CENTRO DE INVESTIGACIÓN Y DE ESTUDIOS AVANZADOS DEL INSTITUTO POLITÉCNICO NACIONAL

UNIDAD ZACATENCO

DEPARTAMENTO DE FARMACOLOGÍA

Participación de la serotonina en la secreción de corticosterona inducida por estrés: investigación en un modelo de estrés crónico en ratas

Tesis que presenta

Neeshu Saroj

Para obtener el grado de

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Director de Tesis

Dr. José Antonio Terrón Sierra

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Involvement of serotonin in stress-induced corticosterone secretion: investigation in a model of chronic stress in rats

Thesis presented by

Neeshu Saroj

To obtain the degree of

Doctor of Science in Pharmacology

Director of Thesis

Dr. José Antonio Terrón Sierra

Mexico City December 2019

DECLARATION

I hereby declare that the PhD project work entitled “Involvement of serotonin in stress-induced corticosterone secretion: investigation in a model of chronic stress in rats” is an authentic work carried out by me at CENTRO DE INVESTIGACIÓN Y DE ESTUDIOS AVANZADOS DEL INSTITUTO POLITÉCNICO NACIONAL, DEPARTAMENTO DE FARMACOLOGÍA, UNIDAD ZACATENCO, under the guidance of Dr. José Antonio Terrón Sierra for fulfillment of the official requirements to obtain the degree of Doctor of Science in Pharmacology

Neeshu Saroj

PhD student

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Abbreviations

AC	Adenylate cyclase
ACCD	Aromatic amino acid decarboxylase
ACTH	Adrenocorticotropic hormone
ANS	Autonomic nervous system
AVP	Arginine vasopressin
BACE1	Beta-site amyloid precursor protein cleaving enzyme 1
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
CORT	Corticosterone
CRH	Corticotropin-releasing hormone
CTRL	Control
CRS	Chronic restraint stress
CREB	cAMP response element-binding protein
DAB	Diaminobenzidine
EDTA	Ethylene-diamino-tetraacetic acid
GAS	General Adaptation Syndrome
GR	Glucocorticoid receptor
GPCR	G-protein-coupled receptor
5-HT	5-Hydroxytryptamine
5-HIAA	5-hydroxyindolacetic acid
HPA	Hypothalamic-pituitary-adrenal
HPLC	High Performance Liquid Chromatography
IML	Intermediolateral cell column

IP3	Inositol 1,4,5-trisphosphate
LAG	Left adrenal gland
LC	Locus coeruleus
mRNA	Messenger RNA
NFAT-1	Nuclear factor of activated T-1
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
pCPA	p-chlorophenylalanine
PLC	Phospholipase C
PPNAD	Primary pigmented nodular adrenocortical disease
PVDF	Polyvinylidene fluoride
PVN	Paraventricular nucleus
RAG	Right adrenal gland
RT-PCR	Reverse transcription-polymerase chain reaction
SAM	Sympatho-adrenomedullary
SDS	Sodium dodecyl sulfate
SRD	Stress related disorders
TBS	Tris-buffered saline
TPH	Tryptophan hydroxylase
VEH	Vehicle

INDEX

INDEX OF FIGURES

Abstract

Chronic restraint stress (CRS) has been shown to magnify acute stress-induced corticosterone (CORT) secretion in rats through a mechanism involving strong 5-HT-like immunoreactivity in adrenocortical cells involved in steroidogenesis along with increased 5-HT content and turnover in the adrenal (AGs). Since hydroxylation of tryptophan by the enzyme tryptophan hydroxylase (TPH) represents the limiting step in the biosynthesis of 5-HT, it was hypothesized that exposure to CRS might induce increased expression and activity of TPH in the AGs, particularly in adrenocortical cells involved in glucocorticoid secretion. On the basis of the above, the purpose of the present study was to investigate the effect of CRS on expression and activity of TPH in AGs and determine the effect of TPH inhibition with *para*-chlorophenylalanine (pCPA) on TPH expression and activity as well as on acute restraint-induced ACTH and CORT secretion. Male Wistar rats were submitted to either home cage control (CTRL) conditions or CRS (20 min/day) for 14 days. These animals were then subdivided into several subgroups to receive vehicle (VEH; 1 ml/kg, i.p.) or pCPA (150 mg/kg, i.p.) for 4 consecutive days (from days 9 to 12 of CTRL and CRS treatments. On day 15, CTRL and CRS animals were processed for collection of tissue samples (AGs and brain) for TPH immunohistochemistry assays in AG sections, Western blot and RT-PCR experiments in whole AGs for TPH protein and mRNA levels measurements, respectively, and TPH activity through measurement of 5-HT and 5-hydroxy-indolacetic acid (5-HIAA) concentrations in AGs and dorsal raphe nucleus by a fluorometric HPLC method. Exposure to CRS induced strong TPH-2-like immunoreactivity (TPH-2-LI) in the *zona glomerulosa* and the outer and inner *zona fasciculata/reticularis* of the adrenal cortex; by contrast, adrenocortical TPH1-like immunoreactivity (TPH1-LI) in CRS animals was modest or absent. This effect of CRS predominantly occurred in adrenals of the left side. In keeping with

these observations, CRS exposure induced a significant increase of TPH-2 but not of TPH-1 protein and mRNA levels in left adrenal glands only. Furthermore, a significantly increase of 5-HT and 5-HIAA levels and turnover (5-HIAA/5-HT ratio) in adrenal glands was detected in AGs from CRS animals. Pretreatment with pCPA significantly inhibited adrenocortical TPH-2-LI as well as TPH2 protein and mRNA levels in left adrenals from CRS animals; pCPA also decreased 5-HT and 5-HIAA concentrations and 5-HT turnover. Finally, the magnified restraint-induced CORT secretion in chronically restrained rats was prevented by pCPA pretreatment thus implying a functional role of TPH (most likely TPH2) in exacerbated restraint-induced CORT secretion. Pretreatment with pCPA restored the decreased restraint-induced ACTH response in CRS animals. These results unravel an important role of adrenocortical TPH-2 expression and function in acute stress-induced ACTH-dissociated CORT secretion in animals with a history of chronic stress and suggest selective TPH-2 inhibitors might have a therapeutic potential in the treatment of stress-related disorders associated with hypercortisolemia.

