in most of the cortical and subcortical regions, including the mPFC (fig. 4a), the hippocampus (CA1 and DG) (fig. 4b), the LaA (fig. 4c), and the thalamus (CMT and DMT) (fig. 4d). No changes in the level of phospho-

ERK1/2 immunoreactivity were observed (fig. 5). These gender-related patterns of neuronal activity were also confirmed at the molecular level, as gene expression analysis illustrates a general up-regulation of gene transcription in the frontal lobe of chronically stressed males, while a differential response was observed in females. It is interesting to note that, in addition to the gender-related dimorphism observed in the level of FOS and phospho-ERK1/2 immunoreactivity following repeated footshock exposure, differential gene expression patterns were also detected between male and female rats, including various key genes underlying neuronal plasticity (table 4).

Amygdala-prefrontocortical involvement in response to chronic footshock stress: a gender comparative view*

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***Adapted from the manuscript in preparation for "Neuroscience"**

Introduction

Exposure to stressful events represents a predisposing factor in the development of depression [153], chronic psychiatric illness characterized by complex cortical and subcortical defects [154,155] and marked gender-related prevalence [156]. These abnormalities include functional and structural deficits, such as neuronal pathology [157], reduced prefrontocortical function [158,159] as well as abnormal amygdala [160,161] and HPA axis activity [162-165]. A troubling aspect of depression involves the prolonged processing of negative emotions and the preferential memory for adverse emotional events [166,167]. Recent clinical findings have confirmed the existence of malfunctions in the coordinated interplay between amygdala and prefrontal cortex as a critical element in the development and maintenance of depressive symptoms, such as recollection of intrusive traumatic memories and persistent low mood [158,168,169].

In the present study, we investigated the cortical-limbic response to prolonged footshock stress in male and female rats, using c-fos (FOS-ir) and phospho-CREB expression as immunohistochemical correlates of cellular activity [170-172] and neuronal plasticity [172-174]. Reliability of these molecular markers to study complex neurocircuits underlying cognitive and emotional regulation [170,175-185] as well as neuronal plasticity changes [186-188] has been established. Analysis of FOS-ir and phospho-CREB expression focused primarily on cortical and limbic regions involved in the modulation of stress, cognitive, and emotional responses such as the prefrontal and anterior cingulate cortices, the hippocampus, amygdala, thalamus, and hypothalamus. This data may contribute to the understanding of the mechanisms underlying gender-related vulnerability to stress and provide new insights into the cortical-limbic circuits involved in the modulation of the response to chronic aversive stimulation.

Materials and Methods

Animals

Adult male (n=12: 212-240 g) and cyclic female (n = 12: 195-212 g) Wistar rats were used in the present investigation. The animals were individually housed (cages 45 x 28 x 20 cm) with food and water available ad libitum and maintained on a 12/12-hr light/dark cycle. They were weighed (09:00) and handled daily for 5-8 min to minimize non-specific stress response. The experiments were carried out in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC), and with the guidelines of the Animal Bio-ethics Committee of the University of Groningen (FDC: 2509).

Chronic footshock paradigm

The rodent footshock-chamber consists of a box containing an animal space placed on a grid floor connected to a shock generator and scrambler. Test rats received one session of 30-60 min/day in the footshock box during which 5 inescapable footshocks were given (0.8 mA in intensity and 8 sec in duration) with different inter-shock intervals in order to make the procedure as unpredictable as possible. This procedure was followed for 20 days. The final day of the experiments (21st day) all rats were placed in the footshock box without being exposed to any painful electric shocks.

Non-stressed rats. Twelve rats (6 males and 6 cyclic females) were used as control animals. These animals were exposed to the same environmental stimuli as stressed rats. They were housed in the same room as stressed rats and regularly exposed to the footshock chamber, although they were never subjected to footshocks or other psychological stressors (such as vocalizations produced by stressed rats) during the entire duration of the experiment.

Physiological and neuroendocrine changes in response to chronic footshock stress

To define the dynamics of the response to chronic footshock stress, changes in physiological and neuroendocrine parameters were measured. Weight gain was monitored on a daily basis throughout the experiment, and upon termination, adrenal glands and thymus were removed and weighed. Graphs were constructed to serve as a reference to verify the severity of stress perceived by the animals during the experiment. In addition, blood samples were taken after the final session (no footshocks were given) and stored at -80°C, until determination of plasma corticosterone, noradrenaline, and adrenaline concentrations by HPLC.

Extraction and Chromatography

Adrenaline and Norepinephrine. Catecholamines were extracted from plasma using liquid/liquid extraction with 3,4-dihydroxybenzylamine as internal standard [189,190]. Briefly, plasma adrenaline and norepinephrine were bound to diphenylborate-ethanolamine at pH 8.6. The extraction was performed with n-heptane (containing 1% octanol and 25%

tetraoctylammoniumbromide). Finally, catecholamines were extracted from the organic phase with diluted acetic acid. Adrenaline and noradrenaline (20µl acetic acid extract) were analyzed using an HPLC/auto-injector (CMA, Sweden) and a Shimadzu LC-10AD pump (Kyoto, Japan) connected to a reversed phase column (Hypersil, C18, 3µm, 150x2.0mm), followed by an electrochemical detector (Antec Leyden, The Netherlands) working at a potential setting of 500mV vs. Ag/AgCl reference. The mobile phase consisted of 50mM acetate buffer, 150mg/l octane sulphonic acid, 150mg/l tetramethylammonium, 15mg/ml Na_2EDTA and 3% methanol, adjusted to pH 4.1. The flow-rate was 0.35ml/min. Temperature was 30°C. The detection limit of the method was 0.1nM.

Corticosterone. For this assay, dexamethason was used as internal standard. After addition of the internal standard, plasma was extracted with 3ml of diethylether, vortexed for 5 min and then centrifuged for 5 min at 3000 x *g*. The extraction procedure was repeated twice. The organic phase was evaporated to dryness in a 50°C waterbath. The residue was reconstituted with 200µL of mobile phase and 50µL was injected into the HPLC system. The mobile phase (flow rate 1.0mL/min) for the determination consisted of acetonitrile in ultrapure water (27:73 v/v). The concentration of both corticosterone and the internal standard was determined with UV detection at a wavelength of 254nm. The detection limit of the method was 10nM.

Immunohistochemistry

Two hours following the beginning of the final session, rats were sacrificed with an overdose of halotane which preceded blood sampling and transcardial perfusion with 4% paraformaldehyde solution in 0.1M sodium phosphate buffer (pH 7.4). The brains were carefully removed and post-fixed in the same solution overnight at 4°C, before being transferred to a potassium phosphate buffer (KPBS 0.02 M, pH 7.4) and stored at 4°C. Following cryoprotection of the brains by overnight immersion in a 30% glucose solution, coronal serial sections of 40µm were prepared on a cryostat microtome. Sections were collected in KPBS with sodiumazide and stored at 4°C.

FOS and phospho-CREB immunohistochemistry

The stainings was performed on free-floating sections under continuous agitation. The sections were preincubated in 0.3% H_2O_2 for 15 min to reduce endogenous peroxidase activity, before being incubated in a primary polyclonal rabbit anti-FOS antibody (Oncogene Research Products, brands of CN Biosciences, Inc, an affiliate of Merck KGaA, Darmstadt, Germany; 1:10000 dilution in KPBS 0.02 M, pH 7.4) or anti-phospho-CREB (Upstate Biotechnology, Charlottesville, VA, USA: www.upstatebiotech.com; 1:1000 dilution in KPBS 0.02 M, pH 7.4). Subsequently, sections were washed with KPBS and incubated at room temperature with biotinylated goat anti-rabbit IgG (Vector Laboratories, Inc., Burlingame, CA, USA; 1:1000 dilution in KPBS 0.02 M, pH 7.4) followed by ABC complex (Vector ABC kit, Vector Laboratories, Burlingame, CA, USA). After another wash, the reaction product

was visualized by adding diaminobenzidine as chromogen and 1% H_2O_2 for 15 min. Finally, sections were washed, mounted on slides, dehydrated and coverslipped with DePex.

Antibody specificity testing. To control for cross-reactivity due to aspecific binding, immunostainings were performed by incubating several sections without the presence of one of the antibodies needed for the reaction (primary, secondary or tertiary antibody), thereby confirming the specificity of all antibodies used. All these reactions were negative confirming the specificity of the antibodies.

Quantification and data analysis

FOS and phospho-CREB positive nuclei were quantified using a computerized imaging analysis system. The quantification was performed by an observer who was blind to group assignment. For counting of the immunoreactive cell nuclei, at least 4-5 sections per each brain area were analyzed. The selected area from regions of interest (ROI) were digitized by using a Sony (SONY Corporation, Tokyo, Japan) charge-coupled device digital camera mounted on a LEICA Leitz DMRB microscope (LEICA, Wetzlar, Germany) at x100 magnification. ROIs included the medial orbitofrontal cortex (mORB: Bregma +4.85 to +3.60), the prefrontal cortex (prelimbic (PrL) and infralimbic (InfraL) area; mPFC: Bregma +3.60 to +1.70), the anterior (AC: Bregma +3.20 to +0.95) and posterior cingulate cortex (postCING: Bregma +1.70 to -1.08); the dentate gyrus (DG: Bregma -2.00 to –3.90); the central (CeA: Bregma –1.53 to –2.85), the lateral (LaA: Bregma –2.00 to -3.70), and the basolateral nucleus of the amygdala (BslA: Bregma –1.78 to –3.25); the paraventricular thalamic (PVT: Bregma –1.33 to –3.90) and hypothalamic nucleus (PVN: Bregma –1.08 to –2.00), the median raphe nucleus (MR: Bregma –9.25 to –10.35) [191]. ROIs were outlined with a digital pen and their areas were measured. Each digitized image was individually set at a threshold to subtract the background optical density, and the numbers of cell nuclei above the background were counted by using the computer-based image analysis system LEICA (LEICA Imaging System Ltd., Cambridge, England). FOS and phospho-CREB positive immunoreactivity was reported as number of positive cells / 0.1mm^2. Although all areas were measured bilaterally, no left-right asymmetry in FOS or phospho-CREB expression was found.

Relative regional cortical-limbic FOS-ir

This analysis allows one to consider individual cortical-limbic structures as parts of a larger, more complex system. In order to perform this calculation, we determined the average regional surface (ARS) of all the regions of interest. The c-fos positive cell densities of each cortical-limbic region was then multiplied by the average regional surface for all animals (regional cell density $_{\text{rat n}}$ * ARS). This was done in order to correct for eventual differences in quantified areas between different rats, thereby providing c-fos positive cell numbers across a similar cortical-limbic quantified surface area in all rats, suitable for comparison.For a detailed description of *relative FOS-ir analysis*, the reader is referred to Chapter 1, Materials and Methods section [192].

Statistics

One-Way-Anova and *F* test of variance were run on numbers of FOS and phospho-CREB immunoreactive cell nuclei from individual brain ROIs from experimental and control conditions. To compare cell counts from individual ROIs, *t* tests for equal or unequal variance were performed. $P<0.05$ was defined as the level of significance between groups.

Results

Physiological and neuroendocrine changes in response to prolonged stress

To define the dynamic of the response to prolonged footshock exposure, physiological and neuroendocrine changes, including body weight gain, adrenal and thymus weight, plasma adrenaline, noradrenaline, and corticosterone levels, were analyzed.

Body weight gain

Body weights were measured daily throughout the experiment in non-stressed and stressed rats (fig. 1). A consistent reduction in body weight gain was observed in chronically stressed males ($F=9.21$, $p<0.013$), while weight gain in control animals continued constantly as expected. No differences were detected between control and stressed females.

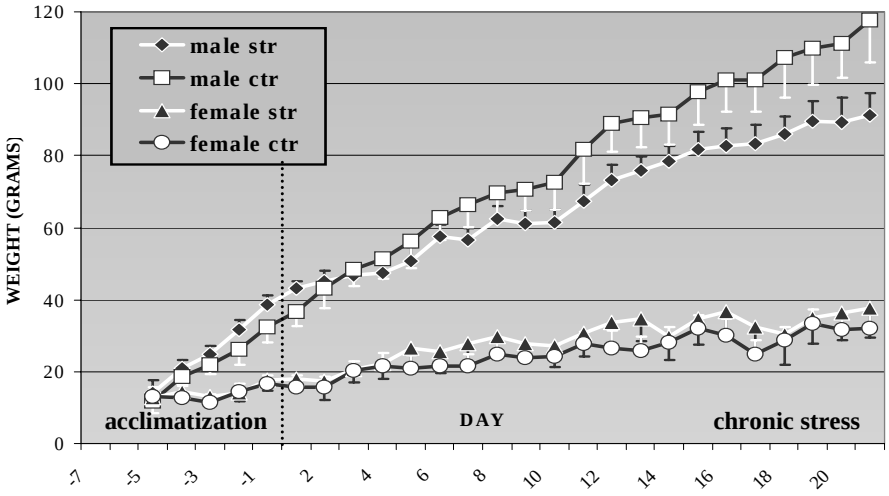

Figure 1. Body weight gain following prolonged stress exposure.

Plasma corticosterone, adrenaline and noradrenaline concentrations

Blood samples were collected by transcardial injection upon termination and corticosterone, adrenaline, and noradrenaline concentrations were subsequently measured by HPLC. Although stressed rats were not exposed to footshocks during the final session, they showed significantly higher plasma catecholamines and corticosterone levels (fig. 2a). Plasma adrenaline ($F=7.34$, $p<0.024$) and corticosterone ($F=5.96$, $p<0.036$) concentrations were significantly higher in chronically stressed males, while increased noradrenaline ($F=6.32$, $p<0.036$) and corticosterone levels ($F=8.1$, $p<0.022$) were observed in stressed females. Chronic stress also induced thymus hypotrophy in females ($F=5.1$, $p<0.048$) and adrenal hypertrophy in both male ($F=26.41$; $p<<0.001$) and female rats ($F=5.94$, $p<0.035$) (fig. 2b). These results suggest the lack of habituation in the response of the HPA axis to repeated

stress but also support the view concerning a persistent hyperactivity of this stress response system following prolonged footshock exposure.

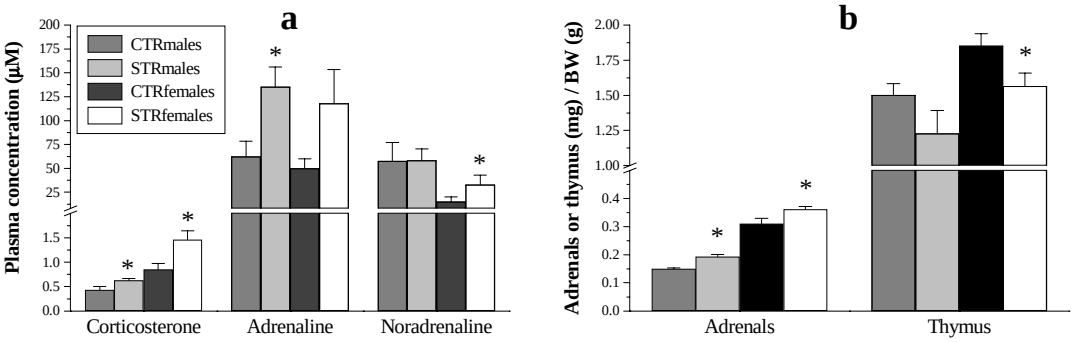

Figure 2. Plasma catecholamine and corticosterone concentrations in chronically stressed male and female rats (a). Prolonged stress exposure also caused a significant adrenal hypertrophy in both genders (b).

Immunohistochemistry

Absolute FOS-ir

In the present study, FOS-ir was quantified throughout various forebrain structures, including the frontal cortex, the hippocampus, the amygdala, the thalamus, and the hypothalamus (fig. 3).

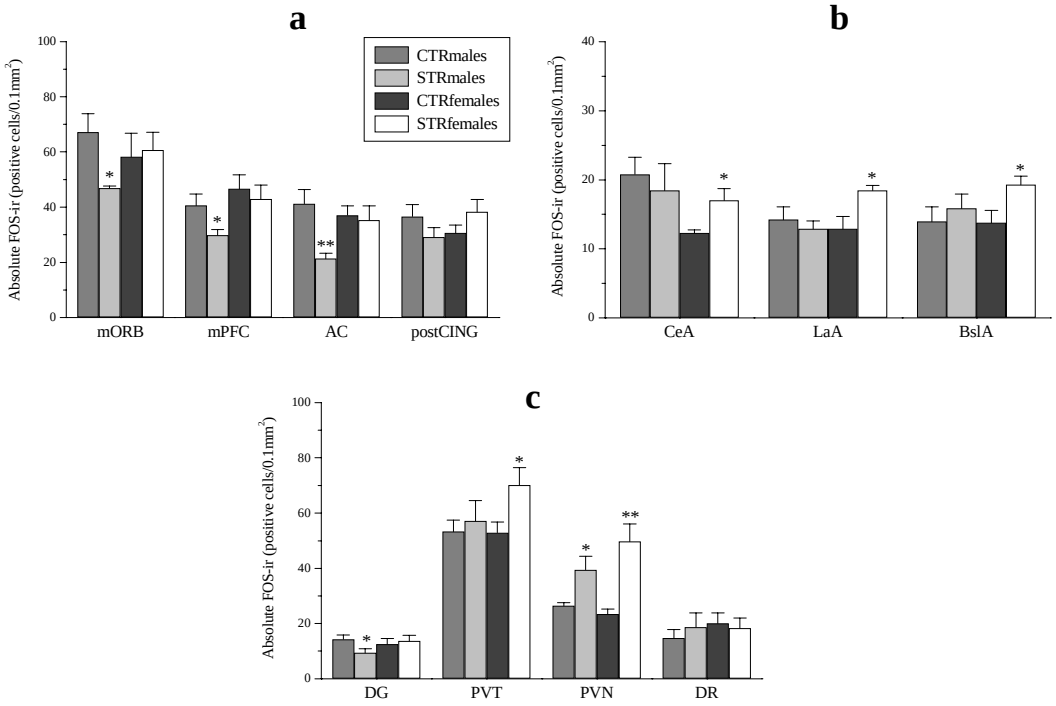

Figure 3. Effect of chronic stress on absolute FOS-ir in: a) medial prefrontal cortex; b) amygdala; c) hippocampal, thalamic and hypothalamic areas.

Chronic footshock stress resulted, in male rats, in a significantly decreased absolute FOS-ir in the mPFC (F=5.17, p<0.046), the mORB (F=5.36, p<0.043), the AC (F=12.92, p<0.0049) (fig. 3a), and the DG (F=5.17, p<0.046) (fig. 3c). Only the PVN showed an opposite tendency, showing a significant induction of FOS-ir (F=6.58, p<0.028) (fig. 3c). Chronically stressed females, in contrast, reported a selective increased FOS-ir in the CeA (F=7.1, p<0.024), the LaA (F=8.25, p<0.017), and the BslA (F=6.2, p<0.032) (fig. 3b). No changes were instead detected in any prefrontocortical region examined (fig. 3a). Likewise males, chronic footshock exposure resulted, in females, in a significantly increased FOS-ir in PVN (F=15.78, p<0.0026) (fig. 3c).

