LONG TERM

SYNAPTIC REORGANIZATION

AFTER IMMOBILIZATION STRESS

A focus on the **HIPPOCAMPUS** and the **TrkB** pathway

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RESUMEN
ABSTRACT

Resumen

El trastorno de estrés postraumático (PTSD) es un trastorno asociado a un trauma y a estrés que se manifiesta después de la exposición a situaciones traumáticas y se caracteriza por la aparición de alteraciones cognitivas tales como trastornos en la memoria. De hecho, la formación hipocampal (FH), una estructura clave en la formación de la memoria, es una de las regiones más afectada en el PTSD, presentando una reducción del volumen y anomalías funcionales. Aunque los efectos del estrés en la plasticidad sináptica se han relacionado con el PTSD, los cambios a largo plazo del estrés en la plasticidad sináptica son desconocidos. El estudio de modelos animales que simulan los síntomas encontrados en pacientes de PTSD puede ser una estrategia efectiva para comprender los efectos a largo plazo del estrés y encontrar nuevas dianas farmacológicas. En este sentido, diversos tratamientos han sido propuestos para el PTSD, sin embargo, ninguno de ellos ha resultado totalmente efectivo. La vía BDNF/TrkB se ha propuesto como una nueva diana debido a la importante implicación de esta vía en modelos animales de PTSD. Además, el estrés y el BDNF están regulados recíprocamente y, por lo tanto, es razonable asumir que el uso de agonistas para la vía de BDNF/TrkB, como la 7,8-dihydroxyflavone (7,8 DHF), puede ser un procedimiento eficaz para atenuar los efectos del estrés.

Para abordar esta cuestión, en un primer lugar analizamos si la IMO presenta efectos anatómicos y conductuales similares a los encontrados en los pacientes de PTSD que puedan determinar si es un modelo putativo de PTSD y la posible implicación de la plasticidad sináptica en los trastornos de la memoria. Por otro lado, investigamos si la administración del 7,8-DHF en la ventana terapéutica del PTSD puede atenuar los efectos de la IMO y si dichos efectos en plasticidad sináptica están relacionados con la alteración de la memoria. La IMO produjo una disminución del volumen de la FH y una alteración de la memoria en tareas dependientes de la misma, efectos similares a los encontrados en otros modelos y en los pacientes de PTSD. Además, la IMO incrementó la densidad de espinas, la LTP y los niveles de BDNF, HCN1 e Iba1. Por lo tanto, es razonable asumir que los efectos de la IMO en plasticidad sináptica están relacionados con las alteraciones en la memoria. Nosotros sugerimos que la IMO intenta compensar los efectos negativos del trauma inicial que derivan en una desconexión y una posterior reconexión de los circuitos implicados en la alteración de la memoria de una forma similar a lo que ocurre durante el desarrollo. El agonista 7,8-DHF previno las alteraciones en memoria espacial y el incremento de LTP sin encontrarse efecto del fármaco per se. Estos resultados sugieren un efecto protector del 7,8-DHF y apoyan la idea del papel neuroprotector de la potenciación de la vía BDNF/TrkB en esta patología y, por lo tanto, muestran que el 7,8-DHF puede constituir un posible tratamiento para los pacientes de PTSD.

Abstract

PTSD is a trauma and stressor-related disorder that occurs after the exposure to traumatic situations. PTSD is characterized by the development of cognitive impairments such as memory dysfunction. In fact, HF, a key structure in memory formation, is one of the most affected areas in PTSD, presenting reduced volume and functional abnormalities. Despite stress effects on synaptic plasticity have been related to PTSD, long term synaptic plasticity stress-related changes remain largely unknown. The study of animal models that mimic symptoms of PTSD patients can be an effective strategy to understand the long term consequences of stress and to find novel drug targets. In this regard, several treatments have been proposed for PTSD. However, none of them has been totally successful. The BDNF/TrkB pathway has been suggested as a novel strategy since animal research has revealed an important implication of this pathway in PTSD animal models. Moreover, stress and BDNF are reciprocally modulated. Hence, it is reasonable to believe that the use of BDNF/TrkB pathway agonists, such as 7,8-dihydroxyflavone, could be an effective way to ameliorate stress effects.

To address this issue we analyzed whether IMO presents similar anatomical and behavioral effects found in PTSD patients to be a putative PTSD model and the possible role of synaptic plasticity as a substrate for memory impairment. Alternatively, we investigated whether 7,8-DHF can ameliorate the PTSD-like effect of the IMO in a therapeutic window administration and whether synaptic plasticity IMO-related effects are causally related to memory impairment. IMO induced a HF volume decrease and HF-dependent task memory impairment similar to those found in others PTSD models or patients. In addition, IMO increased spine density, LTP, BDNF, HCN1 and Iba1 levels. Therefore, it is reasonable to assume that IMO-induced synaptic plasticity is involved in IMO-related memory impairment. We suggest that IMO induces a development-like process trying to compensate the detrimental effects of the initial trauma which derivate in a disconnection and posterior reconnection of the neural network impairing memory. The agonist prevented spatial memory impairment and LTP increase without effect of the drug per se. Taken together, all these results suggest a protective role of 7,8-DHF supporting the idea of a protective role of BDNF/TrkB pathway enhancement in PTSD and hence the value of 7,8-DHF as a putative treatment for PTSD patients.

CONTENTS

ABBREVIATIONS

5-HT: Serotonin
7,8-DHF: 7,8-dihydroxiflavone
ACTH: adrenocorticotropic hormone
AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
Arc: activity-regulated cytoskeleton-associated protein
AVP: arginine-vasopressin
BBB: brain-blood-barrier
BDNF: brain-derived neurotrophic factor
BLA: basolateral amygdala
BNST: bed nuclei of the stria terminalis
BOLD: blood-oxygen-level dependent
CA1-3: cornu ammonis 1-3
CAM: cell adhesion molecules
CaMKII: Calcium/calmodulin-dependent protein kinase II
CeA: central amygdala
CG: cingulated cortex
CNS: central nervous system
CRF or CRH: corticotropin-releasing factor or hormone
CRH-R1: corticotropin-releasing factor receptor 1
CSF: cerebrospinal fluid
DG: dentate gyrus
dlPFC: dorsolateral prefrontal cortex
DTI: diffusion tensor imaging
EAATs: excitatory amino acid transporters
EE: environmental enrichment
EPM: elevated plus maze
EPSP: excitatory postsynaptic potentials
ES: escapable-shock
fMRI: functional magnetic resonance
GABA: gamma-amino butyric acid
GDNF: glial cell line-derived neurotrophic factor
GFAP: glial fibrillary acidic protein
GirKs: G-protein-coupled inwarly-rectifying potassium channels
GluA1-4: AMPA receptors subunits 1-4
GluN1-3: NMDA receptors subunits 1-3
GR: glucocorticoid receptor (or glucocorticoid receptor type II)
HCN: hyperpolarization-activated cyclic nucleotide-gated channels
HF: hippocampal formation
HFS: high frequency stimulation
HPA: hypothalamic-pituitary-adrenal
Iba 1: ionized calcium binding adaptor molecule 1
IEGs: immediate early gens
IgG: immunoglobulin G
Ih: hyperpolarization-activated cation currents
IL: infralimbic cortex
IL-1: interleukin-1
IMO: immobilization on board
IS-LH: inescapable-shock
KO: knockout
LA: lateral amygdala
LFS: low frequency stimulation
LH: learned helplessness
lPFC: lateral prefrontal cortex
LS: lateral septum
LTD: long-term depression
LTP: long-term potentiation
MAPK: mitogen-activated protein kinase
MC2-R: type 2 melanocortin receptor
MeA: medial amygdala
mEPSC: miniature excitatory postsynaptic currents
mGluR1-8: metabotropic receptors 1-8
mpdPVN: medial dorsal paraventricular hypotalamic nucleus of the hypothalamus
mPFC: medial prefrontal cortex
mPVN: magnocellular paraventricular hypotalamic nucleus of the hypothalamus
MR: mineralocorticoid receptor (or glucocorticoid receptor type I)
MRI: magnetic resonance imaging
MRSI or 1H-MRS: magnetic resonance spectroscopic imaging
MWM: Morris water maze
NAA: N-acetylaspartate
NCAM: neural cell adhesion molecules
NGF: nerve growth factor
NMDA: N-methyl-D-aspartate
NT: neurotrophin
NT-3: neurotrophin 3
NT-4/5: neurotrophin 4/5
oPFC: orbital prefrontal cortex
p75ntr: neurotrophin receptor
PFC: prefrontal cortex
PKA: protein kinase A
PL: prelimbic cortex
PNS: peripheral nervous system
POMC: propiomelanocortin
pPVN: parvocellular paraventricular hypotalamic nucleus of the hypothalamus
PSA: polysialic acid
PSD 95: postsynaptic density 95
PSD: postsynaptic density
PTSD: Post-traumatic stress disorder
PVN: paraventricular hypotalamic nucleus of the hypothalamus
RF: radio frequency
SMA: sympathomedulloadrenal

SNP: single nucleotide polymorphism
SPS: single prolonged stress
SSRIs: selective serotonin reuptake inhibitors
STP: short-term potentiation
TMT: 2,3,5-trimethyl-3-thiazoline
Trk: tropomyosin-related tyrosine kinase receptor
TrkA: tropomyosin-related tyrosine kinase receptor A
TrkB: tropomyosin-related tyrosine kinase receptor B
TrkB-FL: full-length TrkB
TrkB-T1: TrkB truncated form 1
TrkB-T2: TrkB truncated form 2
TrkC: tropomyosin-related tyrosine kinase receptor C
VGF: VGF nerve growth factor inducible
vGluts: vesicular glutamate transporters
vmPFC: ventromedial prefrontal cortex
WT: wild-type

GENERAL INTRODUCTION

The concept of stress

Post-Traumatic Stress Disorder

PTSD animal models

Brain plasticity and stress

The concept of stress

The present concept of stress has been established through time. The basic principles of the stress concept are based on the studies of Walter B. Cannon at the beginning of twentieth century (Goldstein & McEwen 2002). He coined the term homeostasis using the concept of *Milieu Interieur,* introduced by Claude Bernard, which refers to the stability of the internal environment. Homeostasis is defined as the coordinated physiological processes that maintain steady states of critical variables in the organism despite environmental challenges. Although the first studies on stress were accomplished by Walter B. Cannon, the first author that coined the term "stress" was Hans Selye. He described the different physiological changes induced by sustained chronic stress, such as adrenal enlargement, gastrointestinal ulceration and thymico-lymphatic involution that happen with different kinds of harmful agents. Selye named these changes "the General Adaptation Syndrome", which presents three phases: alarm, resistance and exhaustion. In his work, he used what it is now considered as systemic (physical) stressors, but nowadays it is known that emotional stressors can also induce these changes.

There is no agreement about the definition of stress. For us, one of the best definitions is that from Vigas (1984): "The response to stress is the response of the organism, developed during evolution, in front of real, anticipated or symbolic events that cannot be solved through normal homeostatic mechanisms". To better understand the concept of stress, it is important to define the terms more precisely. A stressor is the stimulus that provokes a state of stress in the organism, thus resulting in the stress response, which is the set of behavioral and physiological changes that occurs in front of stress.

Stressors are classified in emotional (e.g. holeboard, novel environment, predator odor) and systemic (e.g. cold exposure, hemorrhage, infection). The latter ones represent a real physiological threat to homeostasis, whereas the former ones trigger a similar response, as an anticipation that something dangerous may follow. There is a third category, the mixed stressors (e.g. immobilization on boards (IMO) and forced swim), that contain both emotional and systemic components (mixed nature stressors). Whereas each particular systemic stressor has a well-defined pattern of activation in the Central Nervous System (CNS) (as evaluated with immediate early genes (IEGs) and in particular c-fos (Kovács 1998, Pacák & Palkovits 2001)), most of the emotional or mixed stressors activate a wide range of common brain areas (Armario 2006).

The complex set of responses to emotional stress is under the control of the limbic system (medial prefrontal cortex (mPFC), the hippocampal formation (HF), the amygdala and the lateral septum (LS)) (for review Herman et al. 2005, Jankord & Herman 2008). In order to control the hypothalamic-pituitary-adrenal (HPA) these nuclei send positive or negative inputs through polysynaptic pathways to the paraventricular nucleus of hypothalamus (PVN). Therefore, these limbic areas play an important role in stress response and are key targets in stress-related pathologies like the post-traumatic stress disorder.

The HPA axis

Stress is characterized by the activation of the HPA axis and the sympatho-medullo-adrenal (SMA) system. The HPA axis is one of the most important and well-studied stress markers due to the fact that its physiological and pathological effects are wider and stronger than those of the SMA system. Stressors are processed in several areas of the CNS, but this information always

converges at the PVN, independently of the stressor category. Neurosecretory neurons in the medial parvocellular subdivision of the PVN initiate the stress response by synthesizing and releasing corticotropin-releasing factor or hormone (CRF or CRH) into the pituitary portal blood vessels of the median eminence. CRH interacts with its type 1 receptors (CRH-R1) in the anterior pituitary gland and induces the synthesis of proopiomelanocortin (POMC), the precursor molecule of the adrenocorticotropic hormone (ACTH). ACTH is released into the systemic circulation and arrives to the adrenal cortex, where it activates the synthesis and release of glucocorticoids (corticosterone in rat and mouse and cortisol in most mammals).

The PVN is the initial point of HPA axis. It receives inputs from two major pathways: on one hand, systemic stressors induce HPA axis activation through direct pathways from brain stem and circumventricular areas; on the other hand, emotional stressors act via indirect pathways from telencephalic areas. The PVN nucleus is formed by two main regions: the magnocellular (mPVN) and the parvocellular (pPVN) region (Herman et al. 2003). The pPVN is subdivided in several subregions; however, the most important region for HPA axis is the medial dorsal divisions (mpdPVN). These neurons project to median eminence which is connected to the anterior pituitary gland via the pituitary portal circulation and mainly synthetized CRF and AVP. The mPVN projects to the posterior pituitary and mainly synthesizes arginine-vasopressin (AVP) and oxitocin. AVP is a 9 aminoacid peptide involved in the regulation of osmotic pressure, fluid balance, psychiatric disorders and cognition (Egashira et al. 2009). AVP plays a weak role in ACTH release, but it exerts a positive synergy on CRF effects.

As introduced previously, CRF induces the processing and release of ACTH in the anterior pituitary. This occurs 5-10 min after stress starts. ACTH is a 39 aminoacid peptide that increases the release and the synthesis of glucocorticoids through type 2 melanocortin receptor (MC2-R) in the *zona fasciculata* of the adrenal cortex glands (Adan & Gispen 2000). Glucocorticoids are the main effectors of HPA axis and are involved in the negative effects of stress.

Glucocorticoids signaling

Glucocorticoids have multiple effects in peripheral metabolism. These effects include: stimulation of liver gluconeogenesis, inhibition of insulin secretion and lipolysis. In addition, these steroids reduce food intake, growth and immune function. Finally, glucocorticoids play an important role regulating the HPA axis activity by means of retroinhibitory mechanisms (for review Sapolsky et al. 2000a).

Glucocorticoids act through well-characterized genomic receptors, but also through poorly characterized membrane receptors. There are two types of genomic receptors: type I or mineralocorticoid type (MR) and type II or glucocorticoid type (GR). Genomic receptors differ in their distribution and binding properties (de Kloet et al. 1990). MR are highly expressed in the HF, layer II of the cortex, lymbic systems (LS, medial amygdala (MeA), central amygdala (CeA), olfactory nucleus) and brainstem sensory and motor neurons. GR are widely distributed throughout the brain, but the higher levels are found in the limbic system (HF, septum), pPVN, and supraoptic nucleus, and, to a lesser extent, in ascending monoaminergic neurons of the brainstem. Moderate GR levels are also found in many thalamic nuclei, striatal areas, amygdala and cerebral cortex.

MR has a higher affinity to corticosterone than GR. Both receptors are involved in HPA axis modulation by negative feedback, although the effects of GR are likely to be more important

under most conditions (Oitzl et al. 2010) because most MR are already occupied under basal conditions. While MR are thought to exert a tonic inhibition of the HPA axis, GR are involved in HPA axis retroinhibitory actions in stress conditions. Moreover, both receptors mediate HF modulation of HPA axis. Negative feedback by glucocorticoids is exerted at multiple levels: mPFC, HF, PVN and anterior pituitary gland.

As it has been previously explained, glucocorticoids act through genomic and non-genomic pathways, producing, respectively, slow and fast actions. Genomic receptors, which reside in the cytoplasm, once bound to glucocorticoid translocate to the nucleus and affect gene transcription (Beato & Sanchez-Pacheco 1996). Therefore, their effects are expected to occur between 15 min and several hours, and cannot explain effects observed seconds or minutes after stress, which are not mediated by protein synthesis (Haller et al, 2008). In addition, it has to be noted that some of these glucocorticoids effects are not blocked by MR/GR antagonist and therefore involve other types of receptors. Thus, the non-genomic pathways probably act through poorly characterized mechanism, including G protein-coupled membrane-bound receptors, steroid modulatory sites on plasma membrane neurotransmitter receptors, interaction between cytoplasmatic glucocorticoids receptors and intracellular kinases and/or membrane lipids to activate intracellular cascades or exert direct actions on membrane fluidity (Haller 2008, Riedemann 2010). Reported non-genomics effects in the brain include changes in N-methyl-D-aspartate (NMDA) receptor, gamma-amino butyric acid (GABA) receptors, endocannabinoid receptors, voltage-dependent Ca^{2+} channels and phosphorylation of kinases (Haller et al. 2008, Tasker & Herman, 2011).

Post-traumatic stress disorder (PTSD)

One of the most important pathological consequences of exposure to severe stressors is post-traumatic stress disorder (PTSD). PTSD is an anxiety disorder that develops after single or repeated exposure to traumatic situations that are interpreted as a serious risk of death or serious injury (DSM-IV-TR 2000): e.g. war, violent personal assault, kidnapping, torture, terrorist attack, severe car accident, sexual abuse, witnessing serious injuries or un-expected death of a beloved. The first description of symptoms related to present PTSD was done in 1871 by Jacob Mendez Da Costa, who described a "soldier's heart syndrome" which included tachycardia, anxiety, breathless-ness, and hyper-arousal (Javidi & Yadollahie 2012). The term PSTD appeared for the first time in 1980 in the DSM III as an anxiety disorder. Three types of symptoms are prevalent in PTSD: re-experiencing, avoidance and hyperarousal. Re-experience is indicated by recurring thoughts, flashbacks and nightmares related to the traumatic event. Avoidance is caused by persistent phobia of thoughts, feelings and people related to the trauma. The hyperarousal state causes persistent symptoms of increased arousal, as difficulty in falling or staying asleep, irritability or outbursts of anger, lack of concentration, memory impairment, hypervigilance and exaggerated startle response (DSM-IV-TR 2000). It is hypothesized that all of these symptoms represent the behavioral manifestation of stress-induced changes in brain structures and function (Bremner 2006). Scientific attention to PTSD is constantly increasing. An example of this trend is the introduction in the next DSM V of a new chapter including PTSD "Trauma and stressor related disorders". Moreover, a PTSD subtype will be added: the preschool PTSD (PTSD in children younger than 6 years) (American Psychiatric Association, 2013).

There is high variability in the prevalence and severity of PTSD. Only 5-30% of the victims of a trauma develop PTSD (Mahan & Ressler 2012) and symptomatic patients vary in their intensity.

This variability can be related with several factors that affect PTSD including: genetics (the PTSD presents a high heritability), type and intensity of trauma (there is a direct correlation between intensity of stressor and symptoms severity), early-life experiences (children exposed to PTSD have higher prevalence of PTSD than adults) and gender (PTSD is twice higher in women than men) (Javidi & Yadollahie 2012).

Neuroanatomy of brain regions involved in PTSD

The PTSD neuroanatomy has been widely studied in the literature and involves the PFC, the amygdala and the HF. All these structures are interconnected and each one of them plays a critical role in emotion regulation and memory formation.

The human amygdala is a group of nuclei located in the temporal lobes. It has been related to fear conditioning and emotional memories. The amygdala is formed by an evolutionarily primitive division from the olfactory region named centro-medial cortex associated to a newer region from neocortex, the basolateral complex, whereas the MeA and CeA are from striatal origin. The basolateral (BLA) complex consists of the lateral, basal and accessory basal nuclei. The inhibition of BLA blocks fear-conditioning, particularly, the inhibition of the lateral nucleus, which, during fear conditioning, receives inputs about conditioned stimulus from thalamic and cortical pathways and about unconditioned stimulus from spinal-thalamic and cortical pathways (LeDoux 2000).

The CeA is the main output nucleus for conditioned fear responses. The CeA is under inhibitory control of the lateral nucleus, but also receives modulatory inputs from PFC sensory cortex and brain stem. The CeA projects to hypothalamus, modulating hormonal secretion, and to brain stem, modulating arousal (Paré et al. 2004). It is considered that a deficient inhibition by the PFC leads to a hyperactivation of amygdala in PTSD and increased conditioned emotional responses (Elzinga & Bremner 2002).

The PFC corresponds to the anterior part of the frontal lobes of the human brain. It has been related to working memory, inhibition of irrelevant stimuli responses and inhibition of the amygdala and emotional activation. It is divided in: dorsolateral PFC (dlPFC) (lateral (lPFC) in rodents), orbitofrontal (orbital PFC (oPFC) in rodents) and vmPFC (medial PFC (mPFC) in rodents). However, controversy exists about whether the rat mPFC is homologous to the dlPFC, to the vmPFC or to both. The mPFC in rodents is subdivided into cingulate (CG), prelimbic (PL) and infralimbic (IL) regions. The vmPFC sends projections to the HF, via the entorhinal cortex and the thalamus. There are also dense bidirectional connections between the ventral region of the mPFC and the amygdala (Koenigs & Grafman 2009). Under mild stress conditions, mPFC inhibits amygdala activity. This scenario is found in subjects not exposed to traumatic events, but also in individuals resilient to PTSD. On the contrary, individuals suffering extreme stress show a reduction of the mPFC-mediated amygdala inhibition, causing some of the PTSD symptoms (Pitman et al. 2012).

The third main region involved in PTSD is the HF, located in the medial temporal lobe of human brain. It is involved in learning and memory, particularly, in declarative memories and integration of time and space. Memories are stored initially in the HF and further restructured and moved to cortical areas (Zola-Morgan & Squire 1990). In addition, the HF is involved in emotion and stress responses (Moser & Moser 1998). Anatomically, it is divided in: the Entorhinal Cortex, the dentate gyrus (DG), the *cornu ammonis* (CA1, CA2 and CA3) and the Subicu-

lar Complex. These areas form an organized predominantly unidirectional pathway. These connections are well known and include the following circuit: from enthorhinal cortex, through the perforant pathway, to DG granule cells; through mossy fibers, to CA3 pyramidal neurons; and through Schaffer collaterals to CA1 pyramydal cells and then back to enthorhinal cortex neurons (Kandel 1997, Neves et al. 2008, Squire & Zola-Morgan 1991). Each HF subfield is composed by several layers (Witter and Amaral, 2004).

The DG is composed by:

(i) Polymorphic layer, with interneurons and axons from granule cells that form the mossy fibers.

(ii) Stratum granulosum, which contains the soma of granule cells.

(iii) Stratum moleculare, which contains the axons of the medial perforant pathway from septal nuclei and commissural pathway, from the contralateral DG (internal portion) and perforant pathway from external portion that innervate granule cells dendrites.

CA1, CA2 and CA3 layers are composed by:

(i) Alveus, which contains the axons from pyramidal cells and it is one of the major outputs from HF.

(ii) Stratum oriens, which contains the basal dendrites from pyramidal cells that are innervated by Schaffer collaterals (only in CA1), commissural and septal fibers.

(iii) Stratum pyramidale, which contains pyramidal cells somas and mossy fibers innevation.

(iv) Stratum lucidum, a thin layer of mossy fibers axons, only found in CA3.

(v) Stratum radiatum, which is formed by apical dendrites of pyramidal cells innervated by Schaffer collaterals (only in CA1), commissural and septal fibers.

(vi) Stratum lacunosum-moleculare, which contains dendrites innervated by the perforant pathway.

(vii) Sulcus, a cell-free region which separates CA1 of DG.

Moser and Moser proposed in 1998 that the HF is not a unitary complex. It can be divided into posterior HF (Dorsal HF in rodents) and Anterior HF (Ventral HF in rodents), playing distinct roles (Moser & Moser 1998). Dorsal HF could be involved in cognition and declarative memory through projections to cortical structures and ventral HF could be involved in emotion and stress responses through outputs to bed nuclei of the *stria terminalis* (BNST), *nucleus accumbens*, lateral septum, amygdala, PFC and the ventral subiculum. However, these two HF areas are not isolated and are interconnected (Fanselow & Dong 2010).

Biological bases of PTSD

Work to understand the biological bases of PTSD has focused on structural and functional neuroimaging, psychophysiological and neuroendocrinological variables and genetics.

The magnetic resonance imaging (MRI) allows assessing morphological and also functional studies *in vivo*. MRI is based on exposing the tissue to a magnetic field that orientates all protons in the same direction. Then, by emitting a pulse of radio frequency (RF), protons due to resonance loss their orientation and when the RF pulse stops, protons return to their original state by a relaxation phenomenon. The energy released in relaxation is captured by a solenoid that sends the information to a computer, which interprets it giving an image that is related to the density of protons in each area (Haines 2008).

White matter changes are assessed by diffusion tensor imaging (DTI), which provides information about tissue microstructure and, in particular, about white matter structure and integrity. Fractional anisotropy is one of the DTI values, which quantifies the directional dependence of water diffusion, which in turn is related to the orientation of axons. Fractional anisotropy increase has been related to maturation, development, learning and myelin increase (Zatorre et al. 2012).

Besides lipids and water, MRI analysis can provide information about some metabolites (e.g. N-acetylaspartate (NAA), lactate, choline, taurine, glutamate, creatine, *m*-inositol, GABA) using the magnetic resonance spectroscopic imaging MRSI or ^{1}H-MRS. The content of some metabolites, such as NAA, has become important because it is a marker of functional integrity of neurons. The decrease of NAA has been related to a decrease in the number of neurons and axons (Benarroch 2008).

Brain activity can also be measured by functional MRI (fMRI). This brain activity produces hemodynamic changes, which depend on cerebral blood volume, cerebral blood flow and blood-oxygenation. These hemodynamic changes give rise to the blood-oxygen-level dependent signal (BOLD) contrast. This contrast originates from the regional perturbations of blood. Thus, higher activity induces higher blood-oxygen flow and increases BOLD signal (Ahrens et al. 2002).

The literature about PFC volume in PTSD is limited; however, a volume reduction has been reported (Karl et al. 2006). In addition, a NAA reduction associated with PTSD is also reported in a recent meta-analysis (Karl & Werner 2010). The PFC function in PTSD patients has been studied in several studies. A hypoactivation of the vmPFC has been observed in trauma exposed patients. Thus, the presentation of traumatic-related images (Lanius et al. 2001) and pictures (Bremner et al. 1999a) to PTSD patients decreases the activation of this region, and a negative correlation has been reported between activity in the PFC and severity of PTSD symptoms (Hughes & Shin 2011).

Studies on amygdala volume in patients with PTSD are controversial. Some works presented in the meta-analysis done by Karl and collaborators (2006) show that PTSD patients have a smaller amygdala. These results are in contrast to some studies included in the same meta-analysis, which reported a lack or a low effect of PTSD on amygdala volume. There are no ^{1}H-MRS studies of PTSD patients amygdala. Hyperactivation of amygdala has been reported in several studies. PTSD patients display increased amygdala activity after presentation of trauma-related sounds (Liberzon et al. 1999, Pissiota et al. 2002) and odors (Vermetten et al. 2007). In addition, hyperactivation is found when presenting non-traumatic material (e.g. happy and fearful faces, Rauch 2000). Both positive- and negative-events amygdala-related activation has a positive correlation with PTSD symptoms (Hughes & Shin 2011).

HF has been the most studied structure since the early 90s, when the MRI studies emerged. Since then a significant number of studies have investigated whether HF volume is altered in patients with PTSD. Thus, several meta-analyses supported a lower HF volume in PTSD patients (Karl et al. 2006, Smith 2005). Recently, ^{1}H-MRS studies have also demonstrated a decrease in NAA (Karl & Werner 2010). These data could be associated with some hippocampal related impairments of PTSD patients, such as declarative memory deficits (Elzinga & Bremner 2002). Although it is still unclear whether these changes are a consequence of trauma or they are a risk factor for developing PTSD (Pitman et al. 2012), a seminal study by Gilbertson *et al.* (2002) favors the second possibility. They showed, in an excellent study with combat veterans suffering from PTSD and their twins, who were never mobilized, that these two groups presented similar

HF volume that were smaller as compared to both control groups; combat veterans without PTSD and their respective twins. These data suggest that reduced HF volume precedes PTSD and it is a factor of vulnerability for developing the pathology after being exposed to traumatic experiences.

HF activity also presents controversial data. Some studies showed hypoactivation when presenting traumatic (Bremner et al. 1999b), while others found HF hyperactivation in similar conditions (Shin et al. 2004). There are at least two possible reasons for these discrepancies. First, the diversity of tasks, since different traumatic cues could induce different activation patterns. Second, the dorsal and ventral segmentation of the HF. In this regard, a recent study using network-sensitive functional magnetic resonance has shown that only the posterior (dorsal in rats) hippocampus presents abnormalities (Chen & Etkin 2013).

A recent meta-analysis has reported white matter reductions in corpus callosum and in the cingulum in patients suffering from PTSD. The corpus callosum is a fiber bundle connecting both hemispheres, and the reported reduction suggests a lower connection between hemispheres. The cingulum, the most prominent white matter tract in the limbic system, connects the anterior cingulate and entorhinal gyri and projects to and from the amygdala. Therefore, the reduced cingulum in PTSD patients suggests a reduction in limbic system circuitry (Daniels et al. 2013).

Psychophysiological variables (e.g. heart rate, skin conductance, facial electromyography) have been used to assess symptoms found in PTSD patients and confirm that those subjects present exaggerated startle, impaired extinction and increased sensitivity to trauma-related cues (Pissiota et al. 2002, Pitman et al. 2012). Most neuroendocrinological studies have focused on the HPA axis. It is important to consider that some of the HPA changes present in PTSD patients might exist before the appearance of PTSD and be potential factors of PTSD susceptibility (Armario et al. 2008). Some studies have revealed hypocortisolemia in PTSD patients, but these data have not been replicated in all PTSD population (de Kloet et al. 2006, Yehuda 2006). Similarly, an enhanced negative glucocorticoid feedback has also been reported. ACTH levels present discrepancies. PTSD patients exposed to non-trauma related stimuli showed higher ACTH levels; however, when exposed to stimuli related to combat, ACTH levels did not differ between PTSD patients and healthy controls (de Kloet et al. 2006). Divergences can be explained by the heterogeneity of groups studied, with poor control of certain variables (gender, age, body weight, metabolism, medical illness, mood, substance abuse and environmental stress). Higher levels of CRF in cerebrospinal fluid (CSF) of PTSD patients have been reported (Bremner et al. 1997, Kasckow et al. 2001, Sautter et al. 2003) suggesting central hiperactivity. Alterations of the HPA axis in PTSD patients might be secondary to changes in the influence of the limbic system on this neuroendocrine system.

Since vulnerability to PTSD presents a high heritability, some studies have been focused in the analysis of different genes, including: dopamine receptors, glucocorticoids receptors, CRH receptors, serotonin transporters and receptors, cannabinoid receptors and also neurotrophic factors like the brain-derived neurotrophic factor (BDNF) (Pitman et al. 2012). Dopamine and serotonin receptor genes have been the most assessed, but there are controversial results. Changes in BDNF gene have not been reported. Epigenetic mechanisms have also been studied, and recent studies suggest that PTSD patients display lower levels of DNA methylation in genes related to the immune function (Uddin et al. 2010). However, genetic and epigenetic studies are still preliminary and further studies are required.

PTSD animal models

Development of PTSD animal models are challenging as it requires reproducing psychological symptoms in animals. However, animal models overcome ethics problems associated with human research and provide mechanistic data impossible to obtain in humans. Animal research has used acute and chronic stress exposure as PTSD-like models. However, acute stress fits better to PTSD-causing traumatic situations, where only one exposure to a traumatic event originates PTSD symptoms (Siegmund & Wotjak 2006). Until recently, most of the effects of acute stress exposure were studied in the short-term, whereas PTSD models require to focus on the long-term effects. For us, long-term effects of stress in animals models are those observed two days onwards (Armario et al. 2008).

Siegmund and Wotjak (2006) have proposed three criteria that have to be accomplished by any animal model of PTSD:

(1) **Face validity**: which refers to how similar the causes and consequences are between the animal model and the human pathology; ideally the acute stressor has to induce long-term behavioral effects similar to those suffered by PTSD patients. The severity of stressor should define the severity of the symptoms, and the phenotype should vary between animals (showing affected and non-affected animals).

(2) **Predictive validity**: this refers to the success in the animal model of drug treatments used in humans. For instance, the most effective treatment in PTSD patients, the selective serotonin reuptake inhibitors (SSRIs) administration, should be effective in the animal model.

(3) **Construct validity**: which refers to the fact that animal models have to mimic the functional alterations of the CNS in humans. The traumatic event in animal model should then induce hypofunction of the PFC, hyperfunction of the amygdala and HF impairment.

There are several models of PTSD: learned helplessness (LH), predator or predator odor, single Prolonged Stress (SPS) and IMO. Unfortunately, none of them is devoid of problems (Armario et al. 2008, Miller & McEwen 2006).

In the LH model, animals are typically distributed into three groups (Maier 1984). In the first group, the escapable-shock group (ES), animals are exposed to electric tail-shocks that can be stopped by doing an operant task (wheel turn). In the second group, the inescapable shock group (IS-LH), animals are exposed to the same electric shocks as the first group without the possibility to control them. In the third group, the undisturbed group, animals are slightly restrained, but do not receive any electric shock. After these treatments, the animals are exposed the next day to an escapable task in a shuttle box where animals can avoid electric shocks by crossing to another compartment. Habitually, group differences are not found when a single cross was needed (fixed ratio, FR1), but when two crossings are demanded (FR2) IS group presented more freezing behavior and less escape learning than the other two groups. This behavioral deficit was initially explained by the LH hypothesis: animals exposed to an inescapable shock were less motivated to learn an escape response involving active behavior; consequently, IS-LH animals showed higher escape latency and more escape failure compared to the other groups. Animals also showed signs of depressive-like behavior such as neophobia, decreased social interaction and anhedonia. The main problem of this model is that most of these effects vanish within the 72 h post stress. However, there are other long-lasting effects. For instance, it has been reported that LH model potentiates tone and context fear conditioning a week after LH (Baratta et al. 2007).

It is important to note that other works have reported longer-lasting effects in IS animals. However, in those cases, animals were exposed to foot-shock in the same, or similar, apparatus (shuttle-box) where they were further tested (Maier & Watkins 2005, Murua & Molina 1991). Thus, the longer-lasting effects can be a consequence of the development of contextual fear conditioning.

A short session of foot-shocks has been reported to induce hypoactivity in novel environments four weeks post-stress (Van Dijken et al. 1992). These results cannot be interpreted in terms of depression-like behavior because antidepressant treatment did not reverse hypoactivity (Van Dijken et al. 1992). HPA axis sensitization to novel environments presents controversial data, with no effect (Daviu et al. 2010) or increased HPA sensitization 14 days after the foot-shocks session (van Dijken et al. 1993). These controversial data could be explained by fear conditioning implication, since, as has been commented in the LH model, the re-exposition to the foot-shocks contexts produces more consistent long-lasting effects. In this regard, a short session of foot-shocks resulted in short-term and long-term (8 days) contextual fear conditioning (freezing) that was paralleled by increased HPA activation (Daviu et al. 2012). Although, some studies have found long-term increases in anxiety-like behavior after short sessions of foot-shocks (Van Dijken et al. 1992), but there are controversial data (Armario et al. 2008, Daviu et al. 2010). In addition, Wotjak lab (Siegmund & Wotjak 2007) has developed a putative PTSD mouse model with a single session of foot-shocks that induced long-term fear conditioning, social withdrawal, neophobia and depression-like behavior. Furthermore, fluoxetine, a SSRI, ameliorates PTSD-like symptoms (Siegmund & Wotjak 2007). In this work they compared two mouse strains and only one of them developed symptoms. Therefore strain vulnerability has to be taken into account when discussing PTSD animal models.

Exposures to predators or predator odor are among the most ethological and extensively studied PTSD animal models. Typically a cat or cat's odor is used, considering that cat is the natural predator for rats and mice. In the direct cat exposure model, animal can be, or not be in contact with the cat. The source of odor can be urine or fur/skin, but the exact molecules involved are still unclear. However, recently cat kairomone-like ligands, homologs of the major urinary proteins (mup) family, have been detected in the cat saliva (Papes et al. 2010). These molecules are detected by mice vomeronasal organ sensory system and induce defensive behaviours and ACTH release in mice, similar to those effects observed with cat odors. In some experiments 2,3,5-trimethyl-3-thiazoline (**TMT**) a component of fox odor has been used because, unlike the cat odor, it can be easily dosed. Unfortunately, these different procedures induce different long-lasting effects.