Resumen

El estrés crónico por restricción de movimiento (RMO) aumenta la secreción de corticosterona inducida por estrés agudo en ratas a través de un mecanismo que implica una fuerte inmunorreactividad a la 5-HT en células adrenocorticales involucradas en la esteroidogénesis, junto con un mayor contenido y recambio de 5-HT en las glándulas adrenales (GAs). Dado que la hidroxilación del triptófano por la enzima, triptófano hidroxilasa (TPH), representa el paso limitante en la biosíntesis de 5-HT, se planteó la hipótesis de que la exposición a estrés crónico por RMO podría inducir una mayor expresión y actividad de la TPH en las GAs, particularmente en las células adrenocorticales involucradas en la secreción de glucocorticoides. Por tanto, el objetivo del presente estudio fue investigar el efecto del estrés crónico por RMO sobre la expresión y la actividad de la TPH en las GAs. Asimismo, determinar el efecto de la inhibición de la TPH, mediante un pretratamiento con para-clorofenilalanina (pCPA), sobre la expresión y actividad de la en las GAs y la secreción basal e inducida por estrés agudo de ACTH y CORT. Ratas Wistar macho se sometieron a condiciones control (CTRL) o a estrés crónico por RMO (20 min/día) durante 14 días. Estos animales se subdividieron en varios subgrupos para recibir vehículo (VEH; 1 ml / kg, i.p.) o pCPA (150 mg / kg, ip) durante 4 días consecutivos (del día 9 al 12 de los tratamientos CTRL y de estrés crónico. El día 15 los animales fueron sacrificados y procesados para la obtención de los tejidos (GAs y cerebro) y la subsecuente realización de los ensayos de inmunohistoquímica de TPH en cortes de GAs, de los experimentos de Western blot (niveles de proteína) y RT-PCR (niveles de RNA mensajero) en GAs completas, y medición de las concentraciones de 5-HT y ácido 5-hidroxi-indolacético (5-HIAA) en GAs y en el núcleo del rafé dorsal (NRD) mediante un método fluorométrico de HPLC (determinación de la actividad de la TPH). La exposición a estrés crónico por RMO indujo una fuerte inmunorreactividad a la

TPH2 en la zona glomerulosa, así como en la parte externa e interna de la zona fascicular/ reticular de la corteza suprarrenal; por el contrario, la inmunorreactividad a la TPH1 no se vio alterada o solo lo hizo de manera muy modesta. Este efecto del estrés crónico se produjo predominantemente en las glándulas suprarrenales del lado izquierdo. En concordancia con lo anterior, la exposición a estrés crónico por RMO indujo un aumento significativo de los niveles de proteína y mRNA de la TPH2 -pero no de la TPH1- en las glándulas suprarrenales izquierdas solamente. Además, se detectó un aumento significativo de los niveles y recambio de 5-HT (aumento de la relación de 5-HIAA/5-HT) en las glándulas adrenales de animales expuestos a estrés crónico. El pretratamiento con pCPA inhibió marcadamente la inmunoreactividad a la TPH2 en la corteza adrenal, así como los niveles de proteína y RNA mensajero de la TPH2 en las glándulas adrenales del lado izquierdo provenientes de animales expuestos a estrés crónico. La pCPA también disminuyó las concentraciones de 5-HT y 5-HIAA, así como el recambio de 5-HT (5-HIAA/5-HT). Finalmente, la secreción magnificada de CORT inducida por restricción aguda en animales con historial de estrés crónico fue prevenida por el pretratamiento con pCPA, lo que implica un papel funcional de la TPH (probablemente la TPH2) en la secreción exacerbada de CORT inducida por estrés agudo. El pretratamiento con pCPA restableció la disminución de la respuesta de ACTH inducida por estrés agudo en animales expuestos a estrés crónico por RMO. Estos resultados revelan un papel importante de la expresión y función de la TPH2 adrenocortical en la secreción exacerbada de CORT inducida por estrés agudo independiente de la ACTH, y sugieren que los inhibidores selectivos de la TPH2 podrían tener un potencial terapéutico en el tratamiento de los desordenes relacionados con el estrés asociados con hipercortisolemia.

1. INTRODUCTION

Stress is a natural brain response designed to increase alertness and energy resources to cope with challenging (both positive and negative) situations. If exposure to stress is long-lasting, it may trigger deleterious changes in the body leading to a number of stress-related disorders (SRD), including migraine headaches, coronary artery disease, major depression, anxiety disorders, and Alzheimer's disease, among many others (Esch et al., 2002). The endocrine stress response is controlled by the hypothalamic-pituitary-adrenal (HPA) axis, which is a complex set of direct and feedback interactions among these endocrine glands. Hyperactivity of the HPA axis leading to high circulating levels of glucocorticoids is a common pathophysiological feature of SRD, including major depression (Gold et al., 1986), but the mechanisms underlying dysregulation of the HPA axis however remain unknown. It is believed that most of the pathological alterations in SRD may be accounted for by abnormal secretion of glucocorticoids, as these hormones are well known to regulate a number of functions in the body and regulate the transcription of over two hundred genes (Börcsök et al., 1998). Accumulating evidence suggests that HPA axis dysregulation in SRD may involve a mismatch between ACTH and corticosteroid secretion (Bornstein et al., 2008; Chang et al., 2009) and that ACTH-independent corticosteroid-producing mechanisms might develop during chronic stress (García-Iglesias et al., 2013; Keeney et al., 2006). Our research group has recently reported that exposure to chronic restraint stress (CRS; 20 min/day) for 2 weeks in rats induces HPA axis dysregulation, as reflected by a significant increase of corticosterone (CORT) secretion along with a remarkable decrease of ACTH secretion in response to acute restraint stress thus suggesting dissociation between glucocorticoid production and ACTH secretion (García-Iglesias et al., 2013). Interestingly, magnified stress-induced CORT responses in chronically stressed animals paralleled an increase

in serotonin (5-HT) levels in the adrenal cortex along with increased expression of adrenocortical 5-HT7 receptors (García-Iglesias et al., 2013). In support of a functional involvement of 5-HT7 receptors in stress-induced endocrine responses, magnified stress-induced CORT secretion in animals with a history of chronic stress was significantly inhibited by pretreatment with a 5-HT7 receptor antagonist thus suggesting involvement of 5-HT release and activation of 5-HT7 receptors in the adrenal cortex (García-Iglesias et al., 2013). In fact, we recently reported that chronic stress-induced expression of adrenocortical 5-HT7 receptors as well as adrenal 5-HT content are glucocorticoid-dependent phenomena (Saroj et al., 2019).