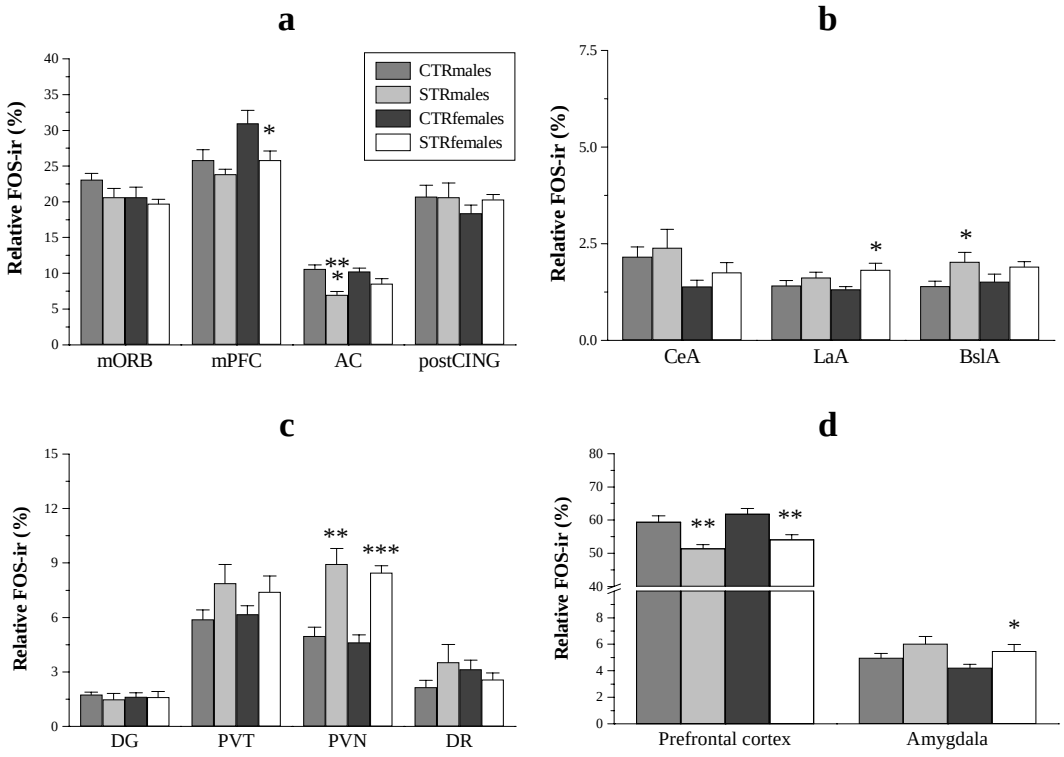

Figure 4. Effect of prolonged stress exposure on relative FOS-ir in: a) medial prefrontal cortex; b) amygdala; c) hippocampal, thalamic and hypothalamic areas; d) prefrontal vs amygdala relative activity.

Relative FOS-ir

Chronic footshock stress was associated, in males, with increased relative FOS-ir in the BslA (F=5.36, p<0.046) (fig. 4b) and PVN (F=16.38, p<0.002) (fig. 4c), while a decreased relative regional activity was detected in the AC (F=25.82, p<<0.001) (fig. 4a). Chronically stressed females illustrated a selective increase of relative FOS-ir in the LaA (F=6.92, p<0.025) (fig. 4b) and PVN (F=8.25, p<0.017) (fig. 4c). In contrast, a significantly decreased relative regional FOS-ir was found in the mPFC (F=5.23, p<0.045) (fig. 4a).

Phospho-CREB immunohistochemistry

In male rats, repeated footshock exposure caused a general reduction of phospho-CREB immunoreactivity in both cortical and subcortical regions (fig. 5). Decreased phospho-CREB immunoreactivity was detected in the mORB ($F=36.06$, $p<<0.001$), the PrL ($F=16.59$, $p<0.002$), the InfraL ($F=38.18$, $p<<0.001$) (fig. 5a), the AC ($F=10.59$, $p<0.009$), the postCING ($F=6.84$, $p<0.026$) (fig. 5b), the hippocampal DG ($F=11.99$, $p<0.006$), the LaA ($F=24.68$, $p<<0.001$), and the BslA ($F=42.32$, $p<<0.001$) (fig. 5c). Surprisingly, no changes in CREB phosphorylation were detected in cyclic female rats with the only exception of the hippocampal dentate gyrus where a marked, although not significant, reduction was observed ($F=4.38$, $p<0.081$) (fig. 5c).

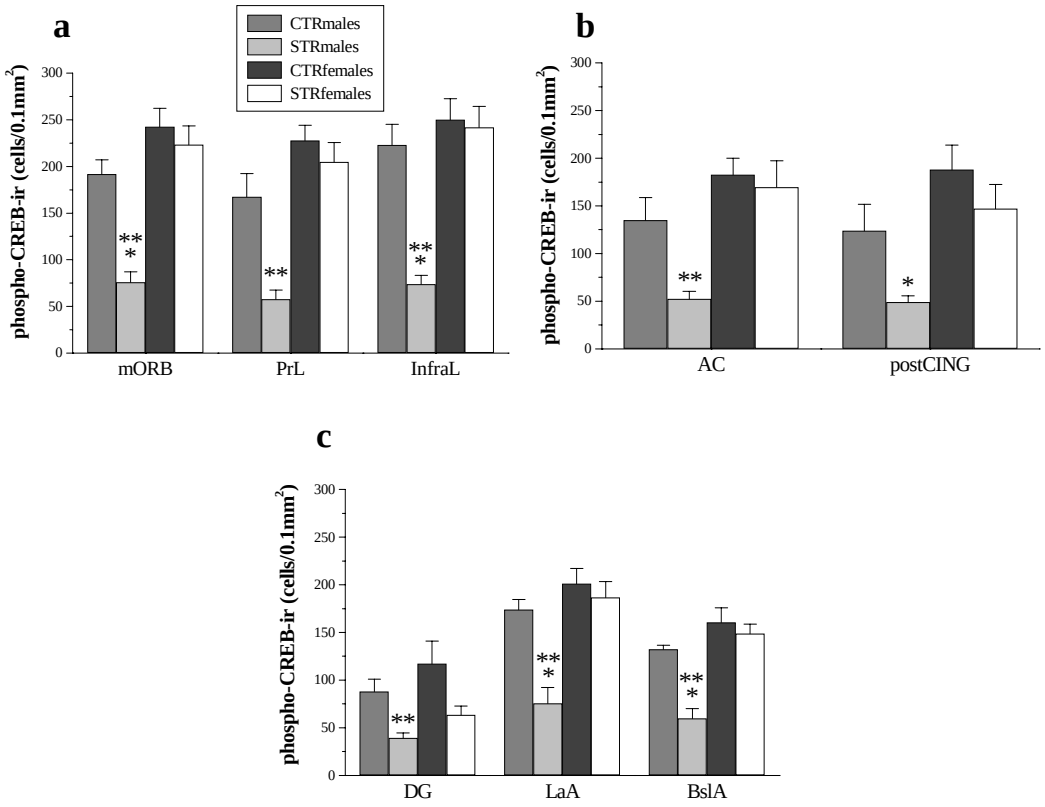

Figure 5. Effect of chronic stress on phospho-CREB immunoreactivity in: a) medial prefrontal cortex; b) cingulate cortex; c) hippocampus and amygdala.

Discussion

Chronic stress has been associated with functional and structural neuronal dysfunctions[139,142,193-195]. In this study, we explored at a cellular level, the alterations of cortical-limbic activity and neuronal plasticity in response to prolonged footshock stress in male and female rats. A significant reduction in body weight gain (fig. 1), elevation of plasma adrenaline and corticosterone levels (fig. 2a), adrenal hypertrophy (fig. 2b), and increased absolute and relative FOS-ir in the PVN (fig. 3c, 4c) were detected in chronically stressed males. Similarly, chronically stressed females reported higher plasma noradrenaline and corticosterone concentrations (fig. 2a), significantly reduced thymus weight, adrenal hypertrophy (fig. 2b), and enhanced absolute and relative PVN FOS-ir (fig. 3c, 4c). Since the PVN plays a key role in HPA axis regulation, the increased activity observed in this nucleus, combined with the increased corticosterone concentrations and adrenal hypertrophy, suggests a prolonged HPA axis hyperactivity in both sexes. It is interesting to note that both stressed male and female rats exhibit similar neuroendocrine responses (fig. 2a,b). However, despite these similarities in the response to stress, differential, gender-related patterns of absolute FOS-ir and phospho-CREB expression were detected (fig. 3, 4, 5). Chronic footshock exposure selectively affected prefrontocortical and hippocampal regions in male rats, causing a significant reduction of absolute regional FOS-ir in the medial orbitofrontal cortex, the medial prefrontal cortex, the anterior cingulate cortex (fig. 3a), and the dentate gyrus (fig. 3c). Interestingly absolute FOS-ir in the amygdala remained unaffected (fig. 3b). A marked reduction of CREB phosphorylation in various cortical and subcortical structures was also observed (fig. 5). In contrast to males, chronically stressed females showed significantly increased absolute FOS-ir in the central, lateral, and basolateral nuclei of the amygdala (fig. 3b). No relevant changes in the level of neuronal activity were observed in cortical regions (fig. 3a) and, surprisingly, only a slightly decreased phospho-CREB immunoreactivity was detected (fig. 5).

The circuitry underlying neuroendocrine regulation has been characterized in both humans and rodents and involves coordinated interactions amongst frontocortical areas (orbital, medial prefrontal and anterior cingulate cortices), the hippocampus, the amygdala, the hypothalamus, and several brainstem nuclei [196,197]. The prefrontal cortex and the hippocampus, in particular, modulate the activity of the HPA axis by maintaining this system under functional inhibition [198,199]. Lesions in prefrontocortical and hippocampal areas result, at least in male rats, in pronounced activation of both HPA axis and sympathetic nervous system [200]. On the contrary, the amygdala stimulates HPA axis activity, either directly through its connections to the PVN and/or indirectly by modulating the activity of various noradrenergic brainstem nuclei [199]. The amygdala also participates in the modulation of acute stress and fear conditioned

responses[177,201,202,203]. Thus, while acute fear is characterized by increased amygdala activity and reduced prefrontal activation [203], the termination of this very same response has been associated with increased frontocortical activity and reduced amygdala activation [204]. Exposure to acute threatening stimuli strongly stimulates the amygdala [203] promoting its activation that prevails over the functional inhibition mediated by cortical and limbic structures, such as the medial prefrontal and the anterior cingulate cortex. As a result, activation of the amygdala may ultimately lead to functional inhibition of prefrontocortical areas [203]. The response profile of the amygdala however, habituates from early to late stages of the aversive response [201]. This structure has been found most consistently activated in the early phases of acute stress or conditioned-fear acquisition and this activation was then found to progressively decrease [205]. It is intriguing to hypothesize that the gradual reduction of amygdala activity may "free" the frontal cortex from the functional inhibition mediated by this limbic structure. In addition, during the final phase of the aversive response, the level of prefrontocortical activation slowly increases, perhaps overcoming amygdala-mediated inhibition and leading in turn to the extinction of the aversive response [204]. In the absence of new adverse stimuli, the prefrontocortical activation remains elevated thereby maintaining the amygdala under inhibition and avoiding the onset of a new "stress" response until the animals are faced with a novel potential threat. Frontocortical deficits have also been shown to release the amygdala from this inhibition [206], resulting possibly in a slower extinction of stress response. It is possible that the reduced frontocortical and hippocampal activation seen in males (reduced absolute FOS-ir) (fig. 3a,b), combined with a general reduction of neuronal plasticity (reduced phospho-CREB immunoreactivity) (fig. 5), may compromise the coordination between frontal and/or subcortical structures, thereby impairing the appropriate regulation of the neuroendocrine response to stress. The probability of an abnormal response to adverse stimuli (slower termination) seems also supported by the prolonged HPA axis hyperactivity, as confirmed by both neuroendocrine (fig. 2) and immunohistochemical findings (fig. 3c, 4c).

This hypothetical framework however, does not explain why the lack of change in the number of FOS-labeled neurons observed in the male amygdala (fig. 3b), since a significant activation would be expected in this limbic structure following stress. However, absolute FOS-ir provides only a general indication of the regional response to specific stimuli since it does not consider, for instance, the individual differences in basal expression of this immediate early gene amongst animals. Therefore, while absolute FOS-ir analysis suggested that long-term footshock stress in males did not engage the amygdala (fig. 3b), relative FOS-ir analysis displayed a different scenario (fig. 4b). Relative analysis revealed a significant chronic stress induced activation of both the BslA and the PVN (fig. 4b,c). In accordance with absolute FOS-ir analysis, a significant reduction of relative FOS-ir was also observed in the mPFC and DG (fig. 4a,c). An

interesting possibility, supported by clinical and preclinical evidence, is the ability of chronic stress to permanently elevate glucocorticoids levels by impairing stress response regulation [162,207-210]. The BslA has been involved in many aspects of stress and conditioned-fear responses [211-214], and a permanent hyperactivity of this nucleus may facilitate, directly and indirectly, HPA axis activation by maintaining the prefrontal cortex under enduring inhibition. Ultimately, amygdala hyperactivity and prefrontocortical hypofunction may act synergistically to promote HPA axis hyperactivity and further elevate glucocorticoid levels. Corticosteroids, in turn, promote stress-induced hippocampal structural impairments [215,216], possibly through inhibitory effects on CREB phosphorylation [143,217]. The combination of these effects may explain the reduced hippocampal absolute FOS-ir and phospho-CREB expression observed in the DG and CA3 (fig. 3c, 5c), important regions for feedback inhibition to the HPA axis[209,218,219]. This complex series of events thus provides a hypothetical pathway by which chronic footshock exposure may lead to HPA axis hyperactivity, elevated glucocorticoid levels, and selective impairment in the circuitry underlying stress response regulation, promoting further glucocorticoid secretion and leading, eventually, to neuronal defects.

Contrary to males, different chronic stress-induced patterns of absolute cortical-limbic FOS-ir (fig. 3) and phospho-CREB expression (fig. 5) were observed in female rats. Stressed females showed a selective increase of absolute FOS-ir in the amygdala (fig. 3b) and PVN (fig. 3c), and display only a slight reduction phospho-CREB immunoreactivity (fig. 5). In the past few years, a growing amount of literature has reported both functional [220-225] and morphological differences [226-232] between the male and the female brain. These gender-related differences might offer important insights into the understanding of the dimorphic patterns of frontocortical-amygdalar FOS-ir and phospho-CREB expression. Sustained stress has been associated with cortical-limbic abnormalities and emotional dysregulation [233-235]. The amygdala, in particular, plays a key role in the modulation of stress response and the pathophysiology of affective disorders [161,169,236]. Whereas such disorders are often associated with marked gender differences [154,155], clinical studies have also revealed sex-related dimorphisms in the involvement of the amygdala during emotional processing [221,223,224,237]. A significant enlargement of this subcortical structure has also been reported in depressed subjects [236]. It is intriguing to speculate that chronic aversive conditions selectively affect the amygdala in females, leading to its abnormal activation (fig. 3b), which in turn may alter the coordinated interactions between cortical and subcortical structures. It is also possible that chronic footshock stress, in addition to the amygdala, targets prefrontocortical regions in females as well, without however causing the state of functional deactivation observed in males, but disrupting the orchestrated coordination between cortical and subcortical regions. Relative FOS-ir analysis seems to facilitate the

interpretation of these results (fig 4). Although marked gender-related differences were seen in the distribution of absolute FOS-ir in response to prolonged stress, the patterns of relative FOS-ir were surprisingly similar in both sexes (fig. 4). Chronic stress resulted in amygdala hyperactivity ($BslA_{males}$ vs. $LaA_{females}$) (fig. 4b) and frontocortical hypoactivity (AC_{males} vs. $mPFC_{females}$) (fig. 4a) in both males and females. Chronic stress might thus impair the ability of frontocortical regions to exert an adequate inhibition on the amygdala, resulting in its functional hyperactivity that causes malfunctions in the modulation of the stress response as well as the impairment of cognitive and emotional regulation. A troubling aspect of depression is represented by the prolonged processing of negative emotions [169]. Intriguingly, the amygdala has been shown to play a central integrative role in the elaboration of emotional stimuli and the retrieval of emotional memories [211,238-240]. One however, may question if the hyperactivity observed in the amygdala is related to the persistent recollection of intrusive emotional memories. An important indication to answer this question may be provided by the analysis of phospho-CREB immunoreactivity. CREB phosphorylation has been reported to be crucial in the modulation of neuronal plasticity and the consolidation of new fear-related memories [181,241]. The phosphorylation of this transcription factor appears to be fundamental for the stability of new and reactivated fear memories [241]. Interestingly, chronically stressed females did not show the general and significant decrease of phospho-CREB expression observed in male rats (fig. 5). We can thus speculate that the availability of phosphorylated CREB in the amygdala may support the prolonged processing of negative emotions leading, in females, to a condition of functional hyperactivity, as documented by the significantly increased absolute and relative FOS-ir (fig. 4b).

The molecular mechanisms underlying this differential effect of chronic footshock stress on phospho-CREB expression remain obscure although an intriguing candidate to explain these differences might be represented by ovarian hormones. As illustrated in chapter 1, chronic footshock stress may cause cortical-limbic abnormalities by impairing the activity of intracellular elements involved in the transduction of neurotrophic signals. By influencing CREB expression and/or phosphorylation, prolonged stress/glucocorticoid exposure may affect neurotrophin availability, reducing neuronal plasticity and increasing the vulnerability of neurons to subsequent insults. Similarly to the previous study, chronically stressed males showed, a marked reduction of phospho-CREB immunoreactivity (fig. 5). Following chronic footshock stress however, no changes of CREB phosphorylation were observed in females (fig. 5). Estrogen and neurotrophins activate similar signaling transduction pathways that culminate with CREB phosphorylation [242,243]. Ovarian hormones also protect neurons from the effects of oxidative stress [244]. It is thus intriguing to hypothesize that ovarian hormones may protect the female brain from the deleterious influence of glucocorticoids on neuronal

plasticity by preventing stress-induced reduction of phospho-CREB immunoreactivity. On the contrary however, ovarian hormone-induced trophic actions may also provide critical substrates (such as phospho-CREB and phospho-ERK1/2) that promote the formation, consolidation and ultimately the recollection of intrusive traumatic memories.

Conclusions

A wide variety of sexual dimorphisms, both structural and functional, between sexes have been described in the brains of many vertebrate species, including humans. In the first part of this chapter, we explored the neurochemical changes induced by prolonged footshock stress in male and cyclic female rats. Remarkably, marked sex differences emerged in the patterns of FOS-ir and ERK1/2 phosphorylation in response to repeated stress. Sex hormones may account for some of the gender discrepancies since cyclic changes in levels of circulating estrogen and progesterone have been established to play a central role in the differences observed in stress-sensitivity and psychopathology between men and women. The neurocircuits underlying cognitive and emotional processes are also prime targets for ovarian hormone action and stress. By influencing the hormonal state of the animals, stress may play a central role in determining gender-related dimorphisms. In a parallel study specifically performed to assess the overall effects of ovarian steroids in central stress integration, immunohistochemical changes associated with sustained stress exposure were analyzed in both cyclic and ovariectomized female rats [245]. Surprisingly, no differences absolute FOS-ir patterns were found between cyclic and ovariectomized females following long-term aversive stimulation in any of the cortical and subcortical regions examined. Therefore, although important, the presence or cyclic fluctuations of sex hormones does not account for all the differences observed between male and female rats.

It is likely however, that the action of ovarian hormones during the early developmental phases along with other factors might also be critical in influencing the structure of the CNS. Morphological sex differences in brain areas underlie sex differences in function, and it has become increasingly clear that male and female brains are two different and separate entities both from a functional and a morphological perspective. Remarkably, while substantial and compelling evidence exists for gender-related differences in brain structure and function, our understanding of the molecular and cellular mechanisms that give rise to these dimorphisms remain poorly explored. Ovarian steroids may prevent the abnormal ERK1/2 phosphorylation that was detected in chronically stressed males. Estrogen and neurotrophins activate similar signaling transduction pathways, including the ERK1/2 cascade [242,243], and protect neurons from the effects of stress by modulating the activity of this pathway [244]. It is thus intriguing to speculate that estrogen and/or progesterone may protect the female brain against some of the consequences of chronic stress by preventing stress-induced alterations of ERK1/2 activity. Sex steroids may substitute the function of neurotrophins reduced by chronic stress, in sustaining the plastic changes needed by the brain when faced with prolonged aversive conditions. The immunohistochemical changes presented here in male and female rats might represent neurochemical evidence for a differential role of stress on

cognitive processing. More importantly, the functional dimorphism within the cortical-limbic network observed following long-term aversive stimulation might illustrate gender-related differences in the sensitivity to the deleterious effects of stress on these neurocircuits. It seems plausible that the reduction of cortical-limbic absolute FOS-ir following acute stress as well as the presence of ovarian hormones may protect female rats from the harmful consequences of chronic footshock stress, thereby preventing both structural (phospho-ERK1/2 hyperphosphorylation) and functional (neuronal hyperactivity) prefrontocortical abnormalities. This view however is in contrast with a growing body of literature, both clinical and preclinical, that supports the notion of higher stress sensitivity in female under both acute and chronic conditions.

