Universitat Autònoma de Barcelona

How the impact of stress changes with simultaneous administration of cocaine in rats?

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ABBREVIATIONS

5-HT: serotonin

Acb: nucleus accumbens

AcbC: accumbens core

AcbSh: accumbens shell

ACTH: adrenocorticotropic hormone

ADX: adrenalectomy

Amg: amygdala

AP-1: activator protein-1

Arc: arcuate nucleus of the hypothalamus

ASR: acoustic startle response

AVP: arginine vasopressin

BLA: basolateral amygdala

BNST: bed nucleus of the stria terminalis

cAMP: cyclic adenosine monophosphate

cDNA: complementary DNA

CeA: central amygdala

CG: central gray

Cg1: cingulate

CNS: central nervous system

CocC: cocaine-control

CocIMO: cocaine-IMO

Cpu: striatum

CpuDL: dorso-lateral striatum

CpuDM: dorso-medial striatum

CpuP: posterior striatum

CpuVL: ventro-lateral striatum

CpuVM: ventro-medial striatum

CREB: cAMP responsive element binding protein

CRF: corticotropin-releasing factor

CRH: corticotropin-releasing hormone

C_t: threshold cycle

CTF: constitutive transcription Factors

DA: dopamine

DAT: DA transporter

DM: dorsomedial nucleus of the hypothalamus

DR: dorsal raphe nucleus

dSTR: dorsal striatum

EPM: elevated plus-maze

Ex: PCR efficiency

FST: forced swimming test

GAPDH: glyceraldahyde-3-phosphate-dehydrogenase

GENLIN: generalized linear model procedure

GLM: general linear model

GR: glucocorticoid receptors

GzLM: generalized linear model

hnRNA: hetero-nuclear ribonucleic acid

HPA: hypothalamic-pituitary-adrenal

i.p.: intraperitoneal

icv: intracerebroventricular

IEG: immediate-early genes

IL: infralimbic

IMO: immobilization in boards

ISH: In situ hybridization

ITF: indulgent transcription Factors

LC: locus coeruleus

LH: lateral hypothalamus

LMM: linear mixed models

LS: lateral septum

LSD: lysergic acid diethylamide

MCN: melanocortin

MDMA: 3.4-methylenedioxymethamphetamine

ME: median eminence

MeA: medial Amygdala

MePO: *median preoptic nucleus*

mPFC: medial prefrontal cortex

MPOA: medial preoptic area

MR: mineralocorticoid receptors

mRNA: messenger ribonucleic acid

NA: noradrenaline

NF-κB: nuclear factor-κB

NGF: nerve growth factor

NMDA: Glutamate

NPY: neuropeptide Y

NTS: nucleus of the solitary tract

Orb: orbital cortex

OVLT: organum vasculosum of the *lamina terminali*

PAG: *periaqueductal gray*

PAGa: anterior *periaqueductal gray*

PAGp: posterior *periaqueductal gray*

PB: parabrachial nucleus

PCR: polymerase chain reaction

peri-PVN: *peri-PVN region*

PFA: paraformaldehyde

PFC: prefrontal cortex

Pir: piriform cortex

PKA: protein kinase

PO: medial preoptic area

POMC: proopiomelanocortin

PrL: prelímbic

PTSD: post-traumatic stress disorders

PVA: paraventricular nucleus of the thalamus

PVN: paraventricular nucleus of the hypothalamus

PVNmp: medial parvocellular of the paraventricular nucleus of the hypothalamus

qRT-PCR: quantitative real-time polymerase chain reaction

R-1h : 1 hour post-IMO

R-2h : 2 hours post-IMO

R-45: 45 minutes post-IMO

R-90: 90 minutes post-IMO

RIA: radioimmunoassay

R-IMO: end of IMO

RMg: magnus raphe nucleus

RNA: ribonucleic acid

RT-PCR: real-time quantitative polymerase chain reaction

SalC: saline-control

SalIMO: saline-IMO

SCN: suprachiasmatic nucleus

SEM: standard error of the mean

SFO: subfornical organ

SMA: sympathetic-adrenal-medullary

SN: substantia nigra

SN-VTA: substancia nigra-ventral tegmental area

SO: supraoptic

TEA: triethanolamine

THC: tetrahydrocannabinol

T_{max}: latency to maximum startle response

UCN: urocortin

V_{Avg}: voltage peak average

V_{max}: voltage maximum startle response or peak

vSUB: ventral subiculum

VTA: ventral tegmental area

FIGURES AND TABLES INDEX

SUMMARY

Exposure to stress increases the acquisition, maintenance and relapse of drug addiction, but the effects of concomitant exposure of drugs and acute severe stressors have not been explored. Our studies were conducted in adult male Sprague-Dawley rats, distributed into four groups in function of cocaine injection and exposure to immobilization (IMO). Animals were injected with saline or cocaine (30 mg/kg, ip) immediately before 1 h of IMO. Results indicated that exposure to IMO induced a prolonged increase in plasma ACTH and corticosterone levels, whereas cocaine only increased corticosterone with a less potent magnitude. Cocaine administration decreased ACTH levels (but not corticosterone) only at the end of IMO, reflecting a mild negative synergism. The long-term anorectic effects of IMO were partially blocked by cocaine injection (negative synergism) which itself also decreased food intake but only transiently and in non-stressed animals. Both IMO and cocaine reduced body-weight gain, although the decrease in weight gain induced by cocaine was delayed more in time and not explained by changes in food intake. The long-term anhedonic-like effects of IMO (measured by the intake of saccharine solutions) were not affected by cocaine administration that itself had no effect. Exposure to IMO induced, twenty-four hours later, an anxiogenic-like effect in the EPM test, accompanied by a decrease in motor activity. Here again cocaine was not able to modify this behaviour alone or concomitantly with IMO. The active behavior in the FST (struggling) was not affected neither by cocaine nor by IMO, but IMO modestly increased mild swimming in non-cocaine injected animals. IMO was able to induce a sensitization of the HPA axis in response to two different heterotypic stressors, although the effects of cocaine in

sensitization were not consistent. The exposure to IMO produced an anxiogenic-like effect in the ASR test, effect that vanished after twenty-four hours, and cocaine injection did not modify this behavior. The animals administered with cocaine in the first exposure to IMO, showed no homotypic adaptation to stress, contrary to the animals that were only exposed to IMO. The main finding of the studies using c-fos as marker of neuronal activation (ISH) indicated that cocaine blocked the activation induced by IMO in the accumbens, the bed nucleus of the stria terminalis and the dorsal Rafe. On the other hand, the studies with RT-PCR showed that the increase in CRF in the amygdala induced by cocaine and by IMO was blocked by the simultaneous exposure to both stimuli. In general, cocaine does appear to protect more than exacerbate the neuroendocrine and behavioral effects of exposure to a severe stressor, suggesting a negative synergy between both stimuli.

SPANISH SUMMARY

La exposición al estrés aumenta la adquisición, mantenimiento y recaída de la adicción a las drogas, pero los efectos de la exposición concomitante a drogas y a estímulos estresantes severos no ha sido explorada. Nuestros estudios han sido realizados en ratas macho adultas Sprague-Dawley, distribuidas en 4 grupos en función de la inyección de cocaína y de la exposición a inmovilización (IMO). Los animales fueron inyectados con salino o con cocaína (30 mg/kg, ip) inmediatamente antes de 1 h de IMO. Los resultados indicaron que la exposición a la IMO indujo un incremento prolongado en los niveles plasmáticos de las hormonas del eje hipotálamo-pituitario-adrenal (HPA), corticosterona y hormona adrenocorticotrópica (ACTH), mientras que la cocaína únicamente incrementó la corticosterona pero con menor magnitud. La administración de cocaína disminuyó los niveles de ACTH (pero no de corticosterona) únicamente al finalizar la IMO, indicando la presencia de una leve sinergia negativa. Los efectos anorexígenos a largo plazo de la IMO se bloquearon parcialmente con la inyección de cocaína (sinergia negativa) la cual por ella misma también disminuía la ingesta de comida en los animales no estresados. Tanto la IMO como la cocaína redujeron la ganancia de peso corporal, aunque la disminución de peso corporal producida por la cocaína se demoró más en el tiempo y no se explicaba por los cambios en la ingesta de comida. Los efectos anhedónicos a largo plazo (medidos por la ingesta de soluciones de sacarina) no se afectaron por la administración de cocaína la cual por sí misma no tenía efecto. La exposición a la IMO indujo, veinticuatro horas después, un efecto ansiogénico en el laberinto elevado, que se acompañó por una disminución en la actividad locomotora. Aquí de nuevo la cocaína administrada

simultáneamente con la IMO no modificó esta conducta. La conducta activa en el test de natación forzada (escape) no se afectó ni por la cocaína ni por la IMO, pero la IMO incrementó de forma modesta la natación suave en los animales no inyectados con cocaína. La IMO indujo también una sensibilización del eje HPA en respuesta a dos estímulos estresantes heterotípicos diferentes, mientras que los efectos de la cocaína sobre dicha sensibilización no fueron consistentes. La exposición a la IMO produjo también un efecto ansiogénico en el test de la respuesta acústica de sobresalto, efecto que se desvaneció después de veinticuatro horas, y la inyección de cocaína no modificó dicha conducta. Los animales administrados con cocaína en la primera exposición a la IMO no mostraron adaptación homotípica al estrés, al contrario de los animales solo expuestos a la IMO. El resultado principal de los estudios que utilizaron el c-fos como marcador de activación neuronal (ISH) indicaron que la cocaína bloqueó la activación inducida por la IMO en el accumbens, en el núcleo del lecho de la estría terminal y el dorsal del Rafe. Por otra parte, los estudios con RT-PCR mostraron que el incremento del factor liberador de la corticotropina (CRF) en la amígdala inducido tanto por la exposición a la cocaína como a la IMO (por separado) se bloqueó por la exposición simultánea a ambos estímulos. En general, la cocaína parece proteger más que exacerbar los efectos neurales, neuroendocrinos y conductuales de la exposición a un estímulo estresante severo, sugiriendo una sinergia negativa entre los dos estímulos.

INTRODUCTION

1. Stress

1.1. The nature of stress

The concept of stress could only be understood after the introduction of the idea that the organisms have physiological mechanisms to maintain an internal balance. Living organisms are not isolated; they are in a constant interaction with a changing, and sometimes challenging, environment. The existence of complex regulatory mechanisms allows the survival and adaptation of the organisms to different external situations. The French physiologist Claude Bernard, back in 1872, introduced the idea that the organisms have a “milieu intérieur” (“internal environment”) and control systems to maintain an internal equilibrium: “*The constancy of the internal environment is the condition that life should be free and independent… So far from the higher animal being indifferent to the external world, it is on the contrary in a precise and informed relation with it, in such a way that its equilibrium results from a continuous and delicate compensation, established as by the most sensitive of balances”*.

In the early twentieth century, Walter Cannon (1932) introduced the term "homeostasis" from the concept of "Milieu Intérieur" proposed by Claude Bernard. Cannon defined homeostasis as a “*the many regulatory processes that maintain the*

stability of various constituents of extracellular fluids within multicellular organisms". Cannon defined the sympathetic-adrenal-medullary (SMA) as key element for the maintenance of homeostasis. Cannon also described the "fight or flight" SMA response as the animal natural reaction to threatening situations. The word "stress" was initially borrowed from physics (Hooke's equation, 1635-1703) by Cannon and others (Kopin, 1995) to refer to agents that can alter and be a threat to homeostasis (Ursin and Olff, 1993; Stratakis and Chrousos, 1995). In the notable work of Cannon, the range of adverse situations that endangered the integrity of the organism, included not only physical stimuli such as heat or hypotension, but also considered as priority psychological stimuli, a fundamental aspect in the current definition of stress (see review in Pacák and Palkovits, 2001, Goldstein and McEwen, 2002).

Probably the biggest name in the field of stress is the endocrinologist Hans Selye (1936), the one who really popularised the concept of "stress" through his numerous scientific studies leading him to define the "stress syndrome" or "general adaptation syndrome" as "*the non-specific response of the organism to any demand upon it*". Selye studied the response of the organisms to various threatening situations such as cold, surgical injury, spinal shock, muscular exercise and intoxications. This syndrome was generally characterised by weight loss, adrenal hypertrophy, gastrointestinal ulceration and thymico-lymphatic involution.

It was in this pathological picture, which occurred in all animals regardless to the type of adverse agent administered, where Selye defined the "general syndrome of adaptation", which included the following 3 stages (Goldstein and Kopin, 2007): (i) the "alarm reaction" characterized by an immediate discharge of adrenaline and noradrenaline (NA) by the sympathetic-adrenal-medullary system (SMA), similar to the "fight or flight" response proposed by Cannon, (ii) the "adaptation phase" or resistance phase, in which changes occur in physiological systems mentioned above and (iii) the

"stage of exhaustion", which happens when the stressful situation persists, leading the organism to death. Later, Selye showed that the physiological changes observed were associated with the release of glucocorticoids by the adrenal cortex that not only contributed to the organism resistance to adverse situations but were also responsible for pathological changes. Therefore, while Cannon emphasis was placed on the activation of the SMA in the maintenance of homeostasis, Selye gave the role to hypothalamic-pituitary-adrenal (HPA) axis as the main effector system in the organism response to harmful or dangerous situations. The two authors build the foundations of what we now understand as the stress response.

Since Selye's initial definition, there have been many attempts to redefine or broaden the concept of stress (Levine, 1985). In the field of stress it is of particular interest the concept of "Allostasis" introduced by McEwen (2000). Briefly, this term states that the maintenance of stability (homeostasis) is only needed in certain critical parameters for the life of the organism (such as pH, osmotic pressure, blood levels of glucose or body temperature). The stability of these variables is maintained through the action of a large number of physiological factors (*i.e.*hormone levels or heart rate) that fluctuate within a wide range to keep the variables constant against environmental perturbations. The price that organisms need to pay to maintain stability of these few critical parameters is what is called the "allostatic load". When this allostatic load is excessive and is prolonged over time gives rise to the pathological consequences associated with stress.

Nowdays, it is generally accepted that the term "stress" comprises a wide range of physiological and/or behavioral changes that have evolved along phylogeny and that take place in the organisms under different challenging situations (Vigas, 1984). This wide range of situations can be either a real, anticipated or symbolic threaten for the integrity of the organism, and cannot be coped by means of normal homeostatic

mechanisms (Vigas, 1984). The stress response, regarded as a positive adaptative process, comprises a set of functional and behavioral reactions to cope with these stressful situations. A coordinated and adequate set of responses to stress is crucial for the survival of the organisms in front of these situations. However, exaggerated responses to stress appear to be closely related to a wide range of physiological and psychological dysfunctions such as cardiovascular (Bohus and Koolhaas, 1993; Steptoe, 1993), sleep disorders (Kant *et al.*, 1995), infertility (Moberg, 1985), anxiety-related disorders and depression (Halbreich, 1987), anorexia (Brambilla, 2001; Donohoe, 1984), or neurodegeneration (Sapolsky, 1992). It has also been shown that stress increases the susceptibility of the organisms to immune-mediated diseases (Munck *et al.*, 1994), cancer (Stefanski, 2001) or addiction to drugs (Piazza and Le Moal, 1996). All these important pathological features of the stress response have triggered an intense study of the different systems activated by stress. In another way stress exposure can be detrimental, because it can create a situation of increased arousal and emotional salience enabling the organism to appropriately respond to the stressor and ensure survival.

1.2. Categorization of a stressful stimulus

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

authors agree that each stressful stimulus has its own central neurochemical and peripheral neuroendocrine "signature" (see Romero and Sapolsky, 1996; Pacák *et al.*, 1998; Palcák and Palkovits, 2001 for a review).

Following the stress definition proposed by Vigas, stressful stimuli have been classified in two main categories: those involving a potential threat or emotional nature (also described as neurogenic, psychological or processive) and those that pose a real danger or systemic nature (also labelled as physical, homeostatic or physiological). Currently are accepted these two categories of stressful stimuli, although certain stimuli have a mixed profile (emotional/physical component). The general criterion is to classify different stressors depending on the main component that characterizes them.

Systemic or physical stressors are those that pose a direct disturbance in homeostasis, such as infections, metabolic or osmotic, hypoxia or hemorrhage. Such stimuli can activate the stress response through reflex mechanisms that do not require conscious awareness or arousal. Physical stressors would range from immunological (endotoxin and cytokine administration) to metabolic and osmotic challenges (insulin and 2-deoxiglucose administration, hypertonic saline injection), among others (ether exposure, exercise, cold exposure, hypoxia, haemorrhage). Anticipatory or emotional stimuli are those that do not constitute a direct threat to the homeostatic balance, but have a certain probability to be followed by a real danger. In animals, stressful stimuli like unfamiliar surroundings, the smell of a predator or social defeat have an emotional nature. Other stimuli such as immobilization in boards (IMO), electric shock or forced swimming test are fundamentally emotional but may have a physical component, so they are considered mixed.

In Vigas stress definition is proposed that the stress response is triggered only when disturbances in the body cannot be done by normal homeostatic mechanisms.

Homeostatic normal response is a specific response and appropriated for each particular situation. When this mechanism is insufficient to restore balance in the organism, starts a nonspecific response of emergency common to many stimuli, known as the stress response. Therefore, when we study the physiological response to a particular situation it is clear that we are observing a mixture of specific and nonspecific responses (see Armario, 2006).

In summary, in all vertebrates exposure to stress mainly involves the activation of two major physiological systems: (i) the autonomic nervous system, especially the sympathetic branch, SMA, with the subsequent release of catecholamines, adrenaline and NA; and (ii) the hypothalamic-pituitary-adrenal (HPA), responsible for the release of glucocorticoids to the bloodstream. Both systems play a crucial role in the stress response, resulting in both physiological and behavioral/cognitive changes indispensable to the survival of the organism.

1.3. Stress and the HPA axis

The HPA axis consists of a complex, well-regulated interaction between the brain, anterior pituitary and adrenal cortex, and is the hormonal system that activates the integrative physiological response to stress, which helps the organism to adapt to increased demands and maintain homeostasis after challenge (Mello and Mendelson, 1997). This endocrine system is also vital for supporting normal physiological functioning, regulates various body processes including digestion, the immune system, mood and sexuality, and energy usage. The general mechanisms of HPA activation in response to stress are shown in Figure 1.

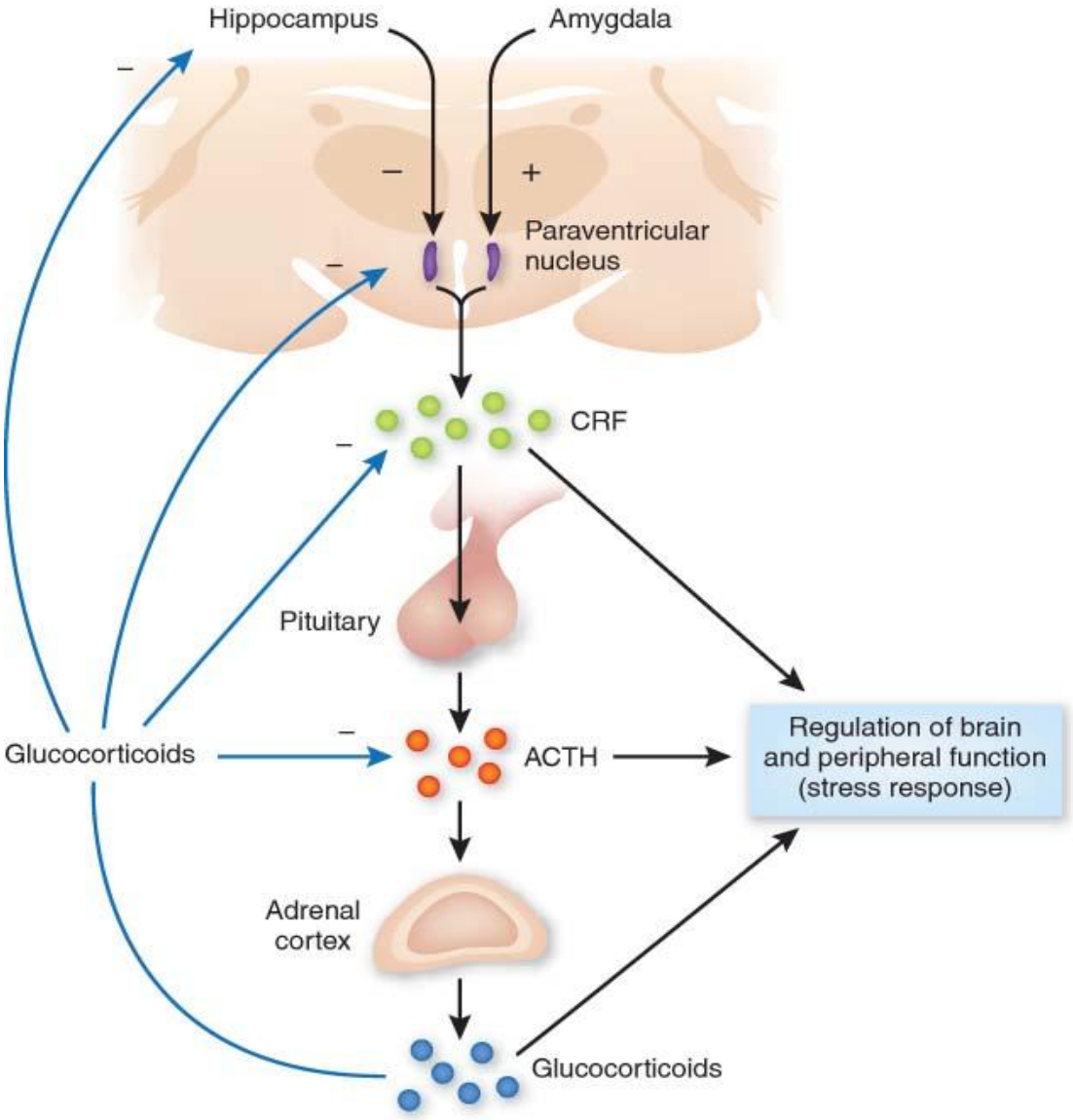


Figure 1. Overview of the functioning of the hypothalamic-pituitary-adrenal or HPA axis. Legend: ⊕ activation/excitatory effect; Θ Supression/inibitory effect (negative feedback). Abbreviations: CRF: corticotropin-releasing factor; ACTH: adrenocorticotropic hormone.

As previously described, stressors are processed by the central nervous system (CNS) and the information converges to the hypothalamus, where is stimulated the synthesis of corticotropin-releasing factor (CRF) from the parvocellular neurosecretory neurons of the paraventricular nucleus of the hypothalamus (PVN; Johnson *et al.*, 1992; Dallman, 1993; Cullinan *et al.*, 1995). Parvocellular neurons in the PVN project to the median eminence and release corticotropin-releasing hormone (CRH) and arginine

vasopressin (AVP), which are then released into the hypophysial portal circulation (Plotsky *et al.*, 1993). CRH and AVP reach the anterior pituitary, where both stimulate the transcription and cleavage of proopiomelanocortin (POMC), the precursor molecule, among other peptides, of adrenocorticotropin hormone (ACTH).

Thus, circulating concentrations of ACTH and glucocorticoids precisely reflect the prior secretion of CRH/AVP by the hypothalamic median eminence (Alexander *et al.*, 1996). Although CRH is required to stimulate ACTH synthesis and secretion from the pituitary, AVP interacts with CRH to potentiate the secretion of ACTH from the corticotrophs (Gillies *et al.*, 1982; Antoni, 1993). The CRH/AVP ratio in parvocellular neurons of the PVN changes under various conditions and in this way can control the amount of ACTH released in response to the stimulation of the PVN (Antoni, 1993).

At the adrenal cortex, ACTH promotes the synthesis and release of glucocorticoids (cortisol in humans, corticosterone in rat) from the zona fasciculata into the systemic circulation (Checkley, 1996). The synthesis and secretion of glucocorticoids (the final product of the HPA axis), in the adrenal cortex is primarily mediated by the action of ACTH on melanocortin-2 receptors integral to cortical cell membranes (Mountjoy *et al.*, 1992), with the adrenal sensitivity changing as a circadian function (Jasper and Engeland, 1994). Normally ACTH concentrations in the circulation are in the low picomolar range (Dallman *et al.*, 1987) and at these concentrations has a specific action in the adrenals, but few extra-adrenal effects.

Glucocorticoids trigger a plethora of actions. In general, they increase hepatic glucose production and release (stimulation of glycogenolysis), as well as cardiovascular muscle tone, but suppress “nonessential systems” for immediate survival, such as the immune, muscle-skeletal and reproductive systems (Herman *et al.*, 1995; Sapolsky *et al.*, 1986).

In fact, glucocorticoids are involved in the suppression of their own release through negative feedback systems that inhibit the release of ACTH (Keller-Wood and Dallman, 1984; Dallman *et al.*, 1987). Feedback systems operate primarily at the level of the parvocellular part of the PVN and anterior pituitary (Keller-Wood and Dallman, 1984; Dallman *et al.*, 1987; Cole *et al.*, 2000), although other brain sites such as the hippocampus and medial prefrontal cortex (mPFC) are also involved in the regulation of HPA axis activity (Kovacs *et al.*, 1986; Jacobson and Sapolsky, 1991; Diorio *et al.*, 1993).

1.3.1. CRF and vasopressin

The principal molecules involved in regulating the release of ACTH are CRF and AVP. Still, it is considered that the release of ACTH in response to stress is not due exclusively to these neuropeptides, but is dependent on the coordinated action of a cocktail of distinct stimulatory factors whose particular composition differs depending on the stressful stimuli (Plotsky, 1991, Romero and Sapolsky, 1996). These additional factors include oxytocin, angiotensin II and catecholamines.

The CRF or CRH is a 41-aminoacid peptide, synthesised in the medial dorsal pPVN in response to a wide variety of stressors that is considered the key component of the HPA axis by being the principal stimulator of the synthesis and release of ACTH from the anterior pituitary.

In addition, CRF is also one of the most widely distributed peptides throughout the CNS (Sawchenko and Swanson, 1990), being found both in the hypothalamus (*e.g.* medial preoptic area (MPOA), supraoptic (SO), lateral hypothalamus (LH)) and other areas of

the brain including the limbic system (*e.g.* bed nucleus of the stria terminalis (BNST), central amygdala (CeA), hippocampus) and the brainstem (*e.g.* Barrington´s nucleus, parabrachial nucleus (PB), inferior olivary complex).

Interestingly, the PVN itself receives CRFergic inputs originated in the hypothalamus (dorsal hypothalamic and perifornical areas, dorsomedial nucleus of the hypothalamus (DM), limbic system (BNST) and brainstem (Barrington´s nucleus, dorsal raphe nucleus (DR)) (Champagne *et al.*, 1998). However, whereas there is also a wide and abundant distribution of CRF mRNA throughout the brain, the CRF primary transcript, a much more sensitive and reliable index for determining the activity of the CRF gene (Herman, *et al.*, 1992), has only been detected in the PVN (Drolet and Rivest, 2001).

The action of CRF is mediated through two types of receptors: CRF1 and CRF2 (with two subtypes: CRF2α and CRF2β) (see Dautzenberg *et al.*, 2001 for a review), which are very differently distributed in the CNS (Chalmers *et al.*, 1995). The CRF1 receptor is widely distributed throughout the brain, including the olfactory bulb, cerebral cortex, limbic system (amygdala, hippocampus), red nucleus, central gray (CG), cerebellum and pituitary (Potter *et al.*, 1994). On the other hand, the CRF2α receptor is generally restricted to limbic structures (lateral septum (LS) and amygdala), whereas the CRF2β receptor is found in non-neuronal elements of the brain (choroid plexus, cerebral blood vessels) and in the periphery (cardiac and skeletal muscle, lung, intestine) (Lovemberg *et al.*, 1995).

Among the family of peptides that bind to CRF receptors are included the urocortin (UCN) I, II and III. The CRF presents higher affinity for CRF-R1, primarily responsible for the synthesis and release of ACTH in response to stress and widely distributed throughout the CNS. The UCN I has a similar affinity for both receptor types, while the UCN II and III bind with greater affinity to CRF-R2, which have a more limited

distribution in the CNS (Vaughan *et al.*, 1995; Kozicz *et al.*, 2004; Korosi and Baram, 2008).

In regard to the HPA axis, although it is unclear the role of CRF-R2, it seems to be important for the maintenance of HPA axis response to stress (Dautzenberg *et al.*, 2001, Dautzenberg and Hauger, 2002, Aguilera *et al.*, 2004, Korosi and Baram, 2008). Both types of receptors are positively coupled to adenylate cyclase, causing an increase in intracellular cyclic adenosine monophosphate (cAMP) and the subsequent activation of cAMP-dependent protein kinase (PKA). The binding of CRF to their CRFR1 receptors in the anterior pituitary leads, by the subsequent cascade of protein phosphorylation, to the transcription of the gene for proopiomelanocortin (POMC), ACTH precursor peptide. In addition, stimulation of these receptors also leads to increases in intracellular Ca2+, stimulating the release of ACTH into the circulation (see Jones and Gillham, 1988; King and Baertschi, 1990 for reviews).

CRF system extends beyond the parvocellular neurons of the PVN. CRF immunoreactive neurons have been observed in other CNS regions, mainly in areas of the basal forebrain (central amygdala and BNST) and in some brainstem regions (nucleus of Barrington, parts of cores raphe), areas that are involved in regulating behavioral responses and autonomic stress responses (Champagne *et al.*, 1998, Morin *et al.*, 1999, Croiset *et al.*, 2000). These neurons are also located in areas of the cerebral cortex and hippocampal formation, although its function is poorly understood. Therefore, the actions of CRF are not restricted to the pituitary action. CRF participate in many other functions in the body. The CRFR1 receptors are involved in processes like attention, learning, memory and emotion processing, whereas CRFR2 is related to basic functions such as eating, playing or defense (Croiset *et al.*, 2000, Smagin and Dunn, 2000, Lowry and Moore, 2006). In addition, in recent years the role of CRFR1 is gaining special relevance, since it has been implicated in the pathophysiology

disorders of fear / anxiety, depression and addictive behavior (Binder and Nemeroff, 2010, Koob, 2010).

The neuropeptide AVP is the other most studied mediator in relation to the regulation of ACTH. AVP is a 9-aminoacid peptide that is principally produced by the posterior-pituitary projecting magnocellular neurons of the PVN and Supraoptic (SO) nucleus of the hypothalamus. AVP exerts its biological effects on 3 types of G protein-coupled receptors: V1a, V1b (both associated with activation of phospholipases) and V2 (coupled to activation of adenylyl cyclase) (Birnbaum, 2000, Itoi *et al.*, 2004). The type V1b (also called V3) acts on corticotrophs cells of the pituitary.

The AVP is involved in functions such as osmotic pressure regulation and fluid balance. The AVP is also involved in cognitive processes and in the pathophysiology of psychiatric disorders (Egashira *et al.*, 2009). Regarding its role in regulating the HPA axis, the AVP itself does not stimulate the synthesis of ACTH and only has a weak effect on the release. However, both in vivo and in vitro, AVP enhance the effects of CRF on the release of ACTH (Rivier and Vale, 1983, see review in Makara *et al.*, 2004). Parvocellular AVP source comes mainly from the same neurons that synthesize CRF.

Under normal conditions, only a fraction of the CRF neurons coexpress AVP, but when the HPA axis is chronically activated, greatly increases the number of CRF neurons that also express AVP (Herman *et al.*, 1989, De Goeij *et al.*, 1992, Makino *et al.*, 1995). Therefore the sharp increase of the AVP activity during chronic stress suggests that it may contribute to maintaining ACTH response in these situations, although this issue is yet to be solved (see review in Aguilera *et al.*, 1994, Makara *et al.*, 2004).

1.3.2. Adrenocorticotropic hormone (ACTH)

As introduced previously, the stress-induced activation of pituitary corticotrophic cells results in the synthesis (from its precursor POMC) and release into the peripheral circulation of the 39-aminoacid peptide ACTH. The ACTH is considered to be the primary peripheral stress hormone, which synthesis and secretion is triggered by a wide range of stressful situations. Once in the systemic circulation, ACTH acts on the cells of the zona fasciculata of the adrenal cortex to stimulate the synthesis and release of glucocorticoids. ACTH acts in its specific cell membrane receptor from the family of melanocortin (MCN) receptors MC2-R, which is positively coupled to Adenylate Cyclase (Tatro, 1996; Adan and Gispen, 2000). The cAMP subsequent increase and PKA activation initiate a cascade of events leading to both synthesis and release of glucocorticoids from the adrenal cortex (corticosterone in rat). Acutely, this pathway leads to the synthesis and release of glucocorticoids, by activating enzymes that are involved in mitochondrial cholesterol transport and steroidogenesis (conversion of cholesterol to pregnenolone).

In addition, ACTH via PKA activation exerts a trophic effect on the adrenal gland, resulting in transcriptional changes in adrenal cells that lead to more complex and lasting changes (Sewer and Waterman, 2003). In this regard, high doses of ACTH have been shown to provoke adrenal hypertrophy and hyperplasia, whereas ACTH depletion induces the atrophy of the adrenal glands. Activation of the HPA axis results in a rapid release of ACTH, reaching the maximum release around 10-15 min. The subsequent release of glucocorticoids need more time, reaching the peak of release arround 15-30 min (Armario, 2000).

Although ACTH is the main regulator of glucocorticoid secretion is well established that there are other mechanisms with a modulatory role in this process, among which include the neural control of adrenal (Vinson *et al.*, 1994).

1.3.3. Glucocorticoids and feedback mechanism

In mammals there are two types of glucocorticoids: cortisol, present in most mammals (including humans) and corticosterone present in rat and other rodents (but also found in humans). The functions of both compounds are essentially similar. Glucocorticoids are named after one of its main metabolic functions, its role in providing the formation of glucose from aminoacids (gluconeogenesis). As mentioned before, these steroids are the main effector molecules of the HPA axis, exerting many functions. The glucocorticoids are involved in most negative consequences associated with stress. However, not all the effects of glucocorticoids are negative. Several studies had demonstrated that under certain conditions glucocorticoids have positive inputs in arousal, learning and memory. Appropriate levels of glucocorticoids are therefore essential for the maintenance of homeostasis in many physiological systems (for review see De Kloet, 2004).

As previously described, glucocorticoids exert multiple actions on the organisms. To further understand the role of glucocorticoids on the response to stress, their actions have been classified in two main categories: modulating and preparative (Sapolsky *et al.*, 2000). Modulating actions of glucocorticoids are those altering the response of the organisms to a stressor, and are further divided into three more categories: permissive, suppressive and stimulating actions. Permissive actions are manifested during the initial phase of the stress response and prepare the defense mechanisms of the organism to cope with stress. This category includes the facilitating effect of the

cardiovascular response, which is determined mainly by other factors (sympathetic activation). Suppressive actions take place after one hour or more after the onset of stress, and prevent the organism from the negative consequences of an excessive response to stress. Through these actions, glucocorticoids suppress the immune system, the inflammatory response and the activity of the HPA axis via feedback inhibition mechanism. On the contrary, stimulating actions, also taking place from about one hour or more after the beginning of stress, enhance the effects of the hormones released after stress, thus helping to mediate the stress response (*i.e.*enhancement of lipolysis and gluconeogenesis). Finally, glucocorticoid preparative actions, which can be either mediating or suppressive, are those modulating the future response of the organisms to stress.

The effects of glucocorticoids may be genomic or nongenomic. It is considered that the rapid effects of glucocorticoids are incompatible with the time required for gene transcription taking place. Although It is postulated that the rapid effects are mediated by the binding to membrane receptors, they are not yet characterized (see review in Makara and Haller 2001, Haller *et al.*, 2008). On the contrary, are well known the two types of intracellular receptors involved in glucocorticoids-mediated genomic changes: type I (or mineralocorticoid, MR), which has high affinity for corticosterone, and type II (or glucocorticoid, GR), which has higher affinity for cortisol and synthetic glucocorticoids such as dexamethasone (Reul and De Kloet, 1985).

Glucocorticoid receptors have a similar structure, with both DNA and steroid-binding domains, and non-occupied receptors are located predominantly in the cytoplasm, forming large heterocomplexes with heat shock proteins, which dissociate after hormone binding. The activated hormone-receptor complex can then translocate into the nucleus and target glucocorticoid response elements found in the promoter region of different genes, thus modulating their transcription. Glucocorticoid receptors can also

modulate gene transcription by direct binding to transcription factors (Nishi and Kawata, 2007) such as activator protein-1 (AP-1), cAMP responsive element binding protein (CREB) and nuclear factor-κB (NF-κB).

Both types of receptors differ not only in their affinity for some ligands but also in peripheral and central distribution (see review in De Kloet *et al.*, 1990). MRs have a high affinity for corticosterone, and their brain expression is abundant in the hippocampal formation, layer II of the cortex, lymbic system (LS, medial Amygdala (MeA), CeA, olfactory nucleus) and brainstem sensory and motor neurons. On the other hand, GRs, with a lower affinity for corticosterone, are widely distributed throughout the CNS. The GR distribution includes the most important areas in relation to negative feedback of the HPA axis: the PVN, anterior pituitary and other extrahypothalamic regions as the medial prefrontal cortex (mPFC) or the hippocampal formation (see review in Armario, 2006b).

On the basis of the differential binding properties and distribution of glucocorticoid receptors, each subtype has been suggested to play a differential role in HPA axis regulation. Due to their high affinity for glucocorticoids, MRs display more than an 80% of occupancy throughout the diurnal cycle of the HPA axis. On the other hand, GRs are partially occupied during the phase of maximal corticosterone secretion of the circadian rhythm (afternoon/evening in the rat) and, more importantly, when there are elevated concentrations of glucocorticoids provoked by exposure to stressful situations (De Kloet, 1991; De Kloet *et al.*, 1993; for review see De Kloet *et al.*, 1998). In general, MRs are thought to mediate the tonic inhibitory control of the HPA axis to control its basal activity throughout the circadian circle, whereas GRs are assumed to be the main contributors to the retroinhibitory effects exerted by glucocorticoids in the pPVN and anterior pituitary to switch-off the HPA axis activity after stress. In addition, the two receptors are thought to mediate coordinately the hippocampal regulation of the HPA

axis activity, and it has been suggested that an adequate MR/GR balance is critical for determining the effects of glucocorticoids on cellular homeostasis, behavioral adaptation and susceptibility to disease (De Kloet, 1991).

As mentioned before, one of the most important functions of glucocorticoids is their regulatory role on the HPA axis through feedback inhibition mechanisms. Depending on their speed of action, feedback mechanisms on the HPA axis have been divided into three categories: fast, intermediate and slow (Keller-Wood and Dallman, 1984). The fast feedback only operates when the rate of glucocorticoids in plasma is rising and its power depends on their own increase in plasma levels. Fast feedback includes the actions of corticosterone on the control of the release of ACTH secretagogues in the Median eminence, and takes place within 10 min after the onset of stress. The speed of this mechanism involves receptors different to the genomic, still uncharacterized, although endocannabinoids seem to play an important role in suppressing glutamatergic stimulatory signals that reach the pPVN (Di *et al.*, 2003, Evanson *et al.*, 2010). Both the intermediate and the slow feedback mechanisms present processes of transcription mediated by GR and MR. These mechanisms also depended of the glucocorticoid levels reached in the hours preceding the exposure to stress. Intermediate feedback mechanisms, developing within 30-60 min after the onset of stress, involve gene-mediated glucocorticoid effects on the coupling of stimulus and secretion, excitability and intracellular signal transduction pathways. Finally, slow feedback develops close to 1 h after the onset of stress and, in contrast to intermediate feedback, may last for several hours. The effects of slow feedback include the blockade of stress-induced CRF, AVP and POMC gene expression.

1.4. Central stress pathways

1.4.1. General concepts

In general, most neurons that project directly to the PVN are located in regions that receive projections from the first or second order somatic nociceptors, visceral afferents or humoral sensory pathways. These neurons are in a position to evoke a reflex and fast activation of the HPA axis. But this response has an energy cost and can not be used in vain without harmful consequences (reviewed in McEwen, 1998). For this reason, the CNS has regulatory mechanisms that adequates the HPA axis response.

This modulation of the response by higher CNS structures is very evident when the organism faces emotional stimuli. In this case, excitatory or inhibitory patterns are generated dependent on learning and memory processes that are able to modulate the stress response. For example, you can reduce the response to a new environment with repeated exposure (habituation), or you can activate the response to innocuous stimuli if they have previously been associated with stressful stimuli (classical conditioning). The control over this range of responses is located in the limbic system, particularly in the hippocampal formation, amygdala and prefrontal cortex (PFC; reviewed in Herman *et al.*, 2003, 2005).

1.4.2. Direct pathways to the PVN

The brainstem catecholaminergic systems play an important role in the HPA axis activation by systemic stimuli. The PVN receives inputs of noradrenaline (NA) and

adrenaline from the nucleus of the solitary tract (NTS) A2/C2 region, which innervate preferentially the PVNmp. In contrast, magnocellular and parvocellular autonomic neurons of the PVN are innervated by the A1/C1 groups of the ventrolateral bulb (Cunningham *et al.*, 1990). The excitatory nature of these projections is confirmed by studies that show an attenuation of the HPA axis response to systemic stimuli after lesions in the bulbar projections to the PVN or by selective lesions of catecholaminergic pathways using 6-hydroxydopamine (Gaillet *et al.*, 1991, Li *et al.*, 1996, Ritter *et al.*, 2003). For example, selective lesions of NA and epinephrine afferents innervating the PVN through microinjection of saporin conjugated to a monoclonal antibody against dopamine β-hydroxylase (an enzyme only present in adrenergic and noradrenergic neurons because is the syntethic enzim for NA), decrease the corticosterone release after glucose deprivation, a systemic stressful stimulus (Ritter *et al.*, 2003). The same lesion also towards glucose deprivation reduces the c-fos mRNA induction in the PVN as well as CRF hetero-nuclear RNA (hnRNA), an intronic fragment of CRF messenger that is rapidly induced and has a very short half-life (the hnRNA is a very useful tool to detect CRF gene expression induced by different stimuli, because the mature messenger is found in large quantities at baseline). On the contrary, the injury does not affect the basal expression of CRF mRNA or the release of corticosterone during the circadian rhythm or in response to an emotional stressor like forced swimming. The NTS is a critical area for the integration of systemic stressful stimuli that stimulate the c-fos activation, a transcription factor used as a neuronal marker of activation after exposure to visceral pain, inflammation or infection, hypovolemia, hypoxia and hypotension (reviewed in Herman *et al.*, 2003). The NTS is also activated by other stimuli of mixed nature such as the restriction of movement, forced swimming test or restraint (Cullinan *et al.*, 1995; for a review see Sawchenko *et al.*, 2000) suggesting that it could also participate in the integration of the response to emotional stimuli, by playing a secondary role in regard to the HPA axis activation.

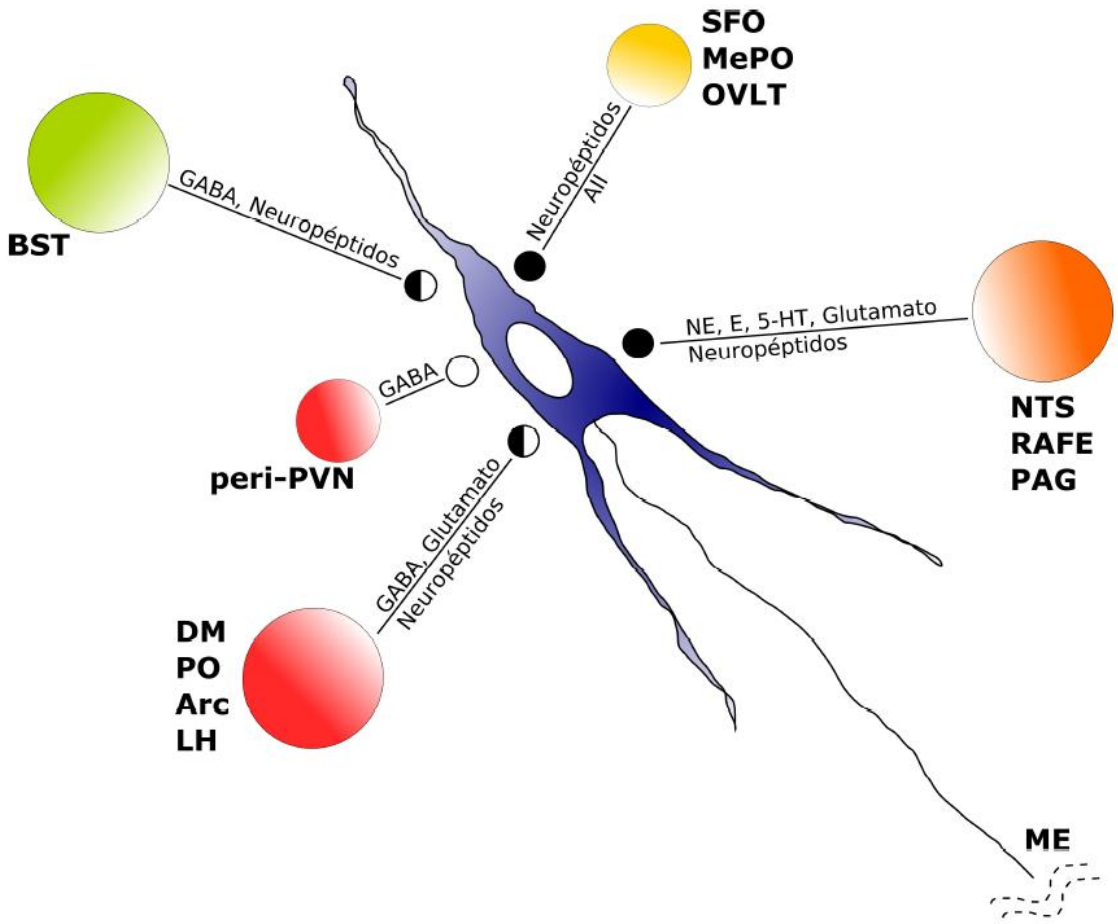


Figure 2. Major direct projections to parvocellular neurons of the paraventricular nucleus of the hypothalamus. The black circles indicate the activating nature of the projections; the white circles indicate the inhibitory nature of the projections. Abbreviations: Arc: arcuate nucleus of the hypothalamus; BNST: bed nucleus of the stria terminalis; DM: dorsomedial nucleus of the hypothalamus; LH: lateral hypothalamus; ME: median eminence; MePO: median preoptic nucleus; NTS: nucleus of the solitary tract; OVLT: organum vasculosum of the lamina terminalis; PAG: periaqueductal gray; peri-PVN: periPVN region; PO: medial preoptic area; SFO: subfornical organ (Adapted from Herman *et al.*, 2003).

On the other hand, it is well established that 5-HT is involved in the HPA axis regulation. Most studies indicate a stimulating role of 5-HT in the secretion of ACTH and corticosterone, which may occur partly in the PVN itself through 5-HT2A receptors and possibly 5-HT1A (Pan and Gilbert, 1992; Van de Kar *et al.*, 2001). However, it is surprising the small number of direct serotonergic projections to the PVN. Those are mostly from the dorsal and medial raphe (Sawchenko *et al.*, 1983) (Figure 2). Most of

the serotonergic fibers are found in adjacent regions to the PVN, suggesting the possibility that the main effect is due to an interaction with GABAergic neurons present in this region (see below). In addition to their direct actions on the PVN, 5-HT innervates structures such as the hippocampal formation, PFC, amygdala and hypothalamus (reviewed in Lowry, 2002), reason why 5-HT might modulate the HPA axis activity through these areas of integration of the stress response. The PVN also receives information from other midbrain areas that are not serotonergic, including the parabrachial nucleus and periaqueductal gray (PAG), both associated with autonomic functions (Saper, 1995).

The PVN receives information directly from the hydric and ionic equilibrium through the system formed by the SFO and OVLT (Figure 2). These regions are involved in controlling cardiovascular and body fluid homeostasis through the coordination of reflexes and behavioral responses such as thirst or salt appetite (Johnson *et al.*, 1996, McKinley *et al.*, 1999). The signals travel through direct projections from the SFO, the median preoptic nucleus (MePO) and the OVLT towards the PVN, innervating both magnocellular and parvocellular neurons, and controlling the secretion of hormones such as AVP. This hormone secretion, along with the sympathetic activation and behavioral responses, helps to maintain cardiovascular homeostasis and fluid balance.

Numerous hypothalamic regions project to the PVN, the majority of these projections are GABAergic (Roland and Sawchenko, 1993) (Figure 2). The PVN is innervated by GABAergic neurons located in close proximity to the peri-PVN region (Boudaba *et al.*, 1996), and thus its activation may result in the inhibition of the HPA axis (Cole and Sawchenko, 2002). The PVNmp region is also innervated by GABAergic neurons located in the DM, although this nucleus also has glutamatergic populations (Ziegler *et al.*, 2002), in a way that might be involved in both activation and inhibition of the PVN parvocellular neurons, depending on the specificity of the stimulus and the neuronal

type activated. Another region that innervates the PVN directly is the medial preoptic area (PO) that, as occurs with DM, also has GABAergic and glutamatergic populations. This area could act as an intermediary on the effects that gonadal steroids exert on the HPA axis. In this regard, it was observed that testosterone administration directly into the PO inhibits the stress response, a phenomenon also observed when administered systemically. The effects of androgens on HPA axis activity appear to be opposed to estrogens, since females show greater corticosterone release against a stressful stimulus. Nevertheless, similar effects of androgens and estrogens on HPA axis are observed in ACTH release (reviewed in Herman *et al.*, 2003). It's important to refer that all these data was observed in rats and thus may exist some differences in humans. Finally, information concerning the energy balance can be transmitted directly to the PVN through the Arc and the lateral hypothalamus (LH) (Figure 2). Arc neurons are sensitive to circulating levels of glucose, leptin and insulin (reviewed in Woods *et al.*, 1998). In Arc the different neuropeptides have complementary roles in respect to food intake, neuropeptide Y (NPY) promotes the intake while the peptides of the POMC family have anorectic effects (reviewed in Woods *et al.*, 1998).

Most direct telencephalic projections to the PVN originate from the bed nucleus of the stria terminalis (BNST), which mainly contains inhibitory GABAergic neurons (Cullinan *et al.*, 1993) (Figure 2). Nevertheless, the effects of BNST on HPA axis activity appear to depend on the stimulated area (Choi *et al.*, 2007). For example, lesions in the posterior BNST increase CRF mRNA expression in the PVN, according to its inhibitory role in regulating the HPA axis, whereas lesions in the anterior BNST decrease CRF mRNA expression (Herman *et al.*, 1994). CRF Intracerebroventricular administration (icv) induces the expression of c-fos and NGFI-B in the PVN (Parkes *et al.*, 1993), suggesting that this neuropeptide could play a role as a positive modulator of the PVN in the response to stress. Although is not known the exact origin of the CRF acting on the PVN, we must highlight the presence of CRF neurons in multiple subdivisions of

the BNST (Ju *et al.*, 1989) that could have a role in this regulation. CRF projections that innervate the PVN, may come not only from the BNST, but also from other hypothalamic areas (perifornical, dorsal hypothalamic nucleus and dorsal hypothalamic area), the dorsal raphe (DR) or Bar (Champagne *et al.*, 1998), without discarding axon-dendrite connections between CRF neurons within the PVN (reviewed in Liposits *et al.*, 1985). Finally, PVN also projects directly to some regions of the thalamus, such as posterior intralaminar and subparafascicular regions, which are involved in the HPA axis activation by stressful auditory stimuli (Campeau and Watson, 2000) or Zona incerta, an area rich in dopaminergic neurons (area A13, Wagner *et al.*, 1995, Cheung *et al.*, 1998). Although there are some data suggesting that dopamine activates the HPA axis (Borowsky and Kuhn, 1992, 1993, Fuertes *et al.*, 2000) it is not known if the effects are by direct action in the PVN (possibly by projections from the Zona incerta) or by indirect action on the superior pathways.

1.4.3. Indirect pathways to the PVN

Regions with a crucial role in the emotional response, such as the PFC, the hippocampal formation, amygdala, septum and midline nuclei of the thalamus, are logical candidates to modulate HPA axis activity (Figure 3). However, none of these structures project directly to the PVN neurons, and thus intermediary neurons are needed to exert their influence on the HPA axis.

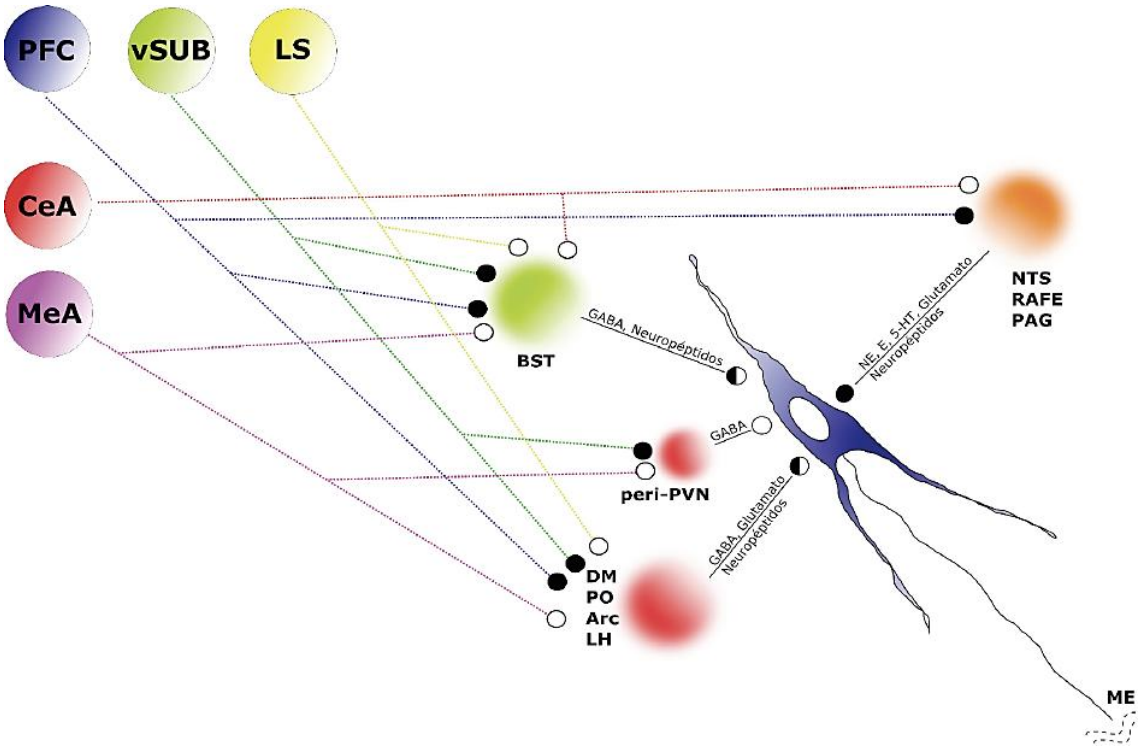


Figure 3. Main indirect projections to parvocellular neurons of the paraventricular nucleus of the hypothalamus. The black circles indicate the activating nature of the projections; the white circles indicate the inhibitory nature of the projections. Abbreviations: Arc: arcuate nucleus of the hypothalamus; BNST: bed nucleus of the stria terminalis; CeA: central amygdala; DM: dorsomedial nucleus of the hypothalamus; LH: lateral hypothalamus; LS: lateral septum; ME: median eminence; MeA: medial Amygdala; MePO: median preoptic nucleus; NTS: nucleus of the solitary tract; OVLT: organum vasculosum of the lamina terminalis; PAG: periaqueductal gray; peri-PVN: periPVN region; PFC: prefrontal cortex; PO: medial preoptic area; SFO: subfornical organ; vSUB: ventral subiculum (Adapted from Herman *et al.*, 2003).