The enzyme tryptophan hydroxylase (TPH) catalyzes the rate-limiting step in the biosynthesis of 5-HT, and an interesting link between high circulating levels of glucocorticoids and the production of adrenocortical 5-HT via TPH expression has been reported in primary pigmented nodular adrenocortical disease (PPNAD), which is a rare cause of ACTH-independent hypercortisolemia (Berger et al., 2009). Indeed, high expression of ectopic TPH2 along with increased amounts of 5-HT have been reported in PPNAD tissue (Bram et al., 2016) thus raising the possibility that exposure to high circulating levels of glucocorticoids might induce TPH2 expression and activity, and thus TPH-mediated synthesis of 5-HT in the adrenal cortex. Remarkably, TPH inhibitors, which are currently under development for the clinical management of carcinoid syndrome, have been suggested as a novel therapeutic strategy for the treatment of PPNAD-associated hypercortisolism (Bram et al., 2016).

1.1. Definition of stress

Stress is the body's natural defense mechanism against predators and dangerous situations. It flushes the body with hormones to prepare systems to evade or confront danger through the

'fight-or-flight' response. As part of the stress response, the body produces substantial quantities of cortisol, adrenaline and noradrenaline, which trigger increased heart rate, heightened muscle preparedness, sweating, and alertness. All these factors improve the ability to respond to a hazardous or challenging situation. It has been described that low levels of stress might be useful and even healthy whereas high levels of stress could result in biological, psychological, and social problems (Tucker et al., 2008). All those environmental factors that trigger this reaction are called stressors. The more stressors are experienced, the more stress and its outcomes result. The concept of stress was first introduced by Hans Selye, who described stress as a 'nonspecific response' of the body to any demand placed upon it (Selye, 1955). On the other hand, stress may also be described as an environmental challenge, either internal or external, that disturbs the state of homeostasis (Leonard, 2005). Thus, the term 'stress' can be used in two ways, either to identify events or circumstances that are perceived adversely (i.e. stressors) or the state induced by such events or circumstances (i.e. the stress response). The stress response is primarily a normal physiological response that allows the organism to respond to environmental challenges and enhance the probability of survival (Sapolsky, 2003).

1.2. The General Adaptation Syndrome

The general adaptation syndrome (GAS) is a three-stage response as proposed by Hans Selye (Selye, 1950).

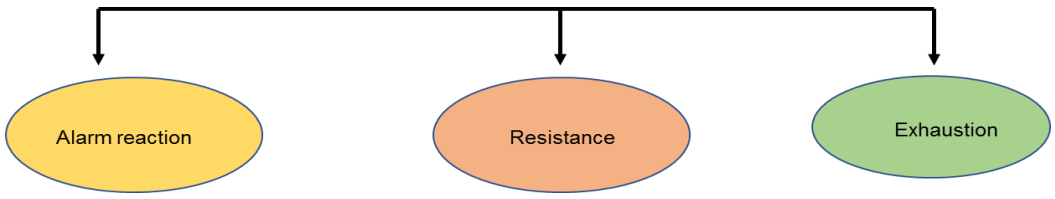


Figure. 1. The stress response

At the alarm reaction stage, a distress signal is sent to the hypothalamic paraventricular nucleus (PVN). The PVN enables the release of glucocorticoids through the release of corticotropin-releasing hormone (CRH) and the action of this on the anterior lobe of the pituitary to induce the release of adrenocorticotripic hormone (ACTH) into the circulation. This hormone in turn reaches the adrenal cortex to induce the synthesis and release of glucocorticoids. These in turn trigger the release of adrenaline from the adrenal medulla to increase heart rate, blood pressure and glucose levels. The physiological changes of the alarm reaction are greatly mediated by the sympathetic arm of the autonomic nervous system (ANS). During the resistance stage, the body attempts to counteract the physiological changes that take place during the alarm reaction. The parasympathetic arm of the ANS plays an important functional role during the resistance stage of the GAS by trying to return the body to homeostasis. This includes a decrease in heart rate and blood pressure. If stress comes to an end during the resistance stage, the body will return to homeostasis. However, if the stressor remains, the body will stay in a state of alertness, and high stress hormone levels will continue up. After an extended period of stress, the organism may go to the exhaustion stage of the GAS. At this point, the body has depleted its energy resources by continually trying but failing to recover from the initial alarm reaction stage. Once it reaches the exhaustion stage, the body is no longer capable of fighting stress. If the individual does not find ways to manage stress levels at this stage, he will be at risk of developing stress-related health conditions, such as depression and anxiety, among many others (Chrousos and Gold, 1992).

1.3. Acute stress

Acute stress is the most generic form of stress. It comes from the demands and pressures of the recent past and anticipated demands and pressures of the near future. Acute stress in small

quantities is exciting in small quantities, but too much of it is exhausting. Acute stress lacks the time to do the extensive damage associated with long-term stress exposure.

1.4. Chronic stress

Chronic stress comes when a person never sees a way out of a difficult situation. It is the stress of unrelenting demands and pressures for seemingly interminable periods. With no hope, the individual gives up searching for solutions. Some chronic stressors stem from traumatic, early childhood experiences that become internalized and remain forever painful and present.

1.5. Chronic stress and the hypothalamic-pituitary-adrenal axis

As described above, chronic stress is generally considered a key risk factor for the development of SRD (Anisman and Zacharko, 1992). Specifically, anxiety and depressive disorders have frequently been associated with preceding periods of chronic stress or stressful life events (Amat et al., 2005). In addition to psychiatric disorders, chronic or severe stress has also been implicated in a variety of other diseases, such as metabolic syndrome including type II diabetes, cardiovascular disease and hypertension (Chrousos and Gold, 1992; Pickering, 2001). The notion that chronic stress exposure could be causal for the development of affective disorders is supported by findings of altered HPA axis function (Chrousos and Gold, 1992; Holsboer, 2000). The HPA axis constitutes one of the fundamental pathways of the mammalian stress response, in which a cascade of events leads to elevations in glucocorticoid hormones.

1.6. The neuroendocrine response to stress

There are three discrete systems required to maintain and reinstate homeostasis during the stress response. The ANS quickly responds within seconds to stress exposure via its sympathetic and parasympathetic divisions, which induce rapid alterations of the physiological state through their neural innervation to target organs. This involves the release of the catecholamines, adrenaline and noradrenaline (the sympatho-adrenomedullary [SAM] response) from the adrenal medulla into the circulation generating the 'fight-or-flight' response (Jansen et al., 1995), which results in a rapid increase of heart rate and blood pressure while enhancing arousal and vigilance. Within the same timeframe, increased secretion of pituitary prolactin and growth hormone, and pancreatic glucagon is often observed following distinct types of stressors (Jones et al., 2012; Lennartsson and Jonsdottir, 2011; Ranabir and Reetu, 2011).