Cat exposure induces long lasting (one to several weeks) increases in anxiety evaluated in the elevated plus maze (EPM) in both, rats (Adamec & Shallow 1993) and mice (Adamec et al. 2004). These anxiety effects are higher than after odor exposure (Adamec et al. 1998). Fur/skin exposure induces immediate defensive behavior and long-term contextual fear conditioning as well as increased anxiety (Blanchard et al. 2003, Muñoz-Abellán et al. 2008), although changes in anxiety are less consistent than contextual fear conditioning (Muñoz-Abellán et al. 2009). However, results from our lab demonstrated that cat odor induces a lower HPA axis activation than IMO in a short exposure (15min), whereas, a week after stress, odor exposed, but not IMO exposed rats showed enhanced anxiety in the EPM. These results are in accordance with previously reported long-term (up to 3 weeks) enhanced anxiety after short single exposure to cat (Adamec et al. 2003). Therefore, there is a clear dissociation between the behavioral and the

HPA axis consequences of predator exposure, suggesting that factors other than the intensity of stressors may be critical for predator exposure consequences. This is in contrast to the general assumption that there is a positive correlation between the severity of symptoms and the intensity of the traumatic event.

Another possible PTSD model is the social defeat. In this model, rats are exposed, during 10-60 min for 1 to 5 days, to a more aggressive and bigger conspecific that entails the defeat of the subject during confrontation (Stam 2007). Defeated animals presented: (i) an enhanced freezing after contextual fear conditioning, (ii) increased anxiety (Buwalda et al. 2005) and (iii) potentiated startle response (Pulliam et al. 2010). In addition, these animals showed depression-like symptoms, such as anhedonia (Rygula et al. 2005) and decreased negative feedback sensitivity of HPA axis (Buwalda et al. 1999). Therefore, it seems that social defeat fits better to depression than to PTSD (Stam 2007).

SPS is another model of PTSD initially proposed by Liberzon and collaborators (1997). They exposed animals to three consecutive stressors: restraint, forced swim and ether. SPS exposure induces an increase in anxiety 7 days after, as evaluated by the startle response (Khan & Liberzon 2004) and the EPM (Imanaka et al. 2006). It also increases foot-shocks-induced fear conditioning (Iwamoto et al. 2007) and spatial memory impairment in the Morris Water Maze (MWM) (Kohda et al. 2007). The increase in the startle response was prevented by topiramate (anticonvulsant) administration (Khan & Liberzon 2004), and the enhanced fear conditioning was prevented by the antidepressant paroxetine (Takahashi et al. 2006) and by d-cycloserine, a partial NMDA receptor agonist (Yamamoto et al. 2008).

IMO

This model consists of immobilizing the animals by taping their four limbs to metal mounts attached to a board (e.g., Márquez et al. 2002, Muñoz-Abellán et al. 2008). This model has been studied in our lab for several years and it is probably among the most severe (predominantly emotional) stressors for rats, even more than high intensity foot-shocks (Márquez et al. 2002).

A single exposure to IMO results in a reduction of HPA response to a second IMO (homotypical stressor), but not to a second novel (heterotypical) stressor. This has been observed days or weeks after the first IMO (Martí et al. 2001). This effect depends on the stimulus intensity, as reduced response to the homotypic stressor is higher with IMO than with other less severe stressors, including foot-shock and restraint (Armario et al. 2004). Importantly, this reduced response has also been found in *c-fos* and *CRF* mRNA expression in PVN (Martí et al. 2001, Vallès et al. 2003). This phenomenon is resistant to several different pharmacological approaches. Thus, only NMDA receptor antagonist (MK-801) (Armario et al. 2004), non-selective D1 and D2 dopamine receptor antagonist (Haloperidol) and a selective D1 antagonist (SCH23390) (Belda et al., unpublished) partially reduced the desensitization of HPA axis response to a second IMO. Some experiments were designed to assess the implication of GR. Thus, it was observed that adrenalectomized rats did not present a desensitization of HPA axis response (Dal-Zotto et al. 2002). Furthermore, the administration of a corticosterone synthesis blocker (metyrapone) before IMO reduced partially the homotypic desensitization after a second IMO (Dal-Zotto et al. 2003), suggesting that this phenomenon is partially dependent on glucocorticoids.

Regarding the putative significance of IMO as a model for PTSD, it is noteworthy that one single exposure to IMO can induce long-term behavioral and neuroendocrine effects. IMO induces a long-term reduction of food intake and body weight gain (Vallès et al. 2000). An increase in anxiety evaluated in the EPM, is sometimes observed in animals exposed to a single IMO even 7 days after the IMO, although the effect vanishes over the next week (Belda et al. 2008). Furthermore, an enhanced startle response 10 days after the IMO has been reported in some experiments (Fuentes et al. unpublished). Interestingly, IMO induces long-term behavioural and neuroendocrine sensitization to novel stressors. Thus, previous exposure to IMO causes a higher HPA axis response to forced swim (Marti et al., 2001) and to novel environments (Belda et al. 2008, Gagliano et al. 2008). Moreover, 10 days after IMO a brief low intensity foot-shock session, that on its own did not increase anxiety, also induces anxiety reappearance conducted in EPM (Belda et al., 2008).

IMO exposure also affects area-specific tasks that mimic some PTSD consequences. IMO-exposed mice have a delayed extinction of a tone-fear conditioning, an amygdala-related task, when conditioning occurred 7 and 9 days after 2 h of IMO (Andero et al. 2011). This effect could be related to hyperactivity of the amygdala and it is prevented by the administration of 7,8-Dihydroxyflavone (7,8-DHF) (Andero et al. 2011), an agonist of the tropomyosin related kinases B receptor (TrkB) (Jang et al. 2010). The HF function after IMO has been assessed using the MWM task. Rats exposed to 2 h of IMO 3 or 9 days before training showed no effect in MWM learning, but they presented a deficit in long-term spatial memory that was prevented by 7,8 DHF administration either before or 8 h after IMO (Andero et al. 2012a). Finally, PFC function has been studied by evaluating a set-shifting task that assessed cognitive flexibility. In this task, rats have to change a prior learned strategy in order to obtain reinforcement. Rat exposed to 2 h of IMO a week before the task presented a higher number of errors and needed more trials to reach criterion (Garrido et al., unpublished).

However, as in other PTSD models, interpretation of the long-term effects of IMO is problematic. One main problem is that IMO-induced changes in anxiety are transient and this stressor is unable to induce fear conditioning measured by endocrine and behavioral variables (Daviu et al. 2012).

Brain plasticity and stress

Santiago Ramon y Cajal's work at the end of the XIX century and early in the XX century laid the foundations of modern neuroscience, describing terms and providing evidence for axonal growth cone and dendritic spines. His research provided data in support of the "neuron doctrine" and described circuitries in several brain areas. He suggested that learning depend on plastic changes at synapses, providing anatomical and conceptual bases to hebbian theory. As Donald O. Hebb literally said "The general idea is an old one, which any two cells or systems of cells that are repeatedly active at the same time will tend to become 'associated', so that activity in one facilitates activity in the other" (Hebb, 1949). Kandel's lab, among others, provided direct evidence for the relation between hebbian theory and learning and memory. Moreover, over the last decades an important concept has been added to explain learning and memory, that of structural plasticity (Caroni et al. 2012). Whereas Hebbian theory is based on pre-existing synapses, structural plasticity encompasses morphological changes (synapses formation and de-

struction) that result in functional consequences. It is accepted that learning and memory involve synaptic plasticity. However, how memory become long-term is poorly understood.

Memory, synaptic plasticity and stress

Memory dysfunction is one of the most characteristic symptoms of PTSD. It has been described in humans (Elzinga & Bremner 2002) and in animal models (Stam 2007). PTSD impairs working memory and induces a failure to inhibit irrelevant data and control of emotions likely to be related to PFC dysfunction. The last aspect is related to reduced amygdala inhibition, thus resulting in enhanced traumatic memory retrieval in PTSD. Finally, regarding the HF-related functions, PTSD reduces declarative memory, fragments memory and increases trauma-related amnesia (Elzinga & Bremner 2002).

Memory is defined as the storage, retention and retrieval of information acquired through learning, and has at least two temporal stages: short-term (minutes to hours) and long-term (days, weeks or longer) memory (Kandel 1997). Memory is classified in declarative (explicit) and non-declarative (implicit). Declarative memory refers to events and facts that are consciously recollected and reproduced. Non-declarative memory refers to habituation, sensitization, conditioning, and skills and procedures that are non-conscious (Squire & Zola-Morgan 1991).

Scientists have been searching for the neurobiological bases of memory for a long time. Squire and Zola-Morgan (1991) proposed the critical role of the temporal lobe, which includes the HF, in memory formation. They proposed this hypothesis due to the compiled data from humans' patients with amnesia and from brain lesions in primates. In the latter case, they performed stereotaxic lesions of the HF and amygdala alone and combined, and compared the effects on different memories tasks. They found that animals with HF lesion performed worse than those without lesion and those with amygdala lesion, while those with the combined amygdala and HF lesion performed even worse than those with HF lesion (Zola-Morgan et al. 1989). They also proposed that memory gradually passes to neocortex in order to allow the HF to acquire new information (Squire & Zola-Morgan 1991).

Morris and collaborators (2003) proposed the "Synaptic Plasticity and Memory" hypothesis: "During learning, spatio-temporal patterns of neural activity that represent events, cause long-lasting changes in the strength of synaptic connections within the brain. Later reactivation of these altered connections causes patterns of cell firing that collectively constitute the experience of memory for these events or the expression of learned changes in behaviour triggered by them". Indeed, synapses are not only connections to transmit information; they also transform it. As Südhof says in a recent review, synapses are "computational devices" and receive and process the action potential (Sudhof 2012). The best characterized cellular substrates of learning and memory-dependent synaptic plasticity are the Long-Term Potentiation (LTP) and Long-term Depression (LTD), which have been assessed mainly in glutamatergic pathways.

In 1973, Tim Bliss and Terje Lømo described for the first time LTP in rabbit hippocampus (Bliss & Lomo 1973). Since then, LTP has been extensively studied by electrophysiological, biochemical and molecular techniques in the HF due to its particular anatomy, defined layers and its organized unidirectional connections, called the trisynaptic pathway. However, HF is not the only structure where LTP has been described during learning and memory processes. The PFC (Auclair et al. 2000) and the amygdala (Roozendaal et al. 2009) also present LTP. The most studied LTP processes in the glutamatergic synapses.

The common protocol for LTP induction in field recording experiments consists of low frequency stimulation (LFS) (<0.1 Hz) of Schaffer collaterals in order to record baseline synaptic responses, which are the excitatory postsynaptic potentials (EPSP), in CA1. Then, LTP is induced by high frequency stimulation (100 Hz) (HFS). This HFS is followed by another period of LFS to record the post-induction EPSP. The LTP is detected by CA1 EPSP increases in amplitude and slope (Otto et al. 1991). LTP can be due to improved synaptic efficacy at pre- and postsynaptic levels (Malenka & Nicoll 1999; Südhof 2012). However, some exceptions exist. For instance, the expression of mossy fibers LTP is mainly presynaptic (Kobayashi et al. 1996), although recent studies demonstrated that the mossy fibers-CA3 synapses also exhibits a novel form of NMDA-dependent LTP (Kerr & Jonas 2008).

A simple explanation of LTP induction at postsynaptic terminals is as follows: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors mediate the EPSP in basal conditions, and are responsible for the inward current associated with monovalent cations permeability (Na^+ and K^+). NMDA receptors present a voltage-dependent activation, a result of extracellular Mg^{2+} ions blockade, such they have a little contribution to basal synaptic response. The repeated HFS stimulation activates several AMPA receptors, which produce a cumulative depolarization of dendritic membrane, leading the repulsion of Mg^{2+} ions and allowing the flow of Na^+ and Ca^{2+} into cell through NMDA receptors. This Ca^{2+} activates signaling cascades that mediate the delivery of AMPA receptors to the synapse (Blitzer et al. 2005). Currently, we can distinguish between early-LTP or short-term potentiation (STP) (minutes or hours), which involves activation of kinases, phosphatases and receptor trafficking; and a late-LTP or long-term potentiation (days or weeks) itself that requires protein synthesis (Kandel 1997).

Although LTP is the most extensively studied, there are other types of activity-dependent plasticity in learning and memory. One of them is LTD. Lynch and collaborators (1977) observed a heterosynaptic long-term depression of synaptic transmission in non-tetanically stimulated pathway of the hippocampus. It is now known that there are two types of LTD: homosynaptic and heterosynaptic. The first one is due to direct low frequency stimulation of synapses (LFS) and therefore, it is activity-dependent. LFS stimulation (1 Hz) is different from the stimulation used to obtain the baseline EPSP (0.1 Hz) and induces a decrease in amplitude and slope of EPSP (Dudek & Bear 1992). This decrease seems to be the consequence of a decreased expression of surface AMPA receptors (Malinow & Malenka 2002). Heterosynaptic LTD occurs in synapses that are inactive during high-frequency stimulation (HFS) of neighboring synapses and is the result of the firing of a distinct modulatory interneuron (Bear & Abraham 1996).

The study of animal PTSD models is important because it can provide new data of how learning and memory work and the biological substrate of some of the PTSD consequences. Stress effects on learning and memory are complex and depend on the stress levels (Kim & Yoon 1998) and the temporal relationship between learning and stress exposure (Sandi 2011). Regarding the stress levels, the relationship appears to follow an inverted U shape pattern: mild and severe stress impair, while medium stress facilitates, learning and memory (Sandi 2011). Unfortunately, most studies have described short-term effects of stress, and few have focused on long-term consequences. However, severe stressors have been shown to produce long-term effects. Henceforth, we are going to address consequences of severe stressors related to synaptic plasticity. In order to better understand corticosterone administration studies, it should be noted that an injection of 5 mg/kg of corticosterone is equivalent to an IMO exposure while oral corti-

costerone administration (20-30 μg/ml in drinking water) to adrenalectomized rats simulates basal corticosterone levels of intact rats.

Glutamate and stress

Glutamate is an excitatory amino acid synthetized *de novo* in neurons from glucose and from glutamine supplied by microglia. Glutamate is packaged in vesicles by the vesicular glutamate transporters (vGluts). Glutamate released to synaptic cleft binds to ionotropic receptors type (NMDA, AMPA or Kainate) to mediate fast excitatory synaptic transmission. Glutamate also binds to metabotropic receptors (mGluR1 to mGluR8) to exert several modulatory effects through G proteins and their second messengers system. Glutamate receptors are located postsynaptically, but also presynaptically and extrasynaptically (Pinheiro & Mulle 2008), as well as in glia (Teichberg 1991).

Ionotropic glutamate receptors are heterotetramers. AMPA is composed by a combination of four subunits (GluA1 to GluA4; formerly GluR 1-4) and NMDA is composed by two obligatory GluN1 (formerly NR1) subunits and two regulatory subunits (GluN2A to GluN2D or GluN3A to GluN3B; formerly NR2A-D or NR3A-B). Long-term synaptic strength is mainly modulated by surface expression of NMDA and AMPA receptors which, in turn, is regulated by degradation, protein synthesis and trafficking between postsynaptic membrane and endosomes. Glutamate is removed from synapses by excitatory amino acid transporters (EAATs) located in glial cells and neurons. In the latter cells, it is converted to glutamine, delivered and taken up by neurons and converted to glutamate closing the glutamate-glutamine cycle. Extracellular glutamate levels result from release-uptake dynamic equilibrium.

Stress effects on glutamate release are divergent depending on the studied area. Thus, acute stress induces a decrease in glutamate release in the hypothalamus (Groeneweg et al. 2012), whereas in the in HF (Venero & Borrell 1999), the PFC (Moghaddam 1993) and the amygdala (Reznikov et al. 2007) stress increases glutamate release. Although it has been proposed that glutamate levels obtained by microdialysis are mainly from non-neuronal origin (Timmerman & Westerink 1997), approaches other than microdialysis have demonstrated a stress-related glutamate increase from neuronal origin. In PFC, the measurement of endogenous glutamate from purified synaptosomes has shown increased glutamate release immediately after acute foot-shock session, which is prevented by antidepressants (Musazzi et al. 2010). The acute treatment of synaptosomes with corticosterone (10μM) evoked glutamate release in HF slices (Wang & Wang 2009). Miniature excitatory postsynaptic currents (mEPSC) frequency, an indirect index of presynaptic facilitation of glutamate release has also been used; corticosterone (10nM) administration on HF slices (Karst et al. 2005) and on the BLA (100nM) increased mEPSC frequency through non-genomic pathway involving membrane located MR. However, no effect were found in the CeA (Karst et al. 2010).

Extracellular glutamate increase is not only due to stress-dependent release. Glutamate uptake can be also modulated by stress. Immediately after tail-shocks, glutamate uptake is impaired in CA1 hippocampal region (Yang et al. 2005) and after 2 h of restraint and acoustic stress during 4 days EAAT-2 protein levels are reduced in the PFC (Zoppi et al. 2011). However, glutamate uptake is increased 30 min after restraint (Gilad et al. 1990) while no effect is observed 24 h after restraint (Fontella et al. 2004).

Forced swim increases NMDA and AMPA mediated currents in PFC and this effect is glucocorticoids-dependent and sustained up to 24h (Yuen et al. 2011). On the contrary, in the HF, corti-

costerone (100 nM) (Karst & Joels 2005) and acute stress induced by fear conditioning (Zhou et al. 2009) affected only AMPA-mediated currents.

A key point to control synaptic efficacy is the NMDA and AMPA receptor endocytosis-exocytosis and the lateral diffusion (Malinow & Malenka 2002, Wenthold et al. 2003). Corticosterone (100 nM) induces an increase of GluA2 containing AMPA receptor in the plasma membrane of the HF as assessed by live imaging and immunocytochemistry (Groc et al. 2008, Martin et al. 2009). It has also been demonstrated that a water maze learning task at 22ºC (a stressful situation) increases AMPA GluA2 in plasma membrane surface (Conboy & Sandi 2010). Moreover, acute stress (forced swim and elevated platform) and corticosterone (100 nM) treatment increase AMPA and NMDA membrane receptors in the PFC (Yuen et al. 2011). The introduction of a synthetic peptide with the aminoacidic sequence of GluA2, which interferes with AMPA receptors recognition and decreases AMPA receptors endocytosis, blocked LTD and prevented memory impairment caused by acute exposure to elevated platform (Wong et al. 2007). It is then possible that stress-induced memory impairment is related to aspects other than AMPA receptors insertion, the increase observed after stress being a compensatory mechanism. Interestingly, chronic administration of corticosterone (20 mg/kg) decreased the total GluN1 protein levels as assessed in synaptosomes analysis of the whole brain and by immunohistochemistry in the HF (Cohen et al. 2011), suggesting that chronic severe stress situations can induce AMPA receptor endocytosis.

These complex and even opposite effects of glucocorticoids and stress in glutamatergic transmission can be related to the complex regulation they exert during learning and memory processes (Sandi 2011). Although the mechanisms by which stress impairs learning and memory are not well known, two parallel consequences of high intensity stressors have been proposed. Stress leads to high concentrations of glucocorticoids that would induce an immediate and sustained glutamate release (García-Bueno et al. 2008, Lowy et al. 1995) by enhancing glutamate release likelihood (Karst et al. 2010) and inhibiting glutamate uptake (Yang et al. 2005). These two effects would cause glutamate spillover that would activate GluN2B NMDA extrasynaptic receptors and increase GluA2 AMPA receptors endocytosis, later enhancing LTD and causing memory impairment (Popoli et al. 2011, Riedemann et al. 2010, Sandi 2011, Wong et al. 2007).

LTP, LTD and stress

The above reviewed stress effects on glutamatergic pathway suggest a substantial modulation of synaptic plasticity by stress. In this regard, several *in vivo* and *in vitro* experiments have assessed the effects of stress on LTP and LTD in the HF. Induction of LTD [TABLE 1] is enhanced immediately after restraint or tail-shock (Chaouloff et al. 2007, Kim et al. 1996, Yang et al. 2005), or one day after forced swim in the dorsal HF (Maggio & Segal 2011). In contrast, one week after SPS impaired HF LTD has been reported (Kohda et al. 2007). Interestingly, on the day after a session of forced swim LTD in the ventral HF is converted from depression to a slow onset potentiation, suggesting a substrate for the differential effects of stress on the HF function: impairment of cognitive and spatial functions related to dorsal HF and enhancement of emotion related to ventral HF (Maggio & Segal 2011).

This overall stress-induced LTD enhancement is prevented by enhancing expression of a glucocorticoids-degrading enzyme (11-β-hydroxysteroid dehydrogenase type II) in hippocampal neurons (Dumas et al. 2010), protein synthesis inhibition (Xu et al. 1998) and NMDA antago-

nists (Wong et al. 2007, Yang et al. 2005). These studies suggest that stress-related LTD enhancement is glucocorticoid and glutamate dependent.

Stress/Stressor	Species/Strain Age or weight at stress start	Region/Time after stress	LTD induction vs Control group	Reference
Tail-shock	Rats SD/ 250-300g	HF/ Immediately	Enhanced (↑)	Yang et al. (2005)
Restraint + tail-shock	Rats Long-Evans/ 290-350g	HF/ Immediately	↑	Kim et al. (1996)
Restraint	Rats SD/ Postnatal day 25-42	HF / 90 min	↑	Chauloff et al. (2007)
Forced swim	Rats Wistar/ 60 days	Dorsal HF/ 1 day	↑	Maggio and Segal (2011)
Social defeat + indivi-dualization	Rats Wistar/ 300-350g	HF/ 7-9 month	↑	Artola et al. (2006)
SPS	Rats SD/ 8-11 weeks	HF/ 1 week	Impaired (↓)	Khoda et al. (2007)
Forced swim	Rats Wistar/ 60 days	Ventral HF/ 1 day	Depression to a slow onset potentiation	Maggio and Segal (2011)
Stress/Stressor	**Species/Strain Age or weight at stress start**	**Region/Time after stress**	**LTP induction vs Control group**	**Reference**
Fear conditioning	Rats SD/ 250-300g	HF / Immedialtely	Impaired (↓)	Li et al. (2005)
Restraint + tail-shock	Rats Long-Evans/ 290-350g	HF/ Immediately	↓	Kim et al. (1996)
Elevated platform	Rats Wistar/ 245-385g	HF/ 3 h	↓	Ryan et al. (2010)
Forced swim	Rats Wistar/ 60 days	Dorsal HF/ 1 day	↓	Maggio and Segal (2011)
Restraint + tail-shock	Rats SD/ 250-350g	HF/ 48 h and 96 h	↓ at 48 h / = at 96 h	Shors et al. (1997)
SPS	Rats SD/ 8-11 weeks	HF/ 1 week	↓	Khoda et al. (2007)
Footshocks (LH)	Rats SD congenitally learned helpless/ 350-540g	HF/ 4 weeks	↓	Ryan et al. (2010)
Forced swim	Rats Wistar/ 60 days	Ventral HF/ 1 day	Enhanced (↑)	Maggio and Segal (2011)

[TABLE 1]. Effects of stress in LTP and LTD

In contrast to LTD, HF LTP induction in brain slices [TABLE 1] is impaired immediately after fear conditioning exposure (Li et al. 2005) and tail-shocks (Kim et al. 1996). The effect appears to persist for one day after fear conditioning (Li et al. 2005) and forced swim (Maggio & Segal 2011) in the dorsal HF, for 4 days after fear conditioning in the re-exposure to the conditioning context (Li et al. 2005) and for one week after SPS (Kohda et al. 2007). Moreover, these effects are also found *in vivo*. Thus, elevated platform exposure inhibited LTP for 3 h (Ryan et al. 2010) and tail-shocks decreased LTP 48 h, but not 96 h, post-stress (Shors et al. 1997). In some cases longer-lasting effects have been found, in that LTP was impaired for 4 weeks in animals exposed to tail-shocks (Ryan et al. 2010). Conversely, in the short-term (hours) some stressors facilitate LTP as reflected in increased mEPSC frequency (Karst et al. 2005), amplitude (Karst & Joels 2005) and number of surface AMPA receptors (Groc et al. 2008).

Stress effects on LTP might depend on the severity of the stressor, the HF region and the time window. There appears to be an inverted-U correlation between the corticosterone levels and LTP magnitude in the HF (Diamond et al. 1992). Disparities are also observed in dorsal and ventral HF (Maggio & Segal 2011) and even within HF subfields: whereas the firing frequency of

CA1-CA3 neurons decreased immediately after 30 min of photic stress, DG granule cells were not affected (Passecker et al. 2011).

Molecular mechanism of synaptic plasticity and stress

Stress-induced changes in glutamatergic synaptic transmission are not restricted to changes in glutamate receptors. There are also other targets for stress-mediated effects. In this section, we will mainly address the effects of stress on some proteins related to glutamatergic modulation.

Presynaptic terminals proteins

Neurotransmitter release involves four main highly regulated functions in presynaptic terminals (Südhof 2012): (i) docking and priming of synaptic vesicles (through SNARE, Sec1/Munc18-like proteins); (ii) recruiting voltage-gated Ca^{2+} channels to enhance vesicle fusion and neurotransmitter release; (iii) allowing correct pre- and postsynaptic interaction through transynaptic adhesion molecules and, finally; (iv) mediating short and long-term plasticity. Synaptophysin is the major resident protein of the vesicle plasma membrane and it has been proposed that this protein regulates the availability of synaptobrevin for interacting with the SNARE complex proteins (Becher et al. 1999, Yelamanchili et al. 2005). It is related to learning and memory, as genetic deletion of synaptophysin gene impaired novel object recognition and spatial learning (Schmitt et al. 2009). Several studies have demonstrated a decrease of synaptophysin protein levels in the HF after chronic unpredictable stress (Briones et al. 2012) and 30 days after a single foot-shock (Herrmann et al. 2012). However, 7 days after a single predator exposure synaptophysin levels were not modified in the PFC and the HF, but they were increased in the amygdala (Campos et al. 2013), in contrast to the decrease observed after chronic unpredictable stress decreased (Luo et al. 2013). These studies suggest the importance of the particular brain region studied and the stress duration.

Postsynaptic terminals proteins

Calcium/calmodulin–dependent protein kinase II (CaMKII)

LTP-associated synaptic plasticity is, in part, due to the increase in intracellular Ca^{2+} concentrations, via NMDA receptor and voltage-gated Ca^{2+} channels (Bliss & Collingridge 1993). This rise in intracellular Ca^{2+} activates several kinases such as protein kinase A (PKA), protein kinase C, mitogen-activated protein kinase (MAPK), tyrosine kinase Src, and CaMKII (Malenka & Nicoll 1999). CaMKII is a molecular key regulator of LTP as its genetic deletion or pharmacological blockade impairs LTP induction (Malenka et al. 1989). Ca^{2+} binds to calmodulin to activate CaMKII that autophosphorylates to maintain its activity beyond the decay of Ca^{2+} levels (Dosemeci & Albers 1996). Moreover, CaMKII phosphorylates AMPA GluA1 subunits to increase their conductance (Derkach et al. 1999).

Little is known about the relationship between stress and CaMKII. Only four works have assessed the effect of stress on CaMKII phosphorylation (pCaMKII). In acute stress, 60 min restraint increased pCaMKII in the HF and this effect is prevented by AMPA, but not by NMDA antagonists (Suenaga et al. 2004). Immediately after restraint, pCaMKII levels increased in the HF in $GluA^{-/-}$, but not wild type, mice (Fumagalli et al. 2011). However, 15 min after a brief (5 min) exposure to forced swim. a decrease in pCaMKII in PFC was observed (Fumagalli et al. 2009). In chronic stress studies, the pCaMKII levels were reduced after chronic intruder social

stress (animals mates are daily changed for 30 days) in CA1 subfield (Gerges et al. 2004), but not by chronic restraint HF (Suenaga et al. 2004). Taken together, these results again suggest that the effect of stress in pCaMKII might depend on the severity of the stressors, their duration and the particular brain area studied.

Activity-regulated cytoskeleton-associated protein (Arc) is an IEG that has been related to memory regulation because is highly modulated by cognitive processes (Okuno et al. 2012). Arc is preferentially maintained at inactive synapses, rather than active, due to a high affinity interaction with CaMKII (Fumagalli et al. 2011, Okuno et al. 2012), suggesting a possible inverse synaptic tagging (Okuno et al. 2012). Arc has been consistently demonstrated to be up-regulated by different types of stressors (Fumagalli et al. 2011, Ons et al. 2004, Trnecková et al. 2007), suggesting a possible role of Arc/CaMKII in stress-related synaptic plasticity.

G-protein-coupled inwardly-rectifying potassium channels (GirKs)

An important modulator of HF transmission is serotonin (5-HT). Dorsal and ventral HF are highly innervated by serotoninergic neurons from median and dorsal raphe, respectively (Mokler et al. 1999), and practically all 5-HT receptors are expressed in the HF (Berumen et al. 2012). 5-HT modulation of glutamatergic pathways is exerted, in part, through 5-HT_{1A} receptor (Saenz del Burgo et al. 2008). Its stimulation in rodents has been related to memory and learning impairment through inhibition of glutamate release (Ogren et al. 2008). This inhibition of glutamate release is mediated by the hyperpolarizing potassium channels, in particular, GirKs (Fairchild 2003, Muma & Beck 1999). GirK family is composed by 4 members. GirK 1-3 is highly expressed in CNS, whereas GirK 4 is primarily found in the heart (Saenz del Burgo et al. 2008). Chronic corticosterone administration in drinking water containing corticosterone (50 μg/ml) decreased GirK2 subunit mRNA expression in the dorsal raphe (Fairchild 2003). However, adrenalectomy reduces GirK protein levels in the HF (Muma & Beck 1999). Although, to our knowledge, there are not stress studies, these results suggest a possible effect of stress in Girk-dependent synaptic modulation.

Hyperpolarization-activated cyclic nucleotide-gated cation channels (HCN)

Other important channels modulating neuronal activity are the hyperpolarization-activated cyclic nucleotide-gated cation channels (HCN). There are four HCN genes (HCN 1-4) and HCN 1 is the most expressed in the HF (Lörincz et al. 2002). HCN underlies the hyperpolarization-activated cation currents (I_h) and has a role in spontaneous pacemaker currents in the brain and the heart (Santoro & Baram 2003). The I_h channels have unusual ion selectivity (they conduct both Na^+ and K^+ ions, but exclude Li^+), and are activated by hyperpolarizing voltage and by cAMP (Robinson & Siegelbaum 2003, Zha et al. 2009). Four physiological functions have been proposed: pacemaker activity, control of resting potential, dendritic integration and regulation of synaptic transmission (Robinson & Siegelbaum 2003). HCN1 appears to participate in learning and memory. Either blockade with specific channel blocker (ZD-7288) or forebrain restricted $HCN1^{-/-}$ enhances HF (Nolan et al. 2004) and PFC-dependent learning (Wang et al. 2007a). One proposed mechanism is that I_h inhibit dendritic excitability (Fan et al. 2005, Tsay et al. 2007). However, in the amygdala, CRF administration produces an increase in resting I_h, mainly due to HCN1 action, and increases neuronal excitability (Giesbrecht et al. 2010). CRF also increased HCN1 protein levels (Tran & Greenwood-Van Meerveld 2012). This opposite effects of I_h on neuronal excitability could be explained by a dual effect, depending on the overall excitability of the neuron (George et al. 2009).

Postsynaptic Density 95 (PSD 95)

The pOstsynaptic density (PSD) is an electro-dense region of the postsynaptic terminal, formed by a multi-protein complex that includes adhesion molecules, glutamate receptors, scaffolding proteins, kinases and trafficking related proteins (Cheng et al. 2006). One of the most common proteins of PSD is PSD 95, also known as synapse-associated 90 (SAP 90) or disc-large homolog 4 (DLG 4) protein. This protein belongs to the group of membrane-associated guanylate kinases (MAGUKs) (Chetkovich et al. 2002) and acts as a scaffolding protein with several protein-protein interaction domains. This allows its binding to several proteins of the PSD including kinases, glutamate receptors, microtubule-associated proteins and cell adhesion molecules (CAM) (Hata & Takai 1999). It has been related to synaptogenesis because during development PSD 95 is one of the first scaffolding proteins to arrive to the spine (Rao et al. 1998). Moreover, this scaffolding protein has a key role in synaptic plasticity as experimental over-expression of PSD95 increases AMPA receptor function and, surprisingly, LTD (Beique & Andrade 2002). Moreover, it increases AMPA function, in part, by increasing AMPA receptor membrane delivery (Ehrlich & Malinow 2004). Accordingly, AMPA function can be blocked in PSD 95-deficient mice (Beique & Andrade 2002).

Exposure to a single restraint (Yang et al. 2008) or odor stress (Zohar et al. 2011) increased HF PSD 95 levels 24 h and 7 days after stress. However, chronic unpredictable stress has been found to decrease amygdala PSD 95 protein levels and this effect is prevented by chronic administration of Neotrofin, a neurotrophin agonist, 30 min prior to stress exposure (Luo 2013).

Structural plasticity and stress

Structural plasticity refers to changes in neuronal dendrites (Tavosanis 2012) and spines (Alvarez & Sabatini 2007). Ramon y Cajal considered that dendrites were a form to enlarge cellular surface and increase synapses (97% of motor neurons surface are dendrites and the 80% of dendrite surface is covered by synapses). Thus, dendritic tree complexity is the result of increasing the number of branching and/or dendrite length and may reflect the propensity of a neuron to increase their synapses (Fiala and Harris, 1999). Dendritic morphology depends on the particular neuron. In particular, pyramidal neurons of the cortex (layer II/III/V) and the HF (CA1/CA3) have two clearly different domains, a basal domain, where dendrites are shorter, and an apical domain with a main longer dendrite, with emerging perpendicular dendrites, ending in an apical tuft (Spruston 2008).

This particular morphology has been related to different inputs onto distinct domains. The soma and the axon receive inhibitory GABAergic inputs, whereas the dendrites receive excitatory inputs from different origins. For instance, CA1 neurons receive innervation from CA3 Schaffer collaterals in the basal and the proximal apical dendrites and from perforant pathway (enthorinal cortex) in the distal apical dendrites (Szirmai et al. 2012, Takács et al. 2012). More precisely, CA3 neurons proximal to CA1 subfield project to basal dendrites, whereas distal CA3 neurons project to CA1 proximal apical dendrites (Ishizuka et al. 1990, Li et al. 1994). Cells, including CA1 neurons, have passive and active mechanisms to integrate their several inputs (Temporal and Spatial summation), which include morphology, voltage-gated ion channels and properties of their synapses (Magee 2000).

In spiny neurons, synapses occur at specialized sites of the dendrites consisting of protrusions that are named spines and were firstly described by Ramon y Cajal. In pyramidal cells (e.g. CA1)

spines have an approximately one-to-one relationship with excitatory synapses. Their functions are to increase the area of contact with the presynaptic terminal (Koch & Zador 1993, Stepanyants et al. 2002), to make a separate biochemical compartment from the dendrites (Harris & Stevens 1989, Koch & Zador 1993) and to regulate electrical properties of the neuron (Spruston 2008, Tsay & Yuste 2004, Yuste & Urban 2005). Spines are highly dynamic and they vary their shape and size constantly. Several spines morphologies have been described, the most common are *filopodia*, which are long and thin very dynamic protrusions; and the simple spines, which are more stable protrusions ending in a bulbous head. Simple spines present different shapes: stubby spines, which do not have neck; thin spines, which have neck and a small head; and finally, mushroom spines, which have neck and a large head (Fiala and Harris, 1999).

Several Golgi staining histological studies has shown dendritic spines motility (Rampon et al. 2000, Schapiro & Vukovich 1970). However, new generation of transgenic animals expressing neuronal fluorescent proteins (Alvarez & Sabatini 2007, Feng et al. 2000), the use two-Photon Laser Scanning Fluorescence Microscopy (Denk et al. 1990) and the new fluorescent intracellular dyes techniques (Moser 1999) have provided live imaging and more suitable quantitative *in vitro* and *in vivo* studies. In particular, long-term live imaging has provided crucial data about differences in spine stability between young and old mice in cortical neurons. The consensus is that in young animals the spines are less persistent than in mature animals (Holtmaat & Svoboda 2009). This fact is due to spine elimination, being higher in young animals than in mature animals. In addition, the spine formation rate remains constant from the postnatal life to adulthood (up to 5 month). This results in a net spine loss in young animals that reaches a plateau in mature animals (Alvarez & Sabatini 2007, Bloss et al. 2011, Holtmaat & Svoboda 2009, Holtmaat et al. 2005, Trachtenberg et al. 2002, Zuo et al. 2005a). Spine loss has been related with microglia both, during development and in adulthood (Chung & Barres 2011). A recent paper has demonstrated that PSD 95 is detected in microglial cytoplasm after synapses elimination. Moreover, mice lacking fractalkine receptor (Cx3cr1), crucial for microglial migration, have higher PSD 95 levels and more dendritic spines, together with characteristics of immature brain assessed by electrophysiology (Paolicelli et al. 2011).