In the second part, we have presented additional evidence in support of the destabilizing effects of chronic stress on the coordinated regulation of cortical-limbic activity and neuronal plasticity. Chronic footshock exposure resulted in a significant reduction of absolute prefrontocortical FOS-ir in males while promoting amygdala hyperactivity in females. Moreover, a general reduction of cortical-limbic phospho-CREB immunoreactivity was observed in chronically stressed male rats, while no changes were found in females. The prefrontal cortex and the amygdala may thus represent primary targets of the dimorphic and detrimental influences of stress. It is possible that chronic footshock stress may induce differential gender-related immunohistochemical changes, due to the divergent and sex-dependent role that cortical and subcortical structures plays during cognitive and emotional assessment. Consequently, the negative impact of prolonged stress exposure may differ depending on the gender of the organism. However, due to the profound interconnections amongst cortical and limbic structures necessary to guarantee a coordinate brain functioning and assure the proper regulation of stress responses, the destabilizing action of stress may lead to similar abnormalities, independently with where the defect primarily appears. In male rats, stress-induced prefrontocortical impairment may release the amygdala and the HPA axis from their functional inhibition, generating an auto-sustaining positive feedback loop in which glucocorticoids stimulate their own release and lead to hippocampal and frontocortical dysfunctions. Female rats, in contrast, possibly due to the protective action of estrogen, did not exhibit the same reduction of neuronal plasticity seen in males. Thus, our data does not support the traditional view of a higher female susceptibility to the effects of stress. In fact, although female rats displayed a higher sensitivity to acute stress, they demonstrated a lower susceptibility to the detrimental influence of prolonged footshock exposure on neuronal plasticity. Nevertheless, we cannot draw definite conclusions with regard to these findings, as it cannot be excluded that the data presented here is the result of the specific markers that were used in this investigation. It remains plausible however, that chronic stress exposure may affect female brain integrity through different molecular and cellular pathways than those found in males.

It is of interest to note that although prolonged stress exposure caused similar neuroendocrine alterations in male and female rats, different changes in FOS-ir were detected in the two investigations presented in this chapter. These discrepancies were particular evident in frontocortical regions of chronically stressed males. Thus, while in the first section ("Immunohistochemical changes induced by repeated footshock stress: revelation of gender-based differences") an increased absolute FOS-ir was observed in prefrontocortical regions in animals exposed to chronic stress, a reduced FOS-ir was instead detected in the second investigation ("Amygdala-prefrontocortical involvement in response to chronic footshock stress: a gender comparative view"). The reason for this discrepancy is unknown, although the two studies markedly differ with regard to the duration of daily aversive sessions, making the footshock procedure in the second investigation (daily sessions ranging from 30 to 60 minutes) more stressful than the one used in the first part of the chapter (daily sessions ranging from 15 to 30 minutes). Besides the previously mentioned differences in absolute FOS-ir patterns, chronically stressed males in both investigations illustrated similar neuroendocrine alterations (adrenal hypertrophy and PVN hyperactivity) which might indicate abnormal HPA axis activity and reduced neuronal plasticity.

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4

Chronic stress and long-term antidepressant administration: neuroendocrine and immunohistochemical changes in the female rat brain

"The greatest lesson in life is to know that
even fools are right sometimes"
Sir Winston Churchill

Antidepressant actions in the female brain

Whereas the previous two chapters provided experimental evidence to substantiate the negative consequences of stressful events on neuronal functioning in male and female rats, this section will focus on the neurochemical adaptations induced by long-term antidepressant treatments in cyclic female rats and their ability to correct stress-induced neuronal abnormalities. Three different classes of antidepressants were tested here including a serotonin reuptake enhancer (tianeptine), a selective serotonin reuptake inhibitor (citalopram), and a selective norepinephrine reuptake inhibitor (reboxetine). These compounds were chosen in consideration of their efficacy in the treatment of stress-related neuropsychiatric disorders, such as panic, anxiety, post-traumatic stress disorder and, more importantly, depression. The decision to combine prolonged stress exposure with simultaneous antidepressant administration stems from the fact that depressed subjects have a long history of stress both before and during pharmacotherapy while experimental animals used to test antidepressants are rarely exposed to concomitant stressful conditions. Clinical evidence suggests that adverse events do not only contribute to the development and/or maintenance of psychopathology in humans [1-3], but also seem to affect the 'therapeutic power" of antidepressants [4]. This investigation was limited to female rats with respect to the fact that most stress-related psychiatric illnesses are characterized by marked gender-related prevalence [5]. Although animal models have provided valuable information regarding possible mechanisms underlying the pathophysiology of these complex diseases [6,7] as well as antidepressants' modes of action [8,9], important discrepancies still exist between experimental models and human psychopathology. For instance, although women have a higher susceptibility to mood disorders, constituting the majority of patients receiving antidepressant treatment, most of the preclinical research has been performed in male animals [10]. In the present chapter, we thus explore the neurochemical changes induced by prolonged footshock exposure and/or concomitant long-term antidepressant treatments, in an attempt to answer a crucial question: are long-term antidepressant treatments similarly effective in preventing/reversing the neurohistochemical changes induced by chronic stress?

Stress and depression: is there a connection?

Depression is a heterogeneous disorder in which different etiological causes, including environmental factors (stressful life events) [3,11] and genetic factors ("vulnerability" or "predisposition") [12], interact in multiple and complex manners [13]. Clinical studies have confirmed the importance of adverse experiences in the development of psychopathology and a strong correlation between stressful life events (SLEs) and the precipitation of depression has been demonstrated, especially in women [3,14,15]. Kendler

and colleagues have speculated that the association between SLEs and major depression accounts for approximately 75% while genetic risk factors account for the remaining 25%[3]. Although it is difficult to attribute a numeric value to the role played by adverse experiences in the development of depression, the significance of this study is owed to the notion that environmental factors may overrule genetic influences and lead to depression independently of genetic vulnerability or predisposition [3,11].

The brain responds to aversive stimuli in a complex yet orchestrated manner. The loss of organization often seen in this response may play a crucial role in the occurrence of neuronal dysfunctions. Preclinical studies have suggested that stress exerts its deleterious influence on the brain by promoting long-term changes in multiple neurotransmitter systems and this action may increase the vulnerability to the development of psychiatric illnesses [16-18]. A leading hypothesis proposes that the impact of stressful events is greater in the initial stage than in the subsequent episode of major depression; the strength of the relationship progressively declines as the number of previous depressive episodes increases (a theory known as the "kindling hypothesis")[19,20,21]. SLEs have been found to be strongly associated with subsequent episodes of depression [3]. The depressogenic effects of adverse experiences seem thus to be concentrated in the period immediately subsequent to the occurrence of such events. Although environmental factors also play a key role in depression, several other investigations have underlined the importance of genetic influences. Two theoretical models that might explain the relationships between these two main risk factors are known as "additive" and "genetic control of sensitivity to the environment model". In the additive model, the increased risk associated with exposure to adverse conditions is similar for individuals with low-risk and high-risk genotypes. This model predicts that the depressogenic impact of SLEs and genetic factors is independent. In contrast, the second model proposes a greater risk of developing depression associated with SLE exposure for those with a high-risk genotype. Genes do influence the risk of depression by altering the individual's sensitivity to the depressogenic effect of SLEs [14]. Clinical evidence seems to support the latter model [15].

Another important aspect in the association between adverse experiences and depression regards the impact of multiple SLEs [22]. To explain this relationship, three plausible theoretical frameworks have been proposed. The most simple or additive model suggests that the impact of SLEs is independent of the occurrence of other events. Multiple SLEs might positively interact and their depressogenic effect increases when it co-occurs with others. The positively interactive model proposes a reservoir of "coping ability" that might withstand the impact of one event but could be overwhelmed by multiple episodes. The negatively interactive model, instead, introduces a threshold for stress that, if exceeded, has no additional impact on depressive risk. Clinical evidence focused on severe SLEs, tends to best support the negatively interactive model. After one

severe SLE, little or no increased risk for depression was found given additional SLEs. Kendler and colleagues, who examined multiple SLEs occurring together in the same month, support the "positively interactive model": the impact of increasing numbers of SLEs on risk for a depressive onset is significantly greater than predicted by an additive model [22].

The monoamine hypothesis of depression

The monoamine hypothesis was coined over 30 years ago [23,24] and suggests an underlying biological basis for depression, namely a deficiency of the monoamine neurotransmitters norepinephrine, serotonin and/or dopamine in the central nervous system [25]. Since the stimulation of the monoaminergic system has been associated with clinical improvement, various classes of antidepressants that act by increasing monoamine levels within the synaptic cleft, either by inhibition of their degradation or by blockade of their reuptake, have been developed [26]. During the past decade, selective serotonin reuptake inhibitors (SSRIs) have become established as the treatment of choice for affective disorders [27]. However, newer antidepressants that selectively elevate norepinephrine levels or act simultaneously on different neurotransmitter systems have also proven to effectively alleviate depressive symptom [28-30].

Although substantial evidence exists to support a role of monoamine systems in the mechanism of action of antidepressants, intensive investigation has failed to find conclusive affirmation of a primary dysfunction in specific monoaminergic systems in subjects with major depressive disorders [31-33]. Moreover, there are several major issues that have not been addressed by the monoamine hypothesis. These problems concern mainly the mode of action of antidepressants acting on serotonergic and noradrenergic systems and include:

- **Efficacy**: in clinical trials, antidepressants, especially the newest generations of drugs including SSRIs, Norepinephrine Reuptake Inhibitors (NARIs), and selective Serotonin/Norepinephrine Reuptake Inhibitors (sSNRI), appear to be effective in approximately 60% of the subjects suffering from depression [34]. Although the first tricyclic agent (TCA) was introduced more than 30 years ago, the newest SSRIs or NARIs have failed to demonstrate an enhanced efficacy compared to these older antidepressants [35,36]. Newest antidepressants however, are better tolerated and do not show the serious cognitive, cardiac, and somatic side effects that characterize long-term TCA treatment [37-44].
- **Selectivity**: it is clear that SSRIs, NARIs, and dual SNRIs act through the stimulation of serotonergic and noradrenergic systems. There is still some confusion however, regarding the specific cellular or molecular targets underlying their therapeutic action, which include neurotransmitter transporters, specific receptors, intracellular proteins, enzymes, and transcription factors [45-48]. Various lines of evidence indicate

that selectivity of these agents dissipates following long-term administration. After several weeks, even highly selective drugs such as SSRIs or NARIs, affect the activity of numerous neurotransmitter systems and brain structures, some of which are not directly linked with the pharmacological profile of the antidepressant [49-51]. An intriguing possibility is thus that this limited selectivity following long-term treatment, rather than their high specificity observed in pharmacological essays, might represent the critical factor for their beneficial effects [52].

- **Mode of action**: an additional question to be addressed concerns the molecular substrates involved in the modulation of antidepressants' therapeutic effects. In contrast to those medications acting through the potentiation of monoaminergic transmission (TCAs, SSRIs, NARIs, and dual SNRIs), other effective antidepressants exert their pharmacological action by enhancing serotonin reuptake (tianeptine) [53,54] or modulating the activity of selective enzymes and/or transcription factors that are not directly linked to monoamine metabolism or signaling transduction pathways (such as lithium and valproate) [55,56].
- **Delayed therapeutic action**: while side-effects are manifested within hours or days, the beneficial effects of antidepressants are delayed and can take several weeks or even months to appear, causing considerable problems with patient compliance [57-59].
- **Monoamine depletion studies**: experimental monoamine depletion exacerbates depressive symptoms only in depressed subjects successfully treated with SSRIs or NARIs. Monoamine depletion failed to induce the same negative effects in medication-free symptomatic patients or healthy subjects. This implies that serotonergic and/or noradrenergic dysfunctions are unlikely to be the primary cause of depression although they may play a critical role in the mechanisms by which antidepressants act [32,33,60,61].

These findings suggests that while the potentiation of monoaminergic neurotransmission is fundamental for the modulation of antidepressants' action, only fragmentary evidence supports a primary role for monoamine deficiencies in depression. Depression is a heterogeneous disease in which numerous factors are involved. Furthermore, the complex nature of this disorder may favor its occurrence in a multiplicity of different forms. Monoaminergic deficiencies may represent just a feature of the "depressive syndrome" and characterize only a limited number of subtypes of depression. Another possibility is that monoamine deficiencies may constitute one of the multiple consequences associated with the course of the disorder. Monoaminergic systems are extensively distributed within the brain [62-64] and it is not surprising that clinical research, throughout the years, has identified, in depressed subjects, abnormalities in the noradrenergic, dopaminergic, and cholinergic system as well as an impaired HPA axis regulation [65-70]. The behavioral and physiological manifestations of this psychiatric illness are complex and undoubtedly mediated by multiple networks of interconnected

neurotransmitter pathways. The abnormal activity of one or more key components may alter the coordinated regulation of an entire system, generating a "domino-like effect" that ultimately disrupts its ability to react to incoming stimuli with appropriate responses. Depression may be better viewed as a complex set of varying symptoms rather then an homogenous disorder, since it exhibits heterogeneous pathology with several different etiological causes yet few common consequences, such as disrupted cortical-limbic function, responsible for most of the deficits associated with the illness (cognitive impairment and emotional dysregulation) [70,71]. Revelations in the understanding of this psychiatric disorder and its treatment call for a clear comprehension of the factors and mechanisms leading to the above-mentioned functional and morphological abnormalities in cortical and subcortical structures.

Molecular and cellular theory of depression: the STRESS-BDNF hypothesis

Although the association between adverse experiences, brain abnormalities, and the occurrence of depression appears to be consistent, much less is known about the neurobiological substrates underlying stress-induced cortical-limbic defects. Stress deeply affects neuronal functional and structural integrity, inducing alterations at the cellular and the molecular level. Molecular changes include modifications of gene expression, protein synthesis and phosphorylation, while cellular changes include dendritic remodeling and/or atrophy, reduced neurogenesis, and possibly neuronal death [72-74]. Advances in molecular techniques have enhanced our insights into the mechanisms underlying the deleterious influence of stress on brain functions as well as the relationships between intracellular abnormalities and psychopathology. In the past few years, a growing number of studies have begun to characterize stress and antidepressant action beyond neurotransmitter and receptor level. This work has demonstrated that multiple intracellular pathways are involved in the transduction and modulation of antidepressant effects [45,75]. Despite the complexity of the intracellular apparatus, growing evidence suggests that the final result of antidepressant action may involve the stimulation of a limited number of "common effectors". One such final mediator, which appears to be a common molecular target of several classes of antidepressants affecting both serotonergic and noradrenergic neurotransmitter systems, is CREB [45,76-78]. CREB regulates the transcription of specific genes, including those codifying for BDNF and TrkB receptor [79-81]. Stress may precipitate depression through its detrimental action on neuronal plasticity achieved by limiting BDNF synthesis and release. Interestingly, experimental data points in the direction of a chronic stress-induced inhibition of CREB phosphorylation and/or BDNF expression. This stress-mediated inhibition may thus provide a theoretical mechanism through which sustained

stress exposure may reduce neuronal plasticity and, ultimately, lead to selective cortical-limbic abnormalities.

Remodeling of synaptic contacts, growth and branching of dendrites are only a few examples of neuronal plasticity. This dynamic process is based on the ability of neuronal systems, brain structures, single neurons, synapses and receptors, to adapt to alterations in the internal and/or external environment by modifying specific structure and functions [82]. To support these dynamic changes, new neurons are also produced in the hippocampus. Neurogenesis has been reported in rats, tree shrews, macaques, and humans, demonstrating that adult-generated neurons are a common feature of the mammalian brain [83,84]. Neurogenesis and neuronal plasticity however, are affected by stress [85,86]. Prolonged stress disrupts dendrite growth and branching [17], causing atrophy[87] and, in severe cases, neuronal death [74,88]. Acute and chronic stress have been shown to suppress neurogenesis, especially in the adult brain [83]. It is important to note that these forms of neuronal plasticity are crucial for proper functioning of the brain and numerous psychiatric disorders are characterized by reduced hippocampal neurogenesis and neuronal atrophy [73,84,89-92]. Reduced hippocampal activity and volume have also been observed in depressed subjects [93,94]. Therefore, although affective disorders have traditionally been conceptualized as neurochemical disorders, there is now considerable literature demonstrating that these illnesses are also associated with significant reductions in regional central nervous system (CNS) volume and cell numbers. Structural changes observed in depression however do not appear to be limited to the hippocampus. Several recent postmortem studies have also documented prefrontocortical abnormalities [95-97], including reductions in the number and density of cortical neurons and glial cells [98]. In the prefrontal cortex, a histological study of area sg24 located in the subgenual prefrontal cortex found striking reductions in glial cell number in patients with familial major depression (24% reduction) and manic-depressive illness (41% reduction), as compared with healthy subjects [95]. Together, these findings provide convincing evidence that decreased regional CNS volume, due to reduction in cell numbers, dendritic atrophy, and/or inhibition of neurogenesis, may lead to cortical-limbic impairments that, ultimately, promote psychopathology.

Neurotrophins participate in a broad range of functions including synaptogenesis, growth, differentiation, and survival [99-101]. Neurotrophic factors such as BDNF have also been shown to enhance the length and complexity of dendritic trees in cortical neurons [102,103]. These crucial activities require the coordinated interactions between multiple mediators, including receptors (Trk receptors), enzymes (PI3K and ERK1/2) and transcription factors (CREB) [104-109]. Activation of Trk receptors following the binding of specific ligands triggers a complex sequence of intracellular events that begins with receptor autophosphorylation, is followed by the activation of several downstream signaling cascades, and culminates with the stimulation and/or inhibition

of the expression of selective genes [107,108,110]. New insights into the role of neurotrophin signaling pathways in the pathophysiology and treatment of depression have been provided by the large number of studies reporting alterations in the expression of one or more members of these cascades in depressed subjects before as well as after antidepressant treatment [76,78,111-116]. Neurotrophin expression as well as intracellular cascades involved in the transduction of trophic signals thus appear to represent common targets of antidepressant action, independent of their pharmacological profile:

- **Serotonin and/or norepinephrine re-uptake inhibitors**. cAMP-mediated regulation of gene transcription has been implicated in the activity of numerous antidepressants acting on serotonin and/or norepinephrine neurotransmitter systems [45]. It has been proposed that CREB might represent the main effector in the modulation of antidepressants' beneficial action [76,78,115]. Chronic SSRI/NARI administration increases CRE-mediated gene expression and CREB phosphorylation in a region- and drug-specific manner [117,118]. The most consistent effects have been observed in the amygdala, hippocampus, and prefrontal cortex [119]. More importantly, induction of CRE-mediated gene expression and CREB phosphorylation were not observed in response to acute pharmacological treatment, which is consistent with the time course of therapeutic action of these drugs [117]. Antidepressant-induced CREB phosphorylation has been reported, as mentioned above, in selective subcortical regions, such as the amygdala [119], and numerous reports have confirmed the importance of this structure in the modulation of some of the behavioral actions of antidepressants [119]. The amygdala also modulates fear-related responses and conditioned avoidance behaviors [120,121]. The possibility that changes in CREB expression and/or phosphorylation may influence the function of this subcortical area is also supported by recent observations, which illustrate that overexpression of CREB in the amygdala alters fear-related memory formation [122,123]. It is also plausible that chronic stress-induced neurochemical changes in the amygdala could promote abnormal cognitive and emotional processing, often observed in depressed subjects, and that long-term antidepressant treatments may correct these alterations [117,119].

  In addition to CREB, another downstream target of both SSRIs and NARIs is BDNF. BDNF expression has been found reduced following stress [124-126] and in depression[114]. Relevance of this neurotrophin in the regulation of neuronal functions has led to the hypothesis that its reduced availability may constitute a critical predisposing factor for the development of neuronal defects and, ultimately, psychopathology [127]. This view is also strongly supported by the antidepressant-like effects of BDNF [128,129] and by the ability of long-term antidepressant treatment to enhance its expression [117,130]. The possibility that antidepressant-induced stimulation of BDNF expression involves CREB is supported by the presence of CRE in the

promoter of BDNF gene [81]. Enhanced CREB expression and phosphorylation induced by long-term antidepressant treatment may ultimately reverse stress-induced reduction of BDNF expression, thereby preventing the deleterious consequences associated with limited availability of this neurotrophin on hippocampal and cortical neurons. A vital role for CREB and BDNF in the pathophysiology of depression and antidepressants' beneficial action has also been suggested by a recent post-mortem investigation documenting reduced CREB and BDNF expression in depressed subjects [112]. More importantly, elevation of cortical CREB levels was found in patients receiving antidepressant medications prior to death [45].

- **Serotonin re-uptake enhancer: tianeptine.** Tianeptine is an atypical antidepressant agent, both in terms of structure (modified tricyclic agent) and pharmacodynamic profile. This antidepressant, unlike traditional TCAs and SSRIs, stimulates the reuptake of serotonin [131]. Despite its alternative neurochemical profile however, tianeptine is effective in the treatment of both major depression and bipolar disorder, with a clinical efficacy similar to TCAs or SSRIs [53,132].

