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").

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.

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. 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. Although sympathetic nervous system activity is regulated by the frontal cortex and the hypothalamus, 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. 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 all contribute to its capacity for autonomous function.

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, including cardiovascular, respiratory, renal, endocrine functions. 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. During severe stress, for instance, the vagus nerve mediates some sympathetic-like effects in the gastrointestinal system, such as the suppression of gastric secretion. Epinephrine is released into the circulation from the adrenal medulla and norepinephrine from postganglionic sympathetic nerves innervating the vascular endothelium. A complex hierarchy of central nervous system elements determines neurosympathetic and adrenomedullary activity. 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. 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 and adrenal gland. 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. 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. The NTS, subsequently, relays this metabolic sensory information to the parabrachial nucleus, the locus coeruleus, the amygdala, and the hypothalamus. Notably plasma epinephrine levels following aversive stimulation vary depending on stressor intensity and although only a small proportion of norepinephrine release diffuses into the bloodstream, circulating norepinephrine is a useful estimate of neurosympathetic activity.

The Hypothalamic-Pituitary-Adrenal (HPA) axis

Prominent amongst the reactions triggered by stress is the release of glucocorticoids by the adrenal glands. 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, as it receives afferent sensory information from several midbrain, cortical, and limbic structures. This nucleus is divided into several clearly distinguishable subregions, 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. The parvocellular subregion also contains the majority of CRF-synthesizing neurons, which modulate the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary. Arginine-vasopressin neurons in PVN also play a role in ACTH secretion.

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) \(^\text{13,23}\). 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 \(^\text{37}\). In non-stressful situations, both CRF and AVP are secreted in the portal system in a circadian, pulsatile fashion \(^\text{38,39}\). During acute stress however, the amplitude and synchronization of CRF and AVP pulsations markedly increase, resulting in increases of ACTH and corticosteroid secretory episodes \(^\text{37}\). 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 \(^\text{40-42}\). They also exert powerful inhibitory influences on ACTH secretion \(^\text{43,44}\). The magnitude of the HPA axis response elicited by hypothalamic neurons is thus limited by neuronal and hormonal mechanisms \(^\text{24,44}\). 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 \(^\text{46}\). 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. There are two different CRF receptors in the brain (CRF₁ and CRF₂). Differential distribution and affinities of these receptors for their specific ligands allow this neuropeptide to exert multiple actions in the brain. The CRF₁ receptor has a higher affinity for its ligand and is most abundant in the neocortex, hypothalamus, amygdala and hippocampus whereas the CRF₂ receptor has a lower affinity and is located in specific subcortical structures such as the amygdala and the hypothalamus.

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. 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). 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.

A fast growing body of clinical and preclinical reports suggests a relationship between alterations in the norepinephrine system and stress. 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. Numerous studies have supported the notion that stress modulates LC sensitivity to CRF, which may thus act as an excitatory neurotransmitter during the initiation of stress responses. Stressors increase CRF concentrations in this midbrain region whereas central administration of this neuropeptide has also been shown to activate the LC. 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.

It is of interest to note that LC neurons exhibit abundant expression of glucocorticoid receptors, 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, whereas neuroanatomical evidence has documented ample norepinephrine-CRF synaptic connections in this nucleus. 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. 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

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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. 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. 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. 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. Glucocorticoid and mineralocorticoid receptors are localized in discrete brain regions, especially in the limbic system, and exert inhibitory control over the HPA axis.

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. 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. The result of this interaction is the suppression of the expression of selective genes, including CRF, 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 through 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. 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. 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. This limbic structure modulates memory consolidation, mediates the storage of emotionally relevant information, and comprises a site of neuronal plasticity during associative learning. Two distinct neural subsystems within the amygdala seem to mediate different types of conditioned fear-related behavior. 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. 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 as this nucleus is thought to represent the final common pathway for the generation of learned fear responses.

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. “New” memories are initially labile and sensitive to disruption before being consolidated into stable long-term memories. The formation of fear-related memories in the amygdala is associated with changes of a broad array of transcriptionally regulated genes. 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\textsuperscript{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\textsuperscript{147}. CREB, in fact, represents a common target for both PI-3 kinase and the MAPK cascade members\textsuperscript{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\textsuperscript{150}.

**The hippocampus**

Several theories have proposed a role for the hippocampus in the acquisition and retrieval of contextual memories\textsuperscript{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\textsuperscript{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\textsuperscript{156}. Recent work has supported the hypothesis that the hippocampal formation modulates contextual fear conditioning by storing a conjunctive representation of context\textsuperscript{157}, assembling contextual representations, and transmitting these representations to the amygdala for association with USs\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{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. 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.