In addition to the above processes, there are also changes in spine shape. *Filopodia* can transform into thin or mushroom spines, but a low number does it and the remaining *filopodia* disappear. On the contrary, spines with a well-defined head are more stable (Alvarez & Sabatini 2007, Holtmaat & Svoboda 2009). In old rats, the number of mushroom spines is equal to that of young rats despite a decrease in total spine density in old rats (Bloss et al. 2011). A similar finding has been reported in humans, whereas large head spine density is similar, the total spine density is lower in an 85 years male as compared to a 40 years male (Benavides-Piccione et al. 2012).

The structural plasticity described above is due to normal developmental processes. However, activity-dependent structural plasticity has also been related to experience and learning. Alvarez and Sabatini (2007) proposed two possible models to explain activity-dependent structural plasticity: i) spines are created due to neuronal activity associated with new experiences (learning) or; ii) spines are created constitutively and activity-driven elimination selects the meaningful connections. These processes appear to be similar to those found during development, which are also activity-dependent (Schafer 2012). Alvarez and Sabatini (2007) proposed that structural plasticity is due to a combination of both models, with the relative contribution of each model depending on the area, the situation that creates the plasticity and the age of the animal. Alt-

hough there is also activity-dependent structural plasticity in presynaptic boutons, it is mainly described in development (Yasuda et al. 2011) and it is less dynamic than in dendritic spines (De Paola et al. 2006).

The most studied models of activity-dependent structural plasticity are sensory deprivation and environmental enrichment (EE). Sensory deprivation, which usually consists of the removal or trimming of whiskers, induces at long-term reduction of spine elimination with no effect in formation (Zuo et al. 2005b), and a less stability in old spines and higher stability of new spines that always formed synapses (Holtmaat et al. 2006), suggests that trimming of whiskers induces adaptive functional changes that probably are involved in remodeling neocortex circuitry. The EE is the enhanced physical, social, and cognitive stimulation of the brain that influences physiology and behavior. EE increased dendrite branching (Faherty et al. 2003, Leggio et al. 2005, Schapiro & Vukovich 1970, Volkmar & Greenough 1972), spine density (Leggio et al. 2005, Rampon et al. 2000, Schapiro & Vukovich 1970), and synapse number (Altschuler 1979, Rampon et al. 2000). These effects of EE are similar to those found in LTP. Moreover, enhanced LTP has been found in EE (Malik & Chattarji 2012). Two elegant studies with time lapse imaging of fluorescent labeled hippocampal neurons revealed that LTP induction was associated with new spines and was blocked by NMDA antagonists (Engert & Bonhoeffer 1999, Maletic-Savatic 1999). LTP-induced enlargement of spine volume is dependent on NMDA receptor, CaMKII, actin cytoskeleton rearrangement (Matsuzaki et al. 2004) and BDNF (Tanaka et al. 2008). On the contrary, LTD induction causes spine loss (Nägerl et al. 2004, Okamoto et al. 2004), reduces spine head diameter and requires NMDA receptors and calcineurin activation (He et al. 2011, Wang et al. 2007b, Zhou et al. 2004). Taken together, these results demonstrate that structural plasticity is influenced by age and changes in sensory inputs, and is associated with LTP and LTD. However, the precise mechanisms involved in the structural changes remain unclear.

One of the most studied effects of stress in the brain are morphological modifications (for review: Gray et al. 2013, McEwen 2012, McEwen & Morrison 2013, Popoli et al. 2011) Chronic stress (e.g. 21 days of daily restraint) causes HF dendrite remodeling, particularly a shrinkage of apical dendrites in CA3 (Mcewen 1999, Orlowski et al. 2011, Vyas et al. 2002). In contrast, chronic stress increases the dendrite length in DG granule cells (Orlowski et al. 2010). Chronic corticosterone administration at very high doses (40 mg/kg) mimics apical dendrite atrophy observed after chronic stress in DG, CA3 (Sousa et al. 2000) and CA1 (Morales-Medina et al. 2009, Sousa et al. 2000). Similar effects on dendritic length have been found in PFC after chronic administration of a high corticosterone dose (25 mg/kg) (Cerqueira et al. 2007) or chronic unpredictable stress (Dias-Ferreira et al. 2009). Corticosterone is also needed for correct morphological features of the HF. Thus, adrenalectomy reduces DG dendrite length and complexity that are restored by basal corticosterone replacement in the drinking water (20 µg/ml) (Sousa et al. 1999). On the contrary, in the amygdala, chronic stress increases dendrite length and branch points (Vyas et al. 2002), although chronic corticosterone does not modify amygdala arborization (Morales-Medina et al. 2009), suggesting that other factors are involved. Chronic stress induces also mPFC morphological remodeling. Decreased branch number and dendritic length was found after one or three weeks of daily restraint (Brown et al. 2005, Cook & Wellman 2004, Martin & Wellman 2011, Radley et al. 2006). This effect is prevented by chronic administration of an NMDA antagonist (Martin & Wellman 2011). Chronic stress also affects spine density. Conversely to the consistent atrophy found in HF dendrites, chronic stress effects on spine density are controversial and do not correlate to dendrite changes. Some works with chronic restraint reported increases in spine density in CA1 neurons (Donohue et al. 2006, Orlowski et al.

2011); whereas in others chronic restraint (Magariños et al. 2011) or chronic corticosterone (40 mg/kg) (Morales-Medina et al. 2009) reduced spine density. In CA3, no effect in spine density (Orlowski et al. 2010, Magariños et al. 2011) or an increase (Sunanda et al. 1995) has been reported. Finally, no effect in spine density has been found in DG (Orlowski et al. 2010). In the PFC there are also controversies in the literature. Thus, while some studies reported a decrease in spine density after chronic restraint (Bloss et al. 2011, Radley et al. 2008), others did not found an effect after chronic unpredictable stress (Dias-Ferreira et al. 2009) or long-term corticosterone administration (Cerqueira et al. 2007). In the amygdala, chronic stress decreased spine density (Bennur et al. 2007), whereas no effect of chronic corticosterone treatment was found (Morales-Medina et al. 2009). These controversial effects could be explained by the use of different quantification techniques, since Golgi staining is not suitable for spine quantification because of the lower resolution of light microscopy (Moser 1999). This problem emphasizes the importance of the use of intracellular dyes followed by confocal (e.g. Radley et al. 2008) or the use of electronic microscopy analysis with posterior 3D reconstruction (e.g. Donohue et al. 2006), which allows the detection of close spines and a 3D perspective. In addition, age of animals has been proposed as a possible explanation, since the spine density differs along lifespan; however, most of the studies used young adult rodents, minimizing the age effect.

Although the vast majority of effect has been described after chronic stress, acute stress also results in structural plasticity. Changes in the amygdala arborization has been described, in particular, dendrite expand has been observed 7 days after exposure to SPS (Cui et al. 2008), predator odor (Zohar et al. 2011) or predator exposure (Adamec et al. 2012). In contrast, after sixteen days of a single exposure to a cat, no changes were observed in the DG (Adamec et al. 2012).

Spine density is also affected by single stress exposure [TABLE 2]. Spine loss has been observed immediately after five hours of a multimodal combined physical/psychological stress in CA3 in mice (Chen et al. 2010), 24 h after fear conditioning exposure in CA1 of mice (Sanders et al. 2012), one week after IS-LH animals in CA1, CA3 and DG of rats (Hajszan et al. 2010) and one week after cat odor exposure of rats (Zohar et al. 2011).

Stress/Stressor	Species/Strain Age or weight at stress start	Region/Time after stress	Spine density vs Control group	Reference
Combined physical and psychological stress	Mice/ 3-4months	CA3/ Immediately	Decrerased (↓)	Chen et al (2010)
Fear conditioning	Mice/ 2-4months	CA1/ 24 h	↓	Snaders et al (2012)
Footshocks (LH)	Rats SD/ 200-250g	CA1, CA3, DG / 1 week	↓	Hajszan et al (2009)
Cat odor	Rats SD/ 150-200g	DG/ 1 week	↓	Zohar (2011)
Restraint + footshock	Rats SD/ 250-350g	CA1/ 24 h	↑	Shors et al (2001); Dalla et al (2009)
Footshocks (LH)	Long Evans/ 5 weeks	DG/ 16 days	=	Adamec et al (2012)

[TABLE 2]. **Effects of stress** in dendritic spines

However, in other studies, a single stress exposure induces a spine density increase or no effect. One day after IS-LH, spine density increased in CA1 in rats (Dalla et al. 2009, Shors et al. 2001), whereas predator exposure caused no effect on DG of rats sixteen days later (Adamec at al.

2012). Combined physical/psychological stress-related spine density decrease in CA3 can be prevented by CRH antagonist administered into the lateral ventricle 30 min before stress (Chen et al. 2010). Moreover, spine elimination has been reported in pyramidal cells of cortical layer 5 by 3 daily administration of 15 mg/kg of corticosterone that is prevented by MR antagonist, but not by GR coadministration with corticosterone (Liston & Gan 2011). It is important to note that acute stress effects depend on stress severity and duration, and even more importantly, on the time window when stress effects are assessed.

Stress-induced structural plasticity appears to be mediated by cell adhesion molecules (CAMs) (McEwen et al. 2011). One of the most studied CAM is the neural cell adhesion molecule (NCAM), which is a cell surface glycoprotein from the immunoglobulin superfamily. There are 3 NCAM isoforms formed by alternative splicing (Cunningham et al. 1987) named NCAM 120, NCAM 140 and NCAM 180 (due to the apparent molecular weight in kilodaltons (kDa). The different isoforms contain the same extracellular domain and only differ in the intracellular domain. NCAM 120 lacks the intracellular domain and is anchored to the plasma membrane via a glycosylphosphatidylinositol residue; NCAM 140 possesses a 30 kDa intracellular domain; and NCAM 180 has the large intracellular domain and it accumulates in postsynaptic densities (Gegelashvili et al. 1993, Kramer et al. 1997). In addition to membrane anchored forms, several soluble NCAM forms have been detected in the CSF. These soluble isoforms inhibited cell binding to immobilized NCAM substrate, suggesting a modulatory role on cell behaviour (Olsen et al. 1993). NCAMs are postranscriptionally modified by polysialic acid (PSA) addition (Krog & Bock 1992).

PSA-NCAM impairs cell adhesion (Rutishauser & Landmesser 1996), but it is required for synaptic plasticity. Removing PSA from NCAM by endoneuraminidase-N enzyme (Endo-N) results in a failure to induce LTP and LTD and impairment of MWM performance (Becker et al. 1996, Muller et al. 1996). PSA synthesis is highly regulated and an important cell adhesion modulator (Senkov et al. 2012). Thus, it is evident the key role of PSA-NCAM in synaptic plasticity (Murase & Schuman 1999, Senkov et al. 2012), spinogenesis and synaptogenesis (Dityatev et al. 2004). In addition, NCAM-KO mice have also LTP impairment (Cremer et al. 2000). Consequently, stress effects on structural plasticity are expected to be accompanied by modulation of both PSA-NCAM and NCAM.

Chronic corticosterone administration (10 mg/kg) decreased NCAM levels in PFC, whereas NCAM HF levels were not affected (Sandi & Loscertales 1999); however, another study showed a decrease in DG levels after chronic administration of high doses of corticosterone in the drinking water (400 μg/ml) (Nacher et al. 2004). This controversial data could be explained by the corticosterone dose and the different methodology used to assess the NCAM levels: ELISA (Sandi & Loscertales 1999) or immunohistochemistry (Nacher et al. 2004). Chronic restraint effects seem to be more consistent, with a reduction of HF NCAM mRNA (Venero et al. 2002) and protein levels (Pham et al. 2003).

A single exposure to predator stress immediately induced a reduction of NCAM levels in HF, PFC and no effect in amygdala (Sandi et al. 2005). In contrast, acute corticosterone administration (10 mg/kg) enhanced NCAM PFC levels, without affecting NCAM HF levels (Sandi & Loscertales 1999). Stress-induced decreases in NCAM levels could be associated to the stress-related memory impairment.

Glia and stress

Glial cells play an important role in brain homeostasis, providing support to neurons and forming myelin. There are two types of glia: microglia and macroglia. The last one includes: astrocytes, oligodendrocytes, ependymal cells and radial glia. Stress and glia have been mainly studied in animals exposed to systemic stressors, but recently, there has been a growing interest in emotional stressors.

Around 40% of hippocampal excitatory synapses are covered by astrocytic processes (Perea et al. 2009). Thus, some glutamatergic synapses have to be considered as a tripartite synapse. Astrocytes respond to synaptic activity by intracellular Ca^{2+} elevations, which induce release of some gliotransmitters and modulators (glutamate, D-serine, ATP, GABA, BDNF, and several peptides and proteins) that affect synaptic transmission (Parpura & Zorec 2010). One of the most used astrocytes marker is the GFAP. Changes in GFAP protein levels have been observed in mood disorders patients, with decreased GFAP-positive cells and protein levels in the PFC (Miguel-Hidalgo et al. 2010, Webster et al. 2001) and the amygdala (Altshuler et al. 2010), but nor in the HF (Webster et al. 2001).

Rats exposed to chronic unpredictable stress present reduced glial proliferation (Banasr et al. 2007) and lower number of GFAP positive cells in the PFC (Banasr & Duman 2008), as well as a decrease in GFAP mRNA and protein in the HF (Liu et al. 2011a). Moreover, corticosterone administration in the drinking water (200 μg/ml) prevents the increase in HF GFAP mRNA levels observed in adrenalectomized rats, suggesting a negative regulation of astrocytes by glucocorticoids (Nichols 1999). However, another study shows that chronic corticosterone administration (implanted pellets with 300 mg for 21 days) induced an increase in GFAP positive cells in CA1, but not CA3 (Bridges et al. 2008). Taken together, these results mainly suggest a reduction of GFAP after chronic stress, although the role of corticosterone is unclear. Acute stress also modulated GFAP levels, since a single session of footshock immediately decreased GFAP expression in the rat cortex, but not in the HF or the hypothalamus (Blandino et al. 2009).

Some authors have associated severe stressors with inflammatory-like processes mediated by high levels of glucocorticoids (Frank et al. 2011, García-Bueno et al. 2008), contrary to the anti-inflammatory role of glucocorticoids in the periphery. These effects might be dependent on the glucocorticoids dose, stress intensity and brain region (for review (Frank et al. 2011, García-Bueno et al. 2008, Sorrells & Sapolsky 2007). Thus, we can suggest that astrocytes could be playing a dual role in stress, releasing pro-inflammatory mediators and modulating glutamatergic synapses through gliotransmitters and modulators release.

Not only astrocytes, but also microglia might be involved in stress induced inflammatory-like process (Frank et al. 2007). Microglia cells are the resident macrophages of CNS and are the main responsible for neuroimmune function. During development, microglia removes apoptotic cells, modulates synaptic connections and contributes to correct brain maturation. In adult healthy brain, microglia cells constitute 10-12% of brain cells, and exist in a "resting" state characterized by a particular morphology with round soma and long branching processes that are constantly testing the brain microenvironment. After brain injury or pathological challenges, microglia changes to reactive state with "amoeboid" morphology able to phagocytize cellular debris or pathogens (Jurgens & Johnson 2012). There is another microglia state, the hyper-ramified, that has been proposed as an activation state that differs from the reactive state; however, literature about this state is sparse (Hinwood et al. 2013).

The effect of stress on neuroimmune function is complex, with evidence of both beneficial and detrimental activation (Jurgens & Johnson 2012). The most used neuroimmune markers are the IL-1 and the microglia. Deak and collaborators (2005) have evaluated the interleukin 1 (IL-1) levels in the hypothalamus after immediately single immobilization and foot-shock, showing an increase of IL1 levels. This suggests that the hypothalamus plays an important role in stress-related immune response. However, a single forced swim fails to induce IL-1 production in the posterior cortex, the HF and in the hypothalamus (Deak et al. 2003). Similarly, 2 h after acute or chronic predator stress does not induce changes (Plata-salaman et al. 2000) in the PFC, the HF, the amygdala and in the hypothalamus. The reasons for the discrepancies among experiments are not known.

Microglia has only been evaluated after chronic stress. Chronic restraint (21 days) induces microglial activation in the PFC, as measured by the ionized calcium-binding adapter molecule 1 (Iba 1, a microglial marker) immunoreactivity (Hinwood et al. 2011)and an increase in the number of branches in microglia, which has been related to activation microglia (Hinwood et al. 2013). Both effects are inhibited by the microglia inhibitor minocycline (Hindwood et al. 2011, 2013). The same protocol induces an increase in Iba1 immunoreactivity in Gerbil hippocampus (Park et al. 2011) and various rat brain areas, including the PFC, BNST, Nucleus accumbens and the CA3 (Tynan et al. 2010). Kopp and collaborators (2013) have compared the differential effects of repeated (chronic restraint) and variable chronic stress (Chronic unpredictable stress) on microglia activation assessed by immunohistochemistry. Surprisingly, chronic unpredictable stress did not alter microglia in the PFC, whereas chronic restraint did. In addition, no effects were found in the PVN by either type of stress. Chronic foot-shocks stress can facilitate the influx, through the brain-blood-barrier (BBB), of monocytes into the HF (Brevet et al. 2010) and the PVN (Ataka et al. 2013). Nair and Bonneau (2006) have demonstrated that the administration of a NMDA receptor antagonist (MK-801), the inhibition of glucocorticoid synthesis or the blockade of GR prevented the chronic restraint-related increase of microglia in the whole brain, suggesting that microglial activation may be mediated by glucocorticoid-induced increases in extracellular glutamate.

Microglia not only participates in the release of pro-inflammatory mediators, but also in synapse pruning (Chung & Barres 2011). Synaptic pruning in immature brain is necessary for appropriate brain development (Paolicelli et al. 2011, Schafer et al. 2012). Furthermore, brain plasticity in the adult brain cannot be understood without synaptic pruning (Ekdahl 2012). Wake and collaborators (Wake et al. 2009) have monitored microglia *in vivo* demonstrating that it makes transient contacts with neuronal synapses and some of these synapses disappear after prolonged microglia contact in cerebral ischemia. Visual experience also modulates microglia interaction and pruning (Tremblay et al. 2010). Long-term (72h) *in vivo* transcranial imaging shows that spine elimination increases in the barrel and primary motor cortex after acute and chronic corticosterone administration (15 mg/kg), and this effect is blocked by MR, but not GR antagonists (Liston & Gan 2011). Finally, as happens with astrocytes, microglia could play a dual role in inflammatory-like processes modulated by stress and in neuronal synapses pruning.

Stress and MRI in animal models

As already discussed (see 2.2 Biological basis of PTSD), in humans, several studies have observed effects of PTSD in PFC, HF and amygdala. Thus, ideally, animal PTSD models should mimic PTSD-like anatomical and functional changes. Although area-specific volumes have been

measured in rats by post mortem stereological studies (Cerqueira et al. 2005, Sousa et al. 1998), MRI imaging allows the assessment of longitudinal and functional studies *in vivo*.

In a longitudinal MRI study with pre-stress and post-stress evaluation, it has been reported that animals exposed to chronic restraint present a reduction of the HF volume compared to the small increase observed in controls (Lee et al. 2009). These results are compatible with stereological studies, where adrenalectomized animals administered over a period of 4 weeks with dexamethasone, a GR agonist, showed a decreased volume of layer II of PFC and CA3 of HF (Cerqueira et al. 2005, Sousa et al. 1998). There is only a single study using a single exposure to stress, which showed a reduction of left HF 74-88 days after foot-shock (Golub et al. 2011).

There are also two studies of DTI analysis in stress. In one of them, animals exposed to chronic restraint stress presented a significant neurite loss density in the HF, specifically in *stratum oriens* and *radiatum* of CA1, in *stratum radiatum* and *lacunosum moleculare* of CA3 and in granule cell layer and molecular layer of DG (Vestergaard-Poulsen et al. 2011). Another study investigated hippocampal volume in animals before fear conditioning, at 1 h and one day after fear conditioning in the HF, the cingulated cortex and the amygdala (Ding et al. 2013). These authors observed that fractional anisotropy in the HF decreased at 1h post-fear conditioning and then returned to pre-fear conditioning values. Fractional anisotropy was increasing in cingulate cortex and adjacent gray matter one hour and one day post-fear conditioning respectively. Finally, in the amygdala significantly increased at 1h and 1 day post-fear conditioning. The authors suggest that these changes in volume of specific brain structures can be related to synaptic plasticity processes.

Few papers have examined the effects of stress on metabolites in brain using MRI. The vast majority of them have used ^{1}H-MRS. A pioneering ^{1}H-MRS study demonstrated that adrenalectomized rats, which not differ in glutamate levels from control rats, after being administered with dexamethasone (0.25 μg/ml in drinking water) for 11 weeks presented higher levels of glutamate in the HF (Schubert et al. 2008). However, chronic unpredictable stress produced a significant decrease of glutamate, glutamine, NAA and GABA levels in the HF and a significant decrease of glutamine and GABA levels in the PFC (Hemanth Kumar et al. 2012). In another study, the chronic unpredictable stress-induced reduction of NAA levels in the HF was reverted by the antidepressant escitalopram (Xi et al. 2011). Similarly, reduced NAA HF levels were found after chronic forced swim that were also prevented by the antidepressant tianeptine (Liu et al. 2011b).

Acute stress also induces changes in some metabolites. In mice, a single fear conditioning protocol significantly decreased NAA levels in the HF at one day and one week after conditioning, whereas in the cingulate cortex the decrease was only observed at one day after conditioning, while no effect at all was observed in the thalamus (Zhou et al. 2012). In another fear conditioning model, that used two levels of foot-shocks (0.5mA vs 1.5 mA); animals were classified according to low or high NAA levels prior to fear conditioning. Low NAA levels in the left dorsal hippocampus predicted persistent PTSD-like symptoms, while animals with pre-traumatic high levels of NAA showed decreased fear conditioning (Siegmund et al. 2009). In a more recent study, animals were classified according to their low, intermediate or high reactivity to stress (on the basis of their HPA response). Interestingly, 10 weeks after a single restraint session high reactive animals presented decreased NAA levels in the right dorsal HF and the PFC (Knapman et al. 2012). Finally, a genetically selected LH rat strain presented lower levels of glutamate in the HF and lower levels of choline-containing compounds in the HF and the PFC (Schulz et al. 2012). Taken together, these data suggest that acute stress induces, at long-term, a general re-

duction of neuronal integrity in the HF and the PFC, which might be related to the hypoactivity and memory impairment described in PTSD patients [TABLE 3].

Stress/Stressor	Species/Strain Age or weight at stress start	Brain region	Metabolite ratios	Reference
Chronic stressors				
Chronic forced swim	Rats/ 200g weight	HF	↓ NAA	Liu et al. (2011)
Chronic High dexametasone	Rats Wistar/ 4,5 months	HF	↑ Glut	Scubert et al. (2008)
CMS	Rats SD/ 3-4 months	PFC and HF	↓ Glut, ↓ Glux and ↓ GABA in PFC ↓ Glut, ↓ NAA, ↓ Glx and ↓ GABA in HF	Hemanth Kumar et al. (2012)
CMUS	Rats SD/ 200g weight	HF	↓ NAA	Xi et al. (2011)
Acute stressors				
Early life stress	Rats wistar/13 days	HF	↓ Glut, ↓ NAA	Llorente et al. (2012)
Footshock (0,5 mA)	Mice C57BL/6N / 23–28g weight	HF, cingulate cortex and thalamus	↓ NAA in HF and cingulate cortex	Zhou et al. (2012)
Footshock (1,5 mA)	Mice C57BL/6N / 8-9 weeks	HF	↓ NAA	Siegmund et al. (2009)
LH	Rats SD/ 8-10 weeks	HF and PFC	↓ Glut in HF and ↓ choline compounds in PFC and HF	Schulz et al. (2011)
Restraint 15min	Mice CD-1 / 8-9 weeks	HF and PFC	↓ NAA in HF and PFC	Knapman et al. (2012)

[TABLE 3]. Effects of stress in ^{1}H-MRS

In two recent works, the immediate effects of stress on brain function and metabolites were assessed using fMRI and ^{1}H-MRS, respectively. Corticosterone administration (intravenously, 68 μg/ml) induced within 0-60 seconds of administration a significant increase in BOLD signal in the HF, the PFC, the striatum, the thalamus, sensory and motor cortex and lateral hypothalamus (Ferris & Stolberg 2010). One hour restraint induced an increase in glutamate levels in the HF and the PFC (Kim et al. 2011). Hence, these results suggest a non-genomic activation induced by corticosterone in PTSD related areas and an altered glutamatergic transmission.

Post-stress brain activation has been tested *ex vivo* by Mn-enhanced MRI. In MRI is common to use contrast agents such as gadolinium or Manganese (Mn^{2+}). Mn^{2+} can be used as a contrast because it is attracted by an external applied magnetic field (paramagnetic ion) (Ahrens et al. 2002), and as a means of monitoring the brain activity due to their similarities to calcium ion (Hankir et al. 2011). However, Mn^{2+} is neurotoxic when accumulates in some brain areas and hence its concentration has to be well-regulated (Fitsanakis et al. 2008). Bangasser and collaborators (2013) used this approach to test brain activation associated with heterotypical sensitization. Animals with or without a prior history of chronic social defeat were exposed to forced swim. As expected, animals with a history of chronic social defeat showed a greater activation of the PFC, the ventral HF (but not the dorsal), the *nucleus accumbens*, the habenula, the amygdala, the BNST, some hypothalamic areas (but not in the PVN), the PAG and the raphe.

To sum up the effects found in anatomical MRI, DTI, ^{1}H-MRS, and functional MRI post stress are consistent with those found in PTSD patients, suggesting that mimic PTSD consequences such as changes in anatomical volume, in white matter volume, in area-specific function, and in metabolites.

Neurotrophins, inducible neuropeptides and stress

Neurotrophins

Neurotrophins (NT) are growth factors involve in neuronal survival and differentiation, structural plasticity and synaptic plasticity (for review: (Chao 2003, Greenberg et al. 2009, Levi et al. 2004, Ohira & Hayashi 2009, Price et al. 2007). Nerve growth factor (NGF) was the first NT to be discovered in 1950 by Rita Levi-Montalcini (Levi-Montalcini 1987), and also the first member of the mammalian NT family that includes NGF, BDNF, neurotrophin 3 (NT-3) and neurotrophin 4/5 (NT-4/5). In addition to the NT family, there are other neurotrophic factors and neuropeptides that are involved in the processes above commented such as the fibroblast growth factor (FGF) family, the glial cell line-derived neurotrophic factor (GDNF) family, the ciliary neurotrophic factor (CNTF) family, the insulin-like growth factors I and II (IGF-I and -II) and even inducible neuropeptides, such as the VGF (non-acronymic). Here, we will focus in BDNF and its receptors, as well as in VGF.

NTs have a 50% of homology in their structure and are found in solution as homodimers or heterodimers (Bothwell 1995, Mowla et al. 2001). NT effects are exerted via two types of receptors classified due to its binding affinity: the low affinity or neurotrophin receptor ($p75^{NTR}$) and the high affinity or tropomyosin-related kinases (Trk). NTs are generated as pre-pro-peptides that are processed by convertases. It is noteworthy that pro-NTs can be processed extracellularly (Lee et al. 2001) and both, pro-NTs and NTs, can exert opposite biological actions: e.g. Pro-BDNF promotes apoptosis and LTD, whereas BDNF induces survival and LTP (Reichardt 2006).

All NTs bind with equal affinity to $p75^{NTR}$ (Roux & Barker 2002), which belongs to the tumor necrosis factor (TNF) receptors family, containing an extracellular cysteine rich domain and a cytoplasmatic death domain (Liepinsh et al. 1997). However, $p75^{NTR}$ differs from TNF receptors because it does not need self-association to signaling (Arévalo & Wu 2006). Recently, it has been described that $p75^{NTR}$ binds with higher affinity to pro-NTs causing cell death (Arévalo & Wu 2006, Nykjaer et al. 2005, Roux & Barker 2002, Teng et al. 2005), but also causing opposite pro-survival effects, enhancing neurite outgrowth, mediating differentiation and myelination and modulating agonist binding to Trk receptors (Reichardt 2006). The lack of intracellular catalytic domain of $p75^{NTR}$ implies that its actions are through associated proteins, exerting its effects via two intracellular cascades: Jun kinase, that promotes the transcription of pro-apoptotic genes and NF-κB, which induces the transcription of pro-survival genes (Reichardt 2006). The $p75^{NTR}$ expression during development is ubiquitous, but postnatally is restricted to magnocellular neurons of the basal forebrain caudate/putamen neurons, motor neurons, cerebellar Purkinje cells, and to ventral premaxillary, mesencephalic trigeminal, hypoglossal, raphe, and suprachiasmatic nuclei (Roux & Barker 2002).

Trk receptor family is formed by three members and NTs bind specifically to one of the three members. NGF binds preferentially to TrkA, BDNF and NT-4 to TrkB and NT-3 to TrkC, although NT-3 binds to the other Trk receptors which less affinity. Moreover, Trk can be activated

in absence of neurotrophins via adenosine receptors (Lee & Chao 2001). All Trk receptors present several isoforms due to alternative splicing, some of them without catalytic domain(Barbacid 1995, Reichardt 2006). Trk structure is formed by an extracellular domains characterized by cysteine-rich regions, leucine-rich regions and immunoglobulin-like (IgG) domains. Contrary to $p75^{NTR}$, it possesses a catalytic intracellular domain. The ligand binding induces Trk dimerization and then autophosphorylation of tyrosine residues, which creates docking sites for proteins promoting the interaction with intracellular cascade proteins (Patapoutian & Reichardt 2001). Thus, Trk induces the activation of three main signaling pathways: mitogen-activated protein kinase/extracellular signal-related kinase (MAPK/ERK), phophoinositide 3-kinase-ν-Akt murine thymoma viral oncogene homolog 1 (PI3K-Akt) and phospholipase C-γ-Protein kinase C (PLCγ–PKC) (Arevalo & Wu 2006, Reichardt 2006).

VGF and stress

One of the most induced mRNA after NGF and BDNF administration is VGF, which was discovered in 1985 by Andrea Levi and collaborators (1985) due to its induction in PC12 after NGF addition. It has been described VGF induction by GDNF, synaptic plasticity, circadian rhythm, estrous cycle, energy balance, water balance, depression and adrenalectomy (for review: (Ferri et al. 2011, Hunsberger et al. 2007, Levi et al. 2004). VGF is processed by the typical neuropeptides route and released by the activity-dependent pathway (Possenti et al. 1989). VGF protein analysis revealed a precursor protein detected by WB as a doublet of 80-90 kDa, which presented post transcriptional modification. Proteolytic post-transcriptional modifications generate diverse VGF polypeptides that are named by the four N-terminal aminoacids and the peptide length (e.g, TLQP-21) (Levi et al. 2004). There is a high VGF hypothalamus expression; although VGF expression is also observed in the HF, the PFC, the thalamus and the amygdala (Snyder & Salton 1998).

Recently, two receptors have been characterized for the TLQP-21 polypeptide: C3a receptor-1 (C3AR1) and C1q receptor (gC1qR). The binding of TLQP-21 to C3AR1, a G protein-coupled receptor protein involved in the complement system, known as the receptor of C3a complement protein (Cassina et al. 2013, Hannedouche et al. 2013), induces an intracellular increase of Ca^{2+} and its binding to gC1qR, the receptor of C1q complement protein, induces an intracellular increase of Ca^{2+} in macrophages and microglia, but not in neurons (Chen et al. 2013). Little is known about other receptors and molecular pathways, and works have been focused in VGF effects on synaptic plasticity modulation. In particular, the analysis of hippocampal cells after BDNF-induced synaptic strengthening shows an increase on the expression of *c-fos*, *Arc* and *vgf*. In addition, VGF peptides enhanced synaptic activity in a dose-dependent manner (Alder et al. 2003). KO VGF mice have impaired LTD, spatial learning and fear conditioning; however, LTP is not impaired and VGF peptides produced a transient potentiation of field EPSP slope decaying after washout in BDNF-dependent manner, since the blockade of TrkB impaired VGF peptides effect (Bozdagi et al. 2008).

To our knowledge, only two groups have tested the effects of stress on VGF. One work demonstrated that TLQP-21 reduced acute restraint effects on plasma adrenaline and noradrenaline and showed that chronic administration of TLQP-21 increased depressive behavior in chronic stress-induced subordinate mice (Razzoli et al. 2012). Moreover, chronic stress has been found to increase TLQP-21 levels in the intermediate lobe of the pituitary gland, an effect blocked by

dopamine receptor antagonist (Tokizane et al. 2013). The effects on the HPA axis remain unknown.

BDNF and TrkB pathway

One of the most studied cellular pathways in CNS is that of BDNF-TrkB, which has an important role during development, but also in adulthood, driving pro-survival signaling and modulating synaptic plasticity (Bramham & Messaoudi 2005). BDNF was described by the first time by Barde and collaborators (1982) as a molecule similar to NGF able to promote survival and fiber outgrowth. BDNF is expressed and released by astrocytes, neurons (Moretto et al. 1994) and microglia (Ferrini & De Koninck 2013). One of the most studied effects of BDNF is its capability to modulate synaptic plasticity. In addition, BDNF effects have been described in pre- and post-synaptic GABA- and glutamatergic terminals (Bramham & Messaoudi 2005, Carvalho et al. 2008). BDNF release has also been described pre- and post-synaptically (Edelmann et al. 2014, Kohara et al. 2001, Lessmann et al. 2003) and it is known that BDNF is transported anterogradely (Altar & DiStefano 1998) and retrogradely (Nawa & Takei 2001).

BDNF as others neuropeptides are transcripted as a pre-pro-peptide; the pre-domain is cleaved off immediately, post transcriptionally modified inside the Golgi apparatus and included in secretory vesicles to be released. The mature BDNF and even the pro-BDNF can be secreted (Lessmann et al. 2003). The release occurs via constitutive pathway (constitutive secretion) or via activity-dependent exocytosis (regulated secretion) in the trans-Golgi network (Lessmann & Brigadski 2009).

Whereas constitutive secretion generates a sustained release of BDNF, modulating its basal levels; the regulated secretion releases momentary high amounts of BDNF, which critically regulates synaptic transmission (Lessmann et al. 2003, Mowla et al. 1999). It has been demonstrated by mutagenesis that the pro-domain is critical for BDNF targeting to be released by regulated secretion (Chen et al. 2004). In addition, a mutation in the val66met polymorphism (a single nucleotide polymorphism (SNP)) in the pro-domain sequence has been found to create a met-BDNF which induces a deficient targeting and is not efficiently included in vesicles of the regulated pathway as compared to the WT val-BDNF. As a result, less BDNF is secreted in activity dependent manner. The SNP val66met is found in humans and produces poorer episodic memory, abnormal hippocampal activation (assessed by fMRI) and lower NAA levels (Egan et al. 2003). Taken together, these data demonstrate the critical implication of BDNF in synaptic plasticity and its deficit could be relevant in some PTSD symptoms.

BDNF actions are exerted basically via TrkB receptor, which has the already described Trk structure: an extracellular domain, a transmembrane region and an intracellular catalytic domain. However, in rats and humans three isoforms are generated by alternative splicing: a TrkB full length (TrkB-FL) and two truncated TrkB (TrkB-T1 and T2) (Middlemas et al. 1991, Stoilov et al. 2002). Although the truncated isoforms lack the catalytic domain, they trigger signal transduction (Baxter et al. 1997). TrkB-FL and TrkB-T1 expression is ubiquitous in the CNS and TrkB-T2 is more restricted (Fryer et al. 1996); however, whereas TrkB-FL expression is higher in development, TrkB-T1 expression is higher in adulthood (Baxter et al. 1997).

TrkB-FL and -T1 are expressed pre- and post-synaptically in neurons (Drake et al. 1999), and also in oligodendrocytes, astrocytes and microglia (Frisén et al. 1993, Fryer et al. 1996, Mizoguchi et al. 2009). BDNF-induced activation of TrkB receptors activates signaling pathways

such as Ras-MAPK/ERK, PI3K-Akt and PLCγ–PKC (Arévalo & Wu 2006, Kaplan & Miller 2000, Reichardt 2006). TrkB-T1 function, despite of being not completely known, has been involved in TrkB-FL modulation because TrkB-T1 reverted BDNF haploinsufficiency (Carim-Todd et al. 2009). Taken together all these data suggest that TrkB-T1 receptors have an important physiological role in BDNF function that are not only restricted to TrkB-FL.