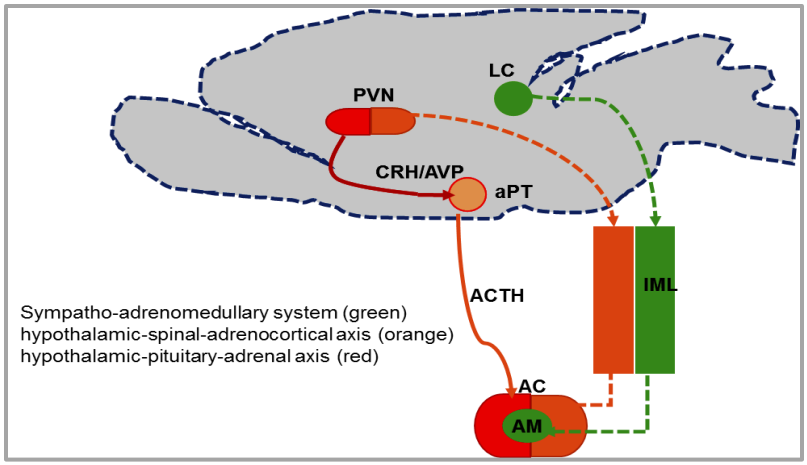


Figure 2. Systems that maintain and reinstate homeostasis during stress(Bonaz et al., 2017)

Activation of the SAM system (Fig. 2; green) through the locus coeruleus (LC in Fig. 2) within the brain stem leads to the release of adrenaline and noradrenaline from the adrenal medulla (AM in Fig. 2) through the intermediolateral cell column (IML in Fig. 2) of the spinal cord. The multisynaptic autonomic hypothalamic-spinal-adrenocortical (HSA) axis (Fig. 2; orange) is concurrently activated and sends projections from the PVN to the IML and, via the splanchnic nerve, to innervate the adrenal cortex (Lowry, 2002). Activation of the HPA axis (Fig. 2; red) stimulates the release of CRH and arginine vasopressin (AVP) from the PVN of the hypothalamus into the pituitary portal circulation. These act concurrently on the anterior pituitary (aPT in Fig. 2), causing the release of ACTH that travels through the systemic circulation to induce glucocorticoid secretion from the adrenal cortex (Gavrilovic and Dronjak, 2005). However, these secondary stress systems are activated as required to meet a stress-induced demand, and those involving prolactin are still incompletely understood, with controversial roles in the dynamics of the stress response. The HSA axis, through direct innervation of the glucocorticoid synthesizing adrenal cortex, has demonstrated essential roles in modulation of ultradian and circadian rhythms and stress-induced neuroendocrine function (Jasper and Engeland, 1994). It has been demonstrated that adrenal splanchnic innervation modulates CORT secretion in rodents by increasing adrenal sensitivity to ACTH (Ulrich-Lai et al., 2006a). Following activation of the SAM system and HSA axis, the stress system is primed, and subsequent activation of the HPA axis results in amplified elevations of circulating corticosteroids with peak plasma levels occurring approximately ten to fifteen minutes after the initiation of stress exposure (Droste et al., 2008). It should be noted that each of these systems has essential roles in normal homeostatic physiology, including basal unstressed conditions. The HPA axis exhibits a prominent daily circadian rhythm characterized by rapid ultradian

oscillations with peak levels of corticosteroids that occur during the active phase over approximately 24 hours (Windle et al., 1998).

1.7. Activation of the hypothalamic-pituitary-adrenal axis and its regulation

Stress-induced activation of the HPA axis involves activation of parvocellular neuroendocrine cells within the PVN, which secrete CRH and AVP into the hypophyseal portal system (Tsigos and Chrousos, 1994). AVP is a potent synergistic factor acting concurrently with CRH on the anterior pituitary, activating their respective transmembrane receptors, vasopressin V1b receptor and CRH receptor 1, both stimulating ACTH release to the systemic circulation (Smith and Vale, 2006). Circulating ACTH subsequently binds to the melanocortin type 2 receptor (MC2R) in the inner adrenal cortex (i.e. *zona fasciculata*) to initiate *de novo* synthesis of glucocorticoids through transcriptional activation of several steroidogenic genes to transport cholesterol across the mitochondrial membrane and ultimately increase the release of corticosteroids (Tsigos and Chrousos, 2002) (Fig. 3). Circulating glucocorticoids can reach every organ of the body, thereby contributing to recovery and adaptation. In the periphery, they exert their functions mainly by mobilizing energy resources, dampening of immune reactions, and increasing the vascular tone (Munck and Náray-Fejes-Tóth, 1992).

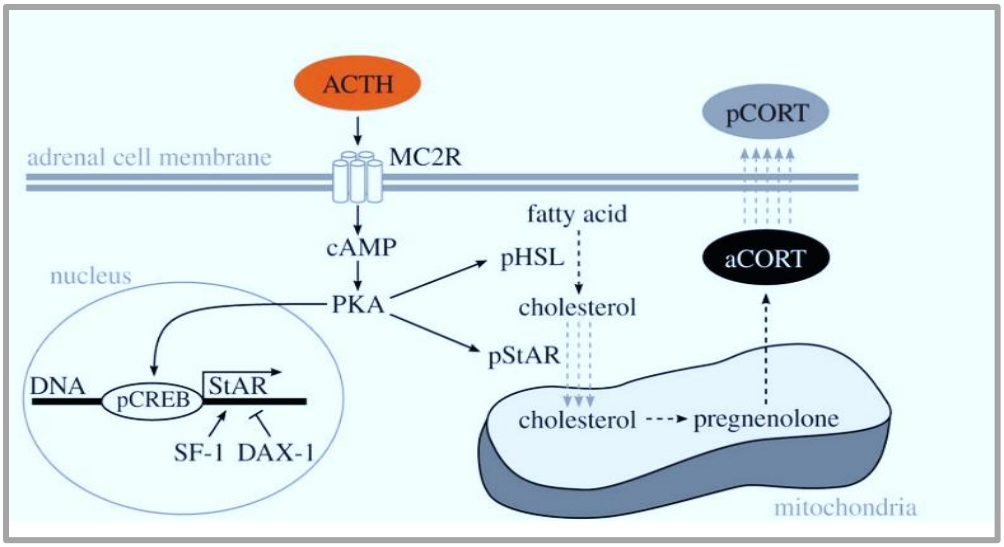


Figure 3. Intracellular mechanisms of ACTH-mediated production and secretion of corticosteroids in the adrenal cortex (Walker et al., 2015)