  The human hippocampus undergoes atrophy in the aftermath of severe stress, recurrent depression, and Cushing's syndrome [89,133-135]. Prolonged psychological stress is also associated with loss of hippocampal neurons in monkeys [136] and with dendritic atrophy in the hippocampal CA3 region in both rodens and primates [137,138]. This atrophy affects apical dendritic trees, comprises a reduction in length and branching, and seems to be the result of alterations of dendritic cytoskeleton [139]. Three main factors mediate hippocampal damage, including endogenous excitatory amino acids, serotonin and glucocorticoids [140,141]. Massive serotonin release occurs in response to stress [142-144]. In addition, stress stimulates the release of excitatory amino acids from mossy fiber synapses and steroid hormones from adrenal glands. These events are not harmful on their own but only when they occur concurrently, since the synergy of their molecular and cellular actions augments their negative effects. Long-term tianeptine administration has been reported to prevent stress-induced atrophy of CA3 pyramidal neurons whereas neither fluoxetine nor desipramine has such effects [54,145]. Tianeptine treatment was also effective in preventing stress-induced learning impairments [54]. Molecular mechanisms underlying the positive action of this atypical antidepressant in preventing stress-mediated hippocampal abnormalities are still a matter of debate however. Several reports have shown the ability of tianeptine to correct stress-induced disturbances of the stress response[146,147]. Hyperactivity of the HPA axis, a common abnormality associated with sustained stress exposure and depression [148,149], may lead to permanently elevated glucocorticoid concentrations. Stress-induced elevation of glucocorticoid level together with massive release of serotonin and glutamate may thus create the

conditions for the development of neuronal defects. Remarkably, the pharmacological profile of tianeptine makes this drug the ideal candidate for the prevention of these abnormalities, since:

1. the serotonin-enhancing nature of this antidepressant allows active removal of serotonin from the synaptic cleft [150];
2. tianeptine has been reported to be effective in preventing stress-induced HPA axis hyperactivity [146,147], which may limit brain exposure to high glucocorticoid levels;
3. recent reports have also presented evidence concerning tianeptine's ability to interfere with glutamatergic transmission [151].

Tianeptine thus seems to exert its actions at multiple levels. It is tempting to speculate that the prevention of interactions between serotonin, glutamate, and glucocorticoids, following the exposure to stressful events, accounts for this antidepressant's ability to limit their harmful synergy, thereby preventing subsequent development of neuronal abnormalities.

Materials and Methods

Animals

Cyclic female Wistar rats were used in this investigation (n° = 25: 195-212 g). The animals were individually housed (cages 45 x 28 x 20 cm) with food and water available ad libitum. They were maintained on a 12/12-hr light/dark cycle, weighed (9:00 hr) and handled daily for 5-8 min to minimize non-specific stress response. The experiments were carried out in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC), and with the guidelines of the Animal Bio-ethics Committee of the University of Groningen (FDC: 2509).

Chronic footshock procedure

The rodent footshock-chamber consists of a box containing an animal space placed on a gridfloor connected to a shock generator and scrambler. Stressed rats received one session of 30-120 min/day in the footshock box during which 5 inescapable footshocks were applied (0.8 mA in intensity and 8 sec in duration) with different inter-shock intervals in order to make the procedure as unpredictable as possible.

Experimental Setup

To investigate the immunohistochemical alterations induced by sustained stress exposure, long-term antidepressant treatments, as well as to examine the interactions between experimental conditions, a 2 X 2 procedure was applied. Rats were randomly assigned to eight groups:

1. **CTR-vehicle** (n° = 6): these rats were exposed, daily, to the footshock box and received vehicle injections. However, these animals were not subjected to footshocks during the experiment;
2. **STRESS-vehicle** (n° = 6): stressed females were exposed, daily, to the footshock procedure (5 electric footshock during an interval of 30-120 minutes) and received vehicle injections;
3. **CTR-tianeptine** (n° = 6): these animals were used as controls to identified neurochemical adaptations induced by long-term tianeptine administration. For this reason, these rats were exposed, daily, to the footshock box and received tianeptine injections. However, they were never exposed to footshocks during the experiment;
4. **STR-tianeptine** (n° = 7): these females were exposed, daily, to the footshock procedure and received tianeptine injections;
5. **CTR-citalopram** (n° = 6): these animals were exposed, daily, to the footshock box and injected with citalopram. They were never subjected to footshocks;
6. **STRESS-citalopram** (n° = 7): stressed females were exposed, daily, to the aversive procedure and received citalopram injections;

7. **CTR-reboxetine** (n° = 6): these rats were exposed, daily, to the footshock box and injected with reboxetine. They did not receive footshocks throughout the experiment.
8. **STR-reboxetine** (n° = 7): these animals were exposed, daily, to the footshock procedure and injected with reboxetine.

On the final day of the experiment all rats were placed in the footshock box. However, during this final session, of the duration of 15 minutes, no footshocks were delivered. It is also important to note that no injections were administered prior of this final session allowing 24-hour washout period.

Pharmacological profile, mode of action, dosage, and route of administration.

Citalopram. The mechanism of action of this SSRI is presumed to be linked to the potentiation of serotonergic activity in the CNS resulting from inhibition of neuronal reuptake of serotonin (5 HT) [152,153]. Citalopram is a racemic mixture (50/50) and the inhibition of serotonin reuptake is primarily due to the (S)-enantiomer. *In vitro* and *in vivo* animal studies suggest that this compound is a highly selective SSRI with minimal effects on norepinephrine and dopamine neuronal reuptake [152,154]. Moreover, this antidepressant has no or very low affinity for 5-$HT_{1A,}$ 5-HT_{2A}, dopamine D_1 and D_2, apha$_1$-, alpha$_2$-, and beta-adrenergic, histamine H_1, gamma aminobutyric acid (GABA), muscarinic cholinergic, and benzodiazepine receptors [154]. Antagonism of muscarinic, histaminergic, and adrenergic receptors has been hypothesized to be associated with various anticholinergic, sedative, and cardiovascular effects of other psychotropic drugs.

Dosage. Citalopram, kindly provided by Lundbeck B.V. (The Netherlands), was dissolved in saline (0.9% NaCl) at a concentration of 20mg*ml^{-1}, and injected intraperitoneously (i.p.) at the dosage of 20mg*kg^{-1}*day^{-1}, for a 21 day-period, 30-45 minutes before exposure to the footshock procedure. The daily dose of 20mg*kg^{-1}*day^{-1} was chosen after reviewing recent pharmacological studies using this antidepressant in a long-term setting [155,156]. These studies suggested that we could rely on the administered dosage of 20mg*kg^{-1} to provide a sufficient plasma concentration of about 250-300 nmol*l^{-1} [156,157], independent of the way of administration (oral vs. osmotic pumps). A plasma concentration of 250-450 nmol*l^{-1} was also observed after chronic citalopram administration through diet (10 and 40 mg*kg^{-1} daily) or oral administration (40 mg*kg^{-1} daily). It is noteworthy that serum citalopram concentrations around 100 nmol*l^{-1} were observed in humans receiving repeated oral doses within a normal dose range [158]. We can thus safely assume that plasma concentrations at least as high as those reported in clinical practice were also reached in the present study using a daily dosage of 20 mg*kg^{-1} administered through i.p. injections for a three-week period. Interestingly, as reported by Kugelberg and colleagues (2001), citalopram levels were constantly higher in the brain compared to those observed in the serum independent of the dosage administered [156]. The ratios between citalopram concentration in the serum and in the brain were constant for each drug concentration administered (10, 20 and 100 mg*kg^{-1}). Furthermore, the

antidepressant levels were 1.5-2 times higher in the cerebral cortex compared to the levels found in the mesencephalon-pons [156].

Tianeptine. Tianeptine is an atypical antidepressant, both structurally and in terms of its neurochemical profile. It is devoid of sedative effects and induces slight stimulation of locomotor activity. In monkeys, it decreases aggressive and emotive states and improves individual behavior [159]. Pharmacological studies have shown that, unlike other antidepressants, tianeptine stimulates the uptake of serotonin and increases 5-hydroxyindoleacetic acid levels in the brain [131]. It does not have anticholinergic effects and is also devoid of any effect on the cardiovascular and neuroendocrine systems [159]. Tianeptine shows no affinity for neurotransmitter receptors and its effects do not seem to depend upon blockade of the neuronal dopamine transporter [160]. Repeated administration increases the responsiveness of the $alpha_1$-adrenergic system [161]. Recent hypotheses on tianeptine's mode of action however, have involved the modulation of excitatory amino acid transmission and new evidence indicates that this antidepressant seems to specifically target the phosphorylation-state of glutamate receptors at the CA3 synapse [151]. Remarkably, tianeptine abolished stress-induced reduction of hypothalamic CRF concentration and markedly reduced stress-related increase of plasma ACTH and corticosterone concentrations[147]. These results suggest that the hypothalamus represents a primary target for antidepressants, a view also supported by the ability of tianeptine to attenuate, in stressed animals, the activation of the HPA axis [147,162]. Tianeptine-induced reduction of hypothalamic-pituitary-adrenal response to stress may constitute one of the mechanisms by which this drug antagonizes stress-induced behavioral deficits as well as prevents atrophy of neuronal dendrites [54,145,163].

Dosage. Tianeptine, kindly provided by the Institut de Recherches Internationales Servier (Paris, France), was dissolved in saline (0.9% NaCl) at a concentration of $10mg*ml^{-1}$, and injected intraperitoneously (i.p.) at the dosage of $10mg*kg^{-1}*day^{-1}$, for a 21 day-period, 30-45 minutes before exposure to the footshock procedure. The daily dose of $10mg*kg^{-1}*day^{-1}$ was chosen based on the indications provided by the drug manufacturer and literature review, as the effective dosage sufficient to prevent behavioral and neurochemical abnormalities in a chronic setting [54,147,151,164-166].

Reboxetine. Reboxetine is a potent and selective norepinephrine reuptake inhibitor without any affinity for neurotransmitter receptors that displays an antidepressant profile in both animal tests and in clinical trials. Unlike desipramine or imipramine, reboxetine has weak affinity for muscarinic, histaminergic H_1, adrenergic $alpha_1$, and dopaminergic D_2 receptors and low toxicity in animals. It is a mixture of (R,R) and (S,S) enantiomers, the latter being more potent although no qualitative difference in pharmacodynamic properties are observed between the two. Humans rapidly absorb reboxetine (t_{max} about 2 hours) with a terminal half-life of elimination ($t_{1/2}$) of 13 hours [167]. *In vivo* action of reboxetine is entirely consistent with the pharmacological action of an antidepressant with preferential action at the norepinephrine reuptake site [168]. Reboxetine has been shown to be an effective first-line

treatment for patients with all grades of depression, effective in preventing relapse and recurrence, and in offering significant benefits in terms of relieving the impaired social functioning associated with depressive disorders [169].

Dosage. Reboxetine, a racemic mixture of R,R- and S,S-([2-[alpha [2-ethoxyphenoxy] benzyl]-morpholine sulfate]) and (+)-(S,S)-reboxetine methanesulfon, was kindly provided by Pharmacia B.V. (The Netherlands). The noradrenaline reuptake inhibitor was dissolved in saline (0.9% NaCl) at a concentration of 20mg*ml^{-1}, and injected intraperitoneously (i.p.) at the dosage of 20mg*kg^{-1}*day^{-1}, for a 21 day-period, 30-45 minutes before exposure to the footshock procedure. The daily doses of 20mg*kg^{-1}*day^{-1} was chosen due to its reported low half-life and on the basis of the review of recent preclinical studies investigating the behavioral and neurochemical changes induced by prolonged administration of this antidepressant using similar dosages in rats [170-173].

Route of administration

In contrast to other studies in which osmotic pumps were used to deliver the drugs, here intraperitoneous injections were chosen as the route of administration. Although osmotic pumps allow a more constant administration, the concentration of antidepressant loaded into each pump must be estimated in advance on the basis of each individual animal's weight. The experimental design used here however presents some problems in establishing body weight since use was made of cyclic female rats and antidepressant treatment has been shown to modify female body weight growth by reducing food intake and, consequently, body weight gain [174,175]. Furthermore, we used a chronic aversive paradigm, which has been reported to strongly affect normal weight gain of animals [176]. As mentioned before, most preclinical studies, including pharmacological testing, have been performed in males, leaving females' response to stress and long-term antidepressant administration poorly explored. The use of osmotic pumps provides a reliable way of administration in males since their responses (in particular body weight changes during a long-term experiment) to different experimental conditions have been well defined. However, as we were unable to reliably predict body weight changes in cyclic female rats during chronic stress and long-term antidepressant administration, we decided to deliver the drugs by i.p. injections to guarantee a fixed and constant dosage throughout the entire duration of the experiment, independent of the effects of experimental conditions on the body weight of the individual animal.

Physiological and neuroendocrine correlates of the chronic stress response

To define the dynamic of the response to prolonged footshock stress and antidepressants, various physiological and neuroendocrine parameters were measured. Weight gain was monitored on a daily basis throughout the experiment, and upon termination, adrenal glands and thymus were removed and weighed. Graphs were constructed to serve as a reference to verify the severity of stress perceived by the animals. In addition, blood samples

were drawn by transcardial puncture immediately upon termination and stored at -80°C. These samples were then used to determine plasma corticosterone concentrations with HPLC.

Corticosterone: extraction and chromatography. For the assay, dexamethason was used as internal standard. After addition of the internal standard, plasma was extracted with 3 ml of diethylether, vortexed for 5 min and then centrifuged for 5 min at 3000 x *g*. The extraction procedure was repeated twice. The organic phase was evaporated to dryness in a 50°C waterbath. The residue was reconstituted with 200µL of mobile phase and 50µL was injected into the HPLC system. The mobile phase (flow rate 1.0mL/min) for the determination consisted of acetonitrile in ultrapure water (27:73 v/v). The concentration of both corticosterone and the internal standard was determined with UV detection at a wavelength of 254nm. The detection limit of the method was 10nM.

Histological procedure

Two hours after the beginning of the final session, the rats were terminated with an overdose of halothane which preceded a transcardial perfusion with 4% paraformaldehyde solution in 0.1M sodium phosphate buffer (pH 7.4). The brains were carefully removed and post-fixed in the same solution overnight at 4°C, before being transferred to a potassium phosphate buffer (KPBS 0.02 M, pH 7.4) and stored at 4°C. Following cryoprotection of the brains by overnight immersion in a 30% glucose solution, coronal serial sections of 40 µm were prepared on a cryostat microtome. Sections were collected in KPBS with sodiumazide and stored at 4°C.

Immunohistochemistry

The staining was performed on free-floating sections under continuous agitation. The sections were preincubated in 0.3% H_2O_2 for 15 min to reduce endogenous peroxidase activity, before being incubated in primary polyclonal rabbit anti-FOS (Oncogene Research Products, brands of CN Biosciences, Inc, an affiliate of Merck KGaA, Darmstadt, Germany; 1:10000 dilution in KPBS 0.02 M, pH 7.4) or anti-phospho-CREB antibodies (Upstate Biotechnology, Charlottesville, VA, USA: www.upstatebiotech.com; 1:1000 dilution in KPBS 0.02 M, pH 7.4) for 60-72 hr at 4°C. Subsequently, sections were washed with KPBS and incubated at room temperature with biotinylated goat anti-rabbit IgG (Vector Laboratories, Inc., Burlingame, CA, USA; 1:1000 dilution) followed by ABC complex (Vector ABC kit, Vector Laboratories, Burlingame, CA, USA). After another wash, the reaction product was visualized by adding diaminobenzidine as chromogen and 1% H_2O_2 for 15 min. Then, the sections were washed, mounted on slides, dehydrated and coverslipped with DePex.

Quantification and data analysis

c-fos and phospho-CREB-labeled nuclei were quantified using a computerized imaging analysis system by an observer who was blind to group assignment. The quantification of the immunoreactive cell nuclei was performed using at least 4-5 sections per each brain area examined. The selected area from regions of interest (ROI) were digitized by using a Sony (SONY Corporation, Tokyo, Japan) charge-coupled device digital camera mounted on a

LEICA Leitz DMRB microscope (LEICA, Wetzlar, Germany) at x100 magnification. ROIs included the medial orbitofrontal cortex (mORB: Bregma +4.85 to +3.60), the medial prefrontal cortex (prelimbic (PrL) and the infralimbic area (InfraL); mPFC: Bregma +3.60 to +1.70), the anterior (AC: Bregma +3.20 to +0.95), and the posterior cingulate cortex (postCING: Bregma +1.70 to -1.08); the hippocampal dentate gyrus (DG: Bregma -2.00 to –3.90) and the CA3 area (CA3: Bregma –2.45 to –4.60); the central (CeA: Bregma –1.53 to –2.85), the lateral (LaA: Bregma –2.00 to -3.70), and the basolateral nucleus of the amygdala (BslA: Bregma –1.78 to –3.25); the paraventricular thalamic nucleus (PVT: Bregma –1.33 to –3.90); the paraventricular hypothalamic nucleus (PVN: Bregma –1.08 to –2.00) [177]. ROIs were outlined with a digital pen. Each digitized image was individually set at a threshold to subtract the background optical density, and the numbers of cell nuclei above the background were counted by using the computer-based image analysis system LEICA (LEICA Imaging System Ltd., Cambridge, England). Only cell nuclei that exceeded a defined threshold were detected by the image analysis system and subsequent counts were reported as number of positive cells/0.1mm^2. Phospho-CREB and FOS positive cells with gray levels below the defined thresholds were thus classified as “negative”. This is important for the understanding of our results, since this method does not allow us to discriminate between negative nuclei with no immunoreactivity and nuclei with (too) low immunoreactivity. In other words this method is not suitable for determining absolute protein levels. All areas were measured bilaterally (no left-right asymmetry of FOS or phospho-CREB immunoreactivity was found). *F* tests of variance were run on numbers of immunoreactive cell nuclei from individual brain regions from experimental and control conditions. That value determined whether *t* tests for equal or unequal variance were performed to compare the cell counts from individual brain regions of control and experimental conditions. $P < 0.05$ was defined as the level of significance between groups.

Results

Physiological and neuroendocrine correlates of the chronic stress response

Physiological parameters, including body weight gain as well as adrenal and thymus size, were measured throughout the experiment or upon termination.

Body weight gain

Vehicle. Body weights were measured daily during the chronic stress procedure. No differences were detected between CTR-vehicle and STR-vehicle females (fig. 1a). This finding is in accordance with previous preclinical data reporting that stress exposure does not affect weight gain in female rats as much as it does in males [178]. Although the lack of difference between CTR-vehicle and STR-vehicle groups, we decided to include this information anyway since the disruption of body weight regulation represents one of the most common side-effects of pharmacotherapy [174,175].

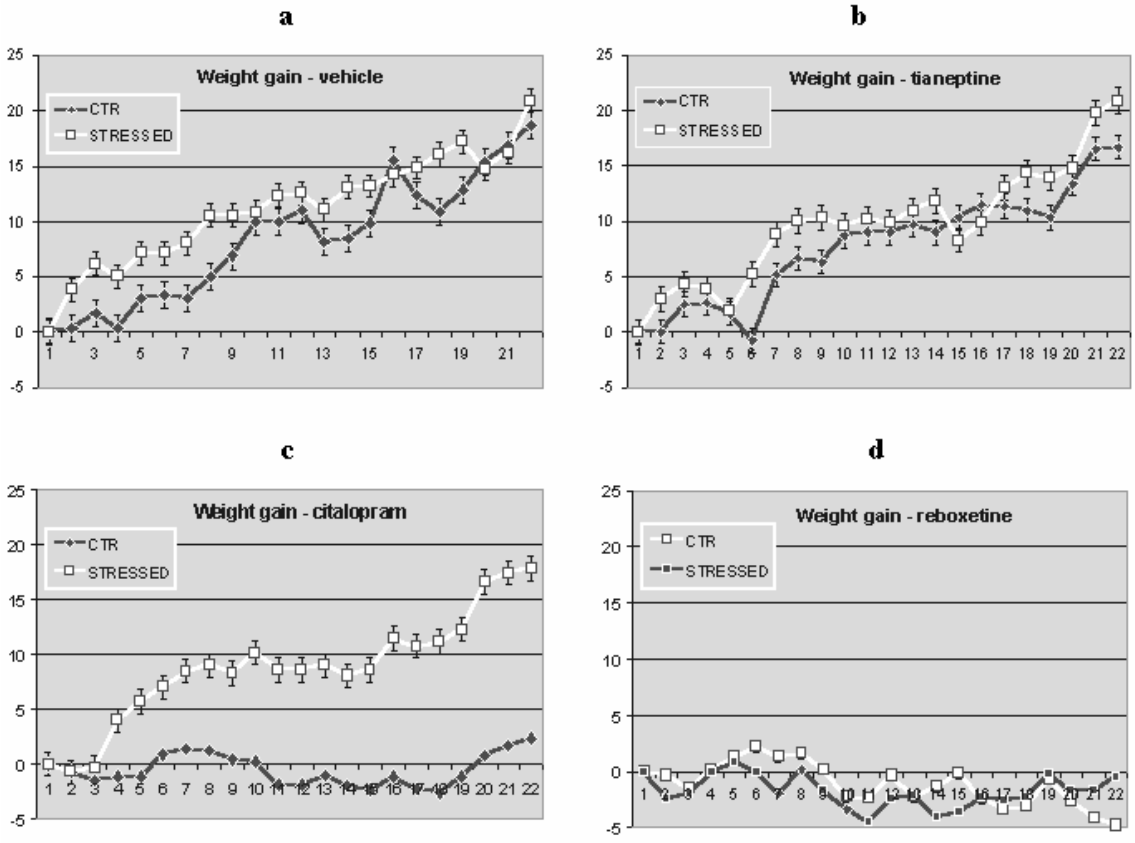


Figure 1. Body weight changes following prolonged stress and/or antidepressant administration.