The hippocampal formation has been related with the terminations of the HPA axis stress response through negative feedback exerted by glucocorticoids (reviewed in Herman and Cullinan, 1997), although there are contradictory data (Jacobson and Sapolsky, 1991, Bradbury *et al.*, 1993, Tuvnes *et al.*, 2003). Not surprisingly in this area exists a high concentration of MR and GR receptors (Reul and De Kloet, 1985; review De Kloet *et al.*, 1990). Several laboratories have observed that lesions in the hippocampal formation prolong ACTH and / or corticosterone secretion after exposure to stressful stimuli such as movement restriction, the paradigm of fear conditioning to a context (measured by freezing), acoustic stimulation or exposure to a new environment

like the open field (reviewed in Herman *et al.*, 2005). On the other hand, the same lesions had no effect on the HPA axis response after exposure to ether or hypoxia, indicating that the involvement of the hippocampal formation in modulating HPA axis activity depends on the stressor (reviewed in Herman *et al.*, 2005). The inhibition of the HPA axis appears to be exerted by a restricted number of neurons, mainly glutamatergic, located in the ventral subiculum (part of the hippocampal formation), which projects to regions such as BNST, MePO and DM, which, in turn, innervate directly to the PVN (for review see Herman and Mueller, 2006) (Figure 3). The subiculum also projects to the peri-PVN region, dominated by GABAergic neurons. Finally, the hippocampal formation could exert control over the PVN through multi-synaptic connections. For example, the LS receives a lot of innervation from the hippocampal formation, while LS projects to the peri-PVN region and other hypothalamic nuclei directly connected with the PVN (reviewed in Herman *et al.*, 2003).

The PFC also modulates HPA axis activity in response to an emotional stressor, with an essentially inhibitory role (Diorio *et al.*, 1993, Figueiredo *et al.*, 2003, McDougall *et al.*, 2004). As the hippocampal formation, the PFC has been linked with the negative feedback exerted by glucocorticoids (Akana *et al.*, 2001). The fibers from the PFC innervate GABAergic regions that project directly to the PVN, such as BNST, perifornical nucleus, LH and DM (reviewed in Herman *et al.*, 2005) (Figure 3). There is also an important innervation of the paraventricular nucleus of the thalamus (PVA), the amygdala, Rafe and NTS, which may modulate the activity of the axis through these areas (reviewed in Herman *et al.*, 2005). In turn, the PFC receives input from other areas involved in the control of the stress response, such as the locus coeruleus (LC), the NTS or the hippocampal formation. Apart from controlling the stress response, the PFC is also involved in the development of addictive behavior (reviewed in Robinson and Kolb, 2004; Feltenstein and See, 2008; George and Koob, 2011). Not surprisingly, PFC receives dopaminergic projections from the ventral tegmental area (VTA) and is

considered as an area where could occur the interaction between the effects of stress and drugs of abuse (reviewed in Montague *et al.*, 2004, Kalivas *et al.*, 2005).

Contrary to what happens with the hippocampal formation and PFC, the amygdala appears to activate the HPA axis (reviewed in Herman *et al.*, 2005). In this regulation different subnuclei of the amygdala are involved: CeA, MeA and basolateral (BLA). Although all subnuclei are involved in the activation of the HPA axis, it appears that each one responds to a concrete stressor. The CeA has been related with the response to systemic stimuli (reviewed in Herman and Cullinan, 1997; Sawchenko *et al.*, 2000; Dayas *et al.*, 2001) and has connections with the brainstem structures that project directly to the PVN, as the NTS and parabrachial nucleus (for review see Alheid, 2003; McDonald, 2003) (Figure 3). There is also evidence of the CeA connection with the BNST where, through GABAergic projections that activate the HPA axis by inhibiting the BNST GABAergic neurons (by disinhibition) that project to the PVN (reviewed in Herman *et al.*, 2003b). Moreover, the MeA could be involved in the integration of emotional stimuli (Cullinan *et al.*, 1995; Dayas *et al.*, 1999, 2001, 2002), using an extensive network of connections with direct projection to the PVN such as the BNST, MePO, PO, anterior hypothalamus and peri-PVN (reviewed in Alheid, 2003; McDonald, 2003) (Figure 3). The projections to these areas are GABAergic, so the activation of the MeA would also result in an activation of the PVN by disinhibition, as occurs with the CeA, although using different circuits (reviewed in Herman *et al.*, 2003b). Finally, the BLA is also activated in response to an emotional stimuli (Cullinan *et al.*, 1995), but mainly projects to other nuclei of the amygdala as the CeA and the MeA. For this reason a complex role of the BLA in the regulation of the stress response is hypotethized, participating in memory processes related to the exposure to stressors and controlling HPA axis activity through other nuclei of the amygdala (Akirav and Richter-Levin, 2006).

LS neurons are clearly activated by emotional stressors, such as novel environments, exposure to a predator or social interaction, showing less activation with systemic stimuli (reviewed in Herman *et al.*, 2003b). Most of these neurons are GABAergic (reviewed in Risold and Swanson, 1997b) and project to the peri-PVN region, the anterior hypothalamus, PO and LH (reviewed in Risold and Swanson, 1997a) (Figure 3). These regions, which contain both glutamatergic and GABAergic neurons, project directly to the PVN (reviewed in Herman *et al.*, 2003b). The LS exerts an inhibitory influence on these areas and is in an excellent position to regulate both activation and inhibition of the PVN. In fact, experimental data support more an inhibitory role since the electrolytic lesion of the LS prolonged corticosterone response to immobilization and increases sensitivity to moderate intensity stimuli (reviewed in Herman *et al.*, 2003). Nevertheless, the effects of these injuries could be caused by the damage produced to the fibers from the fimbria-fornix that passes through this area.

Recent studies have linked various nuclei of the thalamus with HPA axis regulation. In several regions of the midline of the thalamus there is a strong c-fos induction in response to emotional stressors (Cullinan *et al.*, 1995; Emmert and Herman, 1999; Bubser and Deutch, 1999). Among these regions, the PVA may play a key role in the integration of the HPA axis response to daily repeated stimuli. After such daily exposure a phenomenon known as facilitation has been observed, where the response to a new stressful stimulus (heterotypic) is enhanced in the animals that have been previously stressed (Bhatnagar and Dallman, 1998). After a repeated stress regime, the PVA is among the few areas that are selectively activated by heterotypic stimuli and therefore could be involved in the facilitation phenomenon. PVA receives numerous projections from areas sensitive to stress such as ventral subiculum, PFC, BNST, NTS, raphe, parabrachial nucleus and LC. And in turn, the PVN innervates regions that can modulate HPA axis activity like the PFC, BLA and CeA (reviewed in Herman *et al.*, 2003b).

Finally there are several hypothalamic regions capable of interacting with the PVN. Among these, we highlight the suprachiasmatic nucleus (SCN), the HPA axis main regulator of the circadian rhythm (Moore and Speh, 1993), that projects to GABAergic hypothalamic regions such as peri-PVN region or the DM, which in turn innervate the PVN directly (Armstrong, 1995).

2. Cocaine

2.1. The nature of addiction

In DSM-5^{TM} Guidebook (Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition) *American Psychiatric Association* (APA), the terms dependence and addiction are used without distinction. However, the term addiction emphasizes the behavioral connotation of the term and is less prone to be confused with physical dependence. Physical dependence would reflect the physiological adaptation to the effects of a drug while addiction defines uncontrollable consumption thereof. However, physical dependence is neither necessary nor sufficient for a diagnosis of drug addiction. Currently the most accepted definition of addiction described it as a chronic disorder and a relapsing behavior characterized by: (i) seeking and compulsive drug use; (ii) loss of control to limit consumption, despite the negative consequences for the individual; (iii) the appearance of a negative emotional state (dysphoria, irritability, anxiety) when the access to the drug is stopped (Black and Grant, 2014).

According to the United Nations Office on Drugs and Crime (UNODC) about 210 million people consumed illicit substances at least once in 2011, which means 4.8% of the population between the ages of 15 to 64 years old. Among these substances, cannabis is the illicit drug most widely used, followed by psychostimulants like amphetamine and cocaine, and finally by opioids. Therefore, the drug consumption is currently one of the biggest public health challenges of our society.

Despite the addictive potential of some drugs, not all individuals who use or have used any kind of drug become addict. This reveals that the use of a drug does not have to necessarily trigger an addictive process. In fact, only 10-20% of subjects who have been experimenting with a particular drug with addictive potential subsequently develop a dependence disorder (for review see Volkow *et al.*, 2011). There are several factors that predict individual vulnerability to develop an addictive disorder, including genetic factors, age, gender differences, certain individual personality characteristics, pharmacological properties of drugs and multiple environmental factors, such as stress (for review see Le Moal, 2009).

In the characterization of drug consumption there are three distinct phases (for review see Le Moal and Koob, 2007): Use, abuse and addiction. The term use means occasional use, which is associated with certain socio-cultural situations without initially posing a problem for the health and well-being of the subjects, at least in the short term. The term abuse is used when consumption interferes with the individual's everyday life, affecting their health and their social relationships (work, family, etc...). However, unlike addiction, this interference takes place in acute episodes. Finally, the term addiction is characterized by compulsive behavior of drug use despite its negative effects on health and social relations of the individual, the lack of the drug would result in withdrawal (physical or psychological) and often this period ends in relapse and re-entry into addictive process.

2.2. Neuropharmacology of cocaine

Cocaine is a psychostimulant drug, obtained from the leaves of the plant *Erythroxylum coca.* Isolation of cocaine, the coca's active principle, was first described by Albert Niemann in his Ph.D. dissertation titled *"On a New Organic Base in the Coca Leaves",* which was published in 1860 (Karch, 1996). The first reports of cocaine toxicity appeared during the 1880's, when several studies were published describing toxic reactions associated with cocaine and cocaine-related deaths (Karch, 1996). However, none of these negative reports appeared to have much impact. The popularity of coca was not affected and thousands of cocaine-containing patented medicines (local anesthetics) flooded the market, some with truly enormous amounts of cocaine (Karch, 1996). During the early years of the last century it became evident that cocaine was addictive and could produce serious medical complications, especially with the availability of cocaine powder for intranasal or intravenous use. In 1894, cocaine became illegal in the United States and its use waned (Karch, 1996).

Psychostimulants of high abuse potential, such as cocaine and amphetamine, interact initially to block monoamine transporter proteins that are located on monoamonergic nerve terminals (Glowinski and Axeirod, *1965;* Ferris *et al.*, 1972; Iversen, 1973; Ritz *et al.*, 1987). Cocaine inhibits, with about equal potency, the uptake of the three major monoamines neurotransmitters, dopamine (DA), serotonin (5-HT) and noradrenaline (NA) (Rothman *et al.*, 2001), thereby, potentiating monoaminergic transmission (Pettit and Justice, 1989; Broderick *et al.*, 2003). In this way, there is an increase in synaptic levels of these neurotransmitters. The mechanism of action of amphetamine is different

because in addition to induce an inhibition of the uptake is also increasing the release of these neurotransmitters from the presynaptic neuron.

However, the primary neuropharmacological action responsible for its psychomotor stimulant and reinforcing effects appears to be the dopaminergic systems in the CNS (Koob, 1992a). Antidepressant drugs that block NA and/or 5-HT, but not DA reuptake, produce neither significant reinforcement in animal models nor euphoria in humans (Hyman, 1996). Adrenoceptor antagonists, such as phenoxybenzamine and phentolamine also have no effects on cocaine reinforcement, while on the other hand; DA receptor antagonists block cocaine reinforcement (De Wit and Wise, 1977; Roberts *et al.*, 1980).

Brain dopaminergic neurons are organized into two major pathways that originate in the midbrain and project to numerous forebrain and cortical regions. The mesocorticolimbic dopaminergic system projects from the ventral tegmental area (VTA) to the ventral forebrain, including the Acb, olfactory tubercle, frontal cortex, amygdala and septal area. The nigrostriatal dopaminergic system arises primarily from the substantia nigra (SN) and projects to the corpus striatum. This last dopaminergic pathway is implicated in the focused repetitive behavior associated with psychostimulants abuse, called stereotyped behavior (Creese and Iversen, 1974; Robbins and Everitt, 1996). The mesocorticolimbic dopaminergic system has been primarily implicated in cocaine-induced locomotion and in the reinforcing effects of cocaine (Everitt and Wolf, 2002).

The mesolimbic dopaminergic system and its forebrain targets are very old from an evolutionary point of view and are part of the motivational system that regulates responses to natural reinforcers such as food, drink, sex and social interaction (for review see Nestler, 2001). Drugs of abuse affect the brain reward system with a

strength and persistence not seen in natural reinforcers (Nestler, 2001; Robinson and Berridge, 2003).

In particular, the mesoaccumbens dopaminergic pathway, extending from the VTA to the Acb, a brain region thought to be involved in converting emotion into motivated action and movement (for review see Mogenson *et al.*, 1980), seems to be where cocaine act to produce its acute reinforcing actions (Hyman, 1996). Blockage of cocaine induced locomotor activation has been observed following 6-hydroxydopamine lesions of the region of the Acb (Le Moal and Simon, 1991; Sellings *et al.*, 2006). Lesions of the Acb also produce disruption of cocaine self-administration (Roberts *et al.*, 1980; Pettit *et al.*, 1984; Zito *et al.*, 1985). However, 6-hydroxydopamine lesions of the frontal cortex and caudate nucleus fail to significantly alter established cocaine self-administration (Martin-Iverson *et al.*, 1986). Neurochemical studies using *in vivo* studies demonstrate that cocaine increases extracellular DA to a greater extent in the Acb (Carboni *et al.*, 1989; Pettit and Justice, 1989; Cass *et al.*, 1992).

2.3. Behavioral effects of cocaine

In humans, cocaine has potent psychostimulant properties as measured by antifatigue and stimulant actions (Fischman *et al.*, 1983; Romach *et al.*, 1999). Behaviorally, cocaine use in humans has been reported to produce profound subjective feeling of wellbeing (Gawin and Ellinwood, 1989), although feelings of anxiety are also reported (Hart *et al.*, 2004). Some of the major symptoms observed during withdrawal from chronic cocaine intoxication can often include restlessness, agitation and depression (Gawin and Ellinwood, 1989). Interestingly, CRH has been reported to be involved in a

variety of neuropsychiatric disorders including depression and anxiety (Gold *et al.*, 1984; Musselman and Nemeroff, 1996), suggesting that the anxiety associated with cocaine use and withdrawal may depend, in part, on the effects this psychostimulant on the CRH release and subsequent activation of the HPA axis (Goeders, 1997).

In rodents, cocaine increases locomotor activity (Bhattacharyya and Pradhan, 1979; Abel *et al.*, 1989) and decreases food intake (Balopole *et al.*, 1979). Cocaine has also a high abuse potential, and experimental studies have shown that cocaine induces place preference conditioning (Spyraki *et al.*, 1982) and readily acts as reinforcer for drug self-administration (Roberts and Koob, 1982; Goeders *et al.*, 1993). Anxiogenic-like behavior has also been observed in rodents using a variety of behavioral paradigms, following acute administration of cocaine (Costall *et al.*, 1989; Fontana and Commissaris, 1989; Rogerio and Takahashi, 1992) and during withdrawal (Sarnyai *et al.*, 1995).

2.4. From acute to chronic cocaine administration

The acute reinforcing effects of cocaine lead to patterns of drug use that, in vulnerable individuals, eventually result in addiction (Hyman, 1996). Drug addiction is a chronically relapsing disorder characterized by the compulsion to seek and take a drug, with loss of control in limiting intake (Nestler, 2001). Addiction is also characterized by the emergence of a negative emotional state (*e.g.*, dysphoria, anxiety, irritability) when access to the drug is prevented (Koob and Moal, 2006).

Drug-induced neuroadaptations are thought to be critical in the transition to addiction. Extensive studies both in animal models of a variety of species, as well as basic clinical research in humans, using neurochemical, molecular and related behavioral technologies, as well as a variety of imaging techniques, have documented that indeed chronic exposure to drugs does cause alterations in specific aspects of brain function which are persistent over varying periods of time or, in some cases, may even be permanent (Kreek and Koob, 1998; Nestler, 2001). As cocaine use and duration increases, the positive reinforcing effects are diminished while dysphoria (including agitation, anxiety and even panic attacks) increases, suggesting acute tolerance to the arousing and positive mood effects of cocaine (Koob and Moal, 2006). Tolerance can be defined as a given drug producing a decreasing effect with repeated dosing or when larger doses must be administered to produce the same effect (Koob and Moal, 2006).

Tolerance to the reinforcing effects of cocaine may be marked, leading to administration of very high drug doses (Hyman, 1996). However, tolerance does not develop to the stereotyped behavior and psychosis induced by stimulants and, in fact, these behavioral effects appears to show a sensitization (Post *et al.*, 1992; Hyman, 1996). Sensitization is defined as the long-lasting increment in response occurring upon repeated presentation of a stimulus (Nestler, 2001).

The neuroadaptative processes of tolerance and sensitization together with withdrawal symptoms have been proposed as key elements in the development of addiction (Hyman and Malenka, 2001). Withdrawal signs associated with cessation of chronic drug administration usually are characterized by responses that are opposite to the initial effects of the drugs. Withdrawal from chronic or high dose cocaine use in humans is associated with relatively few overt physical signs but a number of motivationally relevant symptoms such as dysphoria and depression, anxiety, anergia, insomnia and

craving (a compelling desire to re-experience the cocaine experience for the drug) (Weddington *et al.*, 1990; Satel *et al.*, 1991; Miller *et al.*, 1993; Koob and Caine, 1999).

There are several theoretical explanations of how drug-induced alterations in psychological function might cause a transition to addiction. The most traditional theoretical explanation of how drug-induced alterations might cause transition to addiction is the hedonic view that drug pleasure and subsequent unpleasant withdrawal symptoms are the main causes of addiction (see discussion in Robinson and Berridge, 2003). It defends that drugs are taken first because they are pleasant, but with repeated drug use homeostatic neuroadaptations lead to tolerance and addiction, such that unpleasant withdrawal symptoms ensue upon the cessation of use, thus, compulsive drug taking is maintaned, to avoid unpleasant withdrawal symptoms (Koob and Moal, *1997).* It is suggested that repeated drug use induces tolerance or downregulation in the mesolimbic dopaminergic system, decreasing the pleasant drug "highs", and that sudden cessation of drug use causes dopaminergic (and serotonergic) neurotransmission to further drop below normal levels, at least for several days, resulting in the dysphoric state of withdrawal (Koob and Moal, 1997). This theoretical explanation also suggests that repeated drug use can activate brain and hormonal stress responses (Koob and Moal, 2006). As a result, addicts who originally take drugs to gain a positive hedonic state are spiraled into a predominantly negative hedonic state, which causes the transition to addiction (Koob and Moal, 1997, 2006).

2.5. Cocaine and dopamine system

As mentioned above, the increase in extracellular DA following acute cocaine administration plays a major role in cocaine reinforcement. Studies using microdialysis have shown that acute cocaine administration leads to an immediate and dose-dependent increase in extracellular DA levels (Church *et al.*, 1987; Carboni *et al.*, 1989; Maisonneuve and Kreek, 1994). Increased synaptic levels of DA will stimulate DA receptors and activate several signal transduction pathways. Pharmacological studies with selective D1, D2 and D3 DA receptors antagonists and knockout studies have shown that all three receptors subtypes appear to mediate the reinforcing effects of cocaine, albeit possibly different components of the response. Low doses of D1 DA receptor antagonist block the reinforcing effects of intravenous cocaine self-administration (Maldonado *et al.*, 1993; Caine and Koob, 1994). D2 DA receptor antagonists block responding for cocaine but also have pronounced motor response inhibitory actions (Koob and Moal, 2006). Decreases in both D1 and D2 receptors have been observed after long-term, heavy exposure to passive administration or self-administration of cocaine in rats (Tsukada *et al.*, 1996; Maggos *et al.*, 1998), nonhuman primates (Moore *et al.*, 1998a, b) and humans (Volkow *et al.*, 1993). D3 DA receptor antagonists block drug-seeking behavior associated with cocaine in second order and progressive-ratio schedules (Koob and Moal, 2006).

Many studies have shown that chronic administration of psychostimulant drugs leads to significant changes in the dopaminergic system. In humans, imaging studies found reduced dopaminergic function in cocaine addicted people (Wu *et al.*, 1997; Volkow *et al.*, 1999). In animal studies, data from continuous self-administration or repeated binge experimenter administered cocaine also show significantly decreased extracellular

basal DA levels (Weiss *et al.*, 1992; Kreek and Koob, 1998). Moreover, as observed during an acute binge pattern of cocaine administration, the extracellular DA levels rise following each dose of cocaine with much greater intensity in the Acb than in the dorsal striatum, after chronic binge pattern cocaine, there is a significant decrease in the extracellular DA levels at the basal time points, and a lower elevation following each cocaine administration, both in the dorsal striatum and in Acb (Maisonneuve *et al.*, 1995). However, chronic administration of cocaine under intermittent schedules rather than a binge-like or continuous pattern results in a rise in the extracellular DA increase with repeated administration (Kalivas and Duffy, 1993; Pierce and Kalivas, 1997a). The data described above show that the response of dopaminergic neurons depended on the doses and pattern of exposure (Koob and Moal, 2006). Continuous access seems to be more likely to produce decreases in firing and transmission, and limited or intermittent access is more likely to produce later increases in firing and DA neurotransmission (Koob and Moal, 2006).

Cocaine works by blocking the DA transporter (DAT) and thereby increasing the availability of free DA within the brain. In addition, the reinforcing and euphoric effects of cocaine are primarily mediated by interaction with the DAT (Mortensen and Amara, 2003). Researchers in several disciplines have empirically demonstrated the integral nature of the DAT protein in both the acute and chronic effects of cocaine and in cocaine addiction (for review see Mash, 2008). In humans intravenous cocaine at doses commonly abused by humans (0.3-0.6 mg/kg) blocked between 60 and 77% of DAT sites. For subjects to perceive cocaine's effects (high feeling state) at least 47% of the DAT had to be blocked. These data demonstrate that in humans the doses used by cocaine abusers lead to significant blockade of DAT, and that this blockade can be associated with the subjective effects of cocaine (Volkov *et al.*, 1997).

Giros and colleagues observed that DAT knockout mice show an attenuated response to cocaine and a reduced preference for cocaine under self-administration paradigms. These mice, however, still self-administer cocaine (although more sessions were needed to meet self-administration criteria) indicating that developmentally compensatory non dopaminergic mechanisms can mediate cocaine-taking behavior in DAT-lacking animals. In *in vitro* studies, cocaine administration did not alter quantitatively measured DA transporter (DAT) mRNA levels in the SN or the VTA following subacute (3 days) binge, chronic (14 days) binge or 10 days withdrawal from a chronic binge administration pattern (Maggos *et al.*, 1997). However, decreases in DAT function including both binding and mRNA levels have been observed in selective brain regions, following 10 days of withdrawal from chronic cocaine administration (Kuhar and Pilotte, 1996).

The changes in brain dopaminergic function are likely to result in decreased sensitivity to natural reinforcers since DA also mediates the reinforcing effects of natural reinforcers, and the disruption of frontal cortical functions, such as the inhibitory control and salience attribution (Volkow *et al.*, 2003). These alterations could contribute to the loss of control and compulsive drug intake that characterize addiction (Volkow *et al.*, 2003). Moreover, reduced DA-mediated reward could explain the high rates of depression, irritability, anxiety, and suicide that have been reported in cocaine-addicted individuals (Dackis and O'Brien, 2001).

2.6. Effects of cocaine on the HPA axis

Cocaine's interactions with the HPA axis system are not yet fully understood. Acute cocaine administration stimulates release of gonadotropins, ACTH, and cortisol or

corticosterone and suppresses prolactin levels. Those effects could evidence that cocaine's effects on the HPA axis may be related to its reinforcing properties (for review see Mello and Mendelson, 1997). An improved understanding of the interactions between the neuroendocrine system and cocaine use and abuse will be helpful in clarifying some aspects of the neurobiology of drug abuse.

There is increasing empirical support for the notion that dysregulation of the HPA axis may increase vulnerability to depression, and a number of other psychiatric disorders, as well as immune dysfunction and cardiovascular disease (Heinrichs *et al.*, 1995; Chrousos and Gold, 1998; Nemeroff, 1998). Cocaine, like stress, modulates HPA axis activity and thereby contributes to the disruption of normal neuroendocrine function.

Acute administration of cocaine stimulates ACTH and cortisol secretion in humans (Baumann *et al.*, 1995; Heesch *et al.*, 1995; Sholar *et al.*, 1998; Elman *et al.*, 1999) and in rhesus monkeys (Heesch *et al.*, 1995; Sarnyai *et al.*, 1996; Broadbear *et al.*, 1999a), and ACTH and corticosterone release in rats (Rivier and Vale, 1987; Borowsky and Kuhn, 1991a; Levy *et al.*, 1991; Saphier *et al.*, 1993). The Ventral Striatum is the main brain area involved in the effects of cocaine on the HPA axis and several neurotransmitters/neuromodulators like DA, Noradrenaline/adrenaline, Acetylcholine (muscarinic), Glutamate (NMDA), Endogenous opioids and CRH are involved in this process (for review see Armario, 2010).

There is considerable evidence that the stimulatory effects of cocaine on HPA axis are mediated by CRH (Mello and Mendelson, 1997). Cocaine stimulates CRH release from hypothalamic tissue in *vitro* (Calogero *et al.*, 1989) and alters CRH levels in different brain areas *in vivo* (Sarnyai *et al.*, 1993). Moreover, cocaine-induced increases in ACTH and corticosterone release are prevented by passive immunization against CRH (Rivier and Vale, 1987; Sarnyai *et al.*, 1992). These results show that the action of

cocaine on the HPA axis is mediated via CRH receptors and depends on the release of endogenous hypothalamic CRH. In addition cocaine can activate the CRH gene expression in the PVN (for review see Armario 2010).

Although the exact mechanisms underlying the effects of cocaine on the HPA axis remain to be clarified, it is increasingly apparent that cocaine-related stimulation of ACTH, and by inference of CRH, is modulated by several interacting neurotransmitter systems (Mello and Mendelson, 2002). Because CRH release is regulated, in part, by DA and 5-HT, antagonists that are selective for DA or 5-HT receptors attenuate cocaine-induced stimulation of ACTH (Borowsky and Kuhn, 1991b; Levy *et al.*, 1991). Moreover, both DA and 5-HT receptor agonists stimulate ACTH release in rats (Borowsky and Kuhn, 1991b; Levy *et al.*, 1994; Baumann *et al.*, 1995).

In contrast to the acute stimulant effects of cocaine in the HPA axis, there are conflicting reports in the literature as to whether chronic cocaine exhibits persistent stimulatory effects on the HPA axis. Repeated cocaine exposure has been reported to produce tolerance (Zhou *et al.*, 1996), sensitization (Schmidt *et al.*, 1995) or no changes (Borowsky and Kuhn, 1991; Levy *et al.*, 1992) in the HPA axis response, depending on the experimental design used (Mantsch *et al.*, 2003). It is suggested that the frequency and duration of cocaine administration, among other variables, may be of critical importance in determining the effects of cocaine on HPA axis activity (Mantsch *et al.*, 2003).

Intermittently cocaine exposure usually does not alter basal levels of ACTH and cortisol or corticosterone, or the hormonal response to acute stimulation with cocaine or synthetic CRH factor (Borowsky and Kuhn, 1991a; Levy *et al.*, 1992; Torres and Rivier, 1992a, b; Laviola *et al.*, 1995; Broadbear *et al.*, 1999). Nevertheless, decreases in CRH receptors have been reported after intermittently cocaine (Goeders *et al.*, 1990;

Mello and Mendelson, 1997). However, tolerance to the stimulatory effects of cocaine on HPA axis function has been reported in rats following a chronic binge pattern administration (Zhou *et al.*, 1996). This tolerance is observed as attenuated corticosterone and ACTH responses to cocaine (Zhou *et al.*, 1996; Zhou *et al.*, 2003). In rats a significant reduction in plasma corticosterone response to cocaine was observed on day 14 of binge administration as compared with day 1 or 3 (Zhou *et al.*, 1996). Moreover, the development of tolerance to the effects of cocaine on the HPA hormone response is associated with a significant reduction in the CRH mRNA levels in the hypothalamus (Zhou *et al.*, 1996). Other group study showed that continuous exposure to cocaine induces behavioral tolerance whereas intermittent administration yields behavioral sensitization (Cain *et al.*, 1993).

During early withdrawal from chronic binge cocaine administration, an activation of the HPA axis of the rat has been observed, as indicated by the significant elevation of plasma ACTH and corticosterone 1 and 4 days after withdrawal (Zhou *et al.*, 2003). However, 10 days after withdrawal, ACTH and corticosterone were at control levels (Zhou *et al.*, 2003). One day after withdrawal from intermittent cocaine administration increased basal levels of corticosterone has been reported (Levy *et al.*, 1994). In contrast, when rats are permitted to self-administer a very high dose of cocaine during long-access sessions, plasma corticosterone is not elevated during acute withdrawal but rather is decreased (Mantsch *et al.*, 2000).

In humans, as already mentioned, cocaine also disrupts HPA axis function. Cocaine administration increases plasma ACTH and cortisol levels in cocaine abusers (Teoh *et al.*, 1994; Baumann *et al.*, 1995; Elman *et al.*, 1999). However, the effects of a challenge dose of cocaine on ACTH secretion are significantly lower in cocaine-dependent men than in occasional cocaine users (Mendelson *et al.*, 1998). Nevertheless, one early study has shown higher basal plasma ACTH and cortisol

levels in cocaine addicts one day after the cessation of cocaine self-administration (Vescovi *et al.*, 1992).

However, after a brief period of abstinence, basal and CRH stimulated ACTH and cortisol levels in cocaine-dependent patients do not show differences from healthy subjects (Vescovi *et al.*, 1992; Baumann *et al.*, 1995b; Mendelson *et al.*, 1998; Jacobsen *et al.*, 2001a).

In general, the previous studies suggest that all addictive drugs activate the HPA axis mainly by acting within the brain. The mechanisms used and brain areas involved (besides the PVN) seem to differ among the various drugs. Acute cocaine acts as a pharmacological stressor in humans and animal models, increasing ACTH levels, although chronic cocaine may affect HPA axis in different ways in function of different factors.

3. Stress and Cocaine

3.1. General concepts

In humans, there is a growing literature that suggests a possible linkage between stress and addiction. A number of studies have identified individuals with dual diagnosis of post-traumatic stress disorders (PTSD) and drug addiction (*e.g.* Zaslav, 1994; Donovan *et al.*, 2001; reviewed by Jacobsen *et al.*, 2001b). Although a causal relationship between exposure to stress and drug addiction has not been clearly

established, prevalence estimates suggest that rates of drug abuse among individuals with PTSD may be as high as 60-80%, suggesting a relationship between stress and increased drug addiction in some cases (Donovan *et al.*, 2001). Exposure to stress or simply the presentation of stress-related imagery have also been identified as potent events for provoking relapse to drug seeking in humans (Lamon and Alonzo, 1997; Sinha *et al.*, 2000; Sinha, 2001). Clinical studies have demonstrated that simple exposure to environmental stimuli or cues previously associated wíth drug taking can produce intense drug craving (Robbins *et al.*, 1999). Preclinical investigations have also demonstrated cue-induced reinstatement (Meil and See, 1996; See, 2002). Within this general context, in this section we will focus in the interacion between stress and cocaine at different levels: endocrine, behavioral, brain activation and gene expression.

3.2. The HPA axis and the behavioral effects of cocaine

The HPA axis seems to have particular importance in drug addiction (Piazza and Le Moal, 1996; Goeders, 1997). The activity of the HPA axis prior and subsequent to cocaine availability appears to be an important determinant of whether or not individuals will engage in cocaine-seeking behavior. Studies show that Corticosterone is necessary during acquisition, as self-administration does not occur unless this stress-related hormone is increased above a threshold critical for reward (Goeders and Guerin, 1996; Goeders 2002). On the other hand, elevated plasma corticosterone facilitates the initiation of cocaine self- administration (Goeders and Guerin, 1996; Mantsch *et al.*, 1998). These data converge to suggest a role for the HPA axis in the modulation of cocaine reinforcement (Goeders, 1997; Goeders, 2002a).

More evidence demonstrating an involvement of the HPA axis in the relapse to cocaine abuse is that corticosterone and CRH are also critical for the stress- and cue-induced reinstatement of extinguished cocaine-seeking behavior (Goeders and Guerin, 1996; Goeders, 1997; Mantsch *et al.*, 1998; Mantsch and Goeders, 1998; Goeders, 2002b). In fact, preclinical studies suggest that the reinforcing properties of cocaine may be influenced by the HPA axis activity (Piazza and Le Moal, 1996; Goeders, 1997; Goeders, 2002a). In humans, the temporal concordance between cocaine-induced stimulation of ACTH, epinephrine and subjective euphoria suggests that these hormonal changes are significant concomitants of the abuse-related effects of cocaine (Mendelson *et al.*, 2002). Although high levels of corticosterone are facilitatory/modulatory factors to maintain self-administration behavior, the increases in corticoesterone levels induced by drug exposure are not critical to maintain self-administration (Marinelli and Piazza, 2002). Thus, self-administration of psychostimulants can dramatically increase glucocorticoid secretion (Baumann *et al.*, 1995b; Broadbear *et al.*, 1999b; Galici *et al.*, 2000), but when this drug-induced increase in glucocorticoids levels is blocked there is no an important effect in the response to cocaine (Deroche *et al.*, 1997). In addition, animals can consistently self-administer cocaine at doses that do not increase corticosterone levels (Broadbear *et al.*, 1999a), further supporting the notion that drug-induced glucocorticoid secretion is not essential to maintain self-administration behavior (Marinelli and Piazza, 2002).

Drug-naive rats that were surgically adrenalectomized did not learn to self-administer cocaine (Goeders and Guerin, 1996b). Dose-response studies have shown that adrenalectomy (ADX) induces a vertical downward shift to the dose-response curve to cocaine during the maintenance phase (Deroche *et al.*, 1997) and this may explain why ADX rats do not learn to self-administrate cocaine. Moreover, metyrapone, which blocks corticosterone synthesis, significantly decreases cocaine-induced locomotion and relapse of cocaine self-administration (Piazza *et al.*, 1994; Goeders and Guerin,

1996a; Goeders and Guerin, 1996b). Pretreatment with a CRH receptor antagonist also produced dose-related decreases in cocaine self-administration in rats (Goeders and Guerin, 2000).

Even though corticosterone plays a facilitatory role on many behavioral responses to psychostimulants, its role in relapse is a little more controversial. Suppression of glucocorticoids does not seem to have important effects on relapse induced by drug priming. Thus, cocaine-induced reinstatement of cocaine self-administration is only minimally decreased by ADX (Erb *et al.*, 1998) and is not modified by ketoconazole, which reduces circulating levels of corticosterone (Mantsch and Goeders, 1998). Instead, corticosterone may play a significant role in stress (electric footshock) and cue-induced reinstatement of cocaine seeking (Goeders, 2002a; Goeders and Clampitt, 2002).

3.3. Interaction between glucocorticoids, dopamine and vulnerability to drugs

It has been hypothesized that exposure to physically or psychologically stressful events may render an individual more sensitive to the reinforcing effects of drugs such as cocaine (Piazza and Le Moal, 1996; Goeders, 1997; Piazza and Le Moal, 1998). This hypothesis is supported by studies where prior exposure to stressful stimuli (electric footshock, social stress and tail pinch) results in an enhancement of behaviors related to drug acquisition (Piazza *et al.*, 1990; Haney *et al.*, 1995; Goeders and Guerin, 1996b), maintenance of cocaine self-administration (Miczek and Mutschler, 1996), and

reinstatement of cocaine-seeking in rats following extinction of operant behavior (Erb *et al.*, 1998).

A faster acquisition of cocaine self-administration has been found in rats exposed to social stress, such as exposure to a resident aggressive animal (Haney *et al.*, 1995; Tidey and Miczek, 1996). Physical stressors can also enhance the propensity to develop cocaine self-administration. This has been shown for repeated tail pinch (Piazza *et al.*, 1990), food restriction (Papasava and Singer, 1985; Carr *et al.*, 2000), and electric footshocks (Goeders and Guerin, 1996b; Xi *et al.*, 2004). Very early life events, such as prenatal stress, can also increase vulnerability to psychostimulants at adulthood (Kippin *et al.*, 2008).

The reinstatement of cocaine seeking behavior can be elicited by exposure to brief periods of intermittent electric footshock or food restriction stress in rats (Shaham *et al.*, 2000; Shalev *et al.*, 2003). However, cocaine reinstatement was prevented in adrenalectomized rats but not in adrenaloctomized animals with corticosterone replacement. In addition, intracerebroventricular infusions of CRH receptor antagonists were also found to block (Erb *et al.*, 1998) or attenuate (Shaham *et al.*, 1998) footshock-induced reinstatement in intact animals and in adreneloctomized animals with corticosterone replacement (Erb *et al.*, 1998). This last example supports the view that CRH acting at extra-hypothalamic sites in the brain also plays an important role in stress-induced reinstatement of cocaine seeking (Shaham *et al.*, 2000). In fact, acute cocaine has been reported to affect CRH-like immunoreactivity in a number of brain regions that are not thought to be directly linked to pituitary-adrenal activity, including basal forebrain and amygdala (Sarnyai *et al.*, 1993; Gardi *et al.*, 1997). Extrahypothalamic CRH systems appear to be involved in certain symptoms of acute drug withdrawal, such as anxiety, and in stress-induced relapse to drug seeking (Sarnyai *et al.*, 2001). Once again, several studies have suggested an important role

for the HPA axis in addiction, showing a link between HPA axis and the ability of environmental cues to stimulate cocaine-seeking behavior in rats. Pretreatment with the corticosterone synthesis inhibitor, ketoconazole, reversed the cue-induced reinstatement of extinguished cocaine-seeking behavior and also attenuated the conditioned increases in plasma corticosterone observed during reinstatement (Goeders and Clampitt, 2002). Pretreatment with a CRH receptor antagonist also resulted in a similar decrease in the ability of environmental cues to stimulate cocaine-seeking behavior (Goeders and Clampitt, 2002).

In contrast to the effects observed during acquisition or reinstatement, neither exposure to footshock (Goeders and Guerin, 1996b) nor exogenous injections of corticosterone affect ongoing cocaine self-administration. This inability to affect maintenance of drug use is probably related to the fact that plasma corticosterone is significantly elevated in a dose-related manner during cocaine self-administration, and further increases in corticosterone are without effect, since a threshold critical for reward has already been crossed (Goeders and Clampitt, 2002). However, low-dose cocaine self- administration (0.25 mg/kg per infusion) can be attenuated by drugs that inhibit the synthesis and/or release of corticosterone, since in this case plasma concentrations of corticosterone is probably reduced below the critical reward threshold (Goeders, 2002a).

It is not inherently intuitive how exposure to a stressor can increase vulnerability to drug self-administration (Goeders and Clampitt, 2002). The preclinical literature suggests that stress increases reinforcement associated with psychomotor stimulants, possibly through a process similar to sensitization (Piazza and Le Moal, 1998; Goeders, 2002b), whereby repeated intermittent injections of cocaine increase the behavioral and neurochemical responses to subsequent exposure to the drug. Interestingly, exposure to stressors or administration of corticosterone can also result in sensitization to the behavioral and neurochemical (*e.g.*, Acb and DA) response to

cocaine (Rouge-Pont *et al.*, 1995; Prasad *et al.*, 1998). These effects are attenuated in adrenalectomized rats (Prasad *et al.*, 1998; Przegalinski *et al.*, 2000) or when corticosterone synthesis is inhibited (Rouge-Pont *et al.*, 1995). The ability of stressors to facilitate the acquisition of drug self-administration may, therefore, result from a similar sensitization phenomenon, perhaps involving DA (Goeders, 1997; Piazza and Le Moal, 1998).

Stress, through activation of the HPA axis and the release of glucocorticoids, influences various regions of the brain including dopaminergic neurons (Piazza and Le Moal, 1996; Piazza and Le Moal, 1997; Piazza and Le Moal, 1998), which express corticosteroid receptors (Harfstrand *et al.*, 1986). In normal situations, glucocorticoids state-dependently increase dopaminergic function, especially in mesocorticolimbic regions, during various consummatory behaviors exhibited in rodent's active period of the light/dark cycle (Piazza and Le Moal, 1996). The interaction of glucocorticoids with mesocorticolimbic dopaminergic system may have a significant impact on vulnerability to self-administer psychostimulant drugs, since increased dopaminergic transmission in these pathways is critical for the reinforcing properties of abusive drugs, rendering the individuals more susceptible to the drug reinforcement (Roberts *et al.*, 1980; Koob and Le Moal, 1997).

GR have been identified in rodent brain on dopaminergic neurons in the VTA and SN as well as in dopaminergic terminal regions including the Acb and PFC (Harfstrand *et al.*, 1986; Diorio *et al.*, 1993; Cintra *et al.*, 1994). Stress-induced elevations in corticosterone may play a critical role in activation of dopaminergic transmission, with corticosterone treatment increasing, and adrenalectomy decreasing extracellular DA levels in Acb and prefrontal cortex of rodents (Imperato *et al.*, 1989; Piazza *et al.*, 1996). This suggests that stress-induced increases in glucocorticoids may interact with mesocorticolimbic brain regions to facilitate drug taking behavior.

4. Immediate early genes (IEGs)

4.1. General concepts

The stimulation of neurons can suppose two different mechanisms through which information is processed and transmitted: the electrophysiological activity and the second messenger cascades. Information about the stimulus is rapidly transmitted and processed through action potentials, while the intracellular messengers involve the production of transcription factors that initiate and/or repress the transcription of other genes, altering the response of neurons to future stimuli.

Due to its rapid induction, which does not require new protein synthesis, these genes are called immediate-early genes (IEG). The IEG encode many functionally different products (Nedivi *et al.*, 1993) such as secretory proteins, cytoplasmic enzymes and transcription factors. The IEG with transcriptional activity are called indulgent transcription Factors (ITF) to distinguish from the rest of IEG without transcriptional activity. The rapid induction of IEG is explained by the presence of preexisting transcription factors in unstimulated cells. This IEG present in unstimulated cells are called constitutive transcription Factors (CTF) (for review see Herdegen and Leah, 1998).

4.2. c-fos

Undoubtedly, the most studied ITF in the CNS is the proto-oncogene c-fos that, by its nature, is a valuable tool in the study of CNS activation against different stimuli. The

viral gene "fos" was isolated in 1982 as an oncogene of the osteosarcoma virus in the mouse Finkel-Biskis-Jiskins (FBJ-MSV) and shortly after was described his cell homologous, c-fos (review in Herdegen and Leah, 1998). In the cell, the c-Fos protein dimerizes with c-Jun family proteins, forming the AP-1 transcription complex (activator protein 1) that regulates the expression of other genes that have in their promoter a binding site for AP-1 (for review see Herdegen and Leah, 1998). The c-fos gene have a complex promoter, sensitive to the action of various second messenger cascades that eventually converge and regulate its expression (Ginty *et al.*, 1994). Multitude of stimuli can induce expression of c-fos, through intracellular messengers like neurotrophic factors, neurotransmitters, depolarization and intracellular increases of Ca^{2+} (for review see Herrera and Robertson, 1996, Herdegen and Leah, 1998). There are also mechanisms of promoter repression or transcription inhibition that regulate the gene activity. In addition to transcription, the activity of c-fos is regulated through the degradation of its own messenger, having its mRNA a half-life that can vary according to circumstances, but with a peak ranging between 30 and 60 min from the beginning of the acute stimulus (Cullinan *et al.*, 1995). Finally, the proteins encoded by ITF are among the more rapidly degraded, in a way that their half-life is very short, 90 to 120 min for c-Fos (for review see Jariel-Encontre *et al.*, 1997). This degradation occurs when the synthesis is over and the majority of c-Fos molecules (90%) are associated Jun proteins (Kovary and Bravo, 1991a, 1991b) since the formation of c-Jun/c-Fos dimers promotes the degradation of c-Fos (Papavassiliou *et al.*, 1992). Due to the rapid synthesis of c-Fos and the delay in the increase of c-Jun, there is a period without Jun after stimulation where protein c-Fos can form heterodimers with the GR receptor and other transcription factors (ATF-4, ATF-2, CBP, p65) influencing the gene expression that occurs immediately after cell activation in the most sensitive period (for review see Herdegen and Leah, 1998). There are also various combinations of Fos and Jun families, in a way that c-Fos/c-Jun complex activate gene expression while the c-Fos/Jun B complex seems to be inhibitor (Sheng and Greenberg, 1990). These

interactions reflect the complex transcriptional changes that occur in the neuron once activated.

The expression of c-fos is considered a good anatomical and functional mapping tool to identify cells and circuits activated in response to different stimuli (reviewed in Hoffman and Lyo, 2002). The expression of c-fos have two important characteristics: the low levels of transcription under basal conditions and its induction by a wide range of stimuli (review in Armario, 2006). Should not be equated the c-fos expression with the increased electrophysiological activity of the neurons because, although both phenomena are commonly coupled they can be dissociated in a way that depolarization can occur without c-fos expression (Luckman *et al.*, 1994). Furthermore, depolarization by itself can not induce the c-fos expression, for that it needs the participation of second messenger cascades (Robertson *et al.*, 1995). The changes in intracellular signaling pathways induce the c-fos expression and depolarization *per se*.

Despite the large amount of information accumulated in functional mapping studies using c-fos as a marker of activation, important information remains to be known about which genes are affected in neurons that present c-fos induction in vivo. In vitro studies show the participation of c-fos in the regulation of different neuropeptides expression and neurotrophic factors such as nerve growth factor (NGF). In *in vivo* studies where the expression of c-fos was inhibited by the administration of antisense oligonucleotide in different areas of CNS (such as the striatum or the central amygdala) allowed to relate the c-fos induction to neuronal plasticity processes that occur in these nuclei after administration of psychostimulants or exposure to stressful stimuli, respectively (reviewed in Chiasson *et al.*, 1997).

Although c-fos is a very powerful tool, its use has certain limitations (reviewed in Hoffman and Lyo, 2002). As mentioned before, we can find neuronal depolarization

withthout c-fos expression, so we cannot rule out the involvement of a particular area in a given process by the lack of c-fos expression. One possible solution is to use other IEG such as c-Jun, ERK, Fos B or NGFI-A with complementary expression patterns (Herdegen *et al.*, 1995, for review see Senba and Ueyama, 1997). Another limitation is a consequence of the c-fos expression dynamics, it works fine to study the activation promoted by acute stimuli with short duration (from minutes to few hours) but it is inconvenient to study long duration stimuli. This is because after a limited period of time, depending on the type of the stimulus, both mRNA and protein return progressively to baseline levels. This phenomenon is very common with high intensity emotional stimuli, such as IMO or movement restriction (Imaki *et al.*, 1992; Senba *et al.*, 1994; Umemoto *et al.*, 1997; Trneckova *et al.*, 2007), but with systemic stimuli, like endotoxin administration (Rivest, 1995) or hypovolemia (Tanimura and Watts, 2000), it can be observed sustained levels (3 to 6 h) of c-fos expression in the PVN. Because of c-fos short half-life, it is better to use other IEG with a longer half-life (like Δ-FosB) to study the effects of prolonged stimuli or long-term effects of acute stimulation.

Another limitation of the use of c-fos is the imposibility to identify the type of activated neurons. Thus, basic questions like whether there are activated GABAergic or inhibited glutamatergic neurons remain unanswered. The combination of c-fos detection with other markers provides additional information on the processes occurring in the CNS. Double labeling techniques can be used to determine the neurotransmitter that is expressed in c-fos activated neurons (*i.e.*Ceccatelli *et al.*, 1989) and through the use of feedback-tracers identify the pathways involved in the activation of a specific group of neurons (*i.e.*Menetrey *et al.*, 1989).

4.3. IEGs in the study of stress

During the last decade the induction of c-fos has been widely used to understand better how the CNS processes and responds to stressors (reviewed in Imaki *et al.*, 1995, Herman and Cullinan, 1997; Kovacs, 1998; Sawchenko *et al.*, 2000, Pacak and Palkovits, 2001, Herman *et al.*, 2003). In fact, the classification of stressful stimuli in systemic and emotional emerges from the results obtained with c-fos expression (reviewed in Sawchenko *et al.*, 2000). This division is based on similarities in the general pattern of activation observed after exposure to a wide range of stressors. In contrast to what happens with the systemic stimuli, the activation maps of the emotional stimuli show large similarities, even when these stimuli differ greatly in their characteristics.

Some brain areas show activation after handling the animal or after exposure to novel environments implicit in some stressors (i.e., box where the electric shocks are applied), regardless to the stressor subsequently presented (reviewed in Kovacs, 1998). Within this group are included some thalamic and amygdala nuclei, cortical and subcortical areas, the LS, anterior BNST, hippocampal formation and PAG. These areas are considered to be related to a general state of activation (arousal) (review Cullinan *et al.*, 1995, Duncan *et al.*, 1996; Campeau and Watson, 1997; Kollack-Walker *et al.*, 1997). This state of alert can be the common factor to most emotional stimuli and it could partially explain the coincidences in the activation pattern. Certainly these are areas that present a low threshold for c-fos induction.

Few works have studied the c-fos response to different intensities of stimuli. It is worth mentioning three papers: Ericsson and colleagues (1994) who administered increasing doses of IL-1; Campeau and colleagues (Campeau and Watson, 1997, Campeau *et al.*,

1997) who used different intensities of noise and fear conditioning paradigms, and Pace and colleagues (2005) who used several novel environments and restraint in a tube. In our laboratory (Ons *et al.*, 2004) we compared the expression of c-fos after exposure to emotional stressors of different intensity: novel environment, forced swimming and immobilization. In our experiments we found areas such as the cortex that respond equally to the three presented stimuli, while the LS, MeA, PVN and LC present a c-fos induction proportional to the intensity of the stimulus. Using an acoustic stimulus as a stressor, Campeau and Watson (1997) found three distinct patterns of c-fos induction: (a) areas that respond to the experimental box (novel environment), regardless of the presented stimulus and with the same intensity; (b) areas related to the auditory system that respond proportionally to the intensity of the stimulus, and finally, (c) areas where c-fos is induced only with higher intensity stimuli (90 and 105 dB): BNST, LS, hippocampus (dentate gyrus) several hypothalamic nuclei (PVN) and stem nuclei as the PAG and Rafe. Pace and colleagues (2005) used three novel environments (a homecage different from the usual, a circular open field and an elevated plataform) and restraint in tube as stressors that differ in intensity judging by ACTH and corticosterone release. They observe that c-fos induction in the PVN follows the same proportional intensity pattern of the stimulus than ACTH and corticosterone release. By contrast, c-fos induction in the hippocampus and cortex seems to be more related with the exploration potential that has the new situation than the stimulus intensity; because it is higher in the elevated platform and open field than with immobilization.

Reviewing the literature concerning the different emotional stimuli, we found areas such as the cortex and thalamus with similar levels of c-fos induction, regardless of the intensity of the presented stimulus. In contrast, c-fos induction in areas such as LS, MeA, some regions of the BNST, the PVN and LC appear to positively discriminate between different intensities of stress. This apparent insensitivity of certain areas of the

CNS to the intensity of the stimulus suggests (Armario, 2006) that: (a) only a limited number of neurons respond to stimulation and c-fos induction in discrete neurons could be an “all or nothing” phenomenon, and (b) the neurons are so sensitive that respond to the slightest stimulus. On the other hand, the existence of areas that respond proportionally to the intensity of emotional stimuli suggests that in these areas larger numbers of neurons are recruited in response to the more severe stimuli and possibly some neurons present different activation thresholds.

To explain how emotional stimuli of clearly distinct nature eventually activate the same areas of the CNS there are two alternative hypotheses (Armario, 2006). The first, and most likely, implies that the same neuronal population is activated regardless of the presented stimulus. This hypothesis fits with the concept of arousal and with the nonspecific activation of the cortex through monoaminergic projections from the brainstem and the signals from the unspecific projection nuclei of the thalamus. The second possibility contemplates a certain level of selectivity, with the activation of circuits anatomically close but functionally separate. That is, in the same area of CNS could coexist neuronal populations with different neurochemical phenotype (especially regarding the neuropeptide used as co-transmitter), and each one of the distinct populations would be activated against a specific stimulus.

4.4. IEGs in the study of cocaine abuse

Classically, the study of CNS areas implicated in reinforcement has been addressed mainly by intracranial electrical stimulation techniques, specific lesions and administration of drugs in specific areas of the CNS. However, since the early 90's, the

IEG have been employed as a tool for the study of anatomical and functional bases of addiction (review in Sumner *et al.*, 2004).

Drugs of abuse trigger the expression of several IEG and each drug has a characteristic pattern of activation (Erdtmann-Vourliotis *et al.*, 1999; Valjent *et al.*, 2004), that also varies depending on the type of administration, acute or chronic (reviewed in Torres and Horowitz, 1999). As already mentioned, all drugs of abuse increase the extracellular levels of dopamine in the AcbSh (Di Chiara and Imperato, 1988), although through distinct mechanisms (review in Geracitano *et al.*, 2006). The PFC and the extended amygdala are involved in both positive and negative reinforcement (reviewed in Koob *et al.*, 1998) and are also important in the response to drugs of abuse (Alheid and Heimer, 1988, Heimer and Alheid, 1991). Therefore, it is expected that all drugs of abuse end up activating the Acb, amygdala and PFC, like all stressful stimuli can activate PVN, regardless of their particularities. However, this does not happen with all drugs or with the same intensity. After acute cocaine administration c-fos expression has been observed not only in the Acb, but also in other areas of the CNS, such as the striatum and the cerebellum (Couceyro *et al.*, 1994; Samaha *et al.*, 2004). Amphetamine induces the expression of c-fos in many areas of the CNS such as the neocortex, caudate-putamen and Acb (Uslaner *et al.*, 2001a), BNST and central amygdala (Day *et al.*, 2001), among others (reviewed in Harlan and Garcia, 1998). However, Valjent and colleagues (2004), using ERK as a marker of neuronal activation, found activation in reward-related areas such as Acb, BNST, CeA and PFC following the administration of cocaine, nicotine, morphine or tetrahydrocannabinol (THC), but not after administration of other substances without addictive potential: antidepressants (desipramine and fluoxetine), scopolamine, caffeine and antipsychotics (haloperidol, raclopride and clozapine).

By contrast, Erdtmann-Vourliotis and collegues (1999), comparing the c-fos expression induced by administration of drugs with low addictive potential such as THC, the 3.4-methylenedioxymethamphetamine (MDMA) and lysergic acid diethylamide (LSD) with other with high potential such as cocaine and morphine have found that drugs with less potential are those that induce c-fos expression with more intensity and in a greater number of areas. All MDMA, THC and LSD are able to induce c-fos in Acb, dorsal striatum, the LS and PVN while cocaine only activate some regions of the caudate-putamen and morphine the LS. Some discrepancies could be due to methodological differences, as the dose, since in some cases, higher doses are required to induce c-fos than to observe effects on behavior. For example, Erdtmann-Vourliotis and collegues (1999) to analyze the induction of c-fos mRNA used doses of 4 to 5 times higher than those used in drug discrimination tests, that model the interoceptive stimuli produced by drugs (eg, Meehan and Schechter, 1998; Schechter, 1998, Shippenberg and Heidbreder, 1995, LSD, MDMA and cocaine, respectively). Differences have also been observed depending on the route and speed of administration, with greater induction of c-fos with rapid intravenous infusions of cocaine (Samaha *et al.*, 2004).

We can expect differences or similarities in the pattern of activation, depending on the pharmacological effects of each drug. For example, drugs that potentiate the serotonergic system, such as LSD or MDMA, can activate discrete areas by direct action on their molecular targets, as occurs in the PAG or Rafe, areas rich in 5-HT (Erdtmann-Vourliotis *et al.*, 1999, Stephenson *et al.*, 1999). The activation of these areas in turn could trigger the activation of other regions of the CNS.

Another common element in the administration of most drugs is the activation of the PVN (reviewed in Lu and Shaham, 2005; Sinha, 2005), although the pathways and mechanisms implicated are unclear. The PVN can be directly activated by drugs or can

be indirectly activated by the action of drugs on areas of the CNS common to the processes of stress and addiction, such as PFC or amygdala.