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. The extracellular signal-regulated kinases (ERKs) and its effector CREB have been shown to play a key role in hippocampal plasticity and memory formation, as documented by a rapid and transient activation of ERK and CREB in response to aversive experiences. 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.

**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. 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. 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. 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.

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. A growing amount of evidence has implicated the prefrontal cortex in the inhibition or
extinction of conditional fear. 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. 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. 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.

Exposure to acute stressful experience has been shown to facilitate classical conditioning in male rats. 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. 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. 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. This evidence supports the involvement of corticosterone in the storage of fear-related stimuli and their consolidation as long-term memories.

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. 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. 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.

Stress-related psychiatric illnesses, such as melancholic depression, have been characterized by persistent HPA axis activation, possibly due to impaired feedback-inhibition. 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. Downregulation of GRs requires extensive and prolonged exposure to extremely high levels of corticosteroids. Interestingly, MR may inhibit GR biosynthesis in the dorsal hippocampus by binding to glucocorticoid response elements present in the GR promoter region. 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. 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. 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. 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). Elevated plasma corticosteroid levels over a prolonged period may stimulate CRF systems. Glucocorticoids may also activate the PVN with a descending CRF projection to brainstem NE-containing neurons. 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. 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. 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. It has been hypothesized that chronic stress-induced GR downregulation may initially lower this “anxiety state.” 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. 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. Most findings support an underactivation of serotonergic function and a complex noradrenergic dysregulation, most consistent with overactivation of this system. 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 immunosuppression. Furthermore, CRF levels in the cerebrospinal fluid (CSF) are also elevated in these subjects. 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, marked hippocampal atrophy, and a small and hypofunctional medial frontal lobe have been reported in depressed patients.
**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 Bioethics 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\textsuperscript{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% tetrachloroquantin). 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 Na₂EDTA 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 \textsuperscript{32}P-labeled first-strand cDNA, 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 68°C 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 68°C for overnight incubation to reach a final probe concentration of 2-5 x 10^6 cpm/ml. Membranes were stringently washed with continuous agitation at 68°C 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 -80°C 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 manufacturer’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% H2O2 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% H2O2 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/0.1mm² (FOS, phospho-ERK1/2, and phospho-CREB immunoreactivity) or number of H+V intersections/0.1mm² (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 *
Neurobiology of stress

The example below illustrates the formulas and their calculations.

<table>
<thead>
<tr>
<th>BRAIN AREAS (ARS)</th>
<th>No stress</th>
<th>Acute stress</th>
<th>Chronic stress</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Absolute</td>
<td>Relative (%)</td>
<td>Absolute</td>
</tr>
<tr>
<td><strong>FRONTAL CORTEX</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prefrontal cortex (6.5mm²)</td>
<td>54±6</td>
<td>61.8±1.9</td>
<td>66±9</td>
</tr>
<tr>
<td>Anterior cingulate (2.9mm²)</td>
<td>15±1</td>
<td>13.1±0.9</td>
<td>41±6</td>
</tr>
<tr>
<td><strong>HIPPOCAMPUS</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dentate gyrus (1.25mm²)</td>
<td>12±2</td>
<td>2.08±0.31</td>
<td>10±1</td>
</tr>
<tr>
<td>CA1 (1.18mm²)</td>
<td>2±1</td>
<td>0.2±0.07</td>
<td>11±4</td>
</tr>
<tr>
<td><strong>AMYGDALA</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Central (1.0mm²)</td>
<td>7±3</td>
<td>0.88±0.31</td>
<td>39±7</td>
</tr>
<tr>
<td>Lateral (1.4mm²)</td>
<td>7±2</td>
<td>1.24±0.24</td>
<td>17±2</td>
</tr>
<tr>
<td>Basolateral (1.6mm²)</td>
<td>6±1</td>
<td>0.96±0.1</td>
<td>17±4</td>
</tr>
<tr>
<td>Medial (2.0mm²)</td>
<td>9±1</td>
<td>3.12±0.34</td>
<td>17±3</td>
</tr>
<tr>
<td><strong>THALAMUS</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Centromedial (0.8mm²)</td>
<td>11±3</td>
<td>0.76±0.29</td>
<td>40±8</td>
</tr>
<tr>
<td>Dorsomedial (1.6mm²)</td>
<td>2±1</td>
<td>0.28±0.08</td>
<td>4±2</td>
</tr>
<tr>
<td>Paraventricular (1.8mm²)</td>
<td>39±6</td>
<td>4.54±0.67</td>
<td>69±6</td>
</tr>
<tr>
<td><strong>HYPOTHALAMUS</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paraventricular (1.5mm²)</td>
<td>27±2</td>
<td>4.66±0.79</td>
<td>115±31</td>
</tr>
<tr>
<td>Dorsomedial (2.0mm²)</td>
<td>44±6</td>
<td>6.4±1.01</td>
<td>52±5</td>
</tr>
<tr>
<td><strong>CORTICAL-LIMBIC SYSTEM</strong></td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td><strong>MIDBRAIN</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medial raphe (MR)</td>
<td>6±1</td>
<td>--</td>
<td>19±5</td>
</tr>
<tr>
<td>Dorsal raphe (DR)</td>
<td>10±2</td>
<td>--</td>
<td>18±3</td>
</tr>
</tbody>
</table>