Tianeptine. No changes in body weight gain were found between CTR-tianeptine and STR-tianeptine animals as well as between tianeptine- and vehicle-treated rats. Tianeptine thus

appears to carry out its action without influencing the normal curve of body weight growth (fig. 1b).

Citalopram. SSRI treatment resulted, in non-stressed females, in a significantly decreased body weight gain ($F=26.8$, $p<<0.001$; CTR-vehicle vs. CTR-SSRI). Serotonin plays a central role in food intake and body weight regulation, especially in the hypothalamus. Long-term SSRI administration has been shown to gradually desensitize the hypothalamic post-synaptic 5-HT_{1A} receptors [179] and this desensitization may affect food intake and weight gain regulation, leading to reduced body weight growth [180]. Surprisingly, chronic stress counteracted this effect ($F=11.06$, $p<0.007$; CTR-SSRI vs. stress-SSRI) restoring a normal weight gain curve (fig. 1c).

Reboxetine. A consistent reduction in body weight gain was observed immediately following the initiation of reboxetine treatment, both in stressed and non-stressed females, compared to vehicle-treated animals. These differences increased gradually throughout the experiment reaching highest significance at the end ($F=45.75$, $p<<0.001$, CTR-vehicle vs. CTR-reboxetine; $F=24.47$, $p<<0.001$, STR-vehicle vs. STR-reboxetine) (fig. 1d).

Plasma corticosterone concentration

Vehicle. No significant differences in corticosterone concentrations were found between any experimental groups with the only exception of STR-reboxetine animals (fig. 2a). Plasma samples were collected only at the time of death, occurring approximately 120 minutes after the final exposure to CSs. Plasma corticosterone levels, in response to stressful conditions, have been reported to reach a peak about 15-30 minutes following the threat and return to basal level in 60-90 minutes [181,182]. Chronically stressed rats however, showed an increased baseline plasma corticosterone concentration compared to control females (+22%).

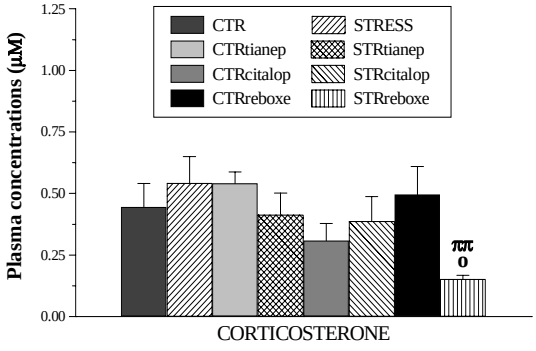


Figure 2a. Plasma corticosterone concentrations

Tianeptine. Although a slight increase of basal corticosterone concentration was observed following tianeptine treatment in non-stressed females (+22%), long-term antidepressant administration prevented the increased glucocorticoid levels detected following chronic footshock exposure (-24%, STR-vehicle vs. STR-tianeptine) (fig. 2a).

Citalopram. Both groups receiving long-term citalopram treatment showed lower basal corticosterone concentrations compared to CTR-vehicle (-69%) and STR-vehicle rats (-40%), respectively (fig. 2a).

Reboxetine. While CTR-reboxetine rats illustrated slightly increased basal serum corticosterone concentrations compared to CTR-vehicle animals (+11%), a significant reduction of plasma glucocorticoid levels was detected in STR-reboxetine females compared

to both STR-vehicle (-72%; F=12.74, p<0.012) and CTR-reboxetine group (-69%; F=21.51, p<0.01) (fig. 2a).

Adrenal and thymus weights

Vehicle. Chronic footshock stress caused marked adrenal hypertrophy (F=6.23, p<0.032) and a slight reduction of thymus weight (F=2.56, p<0.017) (fig. 2b,c).

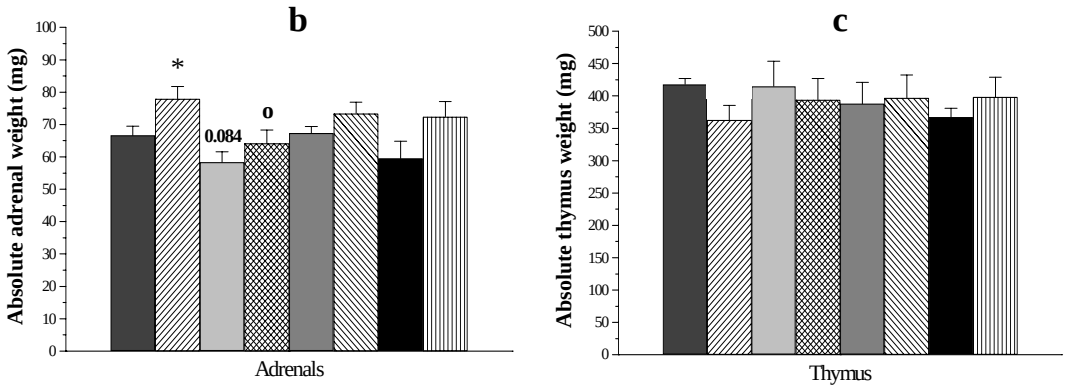


Figure 2b,c. Adrenal and thymus weight following chronic stress and antidepressant treatments

Tianeptine. Preclinical evidence suggests the HPA axis as one of tianeptine targets [147]. In support of this assumption, a marked reduction of adrenal gland weight was observed in non-stressed females following long-term antidepressant treatment (F=3.69, p<0.084, CTR-vehicle vs. CTR-tianeptine). More importantly, tianeptine administration significantly prevented the increased adrenal size seen in response to chronic footshock exposure (F=6.20, p<0.034, STR-vehicle vs. STR-tianeptine). No changes were observed after antidepressant treatment in thymus weight, both in non-stressed and stressed animals (fig. 2b,c).

Citalopram. Long-term SSRI treatment attenuated chronic footshock-induced adrenal hypertrophy. Chronically stressed rats treated with citalopram, in fact, reported a marked but not-significant enlargement of adrenal glands compared to non-stressed animals (F=2.03, p<0.18, CTR-citalopram vs. STR-citalopram; F=1.70, p<0.22, CTR-vehicle vs. STR-citalopram). Citalopram administration did not significantly affected thymus weight (fig. 2b,c).

Reboxetine. Although reboxetine treatment seems able to reduce adrenal weight in non-stressed females compared to vehicle-treated rats (F=1.26, p<0.29), this antidepressant attenuated stress-induced adrenal hypertrophy (F=3.37, p<0.10, CTR-reboxetine vs. STR-reboxetine). Interestingly, reboxetine alone without concurrent exposure to stressful conditions led to a marked reduction of thymus weight (F=2.89, p<0.12, CTR-vehicle vs. CTR-reboxetine). Remarkably, cyclic females, exposed simultaneously to stress and antidepressant treatment, showed only a slight and non-significant thymus weight reduction (F=0.77, p<0.40, CTR-vehicle vs. STR-reboxetine) (fig. 2b,c).

Immunohistochemistry

In the present study, changes of FOS and phospho-CREB immunoreactivity were examined in several cortical and subcortical regions, including the frontal cortex, the hippocampus, the amygdala, the thalamus, and the hypothalamus (fig. 3).

Chronic stress-induced neurochemical changes

Vehicle

FOS-ir. Chronically stressed females showed marked immunohistochemical changes in both cortical and limbic structures, including a significant increase of FOS-ir in the medial orbitofrontal cortex ($F=5.08$, $p<0.048$) (fig. 4a), the prelimbic cortex ($F=5.37$, $p<0.043$) (fig. 4b), the central nucleus of the amygdala ($F=33.09$, $p<<0.001$) (fig. 4h), and the paraventricular nucleus of the hypothalamus ($F=29.83$, $p<<0.001$) (fig. 4l). In contrast, an opposite and significant reduction of FOS-ir was observed in the hippocampal dentate gyrus ($F=5.02$, $p<0.05$) (fig. 4f).

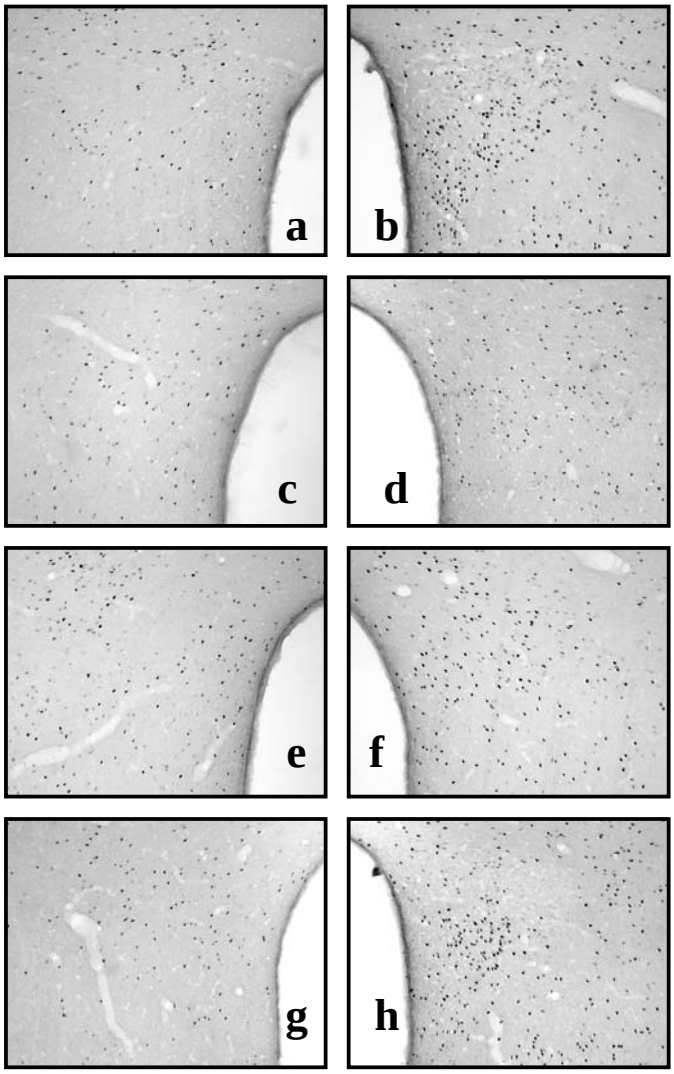


Figure 3. FOS-ir in the PVN following stress and antidepressant administration

Phospho-CREB immunoreactivity. Phospho-CREB expression was examined in cortical and subcortical areas including the medial orbitofrontal, the prelimbic, the infralimbic, the anterior cingulate, the posterior cingulate cortex, the lateral and the basolateral amygdala, the hippocampal dentate gyrus (fig. 5). A marked decreased CREB phosphorylation was observed, following prolonged footshock exposure, in the medial orbitofrontal cortex ($F=4.15$, $p<0.072$) (fig. 6a), the prelimbic cortex ($F=15.01$, $p<0.004$) (fig. 6b), the anterior cingulate cortex ($F=8.93$, $p<0.015$) (fig. 6d), and the DG ($F=10.76$, $p<0.01$) of cyclic female rats (fig. 6f).

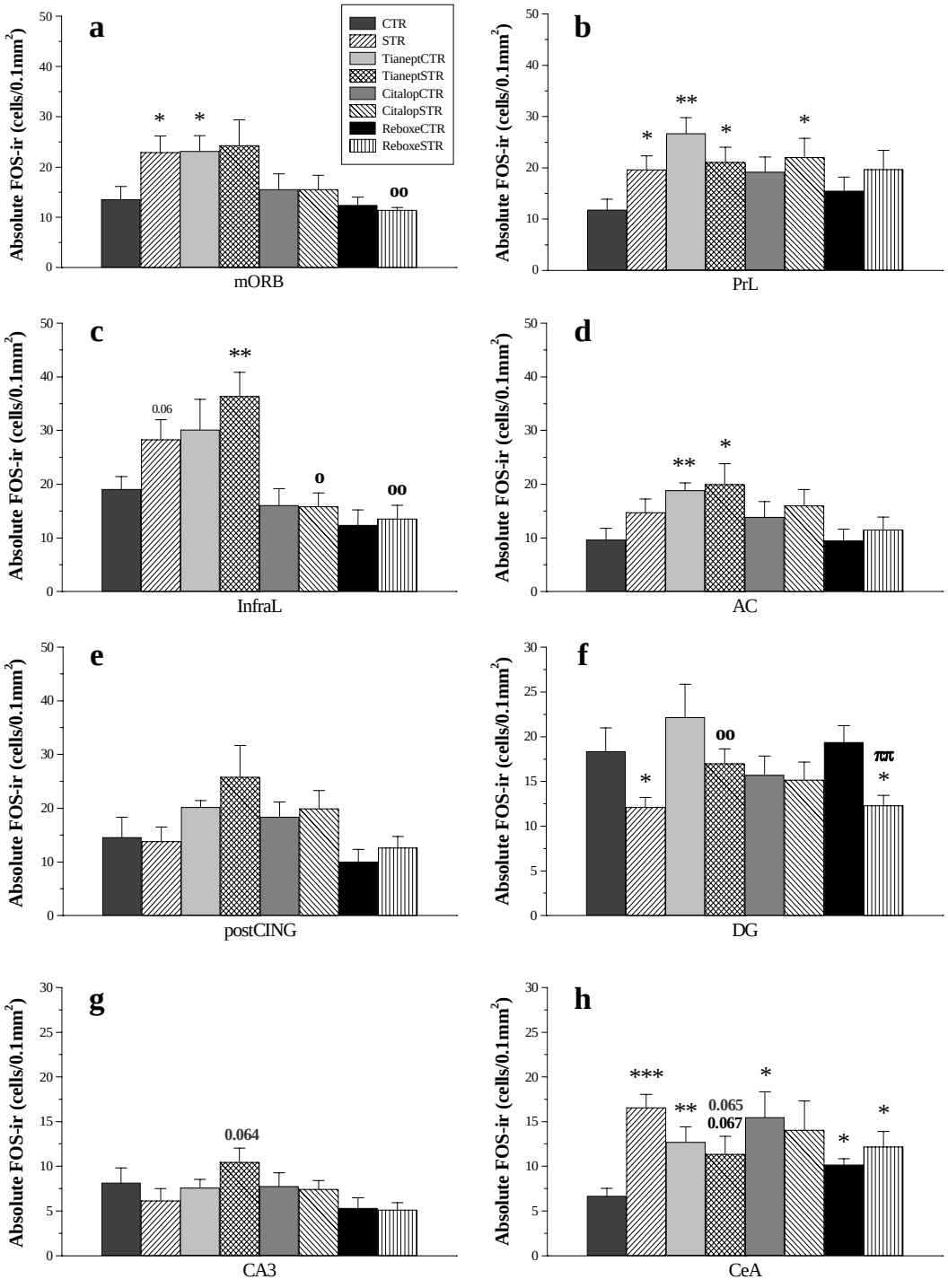
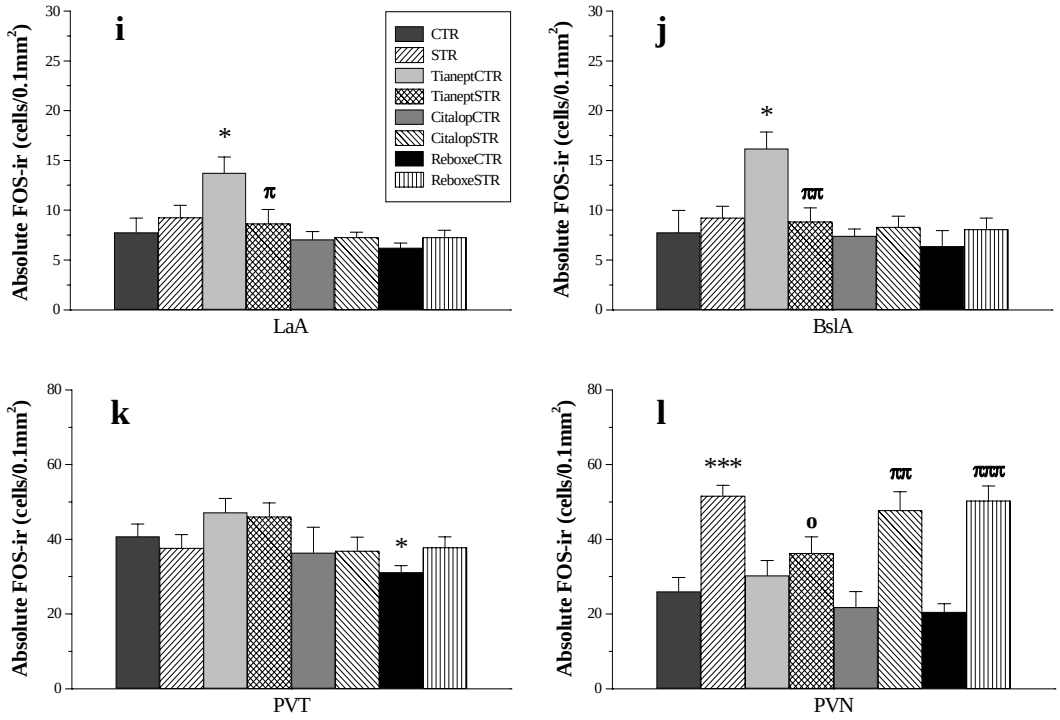


Figure 4. Effect of stress and/or concurrent antidepressant administration on absolute FOS-ir in: a-e) medial prefrontal cortex; f-g) hippocampus; h-j) amygdala; k) thalamus; l) hypothalamus. The symbol * expresses the comparison with CTR-vehicle (*=p<0.05; **=p<0.01; ***=p<0.001). The symbol ^{o} expresses the comparison between STR-vehicle and STR-antidepressant (^{o}=p<0.05; ^{oo}=p<0.01; ^{ooo}=p<0.001). The symbol $^{\pi}$ expresses the comparison between CTR-antidepressant and STR-antidepressant ($^{\pi}$=p<0.05; $^{\pi\pi}$=p<0.01; $^{\pi\pi\pi}$=p<0.001).



Long-term antidepressant administration on basal FOS-ir and CREB phosphorylation (CTR-vehicle vs. CTR-antidepressant)

Tianeptine

FOS-ir. Long-term tianeptine administration caused, in non-stressed animals, a significant increase of basal FOS-ir in the medial orbitofrontal cortex ($F=5.79$, $p<0.037$) (fig. 4a), the prelimbic cortex ($F=16.44$, $p<0.0023$) (fig. 4b), the anterior cingulate ($F=12.96$, $p<0.0049$) (fig. 4d), the central ($F=10.07$, $p<0.01$) (fig. 4h), the lateral ($F=7.79$, $p<0.019$) (fig. 4i), and the basolateral amygdala ($F=9.22$, $p<0.013$) (fig. 4j) compared to vehicle-treated females.

Phospho-CREB immunoreactivity. No significant regional changes in the level of basal cortical-limbic CREB phosphorylation were reported, in non-stressed animals, in response to long-term tianeptine treatment.

Citalopram

FOS-ir. Long-term citalopram administration did not significantly change the level of basal FOS-ir. The only exception was represented by the central nucleus of the amygdala where a marked increase of FOS-ir was observed after prolonged SSRI administration compared to vehicle-treated females ($F=8.70$, $p<0.015$) (fig. 4h).