As shown in Fig. 3, ACTH increases adrenal gland activity via protein kinase A (PKA) activation leading to non-genomic regulation of steroidogenic proteins. This includes phosphorylation of hormone-sensitive lipase (HSL), a protein that increases the levels of intracellular cholesterol (the precursor of steroid hormones) and phosphorylation of steroidogenic acute regulatory protein (StAR), which promotes the transport of cholesterol into the mitochondria, where cholesterol is converted into pregnenolone by the enzyme side-chain cleavage cytochrome P450. This process is followed by several enzymatic reactions within the mitochondria and the endoplasmic reticulum that ultimately leads to glucocorticoid synthesis within the cell (aCORT), which in turn is released into the general circulation (pCORT). PKA also mediates adrenal genomic activity by inducing StAR transcription, which is in turn enhanced or repressed by the transcriptional regulators' steroidogenic factor 1 (SF-1) and DAX-

1, respectively (Walker et al., 2015). In addition, glucocorticoids exert direct negative feedback at various levels of the HPA axis like the PVN and the pituitary, thereby leading to a normalization of HPA axis function. Once released, glucocorticoids bind to two related receptors in the brain, the mineralocorticoid receptors (MR) and the glucocorticoid receptors (GR), albeit with different affinity. MRs bind glucocorticoids with a ten-fold higher affinity as compared to GR and are mostly saturated at low basal corticosteroid levels. GR are additionally activated when glucocorticoid levels are high, i.e., at the circadian peak or after stressful events (Arriza et al., 1988; Reul and de Kloet, 1985). Both receptor types are cytosolic receptors which, upon binding of their ligand, translocate to the nucleus and bind as homodimers, or in regions of coexistence of GR and MR also as heterodimers to a consensus sequence of the DNA, the glucocorticoid responsive element (GRE). Thus, they recruit either co-activators or co-repressors, leading to activation or repression of gene expression (Meijer et al., 2005). As monomers, GR can also directly interact with transcription factors like NFκB, AP-1 or CREB, thereby leading to a reduction of their transcriptional activity (de Kloet et al., 2005). The balance between the occupation of GR and MR is delicate, and any form of dysregulation may increase neuronal vulnerability to stress and adversely influence the stress response (De Kloet et al., 1998).

1.8. The crucial role of adrenal glands in stress

The adrenal glands are wedges of glandular and neuroendocrine tissue adhering to the top of the kidneys by a fibrous capsule. The adrenal glands have a rich blood supply through several arteries branching off the aorta, including the suprarenal and renal arteries, and experience one of the highest rates of blood flow in the body. Adrenal hormones are released into the circulation

via the left and right suprarenal veins. In addition, adrenal cortex produces hormones that are vital to life, such as glucocorticoids, which help regulate metabolism and respond to stress, and aldosterone, which helps control blood pressure (Fig. 4). The inner central part of the gland called the medulla produces the important stress hormones, adrenaline and, to lower extent, noradrenaline.

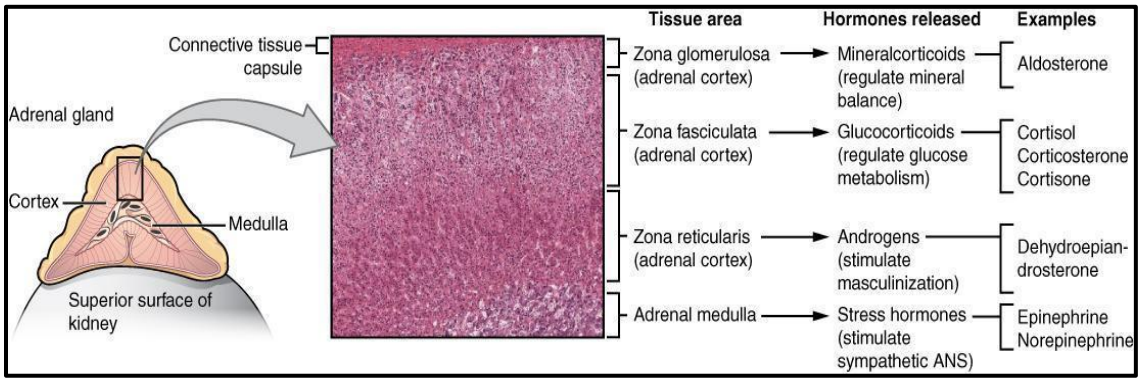


Figure 4. Anatomical and functional features of the adrenal glands (Vinson et al., 1985)

1.9. Hypothalamic-pituitary-adrenal axis dysregulation and depression

A disturbed function of the HPA axis is one of the most consistent neurobiological findings in major depressive disorder (Gold and Chrousos, 2002). In about 50% of the patients suffering from a major depressive episode increased cortisol levels have been reported in plasma, urine and cerebrospinal fluid, as well as enlarged pituitary and adrenal glands (Holsboer, 1999; Sachar et al., 1973); a typical depression however seems to be associated with low HPA axis activity (Matza et al., 2003). In animals, CORT treatment reduced the stress response and facilitated habituation by altering the behavior that was no longer necessary. Thus, HPA axis activity

appears to play a key role in the etiology and progression of depression through its effect on stress responsivity (Stamp and Herbert, 2001).

1.10. Relationship between serotonin and the hypothalamic-pituitary-adrenal axis in stress

The dynamic interplay between 5-HT neurotransmission and the HPA axis has been extensively studied over the past 30 years (Heisler et al., 2007). This complex inter-relationship between 5-HT and the HPA axis has important implications for the current understanding of the endocrinology of the stress response. Indeed, some of the 5-HT neurons arising from the dorsal raphe nucleus (DRN) and the raphe magnus project to the PVN and synapse onto CRH neurons (Tsigos and Chrousos, 1994). 5-HT neurons also project to other brain areas, such as the amygdala and the suprachiasmatic nucleus, which are thought to modulate the function of the PVN (Charnay and Léger, 2010). Dysregulation of the HPA axis and the 5-HT system has been implicated in the pathophysiology of disease states such as affective disorders, anxiety disorders, and obesity (Heisler et al., 2007). Clinical studies have demonstrated that HPA axis overdrive and the accompanying psychopathological outcomes in SRD are improved by drugs that target the brain 5-HT system, such as the selective 5-HT reuptake inhibitors (Blier et al., 2001; Inder et al., 2001). Disturbances in the 5-HT system constitute the neurobiological abnormality most consistently associated with suicide (Duval et al., 2001). This abnormality could be a marker of vulnerability predisposing individuals to auto aggressive and impulsive behavior. However, other abnormalities, including hyperactivity of the HPA axis, have also been described in suicide victims (Corrêa et al., 2002). 5-HT is unable to pass the blood-brain barrier and must be synthesized locally from the precursor molecule L-tryptophan. It has recently been shown that exposure to CRS in rats induces sensitization of stress-induced CORT secretion through an

ACTH-independent mechanism involving increased levels and turnover of 5-HT in adrenal glands (García-Iglesias et al., 2013).