BDNF and TRKB in learning and memory

As it has been explained above, BDNF/TrkB pathway modulates synaptic plasticity (for review Leal et al. 2014, Lu et al. 2008, Panja & Bramham 2014, Tapia-Arancibia et al. 2004, Yamada et al. 2002). It has been described that BDNF increases glutamate transmission through TrkB receptor (Kafitz et al. 1999) and LTP (Figurov et al. 1996, Kang & Schuman 1995) and, conversely, impairs LTD (Kinoshita et al. 1999). Moreover, LTP is impaired in heterozygous BDNF mice (Korte et al. 1995) (homozygous is lethal, due the importance of BDNF for development) and restored by virus-mediated replacement (Korte et al. 1996). In addition, HFS induces BDNF release, suggesting a positive feedback (Patterson et al. 1992). In HF, HFS stimulation (Du et al. 2000) and BDNF in the short-term (Haapasalo et al. 2002) facilitates the insertion of TrkB receptor in cell surface, whereas long BDNF exposure decreases surface receptors (Chen et al. 2005, Haapasalo et al. 2002). Recently, TrkB receptor has become important not only by its implication in early LTP (above described), but also for its role in late LTP related to the "Synaptic Tagging hypothesis". This hypothesis proposes that the synaptic activity generates a tag for those plasticity-related proteins that are needed for the late LTP. Recently, Lu and collaborators have proposed that TrkB could act as a synaptic tag recruiting the plasticity-related proteins required for LTP (Lu et al. 2011).

BDNF plays a critical role in synaptic plasticity in adulthood and during development (Cohen-Cory et al. 2010, Yoshii & Constantine-Paton 2010). Therefore, it is clear that BDNF can modulate structural plasticity. Experiments have demonstrated that sustained BDNF levels are required for spine maintenance and for spatial learning (Vigers et al. 2012). Moreover, the stimulation of BDNF/TrkB pathway increases spine density in CA1 neurons (Tyler & Pozzo-miller 2001), modifies their morphology (Tyler & Pozzo-Miller 2003) and mediates LTP-dependent spine increase (Tanaka et al. 2008). Surprisingly, TrkB inhibition induces an increase of the spine density, although most of the new spines are immature and transient (Chapleau & Pozzo-Miller 2012). Finally, Alonso and collaborators (2004) have demonstrated that BDNF-induced spine increase is MAPK/ERK-dependent and spine elimination is modulated through the $p75^{NTR}$ pathway (Zagrebelsky et al. 2005).

BDNF structural and synaptic plasticity effects have been positively correlated with behavior. Animals trained in a HF-dependent task present higher BDNF mRNA levels than untrained animals (Hall et al. 2000, Kesslak et al. 1998, Schaaf et al. 1999). Accordingly, BDNF deletion causes MWM and object recognition impairment (Heldt et al. 2007, Linnarsson et al. 1997). Furthermore, other brain areas related to learning and memory are affected by BDNF. In this regard, the PFC TrkB mutation and *bdnf* deletion impair learning, which is rescued by TrkB activation (Choi et al. 2012). In the amygdala, fear-conditioning enhances BDNF/TrkB activation (Rattiner et al. 2004) and TrkB activation is needed for amygdala-dependent memory extinction (Chhatwal et al. 2006). In addition, although anxiety is not a learning and memory process, it is also modulated by BDNF and increased anxiety is observed in BDNF-overexpressing

transgenic mice (Govindarajan et al. 2006) and in conditional bdnf knock-out mice, with postmitotic neurons *bdnf* repression (Rauskolb et al. 2010).

BDNF/TRKB pathway in stress

Several studies have demonstrated the importance of stress in BDNF/TrkB pathway, describing reciprocal modulation in multiple brain areas (for review: (Jeanneteau & Chao 2013, Numakawa et al. 2013, Schaaf et al. 2000, Suri & Vaidya 2013). In some studies this modulation has been evaluated by the effects of corticosterone administration on HF BDNF gene expression. Acute corticosterone administration induced a decrease in BDNF expression in DG 2 h after 10 mg/rat administration (Smith et al. 1995), or 3 h after 1mg/kg administration (Schaaf et al. 1998). In the latter case the decreased expression was followed by a decrease in the HF protein levels at 4 and 6 h (Schaaf et al. 1998). Similarly, chronic corticosterone administration also induced a decrease in BDNF expression in the HF in one study (Smith et al. 1995), and in the HF and the PFC in another study (Dwivedi et al. 2006). However, in a third study chronic corticosterone administration at a higher dose 32 mg/kg decreased mRNA and protein in the HF but not in the PFC (Jacobsen & Mørk 2006). Despite the general decrease in BDNF mRNA and protein, there are some discrepancies in these studies, depending on the animal age, time points, corticosterone dose and analyzed brain area. It appears that DG and CA3 are the most affected areas, while CA1 needs a higher dose to be affected (Smith 1995, Schaaf 1998).

Acute stress mimics the consequences of corticosterone administration in BDNF. Several studies have reported that HF BDNF gene expression decrease immediately after IMO (Lee et al. 2008, Smith et al. 1995, Ueyama et al. 1997), at 24 h after restraint (Murakami et al. 2005) and even seven days after a single predator odor exposure (Kozlovsky et al. 2007). Decreases in BDNF protein levels have been found at 5 and 10 h post restraint (Adlard & Cotman 2004), at 24 h after social defeat (Pizarro et al. 2004) and at 24 and 72 h post IS-LH (Greenwood et al. 2007). However, as it happens with corticosterone administration, the time point and the severity/duration of stress are critical. On the contrary, Marmigere and collaborators (2003) have reported a transient increase of BDNF protein levels after one IMO session (180 min) before returning to control levels at 5 h post-IMO.

In contrast to the relative consensus about the effects of acute stress in HF, other brain areas present divergent data. In the amygdala, social defeat decreases BDNF mRNA levels at 24 h post-stress (Pizarro et al. 2004), which differ from data of animals exposed to acute restraint that induces an increase after a single or a 10 day exposure to the stressor v. In the PFC, BDNF increased at 1 h after restraint (Molteni et al. 2001, 2010) and 4-6 h after IS-LH (Greenwood et al. 2007), but no effect on BDNF mRNA was observed 7 days after predator odor stress (Kozlovsky et al. 2007). To summarize, acute severe stressors and corticosterone administration mainly decrease BDNF mRNA and protein in the HF. Whereas increases were observed in the PFC, results are controversial in the amygdala (Naert et al. 2011). BDNF decrease in the HF could be related to some of the negative consequences of stress in plasticity, although, the precise mechanisms are poorly understood.

Despite a first study demonstrated the same post-stress BDNF decrease in DG in adrenalectomized animals and intact animals (Smith 1995), the results of recent studies have demonstrated the interaction between glucocorticoids and BDNF: glucocorticoids have been demonstrated to modulate BDNF transcription (as it has been explained above), to affect translation through

attenuation of a ribosomal protein activation (Shah et al. 2000), and to modulate pre-peptide processing due to prohormone convertases inhibition (Dong et al. 1997).

Chronic restraint has been found to reduce BDNF protein levels in HF at 24 h post-stress (Magariños et al. 2011, Naert et al. 2011, Xu et al. 2006, Yan et al. 2011). This is of interest as BDNF decrease has also been described in HF and PFC of depression patients (Calabrese et al. 2009). Moreover, antidepressant treatment prevents BDNF decrease (Schmidt et al. 2008). However, BDNF levels in PTSD patients evaluated in a few studies presented controversial data: some reported increase (Hauck et al. 2010, Matsuoka et al. 2013), and others decrease (Dell'Osso et al. 2009). In addition, the role of the BDNF SNP val66met has been suggested, but no consistent data have been reported (Frielingsdorf et al. 2010). Three possibilities have been proposed to explain the controversial data regarding PTSD. Firstly, it has been suggested that BDNF could change in a time-dependent manner, increasing in an early PTSD phase, and decreasing in a late phase (Hauck et al. 2010). Secondly, the small size of all the studies reduces the statistical power (Dell'Osso et al. 2009). Finally, the high inter-individual variability of plasma BDNF levels found in PTSD patients (Berger et al. 2010). Therefore, the role of BDNF in PTSD remains unclear and more studies are needed to reveal the role of BDNF in PTSD disease in humans.

BDNF/TrkB pathway-related deficits treatment

Antidepressants show partial efficacy to reduce PTSD symptoms (Steckler & Risbrough 2012). Moreover, on the basis of the above studies, it is reasonable to consider that modulation of the BDNF/TrkB pathway might be relevant to ameliorate stress effects. Given that stress induces mainly a decrease in BDNF levels, we will discuss a possible therapy that involves activation of the BDNF/TrkB pathway.

The use of BDNF/TrkB pathway agonists is the most effective way to modulate the pathway, as TrkB activation enhances intracellular pathways and also increases BDNF release, in a positive feedback loop (Cheng et al. 2011). Several TrkB agonists have been described (Andero & Ressler 2012), but 7,8-DHF presents better pharmacokinetic properties and higher TrkB binding affinity than other agonists and even than BDNF. It is a flavone of plant origin present in fruits and vegetables, which crosses the BBB and induces TrkB dimerization and autophosphorylation, exerting its pro-survival effects via PI3K/Akt and MAPK (Jang et al 2010). 7,8-DHF has been used in a few stress paradigms. In a foot-shock-induced fear conditioning paradigm in mice, the administration of 7,8-DHF before extinction sessions improved extinction learning in both stress-naïve animals, but particularly in those previously exposed to IMO days before conditioning, a procedure that impairs extinction (Andero et al. 2011). 7,8-DHF administration 2 h prior or 8 h after IMO, prevented declarative memory impairment observed several days after IMO in the MWM (Andero et al. 2012). After chronic unpredictable stress, chronic intracerebroventricular administration of 7,8-DHF rescued the observed reduction in HF volume, dendrite length, spines density and cell proliferation (Blugeot et al. 2011).

The mechanisms by which 7,8-DHF, via TrkB activation, exerts its protective effects in stress are not completely known. A possible mechanism is to protect from stress inflammatory-like process (see section Glia and stress) or glutamate release–related negative consequences. Thus, the agonist, 7,8-DHF has demonstrated neuroprotective effects in other diseases, such as ischemia (Uluc et al. 2013), Alzheimer (Devi & Ohno 2012) and glutamate-induced toxicity (Chen et al 2011). In addition, BDNF induces neuroprotective effects in several ischemic, traumatic and

toxic brain injuries (Almeida et al. 2005, Beck et al. 1994, Wu & Pardridge 1999). Thus, TrkB activation by 7,8-DHF exerts similar effects to those produced by BDNF (Jang et al. 2010). Therefore, the characterization of the BDNF/TrkB pathway using this agonist may provide further information of mechanisms that underlie stress effects and also could lead to novel treatment of PTSD and other diseases.

HYPOTHESIS & OBJECTIVES

Nowadays, the knowledge of the neurobiological basis of PTSD is limited and hinders the development of effective treatments. However, the appearance of new animal models can help to understand the underlying mechanism of PTSD, to reveal new insights and to develop new treatments. The main hypothesis of this work is that long-term physiological and behavioral changes found in PTSD could be causally related to enduring changes in synaptic plasticity and may be ameliorated by the administration of the TrkB agonist 7,8-DHF.

In order to assess this hypothesis, we consider these goals:

(1) To analyze long term effects of a single IMO and a chronic IMO on brain and HF volumes and other physiological stress markers, in order to evaluate whether they mimic those found in other PTSD models and in human PTSD patients.

(2) To assess possible long lasting effects of a single IMO on structural plasticity, functional synaptic plasticity and on synaptic plasticity-related proteins, to demonstrate whether PTSD symptoms are causally related to synaptic plasticity.

(3) To study whether a single IMO can produce long lasting memory impairment in a massive trained MWM and whether this memory deficit can reflect possible changes in synaptic plasticity.

(4) To assess whether a single TrkB agonist (7,8-DHF) administration after IMO can prevent changes in functional- and proteins-related synaptic plasticity.

(5) To demonstrate that this long lasting memory impairment can be prevented or reduced by a single administration of this TrkB agonist (7,8-DHF) after stress, reflecting those possible protective effects found in synaptic plasticity.

MATERIALS & METHODS

Subjects

Male Sprague-Dawley rats obtained from the breeding center of the Universitat Autònoma de Barcelona) and from Charles River (France) were used. The animals were about sixty days old (body weight: 350 ± 20 g) at the beginning of the experiments. They were housed in cages of 1000 cm3 (50 x 25 x 15 cm) in standard conditions of temperature (21 ± 1 ºC), humidity (40-60%) and on a 12 h light/dark schedule (lights on at 08:00 h), at least one week before the experiment started. Food and water were available *ad libitum*. The animals were handled 2 days during 6 days for approximately 2 min a day until the beginning of the experiment. The experimental protocol was 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 Communities Council Directive (86/609/EEC).

Stress

IMO was conducted in a separate room from the other animals. Each animal was immobilized by gently restraining their four limbs in a prone position to metal arms attached to a wooden board for 2h. (e.g. Márquez *et al*., 2002; Muñoz-Abellán *et al*., 2008).

Drugs

7,8-DHF (TCI, Tokyo Chemical Industry, ltd. cat #: D1916) was given sc at a 5 mg/kg dose (1 ml/kg) in saline (0.9% NaCl) containing 25% ciclodextrine (Sigma. cat #: H107) or 5% Cremophor (Sigma. cat #: C5135) (Andero et al. 2012) .

ZD7288 (Sigma. cat #:Z3777) was add to aCSF at a final concentration of 10 μM (Tsay 2007).

Western blot

Sample collection

Animals were decapitated and brains were immediately removed and transferred to liquid nitrogen cold Isopenthane. Coronal brain slices (500 μm) were obtained on a Zivic Brain matrix (Zivic Intruments Pittsburgh, PA, USA). PFC, Amygdala, CA1, CA3 and DG areas were dissected. Samples were homogenized by sonication in lysis buffer (TrisHCl 25 mM, NaCl 150 mM, NP40 1 %, PMSF 1 mM, Glicerol 10 %) with protease inhibitor cocktail (Sigma) and phosphatase inhibitor cocktail (Calbiochem). Protein concentration was determined by bicinchoninic acid assay (BCA; Pierce). Samples were stored at -80ºC until processed.

Western blot procedure

Samples were boiled to 95 ºC for 10 min in 4x sample buffer (Invitrogen) and subjected to SDS-PAGE. The SDS-PAGE was transferred to nitrocellulose (Invitrogen) and blocked for 1 h in Tris-NaCl buffer and Tween-20 at 0.1 % (TBS-T) with 5% bovine serum albumin (BSA, Sigma-Aldrich) at room temperature. The membrane was incubated over night at 4ºC in primary antibody in the same blocking buffer. Then after washing with TBS-T, membranes were incubated with secondary antibody coupled to horseradish peroxidise 1 hour at room temperature, which was visualized with enhanced chemiluminescence (ECL Plus, Amersham Bioscience). Images were taken using Quantity One software on a ChemiDoc™ XRS+ System (**Bio Rad**) and quantified by a densitometry program Quantity One (**Bio Rad**). Optic density values were normalized or β-actin. Results are expressed as normalized optic density (OD).

MRI

Animals were initially anesthetized with 4% (v/v) isofluorane in O2 at 1 L/min and then where placed in prone position on a Plexiglas bed, with bite-bar and ear-bars for optimal head immobilization, and with a breathing and temperature sensor. Animals were kept anesthetized with isofluorane via a face mask (1.5% (v/v) isofluorane in O2 at 1 L/min). Breathing and temperature were maintained at physiological rates.

Images were acquired using a 7T Bruker BioSpec 70/30 USR system (Bruker Biospin, Ettlingen, Germany), equipped with a mini-imaging gradient set (400mT/m), a linearly polarized transmit volume coil (72 mm inner diameter) and a dedicated rat brain receive phased array surface coil. Low resolution T2-weighted images using a rapid acquisition with relaxation enhancement (RARE) sequence were initially obtained in axial, sagittal and coronal planes to be used as reference scout images. Imaging parameters for these images were: repetition time (TR) = 2000 ms; effective echo time (TE_{eff}) = 36 ms; RARE factor = 8; field of view (FOV) = 3.5×3.5 cm^2; matrix size (MTX) = 256×256; number of averages (NA) = 1, slice thickness (ST) = 1 cm. Geometry for high resolution images was prescribed such that the first slice starts at the point between the end of the olfactory bulb and the beginning of the cortex and the last stack ends at the beginning of cerebellum. High-resolution T2-weighted images were obtained using a RARE sequence with respiration gating under the following parameters for coronal images: TR = 4000 ms; TE_{eff} = 39 ms; RARE factor = 8; FOV= 3.2×3.2 cm^2; MTX = 320×320, giving an in plane spatial resolution of 100×100 μm^2 acquiring 30 slices with 500 μm slice thickness and 100 μm gap between slices. Total acquisition time was about 25min depending on the respiration rate. The whole brain and HF were manually traced from coronal images using Image J.

For the HF statistical analysis a bregma value was assigned to each stack of the MRI using a standard rat brain atlas (Paxinos & Watson, 2007) to ensure that images that were being compared were equivalent. Only the dorsal HF was used in the statistical analysis (Bregma -1.72 to -3.8 aprox.). HF values were normalized by brain volume. To assess trace variability some animals were repeated after a period of time.

Electrophysiology

Animals were decapitated and brains were immediately removed and slices of 300 μm-thickness were obtained, and maintained in artificial cerebrospinal fluid (aCSF), equilibrated with 95% O2/5% CO2, at room temperature. aCSF consisted of (in mM): 124 NaCl, 2.69 KCl, 26 NaHCO3, 1.25 KH2PO4, 10 glucose, 2.5 CaCl2, 1.2 MgCl2, for LTP, LTD experiments. After 90 min recovery at 32ºC, slices were placed in the recording chamber at 25ºC. fEPSPs were recorded with glass electrodes (filled with aCSF, 0.8–0.2MΩ) placed in the basal dendritic layer (stratum oriens) of CA1 area, stimulating electrode was placed in Schaffer collaterals fibers. At the beginning of each experiment, the basal synaptic transmission (BST) was assessed by an input-output curve increasing the stimulus intensity and measuring the appearance of the population spike. On the basis of these curve, the stimulus was adjusted to elicit a population spike with a slope at which the amplitude of **population spike** reaches **half** of its **maximum** value. Then LTP or LTD was induced. Theta bursting stimulation (TBS) LTP was induced with 5 trains of 10 bursts at 5 Hz; each burst consisting of 4 pulses at 100 Hz. LTD protocol was induced with 1 Hz, 900 pulses. (Benoist et al. 2013).

MWM

The Morris water maze was a black circular pool (Rizhova et al. 2007) (2-m diameter, 45 cm high) filled with water at 25±1°C, and it was divided into four virtual quadrants of equal size. An outer ring in the periphery was divided to study thigmotaxis. A hidden escape platform was placed in the middle of the target quadrant (1.5 cm below the water surface) equidistant from the sidewalls and the middle of the pool. The testing room contained numerous maze cues. The behavior of the animal (latency to reach the platform, total distance swam, and swim speed) was monitored by a video camera mounted on the ceiling above the center of the pool and a computerized tracking system (Ethovision 1.90, Noldus IT, Wageningen, The Netherlands).

MWM procedure consists of 2 days. The first day, animals were exposed to massive training of 8 trials of 120 s, all the animals passed the 4 first trials and then the next 4, with the aim to allow animals to get rest. In the first 4 trials the animal was placed in different start points that were randomly changed in the second 4 trials. If the animals failed to find the platform within 120 s, the experimenter gently guided the animal to the platform. All animals were left on the platform for 30 s and then removed from the pool for a 30-s rest period, after which the next trial commenced.

The second day, animals were exposed to a probe trial of 60 s without platform. After these 60 s animal was removed from the pool and let to rest for 30 s, after which 4 reversal training trials commenced, with the same time and procedure as the initial learning trials, but the platform was then placed in the opposite quadrant than it was in the first day. The analyzed variables in training trials were: latency (time spent to found the platform), average swimming speed, thigmotaxis (% of time spent in the outer area). In probe trials the analyzed variable was the % of time spent in a circular area (concentric to the platform) (PG1) and the equivalent area in the opposite quadrant.

Morphology

Animals were anesthetized with tribromoetanol and transcardially perfused with phosphate buffer (PB) followed by 4% paraformaldehyde (0.1M, pH7.4) prepared in the same buffer. The brains were removed and post-fixed in the same solution for 24 h. Coronal sections (150 μm) were cut on a vibratome and immediately cells in the hippocampus (CA1 and DG area) were individually injected with Alexa 594 (Invitrogen, Eugene, OR) by passing a steady hyperpolarizing current through the electrode (-0.5 to -1.0 nA). The current was applied until the distal tips of each neuron fluoresced brightly. The sections were mounted on a glass slide in fresh ProLong Gold antifade reagent (Invitrogen, Eugene, OR). Slides were left for 24 h in the dark at room temperature for the mounting medium to cure, and the coverslip was then sealed with nail polish. Images were obtained with a confocal microscope (Zeiss LSM510 Meta) and 1 dendrite of 5 differents neurons from stratum oriens were scanned from the soma (96 Neurons from CA1 and 74 from DG). The image stacks (physical size 45 x 45 μm, logical size 1024 x 1024 pixels) consisted of 100-200 image planes and a 63 x glycerol-immersion were used with a calculated optimal zoom factor of 3.2 and a z-step of 0.14 μm.

For each stack, the laser intensity and detector sensitivity were set so fluorescence signal from the spines occupied the full dynamic range of the detector. This meant that some pixels were saturated in the dendritic shaft, but no pixels were saturated within the spines.

Dendritic spine density was determined by tracing the image of the acquired dendrites in three dimensions with Neurolucida (MicroBrightField Inc., Williston, VT,). All protusions were considered spines, applying no correction factors to the spine counts. The reconstructed data were exported to Neurolucida Explorer (MicroBrightField Inc., Williston, VT,) for quantitative analysis, and the spine density was automatically calculated by dividing the number of spines by the dendrite length. Spines were also analyzed as a function of its distance from its origin (Sholl analysis).

Statistical analyses

Statistical analyses were performed with SPSS version 18.0. Generalized linear models (McCulloch & Searle, 2001) and generalized linear model repeated measures analysis (generalized estimating equations models, GEE) (Hardin & Hilbe, 2003) were used where appropriate. In all cases if a statistically significant interaction was found, additional pairwise comparisons (Bonferroni sequential adjustment) were made and the method of estimation was the maximum likelihood (ML). Normality distribution and identity as a link function was always used. In all cases, the significance of the effects was determined by the Wald χ^2 statistic. Student's *t* test was also use when appropriate. Data are presented as mean ± SEM and statistical significance was set at $p<0.05$. Detection of outliers was performed, and when necessary, removed from analyses.

CHAPTER 1

A single stress exposure induces long term synaptic plasticity effects and HF-dependent task impairment

Introduction

PTSD is a trauma and stressor related disorder that occurs after traumatic situations exposure (DSM V). PTSD is characterized by the development of cognitive impairments (Elzinga and Bremner 2002, Karl 2006, Kaouane 2012, Pitman 2012). The most common cognitive impairment in PTSD is the memory dysfunction (Elzinga and Bremner 2002). In fact, HF, a key structure in memory formation, is one of the most affected areas in PTSD, presenting reduced volume (Smith 2005; Karl 2006) and function abnormalities (Hudges and Shin 2011). However, the neurobiological basis of PTSD-related memory impairment has not been elucidated.

Likewise, acute stress exposure in rodents mimics some of the patients PTSD-related memory impairments (Kohda 2007, Stam 2007b, Andero 2011, Andero 2012a, Kaouane 2012). Moreover, some of these effects have been related to short term altered LTP (Ahmed 2006, Chen 2010) and LTD (Wang 2007, Wong 2007), the best characterized cellular model for learning and memory. Several works have provided evidence of the relation between synaptic plasticity and learning and memory (Kandel 1997, Morris 2003). Furthermore, over the last decades an important concept has been added to explain learning and memory, the structural plasticity (Caroni et al., 2012).

Whereas Hebbian theory is based in pre-existing synapses, structural plasticity encompasses morphological changes (synapses formation and destruction) that result as synaptic plasticity consequences. Stress also disrupts structural plasticity (Shors 2001, Dalla 2009). In addition, Diamond et al (2007) proposed "the temporal dynamics model" to explain the stress-induced memory modulation. Suggesting that at early phases stress activates memory consolidation (associated to long term traumatic situation re-experience found in PTSD patients) and then shift to a low plasticity phase (impairing the capacity of memory formation).

However, PTSD long term synaptic plasticity-related changes remain largely unknown. The study of animal models that mimic symptoms of PTSD patients can be a successful strategy to understand the long term consequences of stress and to find novel drug targets.

To address this issue, we first analyzed whether IMO present similar anatomical and behavioral effects found in PTSD patients to be a putative PTSD model, and second, the identification of a possible substrate for the synaptic plasticity memory impairment.

Experimental design

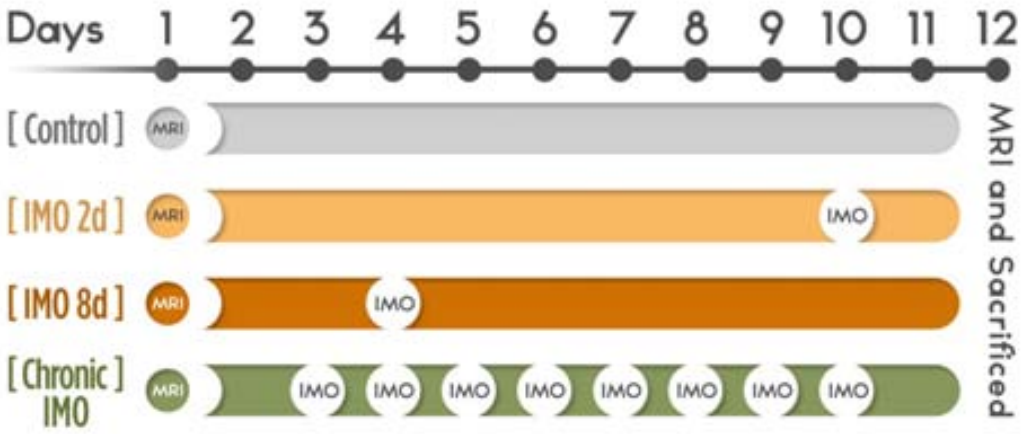
For the analysis of **physiological** and **neuroanatomical** consequences of IMO (**EXPERIMENT 1**), animals after the last day of handling were randomly assigned to groups: (**Control**) Undisturbed animals, animals exposed to 2h of acute IMO and sacrificed either 2 or 8 days (**IMO 2d** or **8d**) respectively, or exposed to 8 consecutive days of 2h IMO and sacrificed 2 days later (**Chronic IMO**).

In MRI animals were exposed to a Pre-stress MRI in day 1 and a post-stress MRI on day 12 and sacrificed immediately after the MRI [FIGURE 1].

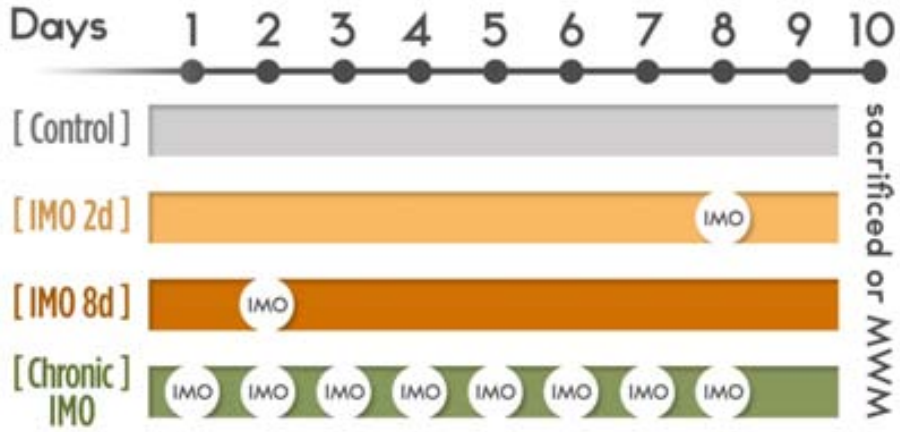
For the analysis of the effects on **structural plasticity** (**EXPERIMENT 2**), **synaptic proteins** (**EXPERIMENT 3**), **activity-related plasticity** (**EXPERIMENT 4**) and **spatial memory** (**EXPERIMENT 5**) induced by stress, animals after the last day of handling were randomly assigned to groups: (**Control**) Undisturbed

animals, animals exposed to 2h of acute IMO and sacrificed either 2 or 8 days (**IMO 2d** or **8d**) respectively, or exposed to 8 consecutive days of 2h IMO and sacrificed 2 days later (**Chronic IMO**).

In the experiment 2 and 5 only groups control, IMO 2d and IMO 8d were used. In the experiment 4 only groups control, IMO 2d was used [**FIGURE 2**].



[**FIGURE 1**]. Experimental design of **EXPERIMENT 1**.



[**FIGURE 2**]. Experimental design of **EXPERIMENTS 2, 3, 4** and **5**.

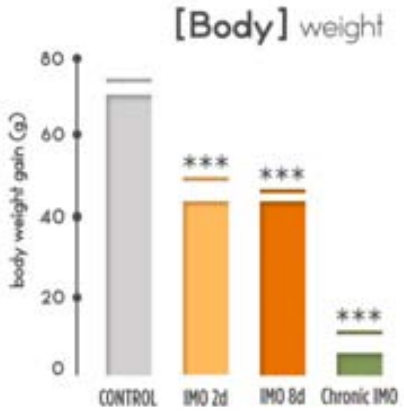
Results

Physiological and neuroanatomical consequences of IMO

BODY WEIGHT analysis [FIGURE 3] revealed a significant **group** effect [Wald $X^2(3) = 104.97$, $p<0.001$]. Both acute (IMO 2d and 8d) and chronic IMO groups showed less body weight gain than controls (always $p<0.001$), but the chronic IMO group also differed from the acute IMO groups ($p<0.001$).

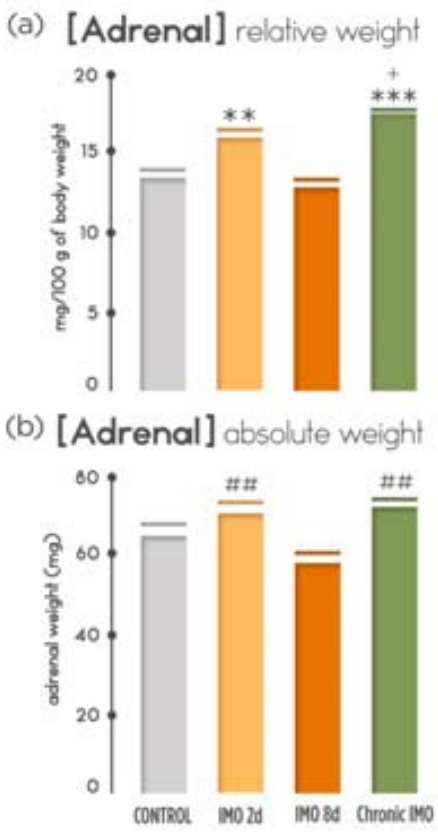
In animals exposed to chronic or acute IMO, the analysis of **RELATIVE** and **ABSOLUTE ADRENAL WEIGHT** [FIGURE 4] revealed a significant **group** effect [Wald $X^2(3) = 37.75$, $p<0.001$; Wald $X^2(3) = 12.81$, $p<0.01$ respectively]. Further analysis showed a significant increase of the relative adrenal weight of IMO 2d *vs* control and IMO 8d groups (both $p<0.01$) and a greater increase in Chronic IMO *vs* control and IMO 8d groups (both $p<0.001$) and *vs* IMO 2d ($p<0.05$). The absolute adrenal weight further analysis showed a significant increase of IMO 8d *vs* 2d post-IMO and chronic IMO groups (both $p<0.01$).

The analysis of **BRAIN VOLUME CHANGES** [FIGURE 5] showed a significant **group** effect [Wald $X^2(3) = 23.44$, $p<0.001$]. Further analysis showed a significant decrease of brain volume on IMO 2d and chronic IMO groups ($p<0.01$ and $p<0.001$, respectively) compared to controls. A tendency to a lower increase of brain volume was found in 8d post-IMO vs control group ($p=0.079$). The percentage of change post-resonance compared to the pre-resonance of control, IMO 2d, IMO 8d and chronic IMO group was 1.15, -0.14, 0.48, -0.5, respectively. The analysis of Brain volume/Body weight ratio changes, showed a significant group effect [Wald $X^2(3) = 56.3$, $p<0.001$]. Further analysis showed a significant decrease of the ratio on IMO 8d and chronic IMO groups ($p<0.05$ and $p<0.001$, respectively) compared to controls and a lower ratio on chronic IMO vs IMO 2d ($p<0.001$).



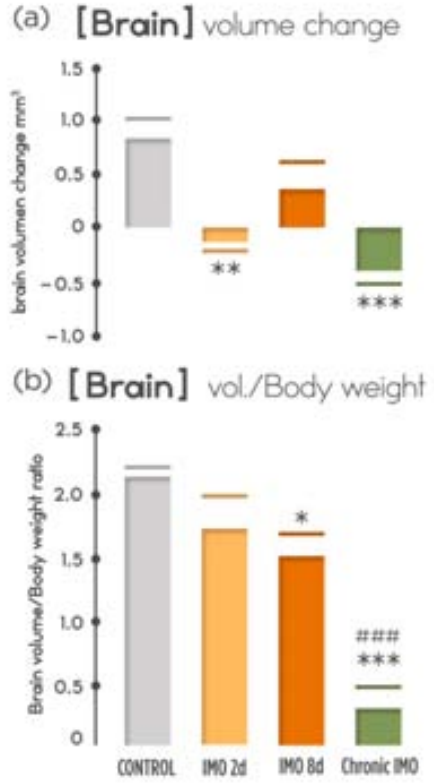
[FIGURE 3]. Body weight gain (gr) between day 1 and 12 of undisturbed animals (Control), animals exposed to 2h of acute IMO and sacrificed either 2 or 8 days (IMO 2d or 8d) respectively, or exposed to 8 consecutive days of 2h IMO and sacrificed 2 days later (Chronic IMO).

****p<0.001 vs control group; $^{+++}$p<0.001 vs Chronic IMO group. Means and S.E.M. are shown.*



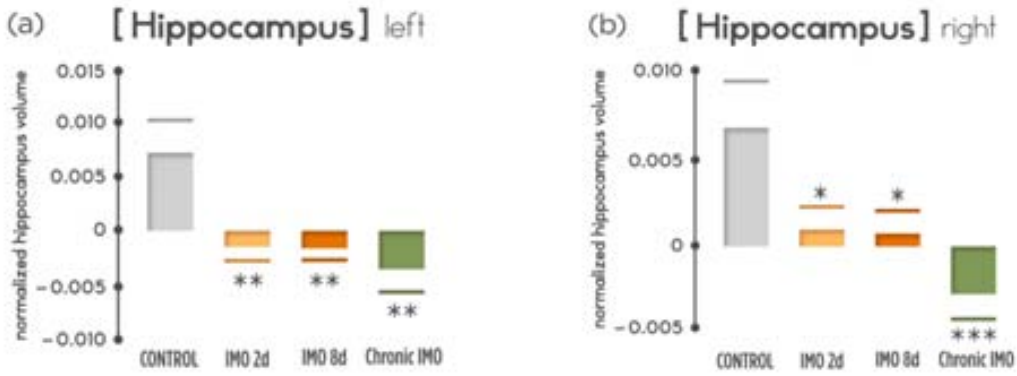
[FIGURE 4]. (a) Relative adrenal weight (mg /100 gr of body weight) and **(b)** Absolute adrenal weight (mg adrenal)of undisturbed animals (Control), animals exposed to 2h of acute IMO and sacrificed either 2 or 8 days (IMO 2d or 8d) respectively, or exposed to 8 consecutive days of 2h IMO and sacrificed 2 days later (Chronic IMO).

**p<0.01 and ***p<0.001 vs control group; *p<0.05 vs IMO 2d group; ##p<0.01 vs IMO 8d group. Means and S.E.M. are shown.



[FIGURE 5]. (a) Brain volume change (mm3) and **(b)** Brain volume/Body weight ratio between day 1 and 12 of undisturbed animals (Control), animals exposed to 2h of acute IMO and sacrificed either 2 or 8 days (IMO 2d or 8d) respectively, or exposed to 8 consecutive days of 2h IMO and sacrifice 2 days later (Chronic IMO).

**p<0.01 and ***p<0.001 vs control group. Means and S.E.M. are shown.



[FIGURE 6]. (a) Left and **(b)** right hippocampal formation volume change normalized to total brain volume of undisturbed animals (Control), animals exposed to 2h of acute IMO and sacrificed either 2 or 8 days (IMO 2d or 8d) respectively, or exposed to 8 consecutive days of 2h IMO and sacrifice 2 days later (Chronic IMO).