IEGs are very useful for the study of simultaneous exposure to several stimuli. The brain activation induced by drugs of abuse is affected by the environmental conditions surrounding the administration, such as stress, what is very important for the aims of the present thesis. Previous studies showed that when amphetamine is given in association with environmental novelty, it induces a much more robust increase in psychomotor activation (Badiani *et al.*, 1995b; Badiani *et al.*, 1998; Badiani *et al.*, 1997; Browman *et al.*, 1998; Crombag et al, 1996; Uslaner *et al.*, 2001) than when it is given in the home cage. In agreement with this fact, it has been shown that administration of psychostimulants in novel (stressful) environments creates a pattern of c-fos expression higher to that found when the administration is in the home-cage (Ostrander *et al.*, 2003). Ostrander and collaborators (2003) findings are very important for the aims of the present study because suggest that psychostimulants may interact with the brain activation induced by stress. Previous studies showed that when amphetamine is given in association with environmental novelty, it induces a much more robust increase in psychomotor activation (Badiani et al., 1995b; Badiani et al., 1998; Badiani et al., 1997; Browman et al., 1998; Crombag et al, 1996; Uslaner et al., 2001) and in striatal c-fos mRNA (Cenci and Bjorklund, 1993) than when it is given in the home cage. In contrast, environmental novelty has no effect on the primary neuropharmacological action of amphetamine in the striatal complex, (*i.e.*its ability to induce dopamine release in the caudate nucleus or in the shell and core of the nucleus accumbens) (Badiani et al., 1998). The ability of amphetamine and cocaine to induce c-fos in the striatum is widely thought to be DA-dependent (Uslaner et al., 2001b).

In some brain areas an additive (summatory) effect or a potentiation (positive synergy) effect could be detected, and in other areas the possibility to detect negative synergies

exists. Summatory or positive synergies have been observed in the mPFC, Cpu and Acb (Badiani *et al.*, 1998; Uslaner *et al.*, 2001), and negative synergies in a subregion of the BNST and in the CeA (Day *et al.*, 2001, 2005, 2008). In our laboratory, using amphetamine and c-fos as a marker, negative synergies have been observed in the PVN (Gómez-Román *et al.*, 2015), the LS and the prelimbic cortex (PrL; Gómez-Román, 2012). In the present thesis we will focus in another psychostimulant, cocaine.

HYPOTHESIS AND OBJECTIVES

In this thesis we specifically studied the effects of the simultaneous exposure to stress and cocaine. We focused on the short and long-term endocrine, behavioral and neural consequences using a severe stressor (IMO), in adult male rats. Whereas the anorectic and putatively anxiogenic effects of cocaine predict that concomitant administration of the drug could exacerbate the negative consequences of stress, the hypothesis of self-medication (*i.e.*Darke, 2012) predicts that cocaine could counteract some of the negative emotional consequences of stress, this property contributing to increase the probability of cocaine consumption under stress or when exposure to stress is anticipated. This project directly tested these two possibilities: (1) Cocaine exacerbates the negative consequences of stress; or (2) Cocaine counteracts some of the negative emotional consequences of stress. The understanding of these interactions may be useful for the prevention and treatment of the comorbidity between diseases related to stress and drug addiction.

Given all the above, the general objective of this work is to characterize the neurobiological substrate of the interaction between cocaine (30 mg/kg) and stress (IMO as an acute severe emotional stressor). We propose the following specific objectives:

1. To study anhedonia-like behavior (two-bottle choice saccharin preference test) and anorexia (food consumption and body-weight gain) in response to IMO and/or the injection of cocaine.

2. To characterize the HPA axis responses induced by IMO and/or cocaine, using ACTH and corticosterone as markers.

3. To study the long-term effects of IMO and/or cocaine in anxiogenic-like behavior (elevated plus-maze and acoustic startle response) and depressive-like behavior (forced swim test).

4. To measure the long-term effects of IMO and/or cocaine in the endocrine sensitization induced by novel (heterotypic) stressors (elevated plus-maze and forced swim test).

5. To evaluate the long-term effects of IMO and/or cocaine in the endocrine desensitization induced by a second exposure to the same (homotypic) stressor (IMO).

6. To describe the activation of the CNS induced by IMO and/or cocaine, using c-fos as a functional anatomical mapping tool, in key areas of the CNS, by in situ hybridization (ISH).

7. To study several mRNA transcripts of dopaminergic-related peptides which are involved in the response to IMO and/or cocaine, in key areas of the CNS, by real-time polymerase chain reaction (RT-PCR).

8. To study several mRNA transcripts of the HPA axis which are involved in the response to IMO and/or cocaine, in key areas of the CNS, by real-time polymerase chain reaction (RT-PCR).

MATERIAL AND METHODS

1. Animals

This study was conducted using adult male Sprague-Dawley rats obtained from the breeding center of the Universitat Autònoma de Barcelona (N=42-50 for each experiment). To fulfill the Doctor Europeus requirements, part of the study was conducted in the Neuroprotection laboratory (Neuropharmacology Group) at the Institute for Molecular and Cell Biology, Porto, Portugal. This parcel of the study was conducted using male Wistar rats acquired from the breeding center of IBMC (strain originally from Charles River Laboratories) (N=48). Nevertheless, the conditions of animal maintenance were equal in the two laboratories. The animals were about sixty days old (body weight: 390 ± 20 g) at the beginning of the experiments. They were housed in pairs in opaque cages of 1000 cm3 (50 x 25 x 15 cm), with standard bedding (Ultrasorb, Panlab SLU), except in Experiment 2 of Chapter I were the animals were singly-housed. Animals were housed in standard conditions of temperature (21 ± 1 ºC), humidity (40-60%) and on a 12 h light / 12 h dark schedule (lights on at 08:00 h), at least one week before the experiment started. Appropriate food (Diet A-04, Panlab SLU) and tap water were available ad libitum.

2. Animal Ethics

The experimental protocols of the experiments run in Spain were approved by the Committee of Ethics of the Universitat Autònoma de Barcelona (CEEAH), by the Generalitat of Catalunya (DARP), following the “Principles of laboratory animal care”, and was carried out in accordance to the European Parlament Parliament and the Council of European Union Directive (2010/63/EU) with BOE 2013 (Real Decreto 53/2013) on the protection of animals used for scientific purposes. The animal facilities were also approved by the Generalitat of Catalunya.

In the experiment conducted in the Neuroprotection group all procedures were approved by the Portuguese Agency for Animal Welfare (General Board of Veterinary Medicine in compliance with the Institutional Guidelines and the European Convention).

All researchers that took part in the study had the appropriate degree to work with life animals (level C awarded by FELASA, Generalitat de Catalunya).

3. General treatments

The animals were handled during six days for approximately 2 min a day until the beginning of the experiment. In addition, animals were previously habituated to the injection protocol and in some experiments to blood sampling procedure before treatments. The subjects were distributed into four groups, in function of cocaine injection and exposure to immobilization on wooden boards (IMO): saline-control

(SalC), cocaine-control (CocC), saline-IMO (SalIMO) and cocaine-IMO (CocIMO). Animals were injected with saline or cocaine (30 mg/kg, i.p.; 4 ml/Kg) immediately prior to the 1 h of exposure to IMO. All experiments started at 8 am. The different experimental groups were counterbalanced to the time of the animals' sacrifice thereby minimizing the possible influence of circadian rhythm on the results.

4. Drugs

The cocaine hydrochloride (provided by Ministerio de Sanidad, Spain) solution was prepared just before the injection and the dose injected was expressed in terms of the base. Cocaine hydrochloride used in the Neuroprotection laboratory was obtained from Sigma Chemicals (St. Louis, MO, USA). A cloth was used to cover the animals during the intraperitoneal (i.p.) injection with Hamilton syringes and sterile 25-G syringe needles.

5. Stress: Immobilization on boards (IMO)

Immediately after injection, IMO rats were stressed for 1 h by taping their four limbs to metal mounts attached to a wooden board (Gagliano *et al.*, 2008; Muñoz-Abellán *et al.*, 2008). Head movements were restricted using two plastic pieces (7 × 6 cm) placed in each side of the head, while the body was subjected to the board by a plastic cloth (10 cm wide) attached with velcro^{R} that surrounds the whole trunk. In the no stress groups,

rats were injected and afterwards returned and maintained undisturbed in the vivarium. Injection and IMO were performed in distinct rooms of the animal facilities. It is accepted that IMO is a moderate stressor with a mainly psychological component, being able to activate the HPA axis in a very consistent manner (Armario *et al.*, 1986; Armario *et al.*, 1988). To study the impact of stress, body weight and food intake were measured every day at approximately the same time.

6. Behavioral analysis

6.1. Two-bottle choice saccharin preference test

The animals were individually housed to measure saccharin and water intake per animal. The saccharine formula used in this study was 2,3-Dihydro-3-oxobenzisosulfonazole sodium salt (SIGMA Ref. S-1002). The objective of this experiment was to obtain a measure of anhedonia-like behavior using a two-bottle choice saccharin preference test during 1 week after IMO. The test consists in tap water versus 0.1% w/v of saccharine diluted in tap water, 24 h/day, with no food or water deprivation. According with previous experiments (Plaznik *et al.*, 1989; Rabasa, 2008), 1 h of stress can produce a significant decrease of saccharin preference. This deficit in saccharin preference can be significantly attenuated by repeated administration of antidepressants (desipramine and citalopram) given prior to the stress session (Plaznik *et al.*, 1989).

Following our group protocol (Pastor-Ciurana *et al.*, 2014) saccharin and water intake were measured every day at approximately the same time. The fluid intake was measured with a scale, since it presents a minor error than volume measurement. The saccharin solution was prepared every day. The positions of saccharin and water bottles changed on a daily basis to avoid a preference bias. Saccharin and water consumption (ml) was also corrected by body weight and the saccharin preference index was also measured to account for possible between-group differences in water consumption (ratio between saccharin consumption and total fluid consumption). Two animals that presented a saccharine preference index lower than 60% during the baseline were excluded from the experiment and another animal was excluded because its intake was very erratic.

6.2. Elevated plus-maze (EPM)

This is one of the most used tests to evaluate anxiety in rodents (Pellow and File, 1986). Its rationale is that rodents avoid open spaces (as the open arms of the maze) and rather prefer the closed arms of the apparatus. Anxiolytics increase the time spent by the animals in the open arms.

The maze consisted of four arms made of black formica, extending from a 10 cm^2 center, positioned 90^o from each other, to form the shape of a plus sign. Each arm is 44 cm long and 10 cm wide. Two of the opposed arms have wooden walls (enclosed arms, 40 cm high) whereas the other two are open arms, with only a 0.5 cm ridge to provide additional grip. The whole maze was elevated 50 cm above the floor. The black room where the test was conducted was illuminated by a red 25 W bulb located 1.3 m above the apparatus. Using two different EPM, the animals from the same home cage

were tested at the same time. Each rat was placed in the center of the maze, facing a closed arm. During the 5 min test, the following measures were taken: (1) the number of open and closed arm entries, (2) total time spent in the open arms, the closed arms and the centre, (3) total number of protected and unprotected head-dips, (4) rearing and (5) defecations. An entry was defined as placing all four paws into a given arm. A head-dip was defined as protruding the head over the ledge of an open arm and down towards the floor while being in the center or in a closed arm (protected) or while being in an open arm (unprotected). Between rats, the apparatus was carefully cleaned with ethanol 5% v/v (diluted in tap water). The behavior was videotaped (camera Sony M388CC and digital video tape JVC VR-716) from the ceiling and later analyzed at blind using the SMART video tracking software version 2.5.19 (Panlab SLU, Barcelona).

6.3. Forced swimming test

Rodents forced to swim in a narrow space from which there is no escape will, after an initial period of vigorous activity, adopt a characteristic immobile posture, making only the necessary movements to keep their heads above the water. Porsolt *et al.* (1977) hypothesized that immobility reflects that animals have learned that escape is not possible and they give up. Immobility was subsequently found to be increased in animal models of depression (for exemple induced by stress or in transgenic rats strains), and decreased by a wide range of clinically active antidepressant drugs (Porsolt *et al.*, 1977, 2001; Petit-Demouliere *et al.*, 2005). This is a test that has been widely used for the validation of drug antidepressants (Porsolt, 1979; Armario *et al.*, 1988; Cryan *et al.*, 2005) and also to evaluate coping active/passive strategies to stressful situations (Armario *et al.*, 1988, Marti and Armario, 1993).

The animals were allocated in transparent cylindrical plastic tanks (height 40 cm, internal diameter 19 cm) containing 22 to 24 cm of water (25 ^{o}C). Four identical tanks were used and four animals, separated by black screens, were exposed simultaneously. The black test room was illuminated by a white 25 W bulb located 30 cm above the apparatus, and the water was changed for each rat. During the 5 min test, (1) struggling, (2) immobility and (3) swimming were measured (Armario *et al.*, 1988). Struggling was defined as diving, jumping or strongly moving all four limbs breaking the surface of the water or scratching the walls. Immobility was considered when the animal remained motionless, keeping their head out of the water. Swimming was deduced by resting the time that the animal spent struggling and immobile from the total time of the test. Swimming was characterized by mild swimming were the animal body was in a more horizontal position and showed no strugling. The behavior was videotaped (camera Sony M388CC and digital video tape JVC VR-716) from the front and later analyzed at blind using Smart software version 2.5.19 (Panlab SLU, Barcelona). The software was calibrated in order that the software data and manual data could present a strong correlation.

6.4. Acoustic startle response (ASR)

Using this procedure (adapted from Meloni and Davis, 2004) we studied the startle response produced by an acoustic stimulus with two startle chambers (SR Labs, San Diego Instruments). The ASR is a transient motor response to an unexpected and intensive stimulus (Figure 4). ASR is a fast twitch of facial and body muscles evoked by a sudden and intense acoustic stimulus. The startle pattern consists of eyelid-closure and a contraction of facial, neck and skeletal muscles (for review see Koch, 1999). The

response is determined by stimulus parameters such as its intensity, rise time and duration. In the last years, several studies have shown that startle response is increased in stressed animals (Cohen *et al.*, 2004, for review see Armario *et al.*, 2008) and in patients with PTSD (Grillon and Baas, 2003; Guthrie and Bryant, 2005, for review see Braff *et al.*, 2001; Marshall and Garakani, 2002; McTeague and Lang, 2012). In addition, basal ASR is decreased in a dose-dependent manner by pretreatment with anxiolytics, in both rodents (Miczek and Vivian, 1993) and humans (Rodríguez-Fornells *et al.*, 1999).

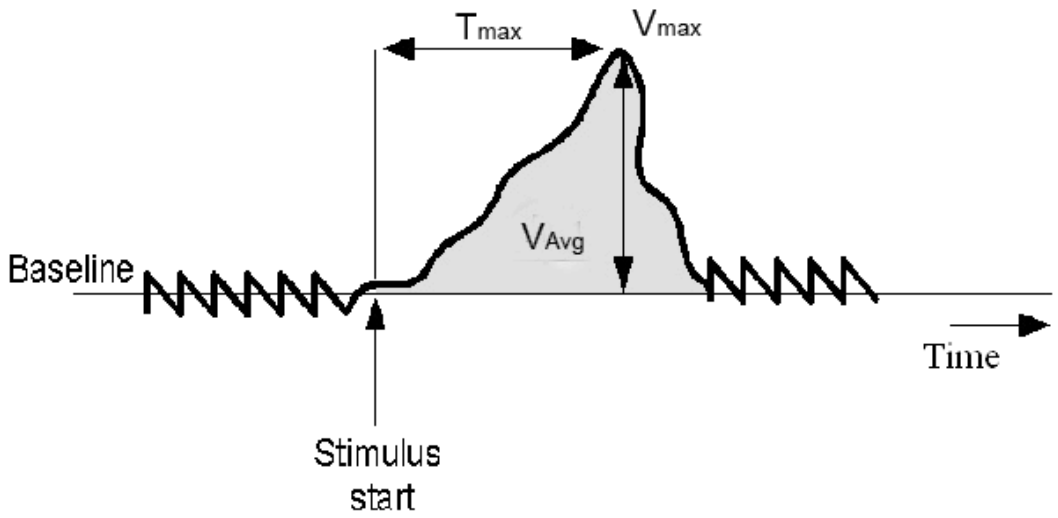


Figure 4. Representation of the acoustic startle response (ASR). The time is represented in the x axis and the force made by the jump of the animal in the platform represented in the y axis. In the Figure are represented some dependent variables such as V_{max} (Maximum startle response or peak), T_{max} (latency to maximum startle response) and V_{Avg} (peak average).

One day after stress, the rats were placed in the startle cages and received a 5 min acclimation period followed (without stimuli) by presentation of 30 startle stimuli during a 15 min interval (ITI: 25-35 s, pulse: 90 dB, pulse duration: 40 ms and background: 50 dB). The total duration of the test was 20 min, and was repeated three more times with an interval of 48 h between each test. The isolation cabinet was 28.3 cm high, 28.8 cm wide and 31 cm deep. The animal enclosure had a cylinder of 9 cm of diameter and 20

cm long where the animal is placed. A tweeter placed 24 cm above the animal provided the background noise and the startle stimuli, which were controlled by SR-LAB software. Startle responses were transduced by a piezoelectric accelerometer placed below the cylinder, digitized, rectified, and recorded in 200 ms readings, starting at the onset of each startle stimulus. The startle chamber was illuminated (white light, 30 W) and was cleaned with ethanol 5% (v/v in tap water) between animals.

The signals were subsequently analyzed using analysis software (SR Labs, San Diego Instruments) programmed to quantify: (1) the maximum startle response (V_{max}), (2) the latency to maximum startle response (T_{max}) and the average of all the startle responses (V_{Avg}) occurring subsequent to startle stimuli. For analysis, only the initial 100 ms after the onset of each startle stimulus (sampled by the cylinder movement and the piezoelectric accelerometer readings), was used. Regarding V_{max} the same results were obtained with 100 ms or 200 ms after the onset of the pulse, but V_{Avg} results were more robust with the 100 ms recordings. The animals were weighed daily to calculate the ratio V_{max}/body weight (Servatius *et al.*, 2005). In this way, the influence of individual animal weight is eliminated from the analysis. Chambers were calibrated daily using a standardization unit from San Diego Instruments and routinely checked with a sonometer (Ref. 2240 BRÜEL & KJAER).

7. Peripheral HPA evaluation by radioimmunoassay (RIA)

7.1. Blood sampling

Blood samples were obtained by tail-nick, which consisted of wrapping the animals gently with a cloth, making a 2 mm incision at the end of the tail artery and massaging the tail while collecting 300 µl of blood into ice-cold EDTA capillary tubes (Sarsted, Granollers, Spain). This procedure has been widely used in our laboratory (García *et al.*, 2000; Martí *et al.*, 2001; Márquez *et al.*, 2002; Belda *et al.*, 2004; Márquez *et al.*, 2006). Vahl and collegues (2005) demonstrated that blood sampling from tail veins can be used in stress studies without confounding the outcome. Even though the procedure involves a minimal stress, the first blood extraction is not affected because it was performed within a maximum period of 2 min for each animal after being removed from the *vivarium,* so that sampling is completed before activation of the HPA axis. For the remaining samples, taken with 1 h interval, the associated stress is usually reduced in animals that have previous experience with the procedure (García *et al.*, 2000; Martí *et al.*, 2001). It has been repeatedly shown in our laboratory that tail-nick sampling along a day offers real resting levels if spaced more than 1 h (Gómez *et al.*, 1998; Márquez *et al.*, 2005).

7.2. Double-antibody radioimmunoassays (RIAs)

Plasma ACTH and corticosterone levels were determined by double-antibody radioimmunoassays (RIAs) as previously reported (Márquez *et al.*, 2006). In brief, ACTH serum was analyzed by a double-antibody RIA using ^{125}I-ACTH (Amersham, Spain) as the tracer, rat synthetic ACTH 1-39 (Sigma, Spain) as the standard and an antibody raised in rabbits against rat ACTH (rb 7) (kindly provided by Dr. W. C. Engeland, Dept. Neuroanatomy, University of Minnesota, Minneapolis, USA). Corticosterone RIA used ^{125}I-carboximethyloxime-tyrosine-methyl ester (ICN-Biolink 2000, Spain) as the tracer, synthetic corticosterone (Sigma, Spain) as the standard and an antibody rose in rabbits against corticosterone–carboximethyloxime-BSA (kindly provided by Dr. G. Makara, Inst. Exp. Med., Budapest, Hungary) and plasma corticosteroid-binding globulin was inactivated by low pH. In all RIA procedures, samples to be compared were run in the same assay to avoid inter-assay variability. The intra-assay coefficient of variation was less than 8 % for ACTH and 6 % for corticosterone. The sensitivity was 12.5 pg/ml for ACTH and 0.1 µg/dl for corticosterone.

8. Histological procedures

8.1. Extraction and preparation of tissue samples

The different experimental groups were counterbalanced to the time of the animals' sacrifice thereby minimizing the possible influence of circadian rhythm on the results.

To avoid possible disturbances, perfusion, IMO and cocaine administration were conducted in different rooms. Immediately after the end of treatment, animals were anesthetized by inhalation in a chamber (Cibertec) saturated with isofluorane (Laboratorios Esteve, Barcelona), and with medical oxygen flow of 0.8L/min (Air Liquide Medicinal). After reaching a deep anesthetized state, it was maintained during the start of the infusion introducing the rat head in a container with a cotton piece soaked in isofluorane. Perfusion was performed transcardially first with a sterilized saline solution (0.9% NaCl) for 2 min and then with a solution of 4% paraformaldehyde (PFA) and 3.8% sodium tetraborate (borax) (PFA / borax) for 10 min. After the perfusion the brains were removed and immersed in PFA / borax to complete fixation. In this manner were maintained at 4 ° C for 16/18 h. After this period the PFA / borax was changed by a 30% solution of sucrose in potassium phosphate buffer of potassium-sodium chloride (KPBS, 0.2 M NaCl, 43 mM potassium phosphate). Then, the brains were maintained at 4 ° C until they were completely embedded in the cryoprotective solution of sucrose (2-3 days). Subsequently the brains were frozen at -80 ° C using isopentane to be preserved at the same temperature until cutted serially in coronal sections of 20 mm by CM3050-S cryostat (Leica Microsystems, Germany). The sections were stored in an antifreeze solution (0.05 M sodium phosphate, pH 7.3, 30% ethylene glycol, 20% glycerol) at -20 ° C until analysis by hybridization "in situ".

8.2. Oligoprobe synthesis (c-fos)

The radioactive riboprobe antisense to c-fos mRNA was generated by in vitro transcription (SP6/T7 Transcription Kit, Roche) from rat cDNA fragments (Dr. I. Verma, The Salk Institute) subcloned in a pBluescript SK-1 plasmid (Stratagene, USA). In each

transcript was used 1 mg of digested plasmid as DNA mold and 35S-UTP as radiolabeled nucleotide (specific activity 1250 Ci / mmol, PerkinElmer). After the transcription and after digestion with 20 U DNase (SP6/T7 Transcription Kit, Roche) were added 40 ml of STE buffer (0.1 M NaCl, 10 mM Tris-Cl, 1 mM EDTA, pH 8.0) and an incubation was made at 65 ° C for 5 min before purification to prevent the formation of secondary structures. Afterwards the removal of the probe was performed using gel filtration columns (mini Quick Spin Columns RNA, Roche). After checking the marking efficiency (> 50%), the eluate of the column containing the radioactive probe was kept at -20 ° C until used in the respective hybridization "in situ" assays.

8.3. In situ hybridization (ISH) of c-fos

Prior to the procedure, the sections were washed with KPBS to remove the antifreeze solution and mounted on microscope slides (Superfrost Plus Slides, Thermo Scientific). Subsequently, sections were dried for about 12 h and stored at -20 ° C in sealed boxes containing a desiccant (Silica Gel PS, Fluka) until the start of the test. The protocol used was adapted from Simmons *et al.* (1989).

All solutions were pre-treated with diethylpyrocarbonate (DEPC) and sterilized by the steam sterilizer before use. The sections were post-fixed in a bath of PFA / borax for 30 min. After this time, two washes of 5 min in KPBS to remove traces of the fixative were performed. Subsequently, protein digestion was performed during 15 min at 37 ° C with Proteinase K (Roche) at a concentration of 0.01 mg / ml in an appropriate solution (100 mM Tris-HCl, pH 8.0, 50 mM EDTA, pH 8.0). Digestion was stopped by washing with water treated with DEPC. Then, sections were conditioned in a triethanolamine solution (TEA 0.1 M, pH 8.0) and acetylated for 10 min in a solution of acetic anhydride at

0.25% in 0.1 M TEA, pH 8.0. After this time, a 5 min wash in a saline solution containing sodium citrate (2X SSC: 0.3 M NaCl, 0.03 M sodium citrate tribasic) was performed. Finally, sections were dehydrated by successive baths in increasing concentrations of ethanol (50%, 75%, 95%, 2 x 100%) allowing them to dry at room temperature.

Once dried, 100 ml of hybridization solution was added over each slide (50% formamide, 0.3 M NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0, 1X Denhardt's solution, dextran sulphate 10 %, yeast tRNA 500 g / l, 10 mM DTT) containing the radioactive probe (106 dpm/100ml) and subsequently covered with coverslips. Sections were incubated for 16-18 h at 60 ° C to allow hybridization. Subsequently, sections were washed in 4 successive baths of 4X SSC, and subjected to digestion with RNase A (Roche) at 0.02 mg / ml in an appropriate solution (0.5 M NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA pH 8.0). After digestion successive washes in decreasing concentrations of SSC (2X to 0.5X); 1 mM DTT, were performed including an astringent wash in SSC 0.1X at a temperature of 60 ° C; and finally a rapid wash in SSC 0,1X at room temperature before dehydration by successive baths in increasing concentrations of ethanol. After allowing the sections to dry at room temperature, they were exposed to Kodak XAR-5 Biomax MR film (Amersham, UK) for the time necessary to detect the radioactive signal. For c-fos depending on the area this time ranged between 17 h and 30 h.

8.4. Image analysis

The stereotactic atlas of Paxinos and Watson (2006) was used to define the coordinates of each test analysis. For analysis were taken from 4-8 photographs per

area and animal. The mRNA levels were determined semiquantitatively in at least two sections per area and animal, by measuring the optical density and the number of pixels in the defined areas with a NIKON, DMX-1200 – Eclipse-E400 system. The analyzed sections were digitalised and quantified using Image software (Scion Corporation). The resulting values (number of pixels x optical density) were expressed in arbitrary units (FLI/mm^2). To verify the exposure time to the films required for proper densitometric analysis, photographs of samples showing a high, medium and low intensity were performed and quantified. The arbitrary units obtained were interpolated in saturation graphics made using an auto radiographic microscale of 14C that was photographed and quantified under the same conditions, verifying that the various settings are located in the linear part of the plot, ideal for quantification. All samples that were statistically compared were processed in the same analysis to avoid inter-assay variability.

9. mRNA expression levels determination by real-time quantitative polymerase chain reaction (RT-PCR)

9.1. Dissection, extraction and preparation of tissue samples

Animals were sacrificed by decapitation and their brain areas dissected using clean surgery material (cleaned with alcohol 70%). The tissue was frozen in liquid nitrogen kept at -80 ºC until extraction of DNA. The stereotactic atlas of Paxinos and Watson (1998) was used to define the coordinates of each area. The dissected areas were amygdala (Amg), dorsal striatum (dSTR), hippocampus, hypothalamus, nucleus

accumbens (Acb), pituitary gland, prefrontal cortex (PFC) and substancia nigra-ventral tegmental area (SN-VTA).

Total RNA was extracted from brain regions related with stress and cocaine responses using the RNeasy® Lipid Tissue Mini kit (Qiagen, Austin, Texas, USA) and following the instructions of the manufacturer. Purity was estimated from the ratio of absorbance readings at 260 and 280 nm and only ratios between 1.8 and 2 were accepted. RNA quality was checked in agarose gel. RNA concentration was determined in a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, Delaware, USA). One pg of RNA was reverse transcribed, using the SuperScriptTM First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA), using oligo(dt) primers and following the instructions of the manufacturer.

9.2. RT-PCR analysis

The expression levels of mRNA transcripts for the different genes studied were measured by quantitative real-time polymerase chain reaction (qRT-PCR). The reference gene, glyceraldahyde-3-phosphate-dehydrogenase (GAPDH) was used as internal standard for normalization. The qRT-PCR reactions, using the equal amounts of total RNA from each sample, were performed on the iQ5 Multicolor Real-time PCR Detection System from Bio-Rad (Hercules, CA, USA), using the iQ™ SYBR® Green Supermix (BioRad Laboratories, Hercules, CA, USA). Product fluorescence was detected at the end of the elongation cycle. All melting curves exhibited a single sharp peak at a temperature characteristic of the primer used. Primer design was performed using the software Beacon Designer (Premier Biosoft International, Palo Alto, CA, USA).

Linearity and efficiency of polymerase chain reaction (PCR) amplification reactions were assessed for all pair of primers using standard curves generated by increasing amounts of cDNA. The relationship between the threshold cycle (C_t) and logarithm of the complementary DNA (cDNA) concentrations were studied according to correlation coefficient and the slopes, calculated by Bio-Rad iQ5 Optical System Software, version 2.0 (Bio-Rad Laboratories, Inc, 2006). For all primer sets, standard curves using four points, diluted over a 100-fold range, always led to a high linearity (correlation coefficients > 1- 0.9875).

The PCR efficiency *(Ex)* was calculated using the equation $Ex = 10^{-1/slope}$**.** Efficiency was presented as a percentage of the template that was amplified in each cycle, calculated by the following equation *%Ex= (EX-1)* x 100. Efficiency close to 100% is the best indicator of a robust, reproducible assay. Efficiency between 90-105% was always used. These amplification efficiencies of PCR assays allow the quantification of mRNA with the comparative C_t quantification method (ΔC_t method) using a reference gene. Following this method, the relative expression of a gene was calculated by the expression: $2^{(Ct\ (reference) - Ct\ (target))}$. This method assumes that both target and reference genes are amplified with efficiencies near 100% and within 5% of each other.

10. Statistical analysis

The “statistical package for social science” (SPSS) program was used (version 17 for Windows). The general strategy used to perform the statistical analysis is summarized in Table 1. For repeated-measures analysis, linear mixed models (LMM) were used.

This procedure expands the general linear model (GLM) so that the error terms and random effects are permitted to exhibit correlated and non-constant variability. For that reason, the linear mixed model provides the flexibility to model not only the mean of a response variable, but its covariance structure as well. Several types of covariance structures (first-order autoregressive, diagonal, compound symmetry, analytic factor, toeplitz and unstructured) were used to compare different models. The chosen "repeated covariance structure" was the one that had presented less number of parameters and lower values of information criteria (-2 Restricted Log Likelihood). As a method of estimation, the maximum likelihood was used in all cases. In case of an interaction between factors, additional pairwise comparisons (based on estimated marginal means) were made. The between-subject factors were cocaine and IMO (each factor with two levels). The within-subject factors were sampling time or day. When only a one-point measure was analyzed (no repeated-measures), a standard GLM was used. Cocaine and IMO were also the between-subject factors. Once more, if a statistical interaction was found, additional pairwise comparisons were made to decompose the interactions. Several of the variables that did not present homogeneity of variances were analyzed by a generalized linear model (GzLM) procedure (GENLIN) (McCulloch and Searle, 2001) with two between factors (cocaine and IMO). Normality distribution and identity as a link function was always used. The significance of the effects was determined by the Wald chi-square statistic. Goodness of fit values (Akaike`s information criterion) was used to compare different models. The generalized linear model is a more flexible statistical tool than the standard general linear model (GLM) because several types of distribution and different covariance structures of the repeated measures data could be chosen. Once more if a statistical interaction was found, additional pairwise comparisons were made to decompose the interactions. To decompose the interactions the strategy was always the same, the group SalC was compared versus CocC and SalIMO, the group CocC versus CocImo, and the group SalIMO versus CocIMO.

Table 1. Summary of the general strategy used to perform the statistical analysis. GLM (general linear model), GzLM (generalized linear model) and LMM (linear mixed models).

TYPE OF DATA			
One-point data	HOMOGENEITY OF VARIANCES (LEVENE)	UNIVARIATE GLM	
	NON-HOMOGENEITY OF VARIANCES	LOG-TRANSFORMATION TO ACHIEVE HOMOGENEITY	GLM
		LOG-TRANSFORMATION DOES NOT REACH HOMOGENEITY	GZLM
Repeated measures	COMPOUND SYMMETRY (MAUCHLY)	REPEATED GLM	
	NON COMPOUND SYMMETRY	LMM	

CHAPTER I

The main aim in this Chapter was to study several parameters related with the intensity of the stress (body weight, food intake, anhedonia, corticosterone and ACTH) after the exposure to cocaine and/or stress (IMO). Anxiety-like behavior was study using an elevated plus-maze (EPM) and the active and passive strategies in front of stressful was study using a forced swimming test (FST). Finally, the study of homotypic long-term desensitization to IMO through peripheral HPA evaluation by radioimmunoassay (RIA) was realized.

1. Introduction

It was shown that cocaine abuse and withdrawal is linked to the development of depressive and/or anxiety-like symptoms (Gawin, 1991; Markou *et al.*, 1992). Moreover, severe anxiety and depression provide part of the negative reinforcement associated with cocaine dependence and are important motivational factors for relapse and maintenance of repetitive cycles of cocaine abuse (Gawin *et al.*, 1989; Markou *et al.*, 1992; Goeders *et al.*, 1993; Shaham *et al.*, 2000; Shalev *et al.*, 2002). The activation induced by the administration of drugs of abuse in areas involved in the stress response or the property of some stressful stimuli to reinstate the search

and drug consumption are some of the evidences of the relationship between stress and addiction (see review in Koob and Le Moal, 2006). In this chapter our objective is focused in the initial interaction between stress and cocaine.

After the evaluation of different physiological responses to stress, the overall conclusion is that there are only few reasonably good indices of stress intensity: increases in plasma levels of catecholamines (particularly adrenaline), glucose, prolactin and HPA hormones and reduction of food intake (Hennessy & Levine, 1978; Natelson *et al.*, 1981; Kant *et al.*, 1983, Armario *et al.*, 1986b; De Boer *et al.*, 1990b), although in the case of HPA hormones, corticosterone is only useful with low intensity stressors (Armario, 2006). In the first experiment of this chapter we checked some of the stress indicators such as, body weight, food intake and anhedonia, to study if these indicators are modified by the simultaneous exposure to cocaine and stress.

The activation of the HPA axis is not only important for its role in an important number of stress-associated pathologies, but also because it is a good marker of stress intensity. One of the most surprising characteristics of the HPA axis is how easy it can be activated by a variety of drugs markedly different from a structural or functional point of view. For instance, virtually all drugs of abuse (amphetamines, cocaine, alcohol, opiates) have been found to activate the HPA axis, despite their clearly heterogeneous mechanisms of action (for review see Goeders, 1997; Armario, 2010). Acute, non-contingent cocaine administration increases plasma levels of ACTH, β-endorphin and corticosterone in rats (Moldow and Fischman, 1987; Forman and Estilow, 1988; Levy *et al.*, 1991; Saphier *et al.*, 1993) and in non-human primates (Sarnyai *et al.*, 1996). Although the mechanisms and site at which cocaine exerts its influence on the HPA axis is presently poorly known, CRF immunoneutralization studies suggest that this effect of cocaine is dependent on

endogenous CRF release into the pituitary portal blood (Rivier and Vale, 1987; Sarnyai *et al.*, 1995). Other studies, using different approaches, showed that the HPA axis activation is in part promoted via serotoninergic and dopaminergic (Levy *et al.*, 1991; Borowsky and Kuhm, 1991) mechanisms. Therefore, in the second experiment it was our goal to evaluate the effects of the combination of stress and cocaine in the HPA axis through the study of the ACTH and corticosterone levels.

Exposure to stress can result in a wide range of physiological and behavioral changes. In general, several studies showed the effects of acute stress on anxiety-related behavior and established that one single exposure to certain severe stressors can induce behavioral changes that lasted for days or weeks (for review see Armario *et al.*, 2008). Most of these changes are reminiscent of enhanced anxiety as reflected by the reduction of time spent in the open arms of the elevated plus-maze (EPM) and the increase of the ASR. Besides these behavioral changes, a single exposure to severe stressors can reduce food intake (Martí *et al.*, 1994; Vallès *et al.*, 2000) and the consumption of sweet solutions (Plaznik *et al.*, 1989; Dess, 1992; Van Dijken *et al.*, 1992) for some days after the stressor. To better study the initial interaction between stress and cocaine, in the third experiment of this chapter the anxiety-like behavior 24 h after treatment using an EPM and the active and passive strategies in front of stressful situations 48 h after treatment using a FST were evaluated. In the fourth experiment anxiety-like behavior using the ASR was also measured.

Furthermore, a single exposure to IMO is able to cause a decrease in rat HPA axis response to the same stimulus applied days or weeks later. Previous experience with IMO, although occasionally it may reduce initial HPA response to the same stimulus, always accelerates recovery of basal levels of ACTH and corticosterone (Martí *et al.*, 2001; Dal-Zotto *et al.*, 2002; 2003; 2004; Vallès *et al.*, 2003). Therefore, in addition to studying the effects produced by acute cocaine administration in animals

immobilized, our aim was also to assess whether this interaction could alter homotypic long-term desensitization.

The purpose to determine whether stress represents a window of vulnerability to the deleterious effects of cocaine or a period of increased resilience to the effects of cocaine leading to long-lasting effects, represents a current and very important issue of investigation.

2. Experimental protocol

Experiment 1: Anhedonia, food intake and weight gain

This experiment was conducted using 44 rats caged individually. This experiment measured the anhedonia-like behavior using a two-bottle choice saccharin preference test (tap water versus 0.1% w/v of saccharine diluted in tap water, 24 h/day, and no food or water deprivation) during 1 week after IMO. Food, saccharin and water intake, as well as body weight were measured every day at approximately the same time (Figure 5).

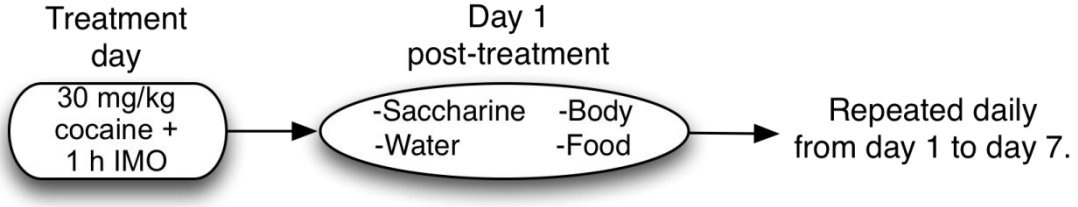


Figure 5. Summary of the experiment 1 taking CocIMO group as example. The aim of this experiment is to evaluate food intake, weight gain and anhedonia in response to stress and / or cocaine.

Experiment 2: Peripheral HPA evaluation by radioimmunoassay (RIA)

This experiment was conducted using 48 rats. Blood samples were collected by tail-nick at 0, 1 and 2 h after IMO to determine plasma ACTH and corticosterone as mentioned before. Anxiety-like behavior was evaluated 24 h after stress and cocaine injection using an EPM (Figure 6). The animals were exposed for 5 min to the EPM. The day after the last handling, blood samples were also taken to determine resting (basal) levels of the two hormones.

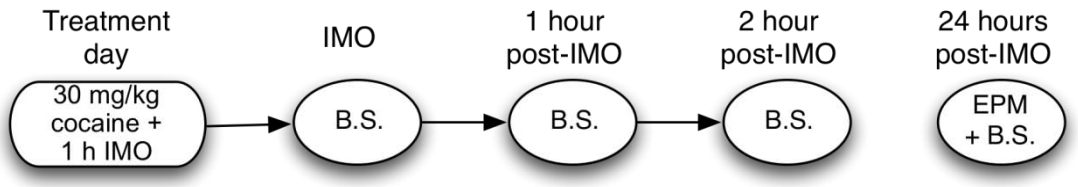


Figure 6. Summary of the experiment 2 taking CocIMO group as example. The aim of this experiment is to evaluate anxiety-like behavior and to determine plasma ACTH and corticosterone in response to stress and / or cocaine.

Experiment 3: EPM and FST

The experiment was conducted using 48 rats. Anxiety-like behavior was evaluated

24 h after IMO and cocaine injection using an EPM. Twenty-four hours later, the FST was done to measure active and passive strategies in front of stressful situations. After the behavioral tests, a blood sample was obtained by tail-nick to measure the HPA response to the test (Figure 7).

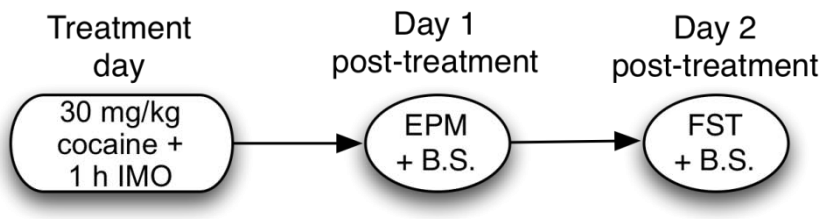


Figure 7. Summary of the experiment 3 taking CocIMO group as example. The aim of this experiment is to evaluate anxiety-like behavior in response to stress and / or cocaine.

Experiment 4: ASR

A total of 50 rats were used in this experiment. The anxiety-like behavior was analyzed by means of the ASR which was measured 24 h after IMO and then repeated each 48 h for a total of 7 days (four tests) (Figure 8).

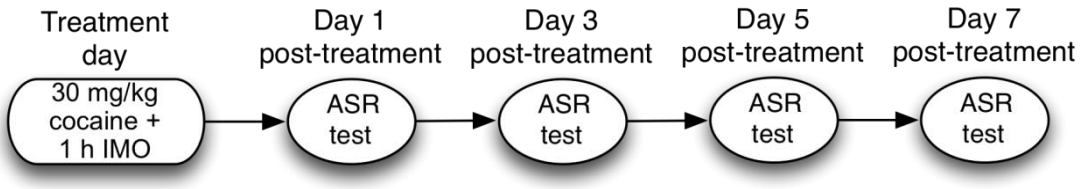


Figure 8. Summary of the experiment 4 taking CocIMO group as example. The aim of this experiment is to evaluate anxiety-like behavior in response to stress and / or cocaine.

Experiment 5: Study of the homotypic long-term desensitization to IMO through peripheral HPA evaluation by radioimmunoassay (RIA)

A total of 50 rats were used in this experiment. The first day, animals were treated according to their group and 8 days after all animals were exposed to IMO (Figure 9). Blood samples were collected by tail-nick at 0, 45 and 90 min after IMO to determine plasma ACTH and corticosterone as mentioned.

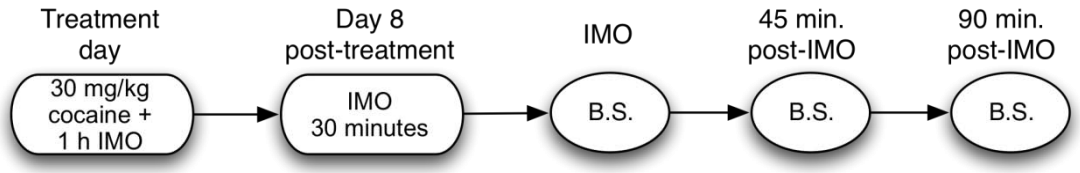


Figure 9. Summary of the experiment 5 taking CocIMO group as example. The aim of this experiment is to study of the desensibilization to IMO through peripheral HPA evaluation by radioimmunoassay (RIA).

3. Results

Experiment 1

Statistical analysis revealed that regarding food intake (Figure 10), the effects of cocaine and stress by itself were not statistically significant. However, the interaction

cocaine x IMO was significant ($F(1,42) = 4.47$, $p < 0.05$). The decomposition of the interaction cocaine x IMO showed that cocaine decreased food intake (regardless of time) only in non-stressed animals ($F(1,42) = 5.4$, $p < 0.05$) and IMO decreased food intake only in saline animals ($F(1,42) = 7.6$, $p < 0.01$). This interaction indicated synergism. Food intake was also affected by day ($F(6,108) = 26.13$, $p < 0.001$). After the treatment, animals decreased their food intake and progressively recovered on the subsequent days. The interactions day x cocaine and day x IMO were also significant statistically ($F(6,108) = 3.90$, $p < 0.001$ and $F(6,108) = 6.78$, $p < 0.001$, respectively). The decomposition of the interaction day x cocaine showed that cocaine decreased food intake on day 1 post-treatment ($F(1,81) = 11.9$, $p < 0.005$, Figure 10). The decomposition of the interaction day x IMO showed that stress decreased food intake on days 1 and 3 ($F(1,81) = 23.2$, $p < 0.005$ and $F(1,81) = 4.6$, $p < 0.05$, respectively) after the treatment. The 2nd day after the treatment IMO presented a tendency to decrease food intake but this was not statistically significant. The decomposition of the interaction cocaine x stress showed that cocaine decreased food intake (regardless of time) only in non-stressed animals ($F(1,42) = 5.4$, $p < 0.05$) and stress decreased food intake only in saline animals ($F(1,42) = 7.6$, $p < 0.01$).

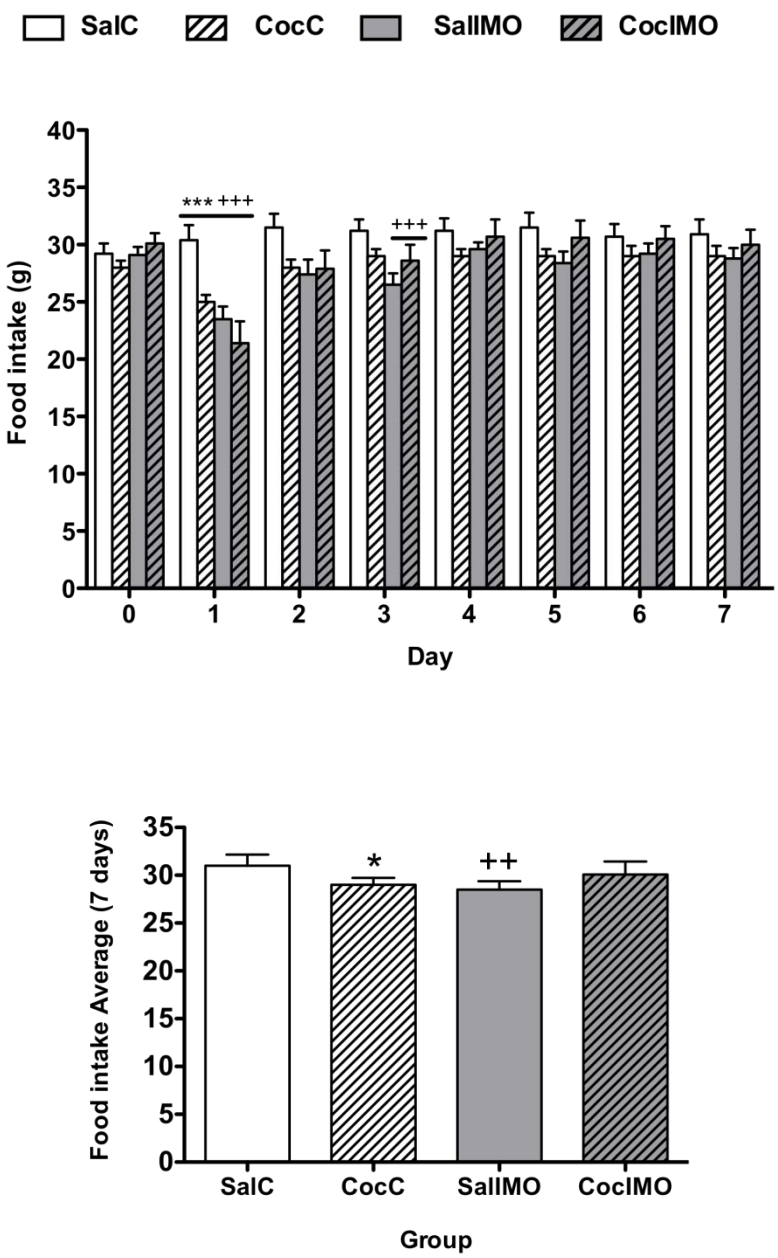


Figure 10. Effects of cocaine administration and IMO on food intake. The data are represented as mean ± SEM of the food (g). The white columns represent the non-stressed groups, the grey columns the IMO groups and the stripped columns the cocaine groups. Food intake was measure daily during 7 days. *: $p<0.05$ *vs* respective saline group (SalC). $^{++}$: $p<0.01$ *vs* respective non-stressed group (SalC) (ANOVA).

Weight gain (Figure 11) was affected negatively by both cocaine ($F(1,38) = 5.7$, $p < 0.05$) and IMO ($F(1,38) = 33.20$, $p < 0.001$). In addition, the weight gain was

affected by days ($F(6,228) = 469.59$, $p < 0.001$) and the interaction day x cocaine was also statistically significant ($F(6,228) = 5.21$, $p < 0.001$). However, the interaction day x IMO was not statistically significant. Therefore, IMO decreased weight gain over all days, whereas the cocaine effect was different in function of the day. The decomposition of the interaction day x cocaine showed a significant effect of cocaine in decreasing weight gain on days 3 ($F(1,55) = 5.88$, $p < 0.05$), 4 ($F(1,55) = 8.44$, $p < 0.01$), 5 ($F(1,55) = 9.30$, $p < 0.01$), 6 ($F(1,55) = 5.56$, $p < 0.05$) and 7 ($F(1,55) = 8.56$ $p < 0.01$), but not on days 1 and 2 post-treatment. The interaction cocaine x IMO was no statistically significant indicating no synergism. Thus, only additive effects were seen.

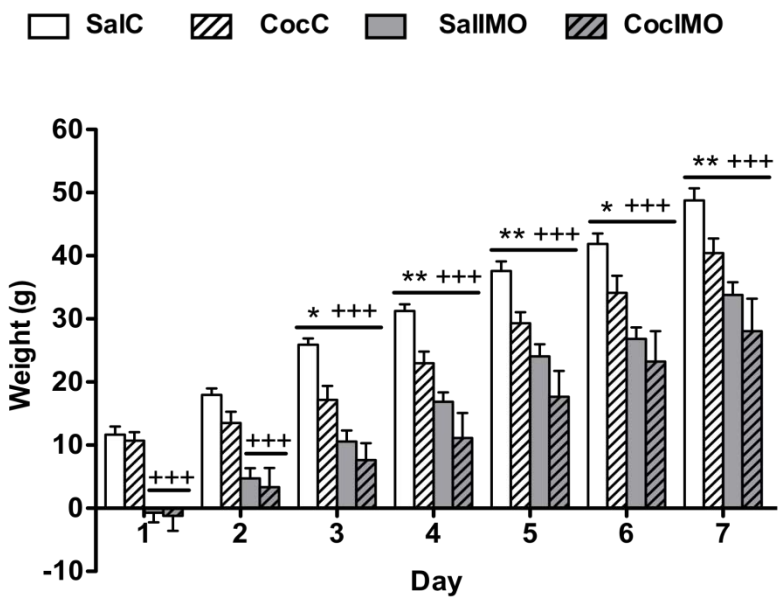


Figure 11. Effects of cocaine administration and IMO on weight gain. The data are represented as mean ± SEM of the weight gain (g). The white columns represent the non-stressed groups, the grey columns the IMO groups and the stripped columns the cocaine groups. $\overset{*}{-}$: $p<0.05$; $\overset{**}{-}$: $p<0.01$ overall effect of cocaine. $\overset{+++}{\text{——}}$: $p<0.001$ overall effect of stress (LMM).

Statistical analysis revealed an overall significant effect of IMO on saccharin intake (Figure 12) over the 7 days subsequent to IMO exposure ($F(1,35) = 5.16$, $p < 0.05$). The lack of significance of the interaction day x IMO indicated that the effect of IMO was basically maintained across time. All the other factors including cocaine were not statistically significant. The same results were observed for the saccharin preference (%), with IMO exposure reducing preference for saccharin (data not shown, $F(1,37) = 5.37$, $p < 0.05$).

After the exposure to IMO, the animals increased their water intake (Figure 12, stress effect: $F(1,35) = 5.04$, $p < 0.05$) over the 7 days after the treatment. All other factors were not statistically significant. No significant differences in total fluid consumption were found between treatments (data not shown).

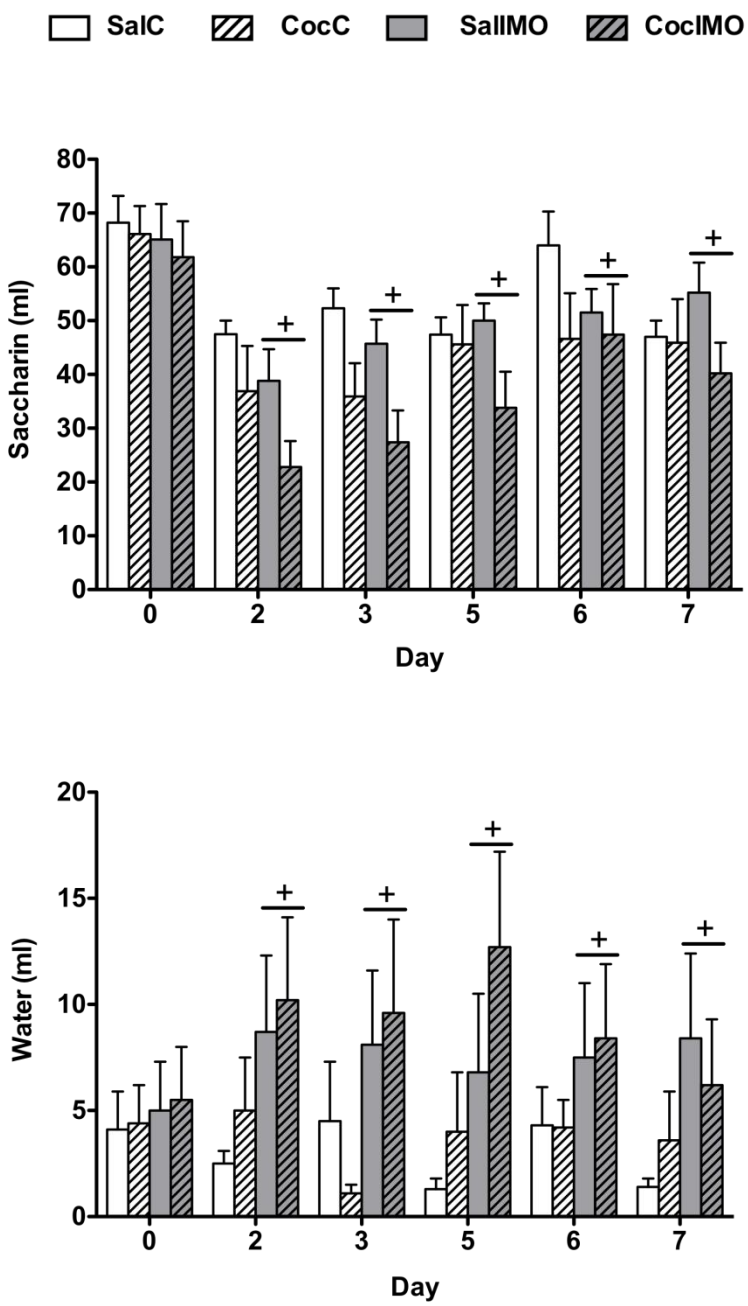


Figure 12. Effects of cocaine administration and IMO on saccharin and water intake. The data are represented as mean ± SEM of saccharin (ml) or water (ml). The white columns represent the non-stressed groups, the grey columns the IMO groups and the stripped columns represent the cocaine groups. $^{+}$: $p<0.05$ overall effect of stress (LMM).

Experiment 2

As Figure 13 shows, the effect of stress (IMO) on ACTH levels was statistically significant ($\chi^2(1) = 254.17$, $p < 0.001$) but the effect of cocaine was not. Moreover, ACTH levels were affected by time ($\chi^2(2) = 299.04$, $p < 0.001$). The interactions cocaine x stress ($\chi^2(1) = 4.45$, $p < 0.05$) and sampling time x stress ($\chi^2(2) = 268.54$, $p < 0.001$) were statistically significant. The interaction between the three factors sampling time x cocaine x stress was also statistically significant ($\chi^2(2) = 7.87$, $p < 0.05$) and therefore was further decomposed.

At the end of IMO (R-IMO), the decomposition of the interaction showed a significant increase of ACTH induced by IMO in the saline ($p < 0.001$) and cocaine ($p < 0.001$) animals comparing with their unstressed controls. In this time period, in the stressed animals, cocaine decreased ACTH levels ($p < 0.05$), indicating the presence of a mild negative synergism. Furthermore, ACTH levels were increased by IMO in both saline and cocaine animals at R-1h and R-2h ($p < 0.001$ in all cases). Finally, at R-2h cocaine slightly increased ACTH levels in comparison to SalC animals ($p < 0.05$).