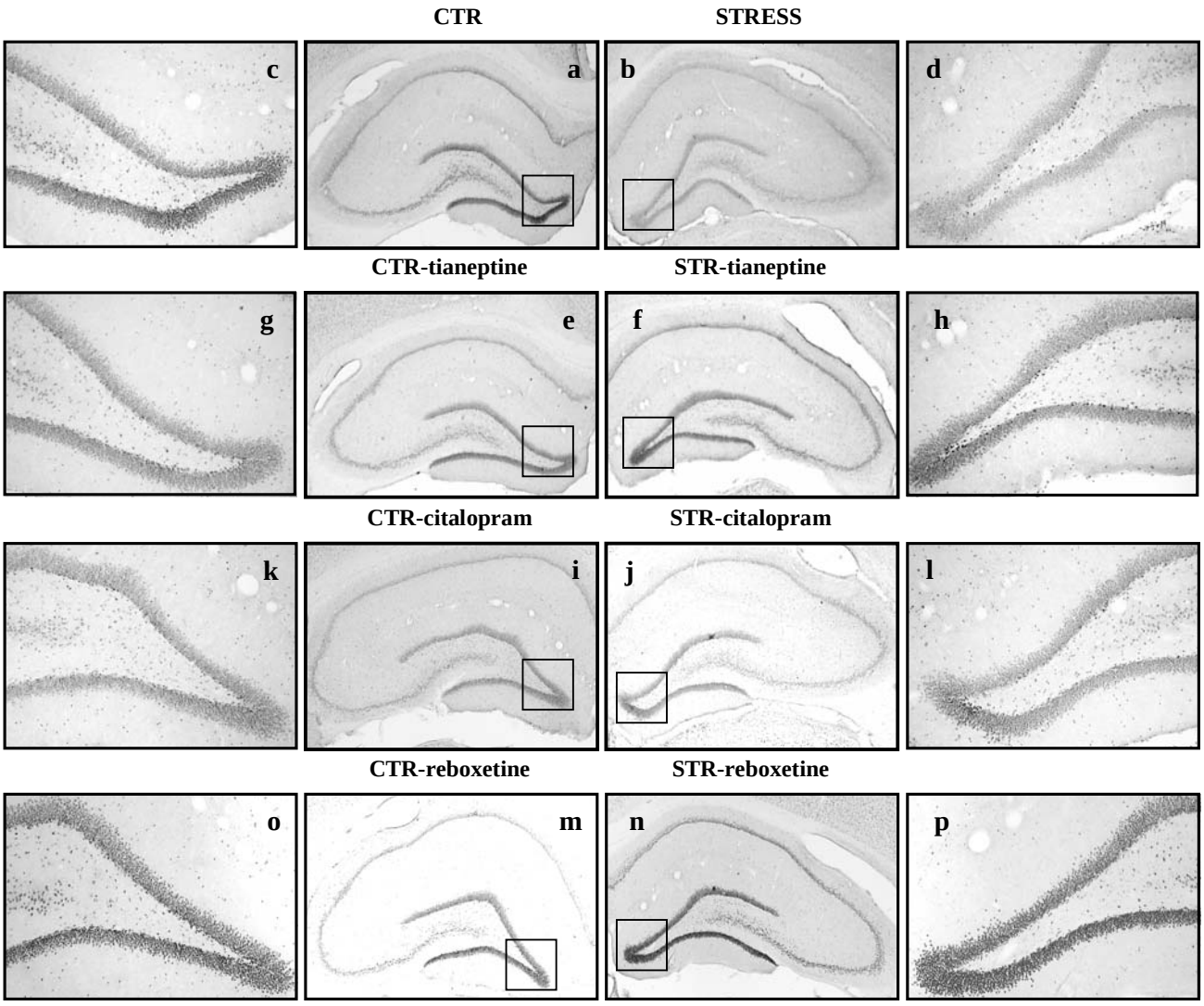


Figure 5. Hippocampal phospho-CREB immunoreactivity following prolonged stress and long-term antidepressant treatments.

Phospho-CREB immunoreactivity. Long-term citalopram treatment resulted in a marked reduction in the level of basal phospho-CREB immunoreactivity (fig. 6). Citalopram-treated females showed a significant reduction of CREB phosphorylation in the posterior cingulate cortex ($F=7.22$, $p<0.023$) (fig. 6e), the DG ($F=17.58$, $p<0.0019$) (fig. 6f), and the BslA ($F=7.53$, $p<0.021$) compared to vehicle-treated rats (fig. 6i).

Reboxetine

FOS-ir. Long-term reboxetine administration caused a significant increase of basal FOS-ir in the CeA ($F=9.74$, $p<0.011$) (fig. 4h), while a reduction was observed in the PVT ($F=6.35$, $p<0.03$) compared to vehicle-treated rats (fig. 4k).

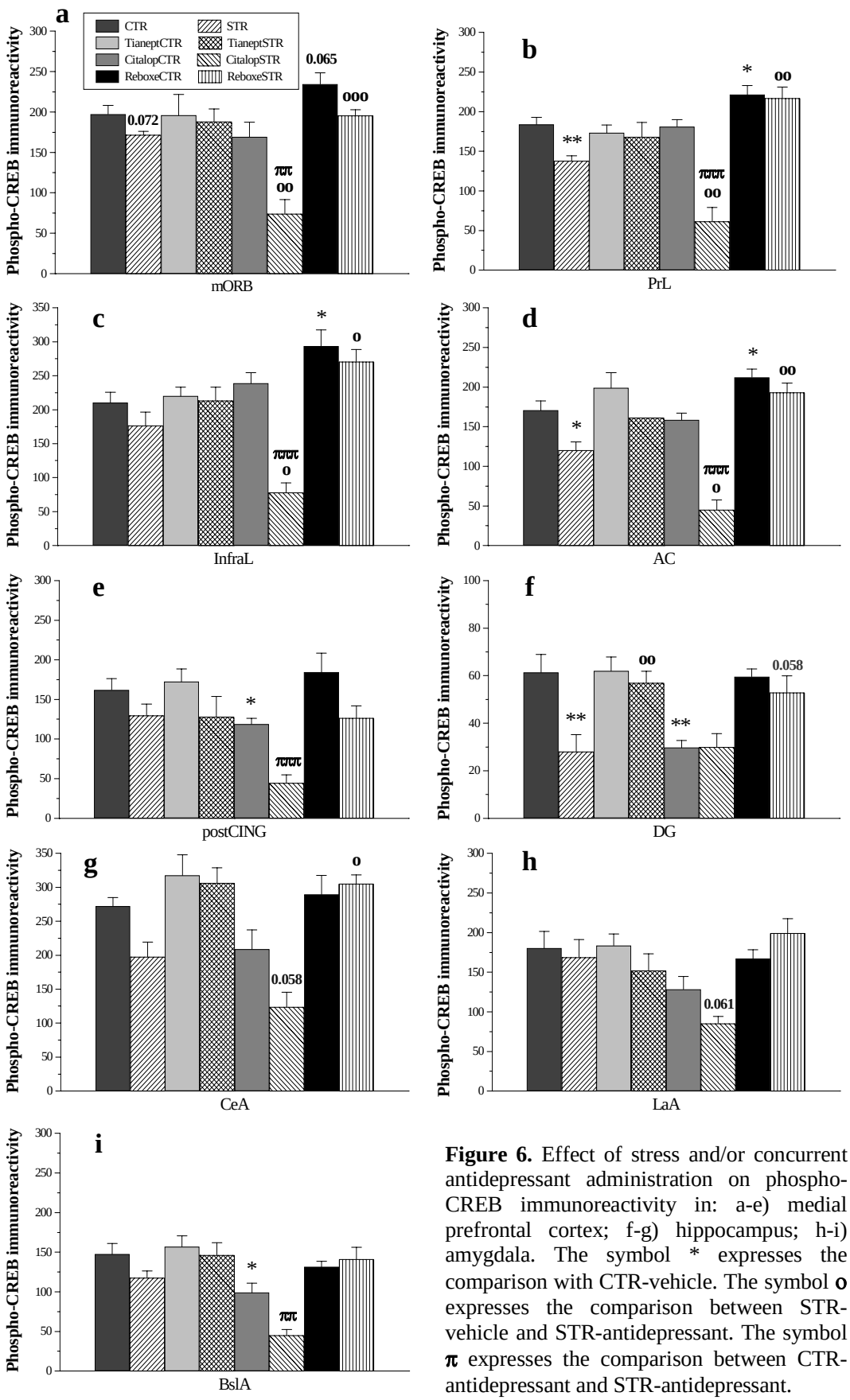


Figure 6. Effect of stress and/or concurrent antidepressant administration on phospho-CREB immunoreactivity in: a-e) medial prefrontal cortex; f-g) hippocampus; h-i) amygdala. The symbol * expresses the comparison with CTR-vehicle. The symbol **o** expresses the comparison between STR-vehicle and STR-antidepressant. The symbol **π** expresses the comparison between CTR-antidepressant and STR-antidepressant.

Phospho-CREB immunoreactivity. Long-term reboxetine treatment markedly enhanced basal cortical-limbic phospho-CREB immunoreactivity. This effect was particularly evident in the medial orbitofrontal cortex ($F=4.30$, $p<0.065$) (fig. 6a), the prelimbic cortex ($F=6.68$, $p<0.027$) (fig. 6b), the infralimbic cortex ($F=8.65$, $p<0.015$) (fig. 6c), and the anterior cingulate cortex ($F=6.88$, $p<0.025$) (fig. 6d).

Immunohistochemical changes induced by chronic stress and concurrent long-term antidepressant administration (STR-vehicle vs. STR-antidepressant)

Tianeptine

FOS-ir. Although long-term tianeptine administration did not normalize chronic stress-induced increased FOS-ir in prefrontocortical regions (fig. 4a-e), this antidepressant was effective in reversing stress-induced immunohistochemical changes in the hippocampal dentate gyrus ($F=5.90$, $p<0.033$) (fig. 4f) and CA3 area ($F=4.25$, $p<0.064$) (fig. 4g).

Phospho-CREB immunoreactivity. Long-term tianeptine administration effectively attenuated footshock-mediated reduction of phospho-CREB immunoreactivity in the DG ($F=13.32$, $p<0.0045$) (fig. 6f) and partially in prefrontocortical areas (fig. 6a-e). No significant immunohistochemical changes were observed in the amygdala (fig. 6g-i).

Citalopram

FOS-ir. Long-term citalopram administration was able to alleviate FOS-ir changes observed in response to chronic footshock exposure. This beneficial effect was particularly evident in the medial orbitofrontal cortex (fig. 4a), the infralimbic cortex ($F=8.22$, $p<0.015$) (fig. 4c), the dentate gyrus (fig. 4f) and the central amygdala (fig. 4h). The only exception was represented by the prelimbic cortex where a significantly increased FOS expression was observed in STR-citalopram rats compared to CTR-vehicle females ($F=5.22$, $p<0.043$) (fig. 4b). However, markedly increased activity was also found in CTR-SSRI animals suggesting that stimulation of serotonergic neurotransmission might have been the main factor responsible for this higher activation. Interestingly, STR-SSRI females illustrated significantly increased FOS-ir in the paraventricular hypothalamic nucleus compared to both CTR-vehicle ($F=11.58$, $p<0.006$) and CTR-SSRI rats ($F=15.36$, $p<0.0024$) (fig. 4l).

Phospho-CREB immunoreactivity. Long-term citalopram administration was not able to attenuate CREB phosphorylation changes associated with chronic footshock exposure. Remarkably, STR-SSRI females reported a marked reduction of phospho-CREB immunoreactivity that was, in several regions, even larger than that detected in both STR-vehicle and CTR-SSRI animals (fig. 6), including the mORB ($F=21.52$, $p<0.0017$, STR-vehicle vs. STR-SSRI; $F=12.18$, $p<0.007$, CTR-SSRI vs. STR-SSRI) (fig. 6a), the PrL ($F=14.2$, $p<0.0044$; $F=32.25$, $p<<0.001$) (fig. 6b), the InfraL ($F=5.7$, $p<0.041$; $F=49.18$, $p<<0.001$) (fig. 6c), the AC ($F=7.43$, $p<0.023$; $F=48.11$, $p<<0.001$) (fig. 6d), the posterior cingulate ($F=29.10$, $p<<0.001$, CTR-SSRI vs. STR-SSRI) (fig. 6e), the CeA ($F=4.69$, $p<0.058$, CTR-SSRI vs. STR-SSRI) (fig. 6g), the LaA ($F=4.59$, $p<0.061$, CTR-SSRI vs. STR-SSRI) (fig. 6h), and the BslA ($F=12.20$, $p<0.0068$, CTR-SSRI vs. STR-SSRI) (fig. 6i).

Reboxetine

FOS-ir. Long-term reboxetine administration attenuated footshock-induced changes, significantly reducing stress-induced FOS upregulation in the mORB (STR-vehicle vs. STR-reboxetine, $F=14.21$, $p<0.0031$) (fig. 4a) and InfraL ($F=11.72$, $p<0.0057$) (fig. 4c) but only partially in the CeA ($F=3.59$, $p<0.085$) (fig. 4h). Antidepressant treatment did not prevent the significant chronic footshock-induced reduction of FOS-ir in the DG ($F=5.17$, $p<0.044$, CTR-vehicle vs. STR-reboxetine; $F=11.51$, $p<0.006$, CTR-reboxetine vs. STR-reboxetine) (fig. 4f) or the increased neuronal activation detected in the PVN ($F=19.35$, $p<0.0011$, CTR-vehicle vs. STR-reboxetine; $F=39.48$, $p<<0.001$ CTR-reboxetine vs. STR-reboxetine) (fig. 4l).

Phospho-CREB immunoreactivity. Interestingly, prolonged reboxetine treatment normalized chronic footshock-mediated reduction of CREB phosphorylation in the medial orbitofrontal cortex (STR-vehicle vs. STR-reboxetine, $F=35.86$, $p<<0.001$) (fig. 6a), the prelimbic cortex ($F=17.87$, $p<0.0024$) (fig. 6b), the infralimbic cortex ($F=9.41$, $p<0.013$) (fig. 6c), the anterior cingulate cortex ($F=15.11$, $p<0.0037$) (fig. 6d), the dentate gyrus ($F=4.59$, $p<0.058$) (fig. 6f), and the central nucleus of the amygdala ($F=15.34$, $p<0.0035$) (fig. 6g).

*Chronic stress-induced neuroendocrine and immunohisto-chemical changes in the female brain: role of long-term citalopram treatment**

A. Trentani, S.D. Kuipers, G.J. Ter Horst, J.A. Den Boer

***Adapted from the manuscript submitted to "Neuropsychopharmacology"**

In the present study, an attempt was made to define the neuroendocrine and immunohistochemical changes induced by prolonged footshock exposure and/or concomitant long-term antidepressant treatment in cyclic female rats. FOS-ir has been widely used as a molecular marker of neuronal activity [181,183,184] and changes in expression of this immediate early gene have been used to explore the neurocircuits underlying various higher-order processes including learning, memory, affective style, emotion and stress response regulation [184-186]. Phospho-CREB immunoreactivity, instead, was used as molecular correlate of neuronal plasticity [105,187,188]. Increased CREB phosphorylation, especially in the amygdala and hippocampus, has been reported to be critical for cognitive processing [122,189-193]. By making use of the properties of these two cellular markers, we investigated the impact of sustained footshock stress on neuronal functioning as well as the ability of long-term citalopram administration to attenuate stress-induced alterations in the female rat brain.

Exposure to chronic and severe stress was confirmed by marked neuroendocrine changes, including increased basal corticosterone levels, adrenal hypertrophy (fig. 2b), reduced thymus weight (fig. 2c), and enhanced FOS expression in the PVN (fig. 3, 4l). This PVN plays a key role in the modulation of HPA axis [194,195] and the increased activation detected in this nucleus following chronic stress (fig. 3, 4l) may illustrate a condition of functional hyperactivity. Interestingly, sustained footshock stress has been shown to strongly activate the HPA axis leading to elevated plasma glucocorticoid levels[196]. The increased PVN activation observed here (fig. 4l) together with the significant adrenal hypertrophy (fig. 2b) seem to substantiate this finding and document the lack of habituation and, more importantly, the persistent activation of HPA axis in response to repeated footshock stress. In addition to the PVN however, a markedly increased FOS-ir was also found in several other cortical and subcortical structures, such as the medial orbitofrontal cortex (fig. 4a), the prelimbic cortex (fig. 4b), the infralimbic cortex (fig. 4c), and the central amygdala (fig. 4h). In contrast, a reduced neuronal activity was found in the hippocampus (in both the dentate gyrus and the CA3 area) (fig. 4f,g). Furthermore, a significantly stress-induced decreased CREB phosphorylation was

observed in the prelimbic cortex (fig. 6b), the anterior cingulate cortex (fig. 6d), and the dentate gyrus (fig. 6f). It is tempting to speculate that these immunohistochemical changes may illustrate the detrimental influence of sustained stress on brain neurochemistry.

Experimental evidence has pointed to the hippocampus as one of the main target of glucocorticoid-mediated actions [90,197,198]. In line with the latter, our results show a significant reduction of FOS-ir (fig. 4f,g) and CREB phosphorylation (fig. 5, 6f) in the hippocampal dentate gyrus of chronically stressed females. It is of interest to note that selective chronic stress-related effects has been reported in the hippocampus of different animal species, including a significant reduction of cell proliferation and dendritic atrophy [54,137,199]. There is some consensus concerning the view that glucocorticoids represent a key mediator in these processes [54] and, for this reason, it is intriguing to relate the reduction of hippocampal FOS-ir (hypoactivity) and phospho-CREB expression (decreased neuronal plasticity) to stress-induced functional and/or morphological impairments. The reduction of CREB phosphorylation however, was not limited to the hippocampus but involved also other forebrain structures (fig. 6). Phosphorylated CREB modulates the transcription of several genes involved in the regulation of neuronal plasticity [187,200,201]. Chronic stress and prolonged exposure to elevated glucocorticoid levels have been reported to reduce both BDNF and CREB expression in the brain [114,124,126,176]. The results presented here, in accordance with previous findings, seem to support an inhibitory action of repeated footshock stress on CREB phosphorylation (fig. 6), an effect possibly related to the persistent activation of the HPA axis.

One possible way to reverse stress-induced abnormalities is by long-term antidepressant treatment [54,202,203]. An important aspect of antidepressants' clinical effectiveness is represented by their ability to attenuate some of the "state-related functional abnormalities" often observed in depressed subjects, such as reduced anterior cingulate and prefrontocortical activity [97,204-207]. It has been proposed that antidepressants, by reversing these state-related abnormalities, may help to correct specific cortical-limbic deficits involved in the development and/or maintenance of affective disorders [85,208]. It is important to mention that the normalization of stress-related dysfunctions constitutes a fundamental step for successful clinical recovery [209,210]. A common abnormality observed following prolonged stress exposure and reported by approximately 50% of depressed subjects is the hyperactivity of the HPA axis [149]. Interestingly, several studies have illustrated a beneficial effect of antidepressants in correcting this disturbance in both humans and animals [54,211,212]. In line with the latter, long-term citalopram administration (20 days, i.p., 20mg*kg^{-1}*day^{-1}), although unable to prevent stress-induced increased FOS-ir in the PVN (fig 4l), effectively reduced basal (-69%) and stress-related corticosterone levels (-40%) (fig. 2a) and, more important,

attenuated footshock-induced adrenal hypertrophy in female rats (fig. 2b). In addition, SSRI treatment partially normalized chronic stress-induced FOS-ir changes in key cortical and subcortical regions primarily involved in the coordination of HPA axis activity, such as the medial orbitofrontal cortex (fig. 4a), the infralimbic cortex [213,214] (fig. 4c), the dentate gyrus [215,216] (fig. 4f), and the central amygdala [217-219] (fig. 4h). Cross-talks between prefrontocortical and limbic structures play a critical role in the modulation of HPA axis activity and the disruption of these coordinated cortical-limbic interactions has been suggested as a central mechanism involved in the development of abnormal stress response regulation [149]. Antidepressants, on the other hand, have been proposed as potential candidates to correct this dysfunction by desensitizing the HPA axis [152]. Since chronically stressed females concurrently treated with citalopram demonstrated an increased PVN FOS-ir (fig. 4l) but no adrenal hypertrophy (fig. 2b), it is intriguing to speculate that long-term antidepressant administration helped to reestablish a coordinated HPA axis regulation and, consequently, limit the adverse effects associated with the persistent stress-induced elevation of glucocorticoid levels. SSRIs have been reported to gradually desensitize the hypothalamic post-synaptic 5-HT_{1A} receptors [179], crucially involved in the regulation of CRF release [220]. One may contemplate whether this citalopram-induced desensitization and reduced CRF secretion might contribute to the decrease of basal and stress-related corticosterone levels, therefore reducing the adrenal hypertrophy otherwise observed following repeated footshock exposure (fig 2b). Although SSRI treatment seemed to attenuate the overall response of the HPA axis, citalopram did not inhibit the significant increase of PVN FOS-ir in chronically stressed females. This finding however, is consistent with previous reports documenting the participation of many different neurotransmitters in the modulation of various aspects of the stress response [221]. An intriguing possibility is that this antidepressant might prevent the development of HPA axis hyperactivity, not by desensitazing hypothalamic receptors but by attenuating the occurrence of selective abnormalities in cortical-limbic regions involved in the modulation of this stress response system. The latter may be illustrated by the ability of citalopram to prevent / reverse stress-induced FOS-ir changes in the prefrontal cortex, the amygdala, and the hippocampus. Prolonged citalopram administration may thus protect the brain against the deleterious effects of abnormal HPA axis modulation and persistently elevated glucocorticoid levels by limiting stress-mediated increase of stimulatory input from the amygdala to the hypothalamus and avoiding footshock-induced disruption of central feedback inhibition governed by the prefrontal cortex and hippocampus.