**p<0.05, **p<0.01 and ***p<0.001 vs control group. Means and S.E.M. are shown.*

The analysis of **LEFT** and **RIGHT HF VOLUME CHANGE**, revealed significant **group** effect [FIGURE 6] for both sides [Wald $X^2(3) = 12.9$, $p<0.01$; Wald $X^2(3) = 13.35$, $p<0.01$ respectively]. In the left HF all stressed animals showed a decrease in HF volume in contrast to the increase observed in controls ($p<0.01$). The left HF percentage of change post-resonance compared to the pre-resonance of control, IMO 2d, IMO 8d and chronic IMO group was 19.7, -0.429, -0.07, -6.26, respectively. In the right HF all stress groups showed significant differences *vs* control group. The chronic IMO group presented a significant volume decrease ($p<0.001$), however, IMO 2d and IMO 8d showed a mild increase *vs* controls ($p<0.05$). The right HF percentage of change post-resonance compared to the pre-resonance of control, IMO 2d, IMO 8d and chronic IMO group was 19.4, 9.11, 5.72, -6.56, respectively.

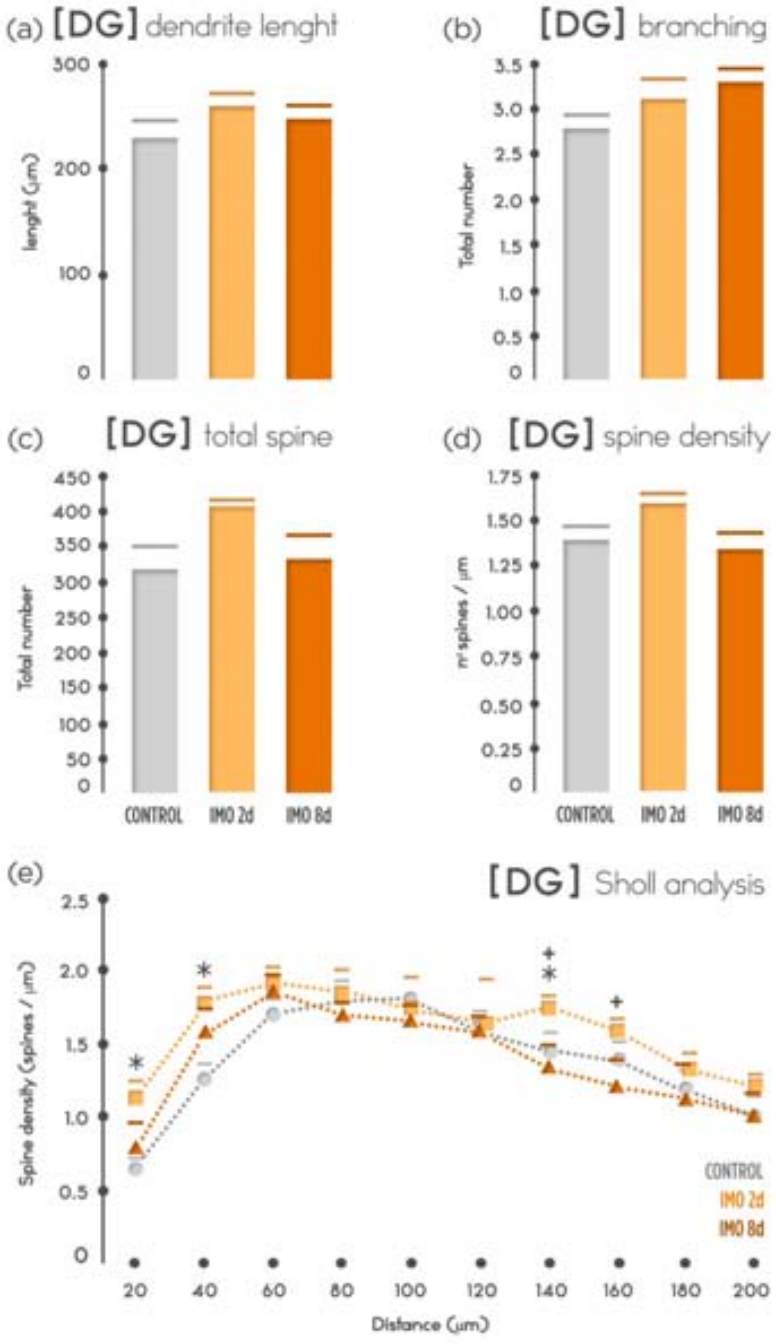
Structural plasticity changes induced by IMO

MORPHOMETRIC ANALYSIS OF DG revealed no significant differences either in total dendritic length [FIGURE 7a], total number of branches per neuron [FIGURE 7b], total spine number [FIGURE 7c] or total spine density [FIGURE 7d]. Sholl analysis of spine density [FIGURE 7e] showed a significant **distance** and **group x distance** interaction [Wald $X^2(9) = 342.88$, $p<0.001$; Wald $X^2(18) = 1255.99$, $p<0.001$, respectively] and a tendency **group** effect [Wald $X^2(2) = 5.23$, $p<0.073$]. The further analysis of group x distance interaction showed an increased spine density in IMO 2d group compared with control group at 20, 40 and 140 μm and compared to IMO 8d group at 140 and 160 μm (all $p<0.05$).

MORPHOMETRIC ANALYSIS OF CA1 revealed no significant differences in total dendritic length [FIGURE 8a] and in total number of branches per neuron [FIGURE 8b]. Total spine number [FIGURE 8c] analysis showed significant group differences in [Wald $X^2(2) = 14.52$, $p<0.001$] Further analysis showed an increase of spine number in the IMO 2d and IMO 8d groups compared with control (both $p<0.001$). Total spine density [FIGURE 8d] analysis showed significant group differences in [Wald $X^2(2) = 16.02$, $p<0.001$] Further analysis showed an increase of spine density in the IMO 2d group compared with control and IMO 8d groups ($p<0.001$ and $p<0.05$), and marginally significant increase in spine density at IMO 8d *vs* control ($p=0.053$). Sholl analysis of spine density [FIGURE 8e] showed a significant **group**, **distance** and **group x distance** interaction [Wald $X^2(2) = 11.106$, $p<0.01$; Wald $X^2(7) = 772.77$, $p<0.001$; Wald $X^2(14) = 68.98$, $p<0.001$, respectively]. The further interaction analysis showed an increase of spine density in IMO 2d compared to control group at the whole distances except at 160 μm (all $p<0.01$, except 20 and 60 μm $p<0.05$). 8d post-IMO groups compared with control rats presented a spine density increase at 40, 60, 80 μm (all $p<0.05$). Spine increase was higher at IMO 2d group than at the IMO 8d at 40 μm ($p<0.05$).

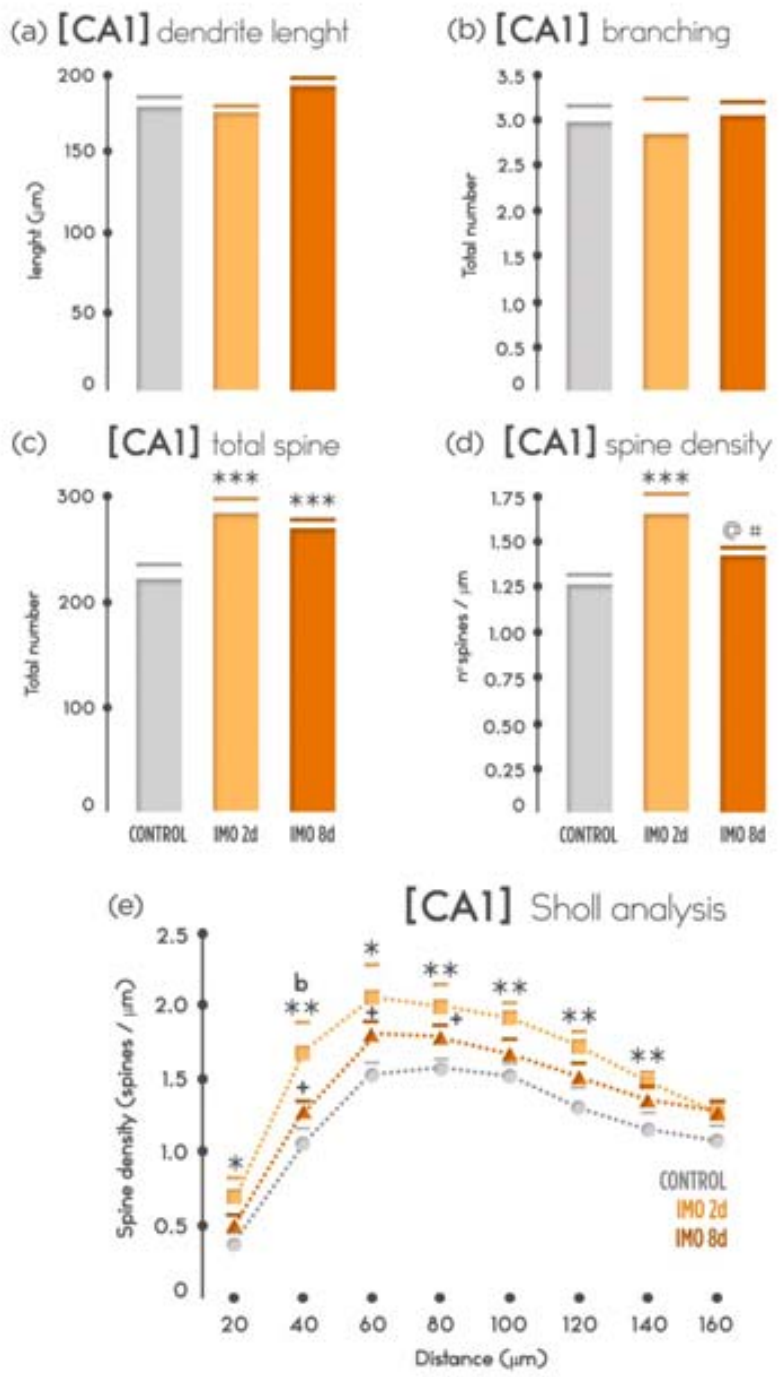
Synaptic proteins changes induced by IMO

BIOCHEMICAL ANALYSES were performed in **DG**, **CA3**, **CA1**, **PFC** and **Amygdala**. HCN1, HCN2 , GirK1, GluA1, Iba1, GFAP, GLUN1 protein levels were evaluated in all the brain areas, whereas in some brain areas were added to the analysis other proteins: in DG pCaMKII, CaMKII, Arc and BDNF, in CA3 and PFC pCaMKII and CaMKII, in CA1 pCaMKII, CaMKII, Arc, BDNF, NCAM and Biochemical analysis of animals exposed to acute **IMO** on **DG** area showed a **group** effect on pCaMKII [FIGURE 9h] [Wald $X^2(2) = 14.01$, $p<0.01$]. Futher analysis showed an increase of the phosphorylation of CaMKII in the IMO 2d group *vs* control ($P<0.01$) and *vs* IMO 8d groups ($P<0.001$). However, no significant differences were found in the other studied proteins TrkB, in the amygdala BDNF, PSD95, Synaptophysin amd VGF.



[FIGURE 7]. Dentate Gyrus morphometric analysis of undisturbed animals (Control), animals exposed to 2h of acute IMO and sacrificed either 2 or 8 days (IMO 2d or 8d) respectively. (a) Total dendritic length. (b) Total number of branches per neuron. (c) Total spine number. (d) Total spine density. (e) Sholl analysis showing the spine density as a function of the distance from the soma.

**p<0.05 IMO 2d vs control group; $^{+}$p<0.05 IMO 2d vs IMO 8d group. Means and S.E.M. are shown.*



[FIGURE 8]. CA1 morphometric analysis of undisturbed animals (Control), animals exposed to 2h of acute IMO and sacrificed either 2 or 8 days (IMO 2d or 8d) respectively. (a) Total dendritic length. (b) Total number of branches per neuron. (c) Total spine number. (d) Total spine density. (e) Sholl analysis showing the spine density as a function of the distance from the soma.

*$^{@}$p<0.05 vs IMO 2d group;$^{\#}$p=0.053 vs control (tendency); *p<0.05, **p<0.01 and ***p<0.001 IMO 2d and 8d vs control group; $^{+}$p<0.05 IMO 8d vs control group;^{b}p<0.05 IMO 8d vs IMO 2d group. Means and S.E.M. are shown.*

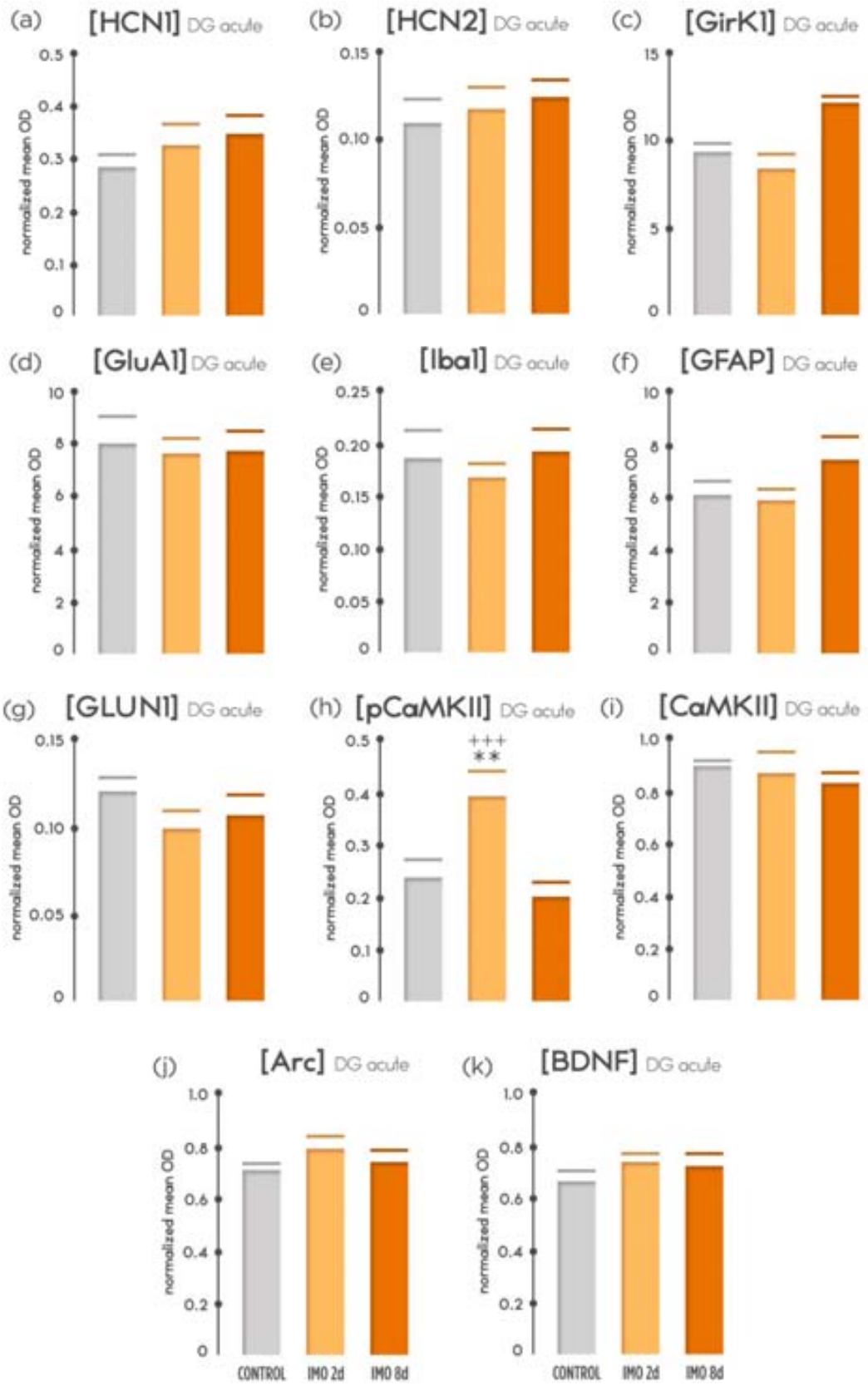
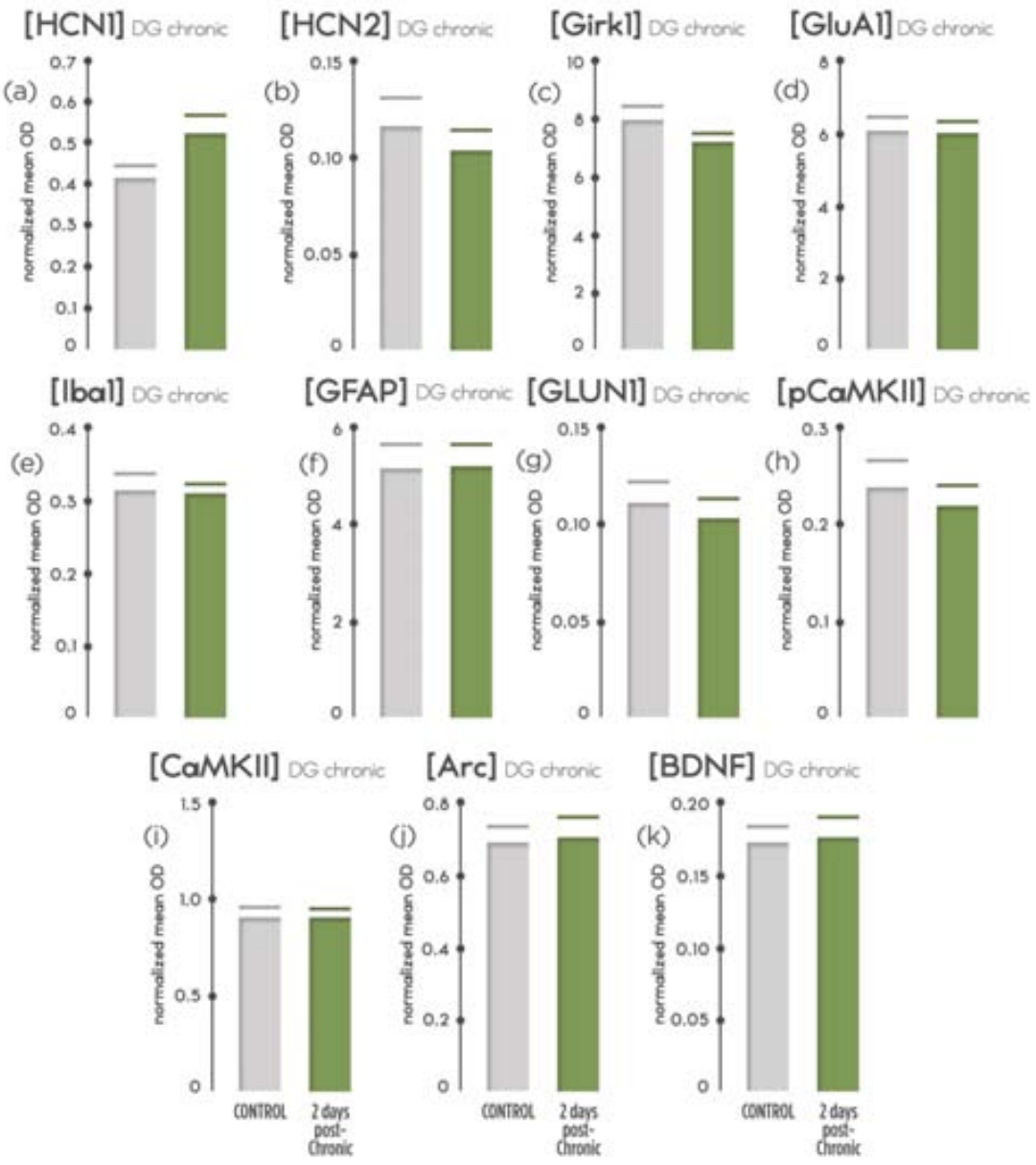


FIGURE 9. Western blot analysis of several synaptic and glial proteins performed on DG of undisturbed animals (Control), animals exposed to 2h of acute IMO and sacrificed either 2 or 8 days (IMO 2d or 8d) respectively. (a) HCN1, (b) HCN2, (c) GirK1, (d) GluA1, (e) Iba1, (f) GFAP, (g) GLUN1, (h) pCaMKII, (i)CaMKII, (j) Arc, (k) BDNF.

***p<0.01 vs control group;$^{+++}$ p<0.001 IMO 8d vs IMO 2d group. Means and S.E.M. are shown*

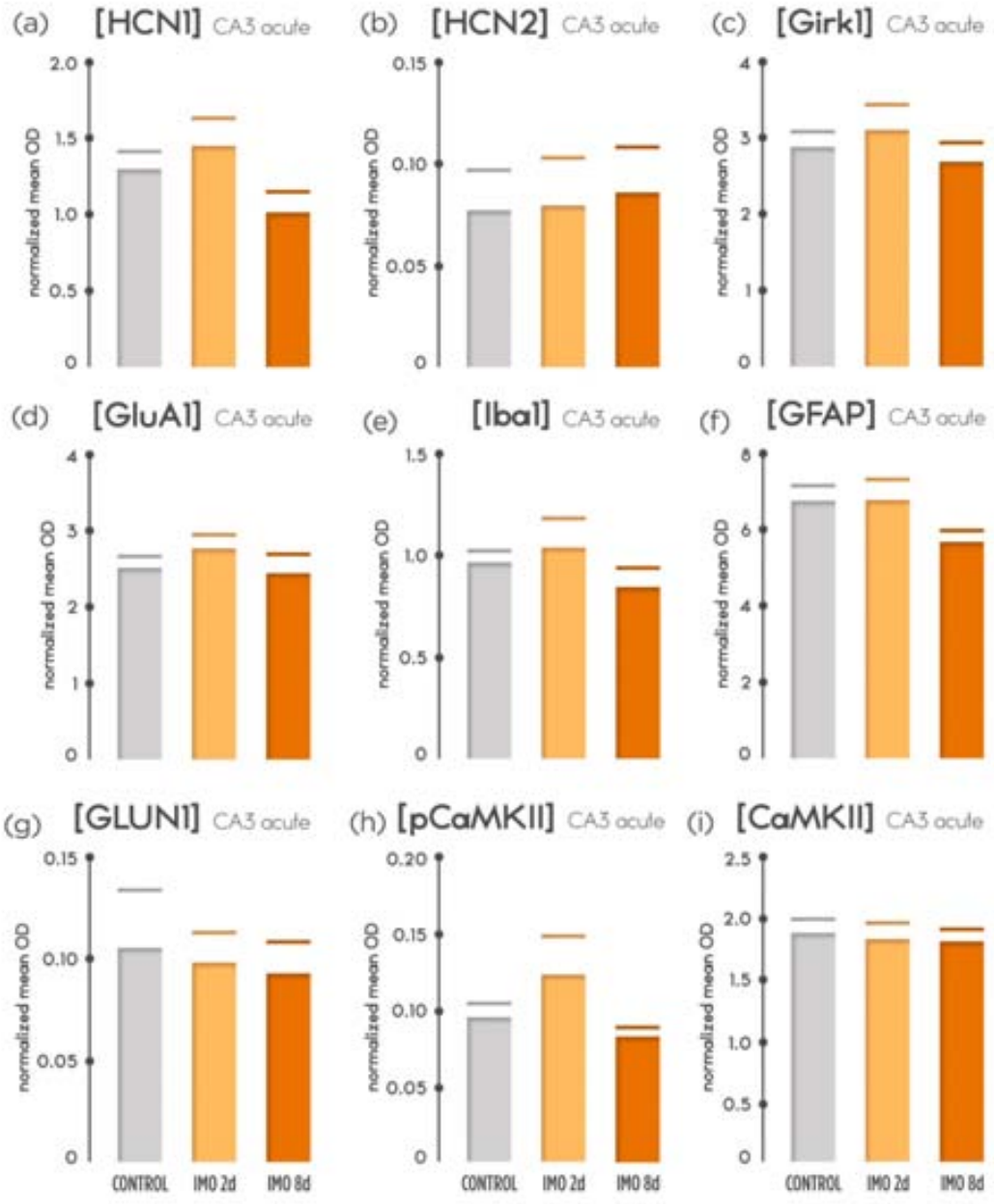
BIOCHEMICAL ANALYSIS of animals exposed to chronic **IMO** on **DG** area [**FIGURE 10**] did not reach significance on any of the studied proteins.



[**FIGURE 10**]. Western blot analysis of several synaptic and glial proteins performed on DG of undisturbed animals (Control) and animals exposed to 2h of chronic IMO and sacrificed after 2 days (Chronic IMO). (a) HCN1, (b) HCN2 , (c) GirK1, (d) GluA1, (e) Iba1, (f) GFAP, (g) GLUN1, (h) pCaMKII, (i)CaMKII, (j) Arc, (k) BDNF.

Means and S.E.M. are shown.

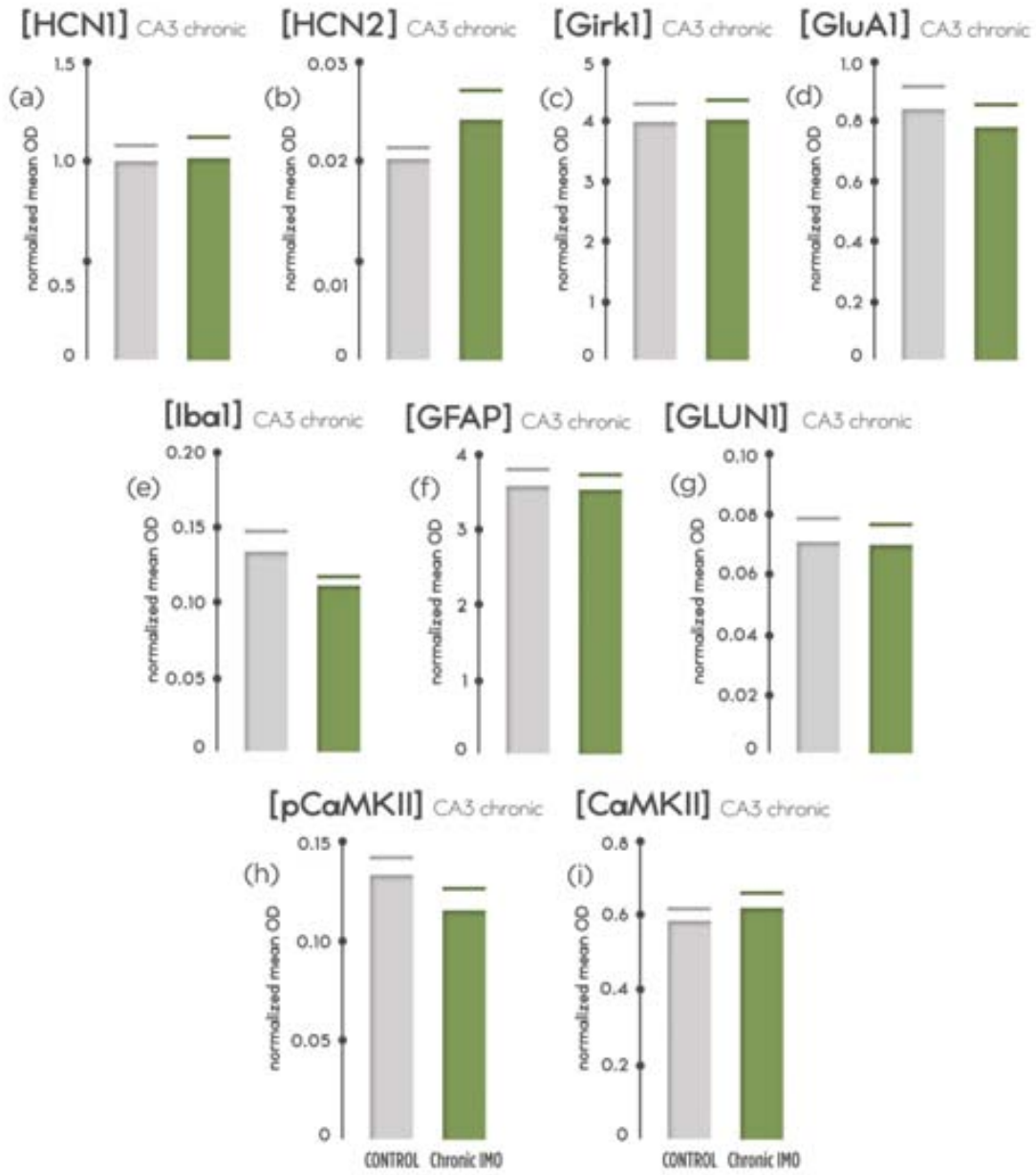
BIOCHEMICAL ANALYSIS of animals exposed to chronic **IMO** on **CA3** area [**FIGURE 11**] did not reach significance on any of the studied proteins.



[**FIGURE 11**]. Western blot analysis of several synaptic and glial proteins performed on CA3 of undisturbed animals (Control), animals exposed to 2h of acute IMO and sacrificed either 2 or 8 days (IMO 2d or 8d) respectively. (a) HCN1, (b) HCN2, (c) GirK1, (d) GluA1, (e) Iba1, (f) GFAP, (g) GLUN1, (h) pCaMKII, (i)CaMKII.

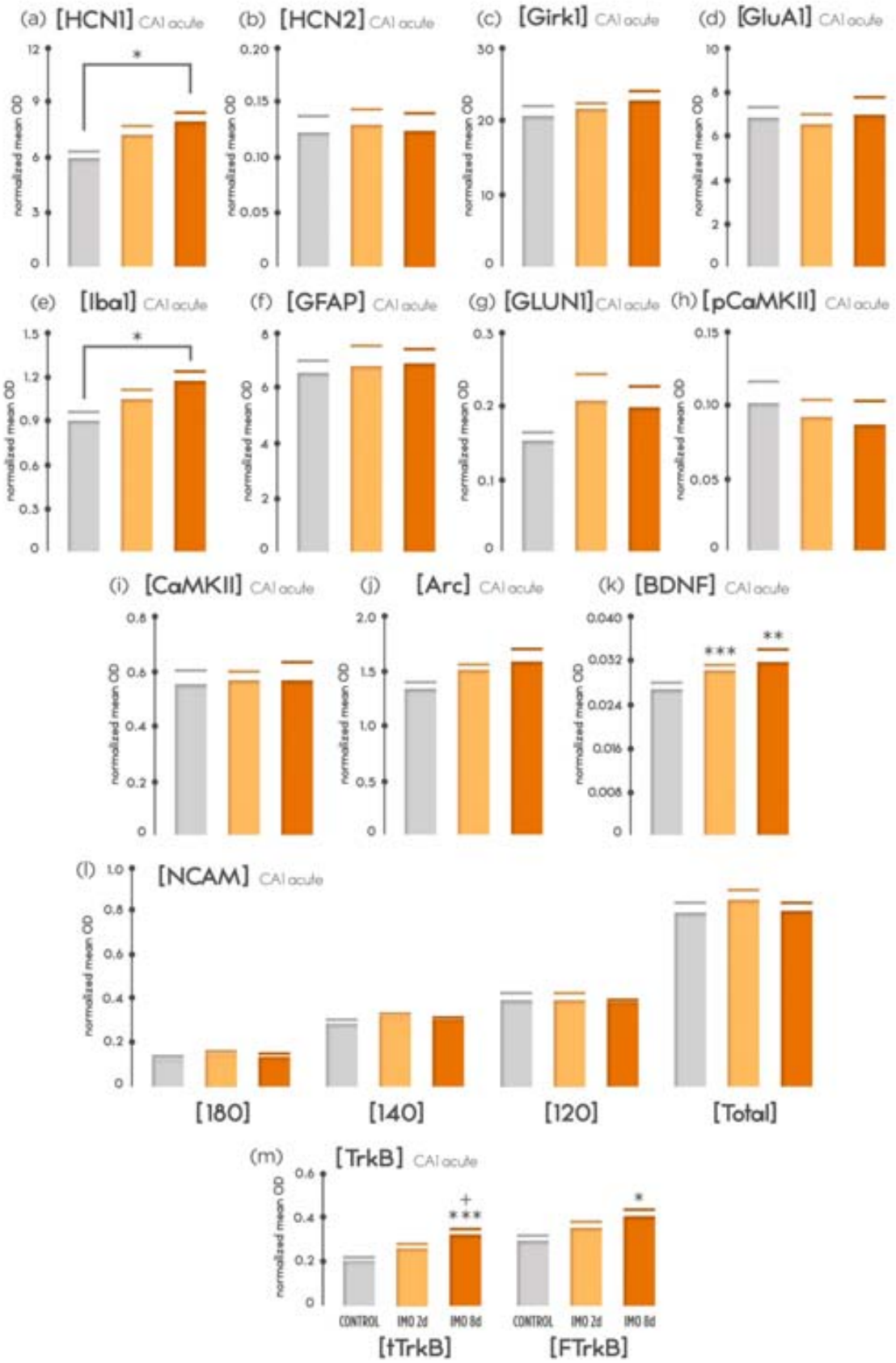
Means and S.E.M. are shown

BIOCHEMICAL ANALYSIS of animals exposed to chronic **IMO** on **CA3** area [**FIGURE 12**] did not reach significance on any of the studied proteins.



[**FIGURE 12**]. Western blot analysis of several synaptic and glial proteins performed on CA3 of undisturbed animals (Control) and animals exposed to 2h of chronic IMO and sacrificed after 2 days (Chronic IMO). (a) HCN1, (b) HCN2 , (c) GirK1, (d) GluA1, (e) Iba1, (f) GFAP, (g) GLUN1, (h) pCaMKII, (i)CaMKII.

Means and S.E.M. are shown.



[FIGURE 13]. Western blot analysis of several synaptic and glial proteins performed on CA1 of undisturbed animals (Control), animals exposed to 2h of acute IMO and sacrificed either 2 or 8 days (IMO 2d or 8d) respectively. (a) HCN1, (b) HCN2, (c) GirK1, (d) GluA1, (e) Iba1, (f) GFAP, (g) GLUN1, (h) pCaMKII, (i)CaMKII. (j) Arc, (k) BDNF, (l) NCAM, (m) TrkB.

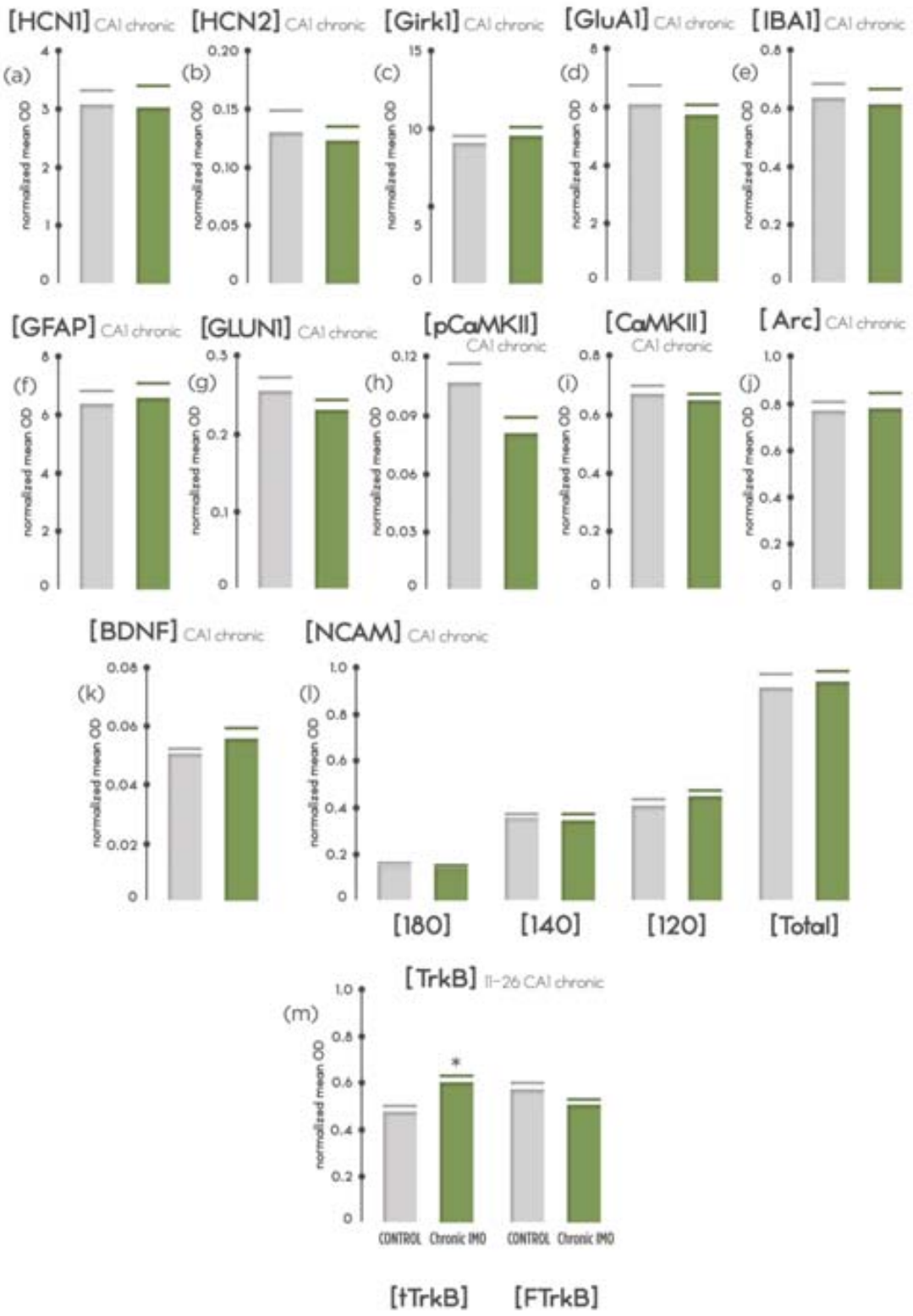
**p<0.05, **p<0.01 and ***p<0.001 vs control group; $^{+}$p<0.05 IMO 8d vs IMO 2d group. Means and S.E.M. are shown*

BIOCHEMICAL ANALYSIS of animals exposed to acute **IMO** on **CA1** area reveled a **group** effect on HCN1 [FIGURE 13a] [Wald $X^2(2) = 7.12$, $p<0.05$]. Futher analysis showed an increase of HCN1 IMO 8d *vs* control ($P<0.05$). The microgial marker Iba1 presented a significant **group** effect [FIGURE 13e] [Wald $X^2(2) = 8.05$, $p<0.05$], Iba1 levels were higher at IMO 8d than in control group. BDNF levels presented a **group** effect [FIGURE 13k] [Wald $X^2(2) = 15.87$, $p\leq0.001$], stress induced a significant increase of BDNF either IMO 2d and IMO 8d as compared to controls ($p<0.01$ and $p<0.001$, respectively). The T1 and the FL form of TrkB presented a significant and a tendency **group** effect respectively [FIGURE 13m] [Wald $X^2(2) = 13.78$, $p\leq0.001$, Wald $X^2(2) = 5.77$, $p=0.056$ respectively]. T1 form analysis revealed higher levels IMO 8d than control and IMO 2d groups ($p<0.001$ and $p<0.05$, respectively) and the FL further showed an increase in the IMO 8d group ($p<0.05$). No significant differences were found in the other proteins.