Plasma corticosterone levels (Figure 13) were increased by cocaine ($\chi^2(1) = 18.64$, $p < 0.001$) and IMO ($\chi^2(1) = 282.21$, $p < 0.001$). No interaction between the two factors (cocaine and stress) was obtained. Nevertheless, corticosterone levels were affected by sampling time ($\chi^2(2) = 24.76$, $p < 0.001$). The interaction sampling time x stress was also statistically significant ($\chi^2(2) = 19.00$, $p < 0.001$). The interaction between the three factors sampling time x cocaine x stress ($\chi^2(2) = 5.13$, $p = 0.077$) approached significance and therefore was further decomposed.

The decomposition of the interaction sampling time x cocaine x stress showed that in non stressed animals, cocaine increased corticosterone levels in R-IMO, R-1h and R-2h ($p < 0.001$; $p < 0.01$; $p < 0.05$, respectively). Furthermore, corticosterone levels were increased by IMO in both saline and cocaine animals, immediately after IMO, at R-1h and at R-2h ($p < 0.001$ in all cases). At the sampling time R-2h cocaine increased corticosterone levels in stressed animals ($p < 0.05$).

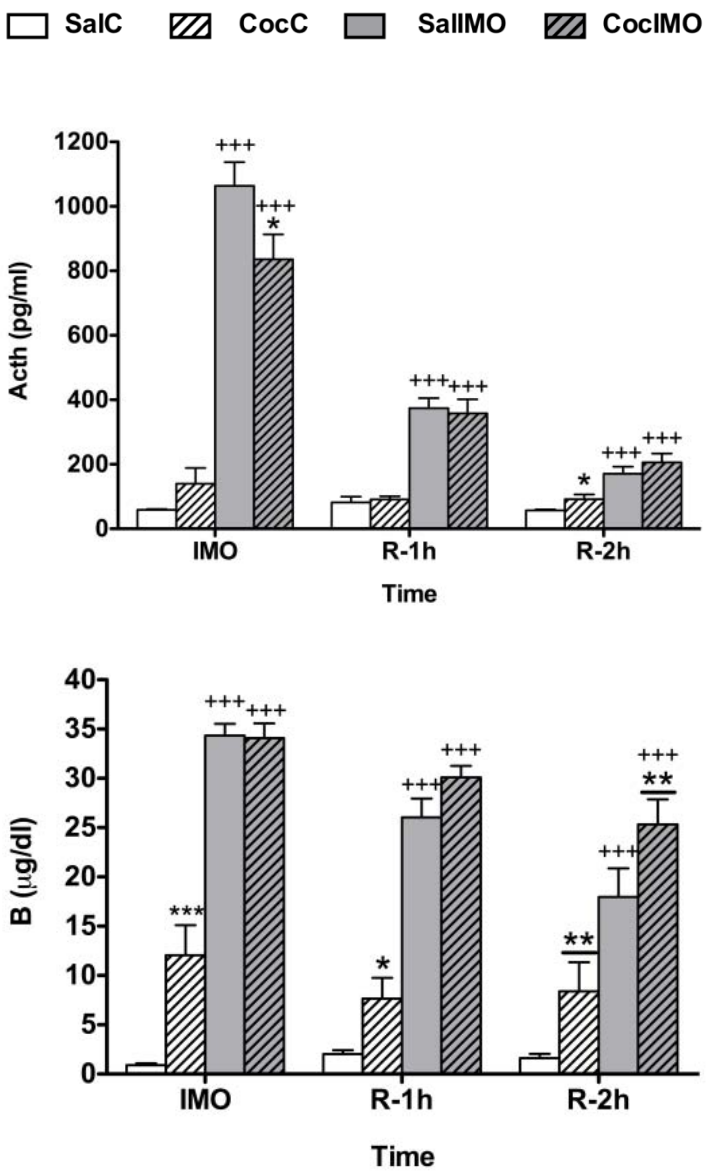


Figure 13. Effects of cocaine administration and IMO on plasma levels of HPA hormones. The data are represented as mean ± SEM of the plasma levels of ACTH and corticosterone (B). The white columns represent the non-stressed groups, the grey columns

the IMO groups, and the stripped columns the cocaine groups. Samples were taken two days before IMO (BL), at the end of IMO (IMO) and 1 and 2 hours after IMO (R-1h and R-2h respectively). These time points correspond to 1, 2 and 3 hours after cocaine. */+: $p<0.05$; **/++: $p<0.01$; ***/+++: $p<0.001$. Not underline symbols represent the significance (but not overall) after the decomposition of the interaction (LMM).

The ACTH and corticosterone responses to the EPM (Figure 14) were significantly increased in the animals previously exposed to IMO ($F(1,39) = 105.79$, $p < 0.001$; $F(1,44) = 48.35$, $p < 0.001$ respectively). In opposition cocaine treatment had no significant effect. The interaction cocaine x IMO was also statistically significant for both ACTH and corticosterone levels ($F(1,39) = 7.55$, $p < 0.01$; $F(1,44) = 5.23$, $p < 0.05$ respectively). The decomposition of the interaction showed that cocaine administration decreased ACTH levels only in stressed animals ($F(1,39) = 6.10$, $p = 0.01$), indicating thus a negative synergism. In addition, both SalIMO and CocIMO groups were statistically different from their respective unstressed groups ($F(1,39) = 100.11$, $p < 0.001$ and $F(1,39) = 24.67$, $p < 0.001$, respectively). In contrast, the decomposition of the interaction showed that cocaine administration increased Corticosterone levels only in non-IMO animals ($F(1,44) = 6.82$, $p = 0.01$). In addition, both SalIMO and CocIMO groups were statistically different from their respective unstressed groups ($F(1,44) = 42.70$, $p < 0.001$ and $F(1,44) = 10.89$, $p < 0.01$, respectively).

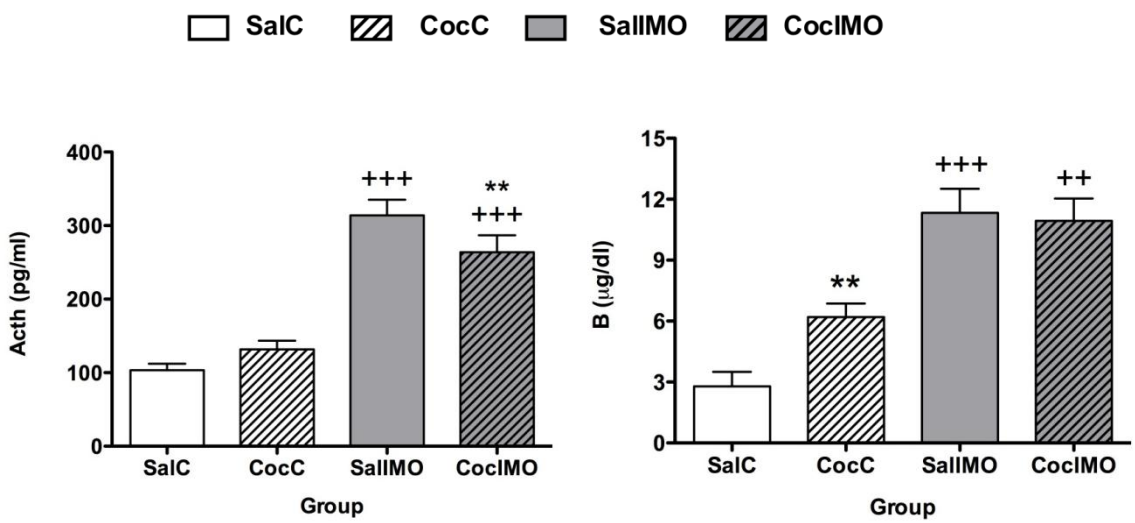


Figure 14. Effects of cocaine administration and IMO on hormonal response to the elevated plus-maze test. The data are represented as mean ± SEM of the plasma ACTH and corticosterone (B). The white columns represent the non-stressed groups, the grey columns the IMO groups and the stripped columns represent the cocaine groups. The decomposition of the cocaine x IMO interaction presented an effect of cocaine. */+: $p<0.05$; **/++: $p<0.01$; ***/+++: $p<0.001$. Underline significance symbols represent the overall effects of the cocaine(*)/IMO(+); not underline symbols represent the significance (but not overall) after the decomposition of the interaction (ANOVA).

Experiment 3

Exposure to IMO decreased the time spent in the open arms of the EPM (Figure 15, Wald X^2 (1) = 4.70, $p < 0.05$). Stress, also reduced the number of entries in the open arms (Figure 15, Wald X^2 (1) = 7.03, $p < 0.01$), the closed arms (F (1,40) = 16.0, $p < 0.001$), and consequently the total entries (F (1,40) = 26.5, $p < 0.001$). The percent of time spent in open arms was also reduced by IMO (Wald X^2 (1) = 4.78, $p < 0.01$), but not by cocaine. Another index of anxiety, percent of open arms entries, was also reduced by IMO (Figure 15, Wald X^2 (1) = 7.29, $p < 0.01$). Furthermore, stress reduced the number of protected (Figure 15, F (1,40) = 9.2, $p < 0.01$) and

unprotected (Wald X^2 (1) = 4.53, $p < 0.05$) head-dipping. Finally, stress increased the number of defecations (Wald X^2 (1) = 4.30, $p < 0.05$) during the EPM test. In all cases, neither cocaine factor nor the interaction cocaine x IMO was statistically significant.

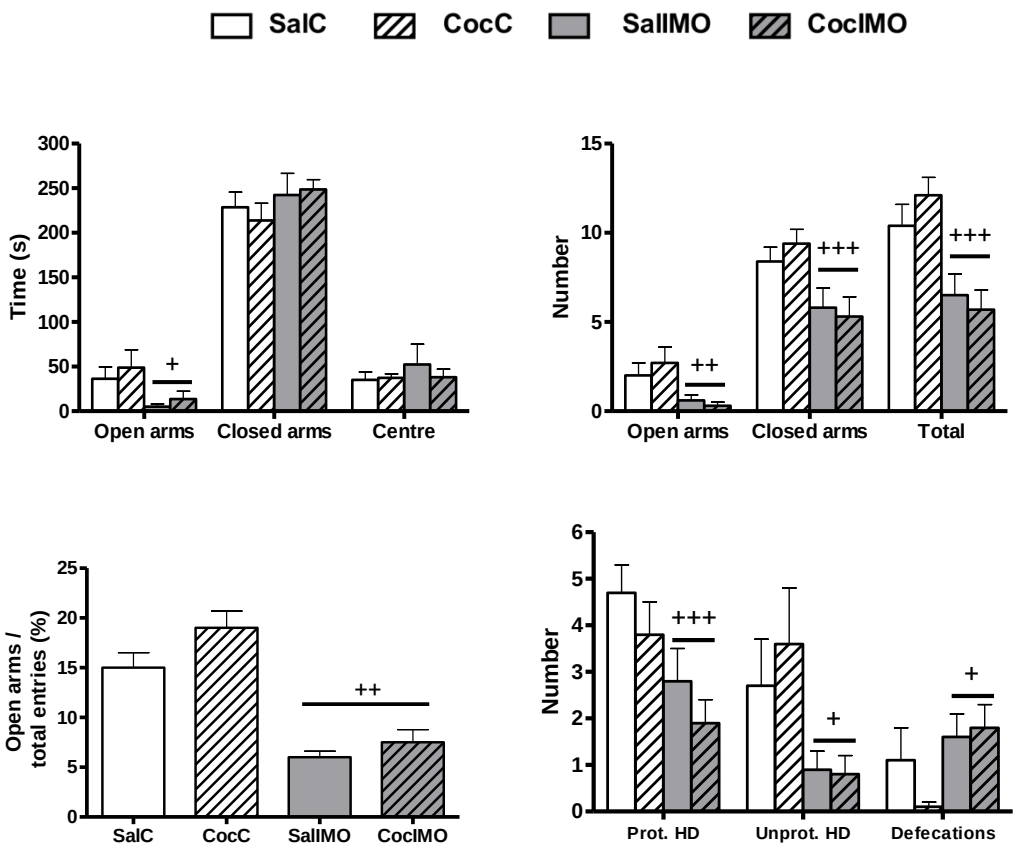


Figure 15. Effects of cocaine administration and IMO on EPM behavior. The data are represented as mean ± SEM. The white columns represent the non-stressed groups, the grey columns the IMO groups and the stripped columns the cocaine groups. $\overline{+}$: $p < 0.05$; $\overline{+++}$: $p < 0.001$; $\overline{+++}$: $p < 0.001$ overall effect of stress (ANOVA or G_ZLM).

In the FST (Figure 16), a significant interaction cocaine x stress was observed regarding mild swimming ($F(1,42) = 4.86$, $p < 0.05$). The decomposition of the interaction cocaine x stress showed that IMO increased swimming time only in saline

animals (F (1,42) = 4.35, $p < 0.05$). No other factor was statistically significant for mild swimming. Concerning immobility and struggling, any treatment effect reached statistical significance.

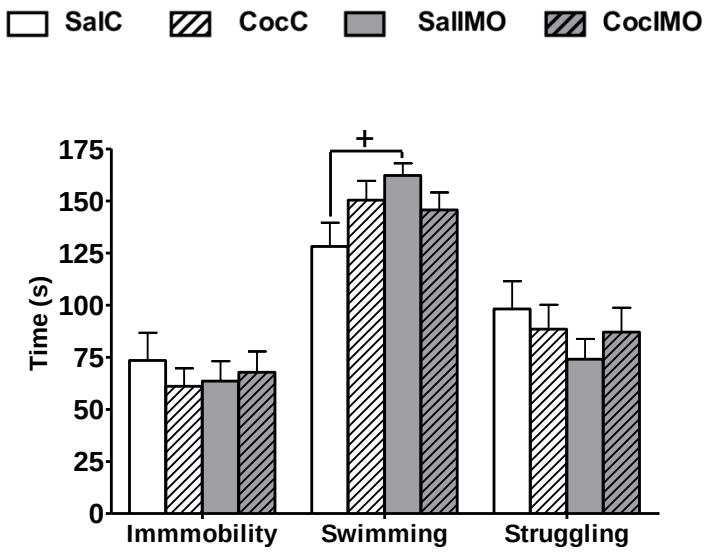


Figure 16. Effects of cocaine administration and IMO on forced swimming test. The data are represented as mean ± SEM of time (s) spent in struggling, swimming and immobility. The white columns represent the non-stressed groups, the grey columns the IMO groups and the stripped columns the cocaine groups. $^{+}$: $p < 0.05$ effect of stress within saline groups (G_ZLM).

The ACTH responses to the EPM and the FST (Figure 17), were significantly increased in the animals previously exposed to IMO (F (1,42) = 86.82, $p < 0.001$; F (1,42) = 23.51, $p < 0.001$ respectively). In the EPM test, the interaction cocaine x IMO was also statistically significant (F (1,42) = 13.13, $p < 0.05$). The decomposition of the interaction showed that cocaine administration increased ACTH levels only in non-stressed animals (F (1,42) = 14.41, $p < 0.001$). In addition, both SalIMO and CocIMO groups were statistically different from their respective unstressed groups (F (1,42) = 73.46, $p < 0.001$ and F (1,42) = 16.79, $p < 0.001$, respectively).

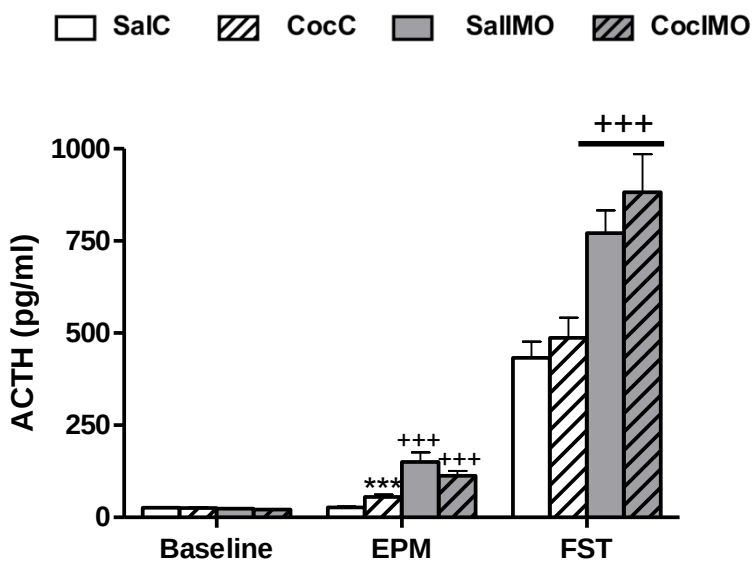


Figure 17. Effects of cocaine administration and IMO on plasma levels of ACTH. The data are represented as mean ± SEM of the plasma ACTH (pg/ml). The white columns represent the non-stressed groups, the grey columns the IMO groups and the stripped columns represent the cocaine groups. In the EPM, cocaine had a significant effect to increase ACTH levels only in saline animals***: $p < 0.001$ *vs* saline group (SalC). $^{+++}$: $p<0.001$ *vs* respective non-stressed group. $\underline{^{+++}}$: $p<0.001$ overall effect of stress (ANOVA).

Experiment 4

Using the V_{max}/weight ratio (Figure 18), the statistical analysis revealed no effect of the main between-subject factors (cocaine and IMO), but a significant interaction day x IMO ($F(3,129) = 3.45$, $p < 0.05$). The interaction cocaine x IMO was not statistically significant. The decomposition of the interaction day x IMO showed that stress increased ASR on day 1 post-treatment ($F(1,137) = 4.57$, $p < 0.05$). The ratio V_{AVG} /weight, another index of ASR, showed a similar pattern of results (data not shown). All factors and interactions were not statistically significant for the variable T_{max}, indicating that the latency of the response was not affected by the treatments.

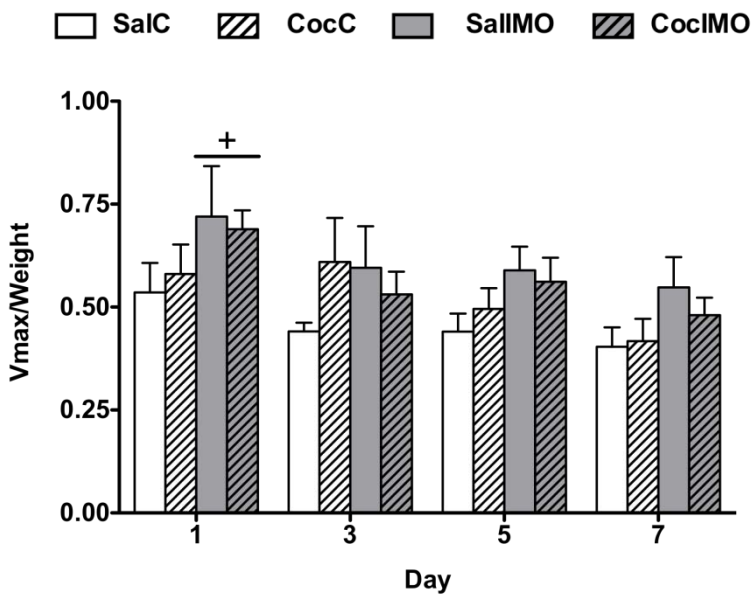


Figure 18. Effects of cocaine administration and IMO on ASR. The data are represented as mean ± SEM of the ratio V_{max} / Weight. The white columns represent the non-stressed groups, the grey columns the IMO groups and the stripped columns the cocaine groups. $^{+}$: $p<0.05$ overall effect of stress (LMM).

Experiment 5

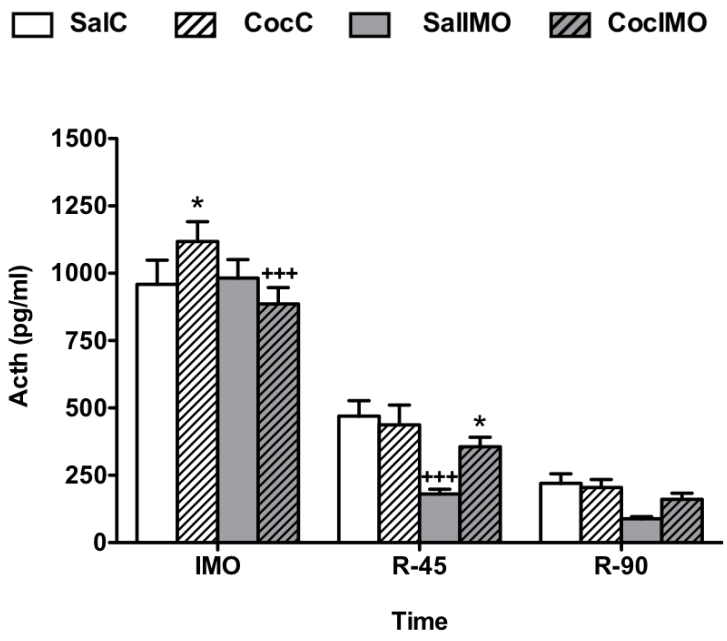
Eight days after treatment, all animals were exposed to 30 min of IMO. Previous pre-exposure to IMO affected ACTH levels response to the new IMO ($F(1,53) = 12.45$, $p < 0.001$) but cocaine pre-exposure did not. Moreover, ACTH levels were affected by time ($F(2,96) = 313.32$, $p < 0.001$). No interaction between the two between-subject factors (cocaine and IMO) was found. The interaction between the three factors sampling time x cocaine x IMO was also statistically significant ($F(2,96) = 7.21$, $p < 0.001$). At the end of IMO (Figure 19), the decomposition of the interaction showed a significant increase of ACTH induced by pre-exposure to cocaine in the control animals ($F(1,131) = 4.68$, $p < 0.05$). In contrast, the pre-exposure to IMO in cocaine

animals decreased ACTH levels ($F(1,131) = 10.87$, $p < 0.001$) in comparison to saline animals pre-exposed to IMO. Furthermore, at 45 min post-IMO (R-45) ACTH levels were decreased by pre-exposure to IMO only in saline animals ($F(1,131) = 14.88$, $p < 0.001$) and pre-exposure to cocaine increased ACTH levels in animals pre-exposed to IMO ($F(1,131) = 5.95$, $p < 0.05$). Thus, the homotypical desensitization induced by a previous exposure to IMO vas blocked by cocaine injected in the first IMO.

No statistical significant differences were observed 90 min after the end of IMO. The groups, SalC, CocC and CocIMO presented statistically significant differences in ACTH levels for each blood sample ($F(2,96) = 53.41$, $p < 0.001$; $F(2,96) = 102.50$, $p < 0.001$; $F(2,96) = 63.83$, $p < 0.001$ respectively). These results show that after IMO, ACTH levels, inside each group, decay with time. The SalIMO group only presented differences between the samples made at the end of the IMO and the following samples that are not different between them ($F(2,96) = 105.60$, $p < 0.001$).

Regarding corticosterone response after the second IMO, the statistical analysis indicated that cocaine and IMO effects were statistically significant ($F(1,51) = 5.84$, $p < 0.05$; $F(1,51) = 8.26$, $p < 0.01$ respectively). Moreover, corticosterone levels were affected by time ($F(2,94) = 94.02$, $p < 0.001$). The double interactions between cocaine x IMO, cocaine x sampling time and IMO x sampling time were also statistically significant ($F(1,51) = 7.42$, $p < 0.01$; $F(2,94) = 4.66$, $p < 0.05$; $F(2,94) = 11.60$, $p < 0.001$ respectively). The interaction between the three factors cocaine x IMO x sampling time was also statistically significant ($F(2,94) = 10.55$, $p < 0.001$). At the end of IMO (Figure 19), the decomposition of the interaction cocaine x IMO x sampling time showed no statistical significant differences in corticosterone levels between groups. At R-45 and at 90 min post-IMO (R-90), corticosterone levels were

decreased by pre-exposure to IMO only in saline animals ($F(1,120) = 18.61$, $p < 0.001$; $F(1,120) = 33.53$, $p < 0.001$ respectively) because pre-exposure to cocaine increased ACTH levels in animals pre-exposed to IMO ($F(1,120) = 24.26$, $p < 0.001$; $F(1,120) = 17.92$, $p < 0.001$ respectively). Therefore the homotypic desensitization in corticosterone levels were blocked also by cocaine. The SalIMO group presented statistically significant differences in corticosterone levels for each blood sample ($F(2,96) = 69.83$, $p < 0.001$). The groups, SalC, CocC and CocIMO only presented differences between the samples made at the end of the IMO and the following samples that are not different between them ($F(2,96) = 8.72$, $p < 0.001$; $F(2,96) = 14.61$, $p < 0.001$; $F(2,96) = 29.51$, $p < 0.001$ respectively).



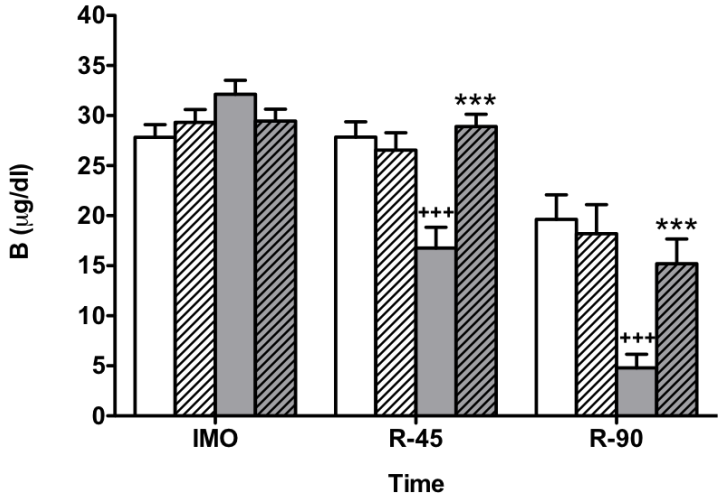


Figure 19. Effects of cocaine administration and IMO on plasma levels of HPA hormones after an additional exposure to IMO. The data are represented as mean ± SEM of the plasma levels of ACTH and corticosterone (B). The white columns represent the previously non-stressed groups, the grey columns the IMO groups, and the stripped columns the cocaine groups. Samples were taken two days before the first IMO, at the end of the second exposure to IMO, 45 and 90 min after (R-45 and R-90 respectively). *: $p<0.05$; ***: $p<0.001$ effect of cocaine. $^{+++}$: $p<0.001$ effect of stress (LMM).

4. Discussion

Exposure to stress and addictive drugs, particularly psychostimulants, can induce some common physiological effects and share activation of some brain areas. These common actions of stress and addictive drugs may constitute the neurobiological substrate explaining the well-known interaction between stress and drug addiction. However, there are almost no studies directly testing the interaction between stress and drugs by simultaneous exposure to both situations. In the present chapter we have studied the neuroendocrine and behavioral consequences of simultaneous exposure to acute IMO and cocaine in adult male rats.

Initial HPA response to IMO and cocaine

Exposure to IMO resulted in a marked activation of the HPA axis (ACTH and corticosterone) in accordance with previous work in our lab and the intensity of the procedure (*e.g.* García *et al.*, 2000; Márquez *et al.*, 2002, 2004). Cocaine administration resulted in a very slight increase in ACTH (1 h after injection), but not significant possibly due to the high values presented by IMO groups. In contrast, a significant corticosterone response induced by cocaine was observed, 1 h, 2 h and 3 h after cocaine administration. The dissociation between ACTH and corticosterone could be explained by small changes in ACTH, that are reflected in more marked effects on corticosterone, because of the high sensitivity of the adrenal glands to small changes in ACTH (Keller-wood *et al.*, 1983). In another experiment (data not shown) using the same protocol but with a higher concentration (30 mg/kg i.p.; 0.5 ml/Kg), cocaine significantly increased corticosterone levels more and more with the passage of time. This may suggest that in the delayed period after cocaine (2-3 h post-injection) hemodynamic changes caused by cocaine may be combined with the small hypovolemia caused by blood sampling, thus resulting in activation of the HPA axis, which is quite sensitive to hypovolemia (Tanimura *et al.*, 1998). The stimulatory effect of acute cocaine administration on the HPA axis is well-established, but activation was usually measured with shorter periods of time after administration (maximum 120 min). Both ACTH and corticosterone increase their levels and reach their maximum peak 20-30 min after a cocaine injection (Ramos-Aliaga and Werner, 1982; Borowsky and Kuhn, 1991; Smith *et al.*, 2004).

The simultaneous exposure to cocaine and IMO resulted in a very mild HPA interaction. At the end of IMO, ACTH levels induced by IMO were reduced by cocaine (negative synergism). Somehow the presence of cocaine seems to block

part of the ACTH release promoted by IMO. The actual mechanisms of this interaction are at present unknown. This small reduction of ACTH levels by cocaine in stressed animals, when translated to humans is in line with some theories that suggested that a subpopulation of chronic cocaine users may actually self-medicate themselves to regulate painful feelings and psychiatric symptoms (Kleber and Gawin, 1984; Khantzian, 1985; Gawin, 1986), as increased rates of affective disorders, anxiety, depression and PTSD are observed in these individuals (Rounsaville *et al.*, 1991; Brady and Lydiard, 1992; Kilbey *et al.*, 1992).

Neuroendocrine and behavioral long-term consequences of IMO and cocaine

Exposure to IMO reduced food intake during the next three days, in accordance with previous data using this stressor and with the anorectic effects of exposure to different severe stressors (Marti *et al.*, 1994; Vallés *et al.*, 2000; Dal-Zotto et al, 2004). An anorectic effect was also observed after cocaine, but this was smaller than that of IMO and was observed only on the first day after injection. These data are also in accordance with the anorectic effects of cocaine and other psychostimulants in the first hours after injection (Bane *et al.*, 1993). Although the magnitude and temporal dynamics of the effects of cocaine on food intake are dependent on the dose, in the present experiment a high dose was used and this could explain that the effect of food intake was observed during the 24 h following drug administration. When food intake during the week post-treatment was followed, concomitant cocaine administration eliminated the reduction caused by IMO (observed by the interaction cocaine x IMO), suggesting a protective effect of cocaine. The mechanisms of this protective effect remain to be studied.

IMO and cocaine not only reduced food intake but also body weight gain. The effects of IMO on body weight gain followed the expected time course, with a stronger reduction on the day after IMO and then a progressive increase in parallel, but always with lower levels than controls. In contrast, cocaine effect on body weight gain progressively accentuated over time in spite of recovery of normal food intake. Apparently, a single dose of cocaine administration was able to induce a quite prolonged reduction of food efficiency, although the mechanisms remain to be characterized.

Exposure to IMO caused a reduction of absolute intake of saccharin that was evident for one week, an effect previously reported by our lab (Rabasa, 2008). Cocaine had no effect and did not interfere with the effects of IMO, suggesting that transient increase in monoamine availability caused by the drug did not alter these negative consequences of the stressor. There are no previous studies about cocaine effects on saccharin intake, making impossible to compare our data with other studies. Since saccharin has not caloric properties, it appears that exposure to IMO is inducing an anhedonic-like state in the animals.

IMO exposure induced an increase in the ACTH response to further different (heterotypic) mild stressors, such as the EPM (novel environment) and the forced-swimming test (FST), 24 h and 48 h (respectively) after IMO. Long-lasting IMO-induced sensitization of the HPA response to a novel environment is in accordance with previous reports using a relatively brief session of electric foot-shock (Van Dijken *et al.*, 1993), IMO (Belda *et al.*, 2008; Gagliano *et al.*, 2008; Muñoz-Abellán *et al.*, 2008; Belda *et al.*, 2012; Daviu *et al.*, 2014) or a long session of electric tail-shock typical of the learned-helplessness paradigm (Johnson *et al.*, 2002; O'Connor *et al.*, 2003, 2004). In the latter case, sensitization was mainly reflected in a faster response

to the acute superimposed stressors and appears to persist for at least 10 days (Johnson *et al.*, 2002). The precise changes responsible for long-term sensitization of the HPA axis caused by IMO are not clearly established, but they may involve reduced negative glucocorticoid feedback (O`Connor *et al.*, 2003) and enhanced inputs to the PVN (O`Connor *et al.*, 2004). Sensitization of the HPA response after a single exposure to IMO is restricted to novel (heterotypic) stressors, as we have repeatedly found that a single exposure to IMO caused a long-term desensitization of the HPA response to the same (homotypic) stressor, which affected both peripheral and central (PVN) components of the HPA axis (see Armario *et al.*, 2004 for a review).

The effects of cocaine in heterotypic sensitization are not clear in the present study. In the experiment 2, ACTH and corticosterone levels in response to a mild stressor (EPM) were measured, and in the experiment 3 corticosterone response to the same stressor (EPM) and to other of higher intensity (FST) was evaluated. To our knowledge, cocaine induced sensitization of the HPA axis to mild stressors has not been previously studied. In one experiment cocaine by itself increased corticosterone (but not ACTH) levels to the EPM, and decreased ACTH sensitization induced by IMO in response to the EPM. However, in the other experiment cocaine by itself increased ACTH response to the EPM but not to the FST, whereas did not modify the HPA sensitization induced by IMO. Thus, additional studies are needed to verify if the effects are consistent.

Exposure to IMO resulted, 24 h later, in lower number of entries and less time spent in the open arms of the EPM, suggesting an anxiogenic-like effect. This anxiogenic effect in the EPM is in accordance with our previous studies and can last for 7 days (Belda *et al.*, 2008), although it is not detected at 14 days post-IMO (Belda *et al.*, 2004). The decrease in time spent in open arms was accompanied by a decrease in

closed arm entries, which suggests that in addition to enhanced anxiety-like behavior, IMO may cause a small and transient reduction of activity in novel environments, also in accordance with previous results (Belda *et al.*, 2008; Daviu, 2008; Rabasa, 2008). This is a phenomenon currently observed with severe stressors that is likely to disappear after 3 days (*e.g.* Woodmansee *et al.*, 1993). For instance, exposure to IMO did not modify neither activity nor exploration in a holeboard when assessed 5 days after the stressor (Gagliano *et al.*, 2008), suggesting that, at this time, IMO-induced inhibition of activity has gone. On the other hand, cocaine had no effect on anxiety-like behavior. Previous results indicated acute anxiogenic effects of cocaine in the EPM (see Introduction) although no previous reports measured anxiety-like behavior at the time evaluated in the present experiment.

Although IMO exposure induced both, a sensitization of the HPA axis and an increase in anxiety-like behavior, both processes are likely to be, at least partially, independent. To our knowledge, only two previous reports have simultaneously studied behavioral and HPA response to novel environments after a previous single exposure to stress (Gagliano *et al.*, 2008, Van Dijken *et al.*, 1993). Van Dijken *et al.* (1993) reported reduced locomotion, increased immobility and a higher ACTH response after exposure to a novel environment in rats previously exposed to a brief session of footshocks 14 days before (corticosterone response did not differ between control and shocked-rats). However, in our lab, previous exposure to IMO resulted in a sensitization of the ACTH response to a holeboard 5–7 days after the stressor, which was not reflected in changes in activity/exploration in the apparatus (Gagliano *et al.*, 2008). In addition, a single exposure to cat fur odor resulted in long-lasting changes in anxiety-like behavior in the EPM with no evidence of HPA sensitization (Muñoz-Abellán *et al.*, 2008). This independence is also corroborated by the lack of statistically significant correlations between hormonal and behavioral data in the present study and in others (Belda *et al.*, 2008).

Exposure to IMO did not modify struggling, the most active behavior in the forced swimming test, when measured 2 days after the stressor. However, IMO did induce a small, but significant, increase in mild swim. In general, forced swim behavior is not very sensitive to acute stressors. When evaluated immediately or very shortly after exposure to stressors, reduction of active behavior or increases in immobility are only observed after very severe stressors and is dependent on the strain of animals (*i.e.*Prince and Anisman, 1984; Shanks and Anisman, 1988; Armario *et al.*, 1991), whereas the delayed effects (24 h) are inconsistent (e. g. Prince and Anisman, 1984; Armario *et al.*, 1991). This differential effect of IMO on struggling and mild swim is in accordance with the hypothesis that they represent two different types of active behaviors in the FST (Armario *et al.*, 1988; Martí and Armario, 1993). Thus, whereas struggling is preferentially increased by administration of antidepressants that inhibit reuptake of noradrenaline, mild swim is enhanced by serotonin reuptake inhibitors (Cryan *et al.*, 2005). Therefore, previous exposure to IMO may enhance for some days brain serotoninergic rather than noradrenergic circuits. Although cocaine did not alter forced swimming behavior, the drug appears to blunt the small effect of IMO on mild swim. In any case, the FST was rather insensitive to acute effects of stress and cocaine, supporting the hypothesis that this test, contrary to other behavioral tests such as the EPM, is likely to reflect a quite stable trait that defines the tendency of animals to adopt active strategies in apparently inescapable situations.

An enhanced ASR is considered to be an index of anxiety (for a review see Davis, 1989). It has been reported that some stressors are able to induce long-lasting (days to weeks) increases in ASR, although the effects are not very consistent as those in the EPM (see Armario *et al.*, 2008, for a review). In the present work, IMO exposure enhanced ASR 24 h after stress, the effect vanished over time and it was independent of cocaine administration, which was also ineffective by itself. Although

exposure to some stressors has been reported to increase ASR for several days, the results are not always consistent (see Armario *et al.*, 2008, for a review), likely because of individual differences in susceptibility to stress and methodological aspects such as pulse intensity (Glowa and Hansen, 1994; Conti and Printz, 2003; Beck and Servatius 2005, 2006; Gonzales *et al.*, 2008) or the need of a longer incubation period (Fuentes *et al.*, 2014).

In conclusion, acute exposure to IMO results in a wide range of neuroendocrine and behavioral consequences that are observed over the week following the exposure to the stressor. These effects were if any weakly affected by simultaneous administration of cocaine, that itself had basically no effect except in food intake and body weight gain. Therefore, the present results do not give a strong support to the hypothesis that cocaine consumption may be mainly explained by action of the drug to relief from the negative consequences of stress. Future studies using chronic administration of cocaine are needed to ascertain whether prolonged exposure to the drug could modify IMO-induced neuroendocrine and behavioral effects.

Disruption of the homotypic long-term desensitization to IMO by cocaine

Repeated exposure to the same stressor will induce a progressive HPA decrease in the response to the stressor (homotypic), while the response to a stressor different (heterotypic) is normal and or even increased (Armario et al, 1984, 1986, 1988). This would indicate that the ability of the HPA axis in response to stress is maintained and therefore adaptation to homotypic stressor is not due to biochemical changes triggered by exposure to stressful situations, such as depletion of the endocrine

glands. In our group we have characterized the long-term effects of a single exposure to stress in response to the homotypic stressor. As in this study, Martí *et al.* (2001) observed a reduction in HPA axis response (desensitization) in animals exposed to IMO when they were re-exposed to the same stimulus for the second time, 8 days later. It seems more appropriate to attribute the decreased response to the reduction of emotional impact produced by familiarizing the animal with repeated stimulation (Martí and Armario, 1998). According to this idea, sensitive variables to stress intensity (ACTH, corticosterone, adrenaline, glucose and prolactin) are those that show a clear reduction in situations of repeated stress (Kvetnansky et al, 1984; Armario et al, 1988; 1990; 1998; De Boer et al, 1990).

It is assumed that the reduction of the stress response to a known stimulus (homotypic), that does not involve a real danger to the organism, can be observed in some cases after a single exposure to the stressor (long term desensitization) or after repeated exposure (stress adaptation) and both cases could represent a learning of adaptive value for the organism (Armario, 2006). In this study we found that cocaine may affect the affective memory of that event. As observed in this study, the animals administered with cocaine in the first exposure to IMO, showed no homotypic adaptation to stress, contrary to the animals that were only exposed to IMO. Given that hypothetically the impact of stress under cocaine exposure is lower (as measured by a decrease in ACTH), it is reasonable to assume that the homotypic desensitization (as a measure of memory of the situation) is also decreased. From this point of view, cocaine when presented to animals during an intense stressful event (such as IMO) seems to have a protective effect.

CHAPTER II

Study of the c-fos induction in the CNS after exposure to cocaine and/or stress (IMO).

1. Introduction

After the study of the interaction between IMO and cocaine in the HPA axis and anxiety related tests, it was important to characterize this interaction in the CNS. The neurobiological basis of the interaction between stress and addiction could be focused in areas of the CNS that show activation with stress and drugs of abuse. As mentioned, the activation of CNS areas related to the stress response induced by the administration of drugs of abuse and the property of some stressful stimuli to reinstate the drug search or consumption, are some of the evidences that have led to propose a relationship between stress and addiction (for a review see Koob and Le Moal, 2006). A single area could show activation of distinct (separate) neuronal populations against various stimuli such as stress and drugs, or on the contrary, both stimuli might activate, at least in part, the same neuronal populations. It seems more likely that stressful stimuli and drugs of abuse interact in specific areas if they activate the same neuronal populations. In this regard, simultaneous exposure to a stressor and cocaine may provide relevant information about the processing of these

stimuli. If the activated neuronal populations are different for each stimulus, we would expect to find that the number of activated neurons by the two stimuli applied jointly be approximately the sum of the activation of each individual stimulus. On the other hand, if the same neuronal populations are activated during simultaneous exposure to stressful stimuli and cocaine, neuronal activation would be similar to the activation caused by the two stimuli separately (unless there is a maximum threshold).

Taking into account the interactions found in the first chapter, it is important to note that as a pharmacological stressor (review in Kovacs, 1998; Sawchenko *et al.*, 2000; Pacak and Palkovits, 2001; Herman *et al.*, 2003) the administration of most drugs of abuse is able to induce c-fos expression in the PVN parvocellular region, where is the largest population of CRF neurons. This is what occurs for cannabinoids (Wenger *et al.*, 1997), morphine (Chang *et al.*, 1995; Laorden *et al.*, 2000), alcohol (Chang *et al.*, 1995; Ryabinin *et al.*, 2000), amphetamine (Engber *et al.*, 1998) and nicotine (Valentine *et al.*, 1996; Matta *et al.*, 1997). Cocaine is a possible exception, because some reports indicate that the drug is not able to induce c-fos expression at the PVN (Torres and Rivier, 1994, Chang *et al.*, 1995; Chocyz *et al.*, 2008) and other find that activation (Ryabinin *et al.*, 2000). In addition, limbic regions are thought to be important in stress and drug responses, and thus are potential sites where psychostimulant drugs and stressful stimuli may interact.

Small doses of cocaine or low intensity stressful stimuli may complicate the understanding of the interactions or additive effects found because c-fos induction in many of the areas analyzed could not be maximum. Under these conditions, some of the neurons could still be capable of being activated after the superposition of two stimuli of moderate intensity, making it difficult to determine if there are or not different populations. To avoid this problem it is necessary to use high intensity stressful stimuli like IMO and high doses of cocaine, capable of causing a strong c-

fos induction. In this way the additive effects or the existence or potentiation or inhibitory effects on the levels of FLI can be better interpreted.

In this chapter we propose to study the effects of simultaneous administration of cocaine and IMO in the CNS, using the induction of c-fos as marker of neuronal activation.

2. Experimental protocol

This experiment was conducted using 64 rats. In the first day, animals were treated according with their group. Half of the animals were sacrificed and perfused at the end of IMO and the other half 1 h after (Figure 20). The brains of the animals were then processed to be evaluated by in situ hybridization analysis as mentioned. Finally, a comparative mapping of the activated brain areas using c-fos as a marker was performed.

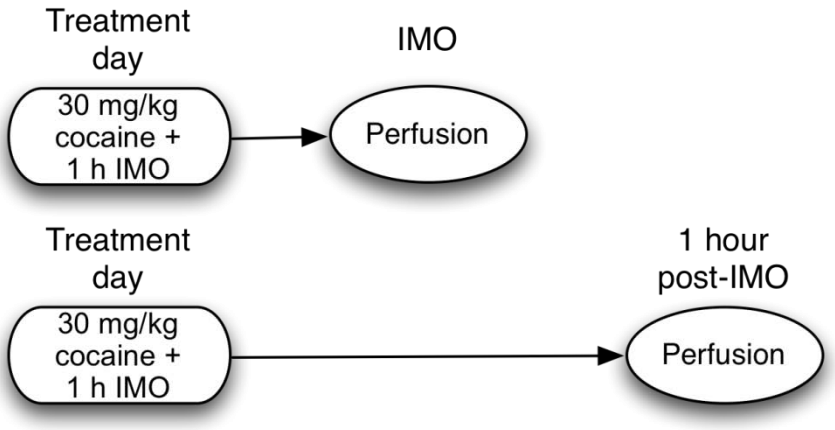
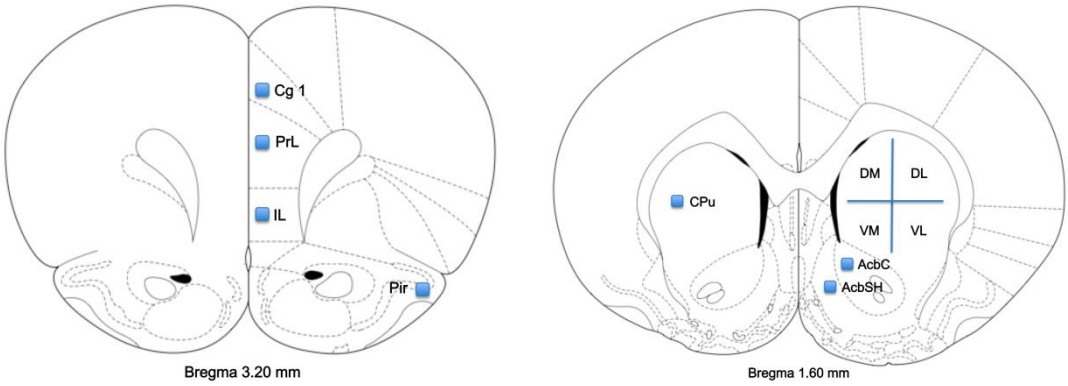


Figure 20. Summary of the experiment taking CocIMO group as example. The aim of this experiment is to evaluate c-fos expression in response to stress and / or cocaine in two time periods.

The stereotactic atlas of Paxinos and Watson (1998) was used to define the coordinates of each area analyzed (Figure 21).



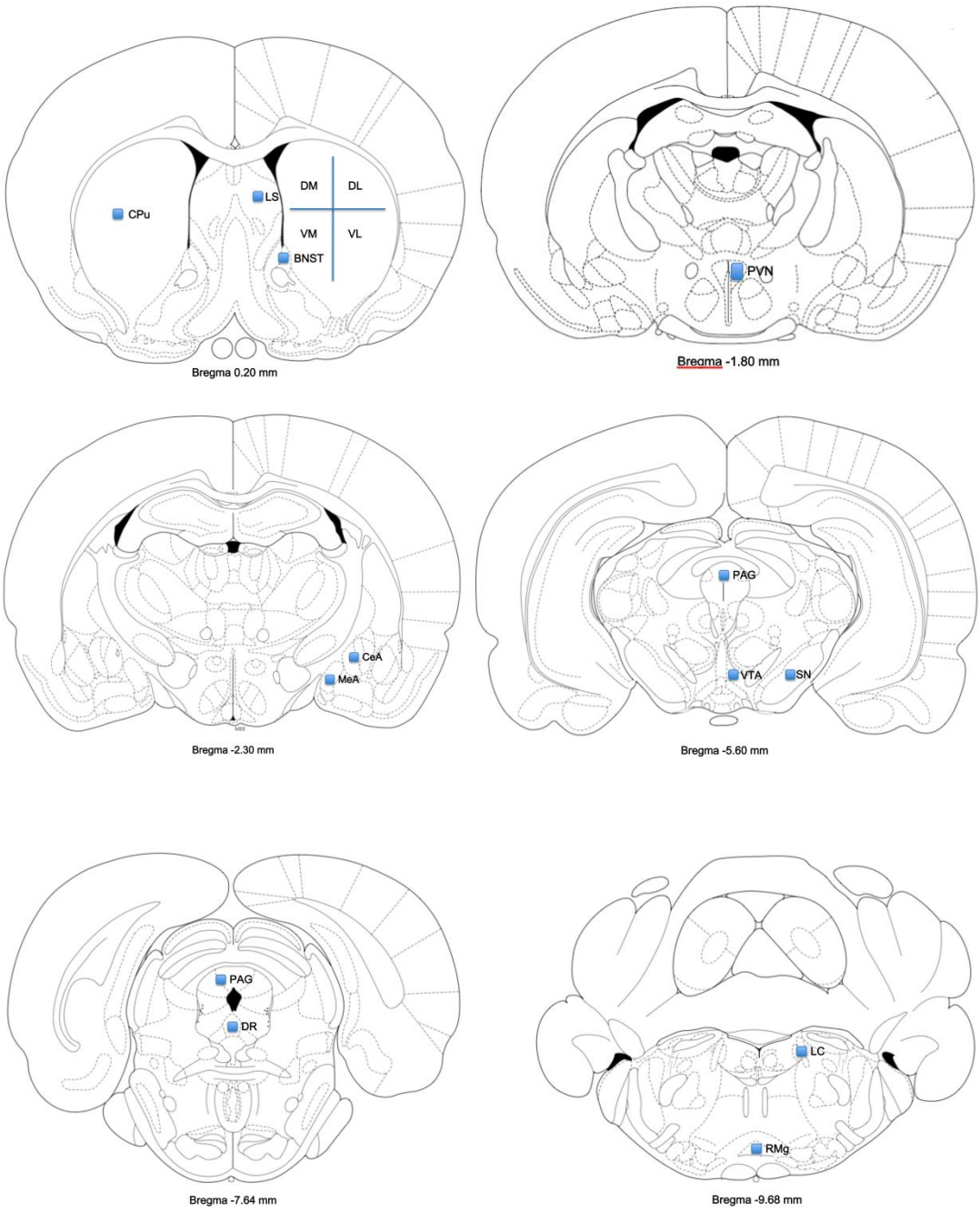


Figure 21. Schematic representation of the stereotactic localization of the CNS areas analyzed (adapted from Paxinos and Watson, 1998). Abbreviations: medial prefrontal cortex (Cg1: cingulate 1, PrL: Prelímbic, IL: infralimbic); Pir: piriform cortex; AcbSh: accumbens shell; AcbC: accumbens core; Cpu: striatum (CpuDM: dorso-medial, CpuDL: dorso-lateral, CpuVM: ventro-medial, CpuVL: ventro-lateral); BNST: bed nucleus of the stria terminalis; LS: lateral septum; PVN: paraventricular nucleus of the hypothalamus; CeA: central Amygdala; MeA: medial amygdala; SN: substantia nigra; VTA: ventral tegmental area; PAG: periaqueductal gray; DR: dorsal raphe nucleus; LC: locus coeruleus; RMg: magnus raphe nucleus.

3. Results

At the end of IMO and regardless cocaine administration, the statistical analysis showed an overall effect of IMO in c-fos expression on Pir, MeA, LC and RMg (Table 2). There was a significant effect of cocaine on anterior and posterior Cpu, MeA, LC and RMg, regardless of exposure to IMO. Finally, statistical analysis showed an interaction IMO x Cocaine in Cg 1, IL, PrL, orbital cortex (Orb), Acb, BNST, LS, PVN, CeA, SN, VTA, PAG and DR.

In the animals sacrificed at R-1h, the statistical analysis showed an overall effect of stress in c-fos expression on Cg 1, Orb, Str (posterior region), LS, CeA, MeA, SN, VTA, PAG and LC, regardless cocaine administration (Table 2). There was a significant effect of cocaine on Cg 1, Obr, Str (posterior and anterior regions), CeA, MeA, SN, VTA, PAG, LC and RMg, despite of the exposure to IMO. Finally, statistical analysis showed an interaction between IMO x Cocaine in Pir, IL, PrL, Acb, BNST, PVN, DR.

Finally, the striatum was separated into four different sub areas as shown in Figure 21 and analyzed according to these divisions. The result for each subarea (data not shown) was equal to the total area of the striatum (only cocaine had a significant effect in c-fos expression).

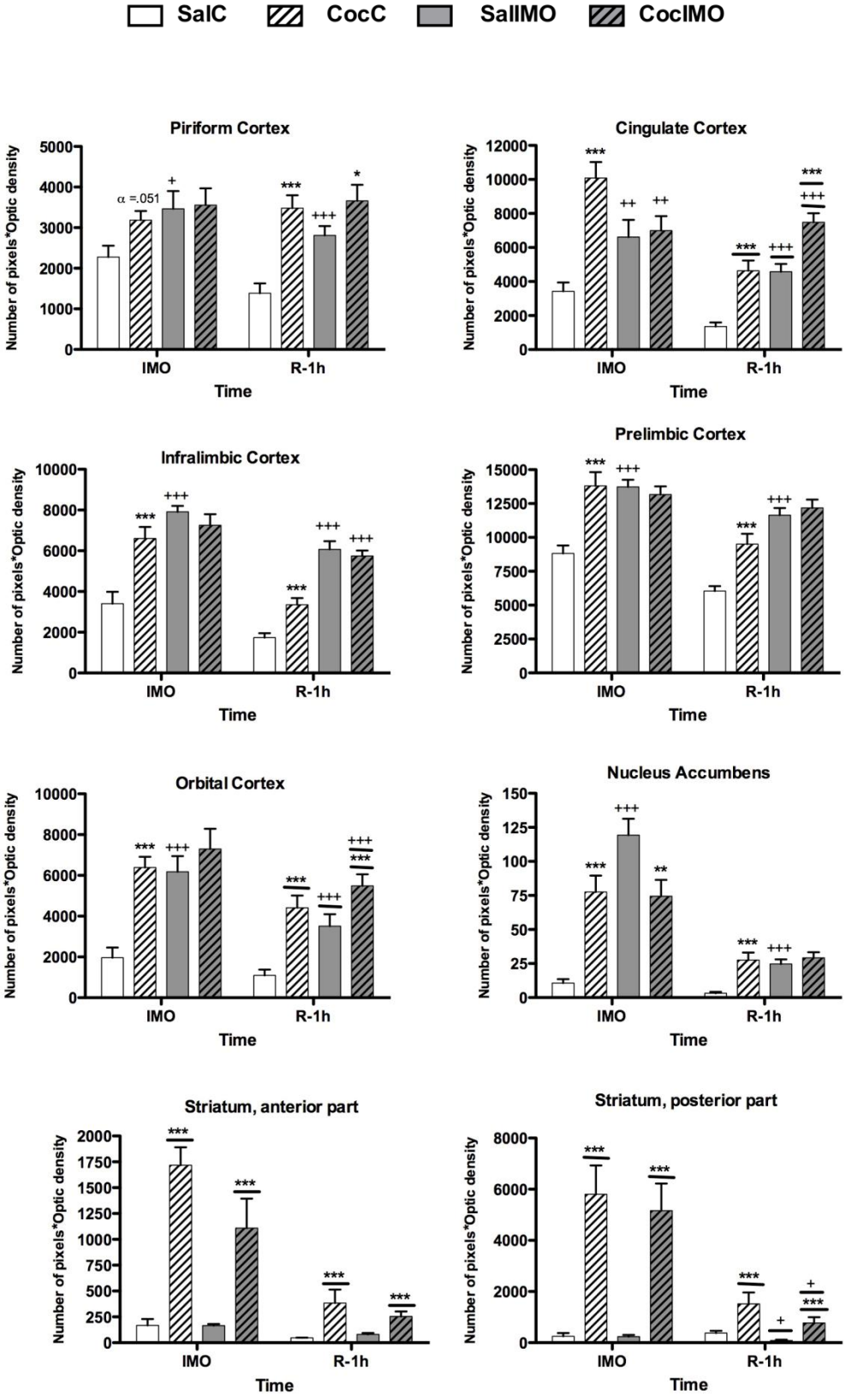
The main effects of the drug and stress in these areas are shown in Table 2 and results from the decomposition of these interactions are shown in Figure 22.

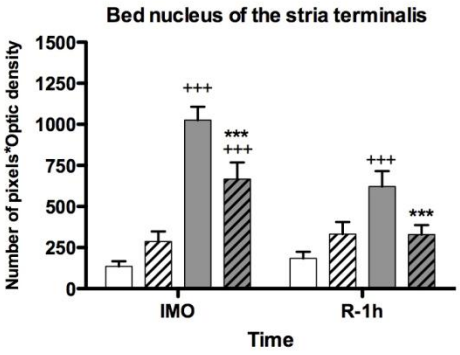
At the end of IMO, the decomposition of the interactions have found the following patterns of FLI: a) areas that were activated equally by IMO and by cocaine, and in which the two stimuli together have no effect different from each one separately (IL, PrL, Orb and PAGa); b) other areas where the response to each of the stimuli is individually different and the combination yields a similar activation to the stimulus that causes greater induction of FLI, whether IMO (LS and PVN) or cocaine (CeA, SN and VTA); c) in the BNST and DR the administration of cocaine in conjunction with IMO, seems to inhibit the activation induced in this area, decreasing the levels of FLI in comparison to the group SalIMO, which shows higher activation; d) finally, other areas where the response to each of the stimuli is individually different and the combination yields a similar activation to the stimulus that causes lower induction of FLI, whether IMO (Cg1) or cocaine (Acb).