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 +
Each ARS has been multiplied by a factor 10 since the regional densities express the number of positive cells/0.1mm² (mPFC: ARS * 10 = 6.5mm² * 10). The regional relative FOS-ir can be thus calculated:

Relative regional activation:

% mPFC rat CTR: \( \frac{(\text{cell density rat CTR} \times \text{ARS})_{\text{mPFC}}}{\text{TOT rat CTR}} = \frac{(54 \times 65)_{\text{mPFC}}}{6669.6} = \frac{3510}{6669.6} = 52.6\% \)

% AC rat CTR: \( \frac{(\text{cell density rat CTR} \times \text{ARS})_{\text{AC}}}{\text{TOT rat CTR}} = \frac{(15 \times 29)_{\text{AC}}}{6669.6} = \frac{435}{6669.6} = 6.52\% \)

Example 2: Rat STR

Total cortical-limbic activation rat STR:

\[ \sum (118 \times 65)_{\text{mPFC}} + (20 \times 29)_{\text{AC}} + (6 \times 12.5)_{\text{DG}} + (4 \times 11.8)_{\text{CA1}} + (16 \times 10)_{\text{CeA}} + (11 \times 14)_{\text{LaA}} + \\
(19 \times 16)_{\text{BslA}} + (16 \times 20)_{\text{MeA}} + (7 \times 8)_{\text{CMT}} + (2 \times 16)_{\text{DMT}} + (40 \times 18)_{\text{PVT}} + (80 \times 20)_{\text{DMH}} + \\
+ (89 \times 15)_{\text{PVN}} = \text{TOT rat STR} 13053.2 \]

Relative regional activation:

% mPFC rat STR: \( \frac{(\text{cell density rat STR} \times \text{ARS})_{\text{mPFC}}}{\text{TOT rat STR}} = \frac{(118 \times 65)_{\text{mPFC}}}{13053.2} = \frac{7670}{13053.2} = 58.76\% \)

% AC rat STR: \( \frac{(\text{cell density rat STR} \times \text{ARS})_{\text{AC}}}{\text{TOT rat STR}} = \frac{(20 \times 29)_{\text{AC}}}{13053.2} = \frac{580}{13053.2} = 4.44\% \)

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).