BIOCHEMICAL ANALYSIS of animals exposed to chronic **IMO** on **CA1** area showed an increase of the T1 TrkB form of chronic IMO *vs* control group [FIGURE 14m] [$t(19) = -2.87$, $p\leq0.001$]. However, no significant differences were found in the other studied proteins.

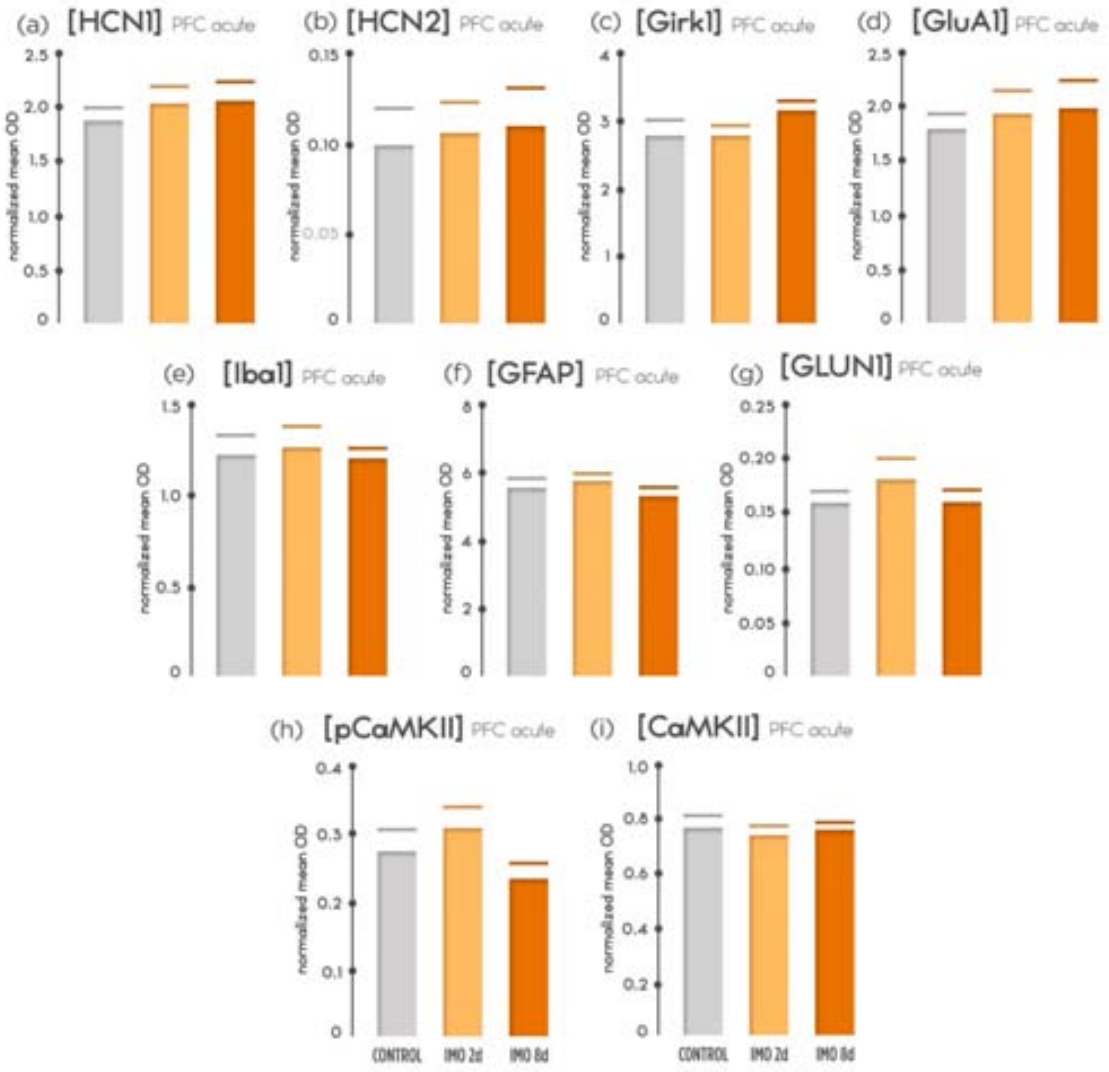
BIOCHEMICAL ANALYSIS of animals exposed to acute **IMO** on **PFC** [FIGURE 15] did not show significant differences on any of the studied proteins. Biochemical analysis of animals exposed to chronic **IMO** on **PFC** area [FIGURE 16] showed an increase of Iba1 levels on chronic IMO *vs* control group [FIGURE 16e] [$t(19) = -2.55$, $p\leq0.05$]. No significant differences were found in the other studied proteins.

BIOCHEMICAL ANALYSIS of animals exposed to acute [FIGURE 17] or chronic [FIGURE 18] **IMO** on **AMY** did not show significant differences on any of the studied proteins.



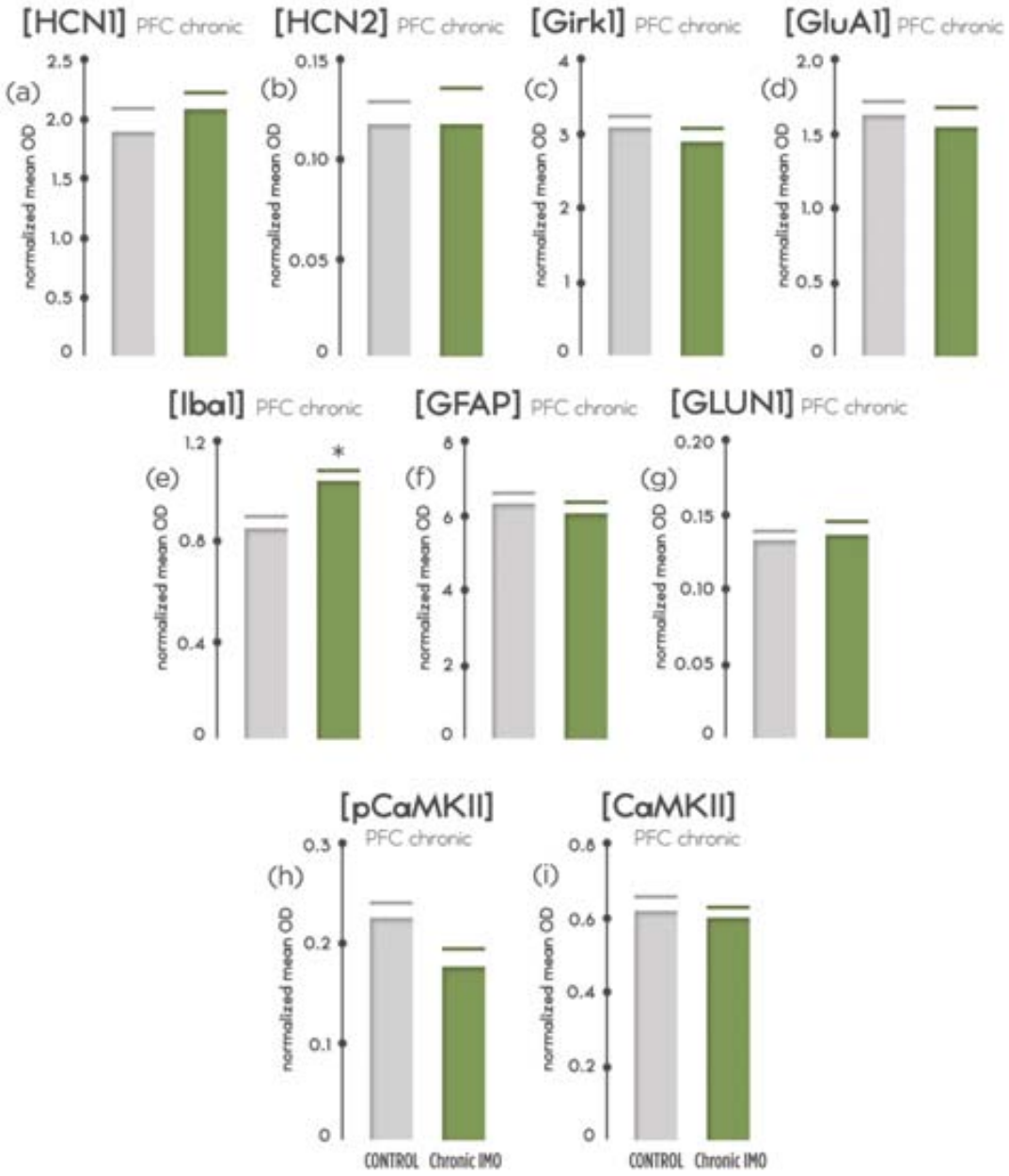
[FIGURE 14]. Western blot analysis of several synaptic and glial proteins performed on CA3 of undisturbed animals (Control) and animals exposed to 2h of chronic IMO and sacrificed after 2 days (Chronic IMO). (a) HCN1, (b) HCN2 , (c) GirK1, (d) GluA1, (e) Iba1, (f) GFAP, (g) GLUN1, (h) pCaMKII, (i)CaMKII, (j) Arc, (k) BDNF, (l) NCAM, (m) TrkB.

**p<0.05vs control group. Means and S.E.M. are shown.*



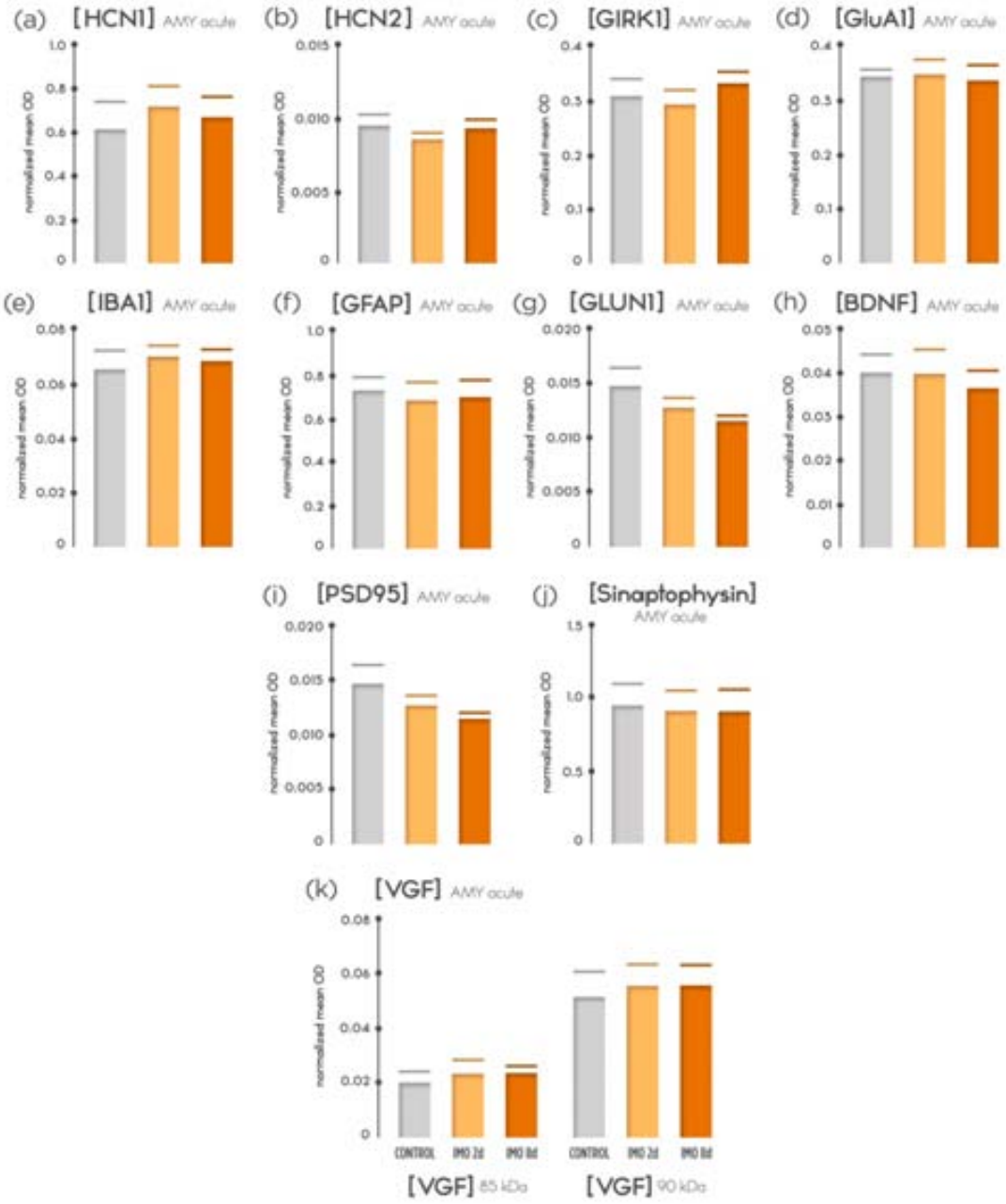
[FIGURE 15]. Western blot analysis of several synaptic and glial proteins performed on PFC of undisturbed animals (Control), animals exposed to 2h of acute IMO and sacrificed either 2 or 8 days (IMO 2d or 8d) respectively. (a) HCN1, (b) HCN2, (c) GirK1, (d) GluA1, (e) Iba1, (f) GFAP, (g) GLUN1, (h) pCaMKII, (i)CaMKII.

Means and S.E.M. are shown



[FIGURE 16]. Western blot analysis of several synaptic and glial proteins performed on PFC of undisturbed animals (Control) and animals exposed to 2h of chronic IMO and sacrificed after 2 days (Chronic IMO). (a) HCN1, (b) HCN2 , (c) GirK1, (d) GluA1, (e) Iba1, (f) GFAP, (g) GLUN1, (h) pCaMKII, (i) CaMKI.

**p<0.05vs control group. Means and S.E.M. are shown.*



[FIGURE 17]. Western blot analysis of several synaptic and glial proteins performed on AMY of undisturbed animals (Control), animals exposed to 2h of acute IMO and sacrificed either 2 or 8 days (IMO 2d or 8d) respectively. (a) HCN1, (b) HCN2, (c) GirK1, (d) GluA1, (e) Iba1, (f) GFAP, (g) GLUN1, (h) BDNF, (i) PSD95, (j) Synaptophysin, (k) VGF.

Means and S.E.M. are shown

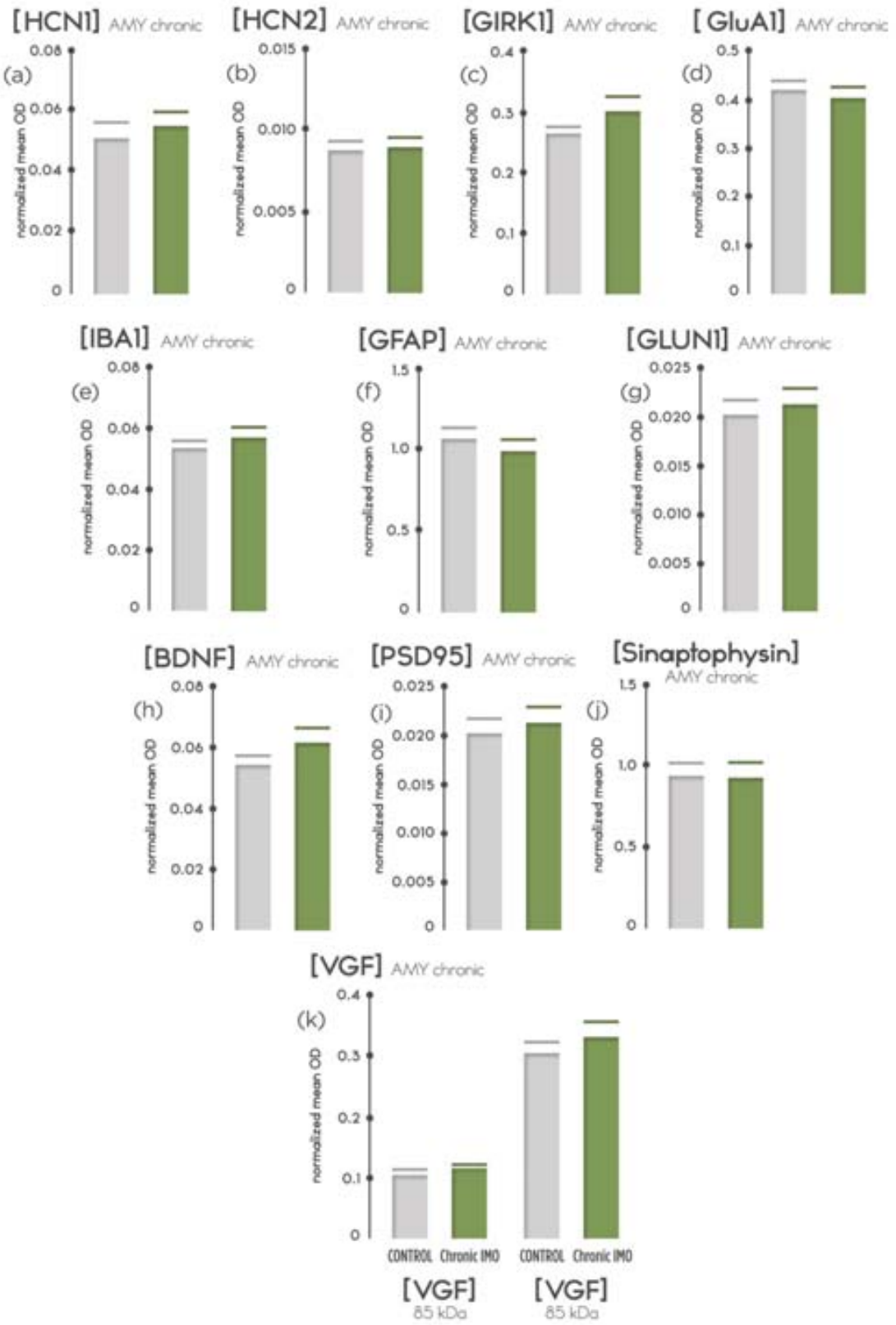
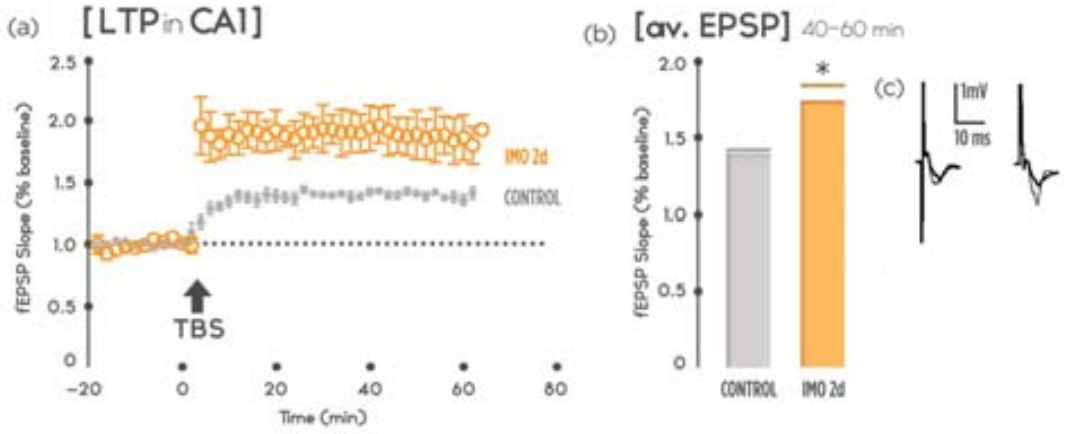


FIGURE 18. Western blot analysis of several synaptic and glial proteins performed on AMY of undisturbed animals (Control) and animals exposed to 2h of chronic IMO and sacrificed after 2 days (Chronic IMO). (a) HCN1, (b) HCN2 , (c) GirK1, (d) GluA1, (e) Iba1, (f) GFAP, (g) GLUN1, (h) BDNF, (i) PSD95, (j) Synaptophysin, (k) VGF.

Means and S.E.M. are shown.

Activity-related plasticity changes induced by IMO

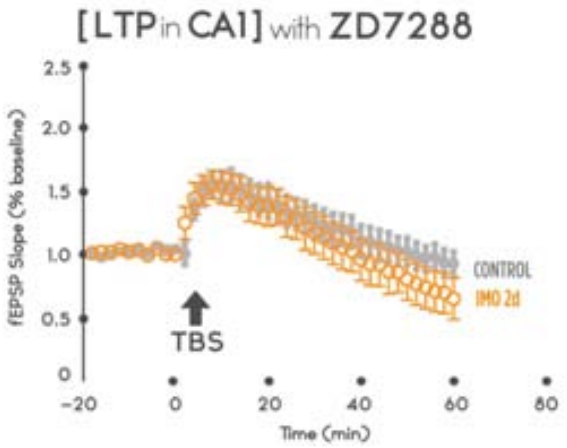
The electrophysiological analysis of animals exposed to IMO on CA1 area 2 days after IMO indicated that TBS protocol was able to induce LTP in both groups [FIGURE 19a]. However, LTP induction was higher in stressed than control animals [FIGURE 19b] [t(9) = -2.29, p<0.05].



[FIGURE 19]. LTP induction from acute slices performed on CA1 of undisturbed animals administered with vehicle (Control) and animals exposed to 2h of acute IMO and sacrificed after 2 days (IMO 2d). (a) LTP induction, (b) Averaged EPSP (40-60 min) (c) Representative traces from immediately before TBS (Thick) and after TBS (Thin).

**p<0.05 vs control group. Means and S.E.M. are shown.*

Electrophysiological analysis of animals exposed to IMO on CA1 area 2 days after IMO in the presence of ZD7288 indicated that TBS protocol was able to induce potentiation, but it was not maintained through time [FIGURE 20].



[FIGURE 20]. LTP induction from acute slices performed on CA1 of undisturbed animals administered with vehicle (Control) and animals exposed to 2h of acute IMO and sacrificed after 2 days (IMO 2d) in the presence of ZD7288.

Means and S.E.M. are shown.

Spatial memory changes induced by IMO

MWM ANALYSIS of animals exposed to acute IMO did not show significant group differences in latency and average swimming speed [**FIGURE 21a & b**], although trial significantly affected to latency [Wald $X^2(11) = 217.68$, $p<0.001$]. Thigmotaxis [**FIGURE 21c**] analysis revealed a **trail** and **group** effect [Wald $X^2(12) = 349.64$, $p<0.001$; Wald $X^2(2) = 6.73$, $p<0.05$, respectively] and a **trial x group** interaction [Wald $X^2(24) = 47.68$, $p<0.01$]. Further decomposition of interaction showed an increase of time spent in outer area of IMO 2d group as compared to control and IMO 8d groups (both $p<0.01$) in trial 4 and 7, on the contrary a decrease in the time spent in outer area in the trial 12 of IMO 8d group *vs* control group ($p<0.05$).

Probe trial analysis [**FIGURE 21d**] revealed a significant **group** and **area** effect [Wald $X^2(2) = 19.65$, $p<0.001$; Wald $X^2(1) = 20.57$, $p<0.001$ respectively] and an **area x group** interaction [Wald $X^2(2) = 9.53$, $p<0.01$]. Further analysis revealed that control and IMO 8d animals spent more time in target area than in opposite ($p<0.001$ and $p<0.01$, respectively), but IMO 2d did not show a significant difference ($p=0.292$). Furthermore, control and IMO 8d groups spent more time than the time of IMO 2d group (both $p<0.01$).

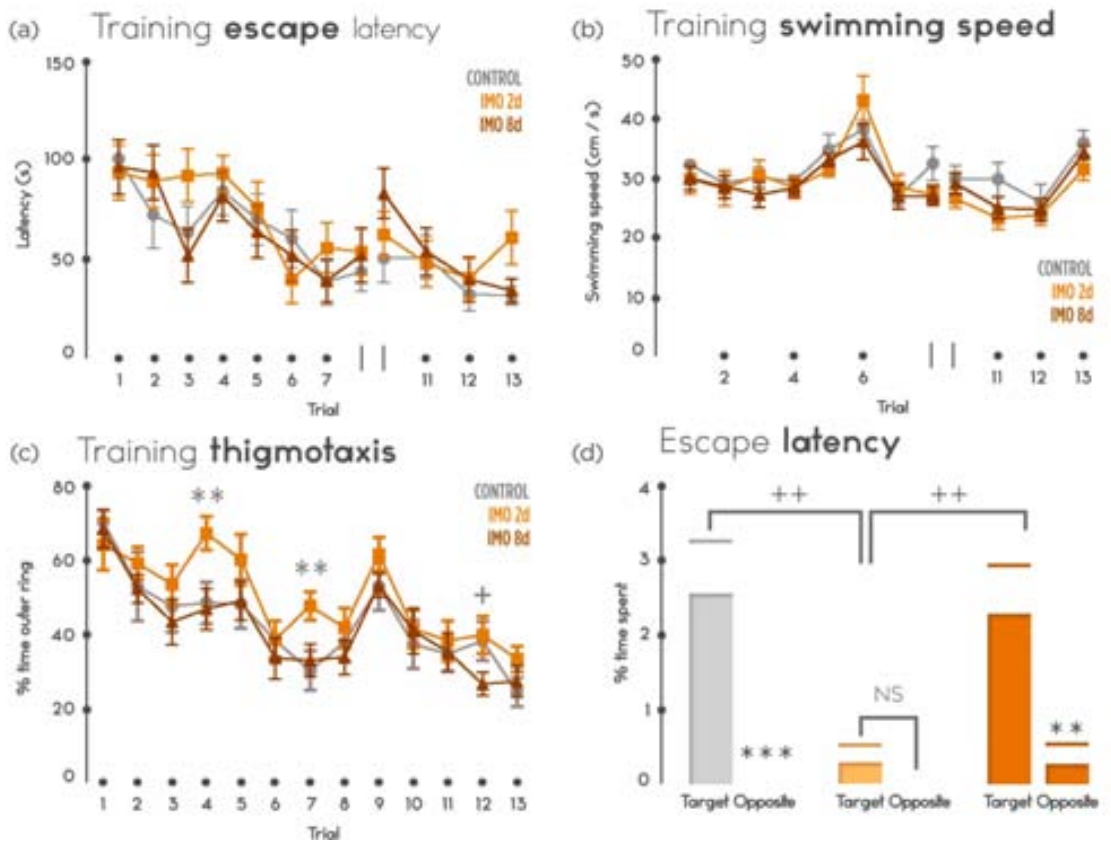


FIGURE 21. MWM performance of undisturbed animals (Control), animals exposed to 2h of acute IMO and sacrificed either 2 or 8 days (IMO 2d or 8d) respectively. (a) Training scape latency, (b) Training average swimming speed, (c) Training thigmotaxis *(*p<0.05 and **p<0.01 vs IMO 2d group; +p<0.05 vs IMO 8d group).* (d) Escape latency *(**p<0.01 and ***p<0.001 vs opposite; ++p<0.01 vs IMO 2d group).*

Means and S.E.M. are shown.

Conclusion

IMO effects on HF volume, physiological variables and HF-dependent task are similar to those found in others PTSD models or patients. However, the plasticity-related factors such as spine density, LTP, BDNF, HCN1 and Iba1 increase seems that follow a development-like process trying to compensate the detrimental effects of the initial trauma.

In summary, we postulate that stress may induce an initial temporal dynamic process, which derived in a disconnection and posterior reconnection of the neural network. Finally, it is reasonable to assume that IMO-induced synaptic plasticity is involved in IMO-induced memory impairment and both effects could be directly or indirectly correlated to the HF abnormality function found in PTSD patients.

CHAPTER 2

7,8-DHF, a TrkB agonist, prevent behavioral and synaptic plasticity stress-induced effects

Introduction

Several treatments have been proposed for PTSD; however, none of them has been totally successful. Antidepressants, the most common PTSD treatment, did not show strong evidence of effectiveness alone or combined with psychotherapy, presenting some controversial results (Hetrick 2010, Andero 2012b, Steckler and Risbrough 2012). Moreover to the treatment of established symptoms, there is a more potential strategy for PTSD treatment: the prevention strategy.

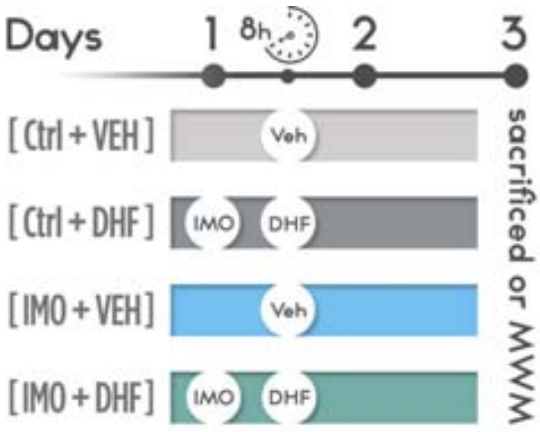
In this regard, BDNF/TrkB pathway has been proposed as a novel strategy (Andero 2012b). Despite the role of BDNF in PTSD patients present some controversy (Dell'Osso 2009, Berger 2010, Frielingsdorf 2010, Hauck 2010), animal research has revealed an important implication of BDNF/TrkB pathway in PTSD animal models and symptoms. Stress and BDNF are directly or indirectly related with the modulation of GC receptors, memory, glutamate release, synaptic plasticity, structural plasticity and also the BDNF/TrkB pathway (Jeanneteau 2008, Blugeot 2011, Andero 2012a, Jeanneteau and Chao 2013, Numakawa 2013). In addition, BDNF is decreased in PTSD animal models (Smith 1995, Ueyama 1997, Pizarro 2004, Greenwood 2007, Kozlovsky 2007, Lee 2008). Hence, it is reasonable to believe that the use of BDNF/TrkB pathway agonists is the most effective way to ameliorate stress effects.

There are several TrkB agonist (Andero 2012b); however, 7,8-dihydroxyflavone (7,8-DHF) presents better pharmacokinetic properties and higher TrkB binding affinity than other agonist and even than BDNF. It is a plant origin flavone present in fruits and vegetables, which crosses the BBB and induces TrkB dimerization and autophosphorylation exerting its pro-survival effects via PI3K/Akt and MAPK (Jang 2010). Furthermore, 7,8-DHF has been used to revert spatial memory impairment (Andero 2012a), rescue an extinction deficit (Andero 2011) and interestingly, the chronic administration prevent chronic stress effects on spines density, dendrite length, cell proliferation, HF volume, dendritic intersections, corticosterone levels, adrenal weight, anhedonia, immobility time (Blugeot 2011).

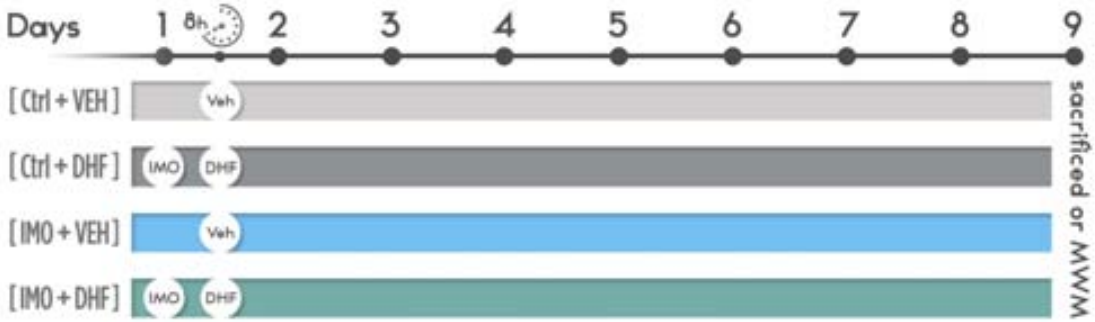
In the present study we investigated whether 7,8-DHF can ameliorate the PTSD-like effect of the IMO in a therapeutic window administration. And to demonstrate whether synaptic plasticity IMO-related effects are causally related to memory impairment.

Experimental design

To demonstrate whether 7,8-DHF can revert the IMO-induced **synaptic proteins (EXPERIMENT 6)**, **activity-related plasticity (EXPERIMENT 7)** and **spatial memory (EXPERIMENT 8)** effects, animals after the last day of handling, rats were randomly assigned to groups: undisturbed animals administered with vehicle (Control+VEH), undisturbed animals administered with DHF (Control+DHF) and animals exposed to 2h of acute IMO and administered with a vehicle (IMO+VEH) or DHF (IMO+DHF) and sacrificed or exposed to MWM after 2 days [FIGURE 1]. In the experiment 6 animals the 8 days after groups were also used [FIGURE 2].



[FIGURE 1]. Experimental design of **EXPERIMENTS 6, 7 AND 8**.



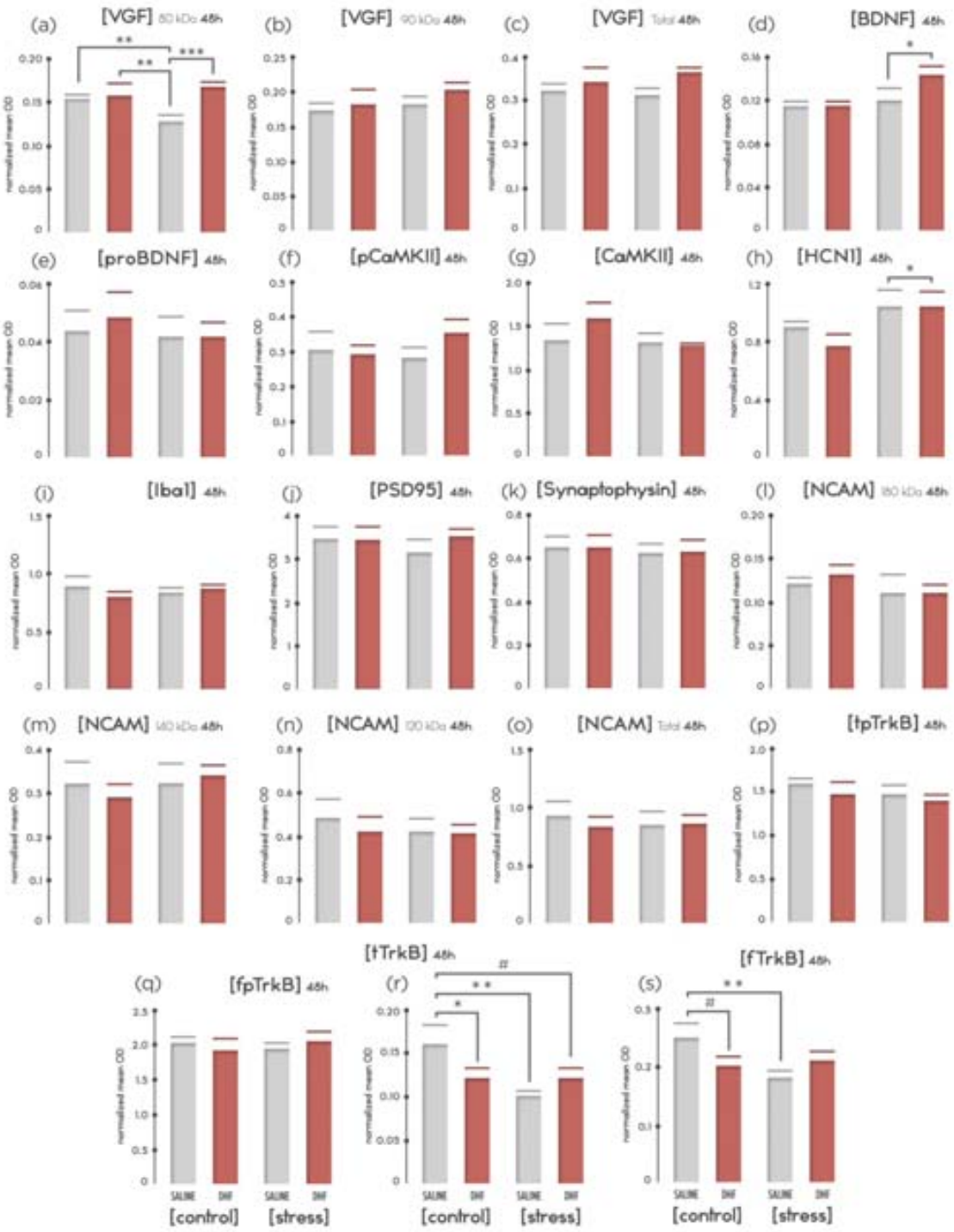
[FIGURE 2]. Experimental design of **EXPERIMENT 6**.