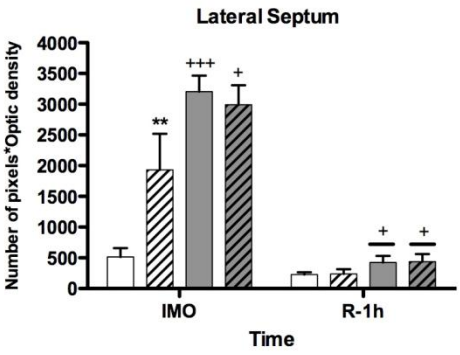
At R-1h, the decomposition of the interactions have found the following patterns of FLI: a) areas that were activated equally by IMO and by cocaine, and in which the two stimuli together have no effect different from each one separately (PrL and Acb); b) other areas where the response to each of the stimuli is individually different and the combination yields a similar activation to the stimulus that causes greater induction of FLI, whether IMO (IL, PVN and DR) or cocaine (Pir); c) finally, in the BNST the administration of cocaine in conjunction with IMO, seems to inhibit the activation in this area, it decreases the levels of FLI in comparison to the group SalIMO, which showed higher activation. This effect seen in the BNST is especially interesting because is observed both immediately after IMO and 1 h later.

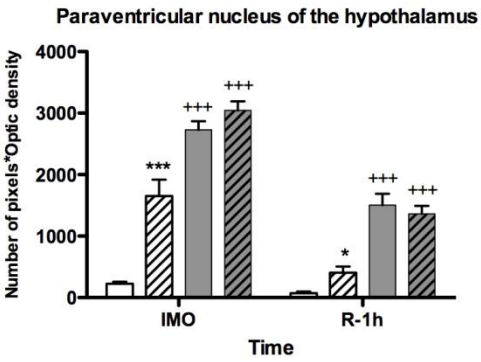
Table 2. Results of Generalized linear models.

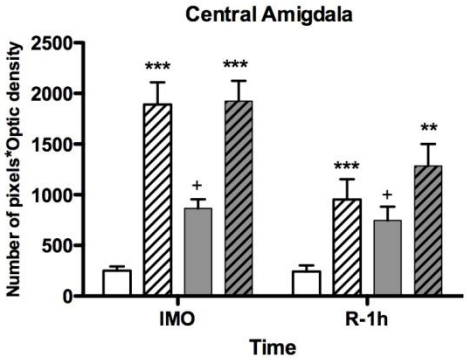
Abbreviations	Areas	Time	df	Cocaine		IMO		Cocaine*IMO	
				Wald	Sig	Wald	Sig	Wald	Sig
Pir	Piriform Cortex	R - IMO	1,28	2,30	**N.S.**	5,59	**,018***	1,54	**N.S.**
		R - 1h	1,28	26,84	**,000*****	7,99	**,005****	4,82	**0,028***
Cg 1	Cingulate Cortex	R - IMO	1,28	19,50	**,000*****	,004	**N.S.**	15,51	**,000*****
		R - 1h	1,28	47,21	**,000*****	45,31	**,000*****	0,163	**N.S.**
IL	Infralimbic Cortex	R - IMO	1,28	7,09	**,008****	29,16	**,000*****	16,21	**,000*****
		R - 1h	1,28	4,95	**,026***	132,8	**,000*****	11,01	**,001*****
PrL	Prelimbic Cortex	R - IMO	1,28	11,24	**,001*****	10,48	**,001*****	17,69	**,000*****
		R - 1h	1,28	13,22	**,000*****	56,39	**,000*****	7,01	**,008****
Orb	Orbital Cortex	R - IMO	1,28	16,72	**,000*****	14,32	**,000*****	5,94	**,015***
		R - 1h	1,28	29,05	**,000*****	12,68	**,000*****	1,85	**N.S.**
Acb	Nucleus Accumbens	R - IMO	1,27	1,16	**N.S.**	26,77	**,000*****	29,87	**,000*****
		R - 1h	1,28	15,60	**,000*****	10,00	**,002****	4,55	**,006****
Cpu	Striatum (anterior part)	R - IMO	1,27	61,54	**,000*****	3,65	**N.S.**	3,64	**N.S.**
		R - 1h	1,28	13,26	**,000*****	,199	**N.S.**	,969	**N.S.**
Cpu	Striatum (posterior part)	R - IMO	1,27	48,84	**,000*****	,183	**N.S.**	,177	**N.S.**
		R - 1h	1,28	14,63	**,000*****	4,644	**,031***	,946	**N.S.**
BNST	Bed nucleus of the stria terminalis	R - IMO	1,26	2,08	**N.S.**	77,77	**,000*****	12,49	**,000*****
		R - 1h	1,28	1,26	**N.S.**	11,23	**,001*****	11,53	**,001*****
LS	Lateral Septum nucleus (intermediate part)	R - IMO	1,25	3,23	**N.S.**	31,35	**,000*****	5,92	**,015***
		R - 1h	1,28	,160	**N.S.**	5,378	**,020***	,000	**N.S.**
PVN	Paraventricular nucleus of the hypothalamus	R - IMO	1,28	30,45	**,000*****	152,2	**,000*****	12,26	**,000*****
		R - 1h	1,28	,490	**N.S.**	66,83	**,000*****	5,96	**,015***
CeA	Central Amigdala nuclei	R - IMO	1,28	85,27	**,000*****	4,91	**,027***	3,988	**,046***
		R - 1h	1,28	17,01	**,000*****	9,02	**,003****	,116	**N.S.**
MeA	Medial Amigdala nuclei	R - IMO	1,28	13,97	**,000*****	8,10	**,004****	2,547	**N.S.**
		R - 1h	1,28	53,12	**,000*****	16,83	**,000*****	,948	**N.S.**
SN	Substantia nigra (reticular)	R - IMO	1,28	68,39	**,000*****	31,65	**,000*****	15,99	**,000*****
		R - 1h	1,28	43,79	**,000*****	18,44	**,000*****	2,08	**N.S.**
VTA	Ventral tegmental area	R - IMO	1,28	198,5	**,000*****	1,76	**N.S.**	16,11	**,000*****
		R - 1h	1,27	173,2	**,000*****	17,22	**,000*****	,107	**N.S.**
PAG	Periaqueductal gray (anterior part)	R - IMO	1,27	15,30	**,000*****	4,92	**,027***	5,45	**,020***
		R - 1h	1,28	34,47	**,000*****	12,74	**,000*****	,015	**N.S.**
PAG	Periaqueductal gray (posterior part)	R - IMO	1,28	20,47	**,000*****	,682	**N.S.**	8,464	**,004****
		R - 1h	1,26	36,23	**,000*****	9,94	**,002****	,596	**N.S.**
DR	Dorsal Raphe nucleus	R - IMO	1,28	,145	**N.S.**	45,51	**,000*****	6,46	**,011***
		R - 1h	1,26	7,27	**,007****	53,96	**,000*****	5,13	**,024***
LC	Locus coeruleus	R - IMO	1,24	24,58	**,000*****	13,88	**,000*****	,288	**N.S.**
		R - 1h	1,27	11,54	**,001*****	35,31	**,000*****	,007	**N.S.**
RMg	Magnus raphe nucleus	R - IMO	1,25	27,18	**,000*****	18,00	**,000*****	,117	**N.S.**
		R - 1h	1,27	8,76	**,003****	2,27	**N.S.**	1,72	**N.S.**

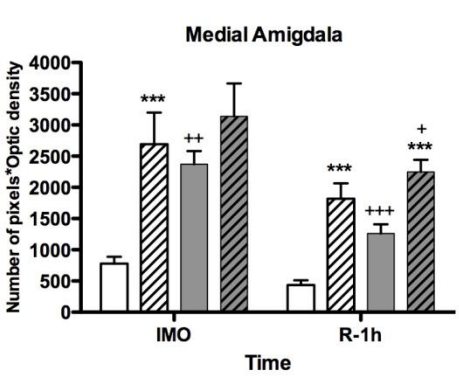

SalC
CocC
SalIMO
CocIMO
Piriform Cortex
Cingulate Cortex
Infralimbic Cortex
Prelimbic Cortex
Orbital Cortex
Nucleus Accumbens
Striatum, anterior part
Striatum, posterior part
Number of pixels*Optic density
IMO
R-1h
Time
α =.051

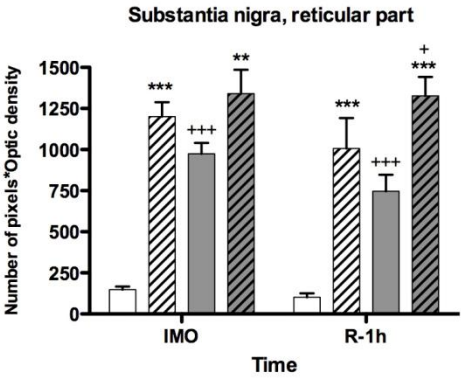

Bed nucleus of the stria terminalis
Number of pixels*Optic density
1500
1250
1000
750
500
250
0
+++
***
+++
+++
***
IMO
R-1h
Time

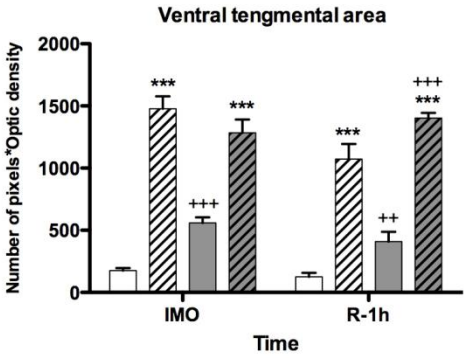

Lateral Septum
Number of pixels*Optic density
4000
3500
3000
2500
2000
1500
1000
500
0
**
+++
+
+
+
IMO
R-1h
Time

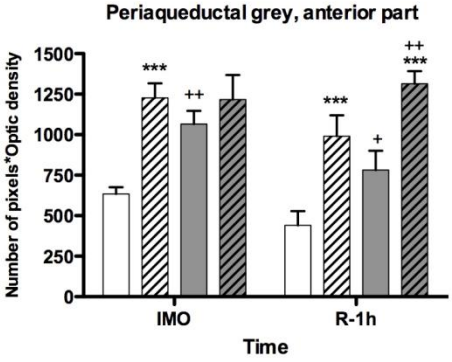

Paraventricular nucleus of the hypothalamus
Number of pixels*Optic density
4000
3000
2000
1000
0
***
+++
+++
*
+++
+++
IMO
R-1h
Time


Central Amigdala
Number of pixels*Optic density
2500
2000
1500
1000
500
0
***
***
+
***
+
**
IMO
R-1h
Time


Medial Amigdala
Number of pixels*Optic density
4000
3500
3000
2500
2000
1500
1000
500
0
***
++
***
+
***
+++
IMO
R-1h
Time


Substantia nigra, reticular part
Number of pixels*Optic density
1500
1250
1000
750
500
250
0
***
+++
**
***
+++
+
***
IMO
R-1h
Time


Ventral tengmental area
Number of pixels*Optic density
2000
1500
1000
500
0
***
+++
***
***
++
+++
***
IMO
R-1h
Time


Periaqueductal grey, anterior part
Number of pixels*Optic density
1500
1250
1000
750
500
250
0
***
++
***
+
++
***
IMO
R-1h
Time

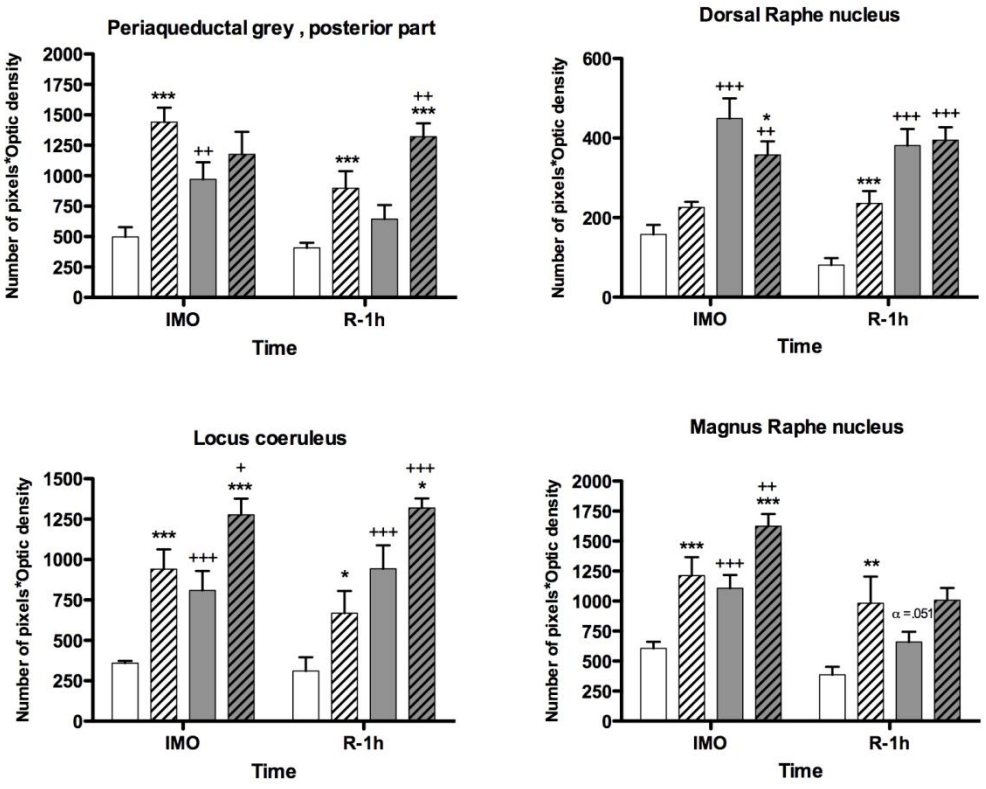


Figure 22. Effects of cocaine administration and IMO on c-fos mRNA levels. The data are represented as mean ± SEM of c-fos (Number of pixels*Optic density). The white columns represent the non-stressed groups, the grey columns the IMO groups, and the stripped columns the cocaine groups. Samples were taken at the end of IMO (IMO) and 1 h after IMO (R-1h). These time points correspond to 1 and 2 h after cocaine. */+: $p<0.05$; **/++: $p<0.01$; ***/+++: $p<0.001$. Underlined significance symbols represent the overall effects of the cocaine(*)/IMO(+); not underlined symbols represent the significance (not overall) after the decomposition of the interaction (G_ZLM).

4. Discussion

Once characterized the possible synergy between cocaine and stress at the peripheral level, we decided to study the possible interaction sites in the CNS using the expression of c-fos as a marker of neuronal activation. We hypothesized that the negative synergy between drug and stress should be observed in key brain areas.

Effects of IMO

Exposure to IMO increased c-fos expression in most of the studied areas: PFC (in the different subregions), BNST, N. accumbens, lateral septum, central and medial amigdala, substantia nigra, VTA, periaqueductal gray, dorsal and magnus raphe nucleus, locus coeruleus and PVN. In contrast, there is almost no c-fos expression in the dorsal striatum. These results are in agreement with other data from our laboratory (Ons *et al.*, 2004, 2010; Vallès *et al.*, 2006) and consistent with other previous studies, using both emotional and systemic stressors (for a review see Armario, 2006). The main difference between both stimuli is the lack of potent c-fos activation in CeA in response to emotional stimuli, especially when c-fos mRNA is evaluated (Cullinan *et al.*, 1995; Campeau *et al.*, 1997; Campeau and Watson, 1997; Bonaz and Rivest, 1998). On the other hand, emotional stressors such as exposure to a new environment, noise, immobilization in tube, the smell of predator or forced swimming induced a marked activation of different regions of the mPFC (Cullinan *et al.*, 1995; Duncan *et al.*, 1996; Campeau and Watson, 1997; Bonaz and Rivest,

1998; Li and Sawchenko, 1998; Dielenberg *et al.*, 2001; Day *et al.*, 2004). As expected, IMO also induced c-fos activation in all regions of the mPFC. This structure appears to be important for the overall processing of emotional stimuli and particularly for the regulation of the HPA axis response to these stimuli. Injury or deactivation of the mPFC causes increased ACTH levels in response to emotional stimuli but not to systemic ones as ether (Diorio *et al.*, 1993; Figueiredo *et al.*, 2003; Radley *et al.*, 2006; 2008).

Effects of cocaine

Cocaine alone activated, as expected based on previous data, most of the areas studied (Pir,Cg1, IL,PrL, Orb, Acb, Cpu, PVN, CeA, MeA, SN, VTA, PAG, LC and RMg), although the degree of activation achieved changed according with the studied area. For example, activation was much more noticeable in mPFC, Cpu, VTA and Acb than in LC, RMg or the PVN itself. The only areas that were not activated by cocaine were the BNST and the LS. All these data are compatible with another mapping study (Zombeck *et al.*, 2010) where cocaine administration in both adolescent and adult mice increased c-fos expression in an extensive number of brain areas.

Moreover, as previously mentioned in the Introduction, most drugs of abuse increase dopamine levels, therefore, we would expect an activation of c-fos in areas related to dopaminergic pathways (Graybiel *et al.*, 1990; Snyder-Keller, 1991, Johansson et al, 1994; Dalia and Wallace, 1995; Konradi et al, 1996; Badiani et al, 1998). In the

context of dopaminergic projections, our study extends previous findings showing that cocaine induced c-Fos expression in Cpu (Graybiel *et al.*, 1990; Steiner and Gerfen, 1993; Couceyro *et al.*, 1994; Torres and Rivier, 1994; Kosofsky *et al.*, 1995; Moratalla *et al.*, 1996; Ryabinin *et al.*, 2000; Jenab *et al.*, 2002, 2003; Willuhn *et al.*, 2003; Kreuter *et al.*, 2004) and Acb (Graybiel *et al.*, 1990; Ryabinin *et al.*, 2000; Szucs *et al.*, 2005; Regier *et al.*, 2012). It is possible to speculate that these areas are responsive for psychomotor effects of cocaine, including development of sensitization. In fact, previous data described a significant correlation between cocaine-induced hyperactivity and c-fos expression in the Acb core and Cpu (Szucs *et al.*, 2005; Zombeck *et al.*, 2010). The role of DA in cocaine-induced c-fos in these areas is demonstrated by studies using D1 mutant mice in which cocaine does not induce c-fos in the Acb and the Cpu (Zhang *et al.*, 2002). Moreover, intrastriatal infusion of a D1 dopamine receptor antagonist also resulted in a dose-dependent blockade of c-fos induction by cocaine (Steiner and Gerfen, 1995).

Another interesting question is whether the pattern of activation induced by cocaine in the striatum is similar to other psychostimulants such as amphetamine. Although it is general assumed that this will be the case, the differences in the mechanisms of action between both drugs suggest that the effects will not be exactly the same. The seminal results of Graybiel *et al.* (1990) already showed that the induction of c-fos by amphetamine was especially important in the striosome, whereas cocaine induced c-fos expression in both, the matrix and the striosome part of the striatum. On the other hand, our results do not show a difference in the pattern of activation of c-fos in response to cocaine between medial and lateral regions of the Cpu (data not shown) and this result is in accordance with Erdtmann-Vourliotis *et al.* (2000) study which also observed no differences between subregions of the Cpu after cocaine administration (50 mg/kg and 10 mg/Kg) in naive rats. Instead of using a medial/lateral subdivision other authors have found a different pattern of activation

induced by cocaine in the caudal/dorsal striatal sectors (maximal activation) in comparison to rostral/ventral (Willhun *et al.*, 2003). Our results are contrary to amphetamine data where a difference in the pattern was observed between the different subdivisions of the Cpu. Amphetamine studies indicated that the dorsal and ventral areas of the medial Cpu are potently activated by the administration of amphetamine, whereas in the same divisions of the lateral part, the activation by the drug is more discrete (Rotllant *et al.*, 2010; Jaber *et al.*, 1995; Turgeon *et al.*, 1996; Hamamura and Ichimaru, 1997). Other differences are that in the ventrolateral and ventromedial quadrants of the Cpu, novelty significantly enhanced the ability of amphetamine to induce c-fos expression but did not modulate the ability of cocaine to induce c-fos mRNA expression. This apparent differential ability of the context to modulate the effects of amphetamine versus cocaine in these specific subregions of the caudate may be due to a number of different factors (see Uslaner *et al.*, 2001 for a discussion). Finally, other authors have also described differences between the pattern of activation induced in the striatum by amphetamine and cocaine, in the sense that amphetamine seems to activate less neurons than cocaine (Johansson *et al.*, 1994).

It is also especially interesting that cocaine administration activates c-fos expression in the CeA. Activation of the CeA has been associated with the response to a large number of stressful stimuli of systemic nature, in several laboratories (Honkaniemi *et al.*, 1992; Ericsson *et al.*, 1994; Rivest and Laflamme, 1995; Rotllant *et al.*, 2002, 2007, 2010) including ours (Vallès *et al.*, 2005) and also in response to a large number of psychoactive drugs (Duncan *et al.*, 1993; Sebens *et al.*, 1995) and drugs of abuse (Matta et al., 1993; McGregor *et al.*, 1998; Navarro *et al.*, 2004; Day *et al.*, 2001, 2005; Rotllant *et al.*, 2007, 2010), including cocaine (Kuzmin and Johansson, 1999). This induction can have a clear functional significance. For example, lesions of the CeA reduced the induction of c-fos in the PVN and SON, among other areas,

after administration of IL-1ß (Xu *et al.*, 1999), while lesions of the MeA decreased the expression of c-fos in response to immobilization in tube (Dayas *et al.*, 1999). All these data support the hypothesis that the CeA would be mainly involved in the control of physiological and behavioral response to stressful systemic stimuli, while the MeA would be involved preferably in emotional responses (Armario, 2006).

As in the Ryabinin *et al.* study (2000), cocaine administration was capable of inducing c-fos expression in the PVN, contrary to others studies (Chang *et al.*, 1995; Torres and Rivier, 1994). All studies were conducted with distinct variables (such as rat strains, cocaine dose, injection volume, measurement hour, gene/protein measurement) as a consequence of different methodologies. Also, as demonstrated by Regier *et al.* (2012), individual differences in personality, such as impulsivity or reward-seeking also determine c-fos brain activation induced by cocaine. As mentioned in the Introduction, several variables may be of critical importance in determining the effects of cocaine on HPA axis activity, including the induction or not of c-fos expression in the PVN. Since several results support the hypothesis that the activation of the HPA axis by cocaine is mediated through the release of endogenous CRF (Sarnayai *et al.*, 1992, 1993; Rivier and Lee, 1994; Gardi *et al.*, 1997), it is important to clarify if this release in mediated by c-fos or other unclear mechanisms.

Interaction between IMO and cocaine

The simultaneous exposure to IMO and cocaine additive effects induced in a vast number of areas (see Table 2), especially at 1 h post-IMO (Cg1, Orb, SN, VTA,

posterior striatum (CpuP),CeA, MeA, PAGa, PAGp, LC) what may suggest that at least partially different populations of neurons are activated. In an important number of other brain areas the simultaneous exposure to both stimuli do not induce a different expression than both individual stimuli or induces an expression equal to the expression induced by the stimulus that produces the greatest activation. In these latter cases there are several explanations related to the subpopulations of neurons activated, including the existence of a ceiling effect that makes difficult the study of positive synergies (potentiation). However, in the present study we have obtained clear negative synergies at the Cg1 (where IMO is inhibiting the activation induced by cocaine, being IMO by itself ineffective) and at the ACb, BNST and DR (where cocaine is inhibiting the activation induced by IMO, being cocaine by itself ineffective).

As mentioned, when animals are exposed simultaneously to drugs and stress several possibilities emerge: an additive effect, a potentiation (positive synergy) or a negative synergy. Previous data using c-fos mRNA indicate additive effects or positive synergies between amphetamine and novelty stress in some brain areas (Cpu, ACb core, mPFC, MeA, basolateral amygdala) (Badiani *et al.*, 1998; Day *et al.,* 2001, 2005, 2008; Uslaner *et al.*, 2001). However, amphetamine and several types of stressors have negative synergistic effects in other brain areas, such as CeA or a subregion of the BNST (oval region), as indicated in several works (Day *et al.*, 2001, 2005, 2008). A similar negative synergy has been observed between cocaine and social stress in PAG, DR and LC (Nikulina *et al.*, 1998). Other studies in our laboratory (Gómez-Román, 2012) have also shown negative synergies between amphetamine and stress in c-fos expression in other brain areas, such as PVN, LS and PrL. This effect in the PVN is especially important because amphetamine strongly inhibited the peripheral HPA axis response to different stressors. However, Gagliano (2016) using a different psychostimulant (methylphenidate) only has

detected a positive synergy (and not negative) between drugs and stress (swim), using c-fos expression, in one brain area, the Cpu. Regarding the lack of negative synergy in c-fos expression at the PVN obtained in Gagliano (2016), when animals are exposed simultaneously to stress (swim) and methylphenidate, and another marker was used (hnRNA-CRF), the inhibition of the stress response induced by methyphenidate was very evident, in agreement with the effects in the peripheral HPA axis induced by that drug. All these data together clearly indicate that negative/positive synergies between psychostimulants and stressors are dependent of several still unknown factors (type of drug, dose, type of stressor, type of marker used to address brain activation).

In the present study, at the end of IMO, stress seems to produce a negative synergy in Cg1 by blocking c-fos expression from some of the neuronal populations activated by cocaine. The c-fos expression for both stimuli together is similar to the expression promoted by IMO alone. Cg1 is an area neuroanatomically connected with limbic structures and is one of the frontal cortical areas most frequently implicated in drug addiction (Goldstein and Volkow, 2002). Cg 1 is also involved in motivational and higher order cognitive functions that are changed in both addicted and PTSD patients (Goldstein and Volkow, 2002; Meng *et al.*, 2014). However, there are no previous data similar to ours, and how stress is blocking cocaine response in this area remains to be studied.

In other regions (Acb, BNST and DR) a different type of negative synergy was observed, cocaine reducing c-fos expression caused by stress. The negative synergy observed in this study between cocaine and IMO in the BNST is in agreement with previous results of Day *et al.* (2001, 2005, 2008), and the observed in the DR is in agreement with Nikulina *et al.* (1998) study. However, regarding Acb, previous data

suggested the existence of positive synergies or additive effects between drugs and stress (Badiani *et al.*, 1998; Uslaner *et al.*, 2001; Ostrander *et al.*, 2003).

As expected, cocaine and IMO increased the number of neurons expressing c-fos in the Abc. The role of Acb in the rewarding effects of drugs of abuse has been extensively studied and it is also a region with the capacity to mediate a diverse range of stress responses by interfacing limbic, cognitive and motor circuitry (Nestler *et al.*, 2002; Lemos *et al.*, 2012). This capacity to mediate stress responses makes the Acb part of the neurobiological substrate of depression and associated pathology (Nestler *et al.*, 2002). Surprisingly, the simultaneous exposure to both stimuli induces a negative synergy that may be related with some of the effects observed in the chapter one, where cocaine reduced some of the negative effects of stress.

In the present study, in animals exposed to cocaine there is no a significant increase in c-fos expression in the BNST but in this area cocaine inhibited certain neuronal populations expressing c-fos in response to IMO. In general, the BNST has been shown to be important for autonomic, neuroendocrine and somatomotor responses during emotional behaviors (Casada and Dafny, 1991; Gray *et al.*, 1993; Dunn and Williams, 1995; Herman and Cullinan, 1997). The BNST has been more involved in anxiety, whereas the CeA may be more important for fear responses (reviewed in Davis, 1998; Davis and Shi, 1999). An important question is to understand which neuronal populations are inhibited by cocaine. Since most BNST cells are GABAergic and they are expected to inhibit target neurons, the hypothesis that cocaine is inhibiting some of the negative effects of stress thru the BNST GABAergig projections deserves to be studied.

As with Acb and BNST, in the DR cocaine reduced the expression of c-fos in response to IMO. The DR is a serotonin-rich nucleus that widely innervates the

forebrain, and increased DR serotonin transmission has traditionally been associated with increased anxiety-like behavior (Gingrich and Hen, 2001). It is well known that cocaine depressed spontaneous cell firing of 5-HT-containing neurons and potentiated the inhibitory effects of 5-HT in the DR. The depressant effects on 5-HT neurons following systemic application of cocaine may result from autoinhibition of 5-HT neurons, presumably as a consequence of cocaine-induced 5-HT reuptake inhibition (Cunningham and Lakoski, 1988). The inhibitory effects of cocaine in the DR are not due to direct effects on the presynaptic autoreceptor but are indirectly produced due to cocaine's inhibition of the 5-HT reuptake system (Black and Lakoski, 1990; Cunningham and Lakoski, 1990). Serotonergic DR neurons may normally mediate a tonic inhibitory effect on cocaine-induced behavior (Herges and Taylor, 1999). The DR is involved in the acute effects of cocaine (motor activity, extracellular levels of dopamine or glutamate), neuroadaptations in the median DR may regulate the long-term consequences of repeated cocaine exposure (Szumlinski *et al.*, 2004). The stress-related and anxiogenic neuropeptide CRF can stimulate the in vitro neuronal firing rates of topographically organized subpopulations of serotonergic neurons within the DR. These findings are consistent with behavioral studies suggesting that serotonergic systems within the DR are involved in the modulation of ongoing anxiety-related behavior and in behavioral sensitization, a process whereby anxiety- and fear-related behavioral responses are sensitized (Abrams *et al.*, 2004). In addition, CRF-related deficits in serotonin activity produced by acute stress may promote the impulsive behavior involved in the initiation of substance abuse (Valentino *et al.*, 2011). Taking into count all the studies it seems that DR is an important brain region to study cocaine addiction and stress related deficits and their interaction.

As cocaine administration decreased ACTH levels induced by IMO we should expect a negative synergy at the PVN. However, in response to simultaneous exposure to

cocaine and IMO, the pattern of activation of PVN basically resembles that of IMO alone rather than the cocaine alone. The group exposed to both stimuli was only statistically different (higher) from the group only injected with cocaine. As mentioned, in previous data from our laboratory (Gómez-Román *et al.*, 2015) a negative synergy regarding c-fos expression between amphetamine and swim stress has been observed. However, Gagliano (2016) using another psychostimulant (methyphenidate) did not find evidences of negative synergies at the PVN using c-fos as the marker (although a negative synergy was detected at the peripheral HPA axis). In the same study of Gagliano (2016) methylphenidate administration was able to decrease CRF expression in the PVN after a swim stress, indicating that future studies are needed with other markers after cocaine administration.

CHAPTER III

Study of the gene expression related to the HPA axis and dopaminergic system after exposure to cocaine and/or stress (IMO).

1. Introduction

In the previous chapter, the study of the c-fos expression showed the interaction between stress and cocaine in some brain areas, and to further characterize these interactions in this chapter we will study mRNA expression of key molecules after both treatments. The objective of this work is to characterize the neurobiological substrate of the interaction between cocaine and stress responsible for the changes in HPA axis hormones levels and dynamics observed in the previous studies. For that, several mRNA transcripts of hormones and peptides that are involved in the response to stress and/or cocaine will be studied in key areas of the CNS by real-time polymerase chain reaction (RT-PCR). Through the study of some genes associated with the activation of the HPA axis (CRH, CRH-R1, POMC, MR, GR) and with the dopaminergic system (TH, MAO-A and D1), is expected to further characterize the interaction between cocaine and stress.

Corticotropin-releasing Hormone (CRH), a 41-amino acid peptide that is mainly

produced in the hypothalamic paraventricular nucleus (PVN), plays a crucial role in stress response and is considered as the central driving force in the activity of hypothalamic-pituitary-adrenal (HPA) axis (Swaab, 2004). CRH is also considered as the central driving force in the stress response and plays a key role in the pathogenesis of depression. In addition to the hypothalamus, CRH neurons are also located in most regions of the prefrontal cortex (PFC) (Swanson *et al.*, 1983), a brain region that is highly associated with the control of emotion and cognition (Miller, 1999). These regions are part of the circuitry involved in modulating ACTH-corticosterone mediated responsivity to stress (Diorio *et al.*, 1993). CRH, produced in the hypothalamic PVN in response to stress, stimulates the synthesis and secretion of ACTH via CRH-R1 in the anterior pituitary of mammals (Kageyama and Suda, 2009; Aguilera *et al.*, 2004, Nikodemova *et al.*, 2002). CRH is implicated not only in the pathophysiology of affective and anxiety disorders but also in aversive states associated with drug withdrawal (Heinrichs *et al.*, 1995; Sarnyai *et al.*, 1995). Although the mechanisms and site at which cocaine exerts its influence on the HPA axis is presently poorly known, CRH immunoneutralization studies suggest that this effect of cocaine is dependent on endogenous CRH release into the pituitary portal blood (Rivier and Vale, 1987; Sarnyai *et al.*, 1995). In addition, a large part of literature also indicates CRH gene expression is regulated in a complex manner by glucocorticoids (Schulkin *et al.*, 1998, 2005). Briefly, it is well known that glucocorticoids negatively regulate the expression of CRH gene in PVN (Keller-Wood and Dallman, 1984; Swanson and Simmons, 1989).

Glucocorticoids exert their action on the brain through two types of central corticosteroid receptors. The mineralocorticoid receptor (MR or type I) is mainly located in limbic structures and shows high affinity for corticosterone (Reul and De Kloet, 1985). The glucocorticoid receptor (or type II) has a more widespread distribution in the brain and has a lower affinity for corticosterone (Reul and De Kloet,

1985). MRs are believed to mediate tonic basal actions of glucocorticoids, whereas GRs appear to mediate phasic responses such as those to stress (McEwen *et al.*, 1986; De Kloet and Reul, 1987). Previous findings suggest both receptors have been implicated in the fear-enhancing effects of glucocorticoids (Oitzl *et al.*, 2001). During stress, MR in coordination with other signals determines the defense against the stressor, hereas GR assists with the recovery and processing of stressful information and the storage of the experience in the memory (Licznerski and Duman, 2013, De Kloet, 2009).

MAO A and B are outer membrane mitochondrial enzymes responsible for the metabolic degradation of biogenic amines in humans (Shih, 1991; Thorpe *et al.*, 1987). MAO-A prefers serotonin (5-HT) and noradrenaline (NA) as substrates, whereas MAO-B prefers phenylethylamine and benzylamine (Shih, 1991; Shih *et al.*, 1999). MAO-A mRNA was also found in forebrain structures, such as the cortex, the hippocampus, the thalamus, and the hypothalamus. The in situ visualization of MAO mRNA demonstrates that MAO-A mRNA synthesis is wide spread in many catecholaminergic and serotonergic cell groups (Jahng *et al.*, 1997). The product of the MAO-A gene is an enzyme that regulates the metabolism of monoamine neurotransmitters, thereby modulating brain function and structure (Fowler *et al.*, 1987; Shih and Thompson, 1999

High numbers of D1 receptors are located within Caudate Putamen (CPu), Nucleus Accumbens (Acb), and Substantia Nigra pars reticulata with a less dense distribution in the amygaloid regions (Savasta *et al.*, 1986). Evidence suggests D1 receptors in the CPu, NAc, and SN facilitate motivated behavior (Norwend *et al.*, 2001; Trevitt *et al.*, 2001), while those in the amygdala are more involved with learning, memory and fear (Macedo *et al.*, 2007; Guarracci *et al.*, 1999). The D1 family is predominately

located within the basolateral amygdala, whereas the central amigdala predominately contains the D2 family (Scibilia *et al.*, 1992). D1 within the amygdala, particularly the basolateral amygdala, are likely involved in modulating motivation for cocaine (Thiel *et al.*, 2010). Other study showed that after two-week cocaine withdrawal, expression of the D1 receptor but not the D2, D3, D4 or D5 was significantly increased (Krishnan *et al.*, 2010). Chocyk and colleagues (2008) study provided evidences for the functional role of dopamine D1 in the PVN and indicate a functional adaptation of dopamine D1-like receptors following a single dose of cocaine without further progression of adaptation or resistance of D1 receptor-mediated genomic function in the course of repeated cocaine intake

2. Experimental protocol

This experiment was conducted using 48 male Wistar rats, obtained from the breeding centre of the Institute for Molecular and Cell Biology (University of Oporto). In the experimental day, animals were treated according with their group. After the treatment, the animals returned and were maintained undisturbed in the vivarium. At the right time animals were sacrificed by decapitation and their brain areas dissected using clean surgery material (cleaned with alcohol 70%). Half of the animals were sacrificed and their brain dissected 4 hours after the end of the IMO and the other half 24 hours after (Figure 23). The stereotactic atlas of Paxinos and Watson (1998) was used to define the areas coordinates. The tissue was kept at -80 ºC until extraction of DNA.

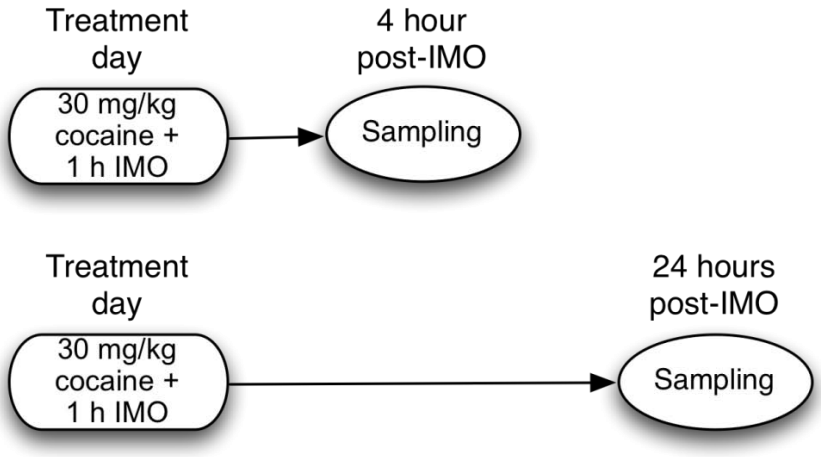


Figure 23. Summary of the experiment taking CocIMO group as example. This experiment was aimed to evaluate the gene expression related to the HPA axis and dopaminergic system after exposure to cocaine and/or stress (IMO).

As previously stated before in the Methodology, RNA was extracted with the kit "RNeasy lipid tissue mini kit" from Quiagen and for the processing of RNA into cDNA was used the "superscript first-strand synthesis system" kit for RT-PCR. The mRNA transcripts related with the dopaminergic system measured were tyrosine hydroxylase (TH), monoamine oxidase-A (MOA-A) and DA receptor 1 (D1). Additionally, the expression levels of mRNA transcripts related with the HPA axis measured were corticotropin-releasing hormone (CRH), CRH receptor 1 (CRH-R1), proopiomelanocortin (POMC), glucocorticoid receptor (GR), mineralocorticoid receptor (MR). Furthermore we also analyzed the mRNA transcripts of Vasopressin (AVP), Oxytocin (OT) and Orexin (ORX), other important Hypothalamic neurotransmitters. The reference gene, glyceraldahyde-3-phosphate-dehydrogenase (GAPDH) was used as internal standard for normalization. Primer sequences and annealing temperatures (Ta) for each gene are presented in Table 3.

Table 3. Primer sets and their sequences, PCR segment size and Ta used in the qRT-PCR to study the expression of dopaminergic-related mRNAs, HPA axis-related mRNAs and other Hypothalamic Neuropeptides

	Gene	Primer sequence	Size (bp)	Tm (°C)	Ta (°C)
STD	**GAPDH**	Forward: 5'-ttc aac ggc aca gtc aag g-3' Reverse; 5'-ctc agc acc agc ate acc-3'	114	75.9	55
Dopaminergic System	**TH**	Forward: 5'-ggc ttc tct gac cag gtg tat c-3' Reverse: 5'-caa tct ctt ccg ctg tgt att cc-3'	112	75.5	55
	MAO-A	Forward: 5-ggc aca gag aca gea aca c-3' Reverse: 5'-cag acc agg cao gga agg-3'	204	77.5	59
	D1	Forward: 5'-act ctg tct gtc ctt ata tcc ttc-3' Reverse: 5'-gtt gte ato etc ggt gte c-3'	114	75.2	55
HPA Axis	**CRH**	Forward: 5'-gga gaa gag aaa gga gaa gag g-3' Reverse: 5'-aga atc ggc tga ggt tgc tg-3'	283	83.3	62
	CRH-R1	Forward: 5'-ctt ctt ctg gat gtt cgg tga g-3' Reverse: 5'-atg agg atg cgg aca atg ttg-3'	279	78.9	59
	POMC	Forward: 5'-gaa gcg gcg ccc tgt gaa-3' Reverse: 5-cte gcc tic cag cte cct ctt-3'	94	78.5	59
	MR	Forward: 5-cta dg tcc tag cat ggt tcg-3 Reverse: 5'-gga agg tca cag gte att gg-3'	231	82.7	59
	GR	Forward: 5'-gga cag cct gac tic ctt gg-3' Reverse: 5'-tcc agg gct tga gta ccc at-3'	76	74.2	55
Hypothalamic Neuropeptides	**AVP**	Forward: 5'-ctc tct gct tgc ttc ctg ag-3' Reverse: 5'-act gtc tca gct cca tgt cg-3'	105	85.0	65
	OT	Forward: 5'-tgc cag gag gag aac tac-3' Reverse: 5'-ccc taa agg tat cat cac aaa g-3'	183	83.8	59
	ORX	Forward: 5'-cgc aga gct aga gcc ata tc-3' Reverse: 5'-cgg ata gaa gac ggg ttc ag -3'	106	83.9	62

Abbreviations: GAPDH: Glyceraldahyde-3-phosphate-dehydrogenase; TH: Tyrosine hydroxilase; MAO-A: Monoamine oxidase A; D1: dopamine receptor 1; CRH: Corticotrophin-releasing hormone; CRH-R1: Corticotrophin-releasing hormone receptor 1; POMC: Proopiomelanocortin; MR: Mineracorticoid receptor; GR: Glucocorticoid receptor; OT: Oxytocin; AVP: Vasopressin; ORX: Orexin; STD: internal standard for normalization; Ta: anneling temperature; Tm: meeting temperature. Ta: anneling temperature; Tm: melting temperature.

The expression levels of mRNA transcripts related with stress and cocaine were measured in amygdala (Amg), dorsal striatum (CPu), hippocampus (Hipp), hypothalamus (Hyp), nucleus accumbens (Acb), pituitary gland (Pit), prefrontal cortex (PFC) and substancia nigra-ventral tegmental area (SN-VTA). The combination of the gene-area-time analysis was chosen according to the expression pattern of each gene (see Table 4).

Table 4. Combination of the gene-area-time analysis according to the expression pattern of the different genes

	Brain Areas	Amg	CPu	Hipp	Hyp	Acb	PFC	SN-VTA	Pit
Dopaminergic System	TH	4h-24h			24h	24h	24h		
	MAO-A	4h-24h	4h-24h	4h-24h		4h-24h	4h-24h	4h-24h	
	D1	4h-24h	4h-24h	4h-24h		4h-24h	4h-24h	4h-24h	
HPA Axis	CRH	4h-24h		4h-24h	4h-24h		4h-24h		
	CRH-R1				4h-24h				4h-24h
	POMC				4h-24h				4h-24h
	GR	4h-24h	4h-24h	4h-24h	4h-24h	4h-24h	4h-24h	4h-24h	4h-24h
	MR				4h-24h				4h-24h

Hypothalamic Neuropeptides	AVP				4h-24h				
	OT				4h-24h				
	ORX				4h-24h				

Abbreviations: CRH: Corticotrophin-releasing hormone; CRH-R1: Corticotrophin-releasing hormone receptor 1; POMC: Proopiomelanocortin; MR: Mineracorticoid receptor; GR: Glucocorticoid receptor; OT: Oxytocin; AVP: Vasopressin; ORX: Orexin; TH: Tyrosine hydroxilase; MAO-A: Monoamine oxidase A; D1: dopamine receptor 1; Amg: amygdala; CPu: dorsal striatum; Hipp: hippocampus; Hyp: hypothalamus; Acb: nucleus accumbens; Pit: pituitary gland; PFC: prefrontal cortex; and SN-VTA: substancia nigra-ventral tegmental area.

3. Results

The main statistical results are shown in Table 5 and Figure 24. Regarding dopaminergic-related mRNAs transcripts expression the statistical analysis showed overall effect of cocaine by increasing the MAO-A expression on Hipp at R-4h, and on SN-VTA at R-24h, regardless IMO exposure. Cocaine also showed a significant effect, decreasing D1 gene expression on SN-VTA at R-4h and on CPu at R-24h. Regardless of cocaine exposure, stress exposure increased MAO-A expression on CPu at R-4h and decreased on Amg at 24h. IMO also showed a significant effect increasing D1 expression on Amg, CPu and Hipp at R-4h and on Hipp at R-24h. There was a significant increase in TH expression promoted by IMO exposure only on Amg at R-4h. In the PFC, IMO promoted a reduction of D1 expression at R-24h and a small tendency on PFC at R-24h. Finally, statistical analysis showed an interaction between the effects of IMO and Cocaine in the MAO-A expression on Acb and SN-VTA at R-4h (see Figure 24). In the Acb the the exposition to both

treatments in simultaneously produced a sum effect int MAO-A expression. In SN-VTA only cocaine reduced the MAO-A expression but when both treatments were presented IMO increased MAOA to normal levels.

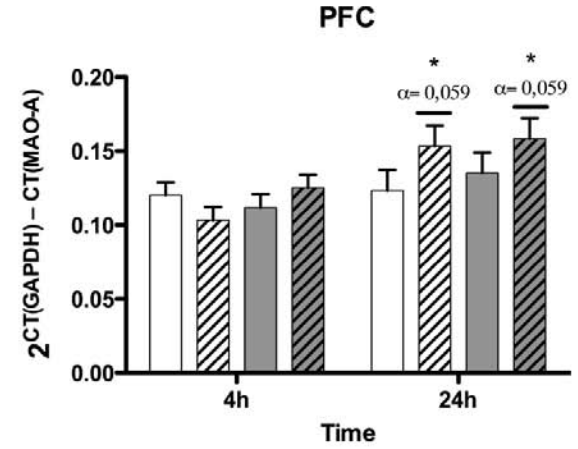
It was also found an interaction between effects in the D1 expression on Acb were a sum of the expression promoted by both stimuli individually were observed.

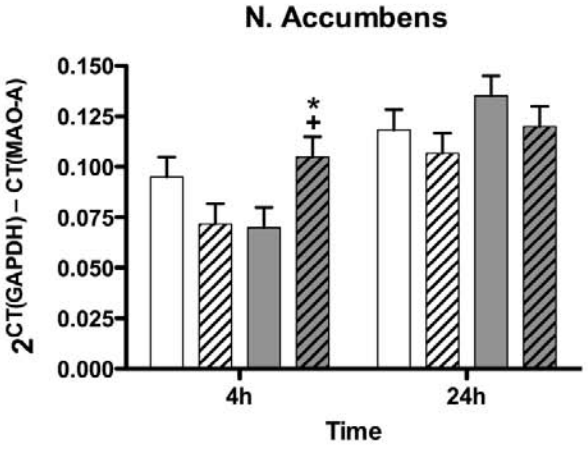
Table 5. Results of Generalized linear models for dopaminergic-related mRNAs expression

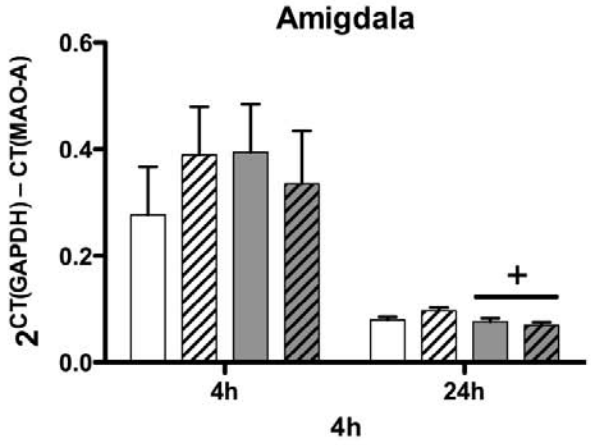
Genes	Areas	Time	df	Cocaine		IMO		Cocaine*IMO	
				Wald	Sig	Wald	Sig	Wald	Sig
MAO-A	PFC	R - 4h	1,20	0,031	N.S.	0,501	N.S.	2,538	N.S.
		R - 24h	1,20	3,568	**0,059 (N.S.)**	0,348	N.S.	0,056	N.S.
	Acb	R - 4h	1,20	0,285	N.S.	0,145	N.S.	7,129	**0,008****
		R - 24h	1,20	0,403	N.S.	1,356	N.S.	0,011	N.S.
	Amg	R - 4h	1,18	0,073	N.S.	0,092	N.S.	0,737	N.S.
		R - 24h	1,20	0,557	N.S.	5,010	**0,025***	3,031	N.S.
	Hipp	R - 4h	1,18	12,796	**0,000*****	1,553	N.S.	0,195	N.S.
		R - 24h	1,19	0,446	N.S.	0,082	N.S.	0,002	N.S.
	CPu	R - 4h	1,17	0,229	N.S.	4,723	**0,030***	2,997	N.S.
		R - 24h	1,19	3,773	**0,052 (N.S.)**	2,526	N.S.	1,788	N.S.
	SN-VTA	R - 4h	1,19	1,839	N.S.	3,363	N.S.	4,738	**0,030***
		R - 24h	1,19	10,120	**0,001*****	0,405	N.S.	0,065	N.S.
D1	PFC	R - 4h	1,19	0,252	N.S.	3,086	N.S.	0,007	N.S.
		R - 24h	1,19	0,370	N.S.	7,682	**0,006****	3,555	**0,059 (N.S.)**
	Acb	R - 4h	1,19	0,003	N.S.	0,040	N.S.	0,286	N.S.
		R - 24h	1,20	0,345	N.S.	0,450	N.S.	5,488	**0,019***
	Amg	R - 4h	1,19	0,035	N.S.	7,074	**0,008****	0,465	N.S.
		R - 24h	1,20	0,000	N.S.	1,716	N.S.	0,042	N.S.
	Hipp	R - 4h	1,19	2,675	N.S.	15,999	**0,000*****	0,858	N.S.
		R - 24h	1,17	3,147	N.S.	4,701	**0,030***	0,350	N.S.
	CPu	R - 4h	1,19	0,088	N.S.	6,470	**0,011***	0,005	N.S.
		R - 24h	1,19	10,986	**0,001*****	1,724	N.S.	0,449	N.S.
	SN-VTA	R - 4h	1,16	6,070	**0,014***	1,308	N.S.	1,038	N.S.
		R - 24h	1,19	0,056	N.S.	0,056	N.S.	0,249	N.S.
TH	PFC	R - 24h	1,18	2,667	N.S.	0,001	N.S.	0,360	N.S.
	Acb	R - 24h	1,20	0,911	N.S.	0,650	N.S.	2,305	N.S.
	Amg	R - 4h	1,18	0,421	N.S.	11,118	**0,001*****	0,147	N.S.
		R - 24h	1,17	0,496	N.S.	2,034	N.S.	1,623	N.S.
	Hyp	R - 24h	1,18	0,727	N.S.	1,519	N.S.	0,247	N.S.

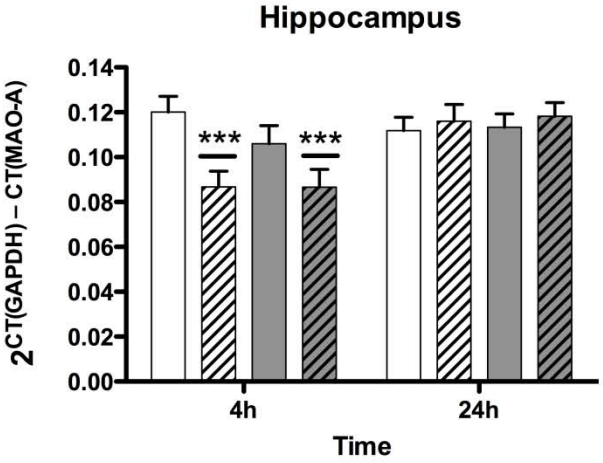
Abbreviations: MAO-A: Monoamine oxidase A; D1: dopamine receptor 1; PFC: prefrontal cortex; Acb: nucleus accumbens; Amg: amygdala; Hipp: hippocampus; CPu: dorsal striatum; and SN-VTA: substancia nigra-ventral tegmental area.