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 ($F_{\text{absolute(ab)}}=6.3$, $p_{\text{absolute(ab)}}<0.03$) (fig. 5a). Absolute FOS-ir was found significantly increased in the AC ($F_{\text{ab}}=14.35$, $p_{\text{ab}}<0.0043$) (fig. 5b), the CA1 ($F_{\text{ab}}=5.39$, $p_{\text{ab}}<0.045$) (fig. 5c), the CeA ($F_{\text{ab}}=13.45$, $p_{\text{ab}}<0.0063$), the LaA ($F_{\text{ab}}=16.39$, $p_{\text{ab}}<0.0037$), the BslA ($F_{\text{ab}}=8.93$, $p_{\text{ab}}<0.017$) and the MeA ($F_{\text{ab}}=6.12$, $p_{\text{ab}}<0.038$) (fig. 5d), the CMT ($F_{\text{ab}}=9.54$, $p_{\text{ab}}<0.013$) and the PVT ($F_{\text{ab}}=11.42$, $p_{\text{ab}}<0.008$) (fig. 5e), the PVN ($F_{\text{ab}}=6.45$, $p_{\text{ab}}<0.032$) (fig. 5f), the MR ($F_{\text{ab}}=6.24$, $p_{\text{ab}}<0.034$) and the DR ($F_{\text{ab}}=8.46$, $p_{\text{ab}}<0.02$) (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 ($F_{\text{relative(re)}}=19.71$, $p_{\text{relative(re)}}<0.0016$) (fig. 6a), the hippocampal CA1 area ($F_{r}=5.93$, $p_{r}<0.038$) (fig. 6b), the central ($F_{r}=13.43$, $p_{r}<0.005$), the lateral ($F_{r}=6.68$, $p_{r}<0.029$) and the basolateral nucleus of the amygdala ($F_{r}=19.41$, $p_{r}<0.0017$) (fig. 6c), and the paraventricular hypothalamic nucleus ($F_{r}=8.64$, $p_{r}<0.016$) (fig. 6e). In contrast, the mPFC ($F_{r}=31.92$, $p_{r}<0.001$) (fig. 6a) and the hippocampal DG ($F_{r}=9.97$, $p_{r}<0.012$) (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 ($F_{\text{ab}}=32.26$, $p_{\text{ab}}<0.001$) (fig. 5a). A general increased absolute FOS-ir was observed in the mPFC ($F_{\text{ab}}=36.90$, $p_{\text{ab}}<0.001$) (fig. 5b), the CeA ($F_{\text{ab}}=6.52$, $p_{\text{ab}}<0.034$), the BslA ($F_{\text{ab}}=14.72$, $p_{\text{ab}}<0.005$) and the MeA ($F_{\text{ab}}=12.25$, $p_{\text{ab}}<0.0081$) (fig. 5d), the DMH ($F_{\text{ab}}=10.05$, $p_{\text{ab}}<0.013$) and the PVN ($F_{\text{ab}}=35.96$, $p_{\text{ab}}<0.001$) (fig. 5f), the MR ($F_{\text{ab}}=9.95$, $p_{\text{ab}}<0.016$) and the DR ($F_{\text{ab}}=28.65$, $p_{\text{ab}}<0.0011$) (fig. 5g). Only the DG showed an opposite effect, such as a significant decrease of absolute FOS-ir following chronic challenge ($F_{\text{ab}}=6.63$, $p_{\text{ab}}<0.033$) (fig. 5c).

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

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.

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.

**Phospho-CREB-ir**

Likewise phospho-ERK1/2 expression, phospho-CREB (ser133) immunoreactivity 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,

![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).](image-url)
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 hyperphosphorylation in medial prefrontocortical dendrites: implications for stress-related cortical pathology?*

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


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. 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. It is intriguing to speculate that repeated stress may disrupt neuronal plasticity either directly through the inhibition of BDNF expression or indirectly by down-regulating CREB phosphorylation and, subsequently, reducing BDNF transcription. 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.

Chronic stress has been shown to exacerbate a number of psychiatric disorders, including depression and anxiety, illnesses characterized by prefrontocortical abnormalities and cognitive deficits. 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. Furthermore, stress has been reported to reduce BDNF expression in the brain, 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. CREB phosphorylation is crucial for its ability to bind DNA and modulate gene expression, and is therefore essential for the transcription of BDNF. 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, 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. Abnormal ERK1/2 activity could ultimately weaken dendritic structure, especially in the synaptic terminals where cytoskeletal proteins are particularly abundant.

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.
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. Acute footshock exposure has been reported to strongly activate this hypothalamic center and elevate plasma corticosteroid levels. Short-term exposure to high glucocorticoid levels positively influence cognitive abilities, enhancing learning and promoting memory consolidation. 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 and dendritic morphology. 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. 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, associative learning and memory formation, due possibly to the role of phosphorylated CREB in the transcription of several genes such as BDNF. An intriguing possibility is that stress may impair neuronal integration through the inhibition of CREB phosphorylation. 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 \cite{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 \cite{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 \cite{280}, which in turn may promote the development of functional and morphological defects \cite{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 \cite{171,292,299}, chronic stress has been associated with reduced phospho-CREB immunoreactivity \cite{225} and BDNF expression \cite{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. Relative 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.
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. 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. 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, 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. 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. 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. 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). 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. A great number of reports have also indicated the medial prefrontal cortex as a functionally dissociable region during cognitive processing in humans. Prevalent mPFC activation has been associated with maintenance and manipulation of information, whereas AC activation has been consistently observed in situations requiring divided attention, novel responses, or the overcoming of a prepotent response. Increased AC activity has also been associated with evaluative processes during the response to conflicts, which occurs when two incompatible reactions are both compelling. Furthermore, anterior cingulate activation has been specifically correlated with the perception of the affective component of pain, particularly its “unpleasantness.” Animal studies have confirmed the involvement of this cortical region in nociceptive and emotional processing, 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. 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. 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 relative 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. 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 as well as reduced AC and hippocampal metabolism, have been detected following chronic stress exposure. 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 elevated 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. 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.
References


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