Results

7,8-DHF administration effect on IMO-related synaptic proteins changes

BIOCHEMICAL ANALYSIS *of animals exposed to both* ***IMO*** *and* ***DHF*** *on* ***CA1*** *area 2 days post-IMO.*

VGF 80 kDa [FIGURE 3a] study revealed a significant **drug** effect [Wald $X^2(1) = 8.44$, $p<0.01$] and the interaction **drug x stress** [Wald $X^2(1) = 6.07$, $p<0.05$]. Decomposition of the interaction showed that IMO exposure decreased VGF levels ($p<0.01$). However, DHF administration did not modify VGF in control group and rescued totally the VGF in the IMO group ($p<0.001$). BDNF levels [FIGURE 3d] analysis showed only a significant **drug** effect [Wald $X^2(1) = 3.91$, $p<0.05$]. BDNF levels were higher in IMO exposed animals. HCN1 levels [FIGURE 3h] analysis revealed only a significant **drug** effect [Wald $X^2(1) = 3.91$, $p<0.05$]. IMO induced an increase of protein levels of HCN1 channel. TrkB-T1 [FIGURE 3r] analysis revealed a significant **stress** effect [Wald $X^2(1) = 4.51$, $p<0.05$] and a significant interaction **drug x stress** [Wald $X^2(1) = 4.15$, $p<0.05$]. Further decomposition showed that IMO exposure and DHF administration decreased TrkB-T1 levels ($p<0.01$ and $p<0.05$ respectively). TrkB-FL [FIGURE 3s] analysis revealed an interaction **drug x stress** [Wald $X^2(1) = 5.23$, $p<0.05$]. Further decomposition showed that IMO exposure and DHF administration decreased TrkB-FL levels ($p\leq0.01$ and $p=0.062$ respectively). The others proteins did not show significant differences.



[FIGURE 3]. Western blot analysis of several synaptic and glial proteins, performed on CA1 of undisturbed animals administered with vehicle (Control+VEH), undisturbed animals administered with DHF (Control+DHF) and animals exposed to 2h of acute IMO and sacrificed after 2 days and administered with a vehicle (IMO+VEH) or DHF (IMO+DHF). (a) VGF 80 kDa, (b) VGF 90 kDa, (c) VGF Total, (d) BDNF, (e) proBDNF, (f) pCaMKII, (g) CaMKII, (h) HCN1, (i)Iba1, (j) PSD95, (k) Synaptophysin, (l) NCAM 180 kDa, (m) NCAM 140 kDa, (n) NCAM 120 kDa, (o) NCAM total, (p) pTrkB-T1, (q) pTrkB-FL, (r) TrkB-T1, (s) TrkB-FL .

**p<0.05, **p<0.01 and ***p<0.001 vs control-VEH group; #p=0.053 vs control-VEH (tendency). Means and S.E.M. are shown*

BIOCHEMICAL ANALYSIS *of* ***CA1*** *area from animals exposed to both* ***IMO*** *and* ***DHF*** *on 8 days post-IMO.*

VGF 90 kDa levels [FIGURE 4b] analysis showed a significant **stress** effect [Wald $X^2(1) = 3.91$, $p<0.05$]. IMO exposed animals presented lower levels of VGF.

VGF total levels [FIGURE 4c] analysis showed only a significant **stress** effect [Wald $X^2(1) = 3.91$, $p<0.05$]. Animals exposed to IMO showed a decrease in VGF levels.

HCN1 [FIGURE 4h] analysis revealed a tendency in interaction **drug x stress** [Wald $X^2(1) = 3.42$, $p=0.064$]. Further decomposition showed that IMO exposure increased HCN1 levels ($p=0.075$ tendency).

Iba1 levels [FIGURE 4i] analysis showed a significant **stress** effect [Wald $X^2(1) = 6.39$, $p<0.05$]. IMO induced an increase of Iba1 channel.

NCAM isoforms 140 kDa [FIGURE 4m] and 120 kDa [FIGURE 4n] as well as total levels of NCAM [FIGURE 4o] presented a significant **stress** effect [Wald $X^2(1) = 4.43$, $p<0.05$; Wald $X^2(1) = 4.48$, $p<0.05$; Wald $X^2(1) = 5.75$, $p<0.05$ respectively]. All NCAM isoforms were increased in the IMO group.No significant differences were found in the other studied proteins.

BIOCHEMICAL ANALYSIS *on* ***DG*** *of animals exposed to both* ***IMO*** *and* ***DHF*** *area 2 days post-IMO.*

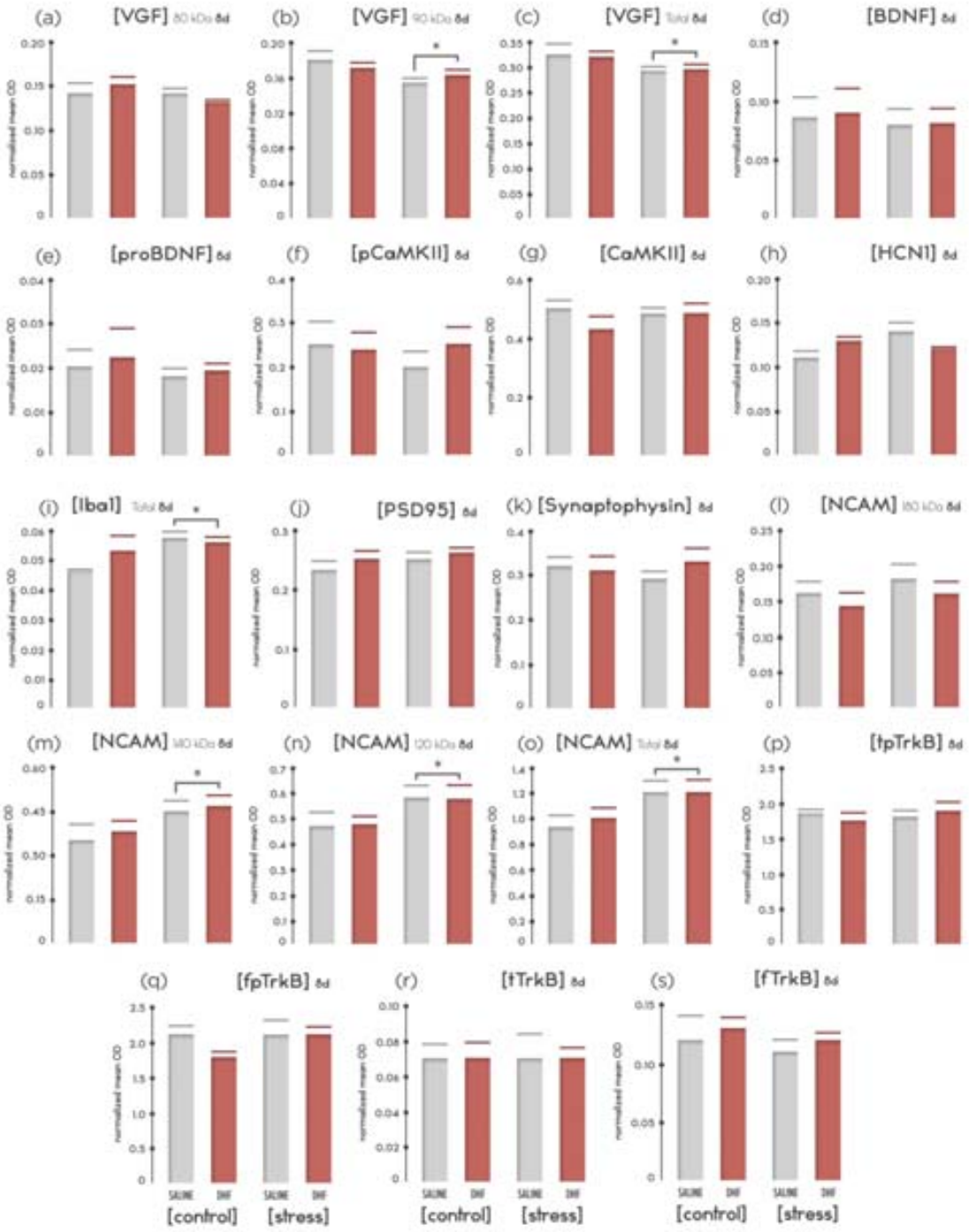
VGF 90 kDa [FIGURE 5b] analysis revealed a significant interaction **drug x stress** [Wald $X^2(1) = 3.92$, $p<0.05$]. Further decomposition showed that IMO exposure and DHF administration together increased VGF 90 kDa ($p<0.01$).

VGF total [FIGURE 5c] analysis revealed a tendency **drug** effect [Wald $X^2(1) = 363$, $p=0.057$]. Animals exposed to IMO had higher levels of VGF total. No significant differences were found in the other studied proteins.

BIOCHEMICAL ANALYSIS *on* ***DG*** *of animals exposed to both* ***IMO*** *and* ***DHF*** *area 8 days post-IMO.*

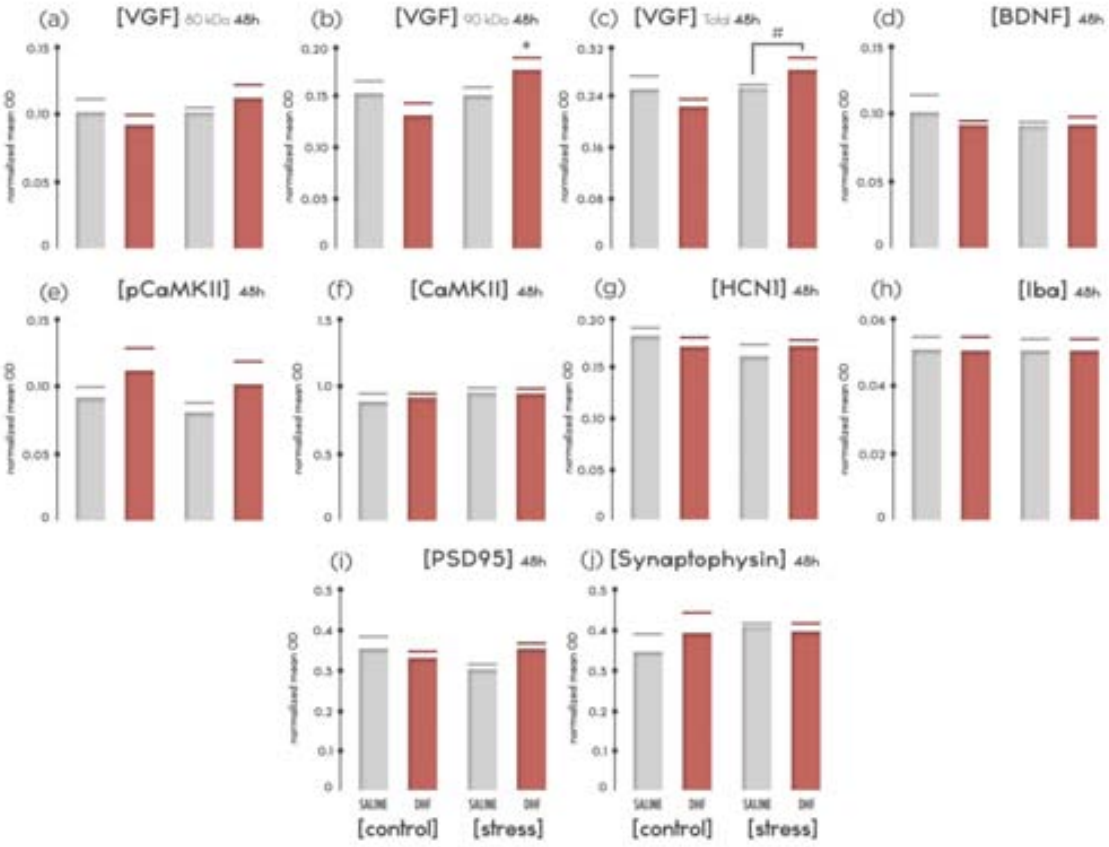
VGF 90 kDa [FIGURE 6a] study revealed a significant **drug** effect [Wald $X^2(1) = 4.009$, $p<0.05$] and the interaction tendency **drug x stress** [Wald $X^2(1) = 3.709$, $p=0.054$]. Decomposition of the interaction showed that DHF administration and IMO exposure increased VGF 90 kDa levels (both $p<0.05$).

HCN1 [FIGURE 6b] analysis showed a significant interaction **drug x stress** [Wald $X^2(1) = 4.1$, $p<0.05$]. Further decomposition showed that IMO exposure reduced HCN1 levels ($p=0.067$ tendency), and DHF administration returned HCN1 to control levels after an IMO exposure ($p<0.05$). The others proteins did not show significant differences.



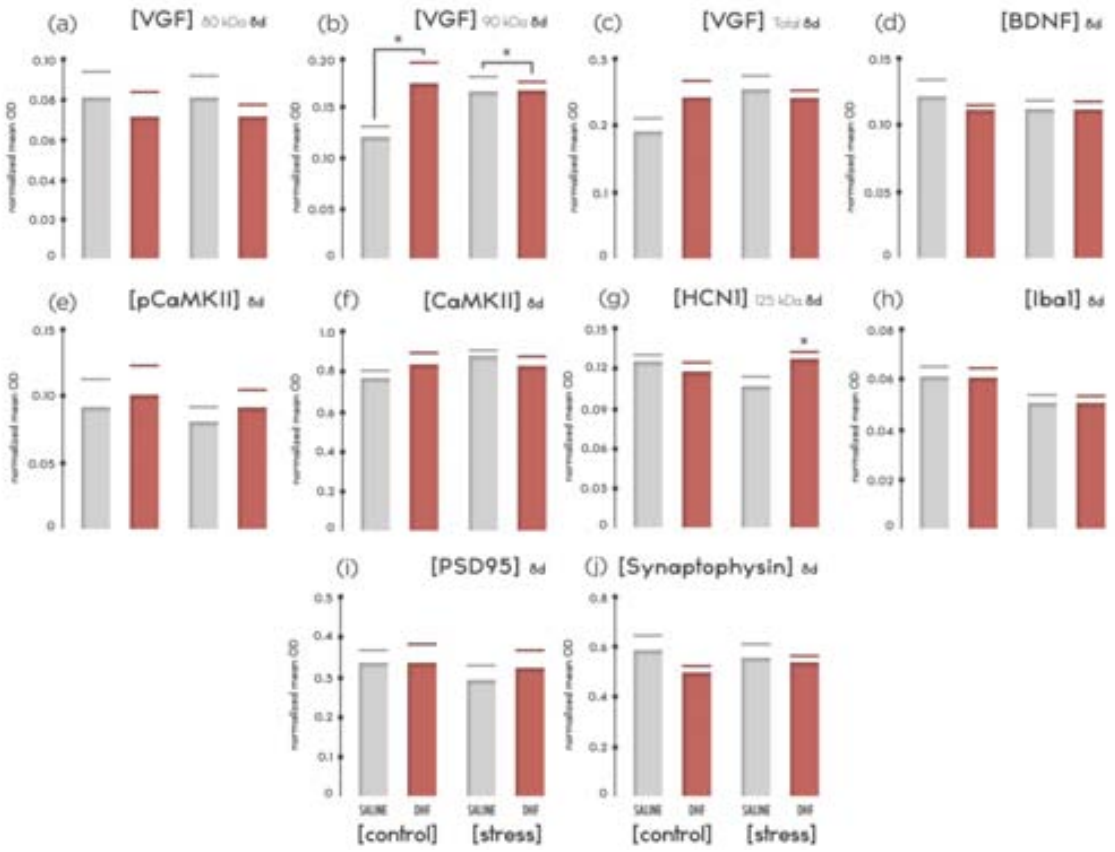
[FIGURE 4]. Western blot analysis of several synaptic and glial proteins, performed on CA1 of undisturbed animals administered with vehicle (Control+VEH), undisturbed animals administered with DHF (Control+DHF) and animals exposed to 2h of acute IMO and sacrificed after 8 days and administered with a vehicle (IMO+VEH) or DHF (IMO+DHF). (a) VGF 80 kDa, (b) VGF 90 kDa, (c) VGF Total, (d) BDNF, (e) proBDNF, (f) pCaMKII, (g) CaMKII, (h) HCN1, (i) Iba1, (j) PSD95, (k) Synaptophysin, (l) NCAM 180 kDa, (m) NCAM 140 kDa, (n) NCAM 120 kDa, (o) NCAM total, (p) pTrkB-T1, (q) pTrkB-FL, (r) TrkB-T1, (s) TrkB-FL .

**p<0.05 vs control group; # p=0.064 vs control-VEH (tendency). Means and S.E.M. are shown.*



[FIGURE 5]. Western blot analysis of several synaptic and glial proteins, performed on DG of undisturbed animals administered with vehicle (Control+VEH), undisturbed animals administered with DHF (Control+DHF) and animals exposed to 2h of acute IMO and sacrificed after 2 days and administered with a vehicle (IMO+VEH) or DHF (IMO+DHF). (a) VGF 80 kDa, (b) VGF 90 kDa, (c) VGF Total, (d) BDNF, (e) pCaMKII, (f) CaMKII, (g) HCN1, (h)Iba1, (i) PSD95, (j) Synaptophysin.

**p<0.05 vs control group; #p=0.057 vs control-VEH (tendency). Means and S.E.M. are shown.*



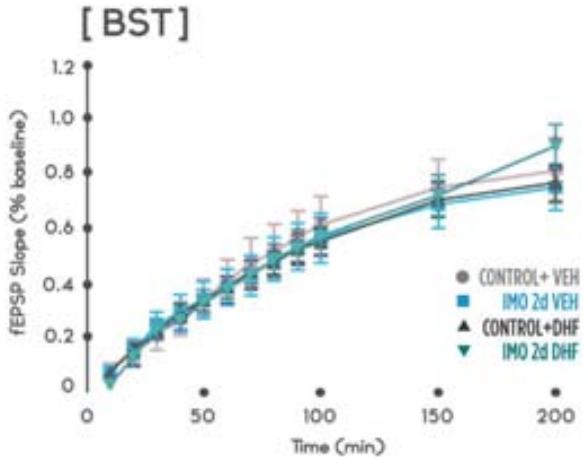
[FIGURE 6]. Western blot analysis of several synaptic and glial proteins, performed on DG of undisturbed animals administered with vehicle (Control+VEH), undisturbed animals administered with DHF (Control+DHF) and animals exposed to 2h of acute IMO and sacrificed after 8 days and administered with a vehicle (IMO+VEH) or DHF (IMO+DHF). (a) VGF 80 kDa, (b) VGF 90 kDa, (c) VGF Total, (d) BDNF, (e) pCaMKII, (f) CaMKII, (g) HCN1, (h)Iba1, (i) PSD95, (j) Synaptophysin.

***p<0.05 vs control group; # p=0.067 vs control-VEH (tendency). Means and S.E.M. are shown.*

7,8-DHF administration effect on activity-related plasticity changes induced by IMO

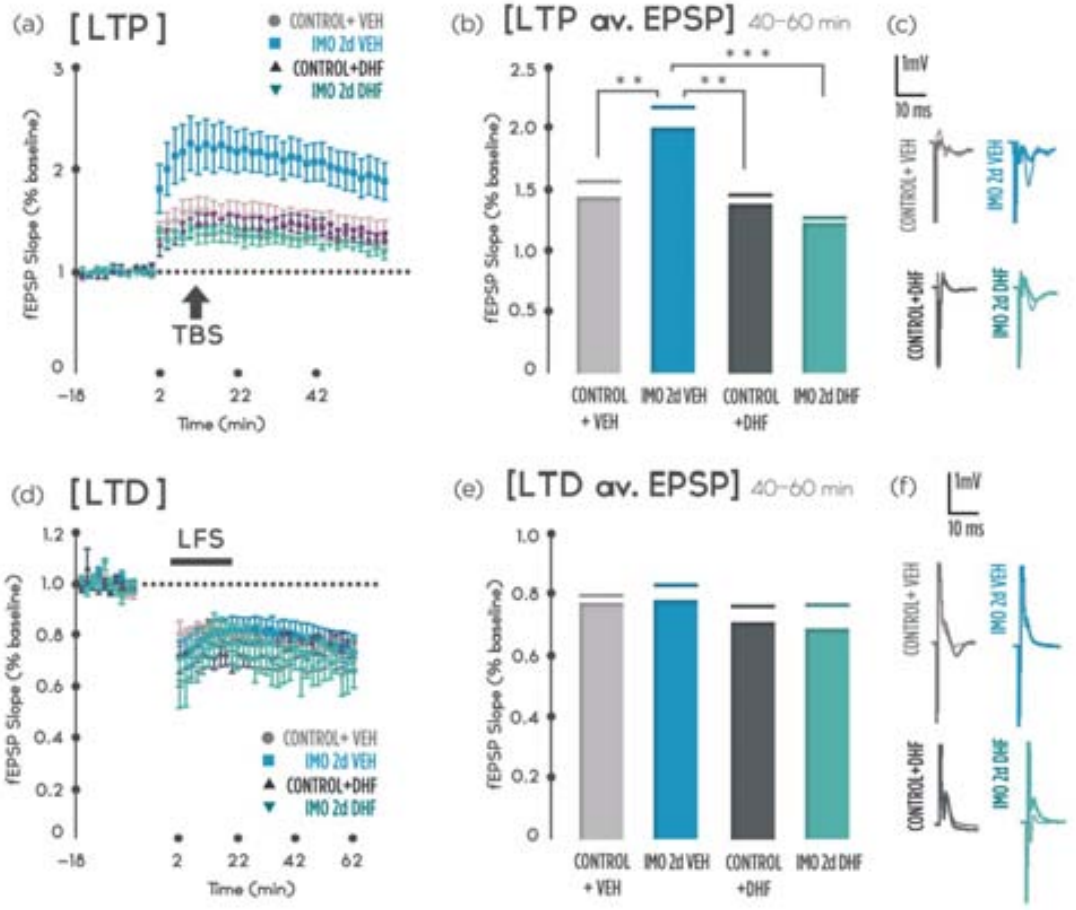
In the **BASAL SYNAPTIC TRANSMISSION ANALYSIS** on **CA1** of animals exposed to both **IMO** and **DHF** area 2 days post-IMO **[FIGURE 7]** no significant changes were found due to stress or DHF.

In the **ELECTROPHYSIOLOGICAL ANALYSIS** on **CA1** area of animals exposed to both **IMO** and **DHF** 2 days post-IMO, LTP **[FIGURE 8a]** study revealed a significant **drug** effect [Wald $X^2(1) = 9.44$, $p<0.01$] and the interaction **drug x stress** [Wald $X^2(1) = 7.57$, $p<0.01$]. Decomposition of the interaction showed that IMO exposure increased LTP levels ($p<0.01$). However, after DHF administration, LTP was totally rescued in the IMO+DHF group ($p<0.001$) and did not modify LTP in control+DHF group.



[FIGURE 7]. BST from acute slices performed on CA1 of undisturbed animals administered with vehicle (Control+VEH), undisturbed animals administered with DHF (Control+DHF) and animals exposed to 2h of acute IMO and sacrificed after 2 days and administered with a vehicle (IMO+VEH) or DHF (IMO+DHF).

Means and S.E.M. are shown.

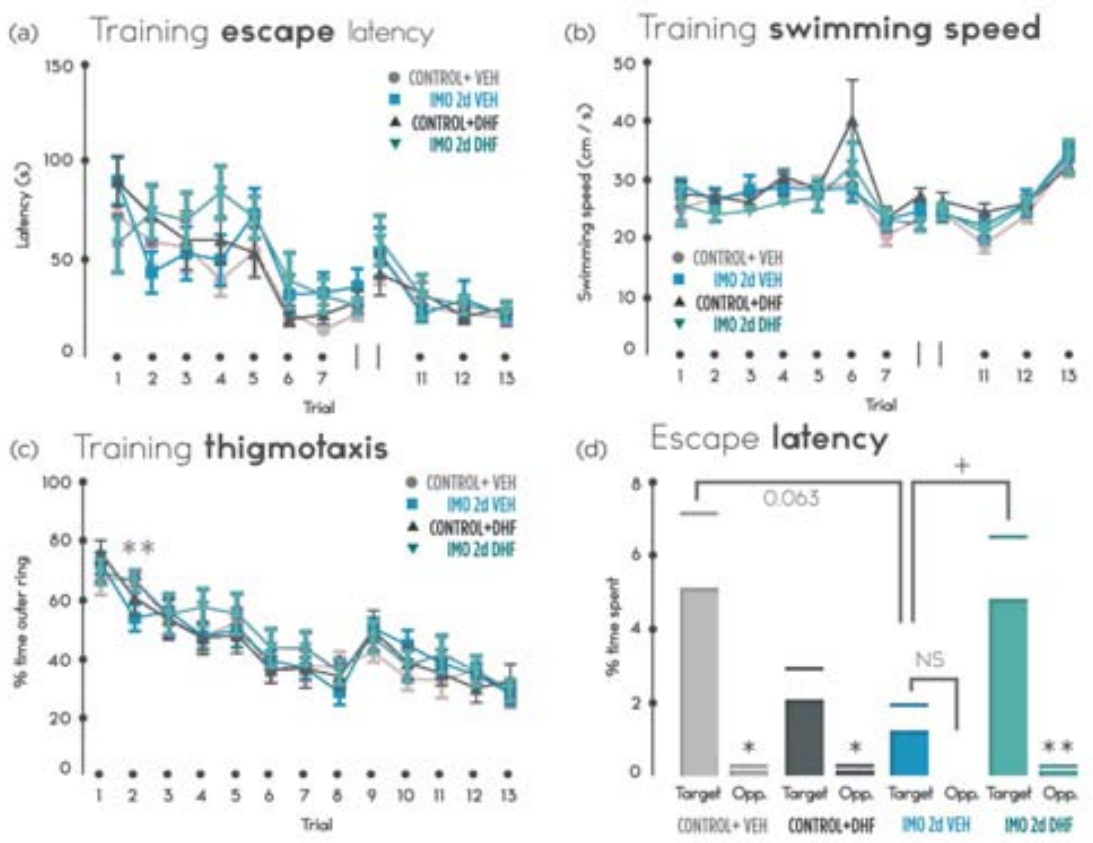


[FIGURE 8]. LTP and LTD induction from acute slices performed on CA1 of undisturbed animals administered with vehicle (Control+VEH), undisturbed animals administered with DHF (Control+DHF) and animals exposed to 2h of acute IMO and sacrificed after 2 days and administered with a vehicle (IMO+VEH) or DHF (IMO+DHF). (a) LTP induction (b) LTP averaged EPSP (40-60 min) (c) LTP representative traces from immediately before TBS (Thick) and after TBS (Thin) (d) LTD induction, (e) LTD averaged EPSP (40-60 min) (f) LTD representative traces from immediately before TBS (Thick) and after TBS (Thin).

***p<0.01 and ***p<0.001 vs control group. Means and S.E.M. are shown.*

7,8-DHF administration effect on spatial memory changes induced by IMO

MWM ANALYSIS of animals exposed to **acute IMO** did not show significant group differences in latency and average swimming speed [**FIGURE 9 a & b**],but latency showed a significant **trial** effect [Wald $X^2(11) = 177.48$, $p<0.001$]. Thigmotaxis [**FIGURE 9 c**] analysis revealed a **trail** effect [Wald $X^2(2) = 6.73$, $p<0.05$] and a **trial x stress x drug** effect [Wald $X^2(12) = 35.38$, $p<0.001$]. Further decomposition of interaction showed a decrease of time spent in outer area of Control+DHF group as compared to control+VEH group ($p<0.01$) in trial 2. Probe trial analysis [**FIGURE 9 d**] revealed a significant **area** and **stress x drug** effect [Wald $X^2(1) = 20.24$, $p<0.001$; Wald $X^2(1) = 6.007$, $p<0.05$ respectively] and a **stress x drug x area** interaction [Wald $X^2(1) = 5.24$, $p<0.05$]. Further analysis revealed that Control+Veh, Control+DHF and IMO+DHF animals spent more time in target area than in opposite ($p<0.05$, $p<0.05$ and $p<0.01$, respectively), but IMO+VEH did not showed a significant difference ($p=0.292$). Furthermore, the time spent in target area of Control+VEH and IMO+DHF was higher than the time of IMO-VEH group ($p=0.063$ (tendency) and $p<0.05$, respectively).



[**FIGURE 9**]. MWM performance of undisturbed animals administered with vehicle (Control+VEH), undisturbed animals administered with DHF (Control+DHF) and animals exposed to 2h of acute IMO and sacrificed after 2 days and administered with a vehicle (IMO+VEH) or DHF (IMO+DHF). (a) Training escape latency, (b) Training average swimming speed, (c) Training thigmotaxis *(**p<0.01 vs control+DHF group).* (d) Probe trial % of time spent *(*p<0.05 and **p<0.01 vs opposite; +p<0.05 and @p=0.063 vs IMO+VEH group).*

Means and S.E.M. are shown.

Conclusion

Although, synaptic protein analysis presented controversies, the drug prevented spatial memory impairment and LTP increase without effect of 7,8-DHF *per se*. The promising results of BDNF/TrkB pathway modulation in preclinical studies support the idea of a protective role of BDNF/TrkB pathway enhancement in PTSD and hence the value of the 7,8-DHF as a putative treatment for PTSD patients. Taken together, all these results suggest a protective role of 7,8-DHF.

GENERAL DISCUSSION

Does IMO really mimic PTSD symptoms ?

Are IMO-related synaptic plasticity effects causally related to behavioral changes ?

Is 7,8-DHF an effective treatment for PTSD ?

Conclusions

The present work studies the long lasting effects of a single exposure to a putative PTSD model, IMO, using multidisciplinary (physiological, behavioral, morphological, electrophysiological and molecular) approaches and also analyzes the role of BDNF/TrkB pathway in the observed effects. In order to prevent the electrophysiological alterations caused by IMO, we administered a TrkB agonist, 7,8-DHF, some hours after stress exposure to test the possibility of this compound to be effective as treatment after a traumatic experience.

Does IMO really mimic PTSD symptoms?

In DSM V, PTSD is classified as a trauma and stressor-related disorder, characterized by some prevalent symptoms: re-experiencing, avoidance, negative cognitions and mood, and hyperarousal. However, some human psychological symptoms are impossible to reproduce in animals. There are several PTSD models in the literature (Siegmund & Wotjak 2006, Stam 2007, Armario et al. 2008) however, we have chosen IMO because is the most severe stressor among those with an emotional component (Marquez et al. 2002) and also accomplishes several critical criteria as it has been extensively explained in the Introduction (section PTSD animal models). In this work we have obtained new data that can help to determine whether the IMO is a putative animal PTSD model.

Physiological consequences of IMO

We found that a single 2h IMO exposure induces long lasting decreases in body weight gain observed 2 days and also 8 days after exposure. This effect is in accordance with previous results from our lab which demonstrated also a marked reduction of food intake (Valles et al. 2000). Similar results have been obtained with another putative PTSD model such as social defeat (P. Meerlo 1997, Ruis et al. 1999). It is difficult to speculate about the meaning of this reduction of food intake as this does not appear to be related to reduced hedonic properties of food (Harris et al. 1998). The severity of IMO is also revealed by the increase in relative adrenal weight found 2 days after a single IMO, an increase similar to that observed 2 days after chronic IMO exposure. However, the lack of effect in absolute adrenal weight and the normalization of relative adrenal weight at 8 days after IMO, indicate that the adrenal increase was transitory.

Neuroanatomical consequences of IMO

It is still unclear whether a reduced HF volume is a consequence of trauma or it is a risk factor for developing PTSD (Pitman et al. 2012), although Gilbertson and collaborators (2002) study favors the second possibility. However, one of the most replicated consequences in human PTSD studies is HF volume abnormality (Pitman et al. 2012). Therefore, although a reduced HF volume might be a factor of vulnerability for developing the pathology after traumatic experiences, it is also possible that traumatic situations could potentiate HF abnormalities. Therefore, a putative animal model of PTSD might induce changes in HF volume. In this work we have designed a longitudinal MRI experiment that has allowed us to assess pre- and post-IMO HF volume, in order to determine whether HF reduction follows stress exposure.

In the 12 day period between the two measurements control animals showed an increase in brain and HF volumes as described in other studies (Lee et al. 2009, Valdes-Hernandez et al. 2011). Considering that brain volume shows a higher correlation with body weight (Valdés-Hernández et al. 2011), we have evaluated brain volume to demonstrate an effect of IMO on

whole brain growth. Animals exposed to IMO showed a reduction of both brain and HF volumes. The effect was found after both single and chronic IMO exposure, supporting the severity of IMO. However, the reduction of brain volume caused by a single IMO exposure was transient because 8 days later no differences with respect to controls were observed. This suggests that the short-term effect is likely to be due to a reduction of animal growth rate that eventually recovers.

When the HF volume corrected by brain volume was analyzed, both left and right HF volume increased in control animals. In contrast, chronic IMO induced a HF volume decrease in both left and right HF. A similar effect has been described after chronic restraint exposure (Lee et al. 2009) and it supports the severity of the IMO. Interestingly, we observed some laterality in the effect of a single IMO exposure on HF as compare to controls: the left HF volume decreased at 2 and 8 days after a single IMO exposure, whereas the right HF showed a mild increase at 2 and 8 days after single IMO as compare to the control group increase. Laterality in the effects of stress on the HF has been described at 74-88 days after a single foot-shock (Golub et al. 2011) and also in PTSD patients (Shu et al. 2013, Zhang et al. 2011), in all cases with a higher left HF reduction. In fact, a recent meta-analysis of the studies that compare left and right HF concludes that the left HF is smaller in PTSD affected group (Woon et al. 2010). However, there are controversies, with another meta-analysis supporting both left and right HF reduction in PTSD patients (Smith 2005). The mechanisms underlying the stress-induced changes in HF volume remain unknown. It has been proposed that chronic high levels of glucocorticoids can play a role by decreasing neurogenesis, neuronal arboritzation (Sapolsky 2000) and HF volume (Sousa et al. 1998). However, HF atrophy in animals exposed to a single traumatic event, such as IMO or other PTSD models (Golub et al. 2011) cannot be explained by glucocorticoids alone, because repeated glucocorticoids bursts appear to be needed to produce long lasting neurotoxicity. Consequently, other factors have to be involved in this process, such as excitotoxicity related to enhanced excitatory transmission or changes in the extracellular volume space.

Structural plasticity effects of IMO

Decreased HF volume after chronic stress involves structural and synaptic changes (Popoli et al. 2011, Sapolsky 2000). Therefore, the above discussed acute stress-induced decreases in HF volume could entail similar consequences, and may be associated with functional HF abnormalities.

The most accepted changes in the HF after chronic stress is the dendritic arboritzation shrinkage (McEwen 1999, Vyas et al. 2002), which has been related to prolonged glucocorticoid exposure (Sapolsky 2000, McEwen 2012). However, we found that a single IMO exposure did not modify either basal dendrite length or dendritic branching in DG or CA1. A similar lack of effect on dendrites has also been found in the DG after a single predator exposure when measured 16 days later (Adamec et al. 2012). The lack of effect of a single IMO may be well explained because a more prolonged glucocorticoid exposure is needed to decrease dendrite length and branching.