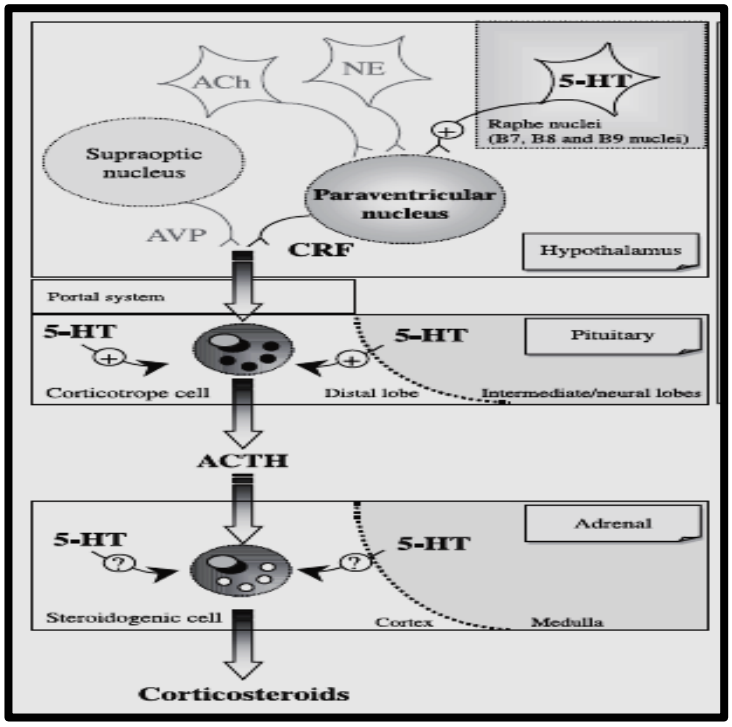


Figure 5. Role of serotonin in activation of the hypothalamic-pituitary-adrenal axis(Contesse et al., 2000)

1.11. Role of serotonin and its receptors in stress

Seven families of 5-HT receptors have been cloned (5-$HT_{1–7}$) (Hoyer et al., 1994). Except for 5-HT3 receptors, which are ligand-gated ion channels, all other 5-HT receptors are seven transmembrane peptides coupled to G proteins. Members of the 5-HT1 receptor family (5-HT1A, 5-HT1B, 5-HT1D, 5-HT1E, 5-HT1F) are mainly coupled to Gi/o/z proteins, members of the 5-HT2 family (5-HT2A, 5-HT2B, 5-HT2C) are coupled to Gq/11 proteins and all other receptor families are coupled to Gs proteins (Albert and Tiberi, 2001; Hoyer et al., 2002) (see Fig. 6). In the serotonergic synapse, the 5-HT receptors are found in pre-synaptic nerve terminals, also called autoreceptors (5-HT1B/1D), and in postsynaptic neurons (most if not all other receptor

families, including 5-HT1B/1D receptors). Additionally, the soma and dendrites of serotonergic neurons contain 5-HT1A receptors that function as negative feedback autoreceptors.

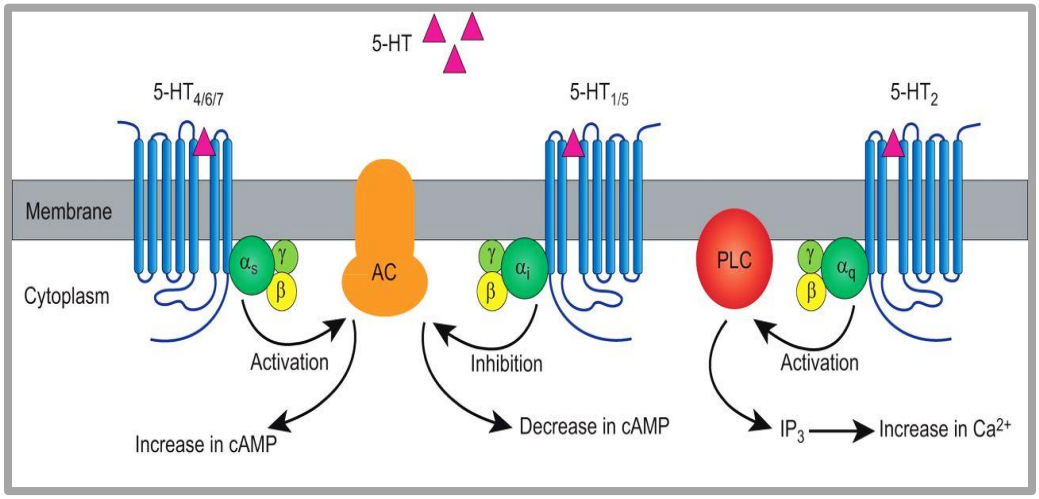


Figure 6. Transduction pathways of 5-HT receptors. Diagram illustrating the receptors depicted are proteins with seven transmembrane α-helixes (blue), a molecular structure shared by all members of the G-protein-coupled receptor (GPCR) superfamily. The receptors bind 5-HT and other ligands on their extracellular side and contain a binding site for G-proteins on the cytoplasmic side. G-proteins are trimeric structures comprising α, β, and γ subunits, each encoded by multiple genes. The binding of 5-HT induces a conformational change in the receptor, which activates G-proteins. Activated G proteins interact with effector proteins, including the enzymes adenylate cyclase (AC) and phospholipase C (PLC), to transduce cellular responses. 5-HT4/6/7 receptors bind G-proteins containing αs subunits, which activate AC, resulting in the production of the second messenger cAMP. 5-HT1/5 receptors bind G-proteins containing αi/o subunits, which inhibit AC, resulting in a decrease in intracellular cAMP. 5-HT2 receptors are linked to G-proteins of the αq family, which activate PLC, resulting in the production of second messengers, including inositol 1,4,5-trisphosphate (IP3). IP3 stimulates the release of Ca2+ from intracellular organelles, leading to an increase in Ca2+ in the cytoplasm. Changes in intracellular cAMP or Ca2+ levels affect numerous cellular processes and regulate gene expression (Tierney, 2018)

It is established that the HPA axis is modulated by the 5-HT system via projections from the dorsal and median raphe nuclei that reach the parvocellular and CRF-producing neurons in the hypothalamic PVN (Larsen et al., 1996; Liposits et al., 1987). Thus, serotonergic inputs

stimulate CRF (Holmes et al., 1982) and ACTH secretion (Kageyama et al., 1998) via 5-HT1A and 5-HT2A/2C receptors (Jørgensen et al., 1999; Pan and Gilbert, 1992). In addition, pharmacological evidence has suggested the involvement of 5-HT7 receptors in HPA axis modulation as the 5-HT1A/5-HT7 receptor agonist, 8-OH-DPAT, elicits neuroendocrine responses (Jørgensen et al., 1999; Vicentic et al., 1998) which, in the case of CORT secretion, are not completely inhibited by the 5-HT1A receptor antagonist.