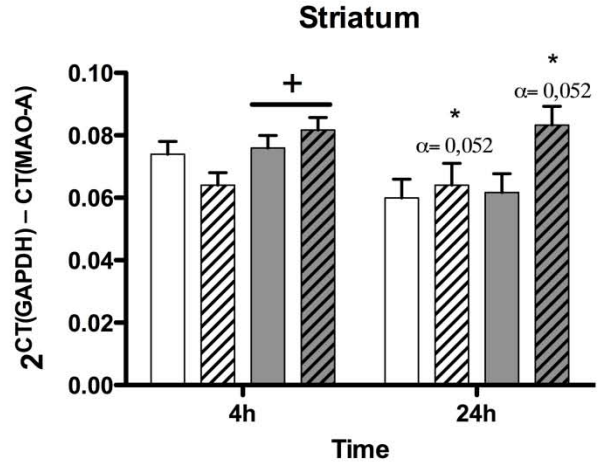
MAO-A

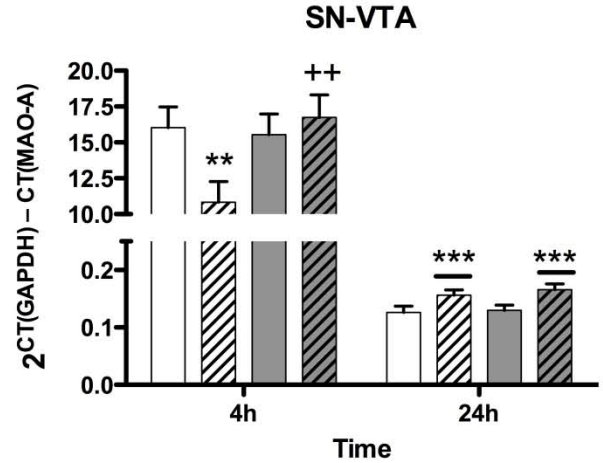




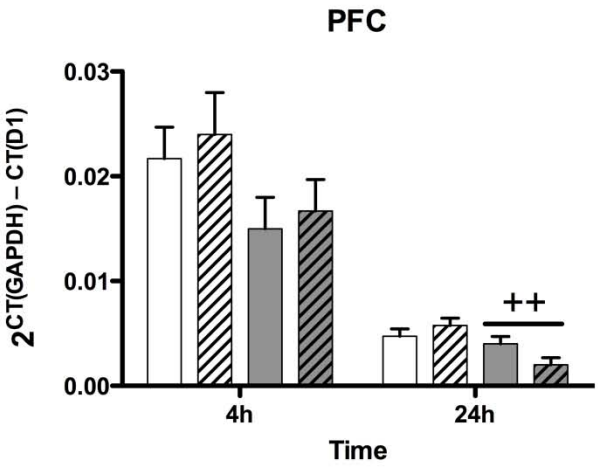


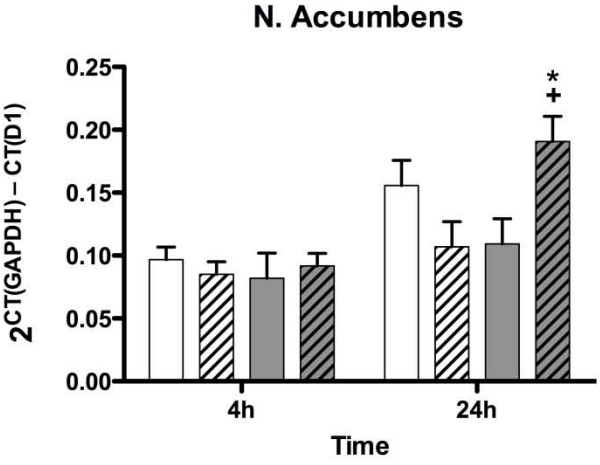

Hippocampus
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
2CT(GAPDH) – CT(MAO-A)
***
***
4h
24h
Time

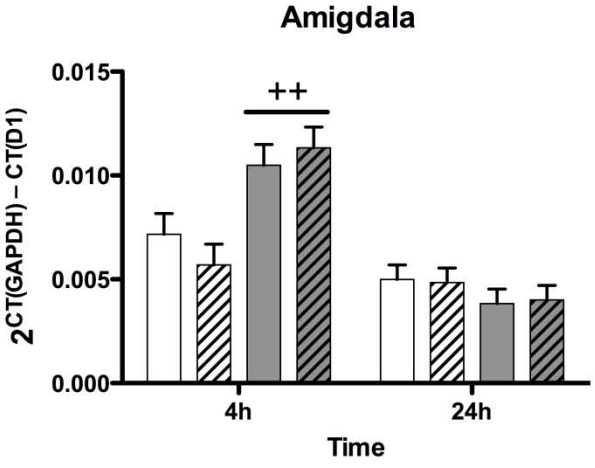

Striatum
0.10
0.08
0.06
0.04
0.02
0.00
2CT(GAPDH) – CT(MAO-A)
+
*
α= 0,052
*
α= 0,052
4h
24h
Time

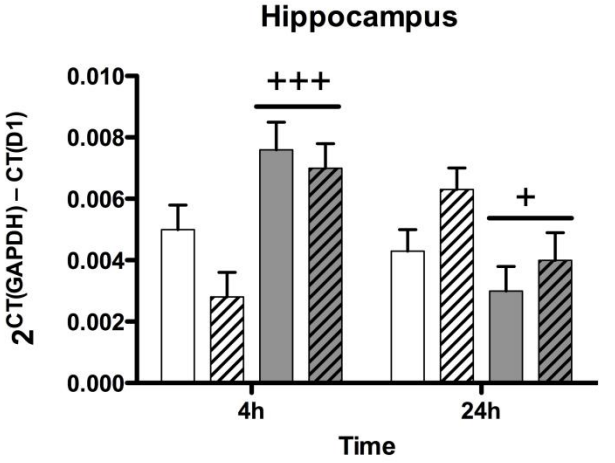

SN-VTA
20.0
17.5
15.0
12.5
10.0
0.2
0.1
0.0
2CT(GAPDH) – CT(MAO-A)
**
++
***
***
4h
24h
Time

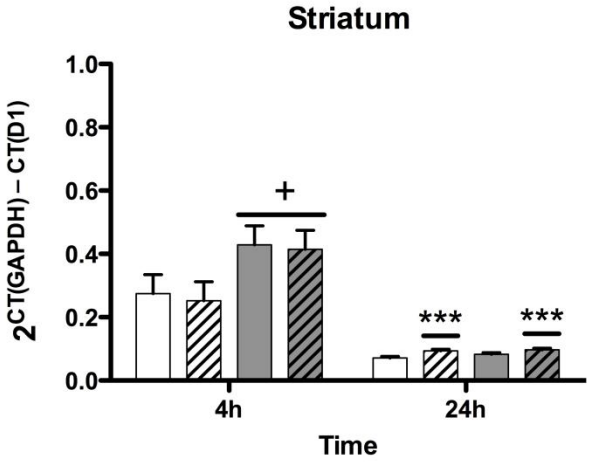
D1

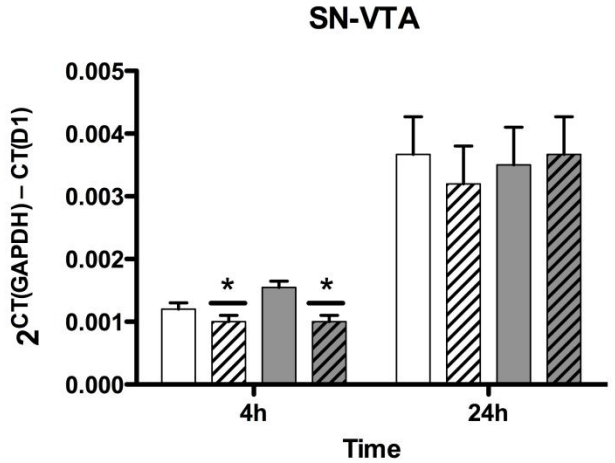

PFC
0.03
0.02
0.01
0.00
2CT(GAPDH) – CT(D1)
++
4h
24h
Time


N. Accumbens
0.25
0.20
0.15
0.10
0.05
0.00
2CT(GAPDH) – CT(D1)
*
+
4h
24h
Time


Amigdala
0.015
0.010
0.005
0.000
2CT(GAPDH) – CT(D1)
++
4h
24h
Time


Hippocampus
0.010
0.008
0.006
0.004
0.002
0.000
2CT(GAPDH) – CT(D1)
+++
+
4h
24h
Time


Striatum
1.0
0.8
0.6
0.4
0.2
0.0
2CT(GAPDH) – CT(D1)
+
***
***
4h
24h
Time


SN-VTA
0.005
0.004
0.003
0.002
0.001
0.000
2CT(GAPDH) – CT(D1)
*
*
4h
24h
Time

TH

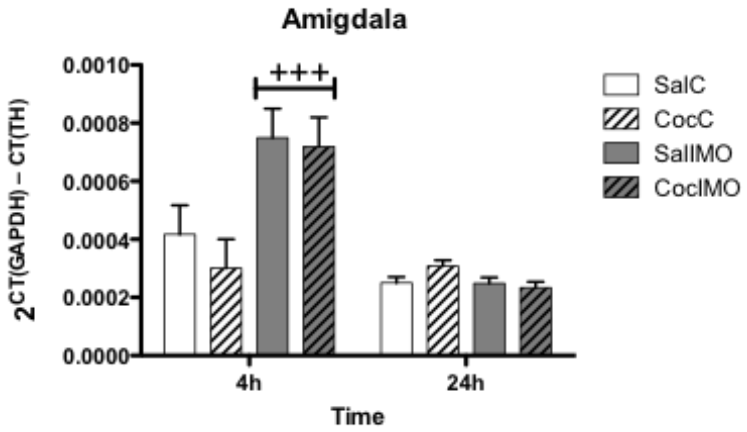


Figure 24. Effects of cocaine administration and IMO on MAO-A, D1 and TH mRNA levels. The data are represented as mean ± SEM of $2^{CT(GAPDH)-CT(target\ gene)}$. The white columns represent the non-stressed groups, the grey columns the IMO groups, and the stripped columns the cocaine groups. Samples were taken 4 hours after the end of IMO (R-4h) and 24 hours after IMO (R-24h). These time points correspond to 5 and 25 hours after cocaine. **: p<0.01; ***: p<0.001 overall effect of cocaine. $^{+}$: p<0.05; $^{++}$: p<0.01; $^{+++}$: p<0.001 overall effect of stress (G_ZLM).

Regarding the statistical analysis of the HPA axis-related mRNAs expression, an overall effect of cocaine was observed, increasing CRH expression on PFC at R-4h. Cocaine also increased MR expression on Pit at R-24h. Regardless cocaine exposure, there was a significant effect of IMO increasing CRH expression on Hyp at R-4h and decreasing on Amg at R-24h. IMO also produced a significant increase in CRH-R1 expression in Pit at R-24h and a small reduction on Hyp at R-4h. In the Hyp a significant effect of IMO was showwed, decreasing the MR, GR and AVP expression at R-24h, regardless cocaine exposure. Finally, an interaction Cocaine x IMO was shown regarding CRH expression in AMG at R-4h; POMC expression in Pit at R-4h and Hyp at R24h; in MR expression in Hyp at R-4h; and in ORX expression in Hyp at 4h. It was also found an interaction Cocaine x IMO in the GR expression in PFC, Amg, CPu and Hyp at R-4h and in Acb at R-24h (Table 6).

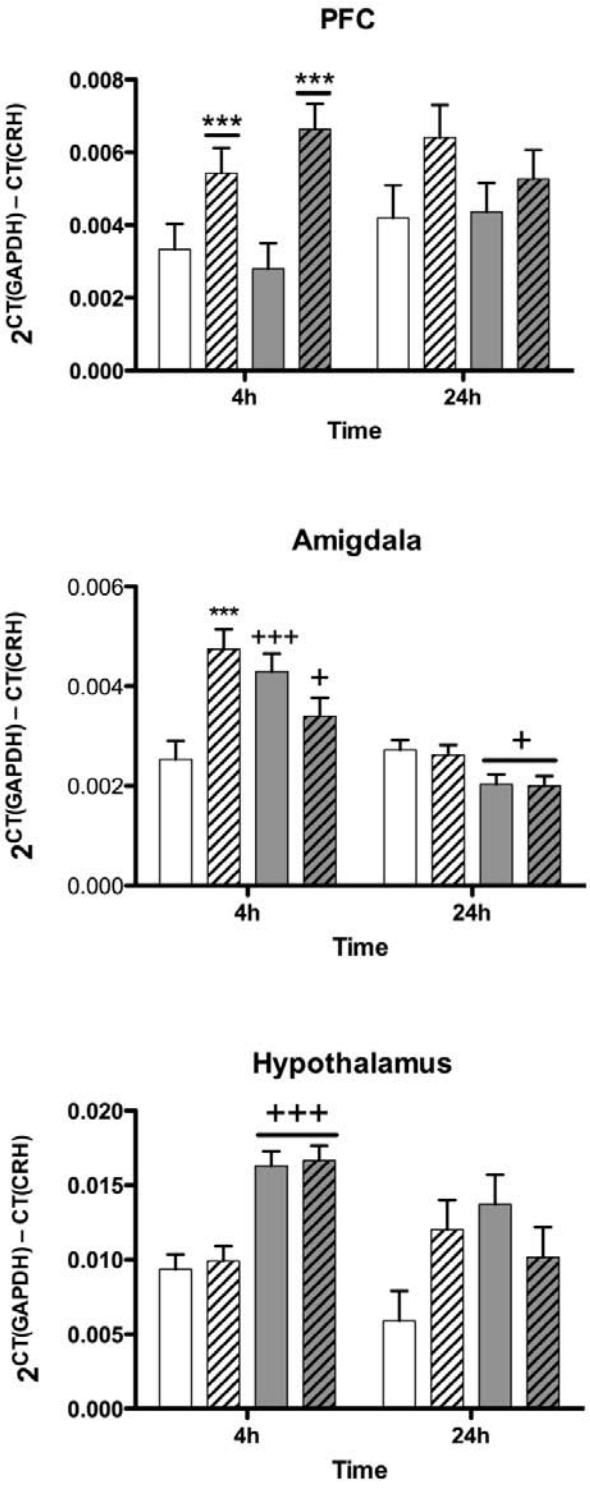
Table 6. Results of Generalized linear models for HPA axis-related mRNAs expression

Genes	Areas	Time	df	Drug		Stress		Drug*Stress	
				Wald	Sig	Wald	Sig	Wald	Sig
CRH	PFC	R - 4h	1,19	17,056	**0,000*** **	0,225	N.S.	1,466	N.S.
		R - 24h	1,18	2,885	N.S.	0,277	N.S.	0,496	N.S.
	Amg	R - 4h	1,19	3,091	N.S.	0,306	N.S.	17,080	**0,000*** **
		R - 24h	1,19	0,052	N.S.	5,479	**0,019***	0,015	N.S.
	Hipp	R - 4h	1,20	2,564	N.S.	0,006	N.S.	0,041	N.S.
		R - 24h	1,18	0,018	N.S.	1,498	N.S.	0,487	N.S.
	Hyp	R - 4h	1,19	0,085	N.S.	18,882	**0,000*** **	0,005	N.S.
		R - 24h	1,18	0,202	N.S.	1,088	N.S.	2,819	N.S.
CRH-R1	Hyp	R - 4h	1,18	0,009	N.S.	3,730	**0,053 (N.S.)**	3,007	N.S.
		R - 24h	1,20	0,350	N.S.	0,301	N.S.	1,579	N.S.
	Pit	R - 4h	1,18	0,014	N.S.	0,387	N.S.	2,669	N.S.
		R - 24h	1,20	3,914	**0,048***	5,719	**0,017***	2,462	N.S.
POMC	Hyp	R - 4h	1,19	1,963	N.S.	0,008	N.S.	2,209	N.S.
		R - 24h	1,19	2,238	N.S.	4,253	**0,039***	6,449	**0,011***
	Pit	R - 4h	1,19	0,271	N.S.	0,419	N.S.	11,304	**0,001*** **
		R - 24h	1,20	0,507	N.S.	1,219	N.S.	0,903	N.S.
MR	Hyp	R - 4h	1,18	3,344	N.S.	2,867	N.S.	13,876	**0,000*** **
		R - 24h	1,18	2,016	N.S.	4,030	**0,045***	1,196	N.S.
	Pit	R - 4h	1,19	0,210	N.S.	0,023	N.S.	0,210	N.S.
		R - 24h	1,18	4,093	**0,043***	3,094	N.S.	2,332	N.S.
GR	PFC	R - 4h	1,17	6,518	N.S.	43,461	**0,000*** **	25,645	**0,000*** **
		R - 24h	1,20	0,001	N.S.	0,766	N.S.	1,416	N.S.
	Acb	R - 4h	1,18	0,635	N.S.	0,439	N.S.	0,074	N.S.
		R - 24h	1,18	0,950	N.S.	0,003	N.S.	6,005	**0,014***
	Amg	R - 4h	1,19	1,281	N.S.	5,070	**0,024***	4,880	**0,027***
		R - 24h	1,20	0,645	N.S.	0,026	N.S.	2,314	N.S.
	Hipp	R - 4h	1,19	0,006	N.S.	0,232	N.S.	0,544	N.S.
		R - 24h	1,17	0,053	N.S.	0,104	N.S.	0,620	N.S.
	CPu	R - 4h	1,19	1,081	N.S.	1,218	N.S.	6,669	**0,010** **
		R - 24h	1,19	0,439	N.S.	0,059	N.S.	0,000	N.S.
	Hyp	R - 4h	1,19	0,167	N.S.	1,783	N.S.	19,046	**0,000*** **
		R - 24h	1,19	0,021	N.S.	7,263	**0,007** **	0,718	N.S.
	Pit	R - 4h	1,18	0,005	N.S.	2,709	N.S.	1,447	N.S.
		R - 24h	1,18	0,239	N.S.	0,649	N.S.	0,413	N.S.
AVP	Hyp	R - 4h	1,18	1,796	N.S.	6,313	**0,012***	0,187	N.S.
		R - 24h	1,18	0,110	N.S.	0,053	N.S.	1,281	N.S.
ORX	Hyp	R - 4h	1,18	0,54	N.S.	0,614	N.S.	4,050	**0,044***
		R - 24h	1,18	2,786	N.S.	0,047	N.S.	2,177	N.S.

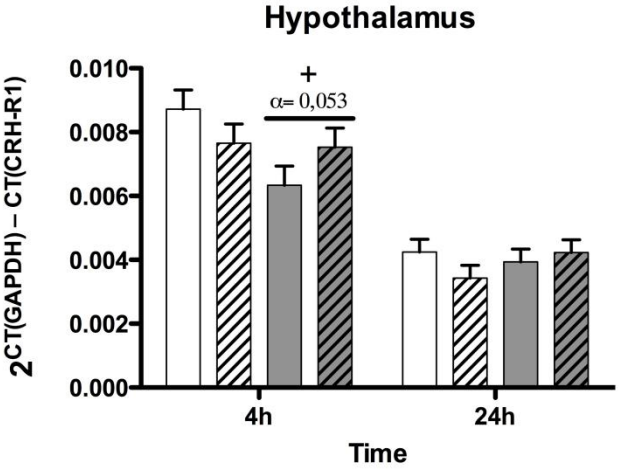
Abbreviations: CRH: Corticotrophin-releasing hormone; CRH-R1: Corticotrophin-releasing hormone receptor 1; POMC: Proopiomelanocortin; MR: Mineracorticoid receptor; GR: Glucocorticoid receptor; AVP: Vasopressin; ORX: Orexin; PFC: prefrontal cortex; Acb: nucleus accumbens; Amg: amygdala; Hipp: hippocampus; CPu: Hyp: hypothalamus; Hyp: hypothalamus; Pit: pituitary gland.

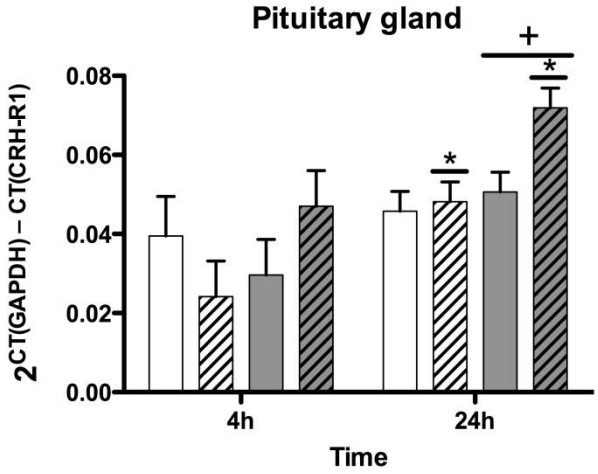
The decomposition of the interactions between drug and stress showed in Table 6 are illustrated in Figure 25.

CRH

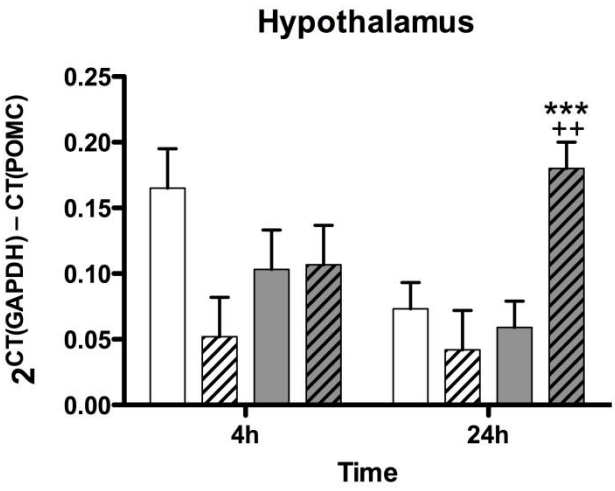


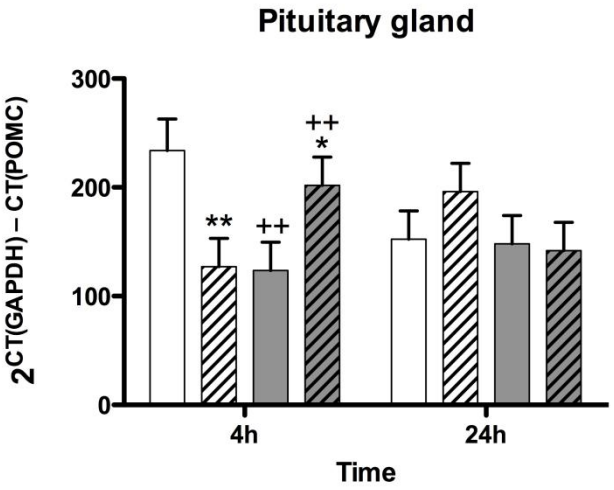
CRH-R1


Hypothalamus
+
α= 0,053
0.010
0.008
0.006
0.004
0.002
0.000
2CT(GAPDH) – CT(CRH-R1)
4h
24h
Time

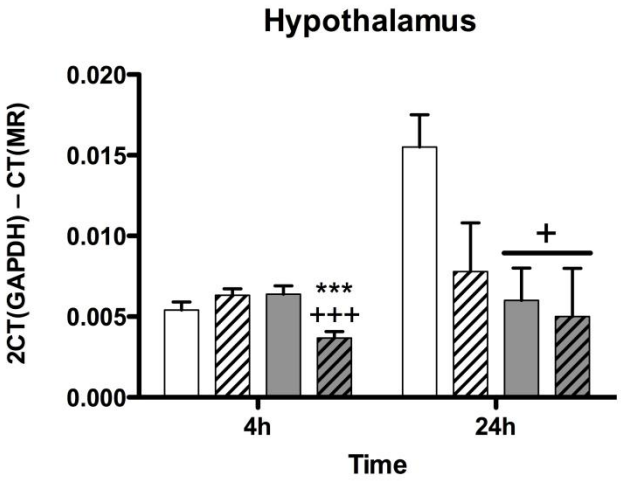

Pituitary gland
+
*
*
0.08
0.06
0.04
0.02
0.00
2CT(GAPDH) – CT(CRH-R1)
4h
24h
Time

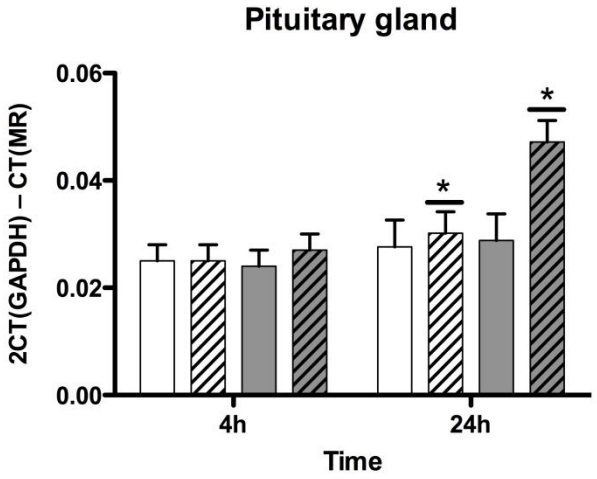
POMC


Hypothalamus
0.25
0.20
0.15
0.10
0.05
0.00
2CT(GAPDH) – CT(POMC)
***
++
4h
24h
Time

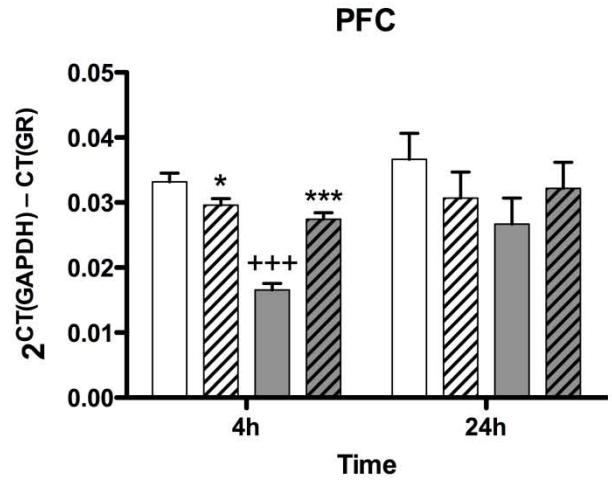

Pituitary gland
300
200
100
0
2CT(GAPDH) – CT(POMC)
**
++
++
*
4h
24h
Time

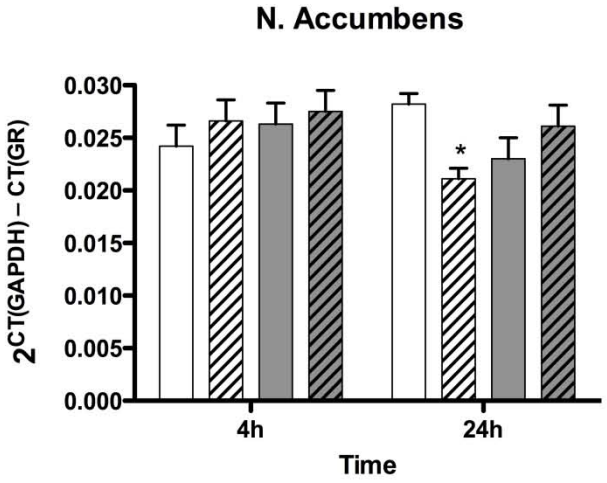
MR

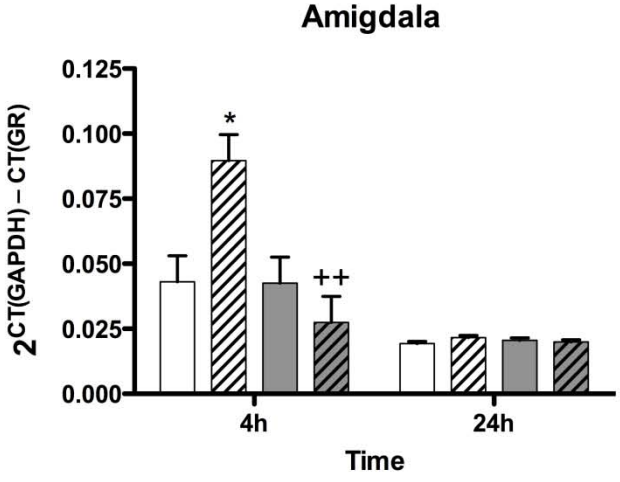

Hypothalamus
0.020
0.015
0.010
0.005
0.000
2CT(GAPDH) – CT(MR)
***
+++
+
4h
24h
Time


Pituitary gland
0.06
0.04
0.02
0.00
2CT(GAPDH) – CT(MR)
*
*
4h
24h
Time

GR


PFC
0.05
0.04
0.03
0.02
0.01
0.00
2CT(GAPDH) – CT(GR)
*
***
+++
4h
24h
Time


N. Accumbens
0.030
0.025
0.020
0.015
0.010
0.005
0.000
2CT(GAPDH) – CT(GR)
*
4h
24h
Time


Amigdala
0.125
0.100
0.075
0.050
0.025
0.000
2CT(GAPDH) – CT(GR)
*
++
4h
24h
Time

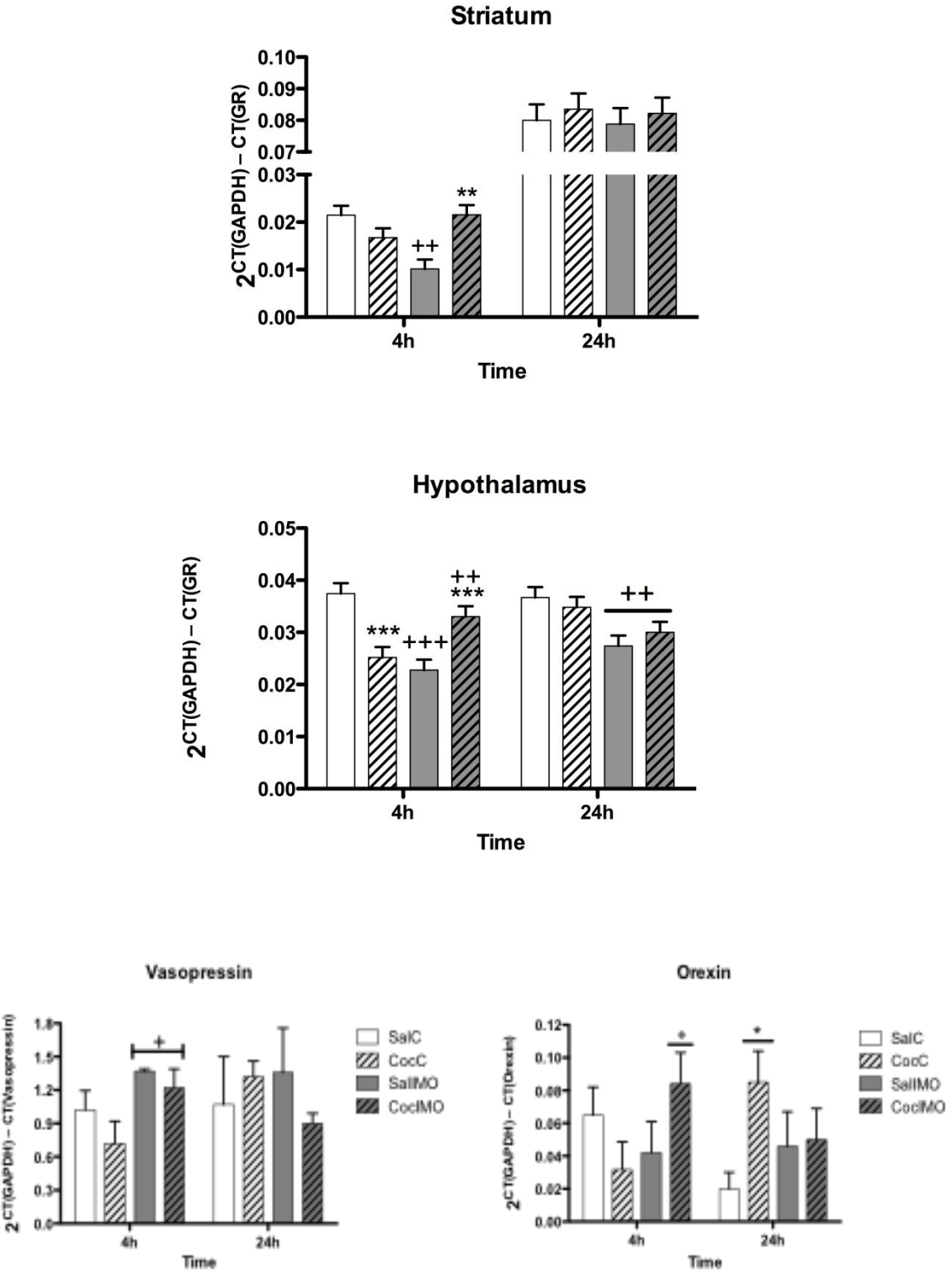


Figure 25. Effects of cocaine administration and IMO on CRH, CRH-R1, POMC, MR and GR mRNA levels. The data are represented as mean ± SEM of $2^{CT(GAPDH)-CT(target\ gene)}$. The white columns represent the non-stressed groups, the grey columns the IMO groups, and the stripped columns the cocaine groups. Samples were taken 4 hours after the end of IMO (R-4h) and 24 hours after IMO (R-24h). These time points correspond to 5 and 25 h after

cocaine. **: p<0.01; ***: p<0.001 overall effect of cocaine. $^{+}$: p<0.05; $^{++}$: p<0.01; $^{+++}$: p<0.001 overall effect of stress (G_ZLM).

The decomposition of the interactions showed a common pattern of gene expression for MAO-A and D1 on Acb both at R-24h; and for POMC on Pit at R-4h. The decomposition of these interactions showed a slightly decrease gene expression promoted by IMO and cocaine separately, but when the two treatments were presented simultaneously (CocIMO), a sum of effects was observed and gene expression presented levels similar to SalC. Cocaine reduced Orexin expression at R-4h and IMO didin´t produced significant changes alone but increased gene expression in CocIMO group.

The decomposition of the interaction found in SN-VTA at R-24h showed a slightly reduction of MAO-A expression promoted by cocaine that disappear when cocaine and IMO are joint together.

In Amg at R-4h, both treatments increased CRH expression, but IMO reduced the CRH expression in animals exposed to cocaine.

In Hyp at R-24h, the POMC expression is not altered by cocaine or IMO but the combination of the two stimuli presented a SUM effect in POMC expression. In opposition, In Hyp at R-4h, the MR expression is not altered by cocaine or IMO but when animals are exposed to both stimuli a decrease in MR expression was observed.

Table 7. Summary of the effets and interacions between Cocaine and IMO

Effects	**R-4h**	**R-24h**
COC effect: decrease (general effect or only in control animals)	**MAO-A**: Hipp, SN-VTA **D1**: SN-VTA **GR**: PFC, Hy **POMC**: Pit	**GR**: Acb
Only COC effect: increase (general effect or only in control animals)	**CRF**: PFC, Amg **GR**: Amg	**MAO-A**: SN-VTA **D1**: CPu **MR**: Pit
Only IMO effect: decrease (general effect or only in control animals)	**GR**: PFC, Hyp **POMC**: Pit	**MAO-A**: Amg **D1**: PFC, Amg, Hipp **MR**: Hyp **GR**: Hyp, Acb
Only IMO effect: increase (general effect or only in control animals)	**D1**: Hipp, CPu **TH**: Amg **CRF**: Hyp, Amy **AVP**: Hyp	**MAO-A**: CPu **CRF**: Amg
Only COC and IMO effects, additive effects (increase)	N/A	**CRF-R1**: Pit
COC x IMO effect, COCIMO higher than SALIMO	**MAO-A**: Acb **GR**: PFC, CPu, Hyp **POMC**: Pit	**D1**: Acb
COC x IMO effect, COCIMO higher than COCC	**MAO-A**: Acb, SN-VTA **GR**: Hyp **POMC**: Pit **Orexin**: Hyp	**D1**: Acb
COC x IMO effect, negative synergy, COCIMO lower than COCC	**MR**: Hyp **GR**: Amg	N/A
COC x IMO effect, negative synergy, COCIMO lower than SALIMO	**CRF**: Amg **MR**: Hyp	N/A
COC x IMO effect, positive synergy (only if COCIMO higher than SALC, and COCIMO higher than COCC or SALIMO)	N/A	**POMC**: Hyp

4. Discussion

The exposure to cocaine and/or IMO induced long-term changes in the expression of key genes in a structure and time-dependent way. We will focus on the more relevant results, paying special attention to the synergies detected with the simultaneous exposure to both stimuli.

Effects of IMO

After IMO, a complex pattern emerged, some key genes were activated and others inhibited, being the effects clearly dependent on the time period analyzed (4 h versus 24 h) and the brain region studied. The exposure to IMO increased at the short term (4 h) the expression of key genes at the hippocampus (D1), caudate-putamen (D1), amygdala (TH, CRF) and hypothalamus (CRF and AVP), whereas a decrease in the expression of other genes was detected at the prefrontal cortex (GR), hypothalamus (GR) and pituitary gland (POMC). One day after IMO, an increase in several genes was also observed in the pituitary gland (CRF-R1), amygdala (CRF) and caudate-putamen (MAO-A), although the predominant effect was a decrease in the expression of other genes, in the amygdala (MAO-A, D1), the prefrontal cortex (D1), the hippocampus (D1), the accumbens (GR) and the hypothalamus (MR and GR).

Our results, in accordance with previous literature, showed 4 h after IMO an increase in CRH mRNA expression in the hypothalamus and amygdala. A wide variety of stressors has been found to increase the expression of the CRH gene in the PVN, such as IMO, restraint stress, footshock, water-maze stress hypovolemia and hypoglycemia (Mamalaki *et al.*, 1992; Harbuz *et al.*, 1994; Kalin *et al.*, 1994;

Paulmyer-Lacroix *et al.*, 1994; Imaki *et al.*, 1995; Herman *et al.*, 1998; Hsu *et al.*, 1998; Skultétyová and Jezová, 1999; Ma *et al.*, 1997; Tanimura *et al.*, 1998; Aguilar-Valles *et al.*, 2005). Early studies already reported that CRH gene expression or release in the amygdala is increased in response to stress (*i.e.*Kalin *et al.*, 1994). Specifically, the subdivision of the amygdala involved is the CeA (Mamalaki *et al.*, 1992; Makino *et al.*, 1994a, b; Watts and Sanchez-Watts, 1995; Hsu *et al.*, 1998). It has been described that this increase in CRF levels in the CeA correlates with increases in anxiety in the elevated plus maze (Shepard *et al.*, 2000). In the present study the increase in CRF expression in the amygdala was transient and 24 h after the injection a decrease in expression was detected which may be a compensatory mechanism after the increase.

On the other hand, as expected, in our study stress promoted a significant increase of AVP mRNA expression in the hypothalamus 4 h after the end of IMO. No significant long-term changes in AVP expression levels were observed. Previous studies showed that acute stress exposure induces transcription of AVP gene in the PVN, and a delayed upregulation in AVP mRNA pools (Herman and Sherman, 1993; Herman, 1995; Kovacs and Sawchenko, 1996a, 1996b).

Induction of the gene expression of POMC (ACTH precursor) in the anterior pituitary is a frequently observed stress response (*i.e.*Lightman and Harbuz, 1993; Watts, 1996**)** reflecting the activation of the HPA axis. Nevertheless most of the previous studies have focused in the anterior pituitary whereas in the present study all the pituitary was analysed. Our study showed that stress reduces the POMC mRNA levels 4 h after IMO and 24 h after levels returned to normal. There are two explanations for these contradictory results. The first possibility is that by analyzing the whole pituitary, the posterior division of the pituitary had a significant reduction in POMC mRNA levels that masked the effects in the anterior division. The second

explanation is the timing of sacrifice, which is posterior to the HPA axis response and adaptation to the stressor (after rapid and slow feedback) and low levels of POMC (that is the ACTH precursor) should be expected.

GR receptors are involved in the negative feedback of the HPA axis (Sapolsky *et al.*, 1984; Herman *et al.*, 1989; Sapolsky *et al.*, 1990) and are located in several brain areas such as the PFC, the hippocampal formation, or the hypothalamus (PVN) and in the pituitary (Herman *et al.*, 2003). Thus, a reduction of GR expression can lead to a reduction of negative feedback if another exposure to stress occurs. Although the inverse relationship between stress-induced levels of glucocorticoids and GR activity in the hippocampus is well established (Sapolsky *et al.*, 1984; Herman *et al.*, 1989; Sapolsky *et al.*, 1990), no changes in GR expression in the whole hippocampus was observed in our study. However, in the present work acute IMO decreased GR mRNA levels in the hypothalamus and the decrease lasted until at least 24 h after IMO. Changes in GR receptors in the hypothalamus are assumed to be due to specific changes in the PVN. In other study, as with GR mRNA, GR heteronuclear RNA levels were decreased in the PVN during acute and chronic IMO, indicating that the GR mRNA levels in this region were regulated at the transcriptional level (Noguchi *et al.*, 2010). A reduction in GR receptors in the prefrontal cortex 4 h after IMO was also observed, although the levels returned to basal 24 h later

The exposure to IMO also induced long-term effects in D1 receptor gene expression. The involvement of dopamine D1 receptors in stress-induced impairment of PFC function has been well described before (Murphy *et al.*, 1996; Zahrt *et al.*, 1997), whereas D1 receptors in the hippocampus are important for modulating the persistence of contextual memories associated with aversive events (*i.e.*Rossato *et al.*, 2009; Kramar *et al.*, 2014). We found a decrease in D1 receptors 24 h after IMO which is compatible with the impairment in working memory induced by stress (Butts

et al., 2011; Devibiss *et al.*, 2012). Moreover, in the present study rats showed an elevated expression of D1 gene in the hippocampus 4 h after IMO but decreased D1 mRNA 24 h after treatment. The decrease in D1 expression in the hippocampus is compatible with the long-term effects of acute stress on hippocampal dependent memories (Andero *et al.*, 2012) and with the mentioned role of D1 receptors in these hippocampal dependent processes (Sariñana *et al.*, 2014).

Effects of cocaine

After the cocaine injection a pattern of activation/inhibition slightly more restricted than after IMO appeared, although the pattern was also clearly dependent on the time period and area analyzed. At 4 h post-injection, cocaine increased the expression of some genes in the prefrontal cortex (CRF) and amygdala (CRF, GR) and decreased the expression in other brain areas: hippocampus (MAO-A), SN-VTA (MAO-A, D1), prefrontal cortex, accumbens and hypothalamus (GR) and pituitary gland (POMC). At 24 h post-injection an increase was observed in the expression of other genes in the SN-VTA (MAO-A), caudate-putamen (D1) and pituitary gland (MR), whereas no decrease was detected. There are no previous data to compare our results, because other studies have used other time periods of sacrifice and/or other patterns of cocaine administration. Early studies already described that following acute cocaine injection there are very diverse time courses of gene expression, being some genes activated shortly after injection and other ones several hours after (Berke *et al.*, 1998). In addition, gene expression following acute (single or binge) or chronic (continuous, intermittent, or binge) is very different (*i.e.*Yuferov *et al.*, 2003; Puig *et al.*, 2012).

In the present study cocaine increases the levels of CRH in the PFC and in the amygdala 4 h after injection. CRH is implicated in the pathophysiology of affective and anxiety disorders and in aversive states associated with drug withdrawal (Heinrichs *et al.*, 1995; Sarnyai *et al.*, 1995). It is known that a single injection of cocaine or an acute binge pattern cocaine administration elevates CRH mRNA levels in the hypothalamus in rats, suggesting that cocaine stimulated CRF synthesis and release in this region (Rivier and Lee, 1994; Zhou *et al.*, 1996, 2004). A single dose of cocaine has also been reported to induce CRH release from the rat central amygdaloid nucleus in vivo (Richter *et al.*, 1995), and acute 'binge' cocaine administration increased CRH mRNA levels in the whole amygdala (Zhou *et al.*, 1996). The same increase in CRF in the amygdala is also seen during withdrawal (Richter and Weiss, 1999; Zhou *et al.*, 2003b).

In the present study, acute cocaine reduced POMC mRNA expression in the hypothalamus 4 h after injection but not 24 h after treatment. In agreement with this fact, previous studies found that in basal anterior pituitary POMC mRNA is reduced following long-access cocaine self administration Mantsch *et al.* (2003) and Zhou *et al.* (2003a) data showed a reduced POMC mRNA level in the hypothalamus after acute 'binge' cocaine administration.

Cocaine in the present work reduced GR mRNA expression in the hypothalamus and in the PFC at least during 4 h, but GR mRNA returned to normal levels 24 h after treatment. Moreover, at 24 h after treatment cocaine produced a significant reduction in GR mRNA expression in the Acb. In contrast to the decrease observed in GR in the hypothalamus, accumbens and PFC, it is noteworthy that GR amygdala levels increase 4 h after treatment. Previous data showed that GR protein expression was also significantly reduced in the dorsomedial hypothalamus (including the PVN) in cocaine self-administering rats, but not in the pituitary gland, ventromedial

hypothalamus, dorsal hippocampus, ventral subiculum, medial prefrontal cortex or amygdala (Mantsch *et al.*, 2007**)**. The other significant change observed in glucocorticoid receptors after cocaine administration in the present work was the increase found in MR expression in the pituitary gland 24 h after treatment, but there are no previous reports to compare our data.

Regarding the dopaminergic system no significant changes were observed in TH expression at the time periods analyzed, whereas MAO-A expression decreased 4 h after injection in the hippocampus and in the SN-VTA, and increased 24 h after injection in the SN-VTA. The temporal changes in SN-VTA are probably compensatory mechanisms. Although there are no previous studies using similar designs to compare our data about TH and MAO-A expression, the effects of cocaine on D1 receptors have been more studied. In the present work, cocaine exposure, in contrast to IMO, promoted 4 h after injection a reduction in D1 mRNA expression in SN-VTA but those alterations were of short duration and vanished 24 h after treatment, time at which an increase in D1 receptors in the caudate-putamen was observed. The specific meaning of those changes is not clear but it is generally accepted that VTA neurons play a critical role in mediating the rewarding effects of acute cocaine and D1 receptors modulate these effects. D1 antagonists microinjection into the VTA reduces cocaine CPP (Galaj *et al.*, 2014), systemic D1 antagonists block the intracranial self-administration of cocaine into the VTA (David *et al.*, 2004), and blockade of D1 receptors into the VTA reduces the rewarding effectiveness of self-administered cocaine (Ranaldi and Wise, 2001). Cocaine-induced locomotor activation is absent in dopamine D1 receptor-mutant mice (Xu *et al.*, 1994) and behavioral sensitization to cocaine is D1-dependent (McCreary and Marsden, 1993; Thomas *et al.*, 1996) and related with an up-regulation of D1-like receptors (Unterwald *et al.*, 1994).

Interaction between IMO and cocaine

What is the most relevant of this chapter is that after the simultaneous exposure to cocaine and IMO several interesting patterns emerged. In one case (CRF-R1 in the pituitary gland), both cocaine and IMO increased the expression of the gene, but only additive effects were observed, and not a potentiation. In most of the cases the COCIMO group has a greater expression of a given gene than SALIMO, at 4 h post-injection (in the accumbens: MAO-A, in the prefrontal cortex, caudate-putamen and hypothalamus: GR, and in the pituitary gland: POMC) or at 24 h post-injection (D1 in the accumbens), or a greater expression than COCC, at 4 h post-injection (in the accumbens and SN-VTA: MAO-A, in the hypothalamus: GR and orexin, and in the pituitary gland: POMC), or at 24 h post-injection (D1 in the accumbens), but the simultaneous exposure was not statistically significant higher than the absolute control group (SALC), suggesting that there were some type of interaction but not a clear potentiation. More interesting was the presence of clear synergies, negative and positive.

At 4 h post-injection/stress the simultaneous exposure to both stimuli resulted in two negative synergies. Cocaine by itself increased GR expression in the amygdala, but the concomitant exposure to stress (by itself ineffective) returned to basal levels this expression. The exact functional meaning of this interaction remains to be determined. On the other hand, either cocaine or stress (both administered alone) increased CRH expression in the amygdala, but the simultaneous exposure to both stimuli blocked this increase. This fact is particularly important because, as mentioned, the increase in CRH in the amygdala has been related to anxiety/disphoria (Zorrilla *et al.*, 2014) and may be a window by which addicts counteract the effects of stress by the intake of drugs. Although in this study drug

injection was acute, it is generally assumed that the acute increase in CRF induced by several drugs, although under some circumstances may develop tolerance with chronic administration, emerges dramatically again during withdrawal being responsible of the negative state that leads to relapse in drug consumption. However, future studies need to ascertain whether this negative synergy during acute intake is also detected during withdrawal.

The studies of Day *et al.* (2001, 2005, 2008) give also support to this negative interaction between stressors and psychostimulants (acute injection, as in the present study) at the amygdala level. As mentioned, the central nucleus of the amygdala releases CRH in response to different acute stressors (*i.e.*Mamalaki *et al.*, 1992; Hsu *et al.*, 1998) and acute drugs, such as alcohol (*i.e.*Lam and Gianoulakis, 2011) or cocaine (*i.e.*Richter *et al.*, 1995; Zhou *et al.*, 1996). Day *et al.* (2001) demonstrated that amphetamine produced a lower c-fos activation in the central nucleus of the amygdala when given in a stressful environment (novelty stress) that when given in the home-cage (non-stressful condition), in contrast to other brain regions were the opposite was found. Although in that study the c-fos activated neurons in the CeA were predominantly enkephalin-containing cells (and no CRH neurons), the possibility remains that only a small subset of the CeA neurons CRH + is critical for the interaction between stress and psychostimulants. Moreover, as proposed by Day *et al.* (2001), as there are several intranuclear connections within the lateral division of the CeA, it may be possible that enkephalin-containing neurons may inhibit CRH cells in this structure. The generality of the effect is confirmed by other data. In other studies, the same group described that amphetamine induced c-fos was decreased after simultaneous exposure to other stressors, such as loud noise and restraint stress (Day *et al.*, 2005) or conditioned fear to a context previously paired to shock (Day *et al.*, 2008). Although the mechanisms of action of this interaction are not known, Day *et al.* (2005) suggested that one possibility is that

stressors inhibit CeA neurons through GABA A or C receptors, leading to functional changes in Pavlovian learning mediated by this structure (Keifer *et al.*, 2015) which are important for the development of craving to drug-associated cues (Everitt, 2014). No negative synergies were observed at 24 h post-injection/stress. However, at 24 h post-treatments a clear positive synergy was observed affecting the expression of POMC in the hypothalamus. The exposure to cocaine or IMO by themselves did not increase POMC expression in the hypothalamus, but the simultaneous exposure to both stimuli lead to a long-term dramatic increase in POMC expression. As our peripheral HPA data (ACTH and corticosterone) was only measured until 2 h post-treatments we do not have complementary evidence of the impact of these changes in plasma levels of HPA hormones.

FINAL CONCLUSIONS

1 Exposure to IMO induced a prolonged increase in plasma ACTH and corticosterone levels, whereas cocaine only increased corticosterone with a less potent magnitude.

2 Cocaine administration decreases ACTH levels at the end of IMO, reflecting a mild negative synergism.

3 The long-term anorectic effects of IMO were partially blocked by cocaine injection (negative synergism) which itself also decreased food intake but only transiently and in non-stressed animals. Both IMO and cocaine reduced body-weight gain, although the decrease in weight gain induced by cocaine was delayed more in time and not explained by changes in food intake.

4 The long-term anhedonic-like effects of IMO (measured by the intake of saccharine solutions) were not affected by cocaine administration that itself had no effect.

5 Exposure to IMO induced, twenty-four hours later, an anxiogenic-like effect in the EPM test, accompanied by a decrease in motor activity. Here again cocaine was not able to modify this behaviour alone or concomitantly with IMO.

6 The active behavior in the FST (struggling) was not affected neither by cocaine nor by IMO. IMO exposure modestly increased mild swimming in non-cocaine injected animals.

7 IMO was able to induce a sensitization of the HPA axis in response to two different heterotypic stressors (EPM and FST). However, the effects of cocaine in the sensitization induced by IMP were not consistent.

8 The exposure to IMO produced an anxiogenic-like effect in the ASR test, effect that vanished after twenty-four hours. Cocaine injection did not modify this behavior.

9 The animals administered with cocaine in the first exposure to IMO, showed no homotypic adaptation to stress, contrary to the animals that were only exposed to IMO.

10 Regarding c-fos expression induced by the simultaneous administration of both stimuli, we have obtained clear negative synergies at the Cg1 (where IMO is inhibiting the activation induced by cocaine, being IMO by itself ineffective) and at the ACb, BNST and DR (where cocaine is inhibiting the activation induced by IMO, being cocaine by itself ineffective).

11 Cocaine by itself increased GR expression in the amygdala, but the concomitant exposure to IMO (by itself ineffective) returned to basal levels this expression. On the other hand, either cocaine or stress (both administered alone) increased CRH expression in the amygdala, but the

simultaneous exposure to both stimuli blocked this increase.

12 In general cocaine does appear to protect more than exacerbate the endocrine, neural and behavioral effects of exposure to a severe stressor, suggesting a small interaction between both stimuli.

REFERENCES

Abel EL, Moore C, Waselewsky D, Zajac C, Russell LD. 1989. Effects of cocaine hydrochloride on reproductive function and sexual behavior of male rats and on the behavior of their offspring. J Androl 10,17-27.

Abrams JK, Johnson PL, Hollis JH, Lowry CA. 2004. Anatomic and functional topography of the dorsal raphe nucleus. Ann N Y Acad Sci 1018,46-57.

Adan RA, Gispen WH. 2000. Melanocortins and the brain: from effects via receptors to drug targets. Eur J Pharmacol 405,13-24.

Aguilar-Valles A, Sanchez E, de Gortari P, Balderas I, Ramirez-Amaya V, Bermudez-Rattoni F, Joseph-Bravo P. 2005. Analysis of the stress response in rats trained in the water-maze: differential expression of corticotropin-releasing hormone, CRH-R1, glucocorticoid receptors and brain-derived neurotrophic factor in limbic regions. Neuroendocrinology 82,306-19.

Aguilera G, Nikodemova M, Wynn PC, Catt KJ. 2004. Corticotropin releasing hormone receptors: two decades later. Peptides 25,319-29.

Aguilera G, Pham Q, Rabadan-Diehl C. 1994. Regulation of pituitary vasopressin receptors during chronic stress: relationship to corticotroph responsiveness. J Neuroendocrinol 6,299-304.

Akana SF, Chu A, Soriano L, Dallman MF. 2001. Corticosterone exerts site-specific and state-dependent effects in prefrontal cortex and amygdala on regulation of adrenocorticotropic hormone, insulin and fat depots. J Neuroendocrinol 13,625-37.

Akirav I, Richter-Levin G. 2006. Factors that determine the non-linear amygdala influence on hippocampus-dependent memory. Dose Response 4,22-37.

Alheid GF. 2003. Extended amygdala and basal forebrain. Ann N Y Acad Sci 985,185-205.

Alheid GF, Heimer L. 1988. New perspectives in basal forebrain organization of special relevance for neuropsychiatric disorders: the striatopallidal, amygdaloid, and corticopetal components of substantia innominata. Neuroscience 27,1-39.

Antelman SM, Eichler AJ, Black CA, Kocan D. 1980. Interchangeability of stress and amphetamine in sensitization. Science 207,329-31.

Antoni FA. 1993. Vasopressinergic control of pituitary adrenocorticotropin secretion comes of age. Front Neuroendocrinol 14,76-122.

Armario A. 2006. The hypothalamic-pituitary-adrenal axis: what can it tell us about stressors? CNS Neurol Disord Drug Targets 5,485-501.

Armario A. 2010. Activation of the hypothalamic-pituitary-adrenal axis by addictive drugs: different pathways, common outcome. Trends Pharmacol Sci 31,318-25.

Armario A, Castellanos JM, Balasch J. 1984. Dissociation between corticosterone and growth hormone adaptation to chronic stress in the rat. Horm Metab Res 16,142-5.

Armario A, Gavalda A, Marti O. 1988. Forced swimming test in rats: effect of desipramine administration and the period of exposure to the test on struggling behavior, swimming, immobility and defecation rate. Eur J Pharmacol 158,207-12.

Armario A, Gil M, Marti J, Pol O, Balasch J. 1991. Influence of various acute stressors on the activity of adult male rats in a holeboard and in the forced swim test. Pharmacol Biochem Behav 39,373-7.

Armario A, Hernandez J, Bluethmann H, Hidalgo J. 1998. IL-6 deficiency leads to increased emotionality in mice: evidence in transgenic mice carrying a null mutation for IL-6. J Neuroimmunol 92,160-9.

Armario A, Lopez-Calderon A, Jolin T, Balasch J. 1986. Response of anterior pituitary hormones to chronic stress. The specificity of adaptation. Neurosci Biobehav Rev 10,245-50.

Armario A, Lopez-Calderon A, Jolin T, Castellanos JM. 1986. Sensitivity of anterior pituitary hormones to graded levels of psychological stress. Life Sci 39,471-5.

Armario A, Marti J, Gil M. 1990. The serum glucose response to acute stress is sensitive to the intensity of the stressor and to habituation. Psychoneuroendocrinology 15,341-7.

Armario A, Marti O, Valles A, Dal-Zotto S, Ons S. 2004. Long-term effects of a single exposure to immobilization on the hypothalamic-pituitary-adrenal axis: neurobiologic mechanisms. Ann N Y Acad Sci 1018,162-72.

Armstrong WE. 1995. Morphological and electrophysiological classification of hypothalamic supraoptic neurons. Prog Neurobiol 47,291-339.

Badiani A, Anagnostaras SG, Robinson TE. 1995. The development of sensitization to the psychomotor stimulant effects of amphetamine is enhanced in a novel environment. Psychopharmacology (Berl) 117,443-52.

Badiani A, Browman KE, Robinson TE. 1995. Influence of novel versus home environments on sensitization to the psychomotor stimulant effects of cocaine and amphetamine. Brain Res 674,291-8.

Badiani A, Camp DM, Robinson TE. 1997. Enduring enhancement of amphetamine sensitization by drug-associated environmental stimuli. J Pharmacol Exp Ther 282,787-94.

Badiani A, Oates MM, Day HE, Watson SJ, Akil H, Robinson TE. 1998. Amphetamine-induced behavior, dopamine release, and c-fos mRNA expression: modulation by environmental novelty. J Neurosci 18,10579-93.

Balopole DC, Hansult CD, Dorph D. 1979. Effect of cocaine on food intake in rats. Psychopharmacology (Berl) 64,121-2.

Bane AJ, McCoy JG, Stump BS, Avery DD. 1993. The effects of cocaine on dietary self-selection in female rats. Physiol Behav 54,509-13.

Baumann MH, Gendron TM, Becketts KM, Henningfield JE, Gorelick DA, Rothman RB. 1995. Effects of intravenous cocaine on plasma cortisol and prolactin in human cocaine abusers. Biol Psychiatry 38,751-5.

Beck KD, Servatius RJ. 2005. Stress-induced reductions of sensory reactivity in female rats depend on ovarian hormones and the application of a painful stressor. Horm Behav 47,532-9.

Beck KD, Servatius RJ. 2006. Interleukin-1beta as a mechanism for stress-induced startle suppression in females. Ann N Y Acad Sci 1071,534-7.

Belda X, Daviu N, Nadal R, Armario A. 2012. Acute stress-induced sensitization of the pituitary-adrenal response to heterotypic stressors: independence of glucocorticoid release and activation of CRH1 receptors. Horm Behav 62,515-24.

Belda X, Marquez C, Armario A. 2004. Long-term effects of a single exposure to stress in adult rats on behavior and hypothalamic-pituitary-adrenal responsiveness: comparison of two outbred rat strains. Behav Brain Res 154,399-408.

Berke JD, Paletzki RF, Aronson GJ, Hyman SE, Gerfen CR. 1998. A complex program of striatal gene expression induced by dopaminergic stimulation. J Neurosci 18,5301-10.

Bernard C. 1972. De la physiologie générale. Librairie Hachette.

Bhatnagar S, Dallman M. 1998. Neuroanatomical basis for facilitation of hypothalamic-pituitary-adrenal responses to a novel stressor after chronic stress. Neuroscience 84,1025-39.

Bhattacharyya AK, Pradhan SN. 1979. Interactions between motor activity and sterotypy in cocaine-treated rats. Psychopharmacology (Berl) 63,311-2.

Binder EB, Nemeroff CB. 2010. The CRF system, stress, depression and anxiety-insights from human genetic studies. Mol Psychiatry 15,574-88.