However, there are other stress-sensitive structural changes that are more directly related to synaptic transmission: spine density. Our results reported an increase in spine density in both the DG and the CA1 two days after single IMO. The effects in the DG were less persistent than in the CA1 where spines were still increased eight days after IMO. Sholl analyses results also reported this less persistent increase in the DG, whereas the CA1 was present since 20 to 140 μm from soma in the DG was limited to 20, 40, 140 and 160 μm from soma. In the present work

CA1 effects were studied in basal dendrites, whereas some previous studies focused on apical dendrites, but it can be taken into account that apical and basal CA1 dendrites received the same afferents. Schaffer already described, in 1892 the Schaffer collaterals and demonstrated that the axons innevate Stratum radiatum but also oriens (Szirmai et al. 2012). Recently, it has been described with anterograde techniques that 91% CA3 axons innervate both CA1 stratum oriens and radiatum pyramidal cells dendritic spines (Takacs et al. 2012). To our knowledge this is the first study describing a long lasting increase in spine density. An increase in spine density has been described in apical spines one day after a single session of foot-shocks (Shors et al. 2001, Dalla et al. 2009).

Studies of the effects of acute stress on spine plasticity in the next 24 h after stressors are controversial some studies showing increases in CA1 after IS-LH (Shors et al. 2001, Dalla et al. 2009), after fear conditioning exposure in CA1 (Sanders et al. 2012) and others decreases after multimodal combined physical/psychological stress in CA3 (Chen et al. 2010). Similarly, long-term effects are controversial. Studies of the LH-IS model described a decrease in spine density in DG and apical dendrites of CA3 and CA1 one week after exposure (Hajszan et al. 2009), whereas predator stress induced no effect in the DG sixteen days after exposure (Adamec et al. 2012). A possible explanation for these discrepancies could be a possible biphasic effect of IS-LH, exerting an increase in spine density (Shors et al. 2001, Dalla et al. 2009) followed by a decrease (Hajszan et al. 2009); however, these possibility is not supported by electrophysiology (See in section Effects of IMO on activity-related plasticity). Another hypothesis could be related with individual susceptibility, because animals exposed to predator stress and subdivided into high, medium and low affected groups (anxiety in EPM), presented disparities in DG spine density, medium and low response animals showing no effect and high responsive animals showing a decrease (Zohar et al. 2011).

IMO-induced increase in spine density can be explained by a combination of two processes. Initially, stress would induce an immediate decrease in spine density (Chen et al. 2010, Magariños et al. 2011, Sanders et al. 2012), resulting in impaired activity-related plasticity and LTP (Kim et al. 1996, Li et al. 2005, Artola et al. 2006, Maggio & Segal 2011). Neurons would attempt to compensate this deficit in activity related plasticity by increasing the amount of spines. This hypothesis is supported by data showing that CA1 neurons are more spiny after an inactivation by tetrodotoxin (Arendt et al. 2013), by glutamate receptor antagonists, sodium and calcium channel blockers and/or a nominally calcium-free medium with high magnesium (Kirov & Harris 1999).

Effects of IMO on activity-related plasticity

It has been extensively reported that stressors can modulate LTP (See Introduction, section LTP, LTD and stress). Therefore, we designed an experiment to assess whether IMO could modulate LTP in the same way as it alters structural plasticity. This experiment was conducted in brain slices and not *in vivo*, due to technical limitations and the possibility brain slices offer to use pharmacological tools. We only analyzed the effects that occurs 2 days post-IMO because it was at that time that IMO induced the highest increase in spine density. We demonstrated that LTP induction in CA1 neurons was enhanced in IMO as compare to control rats. This enhanced LTP was somewhat unexpected because one might assume that the new spines were silent (and did not contribute to LTP). Young, developing, synapses have only NMDA receptors (Kirov & Harris 1999), and are AMPA receptors which are critical for LTP expression (Malinow & Malenka

2002). This paradox could be explained by recent results in that Arendt and collaborators (2013) have demonstrated that inactivation of a neural network with tetrodotoxin is able to enhance LTP as a consequence of the prior induction of silent synapses. They proved that LTP induction produces a high depolarization state, dislodging magnesium and activating all the NMDA receptor in both silent and active synapses. This in turn would induce AMPA receptor insertion into the silent synapses thus turning them active and contributing to increase LTP. Our results of IMO-enhanced LTP are compatible with the observed increase in spine density.

Intriguingly, our results are in contrast to the LTP impairment described 2 days (Shors et al. 1997) and 4 weeks (Ryan et al. 2010) after exposure to the LH-IS model. However, the LH-IS present some controversies because Shors et al (1997) at 96 h reported similar LTP levels between control and stressed rats. These controversies of LH-IS model are also seen in spine density (See section Structural plasticity effects of IMO). Thus, LH-IS consequences in plasticity remain unknown. We suggest that pain associated to LH-IS could induce another type of effect in HF circuitry.

Changes caused by stress in structural and activity-related plasticity are more controversial than those of HF volume or physiological variables, likely because of the complex influence of stressor type, stressor intensity, time, animal sex, strain and experimental procedures (Dalla et al. 2009, Orlowski et al. 2010, Magariños et al. 2011). The present observations of an increase in spine density and LTP associated with a decrease in HF volume are at first sight contradictory. However, both HF volume decrease and LTP increase has been related to memory impairment. Thus, LTP increase have been related with memory impairment due to a saturation of LTP diminishing the plasticity required for memory formation (Martin & Morris 2002, Moser et al. 1998, Pineda et al. 2004). In addition, HF volume decrease (Elzinga et al. 2002, Bremmer et al. 2007) has been associated to verbal memory impairment in PTSD patients. In this regard, as later discussed, IMO also induces impairment of a HF-dependent task.

Synaptic proteins changes induced by stress

After being demonstrated changes at anatomical, structural and physiological levels, we searched for the possible molecular substrate of those changes. With these aim, we analyzed several plasticity-related proteins in critical brain areas: PFC, HF (DG, CA3, CA1) and amygdala. Chronic IMO exposure was used as a reference. Therefore, we will firstly analyze chronic IMO effects and then single IMO effects. In order to discuss the wide range of proteins, analyzed, they will be grouped in proteins related to synaptic activity (HCN1, HCN2, GirK1, GluN1, GluA1, pCaMKII), modulators of structural plasticity (BDNF, TrkB, Arc, NCAM) and glia-related proteins (GFAP and Iba1).

Chronic IMO did not modify any synaptic activity-related proteins, but increased Iba1 levels in PFC, in accordance with previous results using chronic restraint (Tynan et al. 2010, Hinwood et al. 2011, Hinwood et al. 2013). This increase could reflect a pro-inflammatory response, perhaps related to the neurotoxic effects of chronic stress. Stress-induced increase in Iba1 has been found to be glucocorticoids and NMDA receptor dependent (Nair & Bonneau 2006). Microglia function during chronic stress remains unclear. Microglia is involved both pro-inflammatory and anti-inflammatory processes and therefore, it cannot be determined whether this increase reflects a fighting against inflammation or a cause of it. Chronic stress neurotoxicity is compatible with the TrkB-T1 increase observed after chronic IMO in CA1, since TrkB-T1 appears to act as a dominant-negative receptors by forming non-functional heterodimers with TrkB-FL (Eide et

al. 1996). TrkB-T1 is over-expressed in stroke and its downregulation protects against excitotoxicity (Vidaurre et al. 2012).

The acute stress-induced changes in activity-related plasticity led us to think about the possibility that proteins related to glutamatergic modulation could be involved in stress effects. A single IMO exposure increased HCN1 levels eight days after IMO. Blockade of HCN1 channel in PFC has been demonstrated to produce working memory enhancement (Wang et al. 2007a) and a forebrain restricted KO did not have any change in LTP in the HF Schaffer collaterals but shows enhanced LTP in the perforant pathway (Nolan et al. 2004). This suggests a role for HCN1 channels in HF memory impairment which is different from to LTP enhancement caused by IMO. Unfortunately, LTP induction in the presence of a HCN1 channel inhibitor (ZD7288) did not reach reliable data, because LTP did not stabilize after its induction (data not shown).

The levels of pCaMKII increase in DG two days after IMO, and similar increases have been demonstrated immediately after acute restraint in the HF (Suenaga et al. 2004). However, these results were no replicated in 7,8-DHF experiment in which no effect of IMO were found in DG suggesting that is not a consistent result. The lack of effect of pCaMKII in CA1 is at first sight contradictory with LTP increase described in IMO, since CaMKII genetic deletion or pharmacological blockade impairs LTP induction (Malenka et al. 1989). However, this lack of effect could be related support the IMO-related inactivation of HF circuitry and the above commented effect of LTP induction on silent synapses.

IMO did not alter GluN1 and GluA1 levels. However, IMO effects cannot be excluded because the analysis of synaptosomes rather than crude extract analysis could report changes, since stress have been reported to modify NMDA and AMPA receptor endocytosis-exocytosis (Conboy & Sandi 2010, Yuen et al. 2011, Wong et al. 2007). We did not observe any effect of stress in HCN2 and GirK1 and there are no prior stress studies on those proteins.

BDNF/TrkB pathway abnormalities have been considered to be associated with PTSD (Andero & Ressler 2012). In addition, we have prior evidences that negative IMO effects are prevented by activation of this pathway (Andero et al 2011, 2012). We then analyzed BDNF and TrkB protein levels after IMO exposure. BDNF protein levels increased at two and eight days and TrkB-FL and TrkB-T1 levels at eight days after a single IMO session in CA1. Although, astrocytes predominantly express TrkB-T1 (Rose et al. 2003), the observed TrkB-T1 increase is unlikely to be due to an increase in astrocyte number as IMO did not modify GFAP levels.

In the literature, decreases in BDNF levels have been usually reported up to 24h in DG, CA3 and CA1(Aldard & Cotman 2004, Pizzarro et al. 2004), at contrary only one studies have demonstrated a transient increase of BDNF protein levels after one IMO session (180 min) before returning to control levels at 5 h post-IMO (Marmigere et al. 2003). Long-lasting effects of stress on BDNF have been studied in two works presenting decreased levels at 24h and 72 after LH-IS in the whole HF (Greenwood et al. 2007) and seven days after predator odor in CA1 (Kozlovsky et al. 2007). It seems that BDNF levels correlate to spine density levels and even with LTP potentiation after stress, because LH-IS, despite controversies, present a decreased LTP (Shors et al. 1997, Ryan et al. 2010), spine density (Hajszan et al. 2009) and BDNF (Greenwood et al. 2007) after several days after stress; similarly, in predator stressor the BDNF (Kozlovsky et al. 2007) and spine decrease (Zohar et al. 2011) are present after one week of predator exposure. Thus our described BDNF increase correlate to spine density and LTP increase found after stress. We suggest that this stress-related decrease in BDNF levels later give rise to a compensatory mechanism with increased levels two days and up to eight days after IMO, whereas in another stressors

become decreased for long periods. The opposite effects between IMO and LH-IS could be explained because LH is a stressor with a high systemic component and as has been commented above, the pain would induce an opposite effect in HF circuitry. On the other hand, in predator odor both the decrease in BDNF and spine density was observed only in the animals whose behavior was extremely disrupted by exposure to predator odor, suggesting that this effect are not purely a stress consequence and are related to the extreme response to stress (Kozlovsky et al. 2007). Given that stimulation of BDNF/TrkB pathway in CA1 neurons increases spine density (Tyler & Pozzo-Miller 2001), modifies their morphology (Tyler & Pozzo-Miller 2003) and mediates LTP-dependent spine increases (Tanaka et al. 2008), we hypothesized that this BDNF increase at two and eight days after IMO could be responsible for the structural plasticity changes found after IMO.

The IMO-induced increase in the TrkB receptors are in accordance with effects of SPS, another putative animal model of PTSD, which resulted in increased TrkB seven days after exposure in the HF (Takei et al. 2011). It is of note that both isoforms of TrkB receptor are involved in structural plasticity. TrkB-FL signals through MAPK/ERK, PI3K-Akt and PLCγ–PKC pathways to enhance synaptic plasticity and structural plasticity (Fenner 2012). Although the most characterized action of TrkB-T1 is to be a dominant negative inhibitor of TrkB-FL (Eide et al. 1996), it also plays a critical role in structural plasticity by a BDNF-independent filopodia outgrowth in astrocytes and neurons, the is still unknown but is mutually inhibitory with TrkB-FL (Fenner 2012). TrkB-T1 overexpresion in culture HF neurons has been found to enhance formation of filopodia via $p75^{NTR}$ activation (Hartmann et al. 2004). Taken together, all these data suggest that IMO could modulate synaptic plasticity through the BDNF/TrkB pathway.

We did not observe any effect of IMO on Arc levels in the HF and this is in accordance with previous results from our group that did not detected activation of *arc* expression in the HF immediately after exposure to IMO and other stressors, whereas activation was detected in several brain areas (Ons et al. 2004). Similarly, other studies did not find any immediate effect of restraint in CA1 (Mikkelsen & Larsen 2006, Caffino et al. 2011). Interestingly, after forced swim, *arc* expression was not induced in the dorsal hippocampus, but it was in the ventral hippocampus (Luoni et al. 2014), supporting a differential role of those hippocampal areas. The lack of effect of IMO in NCAM levels is in contrast to the reduction found 24 h after a single exposure to predator stress in synaptosomal preparations of the HF, of the PFC, with no effect in amygdala (Sandi et al. 2005). These discrepancies could be due to NCAM stress-related changes being restricted to the synaptic cleft, and therefore, the analysis of synaptosomes could be more appropriate. To our knowledge, no long-lasting effects of stress on NCAM have been reported.

In addition to neurons, others cells in the CNS modulate stress-related synaptic plasticity. Microglia and astrocytes play an important role in the modulation of post-stress pro-inflammatory state and synaptic plasticity (Frank et al. 2007, García-Bueno et al. 2008, Chung & Barres 2011). Thus, we analyzed the effects of a single IMO in Iba1 and GFAP levels. Similarly to chronic stress, an acute IMO increased Iba1 levels in CA1 at eight days but not two days after IMO. Thus, we demonstrated for the first time that a single IMO induces long-term increase in Iba1. Hitherto Iba1 increases have been only described in chronic stress (Hindwood et al. 2011, 2013). We suggest that this microglial Iba1 overexpression could be related to a homeostatic regulation of synaptic plasticity rather than to a pro-inflammatory state in CNS, since in acute stress there is not chronic glucocorticoids exposure to exert an inflammation-like process. We hypothesized that microglia participates in the pruning of spines previously increased by stress. It has been

reported that synaptic pruning by microglia is glucocorticoid-dependent (Liston & Gan 2011) and necessary for DG adult newborn neurons integration (Ekdahl 2012). A role of microglia in homeostatic regulation of synaptic plasticity is compatible with the lack of effect of acute and chronic IMO in GFAP levels, which reveals that to produce a pro-inflammatory state more IMO exposures are needed.

To summarize, Synaptic protein analysis suggest a differential effect of a single and chronic IMO. Acute IMO-induced increases in BDNF and Iba1 are compatible with the increases in spine density and LTP and might contribute to modulate structural changes. Conversely, chronic IMO-induced increases in Iba1 and TrkB-T1 in the PFC and the HF, respectively, might be consistent to chronic stress-induced neurotoxicity as reflected in dendrite and spine shrinkage (McEwen 1999, Sapolsky 2000). In interpreting the results regarding IMO-induced synaptic proteins changes, it should be taken into account technical limitations. Changes in pCaMKII in DG and TrkB-FL and TrkB-T1 changes in CA1 were not replicated in the experiment using 7,8-DHF, suggesting no consistent effects. In addition, due to the lower protein obtain after microdissection, we did not assess the more specific approach of using synaptosome preparations.

In addition to previous data from our lab (see IMO section in the Introduction), the present results added new data in support of the hypothesis that IMO appear to mimic some PTSD-like alterations. IMO effects on physiological variables, HF volume, spine density and LTP are similar or can be related to those found in others PTSD models or in patients. However, the above discussed structural, biochemical and functional changes, do not necessarily implies a casual HF abnormalities. In other words, are IMO-related effects in synaptic plasticity associated with behavioral changes?

Are IMO-related synaptic plasticity effects causally related to behavioral changes?

Stress has a relevant impact on learning and memory. Importantly, high stress levels facilitate implicit/fear conditioning learning (Sandi 2011, Andero et al. 2011, Daviu et al. 2012) whereas impairs spatial/explicit memories. Specifically, acute stress exerts biphasic effects on spatial/explicit memory, with mild stressors improving memory (Sandi 2011) and severe stressors impairing it (de Quervain et al. 1998, Andero et al. 2012). In this regard two of the most described symptoms of PTSD are a resilient memory of the traumatic situation that difficult extinction and an explicit memory impairment (DSM-IV-TR 2000). Thus, it is reasonable to assume that traumatic stressors should induce spatial memory impairment and such effects have been described in some animal PTSD models (Khoda et al. 2007, Andero et al. 2012, Kaouane et al. 2012).

In the present study, we studied spatial learning and memory in the MWM using one day massive training procedure. During the learning trials the IMO exposed animals presented apparently normal performance as compare to controls because no differences were found in the latency to reach the platform, in addition, short memory test evaluated between trial 4 and 5 did not reach differences. Interestingly, the MWM probe trials control and IMO 8d animals spent more time in the area surrounding the position of the hidden platform than in the opposite, but IMO 2d did not show a significant difference. Furthermore, control and IMO 8d groups spent more time in the target area than IMO 2d group, this suggest that after two days IMO impaired

memory. The lack of IMO effect on the latency to reach the platform during learning trials and in the short memory test suggest that the deficit was not due to learning impairment, but it was restricted to long-term (24h) memory. Moreover, IMO groups did not present either sensorimotor skills deficits measured by swimming speed or enhanced anxiety measured by thigmotaxis (trail 9). Interestingly, no effect of IMO was found in reversal learning (the last 4 trials). The reversal learning is a PFC-dependent task, since PFC lesion impairs reversal learning (Cools et al. 2002). Although this is may be compatible with the lack of effect of acute IMO in PFC synaptic plasticity. However, our lab has observed that a single IMO impaired a set-shifting task that assessed cognitive flexibility (Garrido et al., unpublished). This controversy could be explained by the disparities of both tasks.

Our results are consistent with former results from our lab also using IMO, which showed spatial memory impairment in the MWM (Andero et al. 2012). The present MWM procedure was more demanding procedure than in the latter study because we have used massive training on a single day instead of the distributed training throughout several days previously used (Andero et al. 2012). However, surprisingly, in present study IMO 8d animals did not present memory impairment. We suggest that despite a higher number of training days consolidate better the spatial learning than a single day; the sub-chronic exposure to MWM trials (forced swim) could maintain the IMO effects. Since both MWM procedures were conducted with water temperature at 24ºC and can be considered a mild stressful situation inducing an increase of plasma corticosterone immediately after training (Sandi et al 1997).

Spatial memory impairment could be causally related to enduring changes in synaptic plasticity. However, the precise relationship remains unclear. Structural and activity-related consequences of a single IMO appear to be opposite to those expected to impair a HF-dependent task. However, the HF volume decrease and the literature-described memory impairment associated with exaggerated LTP are compatible with the behavioral changes. More precisely, saturation of LTP has been demonstrated to impair memory formation because exaggerated LTP reduces plasticity required for new memory formation (Moser et al. 1998, Martin & Morris 2002, Pineda et al. 2004).

The idea that PTSD causes long-term memory impairment is widely assumed (Elzinaga et al. 2002). In this regard, Diamond and collaborators (2007) have proposed the "Temporal dynamics model" to provide a neurobiological basis for stress-induced amnesia and traumatic memories. They postulate that stress exposure for a brief period of time (minutes) initially facilitates LTP induction, but this is followed by a long period of LTP impairment (hours). Therefore, they suggest that the first phase facilitates those emotional memories related to traumatic memory consolidation. In the second phase the HF shift to a "flashbulb memory" mode that is related to the new memories impairment and the re-experiencing found PTSD patients.

This shift from an initial high neuroplasticity state to a low neuroplasticity state appears to be controversial to our results showing increased spine density and LTP. Our hypothesis to solve this controversy is an extension of the "temporal dynamics model". It emphasizes the long-term consequences of stress, which add the homeostatic synaptic plasticity process proposed by Arendt *et al.* (2013). It has been shown that stressors induce a brief facilitated plasticity phase in the HF. For instance, swim stress facilitated the induction of LTP 15 min and 1h after stress but not 4 h later (Ahmed et al. 2006). We suggest that in the second phase the high glucocorticoids concentration modify this initial facilitation. High glucocorticoids levels induce a decrease of BDNF (Smith et al. 1995, Ueyama et al. 1997, Adlard et al. 2004, Murakami et al. 2005, Lee et al.

2008), an inhibition of glutamate uptake (Yang et al. 2005) and an increase in glutamate release (Venero and Borrell 1999). This release-uptake imbalance would increase extracellular levels of glutamate, glutamate spillover and activation of NR2B NMDA extrasynaptic receptors, producing a mild excitotoxic-like process. This NR2B activation would produce a brief (within hours) spine shrinkage decreasing the spine density (Nagerl et al. 2004, Okamoto et al. 2004, Zhou et al. 2004, Wang et al. 2007b, Chen et al. 2010, He et al. 2011). This latter phase could be related to stress-induced LTD enhancement and LTP impairment (Wong et al. 2007b, Sandi 2011, Riedemann et al. 2010, Popoli et al. 2011), thus blocking network activity.

This blockade of network activity would initiate a non-Hebbian plasticity process, named homeostatic plasticity, in that neurons increasing synaptic strength and spine density to compensate the lack of activity (Kirov & Harris 1999, Arendt et al. 2013). This new synapses, containing only NMDA receptors, would be silent. However, the experimental procedure used to induce LTP would lead to the insertion of AMPA receptors on those silent synapses, transforming them in active and therefore enhancing LTP (Arendt et al. 2013). Therefore, few days after stress the homeostatic plasticity process would trigger a development-like process acting as a compensatory mechanism. This process may consist of a BDNF-dependent synaptogenesis that would increase spine density, in order to reconnect the HF neuronal network. However, it cannot be rule out that these effects are due to a recovery process from stress rather than to a PTSD-like process. Since after a stress-free period the dendrite atrophy induced by chronic stress in HF reverted (Luine et al. 1994, Sousa & Almeida 2012). We hypothesized that these changes would return to the normal state by pruning and refining key connections. In this regard, microglia might prune the previous spine increase, what is compatible with the observed increase in Iba1 levels. In summary we postulate that stress may induce time-dependent dynamic process, which derived in an initial disconnection and a further reconnection of the HF neural network.

Is 7,8-DHF an effective treatment for PTSD?

Several treatments have been proposed for PTSD; however, none of them has been totally successful. In fact, antidepressants, the most common PTSD treatment, did not show strong evidence for effectiveness either alone or combined with psychotherapy (Hetrick et al. 2010, Steckler & Risbrough 2012, Andero & Ressler 2012). Therefore, the development of novel PTSD treatments is required.

BDNF/TrkB pathway is highly related to synaptic plasticity modulation (Yamada et al. 2002, Tapia-Arancibia et al. 2004, Lu et al. 2008, Leal et al. 2014, Panja & Bramham 2014). In addition, recombinant BDNF has been demonstrated that rescues LTP and the BST in BDNF KO mice (Patterson et al. 1996). Moreover, there is a growing interest in the role of BDNF/TrkB pathway in psychiatric disorders (Calabrese et al. 2009, Frielingsdorf et al. 2010). However, the plasmatic levels of BDNF, which present a positive correlation with brain levels in rodents (Karege et al. 2002), has given controversial results in PTSD patients: some reported increase (Hauck et al. 2010, Matsuoka et al. 2013), and others decreases (Dell'Osso et al. 2009). Similarly, animal research related to a possible role of BDNF/TrkB pathway in animal PTSD models has also given controversial results. Decreases in BDNF levels have been usually reported up to 24h after restraint and social defeat, respectively, in DG, CA3 and CA1 (Aldard & Cotman 2004, Pizarro et al. 2004). On the contrary, Marmigere and collaborators (2003) have reported a transient increase of BDNF protein levels after one IMO session (180 min) before returning to control levels

at 5 h post-IMO. In the present work, we observed an increase of BDNF levels at 2 and 8 days after IMO exposure.

REgarless of the controversies on the effects on BDNF levels, we hypothesized that activation of BDNF/TrkB pathway might be beneficial (Andero et al 2011, 2012, Andero & Ressler 2012). We opted for the administration of the TrkB agonist 7,8-DHF, a naturally occurring flavonoid, which presents better pharmacokinetic properties and higher TrkB binding affinity than BDNF, and it has been proposed as a powerful tool for the treatment of neurological diseases (Jang et al. 2010). The 7,8-DHF administration before fear extinction improved extinction in stress-naïve animals, particularly in those previously exposed to IMO some days before conditioning, a procedure that by itself impaired extinction (Andero et al. 2011). Similarly, the administration of 7,8 DHF 2 h prior to or 8 h after IMO prevented the explicit memory impairment observed several days after IMO in the MWM (Andero et al. 2012). In an extensive work, Blugeot and collaborators (2011) exposed animals to combination of stressors (4 days of chronic social defeat and 4 weeks later 3 weeks of chronic mild stress) and classified animals in vulnerable and non-vulnerable depending on the serum BDNF levels after social defeat. Only vulnerable animals presented a decrease in spines density, dendrite length, cell proliferation, HF volume and dendritic intersections. Importantly, the 7,8-DHF administration prevented chronic stress effects in vulnerable group with no effect in the other. It is of note that these effects were achieved by chronic intracerebroventricular administration during all the chronic stress procedure whereas in the other two works from our lab 7,8-DHF was administered acutely and by systemic injections.

In the present work we administered a subcutaneous injection of 7,8-DHF 8h after IMO termination in order to have a therapeutic window after stress exposure. Then we assess behavioral, electrophysiological and molecular consequences two days after IMO. This post-IMO time was chosen because this was the most affected time in prior studies. The MWM probe trial revealed that IMO+VEH did not present differences in the time spent in the target area as compare to the opposite, whereas Control+Veh, Control+DHF and IMO+DHF animals spent more time in the target area. Thus, the drug prevented IMO-induced spatial memory impairment without affecting stress-naïve animals. Again, no group presented sensorimotor skills deficits or altered anxiety. These results are in accordance with our previous results preventing long-term IMO-induced spatial memory impairment in the MWM by 7,8-DHF (Andero et al. 2012). Therefore, activation of the BDNF/TrkB pathway is able to prevent IMO-induced spatial memory deficit. The 7,8-DHF has also been used to recue aging effects on synaptic plasticity (Zeng et al. 2011) and to facilitate LTP induction (Li et al. 2011). Thus, it is reasonable to assume that this effect could be related to the rescue of IMO-induced synaptic plasticity changes via BDNF/TrkB pathway.

The apparent link between 7,8-DHF rescue of behavioral deficit and the activity-related plasticity was clarified in the next experiment. We evaluated whether 7,8-DHF was able to prevent activity-related plasticity. 7,8-DHF reverted the LTP increase found in IMO exposed animals, without affecting the other groups. This is the first time that it is reported that 7,8-DHF prevents stress-induced changes in LTP and the results suggest a causality between changes in LTP and spatial memory impairment. However, memory is a process that requires LTP and LTD (Neves et al. 2008) and both LTP (Kim et al. 1996, Li et al. 2005, Kohda et al. 2007, Maggio & Segal 2011) and LTD (Chaouloff et al. 2007, Kim et al. 1996, Yang et al. 2005, Maggio & Segal 2011) are affected by stress. Therefore, we decided to evaluate the effect of IMO and 7,8-DHF administration on LTD. Surprisingly, no effect of stress or drug was found. This is in contrast to the

general idea that LTD is enhanced by stress; however, this effect has been usually described immediately after stress. In the long-term there is only one study, which found that SPS exposed rats presented a reduction of LTD one week after stress (Khoda et al. 2007). It is possible that LTD is enhanced after a more prolonged period of time between IMO exposure and LTD testing. As discussed above, we have hypothesized that new formed spines are silent. If this is true, one might predict that new spines are not detectable by BST because BST stimulation cannot induce enough depolarization to dislodge the Mg^{2+} from NMDA receptor (Arendt et al. 2013). With this aim, we analyzed the effects of BST. We did not observe any change after IMO, confirming that new synapses are silent.

In order to related IMO-induced biochemical changes with altered functioning, we administered 7,8-DHF 8h after IMO and assessed in CA1 and DG the same proteins as in the previous experiments. Moreover, we included several new ones: VGF, synaptophysin, PSD95. VGF is an inducible neuropeptide and one of the most induced mRNA after NGF and BDNF administration (Levi et al. 2004). Only two groups have tested the effects of stress on VGF. The administration of a VGF peptide TLQP-21 reduced acute restraint effects on plasma adrenaline and noradrenaline and its chronic administration increased depressive behavior in a mouse model of chronic subordination stress (Razzoli et al. 2012). Moreover, chronic stress has been found to increase TLQP-21 levels in the intermediate lobe of the pituitary gland (Tokizane et al. 2013). Synaptophysin is the major resident protein of the vesicle and it is related to learning and memory (Schmitt et al. 2009). Several studies have demonstrated a decrease of synaptophysin protein levels in the HF 24 h after chronic unpredictable stress (Briones et al. 2012) and thirty days after a single foot-shock (Herrmann et al. 2012). However, seven days after a single predator exposure synaptophysin levels were not modified in the PFC and the HF, but they were increased in the amygdala (Campos et al. 2013), in contrast to the decrease observed after chronic unpredictable stress (Luo et al. 2013). We used synaptophysin to determine changes in the presynaptic region. PSD 95 is one of the most common proteins of PSD. It binds to several proteins of the PSD including kinases, glutamate receptors, microtubule-associated proteins and cell adhesion molecules (Hata & Takai 1999). It has been related to synaptogenesis (Rao et al. 1998) and synaptic plasticity (Beique & Andrade 2002). Exposure to a single restraint (Yang et al. 2008) or odor stress (Zohar et al. 2011) increased HF PSD 95 levels 24 h and seven days after stress. We used PSD 95 to determine changes in the postsynaptic region. Although we are aware that other brain regions are involved in PTSD-like changes, we analyzed only CA1 and DG because they were the most affected areas in the previous experiment.

BDNF, HCN1 and Iba1 were the most affected CA1 proteins in the previous experiment. More precisely, in the previous experiment we reported an increase in HCN1 levels at eight days after IMO. However, in the present experiment only a tendency to increase was observed in the IMO+VEH group as compare to control, with no effect in the others groups. In addition, an increase in HCN1 levels was observed two days after IMO in the present experiment in contrast to the lack of effect in the previous one. The proteins from the previous experiment that were not affected by stress did not present changes in the present experiment neither by stress or drug. PSD95 and Synaptophysin did not reveal changes may be because synaptosomes analyses are needed. Conversely, the discrepancies found in pCaMKII presenting no effect in the present experiment and stress effect in the previous one may be due to the low number of animals per group animals, suggesting that more animals are needed to find differences. The same reason can be suggested to explain the discrepancies found in HCN1, which presented an increase eight days after IMO rather than in 2 days after IMO, as found in the present experiment.

In the present experiment BDNF increases at 2 days but not 8 days after IMO, perhaps because at 8 days after stress BDNF changes were less consistent. The 7,8-DHF did not modify its levels. This last result could be explained because the drug is acutely administered and at long-term its effects are dissipated. Since TrkB activation increases BDNF release, in a positive feedback loop (Cheng et al. 2011). The VGF decrease after IMO in CA1 appears to be controversial. Since the neuropeptide VGF is highly related to BDNF, in fact there is a positive feedback between them (Levi et al. 2004). We suggest that other stress-related factors than BDNF are modulating VGF in the present scenario. This descrease was prevented by the drug suggesting a protective role. However, in the DG both drug and IMO induced a VGF increase suggesting different consequences of stress in the DG and the CA1. The NCAM levels gave controversial results among the experiments because no effect was found in the previous experiment and an increase was found at eight days after IMO. The TrkB also presented controversies, in the previous experiment an IMO-related increase was showed whereas a stress-related decrease was found in the present experiment. Both controversies could be due to small changes presented by both proteins, consistent detection of the differences would need higher number of animals per group.

Finally, the increase in Iba1 levels previously observed was replicated in the second experiment. However, 7,8-DHF did not prevent the Iba1 increase, this could be related to the fact that stress on microglia are exerted at eight days and the drug is administered eight hours after stress suggesting that the 7,8-DHF did not prevent all the stress consequences.

Although the changes in synaptic proteins were not consistent, DHF administration prevented spatial memory impairment and LTP increase without effect in stress naïve animals. The promising results of the pharmacological modulation of the BDNF/TrkB pathway in preclinical studies give additional support to the idea of a protective role of BDNF/TrkB pathway in PTSD and hence the putative value of the 7,8-DHF as a treatment for PTSD patients. Nevertheless a main question arises: how does 7,8-DHF modify our proposed model about the long-term consequences of IMO?

We hypothesized that 7,8-DHF in addition to the already described neuroprotective effects (Jang et al. 2010, Chen et al. 2011, Devi & Ohno 2012, Uluc 2013), can prevent the stress-induced inactivation of HF network and the subsequent synaptic plasticity changes. Therefore, we suggest that the drug prevents the functional consequences of the described BDNF reduction immediately after stress (Smith et al. 1995, Ueyama et al. 1997, Adlard et al. 2004, Murakami et al. 2005, Lee et al. 2008). Despite our results support the possible therapeutic role of 7,8-DHF, there are important limitations to this support. We have not assessed the effect of 7,8-DHF on IMO-induced increases in spine density and the discrepancies found in regard to TrkB and other proteins should be clarified in future studies. Lastly, it would be of interest to evaluate effects of IMO at longer lasting times as well as and the IMO-induced changes not only on BDNF but on the activity of the BDNF pathway.

Conclusions

In the present work we have added new data to those previously reported from our lab (see IMO section in the Introduction) in support of the idea that IMO can be a putative animal PTSD model. Thus, using the three criteria proposed by Siegmund and Wotjak (2006), IMO accomplishes:

(1) **Face validity**: a single IMO induced long-term physiological changes, HF volume loss and spatial memory impairment, changes similar to those found in PTSD patients and in some other animal PTSD models. IMO caused one of the most extensively described anatomical consequences of PTSD, HF volume loss, which is also found in some PTSD animal models (Golub et al. 2011).

(2) **Predictive validity**: We prevented spatial memory impairment and LTP increase by the administration of a TrkB agonist (7,8-DHF). However, it should be noted that the 7,8-DHF cannot be considered a clinically validated drug for PTSD, although the modulation of BDNF/TrkB pathway has been proposed as a putative therapy for PTSD (Andero & Ressler 2012).

(3) **Construct validity**: spatial memory impairment found after IMO mimics a described deficit of PTSD patients. Moreover, the plasticity-related changes, such as the increases in spine density and LTP, are compatible with the hyperactivation of the HF described in some PTSD, although it has to be note that there are studies reporting HF hypoactivity (Hudges & Shin 2011).

In addition, we have obtained some evidence that IMO-induced HF abnormalities are related to its effects on synaptic plasticity. We have proposed a neurobiological model based on the "temporal dynamics model" and homeostatic plasticity processes occurring after HF network inactivation. We suggest that IMO causes a transiently reduced glutamate clearance capacity that leads to excitotoxic-like states, which in turn triggers a network-activity blockade. This lack of activity induces a homeostatic plasticity process modifying existing synapses and promoting synaptogenesis that can underlie HF abnormalities.

Finally, our work illustrates the putative value of 7,8-DHF as an effective treatment for PTSD. We have demonstrated that 7,8-DHF prevented the behavioral and electrophysiological effects of IMO and some of the molecular IMO-induced changes. Hence providing some evidence for IMO predictive validity and suggesting 7,8-DHF to be a putative treatment for PTSD.

CONCLUSIONS

(1) **A single exposure to a severe predominantly emotional stressor induced long lasting physiological and HF volume changes** 2 days and 8 days post-IMO which are similar to those found in PTSD patients.

(2) **We demonstrated that HF volume decrease is not present in the first MRI and is only found after IMO,** suggesting that this decrease is a consequence of IMO and not a risk factor.

(3) **Previous exposure to IMO increased spine density** 2 and 8 days post-IMO, being a structural reflection of LTP enhancement observed 2 days after IMO.

(4) **IMO induced significant changes in HF synaptic proteins**, being CA1 the most affected area. On contrary PFC and amygdala did not present changes.

(5) **The plasticity-related factors such as spine density, LTP, BDNF, HCN1 and Iba1 increase seems that follow a development-like process** trying to compensate the detrimental effects of the initial trauma.

(6) **IMO elicited spatial memory impairment 2 days after IMO but not 8 days conducted in MWM.** IMO did not induce any underlying deficit in learning, short-term memory, anxiety or sensorimotor function.

(7) **We demonstrated that IMO effects on synaptic plasticity are causally related to spatial memory impairment** and they can be considered a neurobiological substrate for PTSD-related memory impairment.

(8) **Previous electrophysiological, behavioral and some molecular IMO-induced changes are reverted by a TrkB agonist** (7,8-DHF) administration 8h post-IMO, without effect of the drug *per se*.

(9) **7,8-DHF protective effects suggest that BDNF/TrkB pathway enhancement is a new target in PTSD**. For this reason, 7,8-DHF could be considered as a putative treatment for PTSD.

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