Recent accumulating evidence on the other hand suggests a role of 5-HT7 receptors in stress-induced CORT secretion under conditions of high circulating levels of glucocorticoids such as chronic stress exposure (García-Iglesias et al., 2013). Indeed, activation of 5-HT7 receptors has been shown to mediate sensitized restraint-induced CORT responses, which parallels an increased adrenal content and turnover of 5-HT in the adrenal glands from animals with previous exposure to CRS (García-Iglesias et al., 2013). These findings resemble a number of clinical pathophysiological observations, which have unraveled a role of a stimulatory adrenocortical serotonergic loop in mediating increased cortisol secretion inducing Cushing syndrome, as shown in adrenocortical adenomas/carcinomas (Contesse et al., 2005; Lefebvre et al., 2015; Louiset et al., 2008), primary pigmented nodular adrenal disease (Bram et al., 2016), and ACTH-independent macronodular adrenal hyperplasia (Louiset et al., 2006). Interestingly, secretion of cortisol under these pathological conditions has been shown to be mediated, at least in part, by 'illicit' receptor mechanisms (Lacroix et al., 2001), including the 5-HT7 receptors (Louiset et al., 2008, 2006), and locally-produced 5-HT (Bram et al., 2016), which reportedly induces cortisol secretion with high potency and efficacy in tumorous adrenocortical cells in culture (Contesse et al., 2005). On the basis of these pathophysiological findings, which closely resemble those reported in chronically stressed animals (García-Iglesias et al., 2013; Terrón, 2014), it is thus

likely that exacerbated glucocorticoid secretion involving the aforementioned stimulatory serotonergic loop in adrenocortical cells might represent a more common pathophysiological mechanism of hypercortisolemia in SRD.

1.12. Potential role of TPH in chronic stress-induced adrenocortical synthesis of serotonin

Biosynthesis of 5-HT is a two-step process; the first and rate-limiting step is the conversion of *L*-tryptophan into 5-hydroxytryptophan (5-HTP), which is catalyzed by tryptophan hydroxylase (TPH) (Fig. 7). Two TPH isoforms (TPH1 and TPH2) have been identified and share 71% homology in their protein sequence with theri protein sequences differing at their N-terminal and exhibiting different spatial distribution patterns (Pelosi et al., 2015). Then 5-HTP is subsequently converted into 5-HT through the decarboxylation process mediated by aromatic amino acid decarboxylase (AAAD) (Ichiyama et al., 1970; Lovenberg et al., 1967) (Fig. 7). TPH1 is primarily located in a variety of non-neuronal cells, such as the enterochromaffin cells of the gut and the pineal gland (Zill et al., 2009), whereas TPH2 is predominantly expressed in the myenteric plexus and the serotonergic neurons of the raphe nuclei (Walther and Bader, 2003). Evidence has shown that TPH1 primarily catalyzes the synthesis of peripheral 5-HT, whereas TPH2 functions primarily at central serotonergic neurons (Patel et al., 2004).

An interesting link between high circulating levels of glucocorticoids and the production of adrenocortical 5-HT has been reported in primary pigmented nodular adrenocortical disease (PPNAD), which is a rare cause of ACTH-independent hypercortisolism. Thus, high expression of ectopic TPH2 along with increased amounts of 5-HT have been reported in PPNAD tissue (Bram et al., 2016). It is known that TPH2 exhibits a highly flexible gene expression that is

modulated by a number of internal and external environmental factors including the biological clock, stressors, endogenous hormones and antidepressant therapies (Chen and Miller, 2013). Using the TPH inhibitor, p-chlorophenylalanine (pCPA), a significant decrease in cortisol production by PPNAD tissue was observed (Bram et al., 2016).

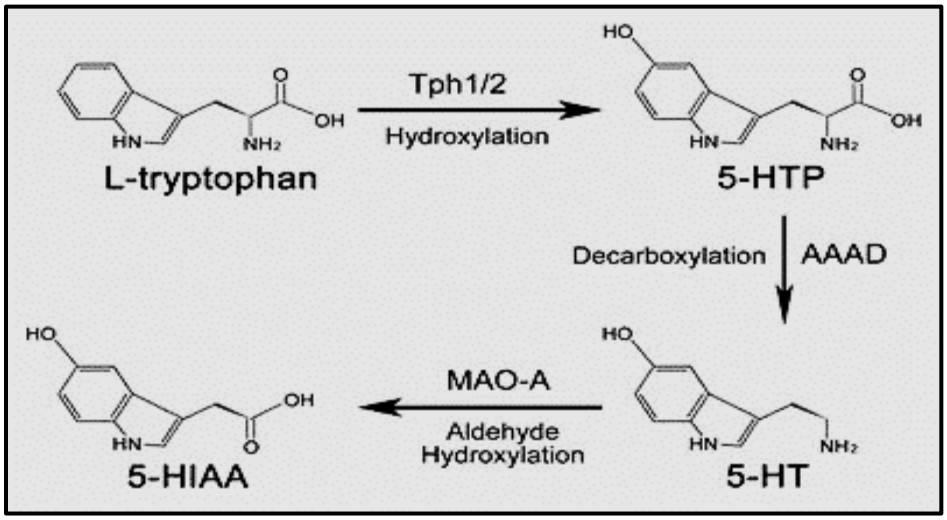


Figure 7. Biosynthesis of serotonin. In animals, serotonin is synthesized from amino acids L-tryptophan. Under the hydroxylation of tryptophan hydroxylase (TPH1/2), L-tryptophan is converted into 5-hydroxytryptophan (5-HTTP), which is subsequently catalyzed into serotonin by aromatic amino acid decarboxylase (AAAD), it is finally metabolized into 5-hydroxy indole acetic acid (5-HIAA) to be removed from the body (Lv and Liu, 2017).

2. Hypothesis

During chronic stress, the serotonergic control of corticosteroid secretion may involve increased expression and/or activity of TPH isoforms (i.e., TPH1 and TPH2) in the adrenal cortex, increased synthesis of 5-HT, and 5-HT-induced activation of adrenocortical 5-HT7 receptors. These alterations might be glucocorticoid-dependent. Pharmacological inhibition of TPH by *p*-chlorophenylalanine (pCPA) pretreatment might reduce CRS-induced sensitization of CORT secretion.