Birnbaumer M. 2000. Vasopressin receptors. Trends Endocrinol Metab 11,404-410.

Black E, Grant J. 2014. The Essencial Companion to the Diagnostic and Statistical Manual of Mental Disorders. Edited by American Psychiatric Association.

Black EW, Lakoski JM. 1990. In Vitro electrophysiology of dorsal raphe serotonergic neurons in subchronic cocaine-treated rats: Development of tolerance to acute cocaine administration. Mol Cell Neurosci 1,84-91.

Bonaz B, Rivest S. 1998. Effect of a chronic stress on CRF neuronal activity and expression of its type 1 receptor in the rat brain. Am J Physiol 275,R1438-49.

Borowsky B, Kuhn CM. 1991. Monoamine mediation of cocaine-induced hypothalamo-pituitary-adrenal activation. J Pharmacol Exp Ther 256,204-10.

Borowsky B, Kuhn CM. 1992. D1 and D2 dopamine receptors stimulate hypothalamo-pituitary-adrenal activity in rats. Neuropharmacology 31,671-8.

Borowsky B, Kuhn CM. 1993. GBR12909 stimulates hypothalamo-pituitary-adrenal activity by inhibition of uptake at hypothalamic dopamine neurons. Brain Res 613,251-8.

Boudaba C, Szabo K, Tasker JG. 1996. Physiological mapping of local inhibitory inputs to the hypothalamic paraventricular nucleus. J Neurosci 16,7151-60.

Bradbury MJ, Strack AM, Dallman MF. 1993. Lesions of the hippocampal efferent pathway (fimbria-fornix) do not alter sensitivity of adrenocorticotropin to feedback inhibition by corticosterone in rats. Neuroendocrinology 58,396-407.

Braff DL, Geyer MA, Swerdlow NR. 2001. Human studies of prepulse inhibition of startle: normal subjects, patient groups, and pharmacological studies. Psychopharmacology (Berl) 156,234-58.

Brambilla F. 2001. Social stress in anorexia nervosa: a review of immuno-endocrine relationships. Physiol Behav 73,365-9.

Broadbear JH, Winger G, Cicero TJ, Woods JH. 1999. Effects of response contingent and noncontingent cocaine injection on hypothalamic-pituitary-adrenal activity in rhesus monkeys. J Pharmacol Exp Ther 290,393-402.

Broadbear JH, Winger G, Cicero TJ, Woods JH. 1999. Effects of self-administered cocaine on plasma adrenocorticotropic hormone and cortisol in male rhesus monkeys. J Pharmacol Exp Ther 289,1641-7.

Broderick PA, Rahni DN, Zhou Y. 2003. Acute and subacute effects of risperidone and cocaine on accumbens dopamine and serotonin release using in vivo microvoltammetry on line with open-field behavior. Prog Neuropsychopharmacol Biol Psychiatry 27,1037-54.

Browman KE, Badiani A, Robinson TE. 1998. Modulatory effect of environmental stimuli on the susceptibility to amphetamine sensitization: a dose-effect study in rats. J Pharmacol Exp Ther 287,1007-14.

Bryant RA, Guthrie RM. 2005. Maladaptive appraisals as a risk factor for posttraumatic stress: a study of trainee firefighters. Psychol Sci 16,749-52.

Bubser M, Deutch AY. 1999. Stress induces Fos expression in neurons of the thalamic paraventricular nucleus that innervate limbic forebrain sites. Synapse 32,13-22.

Butts KA, Weinberg J, Young AH, Phillips AG. 2011. Glucocorticoid receptors in the prefrontal cortex regulate stress-evoked dopamine efflux and aspects of executive function. Proc Natl Acad Sci U S A 108,18459-64.

Cador M, Cole BJ, Koob GF, Stinus L, Le Moal M. 1993. Central administration of corticotropin releasing factor induces long-term sensitization to D-amphetamine. Brain Res 606,181-6.

Cain ST, Griff D, Joyner CM, Ellinwood EH, Nemeroff CB. 1993. Chronic continuous or intermittent infusion of cocaine differentially alter the concentration of neurotensin-like immunoreactivity in specific rat brain regions. Neuropsychopharmacology 8,259-65.

Caine SB, Koob GF. 1994. Effects of dopamine D-1 and D-2 antagonists on cocaine

self-administration under different schedules of reinforcement in the rat. J Pharmacol Exp Ther 270,209-18.

Calogero AE, Gallucci WT, Kling MA, Chrousos GP, Gold PW. 1989. Cocaine stimulates rat hypothalamic corticotropin-releasing hormone secretion in vitro. Brain Res 505,7-11.

Campeau S, Falls WA, Cullinan WE, Helmreich DL, Davis M, Watson SJ. 1997. Elicitation and reduction of fear: behavioural and neuroendocrine indices and brain induction of the immediate-early gene c-fos. Neuroscience 78,1087-104.

Campeau S, Watson SJ. 1997. Neuroendocrine and behavioral responses and brain pattern of c-fos induction associated with audiogenic stress. J Neuroendocrinol 9,577-88.

Cannon W. 1932. The Wisdom of the Body. Paperback

Carboni E, Imperato A, Perezzani L, Di Chiara G. 1989. Amphetamine, cocaine, phencyclidine and nomifensine increase extracellular dopamine concentrations preferentially in the nucleus accumbens of freely moving rats. Neuroscience 28,653-61.

Carr KD, Kim GY, Cabeza de Vaca S. 2000. Chronic food restriction in rats augments the central rewarding effect of cocaine and the delta1 opioid agonist, DPDPE, but not the delta2 agonist, deltorphin-II. Psychopharmacology (Berl) 152,200-7.

Casada JH, Dafny N. 1991. Restraint and stimulation of bed nucleus of the stria terminalis produce similar stress-like behaviors. Brain Res Bull 27,207-12.

Cass WA, Gerhardt GA, Mayfield RD, Curella P, Zahniser NR. 1992. Differences in dopamine clearance and diffusion in rat striatum and nucleus accumbens following systemic cocaine administration. J Neurochem 59,259-66.

Ceccatelli S, Cintra A, Hokfelt T, Fuxe K, Wikstrom AC, Gustafsson JA. 1989. Coexistence of glucocorticoid receptor-like immunoreactivity with neuropeptides in the hypothalamic paraventricular nucleus. Exp Brain Res 78,33-42.

Cenci MA, Bjorklund A. 1993. Transection of corticostriatal afferents reduces amphetamine- and apomorphine-induced striatal Fos expression and turning behaviour in unilaterally 6-hydroxydopamine-lesioned rats. Eur J Neurosci 5,1062-70.

Chalmers DT, Lovenberg TW, De Souza EB. 1995. Localization of novel corticotropin-releasing factor receptor (CRF2) mRNA expression to specific subcortical nuclei in rat brain: comparison with CRF1 receptor mRNA expression. J Neurosci 15,6340-50.

Champagne D, Beaulieu J, Drolet G. 1998. CRFergic innervation of the paraventricular nucleus of the rat hypothalamus: a tract-tracing study. J Neuroendocrinol 10,119-31.

Chang SL, Kenigs V, Moldow RL, Zadina JE. 1995. Chronic treatment with morphine and ethanol, but not cocaine, attenuates IL-1 beta activation of FOS expression

in the rat hypothalamic paraventricular nucleus. Adv Exp Med Biol 373,201-8.

Checkley S. 1996. The neuroendocrinology of depression and chronic stress. Br Med Bull 52,597-617.

Chiasson BJ, Hong MG, Robertson HA. 1997. Putative roles for the inducible transcription factor c-fos in the central nervous system: studies with antisense oligonucleotides. Neurochem Int 31,459-75.

Chocyk A, Czyrak A, Wedzony K. 2008. Dopamine D1-like receptors agonist SKF 38393 increases cFOS expression in the paraventricular nucleus of the hypothalamus--impact of acute and chronic cocaine. J Physiol Pharmacol 59,425-40.

Choi DC, Furay AR, Evanson NK, Ostrander MM, Ulrich-Lai YM, Herman JP. 2007. Bed nucleus of the stria terminalis subregions differentially regulate hypothalamic-pituitary-adrenal axis activity: implications for the integration of limbic inputs. J Neurosci 27,2025-34.

Chrousos GP, Gold PW. 1998. A healthy body in a healthy mind--and vice versa--the damaging power of "uncontrollable" stress. J Clin Endocrinol Metab 83,1842-5.

Church WH, Justice JB, Jr., Byrd LD. 1987. Extracellular dopamine in rat striatum following uptake inhibition by cocaine, nomifensine and benztropine. Eur J Pharmacol 139,345-8.

Cintra A, Zoli M, Rosen L, Agnati LF, Okret S, Wikstrom AC, Gustaffsson JA, Fuxe K. 1994. Mapping and computer assisted morphometry and microdensitometry of glucocorticoid receptor immunoreactive neurons and glial cells in the rat central nervous system. Neuroscience 62,843-97.

Cohen H, Zohar J, Matar MA, Zeev K, Loewenthal U, Richter-Levin G. 2004. Setting apart the affected: the use of behavioral criteria in animal models of post traumatic stress disorder. Neuropsychopharmacology 29,1962-70.

Cole MA, Kim PJ, Kalman BA, Spencer RL. 2000. Dexamethasone suppression of corticosteroid secretion: evaluation of the site of action by receptor measures and functional studies. Psychoneuroendocrinology 25,151-67.

Cole RL, Sawchenko PE. 2002. Neurotransmitter regulation of cellular activation and neuropeptide gene expression in the paraventricular nucleus of the hypothalamus. J Neurosci 22,959-69.

Conti LH, Printz MP. 2003. Rat strain-dependent effects of repeated stress on the acoustic startle response. Behav Brain Res 144,11-8.

Costall B, Kelly ME, Naylor RJ, Onaivi ES, Tyers MB. 1989. Neuroanatomical sites of action of 5-HT3 receptor agonist and antagonists for alteration of aversive behaviour in the mouse. Br J Pharmacol 96,325-32.

Couceyro P, Pollock KM, Drews K, Douglass J. 1994. Cocaine differentially regulates activator protein-1 mRNA levels and DNA-binding complexes in the rat striatum and cerebellum. Mol Pharmacol 46,667-76.

Creese I, Iversen SD. 1974. The role of forebrain dopamine systems in amphetamine induced stereotyped behavior in the rat. Psychopharmacologia 39,345-57.

Croiset G, Nijsen MJ, Kamphuis PJ. 2000. Role of corticotropin-releasing factor, vasopressin and the autonomic nervous system in learning and memory. Eur J Pharmacol 405,225-34.

Crombag HS, Badiani A, Robinson TE. 1996. Signalled versus unsignalled intravenous amphetamine: large differences in the acute psychomotor response and sensitization. Brain Res 722,227-31.

Cryan JF, Valentino RJ, Lucki I. 2005. Assessing substrates underlying the behavioral effects of antidepressants using the modified rat forced swimming test. Neurosci Biobehav Rev 29,547-69.

Cullinan WE, Herman JP, Battaglia DF, Akil H, Watson SJ. 1995. Pattern and time course of immediate early gene expression in rat brain following acute stress. Neuroscience 64,477-505.

Cullinan WE, Herman JP, Watson SJ. 1993. Ventral subicular interaction with the hypothalamic paraventricular nucleus: evidence for a relay in the bed nucleus of the stria terminalis. J Comp Neurol 332,1-20.

Cunningham KA, Lakoski JM. 1988. Electrophysiological effects of cocaine and procaine on dorsal raphe serotonin neurons. Eur J Pharmacol 148,457-62.

Cunningham KA, Lakoski JM. 1990. The interaction of cocaine with serotonin dorsal raphe neurons. Single-unit extracellular recording studies. Neuropsychopharmacology 3,41-50.

Dackis CA, O'Brien CP. 2001. Cocaine dependence: a disease of the brain's reward centers. J Subst Abuse Treat 21,111-7.

Dal-Zotto S, Marti O, Armario A. 2003. Glucocorticoids are involved in the long-term effects of a single immobilization stress on the hypothalamic-pituitary-adrenal axis. Psychoneuroendocrinology 28,992-1009.

Dal-Zotto S, Martí O, Armario A. 2002. Is repeated exposure to immobilization needed to induce adaptation of the hypothalamic-pituitary-adrenal axis? Influence of adrenal factors. Behav Brain Res. ,187-95.

Dal-Zotto S, Martí O, Delgado R, Armario A. 2004. Potentiation of glucocorticoid release does not modify the long-term effects of a single exposure to immobilization stress. Psychopharmacology 230-7.

Dalia A, Wallace LJ. 1995. Amphetamine induction of c-fos in the nucleus accumbens is not inhibited by glutamate antagonists. Brain Res 694,299-307.

Dallman MF. 1993. Stress update Adaptation of the hypothalamic-pituitary-adrenal axis to chronic stress. Trends Endocrinol Metab 4,62-9.

Dallman MF, Akana SF, Jacobson L, Levin N, Cascio CS, Shinsako J. 1987. Characterization of corticosterone feedback regulation of ACTH secretion. Ann N Y Acad Sci 512,402-14.

Darke S. 2012. Pathways to heroin dependence: time to re-appraise self-medication. Addiction 108,659-67.

Dautzenberg FM, Braun S, Hauger RL. 2001. GRK3 mediates desensitization of

CRF1 receptors: a potential mechanism regulating stress adaptation. Am J Physiol Regul Integr Comp Physiol 280,R935-46.

David V, Segu L, Buhot MC, Ichaye M, Cazala P. 2004. Rewarding effects elicited by cocaine microinjections into the ventral tegmental area of C57BL/6 mice: involvement of dopamine D1 and serotonin1B receptors. Psychopharmacology 174(3).

Davis M. 1998. Are different parts of the extended amygdala involved in fear versus anxiety? Biol Psychiatry 44,1239-47.

Davis M, Shi C. 1999. The extended amygdala: are the central nucleus of the amygdala and the bed nucleus of the stria terminalis differentially involved in fear versus anxiety? Ann N Y Acad Sci 877,281-91.

Daviu N, Rabasa C, Nadal R, Armario A. 2014. Comparison of the effects of single and daily repeated immobilization stress on resting activity and heterotypic sensitization of the hypothalamic-pituitary-adrenal axis. Stress 17,176-85.

Daviu NA. 2008. Efects de l'estrès crònic sobre l'ansietat i l'activitat de l'eix hipotalàmic-pituitari-adrenal en rates: establiment de condicionament a un context. . Master Thesis. Unpublished.

Day HE, Badiani A, Uslaner JM, Oates MM, Vittoz NM, Robinson TE, Watson SJ, Jr., Akil H. 2001. Environmental novelty differentially affects c-fos mRNA expression induced by amphetamine or cocaine in subregions of the bed nucleus of the stria terminalis and amygdala. J Neurosci 21,732-40.

Day HE, Kryskow EM, Nyhuis TJ, Herlihy L, Campeau S. 2008. Conditioned fear inhibits c-fos mRNA expression in the central extended amygdala. Brain Res 1229,137-46.

Day HE, Masini CV, Campeau S. 2004. The pattern of brain c-fos mRNA induced by a component of fox odor, 2,5-dihydro-2,4,5-trimethylthiazoline (TMT), in rats, suggests both systemic and processive stress characteristics. Brain Res 1025,139-51.

Day HE, Nebel S, Sasse S, Campeau S. 2005. Inhibition of the central extended amygdala by loud noise and restraint stress. Eur J Neurosci 21,441-54.

Dayas CV, Buller KM, Crane JW, Xu Y, Day TA. 2001. Stressor categorization: acute physical and psychological stressors elicit distinctive recruitment patterns in the amygdala and in medullary noradrenergic cell groups. Eur J Neurosci 14,1143-52.

Dayas CV, Buller KM, Day TA. 1999. Neuroendocrine responses to an emotional stressor: evidence for involvement of the medial but not the central amygdala. Eur J Neurosci 11,2312-22.

De Boer SF, De Beun R, Slangen JL, Van der Gugten J. 1990. Dynamics of plasma catecholamine and corticosterone concentrations during reinforced and extinguished operant behavior in rats. Physiol Behav 47,691-8.

De Boer SF, Koopmans SJ, Slangen JL, Van der Gugten J. 1990. Plasma catecholamine, corticosterone and glucose responses to repeated stress in

rats: effect of interstressor interval length. Physiol Behav 47,1117-24.

De Goeij DC, Jezova D, Tilders FJ. 1992. Repeated stress enhances vasopressin synthesis in corticotropin releasing factor neurons in the paraventricular nucleus. Brain Res 577,165-8.

De Kloet ER. 2004. Hormones and the stressed brain. Ann N Y Acad Sci 1018,1-15.

De Kloet ER. 2009. [Stress: a neurobiological perspective]. Tijdschr Psychiatr 51,541-50.

De Kloet ER, Oitzl MS, Joels M. 1993. Functional implications of brain corticosteroid receptor diversity. Cell Mol Neurobiol 13,433-55.

De Kloet ER, Reul JM. 1987. Feedback action and tonic influence of corticosteroids on brain function: a concept arising from the heterogeneity of brain receptor systems. Psychoneuroendocrinology 12,83-105.

De Kloet ER, Reul JM, Sutanto W. 1990. Corticosteroids and the brain. J Steroid Biochem Mol Biol 37,387-94.

De Kloet ER, Sutanto W, Rots N, van Haarst A, van den Berg D, Oitzl M, van Eekelen A, Voorhuis D. 1991. Plasticity and function of brain corticosteroid receptors during aging. Acta Endocrinol (Copenh) 125 Suppl 1,65-72.

De Kloet ER, Vreugdenhil E, Oitzl MS, Joels M. 1998. Brain corticosteroid receptor balance in health and disease. Endocr Rev 19,269-301.

De Kloet R, Wallach G, McEwen BS. 1975. Differences in corticosterone and dexamethasone binding to rat brain and pituitary. Endocrinology 96,598-609.

De Wit H, Wise RA. 1977. Blockade of cocaine reinforcement in rats with the dopamine receptor blocker pimozide, but not with the noradrenergic blockers phentolamine or phenoxybenzamine. Can J Psychol 31,195-203.

Deroche V, Marinelli M, Le Moal M, Piazza PV. 1997. Glucocorticoids and behavioral effects of psychostimulants. II: cocaine intravenous self-administration and reinstatement depend on glucocorticoid levels. J Pharmacol Exp Ther 281,1401-7.

Deroche V, Piazza PV, Casolini P, Le Moal M, Simon H. 1993. Sensitization to the psychomotor effects of amphetamine and morphine induced by food restriction depends on corticosterone secretion. Brain Res 611,352-6.

Dess NK. 1992. Divergent responses to saccharin vs. sucrose availability after stress in rats. Physiol Behav 52,115-25.

Devilbiss DM, Jenison RL, Berridge CW. 2012. Stress-induced impairment of a working memory task: role of spiking rate and spiking history predicted discharge. PLoS Comput Biol 8,e1002681.

Di Chiara G, Imperato A. 1988. Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. Proc Natl Acad Sci U S A 85,5274-8.

Di S, Malcher-Lopes R, Halmos KC, Tasker JG. 2003. Nongenomic glucocorticoid inhibition via endocannabinoid release in the hypothalamus: a fast feedback

mechanism. J Neurosci 23,4850-7.

Dielenberg RA, Hunt GE, McGregor IS. 2001. "When a rat smells a cat": the distribution of Fos immunoreactivity in rat brain following exposure to a predatory odor. Neuroscience 104,1085-97.

Diorio D, Viau V, Meaney MJ. 1993. The role of the medial prefrontal cortex (cingulate gyrus) in the regulation of hypothalamic-pituitary-adrenal responses to stress. J Neurosci 13,3839-47.

Donohoe TP. 1984. Stress-induced anorexia: implications for anorexia nervosa. Life Sci 34,203-18.

Donovan B, Padin-Rivera E, Kowaliw S. 2001. "Transcend": initial outcomes from a posttraumatic stress disorder/substance abuse treatment program. J Trauma Stress 14,757-72.

Drolet G, Rivest S. 2001. Corticotropin-releasing hormone and its receptors; an evaluation at the transcription level in vivo. Peptides 22,761-7.

Duncan GE, Johnson KB, Breese GR. 1993. Topographic patterns of brain activity in response to swim stress: assessment by 2-deoxyglucose uptake and expression of Fos-like immunoreactivity. J Neurosci 13,3932-43.

Duncan GE, Knapp DJ, Breese GR. 1996. Neuroanatomical characterization of Fos induction in rat behavioral models of anxiety. Brain Res 713,79-91.

Dunn JD, Williams TJ. 1995. Cardiovascular responses to electrical stimulation of the bed nucleus of the stria terminalis. J Comp Neurol 352,227-34.

Egashira N, Mishima K, Iwasaki K, Oishi R, Fujiwara M. 2009. New topics in vasopressin receptors and approach to novel drugs: role of the vasopressin receptor in psychological and cognitive functions. J Pharmacol Sci 109,44-9.

Elman I, Breiter HC, Gollub RL, Krause S, Kantor HL, Baumgartner WA, Gastfriend DR, Rosen BR. 1999. Depressive symptomatology and cocaine-induced pituitary-adrenal axis activation in individuals with cocaine dependence. Drug Alcohol Depend 56,39-45.

Emmert MH, Herman JP. 1999. Differential forebrain c-fos mRNA induction by ether inhalation and novelty: evidence for distinctive stress pathways. Brain Res 845,60-7.

Engber TM, Koury EJ, Dennis SA, Miller MS, Contreras PC, Bhat RV. 1998. Differential patterns of regional c-Fos induction in the rat brain by amphetamine and the novel wakefulness-promoting agent modafinil. Neurosci Lett 241,95-8.

Erb S, Shaham Y, Stewart J. 1998. The role of corticotropin-releasing factor and corticosterone in stress- and cocaine-induced relapse to cocaine seeking in rats. J Neurosci 18,5529-36.

Erdtmann-Vourliotis M, Mayer P, Riechert U, Hollt V. 1999. Acute injection of drugs with low addictive potential (delta(9)-tetrahydrocannabinol, 3,4-methylenedioxymethamphetamine, lysergic acid diamide) causes a much higher c-fos expression in limbic brain areas than highly addicting drugs (cocaine and morphine). Brain Res Mol Brain Res 71,313-24.

Ericsson A, Kovacs KJ, Sawchenko PE. 1994. A functional anatomical analysis of central pathways subserving the effects of interleukin-1 on stress-related neuroendocrine neurons. J Neurosci 14,897-913.

Evanson NK, Tasker JG, Hill MN, Hillard CJ, Herman JP. 2010. Fast feedback inhibition of the HPA axis by glucocorticoids is mediated by endocannabinoid signaling. Endocrinology 151,4811-9.

Everitt BJ. 2014. Neural and psychological mechanisms underlying compulsive drug seeking habits and drug memories--indications for novel treatments of addiction. Eur J Neurosci 40,2163-82.

Everitt BJ, Wolf ME. 2002. Psychomotor stimulant addiction: a neural systems perspective. J Neurosci 22,3312-20.

Feltenstein MW, See RE. 2008. The neurocircuitry of addiction: an overview. Br J Pharmacol 154,261-74.

Ferris RM, Tang FL, Maxwell RA. 1972. A comparison of the capacities of isomers of amphetamine, deoxypipradrol and methylphenidate to inhibit the uptake of tritiated catecholamines into rat cerebral cortex slices, synaptosomal preparations of rat cerebral cortex, hypothalamus and striatum and into adrenergic nerves of rabbit aorta. J Pharmacol Exp Ther 181,407-16.

Figueiredo HF, Bruestle A, Bodie B, Dolgas CM, Herman JP. 2003. The medial prefrontal cortex differentially regulates stress-induced c-fos expression in the forebrain depending on type of stressor. Eur J Neurosci 18,2357-64.

Fischman MW, Schuster CR, Hatano Y. 1983. A comparison of the subjective and cardiovascular effects of cocaine and lidocaine in humans. Pharmacol Biochem Behav 18,123-7.

Fontana DJ, Commissaris RL. 1989. Effects of cocaine on conflict behavior in the rat. Life Sci 45,819-27.

Forman LJ, Estilow S. 1988. Cocaine influences beta-endorphin levels and release. Life Sci 43,309-15.

Fowler JS, MacGregor RR, Wolf AP, Arnett CD, Dewey SL, Schlyer D, Christman D, Logan J, Smith M, Sachs H and others. 1987. Mapping human brain monoamine oxidase A and B with 11C-labeled suicide inactivators and PET. Science 235,481-5.

Fuentes S, Carrasco J, Armario A, Nadal R. 2014. Behavioral and neuroendocrine consequences of juvenile stress combined with adult immobilization in male rats. Horm Behav 66,475-86.

Fuertes G, Milanes MV, Rodriguez-Gago M, Marin MT, Laorden ML. 2000. Changes in hypothalamic paraventricular nucleus catecholaminergic activity after acute and chronic morphine administration. Eur J Pharmacol 388,49-56.

Gagliano H, Fuentes S, Nadal R, Armario A. 2008. Previous exposure to immobilisation and repeated exposure to a novel environment demonstrate a marked dissociation between behavioral and pituitary-adrenal responses. Behav Brain Res 187,239-45.

Gagliano H, 2016. ¿Puede el metilfenidato atenuar el impacto del estrés? Un modelo experimental en rata. Non published PhD dissertation. Universitat Autònoma de Barcelona.

Gaillet S, Lachuer J, Malaval F, Assenmacher I, Szafarczyk A. 1991. The involvement of noradrenergic ascending pathways in the stress-induced activation of ACTH and corticosterone secretions is dependent on the nature of stressors. Exp Brain Res 87,173-80.

Galaj E, Manuszak M, Arastehmanesh D, Ranaldi R. 2014. Microinjections of a dopamine D1 receptor antagonist into the ventral tegmental area block the expression of cocaine conditioned place preference in rats. Behav Brain Res 272,279-85.

Galici R, Pechnick RN, Poland RE, France CP. 2000. Comparison of noncontingent versus contingent cocaine administration on plasma corticosterone levels in rats. Eur J Pharmacol 387,59-62.

Garcia A, Armario A. 2001. Individual differences in the recovery of the hypothalamic-pituitary-adrenal axis after termination of exposure to a severe stressor in outbred male Sprague-Dawley rats. Psychoneuroendocrinology 26,363-74.

Garcia A, Marti O, Valles A, Dal-Zotto S, Armario A. 2000. Recovery of the hypothalamic-pituitary-adrenal response to stress. Effect of stress intensity, stress duration and previous stress exposure. Neuroendocrinology 72,114-25.

Gardi J, Biro E, Sarnyai Z, Vecsernyes M, Julesz J, Telegdy G. 1997. Time-dependent alterations in corticotropin-releasing factor-like immunoreactivity in different brain regions after acute cocaine administration to rats. Neuropeptides 31,15-8.

Gawin FH. 1986. Neuroleptic reduction of cocaine-induced paranoia but not euphoria? Psychopharmacology (Berl) 90,142-3.

Gawin FH. 1991. Cocaine addiction: psychology and neurophysiology. Science 251,1580-6.

Gawin FH, Ellinwood EH, Jr. 1989. Cocaine dependence. Annu Rev Med 40,149-61.

Gawin FH, Kleber HD. 1986. Abstinence symptomatology and psychiatric diagnosis in cocaine abusers. Clinical observations. Arch Gen Psychiatry 43,107-13.

Gawin FH, Morgan C, Kosten TR, Kleber HD. 1989. Double-blind evaluation of the effect of acute amantadine on cocaine craving. Psychopharmacology (Berl) 97,402-3.

George O, Koob GF. 2011. Individual differences in prefrontal cortex function and the transition from drug use to drug dependence. Neurosci Biobehav Rev 35,232-47.

Geracitano R, Federici M, Bernardi G, Mercuri NB. 2006. On the effects of psychostimulants, antidepressants, and the antiparkinsonian drug levodopa on dopamine neurons. Ann N Y Acad Sci 1074,320-9.

Gillies GE, Linton EA, Lowry PJ. 1982. Corticotropin releasing activity of the new

CRF is potentiated several times by vasopressin. Nature 299,355-7.

Gingrich JA, Hen R. 2001. Dissecting the role of the serotonin system in neuropsychiatric disorders using knockout mice. Psychopharmacology (Berl) 155,1-10.

Ginty DD, Bonni A, Greenberg ME. 1994. Nerve growth factor activates a Ras-dependent protein kinase that stimulates c-fos transcription via phosphorylation of CREB. Cell 77,713-25.

Glowa JR, Hansen CT. 1994. Differences in response to an acoustic startle stimulus among forty-six rat strains. Behav Genet 24,79-84.

Glowinski J, Axelrod J. 1965. Effect of Drugs on the Uptake, Release, and Metabolism of H3-Norepinephrine in the Rat Brain. J Pharmacol Exp Ther 149,43-9.

Goeders NE. 1997. A neuroendocrine role in cocaine reinforcement. Psychoneuroendocrinology 22,237-59.

Goeders NE. 2002. Stress and cocaine addiction. J Pharmacol Exp Ther 301,785-9.

Goeders NE. 2002. The HPA axis and cocaine reinforcement. Psychoneuroendocrinology 27,13-33.

Goeders NE, Bienvenu OJ, De Souza EB. 1990. Chronic cocaine administration alters corticotropin-releasing factor receptors in the rat brain. Brain Res 531,322-8.

Goeders NE, Clampitt DM. 2002. Potential role for the hypothalamo-pituitary-adrenal axis in the conditioned reinforcer-induced reinstatement of extinguished cocaine seeking in rats. Psychopharmacology (Berl) 161,222-32.

Goeders NE, Guerin GF. 1996. Role of corticosterone in intravenous cocaine self-administration in rats. Neuroendocrinology 64,337-48.

Goeders NE, Guerin GF. 1996. Effects of surgical and pharmacological adrenalectomy on the initiation and maintenance of intravenous cocaine self-administration in rats. Brain Res 722,145-52.

Goeders NE, Guerin GF. 2000. Effects of the CRH receptor antagonist CP-154,526 on intravenous cocaine self-administration in rats. Neuropsychopharmacology 23,577-86.

Goeders NE, McNulty MA, Guerin GF. 1993. Effects of alprazolam on intravenous cocaine self-administration in rats. Pharmacol Biochem Behav 44,471-4.

Gold PW, Chrousos G, Kellner C, Post R, Roy A, Augerinos P, Schulte H, Oldfield E, Loriaux DL. 1984. Psychiatric implications of basic and clinical studies with corticotropin-releasing factor. Am J Psychiatry 141,619-27.

Goldstein DS, Kopin IJ. 2007. Evolution of concepts of stress. Stress 10,109-20.

Goldstein DS, McEwen B. 2002. Allostasis, homeostats, and the nature of stress. Stress 5,55-8.

Goldstein RZ, Volkow ND. 2002. Drug addiction and its underlying neurobiological basis: neuroimaging evidence for the involvement of the frontal cortex. Am J

Psychiatry 159,1642-52.

Gomez F, De Kloet ER, Armario A. 1998. Glucocorticoid negative feedback on the HPA axis in five inbred rat strains. Am J Physiol 274,R420-7.

Gomez-Roman. 2012. La exposición simultánea al estrés y a la Anfetamina revela sinergias negativas que afectan a la respuesta neuroendocrina y a la activación del sistema nervioso central. . PhD Thesis. Unpublished.

Gomez-Roman A, Ortega-Sanchez JA, Rotllant D, Gagliano H, Belda X, Delgado-Morales R, Marin-Blasco I, Nadal R, Armario A. 2015. The neuroendocrine response to stress under the effect of drugs: Negative synergy between amphetamine and stressors. Psychoneuroendocrinology 63,94-101.

Gonzales M, Garrett C, Chapman CD, Dess NK. 2008. Stress-induced attenuation of acoustic startle in low-saccharin-consuming rats. Biol Psychol 79,193-9.

Gray TS, Piechowski RA, Yracheta JM, Rittenhouse PA, Bethea CL, Van de Kar LD. 1993. Ibotenic acid lesions in the bed nucleus of the stria terminalis attenuate conditioned stress-induced increases in prolactin, ACTH and corticosterone. Neuroendocrinology 57,517-24.

Graybiel AM, Moratalla R, Robertson HA. 1990. Amphetamine and cocaine induce drug-specific activation of the c-fos gene in striosome-matrix compartments and limbic subdivisions of the striatum. Proc Natl Acad Sci U S A 87,6912-6.

Grillon C, Baas J. 2003. A review of the modulation of the startle reflex by affective states and its application in psychiatry. Clin Neurophysiol 114,1557-79.

Guarraci FA, Frohardt RJ, Kapp BS. 1999. Amygdaloid D1 dopamine receptor involvement in Pavlovian fear conditioning. Brain Res 827,28-40.

Halbreich U, Goldstein S, Sharpless N, Asnis G, Kang BJ, Ryan J. 1987. Diurnal rhythm of plasma MHPG in endogenous depression. Psychiatry Res 20,79-81.

Haller J, Mikics E, Makara GB. 2008. The effects of non-genomic glucocorticoid mechanisms on bodily functions and the central neural system. A critical evaluation of findings. Front Neuroendocrinol 29,273-91.

Hamamura T, Ichimaru Y. 1997. Amphetamine sensitization augments amphetamine-induced Fos expression in the lateral habenula. Brain Res 767,140-3.

Haney M, Maccari S, Le Moal M, Simon H, Piazza PV. 1995. Social stress increases the acquisition of cocaine self-administration in male and female rats. Brain Res 698,46-52.

Harbuz MS, Jessop DS, Lightman SL, Chowdrey HS. 1994. The effects of restraint or hypertonic saline stress on corticotrophin-releasing factor, arginine vasopressin, and proenkephalin A mRNAs in the CFY, Sprague-Dawley and Wistar strains of rat. Brain Res 667,6-12.

Harfstrand A, Fuxe K, Cintra A, Agnati LF, Zini I, Wikstrom AC, Okret S, Yu ZY, Goldstein M, Steinbusch H and others. 1986. Glucocorticoid receptor immunoreactivity in monoaminergic neurons of rat brain. Proc Natl Acad Sci U S A 83,9779-83.

Harlan RE, Garcia MM. 1998. Drugs of abuse and immediate-early genes in the forebrain. Mol Neurobiol 16,221-67.

Hart CL, Ward AS, Collins ED, Haney M, Foltin RW. 2004. Gabapentin maintenance decreases smoked cocaine-related subjective effects, but not self-administration by humans. Drug Alcohol Depend 73,279-87.

Heimer L, Alheid GF. 1991. Piecing together the puzzle of basal forebrain anatomy. Adv Exp Med Biol 295,1-42.

Heinrichs SC, Menzaghi F, Merlo Pich E, Britton KT, Koob GF. 1995. The role of CRF in behavioral aspects of stress. Ann N Y Acad Sci 771,92-104.

Hennessy MB, Levine S. 1978. Sensitive pituitary-adrenal responsiveness to varying intensities of psychological stimulation. Physiol Behav 21,295-7.

Herdegen T, Kovary K, Buhl A, Bravo R, Zimmermann M, Gass P. 1995. Basal expression of the inducible transcription factors c-Jun, JunB, JunD, c-Fos, FosB, and Krox-24 in the adult rat brain. J Comp Neurol 354,39-56.

Herdegen T, Leah JD. 1998. Inducible and constitutive transcription factors in the mammalian nervous system: control of gene expression by Jun, Fos and Krox, and CREB/ATF proteins. Brain Res Brain Res Rev 28,370-490.

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

Herman JP, Cullinan WE. 1997. Neurocircuitry of stress: central control of the hypothalamo-pituitary-adrenocortical axis. Trends Neurosci 20,78-84.

Herman JP, Cullinan WE, Watson SJ. 1994. Involvement of the bed nucleus of the stria terminalis in tonic regulation of paraventricular hypothalamic CRH and AVP mRNA expression. J Neuroendocrinol 6,433-42.

Herman JP, Cullinan WE, Young EA, Akil H, Watson SJ. 1992. Selective forebrain fiber tract lesions implicate ventral hippocampal structures in tonic regulation of paraventricular nucleus corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) mRNA expression. Brain Res 592,228-38.

Herman JP, Dolgas CM, Carlson SL. 1998. Ventral subiculum regulates hypothalamo-pituitary-adrenocortical and behavioural responses to cognitive stressors. Neuroscience 86,449-59.

Herman JP, Figueiredo H, Mueller NK, Ulrich-Lai Y, Ostrander MM, Choi DC, Cullinan WE. 2003. Central mechanisms of stress integration: hierarchical circuitry controlling hypothalamo-pituitary-adrenocortical responsiveness. Front Neuroendocrinol 24,151-80.

Herman JP, Mueller NK. 2006. Role of the ventral subiculum in stress integration. Behav Brain Res 174,215-24.

Herman JP, Ostrander MM, Mueller NK, Figueiredo H. 2005. Limbic system mechanisms of stress regulation: hypothalamo-pituitary-adrenocortical axis. Prog Neuropsychopharmacol Biol Psychiatry 29,1201-13.

Herman JP, Patel PD, Akil H, Watson SJ. 1989. Localization and regulation of glucocorticoid and mineralocorticoid receptor messenger RNAs in the hippocampal formation of the rat. Mol Endocrinol 3,1886-94.

Herman JP, Sherman TG. 1993. Acute stress upregulates vasopressin gene expression in parvocellular neurons of the hypothalamic paraventricular nucleus. Ann N Y Acad Sci 689,546-9.

Herrera DG, Robertson HA. 1996. Activation of c-fos in the brain. Prog Neurobiol 50,83-107.

Hoffman GE, Lyo D. 2002. Anatomical markers of activity in neuroendocrine systems: are we all 'fos-ed out'? J Neuroendocrinol 14,259-68.

Honkaniemi J, Fuxe K, Rechard L, Koistinaho J, Isola J, Gustafsson JA, Okret S, Pelto-Huikko M. 1992. Colocalization of fos- and glucocorticoid receptor-like immunoreactivities in the rat amygdaloid complex after immobilization stress. J Neuroendocrinol 4,547-55.

Hsu DT, Chen FL, Takahashi LK, Kalin NH. 1998. Rapid stress-induced elevations in corticotropin-releasing hormone mRNA in rat central amygdala nucleus and hypothalamic paraventricular nucleus: an in situ hybridization analysis. Brain Res 788,305-10.

Hyman SE. 1996. Addiction to cocaine and amphetamine. Neuron 16,901-4.

Hyman SE, Malenka RC. 2001. Addiction and the brain: the neurobiology of compulsion and its persistence. Nat Rev Neurosci 2,695-703.

Imaki T, Shibasaki T, Hotta M, Demura H. 1992. Early induction of c-fos precedes increased expression of corticotropin-releasing factor messenger ribonucleic acid in the paraventricular nucleus after immobilization stress. Endocrinology 131,240-6.

Imaki T, Xiao-Quan W, Shibasaki T, Yamada K, Harada S, Chikada N, Naruse M, Demura H. 1995. Stress-induced activation of neuronal activity and corticotropin-releasing factor gene expression in the paraventricular nucleus is modulated by glucocorticoids in rats. J Clin Invest 96,231-8.

Imperato A, Angelucci L. 1989. The effects of clozapine and fluperlapine on the in vivo release and metabolism of dopamine in the striatum and in the prefrontal cortex of freely moving rats. Psychopharmacol Bull 25,383-9.

Itoi K, Jiang YQ, Iwasaki Y, Watson SJ. 2004. Regulatory mechanisms of corticotropin-releasing hormone and vasopressin gene expression in the hypothalamus. J Neuroendocrinol 16,348-55.

Iversen LL. 1973. Catecholamine uptake processes. Br Med Bull 29,130-5.

Jaber M, Cador M, Dumartin B, Normand E, Stinus L, Bloch B. 1995. Acute and chronic amphetamine treatments differently regulate neuropeptide messenger RNA levels and Fos immunoreactivity in rat striatal neurons. Neuroscience 65,1041-50.

Jacobsen LK, Giedd JN, Kreek MJ, Gottschalk C, Kosten TR. 2001. Quantitative medial temporal lobe brain morphology and hypothalamic-pituitary-adrenal axis

function in cocaine dependence: a preliminary report. Drug Alcohol Depend 62,49-56.

Jacobsen LK, Southwick SM, Kosten TR. 2001. Substance use disorders in patients with posttraumatic stress disorder: a review of the literature. Am J Psychiatry 158,1184-90.

Jacobson L, Sapolsky R. 1991. The role of the hippocampus in feedback regulation of the hypothalamic-pituitary-adrenocortical axis. Endocr Rev 12,118-34.

Jahng JW, Houpt TA, Wessel TC, Chen K, Shih JC, Joh TH. 1997. Localization of monoamine oxidase A and B mRNA in the rat brain by in situ hybridization. Synapse 25,30-6.

Jariel-Encontre I, Salvat C, Steff AM, Pariat M, Acquaviva C, Furstoss O, Piechaczyk M. 1997. Complex mechanisms for c-fos and c-jun degradation. Mol Biol Rep 24,51-6.

Jasper MS, Engeland WC. 1994. Splanchnic neural activity modulates ultradian and circadian rhythms in adrenocortical secretion in awake rats. Neuroendocrinology 59,97-109.

Jenab S, Festa ED, Russo SJ, Wu HB, Inturrisi CE, Quinones-Jenab V. 2003. MK-801 attenuates cocaine induction of c-fos and preprodynorphin mRNA levels in Fischer rats. Brain Res Mol Brain Res 117,237-9.

Jenab S, Niyomchai T, Chin J, Festa ED, Russo SJ, Perrotti LI, Quinones-Jenab V. 2002. Effects of cocaine on c-fos and preprodynorphin mRNA levels in intact and ovariectomized Fischer rats. Brain Res Bull 58,295-9.

Johansson B, Lindstrom K, Fredholm BB. 1994. Differences in the regional and cellular localization of c-fos messenger RNA induced by amphetamine, cocaine and caffeine in the rat. Neuroscience 59,837-49.

Johnson AK, Cunningham JT, Thunhorst RL. 1996. Integrative role of the lamina terminalis in the regulation of cardiovascular and body fluid homeostasis. Clin Exp Pharmacol Physiol 23,183-91.

Johnson AK, Zardetto-Smith AM, Edwards GL. 1992. Integrative mechanisms and the maintenance of cardiovascular and body fluid homeostasis: the central processing of sensory input derived from the circumventricular organs of the lamina terminalis. Prog Brain Res 91,381-93.

Johnson DR, Westermeyer J, Kattar K, Thuras P. 2002. Daily charting of posttraumatic stress symptoms: a pilot study. J Nerv Ment Dis 190,683-92.

Ju G, Han ZS. 1989. Coexistence of corticotropin releasing factor and neurotensin within oval nucleus neurons in the bed nuclei of the stria terminalis in the rat. Neurosci Lett 99,246-50.

Kageyama K, Suda T. 2009. Regulatory mechanisms underlying corticotropin-releasing factor gene expression in the hypothalamus. Endocr J 56,335-44.

Kalin NH, Takahashi LK, Chen FL. 1994. Restraint stress increases corticotropin-releasing hormone mRNA content in the amygdala and paraventricular nucleus. Brain Res 656,182-6.

Kalivas PW, Duffy P. 1993. Time course of extracellular dopamine and behavioral sensitization to cocaine. I. Dopamine axon terminals. J Neurosci 13,266-75.

Kalivas PW, Volkow N, Seamans J. 2005. Unmanageable motivation in addiction: a pathology in prefrontal-accumbens glutamate transmission. Neuron 45,647-50.

Kant GJ, Bunnell BN, Mougey EH, Pennington LL, Meyerhoff JL. 1983. Effects of repeated stress on pituitary cyclic AMP, and plasma prolactin, corticosterone and growth hormone in male rats. Pharmacol Biochem Behav 18,967-71.

Kant GJ, Pastel RH, Bauman RA, Meininger GR, Maughan KR, Robinson TN, 3rd, Wright WL, Covington PS. 1995. Effects of chronic stress on sleep in rats. Physiol Behav 57,359-65.

Karch SB. 1996. The pathology of drug abuse. Boca Raton: CRC Press, INC,10.

Keifer OP, Jr., Hurt RC, Ressler KJ, Marvar PJ. 2015. The Physiology of Fear: Reconceptualizing the Role of the Central Amygdala in Fear Learning. Physiology (Bethesda) 30,389-401.

Keller-Wood ME, Dallman MF. 1984. Corticosteroid inhibition of ACTH secretion. Endocr Rev 5,1-24.

Keller-Wood ME, Shinsako J, Dallman MF. 1983. Inhibition of the adrenocorticotropin and corticosteroid responses to hypoglycemia after prior stress. Endocrinology 113,491-6.

Khantzian EJ. 1985. The self-medication hypothesis of addictive disorders: focus on heroin and cocaine dependence. Am J Psychiatry 142,1259-64.

Kilbey MM, Breslau N, Andreski P. 1992. Cocaine use and dependence in young adults: associated psychiatric disorders and personality traits. Drug Alcohol Depend 29,283-90.

Kippin TE, Szumlinski KK, Kapasova Z, Rezner B, See RE. 2008. Prenatal stress enhances responsiveness to cocaine. Neuropsychopharmacology 33,769-82.

Kleber HD, Gawin FH. 1984. The spectrum of cocaine abuse and its treatment. J Clin Psychiatry 45,18-23.

Koch M. 1999. The neurobiology of startle. Prog Neurobiol 59,107-28.

Kolber BJ, Muglia LJ. 2009. Defining brain region-specific glucocorticoid action during stress by conditional gene disruption in mice. Brain Res 1293,85-90.

Kollack-Walker S, Watson SJ, Akil H. 1997. Social stress in hamsters: defeat activates specific neurocircuits within the brain. J Neurosci 17,8842-55.

Koob GF. 1992. Neural mechanisms of drug reinforcement. Ann N Y Acad Sci 654,171-91.

Koob GF. 2010. The role of CRF and CRF-related peptides in the dark side of addiction. Brain Res 1314,3-14.

Koob GF, Caine SB. 1999. Cocaine addiction therapy--are we partially there? Nat Med 5,993-5.

Koob GF, Le Moal M. 1997. Drug abuse: hedonic homeostatic dysregulation.

Science 278,52-8.

Koob GF, Le Moal M. 2006. What is addiction? Elsevier. London.

Koob GF, Sanna PP, Bloom FE. 1998. Neuroscience of addiction. Neuron 21,467-76.

Kopin IJ. 1995. Definitions of stress and sympathetic neuronal responses. Ann N Y Acad Sci 771,19-30.

Korosi A, Baram TZ. 2008. The central corticotropin releasing factor system during development and adulthood. Eur J Pharmacol 583,204-14.

Kosofsky BE, Genova LM, Hyman SE. 1995. Substance P phenotype defines specificity of c-fos induction by cocaine in developing rat striatum. J Comp Neurol 351,41-50.

Kovacs K, Kiss JZ, Makara GB. 1986. Glucocorticoid implants around the hypothalamic paraventricular nucleus prevent the increase of corticotropin-releasing factor and arginine vasopressin immunostaining induced by adrenalectomy. Neuroendocrinology 44,229-34.

Kovacs KJ. 1998. c-Fos as a transcription factor: a stressful (re)view from a functional map. Neurochem Int 33,287-97.

Kovacs KJ, Sawchenko PE. 1996. Regulation of stress-induced transcriptional changes in the hypothalamic neurosecretory neurons. J Mol Neurosci 7,125-33.

Kovacs KJ, Sawchenko PE. 1996. Sequence of stress-induced alterations in indices of synaptic and transcriptional activation in parvocellular neurosecretory neurons. J Neurosci 16,262-73.

Kovary K, Bravo R. 1991. The jun and fos protein families are both required for cell cycle progression in fibroblasts. Mol Cell Biol 11,4466-72.

Kovary K, Bravo R. 1991. Expression of different Jun and Fos proteins during the G0-to-G1 transition in mouse fibroblasts: in vitro and in vivo associations. Mol Cell Biol 11,2451-9.

Kozicz T, Korosi A, Korsman C, Tilburg-Ouwens D, Groenink L, Veening J, van Der Gugten J, Roubos E, Olivier B. 2004. Urocortin expression in the Edinger-Westphal nucleus is down-regulated in transgenic mice over-expressing neuronal corticotropin-releasing factor. Neuroscience 123,589-94.

Kramar CP, Chefer VI, Wise RA, Medina JH, Barbano MF. 2014. Dopamine in the dorsal hippocampus impairs the late consolidation of cocaine-associated memory. Neuropsychopharmacology 39,1645-53.

Kreek MJ, Koob GF. 1998. Drug dependence: stress and dysregulation of brain reward pathways. Drug Alcohol Depend 51,23-47.

Kreuter JD, Mattson BJ, Wang B, You ZB, Hope BT. 2004. Cocaine-induced Fos expression in rat striatum is blocked by chloral hydrate or urethane. Neuroscience 127,233-42.

Kuhar MJ, Pilotte NS. 1996. Neurochemical changes in cocaine withdrawal. Trends

Pharmacol Sci 17,260-4.

Kuzmin A, Johansson B. 1999. Expression of c-fos, NGFI-A and secretogranin II mRNA in brain regions during initiation of cocaine self-administration in mice. Eur J Neurosci 11,3694-700.

Kvetnansky R, Németh S, Vigas M, Oprsalová Z, Jurcovicova J. Plasma catecolamines in rats during adaptation to intermitent exposure to different stressors. In:Usdin E, Kvetnansky R, Axelrod J, editors. Stress. The role of catecholamines and other neurotransmitters. New York: Gordon and Breach Science Publishers; 1984.p. 537–62.

Lam MP, Gianoulakis C. 2011. Effects of acute ethanol on corticotropin-releasing hormone and beta-endorphin systems at the level of the rat central amygdala. Psychopharmacology (Berl) 218,229-39.

Lamon BC, Alonzo A. 1997. Stress among males recovering from substance abuse. Addict Behav 22,195-205.

Laorden ML, Fuertes G, Gonzalez-Cuello A, Milanes MV. 2000. Changes in catecholaminergic pathways innervating paraventricular nucleus and pituitary-adrenal axis response during morphine dependence: implication of alpha(1)- and alpha(2)-adrenoceptors. J Pharmacol Exp Ther 293,578-84.

Laviola G, Wood RD, Kuhn C, Francis R, Spear LP. 1995. Cocaine sensitization in periadolescent and adult rats. J Pharmacol Exp Ther 275,345-57.

Le Moal M. 2009. Drug abuse: vulnerability and transition to addiction. Pharmacopsychiatry 42 Suppl 1,S42-55.

Le Moal M, Koob GF. 2007. Drug addiction: pathways to the disease and pathophysiological perspectives. Eur Neuropsychopharmacol 17,377-93.

Le Moal M, Simon H. 1991. Mesocorticolimbic dopaminergic network: functional and regulatory roles. Physiol Rev 71,155-234.

Lemos JC, Wanat MJ, Smith JS, Reyes BA, Hollon NG, Van Bockstaele EJ, Chavkin C, Phillips PE. 2012. Severe stress switches CRF action in the nucleus accumbens from appetitive to aversive. Nature 490,402-6.

Levine HG. 1985. Situational anxiety and everyday life experiences of mildly mentally retarded adults. Am J Ment Defic 90,27-33.

Levy AD, Li Q, Alvarez Sanz MC, Rittenhouse PA, Kerr JE, Van de Kar LD. 1992. Neuroendocrine responses to cocaine do not exhibit sensitization following repeated cocaine exposure. Life Sci 51,887-97.

Levy AD, Li QA, Kerr JE, Rittenhouse PA, Milonas G, Cabrera TM, Battaglia G, Alvarez Sanz MC, Van de Kar LD. 1991. Cocaine-induced elevation of plasma adrenocorticotropin hormone and corticosterone is mediated by serotonergic neurons. J Pharmacol Exp Ther 259,495-500.

Levy AD, Rittenhouse PA, Li Q, Yracheta J, Kunimoto K, Van de Kar LD. 1994. Influence of repeated cocaine exposure on the endocrine and behavioral responses to stress in rats. Psychopharmacology (Berl) 113,547-54.

Li HY, Ericsson A, Sawchenko PE. 1996. Distinct mechanisms underlie activation of hypothalamic neurosecretory neurons and their medullary catecholaminergic afferents in categorically different stress paradigms. Proc Natl Acad Sci U S A 93,2359-64.

Li HY, Sawchenko PE. 1998. Hypothalamic effector neurons and extended circuitries activated in "neurogenic" stress: a comparison of footshock effects exerted acutely, chronically, and in animals with controlled glucocorticoid levels. J Comp Neurol 393,244-66.

Licznerski P, Duman RS. 2013. Remodeling of axo-spinous synapses in the pathophysiology and treatment of depression. Neuroscience 251,33-50.

Lightman SL, Harbuz MS. 1993. Expression of corticotropin-releasing factor mRNA in response to stress. Ciba Found Symp 172,173-87; discussion 187-98.

Liposits Z, Paull WK, Setalo G, Vigh S. 1985. Evidence for local corticotropin releasing factor (CRF)-immunoreactive neuronal circuits in the paraventricular nucleus of the rat hypothalamus. An electron microscopic immunohistochemical analysis. Histochemistry 83,5-16.

Lovenberg TW, Chalmers DT, Liu C, De Souza EB. 1995. CRF2 alpha and CRF2 beta receptor mRNAs are differentially distributed between the rat central nervous system and peripheral tissues. Endocrinology 136,4139-42.

Lowry CA. 2002. Functional subsets of serotonergic neurones: implications for control of the hypothalamic-pituitary-adrenal axis. J Neuroendocrinol 14,911-23.

Lowry CA, Moore FL. 2006. Regulation of behavioral responses by corticotropin-releasing factor. Gen Comp Endocrinol 146,19-27.

Lu, Shahan. 2005. The role of stress in opiate and psycostimulant addiction: evidence from animals models. In Steckler, T et al., (Eds.) Handbook of stress and the brain. Elsevier 15,315-332.

Luckman SM, Dyball RE, Leng G. 1994. Induction of c-fos expression in hypothalamic magnocellular neurons requires synaptic activation and not simply increased spike activity. J Neurosci 14,4825-30.

Ma XM, Levy A, Lightman SL. 1997. Rapid changes in heteronuclear RNA for corticotrophin-releasing hormone and arginine vasopressin in response to acute stress. J Endocrinol 152,81-9.

Macedo CE, Martinez RC, Albrechet-Souza L, Molina VA, Brandao ML. 2007. 5-HT2- and D1-mechanisms of the basolateral nucleus of the amygdala enhance conditioned fear and impair unconditioned fear. Behav Brain Res 177,100-8.

Maggos CE, Spangler R, Zhou Y, Schlussman SD, Ho A, Kreek MJ. 1997. Quantitation of dopamine transporter mRNA in the rat brain: mapping, effects of "binge" cocaine administration and withdrawal. Synapse 26,55-61.

Maggos CE, Tsukada H, Kakiuchi T, Nishiyama S, Myers JE, Kreuter J, Schlussman SD, Unterwald EM, Ho A, Kreek MJ. 1998. Sustained withdrawal allows normalization of in vivo [11C]N-methylspiperone dopamine D2 receptor binding

after chronic binge cocaine: a positron emission tomography study in rats. Neuropsychopharmacology 19,146-53.

Maisonneuve IM, Kreek MJ. 1994. Acute tolerance to the dopamine response induced by a binge pattern of cocaine administration in male rats: an in vivo microdialysis study. J Pharmacol Exp Ther 268,916-21.

Makara GB, Haller J. 2001. Non-genomic effects of glucocorticoids in the neural system. Evidence, mechanisms and implications. Prog Neurobiol 65,367-90.

Makara GB, Mergl Z, Zelena D. 2004. The role of vasopressin in hypothalamo-pituitary-adrenal axis activation during stress: an assessment of the evidence. Ann N Y Acad Sci 1018,151-61.

Makino S, Gold PW, Schulkin J. 1994. Effects of corticosterone on CRH mRNA and content in the bed nucleus of the stria terminalis; comparison with the effects in the central nucleus of the amygdala and the paraventricular nucleus of the hypothalamus. Brain Res 657,141-9.

Makino S, Gold PW, Schulkin J. 1994. Corticosterone effects on corticotropin-releasing hormone mRNA in the central nucleus of the amygdala and the parvocellular region of the paraventricular nucleus of the hypothalamus. Brain Res 640,105-12.

Makino S, Smith MA, Gold PW. 1995. Increased expression of corticotropin-releasing hormone and vasopressin messenger ribonucleic acid (mRNA) in the hypothalamic paraventricular nucleus during repeated stress: association with reduction in glucocorticoid receptor mRNA levels. Endocrinology 136,3299-309.