An additional mechanism underlying the therapeutic action of antidepressants involves the attenuation of structural alterations observed in depressed subjects, such as dendritic atrophy and neuronal pathology [95,98]. Remarkably, structural deficits similar to those observed in depression have also been reported in chronically stressed

animals[54,137]. As mentioned above, repeated stress was associated with a marked reduction of CREB phosphorylation, particularly in the medial orbitofrontal cortex (fig. 6a), the prelimbic cortex (fig. 6b), the anterior cingulate (fig. 6d), and the hippocampal dentate gyrus (fig. 6f). Sustained HPA axis hyperactivity as well as persistently high glucocorticoid levels have been reported to inhibit the phosphorylation of this transcription factor [176,222], suggesting an abnormal activity of this stress response axis as a key factor in the development of morphological abnormalities. It is interesting to note that while several studies have emphasized the ability of the atypical antidepressant tianeptine to correct stress-induced structural defects [54,199], preclinical investigations evaluating the effects of TCAs and SSRIs have failed to reveal any beneficial action of these latter antidepressants [145]. The results presented here seem in line with these findings, as long-term citalopram treatment failed to prevent stress-induced reduction of phospho-CREB expression in cyclic female rats (fig. 6). Notably, citalopram administration strengthened stress-induced inhibition of CREB phosphorylation. The biological importance of this effect remains obscure. A possibility however may lie in the synergistic action between serotonin reuptake inhibition and glucocorticoids. Under severe stressful conditions, the interactions between elevated serotonin and glucocorticoid levels may represent a critical event in the process that leads to the reduction of neuronal plasticity and the development of stress-induced structural impairments [54]. Citalopram, by inhibiting serotonin reuptake, prolongs the presence of this neurotransmitter in the synaptic cleft. During chronic stress however, the simultaneous exposure to elevated serotonin and glucocorticoid concentrations may result in the combination of their individual effects and have deleterious consequences for neuronal plasticity. This synergistic action may result in greater reduction of phospho-CREB expression in cyclic female rats concurrently exposed to chronic footshock and SSRI treatment, especially in frontocortical territories (fig. 6a-e), compared to chronically stressed vehicle treated animals.

*Normalization of chronic stress-induced hippocampal-HPA axis dysregulation by long-term tianeptine administration**

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***Adapted from the manuscript submitted to "Molecular Pharmacology"**

As previously stated, prolonged HPA axis activation may lead to the development of structural abnormalities in the hippocampus and prefrontal cortex [87,90,197,198]. Preclinical studies however, have also suggested a critical role of other key players, besides glucocorticoids, in the modulation of negative effects of chronic stress on neuronal plasticity. Excessive glutamate exposure, for instance, has also been associated with neurotoxicity and dendritic atrophy [73,92,198]. Accordingly, pharmacological treatments selectively targeting glutamatergic neurotransmission have been reported to be able to effectively prevent the occurrence of stress-induced structural defects [73,74,223].

Tianeptine is an effective antidepressant that, in contrast to SSRIs and tricyclic agents, does not act through the inhibition of serotonin reuptake. On the contrary, one mechanism by which this atypical antidepressant is believed to exert its beneficial effect is by stimulating the removal of serotonin from neuronal terminals [150]. Recently however, some contention has emerged regarding tianeptine's mode of action, raising some doubts concerning its selectivity for serotonergic transmission. New preclinical data, for instance, seem to implicate the excitatory amino acid glutamate as an additional target of this drug [54,151]. It is of interest to note that serotonergic and glutamatergic systems are closely interconnected and superimposed. Moreover, serotonin plays a central role in the modulation of glutamate release [54]. Despite the specific nature of the molecular substrates underlying tianeptine's therapeutic action, this relationship between serotonin and glutamate remains of particular interest. The latter holds particularly true with respect to stressful conditions as these are associated with massive release of serotonin, glutamate, and corticosteroids [54]. Under sustained stressful conditions, prolonged interactions between stress hormones and neurotransmitters may thus present a serious neuronal caveat. It has been suggested that the simultaneous and persistent elevation of serotonin, glutamate, and glucocorticoids levels may facilitate the development of neuronal defects [54,83,88,224,225]. Through the removal of serotonin from the synaptic cleft and/or targeting the glutamatergic system, tianeptine may thereby attenuate the deleterious interaction between neurotransmitters and stress hormones and

the occurrence of structural defects such as dendritic atrophy and reduced granule cell proliferation.

Long-term tianeptine treatment resulted, in cyclic female rats, in a significant increase of basal FOS-ir in the medial orbitofrontal cortex (fig. 4a), the prelimbic cortex (fig. 4b), the anterior cingulate cortex (fig. 4d), the central (fig. 4h), the lateral (fig. 4i), and the basolateral amygdala (fig. 4j). These effects might be related to the "serotonin reuptake-enhancing" nature of this antidepressant. Long-term tianeptine administration appears to prevent footshock-induced immunohistochemical changes in the hippocampus and hypothalamus, supporting previous reports concerning the beneficial effect of this antidepressant on stress-induced neuronal abnormalities in males [54,199]. More important, chronically stressed females concomitantly treated with this atypical antidepressant showed a limited increase of FOS-ir in the PVN compared to both vehicle treated non-stressed and stressed animals (fig. 4l). In further support of this "anti-stress" effect, tianeptine markedly decreased adrenal weight in non-stressed females and, more importantly, prevented footshock-induced adrenal hypertrophy in chronically stressed animals (fig. 2b).

Prevention of stress-induced HPA axis hyperactivity seems to represent a fundamental step in tianeptine's mechanism of action. Whereas both acute and prolonged footshock exposure have been related to increased PVN and HPA axis activation (see chapter 1 and 2), long-term tianeptine administration significantly lowered stress-induced FOS-ir in this hypothalamic nucleus (fig. 4l) and prevented footshock-induced adrenal hypertrophy (fig. 2b). The pharmacological profile of this antidepressant, together with analysis of its neurochemical effects, may provide valuable insights into the mechanisms through which tianeptine regulates HPA axis function. While serotonin is known to play a central role in the modulation of HPA axis activity in response to stress [202,226,227], tianeptine-induced enhancement of serotonin reuptake may directly limit stress-induced HPA axis activation. Alternatively, prolonged antidepressant treatment may also interfere with glutamate action in the synaptic cleft[151]. Like serotonin, this excitatory amino acid is involved in the regulation of stress response[194,195,228], yet it is also a major candidate in the modulation of stress-induced hippocampal neurotoxicity [54,83,88,224,225]. By targeting simultaneously serotonin and glutamate neurotransmission, tianeptine may attenuate the deleterious effects associated with massive neurotransmitter release following stressful conditions. Besides the role played by serotonin and glutamate however, sustained exposure to high glucocorticoid concentrations represents another key factor in the development of neuronal defects [54,87]. Chronically elevated glucocorticoid levels may result from an abnormal modulation of HPA axis response, possibly caused by impaired hippocampal feedback inhibition [229-231]. Stress and glucocorticoid have been reported to reduce BDNF expression [125,126,232], a fundamental element in the regulation of hippocampal neurogenesis and plasticity. This

action may ultimately lead to dendritic atrophy and reduced hippocampal metabolism, disrupting the ability of this limbic structure to efficiently regulate HPA axis function. A possible molecular mechanism underlying tianeptine's beneficial action in the hippocampus involves the attenuation of stress-induced reduction of CREB phosphorylation (fig. 6f). Our results confirm the inhibitory influence of repeated footshock stress on CREB phosphorylation in both male [176] and female rats (fig. 6). Given the critical role of CREB in the modulation of BDNF transcription [81], this finding may provide a possible molecular pathway underlying stress-induced hippocampal structural defects. Long-term tianeptine treatment, on the other hand, significantly prevented stress-induced reduction of CREB phosphorylation in the dentate gyrus (fig. 6f). It is intriguing to speculate that tianeptine, by attenuating stress-induced reduction of neuronal plasticity, may prevent the development of structural and functional abnormalities in the hippocampus, restoring its ability to efficiently regulate the activity of the HPA axis and thereby limiting footshock-induced hyperactivity of this critical stress response system.

*Prevention of chronic stress-induced reduction of neuronal plasticity by long-term reboxetine treatment**

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Although in the past decade attention has been largely focused on the involvement of serotonin in the pathophysiology and treatment of depression, the abnormal activity of other neurotransmitter systems might underlie some of the functional and structural deficits reported in depressed subjects. As growing clinical literature has proven, affective disorders are not merely serotonin-related illnesses but diverse and complex pathologies involving dysfunctions in multiple neurotransmitter systems. Abnormalities of noradrenergic function, for instance, have already been established in depression and this neurotransmitter system is becoming an interesting candidate in the development of new and, hopefully, more efficient pharmacological treatments [168,233]. Interest in these compounds is mostly attributable to the central role of norepinephrine in the modulation of stress response [234-236] and strong strong evidence supporting the relationship between alterations in the noradrenergic system and behaviors of fear and anxiety [237,238]. These findings have thus generated the hypothesis that some of the symptoms seen in subjects with affective disorders may be related to abnormalities of noradrenergic neurotransmission [239-243]. Since abnormal noradrenergic regulation may participate to the development of stress-related psychiatric disorders, we decided to explore the neurochemical changes induced by long-term reboxetine administration and examine the ability of this antidepressant to correct chronic footshock-induced cortical-limbic alterations.

Reboxetine is a highly selective noradrenaline reuptake inhibitor and represents the first of a new generation of antidepressant agents with specificity for the noradrenergic system [168,233]. More importantly, the selectivity of this compound allows the study of neurochemical alterations induced by long-term stimulation of the noradrenergic system. Due to the recent introduction of reboxetine in clinical practice however, only a few preclinical studies have investigated the neurochemical alterations associated with its prolonged administration [171,173,212]. This lack of data has limited our understanding of the intracellular cascades involved in the modulation of the therapeutic effects of this antidepressant. Surprisingly, a reduction of cortical phospho-CREB expression has previously been documented in response to long-term reboxetine administration in rats [244]. This finding however, seems in conflict with various studies reporting a general increased CREB transcription and phosphorylation in different

cortical and subcortical structures, including the frontal cortex and the hippocampus, following long-term antidepressant treatments [76,77,117,119]. Our results, in non-stressed cyclic females, seem in line with these latter findings, as illustrated by the selectively enhancement of CREB phosphorylation, following chronic reboxetine administration, in the medial orbitofrontal (fig. 6a), the prelimbic (fig. 6b), the infralimbic (fig. 6c) and the anterior cingulate cortex (fig. 6d). No changes were found in other cortical or subcortical structures (fig. 6). The discrepancy between our and Manier's results remains unknown. However, while Manier and colleagues performed their study in male rats [244], we decided to carry out our investigation in cyclic female animals. This important methodological difference may thus account for the discrepancy observed between the two studies. Ovarian hormones have been reported to affect the intracellular pathways modulating synaptic plasticity [245-247] and stimulate the transcription of several genes including BDNF and tyrosine hydroxylase [248-253], the limiting enzyme in the biochemical cascade regulating norepinephrine synthesis. It is plausible that the presence of estrogen and/or progesterone might affect the immunohistochemical changes induced by long-term reboxetine administration. Current studies are being performed to investigate the role of ovarian hormones in the transduction of stress-related signals and the modulation of reboxetine-mediated neurochemical adaptations.

Long-term reboxetine treatment attenuated chronic footshock-induced increased FOS-ir in cortical regions, including the medial orbitofrontal (fig. 4a) and infralimbic cortex (fig. 4c). However, it did not normalize stress-induced FOS-ir changes in subcortical regions, as documented by the significant increased neuronal activity detected in the central amygdala (fig. 4h) and paraventricular hypothalamic nucleus (fig. 4l). The amygdala and the PVN, together with the locus coeruleus, represent key components of the noradrenergic stress response system [234,237,254]. It is of interest to note that the increased neuronal activity in the central amygdala following reboxetine treatment seems to be related to the stimulation of noradrenergic function caused by the norepinephrine reuptake-enhancing nature of the antidepressant, as both control and stressed rats reported a similar induction FOS-ir (fig. 4h). In contrast, the enhanced FOS-ir in the PVN appears to depend on the activation of this nucleus by exposure to threatening conditions and not upon the pharmacological profile of the drug, as the increased neuronal activity was found only in chronically stressed rats (fig. 4l). Moreover, long-term reboxetine treatment was not able to correct the significant reduction of FOS-ir detected in the hippocampus following repeated footshock stress (fig. 4f,g).

In addition to altered patterns of FOS-ir, norepinephrine reuptake inhibition also induced marked changes in the level of CREB phosphorylation. Chronically stressed females treated with reboxetine showed a significant enhancement of phospho-CREB expression in the medial orbitofrontal cortex (fig. 6a), the prelimbic cortex (fig. 6b), the

infralimbic cortex (fig. 6c), the anterior cingulate cortex (fig. 6d), the hippocampal dentate gyrus (fig. 6f), and the central nucleus of the amygdala (fig. 6g) compared to stressed animals only treated with vehicle. Long-term antidepressant administration also enhanced phospho-CREB immunoreactivity above baseline in various prefrontocortical areas such as the medial orbitofrontal cortex (fig. 6a), the prelimbic cortex (fig. 6b), the infralimbic cortex (fig. 6c), and the anterior cingulate cortex (fig. 6d). Remarkably, reboxetine administration reversed footshock-induced reduction of CREB phosphorylation in both cortical and subcortical structures (fig. 6). These findings seem thus to substantiate a positive action of reboxetine on neuronal plasticity, counteracting the detrimental influences of persistent footshock stress. Prolonged stress as well as sustained elevation of glucocorticoid levels have been reported to down-regulate BDNF expression [125,126,232]. Reboxetine, by reversing footshock-induced reduction of CREB phosphorylation, might attenuate stress-induced inhibition of BDNF expression. This may in turn provide selective cortical and subcortical structures, involved in the modulation of stress response, with the necessary plasticity needed to cope with persistent adverse conditions and avoid the development of abnormal HPA axis activity. It is also important to note that prevention of HPA axis hyperactivity is not obtained by desensitizing the PVN, as shown by the significant induction of FOS-ir in this hypothalamic nucleus in females simultaneously subjected to footshock and reboxetine treatment.

Conclusions and limitations

Limitations

Before drawing any final conclusions, one must first always consider the limitations of the experimental design. A few points of interest are discussed below.

A major point of consideration lies in the fact that, in this study, use was made of cyclic female rats. A confounding element in the interpretation of the results was represented by the hormonal state of the animals. Intracellular transduction cascades as well as the neurocircuits regulating stress response are prime targets for ovarian hormone action [255-257]. In turn, release of sex steroids is also influenced by adverse experiences [258]. Estrogen and progesterone may thus play a critical role in determining the immunohistochemical adaptations observed here following long-term footshock exposure and / or antidepressant treatment.

Although not a drawback of the experimental design, we have chosen not to address several issues. For instance, immunohistochemical changes induced by antidepressant treatments in midbrain regions such as the raphe nuclei and the locus coeruleus were not discussed here. As these regions contain the majority of serotonergic and noradrenergic cell bodies, these could provide important insights into the acute actions of stress and antidepressant treatments. Nevertheless, we preferred to focus on those alterations occurring in cortical and subcortical regions instead, since our goal was to investigate the neurobiological mechanisms underlying long-term stress exposure and / or antidepressant administration. Cortical-limbic regions, besides receiving strong serotonergic and noradrenergic projections, are also fundamentally involved in the regulation of stress, cognitive and emotional responses. They therefore most likely represent more suitable targets in the understanding of the molecular events involved in the development of neuronal impairments and the neurobiological mechanisms mediating antidepressant response.

An additional point of consideration regards the extent of this data analysis. In this paper we do not provide inter-drug comparisons (as stated earlier we used a 2x2 analysis) at the molecular level since our main objective was limited to exploring the ability of specific antidepressants to attenuate chronic stress-induced neurohistochemical abnormalities. Future research however would benefit from an in-depth investigation consisting of a comparative view between their underlying neurochemistry.

As it goes beyond the scope of this study to elaborate upon these latter issues, we acknowledge their relevance within this research. Additional studies are currently being performed to highlight these aspects.

Conclusions

In the present chapter, we illustrate how different antidepressants, characterized by selective and sometimes antagonistic pharmacological profiles, carry out their neurochemical effects by targeting similar intracellular substrates albeit through different mechanisms. Our findings suggest that a critical step in citalopram's mode of action may be constituted by the attenuation of stress-induced functional cortical and subcortical abnormalities. Although not effective in reversing stress-induced phospho-CREB expression changes, long-term SSRI administration did demonstrate positive effects in correcting footshock-induced FOS-ir changes and preventing HPA axis hyperactivity in female rats. The reduction of stress-induced PVN activation and the prevention of hippocampal abnormalities may represent a central step in tianeptine's therapeutic action. These effects may account for tianeptine's ability to prevent the development of HPA axis hyperactivity and avoid persistent exposure to elevated glucocorticoid concentrations. By strongly enhancing CREB phosphorylation, reboxetine may reverse chronic stress-induced reduction of neuronal plasticity, thereby promoting brain structural flexibility and rapid adaptive changes of internal homeostasis in response to prolonged stress exposure. It is of interest to note that all three antidepressant prevented the development of abnormal HPA axis activity.

The maladaptive consequences of stress on neuronal integrity render it one of the primary pathological factors involved in the etiology of stress-related psychiatric disorders. Its detrimental influences on neuronal functioning however, may also account in part for the limited therapeutic power of antidepressants. Given the complex nature of the association between stress, psychiatric disorders, and the mode of action of antidepressants, elucidation of these interactions is of great relevance yet unlikely to be unraveled in the near future. Considering the limited efficacy of today's antidepressants, a full understanding of the mechanisms underlying the contribution of stress on psychopathology, is essential for the development of novel, more successful treatments. This however, shall not prove an easy task, due primarily to our poor comprehension of the mechanisms involved in the development of these psychiatric illnesses and the lack of a clear understanding of the neurobiological substrates mediating the therapeutic effects of antidepressants.

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5

Summary and Conclusions

"Imagination is more important than knowledge"

Albert Einstein

Current treatments for depression are inadequate for many individuals and progress in understanding the neurobiology of this or other affective disorders is slow. Nevertheless, several promising hypotheses concerning the mechanisms underlying stress-induced pathology and antidepressant response have recently been formulated. These have been largely based on the dysregulation of the HPA axis and implicate abnormal glucocorticoids, neurotrophic factors and CREB regulation. The data presented in this thesis seem to corroborate many of these aspects, although our findings also suggest some surprising effects of stress with regard to gender discrepancies. The fact that stress, particularly when prolonged and severe, affects both functional and structural neuronal integrity has been well established and is illustrated in chapter 1. However, the molecular mechanisms involved in these stress-mediated adaptations appear to differ between male and female rats as presented in chapter 2. Nevertheless, following long-term antidepressant treatments, females seem to express, similar to males, attenuation of some of the deleterious consequences associated with sustained stress exposure (chapter 3).