Maldonado R, Robledo P, Chover AJ, Caine SB, Koob GF. 1993. D1 dopamine receptors in the nucleus accumbens modulate cocaine self-administration in the rat. Pharmacol Biochem Behav 45,239-42.

Mamalaki E, Kvetnansky R, Brady LS, Gold PW, Herkenham M. 1992. Repeated immobilization stress alters tyrosine hydroxylase, corticotropin-releasing hormone and corticosteroid receptor messenger ribonucleic Acid levels in rat brain. J Neuroendocrinol 4,689-99.

Mantsch JR, Cullinan WE, Tang LC, Baker DA, Katz ES, Hoks MA, Ziegler DR. 2007. Daily cocaine self-administration under long-access conditions augments restraint-induced increases in plasma corticosterone and impairs glucocorticoid receptor-mediated negative feedback in rats. Brain Res 1167,101-11.

Mantsch JR, Goeders NE. 1998. Generalization of a restraint-induced discriminative stimulus to cocaine in rats. Psychopharmacology (Berl) 135,423-6.

Mantsch JR, Saphier D, Goeders NE. 1998. Corticosterone facilitates the acquisition of cocaine self-administration in rats: opposite effects of the type II glucocorticoid receptor agonist dexamethasone. J Pharmacol Exp Ther 287,72-80.

Mantsch JR, Schlussman SD, Ho A, Kreek MJ. 2000. Effects of cocaine self-administration on plasma corticosterone and prolactin in rats. J Pharmacol Exp Ther 294,239-47.

Mantsch JR, Yuferov V, Mathieu-Kia AM, Ho A, Kreek MJ. 2003. Neuroendocrine alterations in a high-dose, extended-access rat self-administration model of escalating cocaine use. Psychoneuroendocrinology 28,836-62.

Marinelli M, Piazza PV. 2002. Interaction between glucocorticoid hormones, stress and psychostimulant drugs. Eur J Neurosci 16,387-94.

Markou A, Hauger RL, Koob GF. 1992. Desmethylimipramine attenuates cocaine withdrawal in rats. Psychopharmacology (Berl) 109,305-14.

Marquez C, Belda X, Armario A. 2002. Post-stress recovery of pituitary-adrenal hormones and glucose, but not the response during exposure to the stressor, is a marker of stress intensity in highly stressful situations. Brain Res 926,181-5.

Marquez C, Nadal R, Armario A. 2004. The hypothalamic-pituitary-adrenal and glucose responses to daily repeated immobilisation stress in rats: individual differences. Neuroscience 123,601-12.

Marquez C, Nadal R, Armario A. 2005. Responsiveness of the hypothalamic-pituitary-adrenal axis to different novel environments is a consistent individual trait in adult male outbred rats. Psychoneuroendocrinology 30,179-87.

Marquez C, Nadal R, Armario A. 2006. Influence of reactivity to novelty and anxiety on hypothalamic-pituitary-adrenal and prolactin responses to two different novel environments in adult male rats. Behav Brain Res 168,13-22.

Marshall RD, Garakani A. 2002. Psychobiology of the acute stress response and its relationship to the psychobiology of post-traumatic stress disorder. Psychiatr Clin North Am 25,385-95.

Marti J, Armario A. 1993. Effects of diazepam and desipramine in the forced swimming test: influence of previous experience with the situation. Eur J Pharmacol 236,295-9.

Marti O, Armario A. 1998. Anterior pituitary response to stress: time-related changes and adaptation. Int J Dev Neurosci 16,241-60.

Marti O, Garcia A, Valles A, Harbuz MS, Armario A. 2001. Evidence that a single exposure to aversive stimuli triggers long-lasting effects in the hypothalamus-pituitary-adrenal axis that consolidate with time. Eur J Neurosci 13,129-36.

Marti O, Marti J, Armario A. 1994. Effects of chronic stress on food intake in rats: influence of stressor intensity and duration of daily exposure. Physiol Behav 55,747-53.

Martin-Iverson MT, Szostak C, Fibiger HC. 1986. 6-Hydroxydopamine lesions of the medial prefrontal cortex fail to influence intravenous self-administration of cocaine. Psychopharmacology (Berl) 88,310-4.

Mash DC. 2008. Dopamine transporter, disease states and pathology. Chemistry,Biology and Pharmacology. M.L. Trudell & S. Izenwasser, Eds.: 29–47.Wiley. Hoboken, NJ.

Matta SG, Foster CA, Sharp BM. 1993. Nicotine stimulates the expression of cFos protein in the parvocellular paraventricular nucleus and brainstem catecholaminergic regions. Endocrinology 132,2149-56.

Matta SG, Valentine JD, Sharp BM. 1997. Nicotinic activation of CRH neurons in extrahypothalamic regions of the rat brain. Endocrine 7,245-53.

McCreary AC, Marsden CA. 1993. Cocaine-induced behaviour: dopamine D1 receptor antagonism by SCH 23390 prevents expression of conditioned sensitisation following repeated administration of cocaine. Neuropharmacology 32,387-91.

McCulloch, Searle. 2001. Generalized linear and mixed models. New York: Wiley and Sons.

McDonald AJ. 2003. Is there an amygdala and how far does it extend? An anatomical perspective. Ann N Y Acad Sci 985,1-21.

McDougall SJ, Widdop RE, Lawrence AJ. 2004. Medial prefrontal cortical integration of psychological stress in rats. Eur J Neurosci 20,2430-40.

McEwen BS. 1998. Protective and damaging effects of stress mediators. N Engl J Med 338,171-9.

McEwen BS. 2000. The neurobiology of stress: from serendipity to clinical relevance. Brain Res 886,172-189.

McEwen BS, De Kloet ER, Rostene W. 1986. Adrenal steroid receptors and actions in the nervous system. Physiol Rev 66,1121-88.

McGregor IS, Arnold JC, Weber MF, Topple AN, Hunt GE. 1998. A comparison of delta 9-THC and anandamide induced c-fos expression in the rat forebrain. Brain Res 802,19-26.

McKinley MJ, Gerstberger R, Mathai ML, Oldfield BJ, Schmid H. 1999. The lamina terminalis and its role in fluid and electrolyte homeostasis. J Clin Neurosci 6,289-301.

Meehan SM, Schechter MD. 1998. LSD produces conditioned place preference in male but not female fawn hooded rats. Pharmacol Biochem Behav 59,105-8.

Meil WM, See RE. 1996. Conditioned cued recovery of responding following prolonged withdrawal from self-administered cocaine in rats: an animal model of relapse. Behav Pharmacol 7,754-763.

Mello NK, Bowen CA, Mendelson JH. 2002. Comparison of plasma cocaine levels during a "binge" pattern of cocaine administration in male and female rhesus monkeys. Psychopharmacology (Berl) 164,19-26.

Mello NK, Mendelson JH. 1997. Cocaine's effects on neuroendocrine systems: clinical and preclinical studies. Pharmacol Biochem Behav 57,571-99.

Meloni EG, Davis M. 2004. The substantia nigra pars reticulata mediates the enhancement of startle by the dopamine D1 receptor agonist SKF 82958 in rats. Psychopharmacology (Berl) 174,228-36.

Mendelson JH, Mello NK, Sholar MB, Siegel AJ, Mutschler N, Halpern J. 2002. Temporal concordance of cocaine effects on mood states and neuroendocrine hormones. Psychoneuroendocrinology 27,71-82.

Mendelson JH, Sholar M, Mello NK, Teoh SK, Sholar JW. 1998. Cocaine tolerance:

behavioral, cardiovascular, and neuroendocrine function in men. Neuropsychopharmacology 18,263-71.

Menetrey D, Gannon A, Levine JD, Basbaum AI. 1989. Expression of c-fos protein in interneurons and projection neurons of the rat spinal cord in response to noxious somatic, articular, and visceral stimulation. J Comp Neurol 285,177-95.

Meng Y, Qiu C, Zhu H, Lama S, Lui S, Gong Q, Zhang W. 2014. Anatomical deficits in adult posttraumatic stress disorder: a meta-analysis of voxel-based morphometry studies. Behav Brain Res 270,307-15.

Miczek KA, Mutschler NH. 1996. Activational effects of social stress on IV cocaine self-administration in rats. Psychopharmacology (Berl) 128,256-64.

Miczek KA, Vivian JA. 1993. Automatic quantification of withdrawal from 5-day diazepam in rats: ultrasonic distress vocalizations and hyperreflexia to acoustic startle stimuli. Psychopharmacology (Berl) 110,379-82.

Miller EK. 1999. The prefrontal cortex: complex neural properties for complex behavior. Neuron 22,15-7.

Miller NS, Summers GL, Gold MS. 1993. Cocaine dependence: alcohol and other drug dependence and withdrawal characteristics. J Addict Dis 12,25-35.

Moberg PJ, Nord CE. 1985. Anaerobic bacteria in urine before and after prostatic massage of infertile men. Med Microbiol Immunol 174,25-8.

Mogenson GJ, Jones DL, Yim CY. 1980. From motivation to action: functional interface between the limbic system and the motor system. Prog Neurobiol 14,69-97.

Moldow RL, Fischman AJ. 1987. Cocaine induced secretion of ACTH, beta-endorphin, and corticosterone. Peptides 8,819-22.

Montague PR, Hyman SE, Cohen JD. 2004. Computational roles for dopamine in behavioural control. Nature 431,760-7.

Moore RJ, Vinsant SL, Nader MA, Porrino LJ, Friedman DP. 1998. Effect of cocaine self-administration on dopamine D2 receptors in rhesus monkeys. Synapse 30,88-96.

Moore RJ, Vinsant SL, Nader MA, Porrino LJ, Friedman DP. 1998. Effect of cocaine self-administration on striatal dopamine D1 receptors in rhesus monkeys. Synapse 28,1-9.

Moore RY, Speh JC. 1993. GABA is the principal neurotransmitter of the circadian system. Neurosci Lett 150,112-6.

Moratalla R, Elibol B, Vallejo M, Graybiel AM. 1996. Network-level changes in expression of inducible Fos-Jun proteins in the striatum during chronic cocaine treatment and withdrawal. Neuron 17,147-56.

Morin SM, Ling N, Liu XJ, Kahl SD, Gehlert DR. 1999. Differential distribution of urocortin- and corticotropin-releasing factor-like immunoreactivities in the rat brain. Neuroscience 92,281-91.

Mountjoy KG, Robbins LS, Mortrud MT, Cone RD. 1992. The cloning of a family of

genes that encode the melanocortin receptors. Science 257,1248-51.

Munoz-Abellan C, Andero R, Nadal R, Armario A. 2008. Marked dissociation between hypothalamic-pituitary-adrenal activation and long-term behavioral effects in rats exposed to immobilization or cat odor. Psychoneuroendocrinology 33,1139-50.

Murphy BL, Arnsten AF, Goldman-Rakic PS, Roth RH. 1996. Increased dopamine turnover in the prefrontal cortex impairs spatial working memory performance in rats and monkeys. Proc Natl Acad Sci U S A 93,1325-9.

Musselman DL, Nemeroff CB. 1996. Depression and endocrine disorders: focus on the thyroid and adrenal system. Br J Psychiatry Suppl,123-8.

Natelson BH, Tapp WN, Adamus JE, Mittler JC, Levin BE. 1981. Humoral indices of stress in rats. Physiol Behav 26,1049-54.

Navarro JF, Rivera A, Maldonado E, Cavas M, de la Calle A. 2004. Anxiogenic-like activity of 3,4-methylenedioxy-methamphetamine ("Ecstasy") in the social interaction test is accompanied by an increase of c-fos expression in mice amygdala. Prog Neuropsychopharmacol Biol Psychiatry 28,249-54.

Nedivi E. 1999. Molecular analysis of developmental plasticity in neocortex. J Neurobiol 41,135-47.

Nemeroff CB. 1998. Psychopharmacology of affective disorders in the 21st century. Biol Psychiatry 44,517-25.

Nestler EJ. 2001. Molecular basis of long-term plasticity underlying addiction. Nat Rev Neurosci 2,119-28.

Nestler EJ, Barrot M, DiLeone RJ, Eisch AJ, Gold SJ, Monteggia LM. 2002. Neurobiology of depression. Neuron 34,13-25.

Nikodemova M, Diehl CR, Aguilera G. 2002. Multiple sites of control of type-1 corticotropin releasing hormone receptor levels in the pituitary. Arch Physiol Biochem 110,123-8.

Nikulina EM, Marchand JE, Kream RM, Miczek KA. 1998. Behavioral sensitization to cocaine after a brief social stress is accompanied by changes in fos expression in the murine brainstem. Brain Res 810,200-10.

Nishi M, Kawata M. 2007. Dynamics of glucocorticoid receptor and mineralocorticoid receptor: implications from live cell imaging studies. Neuroendocrinology 85,186-92.

Noguchi T, Makino S, Matsumoto R, Nakayama S, Nishiyama M, Terada Y, Hashimoto K. 2010. Regulation of glucocorticoid receptor transcription and nuclear translocation during single and repeated immobilization stress. Endocrinology 151,4344-55.

Nowend KL, Arizzi M, Carlson BB, Salamone JD. 2001. D1 or D2 antagonism in nucleus accumbens core or dorsomedial shell suppresses lever pressing for food but leads to compensatory increases in chow consumption. Pharmacol Biochem Behav 69,373-82.

O'Connor KA, Ginsberg AB, Maksimova E, Wieseler Frank JL, Johnson JD, Spencer RL, Campeau S, Watkins LR, Maier SF. 2004. Stress-induced sensitization of the hypothalamic-pituitary adrenal axis is associated with alterations of hypothalamic and pituitary gene expression. Neuroendocrinology 80,252-63.

O'Connor KA, Johnson JD, Hansen MK, Wieseler Frank JL, Maksimova E, Watkins LR, Maier SF. 2003. Peripheral and central proinflammatory cytokine response to a severe acute stressor. Brain Res 991,123-32.

Oitzl MS, Reichardt HM, Joels M, De Kloet ER. 2001. Point mutation in the mouse glucocorticoid receptor preventing DNA binding impairs spatial memory. Proc Natl Acad Sci U S A 98,12790-5.

Ons S, Marti O, Armario A. 2004. Stress-induced activation of the immediate early gene Arc (activity-regulated cytoskeleton-associated protein) is restricted to telencephalic areas in the rat brain: relationship to c-fos mRNA. J Neurochem 89,1111-8.

Ons S, Rotllant D, Marin-Blasco IJ, Armario A. 2010. Immediate-early gene response to repeated immobilization: Fos protein and arc mRNA levels appear to be less sensitive than c-fos mRNA to adaptation. Eur J Neurosci 31,2043-52.

Ostrander MM, Richtand NM, Herman JP. 2003. Stress and amphetamine induce Fos expression in medial prefrontal cortex neurons containing glucocorticoid receptors. Brain Res 990,209-14.

Pacak K, Baffi JS, Kvetnansky R, Goldstein DS, Palkovits M. 1998. Stressor-specific activation of catecholaminergic systems: implications for stress-related hypothalamic-pituitary-adrenocortical responses. Adv Pharmacol 42,561-4.

Pacak K, Palkovits M. 2001. Stressor specificity of central neuroendocrine responses: implications for stress-related disorders. Endocr Rev 22,502-48.

Pace TW, Gaylord R, Topczewski F, Girotti M, Rubin B, Spencer RL. 2005. Immediate-early gene induction in hippocampus and cortex as a result of novel experience is not directly related to the stressfulness of that experience. Eur J Neurosci 22,1679-90.

Pan L, Gilbert F. 1992. Activation of 5-HT1A receptor subtype in the paraventricular nuclei of the hypothalamus induces CRH and ACTH release in the rat. Neuroendocrinology 56,797-802.

Papasava M, Singer G. 1985. Self-administration of low-dose cocaine by rats at reduced and recovered body weight. Psychopharmacology (Berl) 85,419-25.

Papavassiliou AG, Treier M, Chavrier C, Bohmann D. 1992. Targeted degradation of c-Fos, but not v-Fos, by a phosphorylation-dependent signal on c-Jun. Science 258,1941-4.

Parkes D, Rivest S, Lee S, Rivier C, Vale W. 1993. Corticotropin-releasing factor activates c-fos, NGFI-B, and corticotropin-releasing factor gene expression within the paraventricular nucleus of the rat hypothalamus. Mol Endocrinol 7,1357-67.

Pastor-Ciurana J, Rabasa C, Ortega-Sanchez JA, Sanchis-Olle M, Gabriel-Salazar

M, Ginesta M, Belda X, Daviu N, Nadal R, Armario A. 2014. Prior exposure to repeated immobilization or chronic unpredictable stress protects from some negative sequels of an acute immobilization. Behav Brain Res 265,155-62.

Paulmyer-Lacroix O, Anglade G, Grino M. 1994. Insulin-induced hypoglycaemia increases colocalization of corticotrophin-releasing factor and arginine vasopressin mRNAs in the rat hypothalamic paraventricular nucleus. J Mol Endocrinol 13,313-20.

Paxinos G, Watson C. 1998. The rat brain in stereotaxic coordinates. Academic press, INC., London.

Paxinos GW, C. 2006. The Rat Brain in Stereotaxic Coordinates.456.

Pellow S, File SE. 1986. Anxiolytic and anxiogenic drug effects on exploratory activity in an elevated plus-maze: a novel test of anxiety in the rat. Pharmacol Biochem Behav 24,525-9.

Petit-Demouliere B, Chenu F, Bourin M. 2005. Forced swimming test in mice: a review of antidepressant activity. Psychopharmacology (Berl) 177,245-55.

Pettit HO, Ettenberg A, Bloom FE, Koob GF. 1984. Destruction of dopamine in the nucleus accumbens selectively attenuates cocaine but not heroin self-administration in rats. Psychopharmacology (Berl) 84,167-73.

Pettit HO, Justice JB, Jr. 1989. Dopamine in the nucleus accumbens during cocaine self-administration as studied by in vivo microdialysis. Pharmacol Biochem Behav 34,899-904.

Piazza PV, Barrot M, Rouge-Pont F, Marinelli M, Maccari S, Abrous DN, Simon H, Le Moal M. 1996. Suppression of glucocorticoid secretion and antipsychotic drugs have similar effects on the mesolimbic dopaminergic transmission. Proc Natl Acad Sci U S A 93,15445-50.

Piazza PV, Deminiere JM, le Moal M, Simon H. 1990. Stress- and pharmacologically-induced behavioral sensitization increases vulnerability to acquisition of amphetamine self-administration. Brain Res 514,22-6.

Piazza PV, Le Moal M. 1997. Glucocorticoids as a biological substrate of reward: physiological and pathophysiological implications. Brain Res Brain Res Rev 25,359-72.

Piazza PV, Le Moal M. 1998. The role of stress in drug self-administration. Trends Pharmacol Sci 19,67-74.

Piazza PV, Le Moal ML. 1996. Pathophysiological basis of vulnerability to drug abuse: role of an interaction between stress, glucocorticoids, and dopaminergic neurons. Annu Rev Pharmacol Toxicol 36,359-78.

Piazza PV, Marinelli M, Jodogne C, Deroche V, Rouge-Pont F, Maccari S, Le Moal M, Simon H. 1994. Inhibition of corticosterone synthesis by Metyrapone decreases cocaine-induced locomotion and relapse of cocaine self-administration. Brain Res 658,259-64.

Plaznik A, Stefanski R, Kostowski W. 1989. Restraint stress-induced changes in saccharin preference: the effect of antidepressive treatment and diazepam.

Pharmacol Biochem Behav 33,755-9.

Plotsky PM. 1991. Pathways to the secretion of adrenocorticotropin: a view from the portal*. J Neuroendocrinol 3,1-9.

Plotsky PM, Thrivikraman KV, Meaney MJ. 1993. Central and feedback regulation of hypothalamic corticotropin-releasing factor secretion. Ciba Found Symp 172,59-75; discussion 75-84.

Porsolt RD. 1979. Animal model of depression. Biomedicine 30,139-40.

Porsolt RD, Le Pichon M, Jalfre M. 1977. Depression: a new animal model sensitive to antidepressant treatments. Nature 266,730-2.

Post RM, Weiss SR, Fontana D, Pert A. 1992. Conditioned sensitization to the psychomotor stimulant cocaine. Ann N Y Acad Sci 654,386-99.

Potter E, Sutton S, Donaldson C, Chen R, Perrin M, Lewis K, Sawchenko PE, Vale W. 1994. Distribution of corticotropin-releasing factor receptor mRNA expression in the rat brain and pituitary. Proc Natl Acad Sci U S A 91,8777-81.

Prasad BM, Ulibarri C, Sorg BA. 1998. Stress-induced cross-sensitization to cocaine: effect of adrenalectomy and corticosterone after short- and long-term withdrawal. Psychopharmacology (Berl) 136,24-33.

Prince CR, Anisman H. 1984. Acute and chronic stress effects on performance in a forced-swim task. Behav Neural Biol 42,99-119.

Przegalinski E, Filip M, Siwanowicz J, Nowak E. 2000. Effect of adrenalectomy and corticosterone on cocaine-induced sensitization in rats. J Physiol Pharmacol 51,193-204.

Puig S, Noble F, Benturquia N. 2012. Short- and long-lasting behavioral and neurochemical adaptations: relationship with patterns of cocaine administration and expectation of drug effects in rats. Transl Psychiatry 2,e175.

Rabasa CP. 2008. Una història prèvia d'estrès crònic redueix l'impacte de l'exposició a una situaciò d'estrès agut d'elevada intensitat. Master Thesis. Unpublished.

Radley JJ, Arias CM, Sawchenko PE. 2006. Regional differentiation of the medial prefrontal cortex in regulating adaptive responses to acute emotional stress. J Neurosci 26,12967-76.

Radley JJ, Williams B, Sawchenko PE. 2008. Noradrenergic innervation of the dorsal medial prefrontal cortex modulates hypothalamo-pituitary-adrenal responses to acute emotional stress. J Neurosci 28,5806-16.

Ramos-Aliaga R, Werner G. 1992. Pituitary-Adrenocortical response in acutely and chronically cocaine-treatment rats. Research Communications in substances Abuse,29-48.

Ranaldi R, Wise RA. 2001. Blockade of D1 dopamine receptors in the ventral tegmental area decreases cocaine reward: possible role for dendritically released dopamine. J Neurosci 21,5841-6.

Regier PS, Carroll ME, Meisel RL. 2012. Cocaine-induced c-Fos expression in rats selectively bred for high or low saccharin intake and in rats selected for high or

low impulsivity. Behav Brain Res 233,271-9.

Reul JM, De Kloet ER. 1985. Two receptor systems for corticosterone in rat brain: microdistribution and differential occupation. Endocrinology 117,2505-11.

Richter RM, Pich EM, Koob GF, Weiss F. 1995. Sensitization of cocaine-stimulated increase in extracellular levels of corticotropin-releasing factor from the rat amygdala after repeated administration as determined by intracranial microdialysis. Neurosci Lett 187,169-72.

Richter RM, Weiss F. 1999. In vivo CRF release in rat amygdala is increased during cocaine withdrawal in self-administering rats. Synapse 32,254-61.

Risold PY, Swanson LW. 1997. Connections of the rat lateral septal complex. Brain Res Brain Res Rev 24,115-95.

Ritter S, Watts AG, Dinh TT, Sanchez-Watts G, Pedrow C. 2003. Immunotoxin lesion of hypothalamically projecting norepinephrine and epinephrine neurons differentially affects circadian and stressor-stimulated corticosterone secretion. Endocrinology 144,1357-67.

Ritz MC, Lamb RJ, Goldberg SR, Kuhar MJ. 1987. Cocaine receptors on dopamine transporters are related to self-administration of cocaine. Science 237,1219-23.

Rivest S, Laflamme N. 1995. Neuronal activity and neuropeptide gene transcription in the brains of immune-challenged rats. J Neuroendocrinol 7,501-25.

Rivier C, Lee S. 1994. Stimulatory effect of cocaine on ACTH secretion: role of the hypothalamus. Mol Cell Neurosci 5,189-95.

Rivier C, Vale W. 1983. Interaction of corticotropin-releasing factor and arginine vasopressin on adrenocorticotropin secretion in vivo. Endocrinology 113,939-42.

Rivier C, Vale W. 1987. Cocaine stimulates adrenocorticotropin (ACTH) secretion through a corticotropin-releasing factor (CRF)-mediated mechanism. Brain Res 422,403-6.

Robbins SJ, Ehrman RN, Childress AR, O'Brien CP. 1999. Comparing levels of cocaine cue reactivity in male and female outpatients. Drug Alcohol Depend 53,223-30.

Robbins TW, Everitt BJ. 1996. Neurobehavioural mechanisms of reward and motivation. Curr Opin Neurobiol 6,228-36.

Roberts DC, Koob GF. 1982. Disruption of cocaine self-administration following 6-hydroxydopamine lesions of the ventral tegmental area in rats. Pharmacol Biochem Behav 17,901-4.

Roberts DC, Koob GF, Klonoff P, Fibiger HC. 1980. Extinction and recovery of cocaine self-administration following 6-hydroxydopamine lesions of the nucleus accumbens. Pharmacol Biochem Behav 12,781-7.

Robertson LM, Kerppola TK, Vendrell M, Luk D, Smeyne RJ, Bocchiaro C, Morgan JI, Curran T. 1995. Regulation of c-fos expression in transgenic mice requires multiple interdependent transcription control elements. Neuron 14,241-52.

Robinson TE, Berridge KC. 2003. Addiction. Annu Rev Psychol 54,25-53.

Robinson TE, Kolb B. 2004. Structural plasticity associated with exposure to drugs of abuse. Neuropharmacology 47 Suppl 1,33-46.

Rodriguez-Fornells A, Riba J, Gironell A, Kulisevsky J, Barbanoj MJ. 1999. Effects of alprazolam on the acoustic startle response in humans. Psychopharmacology (Berl) 143,280-5.

Rogerio R, Takahashi RN. 1992. Anxiogenic properties of cocaine in the rat evaluated with the elevated plus-maze. Pharmacol Biochem Behav 43,631-3.

Roland BL, Sawchenko PE. 1993. Local origins of some GABAergic projections to the paraventricular and supraoptic nuclei of the hypothalamus in the rat. J Comp Neurol 332,123-43.

Romach MK, Glue P, Kampman K, Kaplan HL, Somer GR, Poole S, Clarke L, Coffin V, Cornish J, O'Brien CP and others. 1999. Attenuation of the euphoric effects of cocaine by the dopamine D1/D5 antagonist ecopipam (SCH 39166). Arch Gen Psychiatry 56,1101-6.

Romero LM, Sapolsky RM. 1996. Patterns of ACTH secretagog secretion in response to psychological stimuli. J Neuroendocrinol 8,243-58.

Rothman RB, Baumann MH, Dersch CM, Romero DV, Rice KC, Carroll FI, Partilla JS. 2001. Amphetamine-type central nervous system stimulants release norepinephrine more potently than they release dopamine and serotonin. Synapse 39,32-41.

Rotllant D, Marquez C, Nadal R, Armario A. 2010. The brain pattern of c-fos induction by two doses of amphetamine suggests different brain processing pathways and minor contribution of behavioural traits. Neuroscience 168,691-705.

Rotllant D, Nadal R, Armario A. 2007. Differential effects of stress and amphetamine administration on Fos-like protein expression in corticotropin releasing factor-neurons of the rat brain. Dev Neurobiol 67,702-14.

Rotllant D, Ons S, Carrasco J, Armario A. 2002. Evidence that metyrapone can act as a stressor: effect on pituitary-adrenal hormones, plasma glucose and brain c-fos induction. Eur J Neurosci 16,693-700.

Rouge-Pont F, Marinelli M, Le Moal M, Simon H, Piazza PV. 1995. Stress-induced sensitization and glucocorticoids. II. Sensitization of the increase in extracellular dopamine induced by cocaine depends on stress-induced corticosterone secretion. J Neurosci 15,7189-95.

Rounsaville BJ, Anton SF, Carroll K, Budde D, Prusoff BA, Gawin F. 1991. Psychiatric diagnoses of treatment-seeking cocaine abusers. Arch Gen Psychiatry 48,43-51.

Ryabinin AE, Wang YM, Bachtell RK, Kinney AE, Grubb MC, Mark GP. 2000. Cocaine- and alcohol-mediated expression of inducible transcription factors is blocked by pentobarbital anesthesia. Brain Res 877,251-61.

Samaha AN, Mallet N, Ferguson SM, Gonon F, Robinson TE. 2004. The rate of

cocaine administration alters gene regulation and behavioral plasticity: implications for addiction. J Neurosci 24,6362-70.

Saper C. 1995. Central autonomic system. In: Paxinos, G. (Ed.), The Rat Nervous System (2ed). Academic Press, Sidney,107-135.

Saphier D, Welch JE, Farrar GE, Goeders NE. 1993. Effects of intracerebroventricular and intrahypothalamic cocaine administration on adrenocortical secretion. Neuroendocrinology 57,54-62.

Sapolsky RM. 1992. Do glucocorticoid concentrations rise with age in the rat? Neurobiol Aging 13,171-4.

Sapolsky RM, Armanini MP, Packan DR, Sutton SW, Plotsky PM. 1990. Glucocorticoid feedback inhibition of adrenocorticotropic hormone secretagogue release. Relationship to corticosteroid receptor occupancy in various limbic sites. Neuroendocrinology 51,328-36.

Sapolsky RM, Krey LC, McEwen BS. 1984. Glucocorticoid-sensitive hippocampal neurons are involved in terminating the adrenocortical stress response. Proc Natl Acad Sci U S A 81,6174-7.

Sapolsky RM, Krey LC, McEwen BS. 1986. The neuroendocrinology of stress and aging: the glucocorticoid cascade hypothesis. Endocr Rev 7,284-301.

Sapolsky RM, Romero LM, Munck AU. 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocr Rev 21,55-89.

Sarinana J, Kitamura T, Kunzler P, Sultzman L, Tonegawa S. 2014. Differential roles of the dopamine 1-class receptors, D1R and D5R, in hippocampal dependent memory. Proc Natl Acad Sci U S A 111,8245-50.

Sarnyai Z, Biro E, Babarczy E, Vecsernyes M, Laczi F, Szabo G, Krivan M, Kovacs GL, Telegdy G. 1992. Oxytocin modulates behavioural adaptation to repeated treatment with cocaine in rats. Neuropharmacology 31,593-8.

Sarnyai Z, Biro E, Gardi J, Vecsernyes M, Julesz J, Telegdy G. 1993. Alterations of corticotropin-releasing factor-like immunoreactivity in different brain regions after acute cocaine administration in rats. Brain Res 616,315-9.

Sarnyai Z, Biro E, Gardi J, Vecsernyes M, Julesz J, Telegdy G. 1995. Brain corticotropin-releasing factor mediates 'anxiety-like' behavior induced by cocaine withdrawal in rats. Brain Res 675,89-97.

Sarnyai Z, Mello NK, Mendelson JH, Eros-Sarnyai M, Mercer G. 1996. Effects of cocaine on pulsatile activity of hypothalamic-pituitary-adrenal axis in male rhesus monkeys: neuroendocrine and behavioral correlates. J Pharmacol Exp Ther 277,225-34.

Sarnyai Z, Shaham Y, Heinrichs SC. 2001. The role of corticotropin-releasing factor in drug addiction. Pharmacol Rev 53,209-43.

Satel SL, Price LH, Palumbo JM, McDougle CJ, Krystal JH, Gawin F, Charney DS, Heninger GR, Kleber HD. 1991. Clinical phenomenology and neurobiology of cocaine abstinence: a prospective inpatient study. Am J Psychiatry 148,1712-6.

Savasta M, Dubois A, Scatton B. 1986. Autoradiographic localization of D1 dopamine receptors in the rat brain with [3H]SCH 23390. Brain Res 375,291-301.

Sawchenko PE, Arias C. 1995. Evidence for short-loop feedback effects of ACTH on CRF and vasopressin expression in parvocellular neurosecretory neurons. J Neuroendocrinol 7,721-31.

Sawchenko PE, Li HY, Ericsson A. 2000. Circuits and mechanisms governing hypothalamic responses to stress: a tale of two paradigms. Prog Brain Res 122,61-78.

Sawchenko PE, Pfeiffer SW. 1995. Ultrastructural localization of inhibin beta- and somatostatin-28-immunoreactivities in the paraventricular and supraoptic nuclei. Brain Res 694,233-45.

Sawchenko PE, Swanson LW, Steinbusch HW, Verhofstad AA. 1983. The distribution and cells of origin of serotonergic inputs to the paraventricular and supraoptic nuclei of the rat. Brain Res 277,355-60.

Schechter MD. 1998. MDMA-like stimulus effects of hallucinogens in male Fawn-Hooded rats. Pharmacol Biochem Behav 59,265-70.

Schmidt ED, Tilders FJ, Janszen AW, Binnekade R, De Vries TJ, Schoffelmeer AN. 1995. Intermittent cocaine exposure causes delayed and long-lasting sensitization of cocaine-induced ACTH secretion in rats. Eur J Pharmacol 285,317-21.

Schulkin J, Gold PW, McEwen BS. 1998. Induction of corticotropin-releasing hormone gene expression by glucocorticoids: implication for understanding the states of fear and anxiety and allostatic load. Psychoneuroendocrinology 23,219-43.

Schulkin J, Morgan MA, Rosen JB. 2005. A neuroendocrine mechanism for sustaining fear. Trends Neurosci 28,629-35.

Scibilia RJ, Lachowicz JE, Kilts CD. 1992. Topographic nonoverlapping distribution of D1 and D2 dopamine receptors in the amygdaloid nuclear complex of the rat brain. Synapse 11,146-54.

Sebens JB, Koch T, Ter Horst GJ, Korf J. 1995. Differential Fos-protein induction in rat forebrain regions after acute and long-term haloperidol and clozapine treatment. Eur J Pharmacol 273,175-82.

See RE. 2002. Neural substrates of conditioned-cued relapse to drug-seeking behavior. Pharmacol Biochem Behav 71,517-29.

Sellings LH, McQuade LE, Clarke PB. 2006. Evidence for multiple sites within rat ventral striatum mediating cocaine-conditioned place preference and locomotor activation. J Pharmacol Exp Ther 317,1178-87.

Selye H. 1936. A Syndrome Produced by Diverse Nocuous Agents. The Journal of Neuropsychiatry & Clinical Neurosciences.

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

Senba E, Umemoto S, Kawai Y, Noguchi K. 1994. Differential expression of fos family and jun family mRNAs in the rat hypothalamo-pituitary-adrenal axis after immobilization stress. Brain Res Mol Brain Res 24,283-94.

Servatius RJ, Beck KD, Moldow RL, Salameh G, Tumminello TP, Short KR. 2005. A stress-induced anxious state in male rats: corticotropin-releasing hormone induces persistent changes in associative learning and startle reactivity. Biol Psychiatry 57,865-72.

Sewer MB, Waterman MR. 2003. ACTH modulation of transcription factors responsible for steroid hydroxylase gene expression in the adrenal cortex. Microsc Res Tech 61,300-7.

Shaham Y, Erb S, Leung S, Buczek Y, Stewart J. 1998. CP-154,526, a selective, non-peptide antagonist of the corticotropin-releasing factor1 receptor attenuates stress-induced relapse to drug seeking in cocaine- and heroin-trained rats. Psychopharmacology (Berl) 137,184-90.

Shaham Y, Erb S, Stewart J. 2000. Stress-induced relapse to heroin and cocaine seeking in rats: a review. Brain Res Brain Res Rev 33,13-33.

Shalev U, Grimm JW, Shaham Y. 2002. Neurobiology of relapse to heroin and cocaine seeking: a review. Pharmacol Rev 54,1-42.

Shalev U, Marinelli M, Baumann MH, Piazza PV, Shaham Y. 2003. The role of corticosterone in food deprivation-induced reinstatement of cocaine seeking in the rat. Psychopharmacology (Berl) 168,170-6.

Shanks N, Anisman H. 1988. Stressor-provoked behavioral changes in six strains of mice. Behav Neurosci 102,894-905.

Sheng M, Greenberg ME. 1990. The regulation and function of c-fos and other immediate early genes in the nervous system. Neuron 4,477-85.

Shih JC. 1991. Molecular basis of human MAO A and B. Neuropsychopharmacology 4,1-7.

Shih JC, Chen K, Ridd MJ. 1999. Role of MAO A and B in neurotransmitter metabolism and behavior. Pol J Pharmacol 51,25-9.

Shih JC, Thompson RF. 1999. Monoamine oxidase in neuropsychiatry and behavior. Am J Hum Genet 65,593-8.

Shippenberg TS, Heidbreder C. 1995. Sensitization to the conditioned rewarding effects of cocaine: pharmacological and temporal characteristics. J Pharmacol Exp Ther 273,808-15.

Sholar MB, Mendelson JH, Mello NK, Siegel AJ, Kaufman MJ, Levin JM, Renshaw PF, Cohen BM. 1998. Concurrent pharmacokinetic analysis of plasma cocaine and adrenocorticotropic hormone in men. J Clin Endocrinol Metab 83,966-8.

Simmons DM, Arriza JL, Swanson LW. 1989. A Complete Protocol for In Situ Hybridization of Messenger RNAs in Brain and Other Tissues with Radio-Labelled Single-Stranded RNA Probes. J. Histotechnol. 12,169-181.

Simmons DM, Swanson LW. 2009. Comparing histological data from different brains:

sources of error and strategies for minimizing them. Brain Res Rev 60,349-67.

Sinha R. 2001. How does stress increase risk of drug abuse and relapse? Psychopharmacology (Berl) 158,343-59.

Sinha R. 2005. Stress and drug abuse. In: Steckler, T. et al. (Eds.), Handbook of stress and brain. Elsevier 15,333-356.

Sinha R, Fuse T, Aubin LR, O'Malley SS. 2000. Psychological stress, drug-related cues and cocaine craving. Psychopharmacology (Berl) 152,140-8.

Skultetyova I, Jezova D. 1999. Dissociation of changes in hypothalamic corticotropin-releasing hormone and pituitary proopiomelanocortin mRNA levels after prolonged stress exposure. Brain Res Mol Brain Res 68,190-2.

Smagin GN, Heinrichs SC, Dunn AJ. 2001. The role of CRH in behavioral responses to stress. Peptides 22,713-24.

Smith SM, Vaughan JM, Donaldson CJ, Rivier J, Li C, Chen A, Vale WW. 2004. Cocaine- and amphetamine-regulated transcript activates the hypothalamic-pituitary-adrenal axis through a corticotropin-releasing factor receptor-dependent mechanism. Endocrinology 145,5202-9.

Snyder-Keller AM. 1991. Striatal c-fos induction by drugs and stress in neonatally dopamine-depleted rats given nigral transplants: importance of NMDA activation and relevance to sensitization phenomena. Exp Neurol 113,155-65.

Spyraki C, Fibiger HC, Phillips AG. 1982. Cocaine-induced place preference conditioning: lack of effects of neuroleptics and 6-hydroxydopamine lesions. Brain Res 253,195-203.

Stefanski V. 2001. Social stress in laboratory rats: behavior, immune function, and tumor metastasis. Physiol Behav 73,385-91.

Steiner H, Gerfen CR. 1993. Cocaine-induced c-fos messenger RNA is inversely related to dynorphin expression in striatum. J Neurosci 13,5066-81.

Steiner H, Gerfen CR. 1995. Dynorphin opioid inhibition of cocaine-induced, D1 dopamine receptor-mediated immediate-early gene expression in the striatum. J Comp Neurol 353,200-12.

Stephenson CP, Hunt GE, Topple AN, McGregor IS. 1999. The distribution of 3,4-methylenedioxymethamphetamine "Ecstasy"-induced c-fos expression in rat brain. Neuroscience 92,1011-23.

Steptoe A, Kearsley N, Walters N. 1993. Cardiovascular activity during mental stress following vigorous exercise in sportsmen and inactive men. Psychophysiology 30,245-52.

Stratakis CA, Chrousos GP. 1995. Neuroendocrinology and pathophysiology of the stress system. Ann N Y Acad Sci 771,1-18.

Sumner BE, Cruise LA, Slattery DA, Hill DR, Shahid M, Henry B. 2004. Testing the validity of c-fos expression profiling to aid the therapeutic classification of psychoactive drugs. Psychopharmacology (Berl) 171,306-21.

Swaab DF. 2004. Neuropeptides in hypothalamic neuronal disorders. Int Rev Cytol

240,305-75.

Swanson LW, Sawchenko PE, Rivier J, Vale WW. 1983. Organization of ovine corticotropin-releasing factor immunoreactive cells and fibers in the rat brain: an immunohistochemical study. Neuroendocrinology 36,165-86.

Szucs RP, Frankel PS, McMahon LR, Cunningham KA. 2005. Relationship of cocaine-induced c-Fos expression to behaviors and the role of serotonin 5-HT2A receptors in cocaine-induced c-Fos expression. Behav Neurosci 119,1173-83.

Szumlinski KK, Frys KA, Kalivas PW. 2004. Dissociable roles for the dorsal and median raphe in the facilitatory effect of 5-HT1A receptor stimulation upon cocaine-induced locomotion and sensitization. Neuropsychopharmacology 29,1675-87.

Tanimura SM, Watts AG. 1998. Corticosterone can facilitate as well as inhibit corticotropin-releasing hormone gene expression in the rat hypothalamic paraventricular nucleus. Endocrinology 139,3830-6.

Tanimura SM, Watts AG. 2000. Adrenalectomy dramatically modifies the dynamics of neuropeptide and c-fos gene responses to stress in the hypothalamic paraventricular nucleus. J Neuroendocrinol 12,715-22.

Tatro JB. 1996. Receptor biology of the melanocortins, a family of neuroimmunomodulatory peptides. Neuroimmunomodulation 3,259-84.

Teoh SK, Mendelson JH, Woods BT, Mello NK, Hallgring E, Anfinsen P, Douglas A, Mercer G. 1993. Pituitary volume in men with concurrent heroin and cocaine dependence. J Clin Endocrinol Metab 76,1529-32.

Thiel KJ, Wenzel JM, Pentkowski NS, Hobbs RJ, Alleweireldt AT, Neisewander JL. 2010. Stimulation of dopamine D2/D3 but not D1 receptors in the central amygdala decreases cocaine-seeking behavior. Behav Brain Res 214,386-94.

Thomas WL, Jr., Cooke ES, Hammer RP, Jr. 1996. Cocaine-induced sensitization of metabolic activity in extrapyramidal circuits involves prior dopamine D1-like receptor stimulation. J Pharmacol Exp Ther 278,347-53.

Thorpe LW, Westlund KN, Kochersperger LM, Abell CW, Denney RM. 1987. Immunocytochemical localization of monoamine oxidases A and B in human peripheral tissues and brain. J Histochem Cytochem 35,23-32.

Tidey JW, Miczek KA. 1996. Social defeat stress selectively alters mesocorticolimbic dopamine release: an in vivo microdialysis study. Brain Res 721,140-9.

Torres G, Horowitz JM. 1999. Drugs of abuse and brain gene expression. Psychosom Med 61,630-50.

Torres G, Rivier C. 1992. Cocaine-induced ACTH secretion: dependence of plasma levels of the drug and mode of exposure. Brain Res Bull 29,51-6.

Torres G, Rivier C. 1992. Differential effects of intermittent or continuous exposure to cocaine on the hypothalamic-pituitary-adrenal axis and c-fos expression. Brain Res 571,204-11.

Torres G, Rivier C. 1994. Induction of c-fos in rat brain by acute cocaine and fenfluramine exposure: a comparison study. Brain Res 647,1-9.

Trevitt JT, Carlson BB, Nowend K, Salamone JD. 2001. Substantia nigra pars reticulata is a highly potent site of action for the behavioral effects of the D1 antagonist SCH 23390 in the rat. Psychopharmacology (Berl) 156,32-41.

Trneckova L, Rotllant D, Klenerova V, Hynie S, Armario A. 2007. Dynamics of immediate early gene and neuropeptide gene response to prolonged immobilization stress: evidence against a critical role of the termination of exposure to the stressor. J Neurochem 100,905-14.

Tsukada H, Kreuter J, Maggos CE, Unterwald EM, Kakiuchi T, Nishiyama S, Futatsubashi M, Kreek MJ. 1996. Effects of binge pattern cocaine administration on dopamine D1 and D2 receptors in the rat brain: an in vivo study using positron emission tomography. J Neurosci 16,7670-7.

Turgeon SM, Pollack AE, Schusheim L, Fink JS. 1996. Effects of selective adenosine A1 and A2a agonists on amphetamine-induced locomotion and c-Fos in striatum and nucleus accumbens. Brain Res 707,75-80.

Tuvnes FA, Steffenach HA, Murison R, Moser MB, Moser EI. 2003. Selective hippocampal lesions do not increase adrenocortical activity. J Neurosci 23,4345-54.

Umemoto S, Kawai Y, Ueyama T, Senba E. 1997. Chronic glucocorticoid administration as well as repeated stress affects the subsequent acute immobilization stress-induced expression of immediate early genes but not that of NGFI-A. Neuroscience 80,763-73.

Unterwald EM, Ho A, Rubenfeld JM, Kreek MJ. 1994. Time course of the development of behavioral sensitization and dopamine receptor up-regulation during binge cocaine administration. J Pharmacol Exp Ther 270,1387-96.

Ursin H, Olff M. 1993. Psychobiology of coping and defence strategies. Neuropsychobiology 28,66-71.

Uslaner J, Badiani A, Day HE, Watson SJ, Akil H, Robinson TE. 2001. Environmental context modulates the ability of cocaine and amphetamine to induce c-fos mRNA expression in the neocortex, caudate nucleus, and nucleus accumbens. Brain Res 920,106-16.

Uslaner J, Badiani A, Norton CS, Day HE, Watson SJ, Akil H, Robinson TE. 2001. Amphetamine and cocaine induce different patterns of c-fos mRNA expression in the striatum and subthalamic nucleus depending on environmental context. Eur J Neurosci 13,1977-83.

Vahl TP, Ulrich-Lai YM, Ostrander MM, Dolgas CM, Elfers EE, Seeley RJ, D'Alessio DA, Herman JP. 2005 Comparative analysis of ACTH and corticosterone sampling methods in rats. Am J Physiol

Endocrinol Metab,289:E823-828.

Valentine JD, Matta SG, Sharp BM. 1996. Nicotine-induced cFos expression in the hypothalamic paraventricular nucleus is dependent on brainstem effects:

correlations with cFos in catecholaminergic and noncatecholaminergic neurons in the nucleus tractus solitarius. Endocrinology 137,622-30.

Valentino RJ, Reyes B, Van Bockstaele E, Bangasser D. 2011. Molecular and cellular sex differences at the intersection of stress and arousal. Neuropharmacology 62,13-20.

Valjent E, Pages C, Herve D, Girault JA, Caboche J. 2004. Addictive and non-addictive drugs induce distinct and specific patterns of ERK activation in mouse brain. Eur J Neurosci 19,1826-36.

Valles A, Marti O, Armario A. 2003. Long-term effects of a single exposure to immobilization stress on the hypothalamic-pituitary-adrenal axis: transcriptional evidence for a progressive desensitization process. Eur J Neurosci 18,1353-61.

Valles A, Marti O, Armario A. 2005. Mapping the areas sensitive to long-term endotoxin tolerance in the rat brain: a c-fos mRNA study. J Neurochem 93,1177-88.

Valles A, Marti O, Armario A. 2006. Long-term effects of a single exposure to immobilization: a c-fos mRNA study of the response to the homotypic stressor in the rat brain. J Neurobiol 66,591-602.

Valles A, Marti O, Garcia A, Armario A. 2000. Single exposure to stressors causes long-lasting, stress-dependent reduction of food intake in rats. Am J Physiol Regul Integr Comp Physiol 279,R1138-44.

Van de Kar LD, Javed A, Serres F, Raap DK, Gray TS. 2001. 5-HT2A receptors stimulate ACTH, corticosterone, oxytocin, renin, and prolactin release and activate hypothalamic CRF and oxytocin-expressing cells. J. Neurosci.,3572-3579.

van Dijken HH, de Goeij DC, Sutanto W, Mos J, De Kloet ER, Tilders FJ. 1993. Short inescapable stress produces long-lasting changes in the brain-pituitary-adrenal axis of adult male rats. Neuroendocrinology 58,57-64.

van Dijken HH, Mos J, van der Heyden JA, Tilders FJ. 1992. Characterization of stress-induced long-term behavioural changes in rats: evidence in favor of anxiety. Physiol Behav 52,945-51.

Vaughan J, Donaldson C, Bittencourt J, Perrin MH, Lewis K, Sutton S, Chan R, Turnbull AV, Lovejoy D, Rivier C and others. 1995. Urocortin, a mammalian neuropeptide related to fish urotensin I and to corticotropin-releasing factor. Nature 378,287-92.

Vescovi PP, Coiro V, Volpi R, Passeri M. 1992. Diurnal variations in plasma ACTH, cortisol and beta-endorphin levels in cocaine addicts. Horm Res 37,221-4.

Vigas M. 1984. Problems of definition of stress stimulus and specificity of stress response. In: Usdin E et al. (Eds), Stress, the role of catecholamines and other neurotransmitters: proceedings of the third international symposium on catecholamines and other neurotransmitters in stress, Gordon and Breach Sci Publ. 23-25.

Vinson GP, Hinson JP, Toth IE. 1994. The neuroendocrinology of the adrenal cortex.

J Neuroendocrinol 6,235-46.

Volkow ND, Fowler JS, Wang GJ. 1999. Imaging studies on the role of dopamine in cocaine reinforcement and addiction in humans. J Psychopharmacol 13,337-45.

Volkow ND, Fowler JS, Wang GJ. 2003. The addicted human brain: insights from imaging studies. J Clin Invest 111,1444-51.

Volkow ND, Fowler JS, Wang GJ, Hitzemann R, Logan J, Schlyer DJ, Dewey SL, Wolf AP. 1993. Decreased dopamine D2 receptor availability is associated with reduced frontal metabolism in cocaine abusers. Synapse 14,169-77.

Volkow ND, Wang GJ, Fischman MW, Foltin RW, Fowler JS, Abumrad NN, Vitkun S, Logan J, Gatley SJ, Pappas N and others. 1997. Relationship between subjective effects of cocaine and dopamine transporter occupancy. Nature 386,827-30.

Volkow ND, Wang GJ, Fowler JS, Tomasi D. 2011. Addiction circuitry in the human brain. Annu Rev Pharmacol Toxicol 52,321-36.

Watts AG, Sanchez-Watts G. 1995. Region-specific regulation of neuropeptide mRNAs in rat limbic forebrain neurones by aldosterone and corticosterone. J Physiol 484 (Pt 3),721-36.

Watts AG, Swanson LW. 1987. Efferent projections of the suprachiasmatic nucleus: II. Studies using retrograde transport of fluorescent dyes and simultaneous peptide immunohistochemistry in the rat. J Comp Neurol 258,230-52.

Weddington WW, Brown BS, Haertzen CA, Cone EJ, Dax EM, Herning RI, Michaelson BS. 1990. Changes in mood, craving, and sleep during short-term abstinence reported by male cocaine addicts. A controlled, residential study. Arch Gen Psychiatry 47,861-8.

Weiss F, Markou A, Lorang MT, Koob GF. 1992. Basal extracellular dopamine levels in the nucleus accumbens are decreased during cocaine withdrawal after unlimited-access self-administration. Brain Res 593,314-8.

Wenger T, Jamali KA, Juaneda C, Leonardelli J, Tramu G. 1997. Arachidonyl ethanolamide (anandamide) activates the parvocellular part of hypothalamic paraventricular nucleus. Biochem Biophys Res Commun 237,724-8.

Willuhn I, Sun W, Steiner H. 2003. Topography of cocaine-induced gene regulation in the rat striatum: relationship to cortical inputs and role of behavioural context. Eur J Neurosci 17,1053-66.

Woodmansee WW, Silbert LH, Maier SF. 1993. Factors that modulate inescapable shock-induced reductions in daily activity in the rat. Pharmacol Biochem Behav 45,553-9.

Woods SC, Seeley RJ, Porte D, Jr., Schwartz MW. 1998. Signals that regulate food intake and energy homeostasis. Science 280,1378-83.

Wu JC, Bell K, Najafi A, Widmark C, Keator D, Tang C, Klein E, Bunney BG, Fallon J, Bunney WE. 1997. Decreasing striatal 6-FDOPA uptake with increasing duration of cocaine withdrawal. Neuropsychopharmacology 17,402-9.

Xi ZX, Gilbert J, Campos AC, Kline N, Ashby CR, Jr., Hagan JJ, Heidbreder CA, Gardner EL. 2004. Blockade of mesolimbic dopamine D3 receptors inhibits stress-induced reinstatement of cocaine-seeking in rats. Psychopharmacology (Berl) 176,57-65.

Xu M, Moratalla R, Gold LH, Hiroi N, Koob GF, Graybiel AM, Tonegawa S. 1994. Dopamine D1 receptor mutant mice are deficient in striatal expression of dynorphin and in dopamine-mediated behavioral responses. Cell 79,729-42.

Xu Y, Day TA, Buller KM. 1999. The central amygdala modulates hypothalamic-pituitary-adrenal axis responses to systemic interleukin-1beta administration. Neuroscience 94,175-83.

Yuferov V, Kroslak T, Laforge KS, Zhou Y, Ho A, Kreek MJ. 2003. Differential gene expression in the rat caudate putamen after "binge" cocaine administration: advantage of triplicate microarray analysis. Synapse 48,157-69.

Zahrt J, Taylor JR, Mathew RG, Arnsten AF. 1997. Supranormal stimulation of D1 dopamine receptors in the rodent prefrontal cortex impairs spatial working memory performance. J Neurosci 17,8528-35.

Zaslav MR. 1994. Psychology of comorbid posttraumatic stress disorder and substance abuse: lessons from combat veterans. J Psychoactive Drugs 26,393-400.

Zhang D, Zhang L, Lou DW, Nakabeppu Y, Zhang J, Xu M. 2002. The dopamine D1 receptor is a critical mediator for cocaine-induced gene expression. J Neurochem 82,1453-64.

Zhou Y, Spangler R, Ho A, Kreek MJ. 2003. Increased CRH mRNA levels in the rat amygdala during short-term withdrawal from chronic 'binge' cocaine. Brain Res Mol Brain Res 114,73-9.

Zhou Y, Spangler R, LaForge KS, Maggos CE, Ho A, Kreek MJ. 1996. Corticotropin-releasing factor and type 1 corticotropin-releasing factor receptor messenger RNAs in rat brain and pituitary during "binge"-pattern cocaine administration and chronic withdrawal. J Pharmacol Exp Ther 279,351-8.

Zhou Y, Spangler R, LaForge KS, Maggos CE, Ho A, Kreek MJ. 1996. Modulation of CRF-R1 mRNA in rat anterior pituitary by dexamethasone: correlation with POMC mRNA. Peptides 17,435-41.

Zhou Y, Spangler R, Schlussman SD, Ho A, Kreek MJ. 2003. Alterations in hypothalamic-pituitary-adrenal axis activity and in levels of proopiomelanocortin and corticotropin-releasing hormone-receptor 1 mRNAs in the pituitary and hypothalamus of the rat during chronic 'binge' cocaine and withdrawal. Brain Res 964,187-99.

Zhou Y, Spangler R, Yuferov VP, Schlussmann SD, Ho A, Kreek MJ. 2004. Effects of selective D1- or D2-like dopamine receptor antagonists with acute "binge" pattern cocaine on corticotropin-releasing hormone and proopiomelanocortin mRNA levels in the hypothalamus. Brain Res Mol Brain Res 130,61-7.

Ziegler DR, Cullinan WE, Herman JP. 2002. Distribution of vesicular glutamate transporter mRNA in rat hypothalamus. J Comp Neurol 448,217-29.

Zito KA, Vickers G, Roberts DC. 1985. Disruption of cocaine and heroin self-administration following kainic acid lesions of the nucleus accumbens. Pharmacol Biochem Behav 23,1029-36.

Zombeck JA, Lewicki AD, Patel K, Gupta T, Rhodes JS. 2010. Patterns of neural activity associated with differential acute locomotor stimulation to cocaine and methamphetamine in adolescent versus adult male C57BL/6J mice. Neuroscience 165,1087-99.

Zorrilla EP, Logrip ML, Koob GF. 2014. Corticotropin releasing factor: a key role in the neurobiology of addiction. Front Neuroendocrinol 35,234-44.