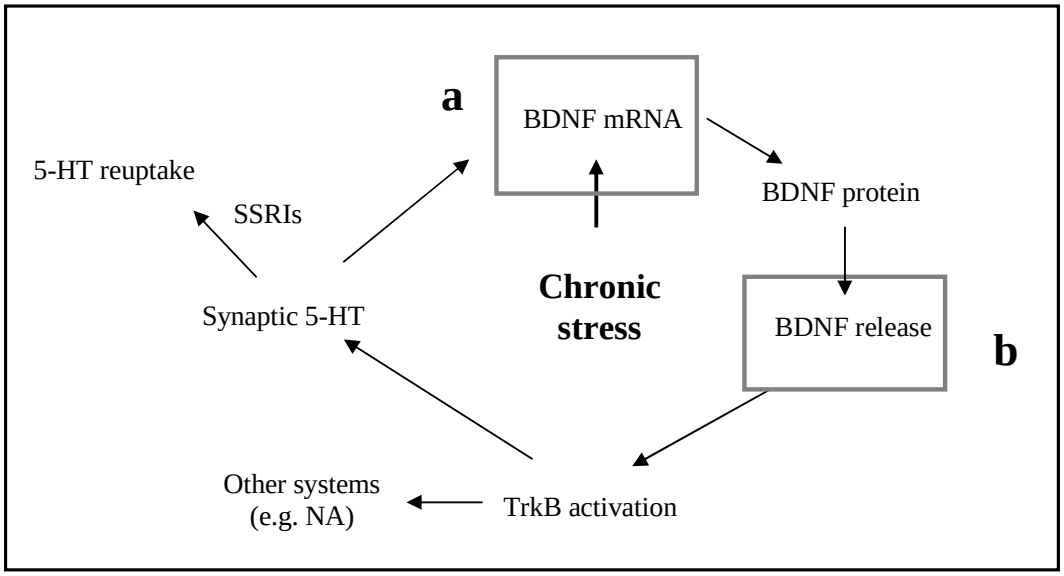

Figure 1

With respect to the HPA axis, CREB and neurotrophin aspects of stress-induced pathology, the data illustrated in chapter 1 seems in line with reports in support of the neurotrophin hypothesis of depression (figure 1). Repeated stress, in fact, selectively targeted the regulation of (BDNF)-ERK1/2-CREB cascade in male rats. In general chronic stress has been proposed to cause neuronal abnormalities by impairing coordinated HPA axis regulation [1-3]. Accordingly, neuroendocrine changes observed in chronically stressed males were in line with persistent HPA axis hyperactivity. One might speculate that prolonged elevation of glucocorticoid concentrations could represent a crucial predisposing factor in the development of multiple abnormalities in the regulation of

BDNF-ERK1/2-CREB pathway, such as persistent, and possibly uncontrolled, ERK1/2 activation and reduced CREB phosphorylation.

ERK1/2 and CREB play fundamental roles in the transduction of neurotrophin-related signals [5-9]. Recent studies however, have also implicated these proteins in the modulation of cognitive processes and the induction of long-lasting neuronal plasticity[10-13]. A crucial aspect of these complex processes involves activity-driven induction of new gene expression. This in turn is required for long-lasting potentiation of synaptic transmission associated with learning and memory [9,14-16]. Transcription of genes such as BDNF is regulated by patterns of CREB phosphorylation [9].

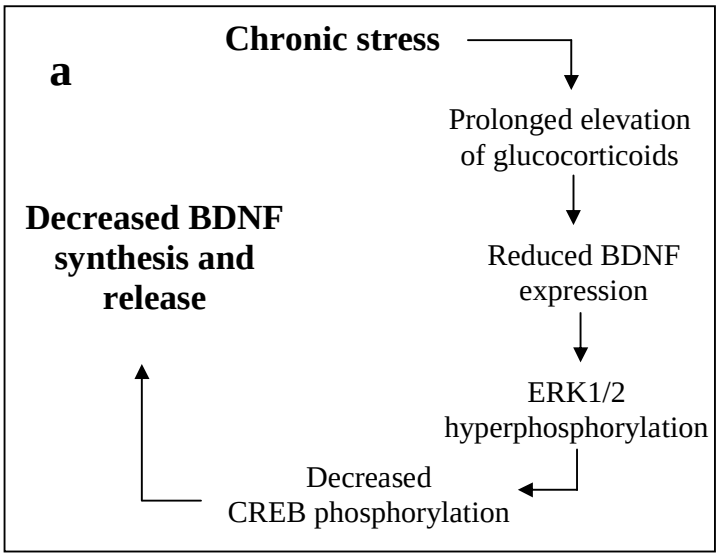

Figure 2

Due to the dependency between CREB phosphorylation and BDNF expression, we propose that the observed reduction of phosporylated CREB levels might result in downregulated BDNF transcription, thereby depriving neurons of this fundamental neurotrophic factor (figure 2). CREB phosphorylation depends by the activation of selective protein kinases such as ERK-1 and -2 [17,18] and an abnormal interaction between these kinases and their effector may also result in a reduction of BDNF expression. In accordance, prolonged ERK phosphorylation has been reported to negatively regulate CREB expression and phosphorylation [19]. Furthermore, due to the role of ERK1/2 in the regulation of cytoskeletal integrity, persistent and uncontrolled activation of these kinases may lead to hyperphosphorylation of various cytoskeletal proteins that could ultimately weaken dendritic structure, especially in synaptic terminals where cytoskeletal proteins are particularly abundant [20-22]. In conclusion, chronic stress-induced phospho-ERK1/2 and phospho-CREB abnormalities may limit BDNF availability, disrupting neuronal plasticity and facilitating the development of neuronal defects.

To date, the exact mechanisms involved in the development of stress-induced ERK1/2 and CREB abnormalities remain largely unknown. As previously stated however, an important factor in this process includes the persistent elevation of circulating glucocorticoids. Adrenal steroids have been reported to inhibit BDNF expression [23] and although short-term reductions of BDNF availability may be compensated for by alternative adaptations, we believe that prolonged downregulation of this neurotrophin results in cellular dysfunctions. When such disturbances continuously occur in the BDNF pathway, this could in turn lead to persistently decreased BDNF synthesis and release and, ultimately, reduced neuronal plasticity.

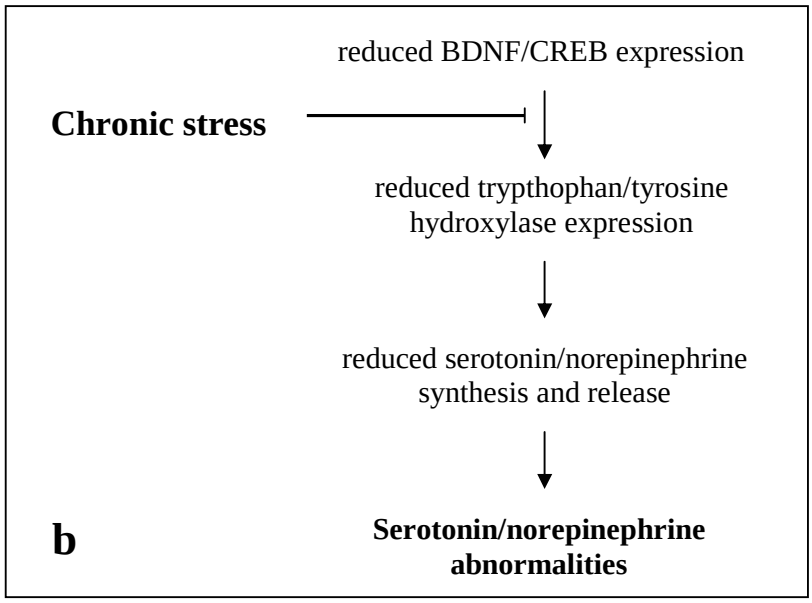

Figure 3

In human studies, prolonged stress exposure has been associated with impaired neuronal plasticity and psychopathology. The link between these two phenomena has always been fragmentary, although it has been well established that stress represents an important predisposing factor for the development of a wide variety of psychiatric illnesses, including anxiety and depression. These disorders are characterized by multiple serotonergic/noradrenergic defects and marked gender-related prevalence. It is of interest to note that CREB and BDNF play a central role in the expression of two enzymes involved in serotonin and norepinephrine synthesis, such as tryptophan and tyrosine hydroxylase [24-26] (figure 3). In addition to direct negative effects on neuronal plasticity (reduced CREB and BDNF expression), chronic stress may also affect neurotransmitter synthesis, which could indirectly lead to impaired serotonergic and noradrenergic function. Recent reports of significantly reduced BDNF concentrations in the CSF of depressed subjects support the link between decreased availability of this neurotrophin and associated psychopathology.

Despite a growing body of literature that emphasizes the greater female sensitivity to stress [27-29] and the higher prevalence of psychiatric disorders in women [30,31], our results suggest a more complex situation. Whereas chronically stressed males revealed increased cortical-limbic FOS-ir, abnormal prefrontocortical ERK1/2, and reduced cortical and subcortical phospho-CREB expression, stressed females only showed a decreased cortical-limbic FOS-ir. These findings might thus implicate that repeated footshock exposure represents a more severe stressor for male than for female rats. As it has been previously suggested that responses to stress could be stressor-specific [32,33], it cannot be excluded that the differential gender-dependent patterns of protein expression and phosphorylation seen here are specific to this particular paradigm and/or the immunohistochemical markers used in this analysis. Studies reporting higher stress sensitivity in female rats and greater prevalence of stress-related psychiatric disorders in women [30,31], are thus in stride with the data presented in this thesis which indicate milder stress-induced abnormalities in cyclic female rats (chapter 2). A putative explanation for these conflicting results is provided by the presence, in females, of ovarian hormones, which may attenuate some of the deleterious effects of stress on neuronal plasticity. Sex steroids have been shown to protect neurons from a wide variety of insults and their intracellular actions appear to be mediated by the same signaling pathways underlying neuronal plasticity such as the MAPK cascade. Accordingly, estrogen has also been reported to stimulate CREB phosphorylation [34-36]. It is thus possible that the negative influences of repeated footshock exposure on BDNF expression, ERK1/2 activation and CREB phosphorylation might have been attenuated by the presence of ovarian hormones (chapter 2). The protective effects of estrogen upon increased severity of the stressor, due to longer exposure to aversive conditions, may however be insufficient to counteract or override the negative influences under more severe stressful circumstances (chapter 3). Remarkably, the established gender-related prevalence of affective disorders begins with puberty and continues until menopause, a period during which ovarian hormones cyclically fluctuate. Despite their reported neuroprotective effects, sex hormones have also been considered as main candidates for this differential susceptibility to psychiatric illnesses, yielding a paradox.

Despite extensive research during the past two decades, our knowledge of the etiological factors and molecular mechanisms underlying the differential sensitivity to stress between males and females still remains fragmentary as well as the link between ovarian hormones and psychopathology. Although implicated, ovarian hormones do not account for all of the differences between male and female brains. Other factors besides simple hormonal fluctuations may also play a contributing role, such as:

- gender-related structural and functional differences between the male and the female brain;
- gender-related differences in intracellular transduction pathways modulating specific neuronal functions;
- greater HPA axis activity and higher glucocorticoid levels in females.

Antidepressants such as selective monoamine reuptake inhibitors represent the treatment of choice for affective disorders such as anxiety and depression. Selective serotonin and norepinephrine reuptake inhibitors (SSRIs and NARIs) have received particular interest since stimulation of serotonergic and/or noradrenergic transmission has been associated with clinical recovery [37]. Recently however, newer medications characterized by atypical pharmacological profiles (i.e. tianeptine) have been added to the already large number of drugs available for the treatment of mood disorders. Altering monoamine availability in the synaptic cleft represents only one of the mechanisms by which these compounds exert their beneficial effects. By modifying monoamine levels, antidepressants also enhance neurogenesis and neuronal plasticity[38,39], which may in turn help to correct functional and structural dysfunctions involved in the development and/or maintenance of affective disorders [40-43]. It is interesting to note that although characterized by different and, sometime, opposite mechanisms of action, citalopram, tianeptine, and reboxetine share surprisingly similar clinical efficacy. This suggests that different antidepressants may correct complex neuronal dysfunctions by acting at different levels. Tianeptine, for instance, a serotonin reuptake enhancer, appears to selectively target the HPA axis and the hippocampus, limiting stress-induced activation of the former and protecting the latter from the deleterious consequences of elevated glucocorticoid concentrations. Similarly, our findings show that citalopram, a serotonin reuptake inhibitor, was able to attenuate stress-induced abnormal HPA axis regulation. Besides the hippocampus however, citalopram may exert its beneficial effects by correcting malfunctions in the neurocircuitry underlying the modulation of this critical stress response system. Reboxetine, a norepinephrine reuptake inhibitor, appears instead to exert its positive action by counteracting the negative influence of repeated stress on neuronal plasticity, an effect that appears to be mediated by the stimulation of CREB phosphorylation. These findings suggest that antidepressants characterized by different pharmacological profiles may all target those “state-related functional and/or structural abnormalities” involved in the development and/or maintenance of psychopathology.

It is clear from this discussion that although research is progressing, we are still a long way from fully understanding the neurobiological substrates controlling mood and emotions under normal and pathological conditions. Given the pervasive deficits that characterize affective disorders, it is likely that the mechanisms by which currently

available treatments attenuate depressive symptoms involve the modulation of the activity of numerous brain regions, neurotransmitter systems, and various peptides besides the ones discussed here. Progress in functional brain imaging might represent a powerful tool to identify differential activation patterns and gross circuits in the brain that are affected in depressed subjects. In addition to gender-related dimorphisms in the healthy and pathological brain, the ability to image the living human brain might one day even allow us to investigate BDNF, CREB and newly born neurons, thereby helping direct research into the molecular and cellular mechanisms involved. Ultimately however, the key to elucidating the riddle of the specific disorder mysteries lies in genetics and prevention. Due to the heterogeneous nature of such disorders, many patients remain treatment-resistant. Identifying specific genetic variations that confer risk or resistance for depression will likely represent the essential step in categorizing depression based on underlying biology. In turn, these advances will lead to new approaches to stress-pathology research and perhaps development of definite treatments and eventually cures or, even better, preventive measures.

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Samenvatting

Stress heeft invloed op zowel de fysiologie als de biochemie van de hersenen. De normale stressreactie is van relatief korte duur, van enkele uren tot dagen, en bestaat uit een korte autonome respons die wordt gevolgd door een neuroendocriene reactie van langere duur. Pathologische (chronische) stress gaat gepaard met een langdurige neuroendocriene respons, o.a. verhoging van basale plasma cortisol (corticosteron) spiegel, en heeft schadelijke gevolgen voor het functioneren en de anatomie van de hersenen. Chronische stress en de daaraan gerelateerde structurele en functionele veranderingen in de hersenen worden gezien als de belangrijkste oorzaak van psychiatrische stoornissen zoals angst en depressie.

Het eerste hoofdstuk van dit proefschrift beschrijft de neuroendocriene en immunohistochemische veranderingen die worden geïnduceerd in de hersenen van mannelijke ratten na acute en chronische stress. Voor de inductie van stress pathologie is gebruik gemaakt van een paradigma dat wordt gekarakteriseerd door zowel fysieke als psychologische stress. De dieren werden gedurende 3, 14 of 21 dagen in een voetschok-kooi gezet, waarin ze dagelijks 5 electrische schokken kregen (fysieke stress) en gedurende 15 tot 120 minuten verbleven (psychologische stress). De gevolgen van deze stress op cellulair en moleculair niveau zijn immunocytochemisch onderzocht met antilichamen gericht tegen eiwitten die tot expressie worden gebracht na neuronale activatie (Fos) of een rol spelen bij de regulatie van neuronale plasticiteit (phospho-ERK1/2 and phospho-CREB). De resultaten van deze studies leveren aanwijzingen op voor het ontstaan van stressgeïnduceerde morfologische en functionele defecten in corticale en limbische gebieden, waaronder de prefrontale cortex, hippocampus, en hypothalamus. Chronische stress ging gepaard met verhoogde Fos expressie in de paraventriculaire nucleus van de hypothalamus (PVN) en hypertrofie van de bijnier. Beide zijn aanwijzingen voor hypothalamo-pituitary-adrenal (HPA) hyperactiviteit die ook depressieve patiënten karakteriseert. Een verhoogde prefrontale neuronale activiteit na chronische stress, gemeten met Fos expressie, was geassocieerd met een selectieve accumulatie van pERK1/2 in de distale dendriet. Fos en phospho-CREB expressie in de cingulate cortex was gereduceerd na chronische stress. Daarnaast worden data gepresenteerd die wijzen op een gecompromitteerde cortico-limbische functie en neuronale plasticiteit.

Klinisch onderzoek toont dat de zogenaamde “life events” - chronische stress door het verlies van een partner of kind, werk, of andere traumatische ervaring - een belangrijke rol spelen bij het ontstaan van affectieve stoornissen; psychiatrische ziektes die worden gekenmerkt door disfunctioneren van corticale en subcorticale neuronale substraten en een sterke geslachtsgerelateerde prevalentie. Bij vrouwen komen deze aandoeningen 2 tot 3 keer vaker voor dan bij mannen. Over de reden voor deze verschillen in prevalentie, en de moleculaire en cellulaire gevolgen van chronische stress bij mannelijke en vrouwelijke proefdieren is nog weinig bekend en zijn daarom onderwerp van studie in hoofdstuk 2. Geslachtsspecifieke veranderingen in de cerebrale Fos, phospho-ERK1/2, en phospho-CREB expressie suggereert dat verschillende cellulaire mechanismen betrokken zijn bij stress adaptatie in mannelijke en vrouwelijke ratten. In de discussie wordt ingegaan op de

mogelijke klinische betekenis van deze waarnemingen, voor stress gevoeligheid van mannen en vrouwen en ontstaan van psychopathologie.

Een belangrijk aspect van de etiologie van affectieve stoornissen is een verstoorde functie van het serotonerge (5-HT) neurotransmitter systeem. Klinische studies hebben aangetoond dat verbetering van de serotonine huishouding in de hersenen gepaard gaat met een vermindering van symptomen van affectieve stoornissen en de serotonine heropname remmers (SSRI's) zijn daarom eerste keuze antidepressiva voor de behandeling van deze psychiatrische stoornissen. Echter de psychiater heeft tegenwoordig de beschikking over een breed arsenaal aan antidepressiva met verschillende, soms met aan SSRI's tegengestelde werkingsmechanismen. Het belangrijkste, maar zeker niet het enige effect van antidepressiva is de verhoging van de beschikbaarheid van de neurotransmitter serotonine in de synaps. Daarnaast beïnvloeden antidepressiva de neurogenese – het proces van vorming van nieuwe neuronen in de hippocampus en prefrontale cortex – en ze worden in verband gebracht met handhaving en regulatie van neuronale plasticiteit. Met name deze laatst genoemde processen worden geassocieerd met een herstel van structurele en functionele defecten in de hersenen van depressieve patiënten. Diermodellen hebben een waardevolle bijdrage geleverd aan het begrip van cellulaire en moleculaire pathofysiologische mechanismen die ten grondslag liggen aan het ontstaan van affectieve stoornissen en de mogelijke rol in de cellulaire fysiologie van antidepressiva. Maar, er zijn grote discrepanties tussen diermodellen en de pathofysiologie van deze psychiatrische stoornissen. Het meeste preklinische onderzoek is verricht in mannelijke proefdieren terwijl vrouwen de hoogste prevalentie van affectieve stoornissen rapporteren en de belangrijkste gebruikers zijn van antidepressiva. Ook worden antidepressiva zelden getest onder omstandigheden waarin de dieren voor of tijdens de behandeling zijn blootgesteld aan chronische stress, terwijl patiënten zowel voor als tijdens de farmacotherapeutische behandeling ervaring van stress rapporteren. Dit impliceert dat stress mogelijk niet alleen het ontstaan van affectieve stoornissen verklaart maar ook invloed heeft op de kans van slagen van farmacotherapie. In hoofdstuk 3 wordt het effect beschreven van drie antidepressiva met verschillende werkingsmechanismen, citalopram (SSRI), tianeptine (een serotonine reuptake enhancer), en reboxitine (een selectieve noradrenaline reuptake inhibitor), op cellulaire en moleculaire neurobiologische processen in vrouwelijke proefdieren die tijdens de farmacotherapie werden gestresst. De studie toont significante verschillen in de cerebrale Fos en phospho-CREB expressie van deze antidepressiva. Tianeptine toonde onder deze omstandigheden een selectief effect in systemen die zijn betrokken bij de regulatie van de HPA-as. Het proefschrift eindigt met een algemene discussie waarin wordt beschreven welke betekenis de waarnemingen hebben voor regulatie/handhaving van neuronale plasticiteit in relatie tot het ontstaan en de behandeling van affectieve stoornissen, in het bijzonder depressie.

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