3. General aim

To investigate the effect of CRS exposure on TPH expression and activity in the adrenal glands and determined the role of TPH on chronic stress-induced endocrine dysregulation in rats.

4. Specific aims

4.1. Examine the effect of CRS as compared to that of control conditions on expression of tryptophan hydroxylase isoforms (TPH1 and TPH2) in the adrenal glands.

4.2. Analyze the effect of a chronic 14-day treatment with CORT (20 mg/kg, s.c., per day), as compared to that of vehicle (1 ml/kg, s.c., per day), on a) TPH isoform and 5-HT7 receptor expression in adrenal glands; and b) acute stress-induced ACTH and CORT secretion.

4.3. Investigate the effect of TPH inhibition by p-chlorophenylalanine pretreatment (150 mg/kg i.p) on: a)5-HT-like immunoreactivity and 5-HT level by using HPLC; And b)TPH isoform gene and protein expression in the adrenal glands; and c) TPH activity in the adrenal glands; d) acute stress-induced ACTH and corticosterone (CORT) secretion.

4.4. To prospect the effect of CRS and chronic 14-day treatment with CORT (20 mg/kg, s.c., per day), as compared to that of a vehicle (1 ml/kg, s.c., per day) and pretreatment with pCPA (150mg/kg) on mRNA expression of (cREB) and GR in the adrenals.

5. METHODS

5.1 Animals

Male Wistar rats (200-220 and 150-200 g of body weight) from our own institutional inbred facilities were used. Animals (five per cage) were kept at constant temperature (22±1ºC) and humidity (50-55%) under a 12:12 h: light/dark cycle (lights on 06:00-18:00 h) with food and water available *ad libitum*. All the procedures and protocols complied with Federal regulations and were approved by the CINVESTAV-IPN ethics committee (Comité Interno para el Cuidado y Uso de Los Animales de Lab oratorio; CICUAL). Efforts were made to minimize unnecessary suffering of the animals and their numbers.

5.2. Chronic restraint stress

Treatment with CRS consisted of 20 min daily sessions of restraint (between 8:00 and 11:00 h) for 14 days, which was induced by placing the animals in well-ventilated adjustable-length cylindrical plexiglass tubes (6.5 cm in internal diameter and 20 cm in length). Another group of animals, which served as the control (CTRL) group, was daily taken from the tail for 20 s and returned to their home cages for 14 days. Since animals submitted to CRS were placed in the tubes by taking them from the tail, tail lifting was performed in CTRL animals to exclude the effect of tail lifting itself on potential chronic restraint-induced changes in the HPA system.

5.3. Animal groups and chronic corticosterone treatment

A 14-day chronic treatment with CORT (20 mg/kg, subcutaneously (s.c.) per day) or its vehicle (VEH; 20% 2–hydroxypropyl--cyclodextrin; 1 mL/kg, s.c. per day) was given to the animals (between 0800 and 1200). To reduce stress, both VEH and CORT were administered s.c. At the

nape of the neck, with the animals being slightly restrained. A control (CTRL), home cage animal group, was included for most experimental comparisons.

5.4. TPH inhibition with p-chlorophenylalanine pretreatment

The animal group was taken as a control without treatment, and chronic restraint stress will consist of 20 min daily sessions of restraint for 14 min days on the day of the middle (9 to 12). Animals were taken pCPA (150 mg/kg, i.p. 4 days), and water 1ml/kg was given to each group of animals (between 8:00 and 12:00 h). In order to reduce stress-induced by injection, the peritoneal route of administration will be used.

5.5. Determination of body, adrenal gland and thymus weight

Total body weight of the animals was measured and recorded every day during the entire course of the 14-day treatments. Body weight gain was calculated by subtracting the body weight recorded each day by the body weight recorded on day one, and then this value was multiplied by 100. The adrenal glands from both sides and thymus will be removed and weighed for subsequent determination of relative organ weight (i.e. mg/100 g of body weight). These somatometric parameters were also recorded in a CTRL animal group.

5.6. Immunohistochemistry assays

For immunohistochemistry assays, one day after treatments (i.e. on day 15) animals of each group were anesthetized with sodium pentobarbital (60-70 mg/kg, i.p.) and perfused via the ascending aorta with 0.1M phosphate-buffered saline (PBS; pH 7.4) followed by 0.1 M phosphate-buffered 4% paraformaldehyde (pH 7.4). A CTRL animal group was included for

comparison. The adrenal glands were removed, post-fixed for 24 h at 4ºC, and stored in 0.1 M PBS containing 30% sucrose at 4ºC until use; only the left adrenals were used for the assays. Adrenal sections (35 μm-thick) were obtained using a freezing microtome and collected in culture wells containing 0.1 M PBS and stored at 4ºC overnight. On the day of the assay, sections were incubated in 0.1 M PBS for 10 min and then washed four times (10 min each, twice with 0.1 M PBS and twice with 0.1 M PBS containing 0.3% Triton X-100). For antigen retrieval, sections were incubated in citrate buffer for 10 sec in microwave, allowed to cool for 20 min at room temperature, washed three times (5 min each; twice with 0.1 M PBS and once with 0.1 M PBS containing 0.3% Triton X-100) and then incubated in 0.1 M PBS containing 3% hydrogen peroxide for 30 min. After three more washes (5 min each; twice with 0.1 M PBS and once with 0.1 M PBS containing 0.3% Triton X-100), sections were incubated with 0.3% bovine serum albumin (BSA) in 0.1 M PBS containing 0.1% Triton X-100 for two hr. at room temperature. Next, sections were incubated with a primary antibody.

Primary antibody	Dilution	Secondary antibody	Dilution
5-HT (Goat 20079 lot 1530001) I. Star	1 :200	Rabbit Anti- Goat (Invitrogen)	1:1000
5-HT7 R (Rabbit 244330) Immuno star	1 :200	Goat Anti – Rabbit (Jackson)	1:1000
TPH -1 (rabbit AB- 15570) Millipore	1:300	Goat Anti – Rabbit (Jackson)	1:1000
TPH- 2 (Rabbit ABN-60 2452473 M.P.	1:500	Goat Anti – Rabbit (Jackson)	1:1000

After three washes (twice with 0.1 M PBS and once with 0.1 M PBS containing 0.3% Triton X-100; 10 min each), sections were incubated with an HRP-conjugated secondary antibody for two h at room temperature. Sections were revealed with 3,3′-diaminobenzidine (DAB substrate kit; Vector Laboratories, Burlingame, CA, USA) in 0.1 M PBS for 1-2 min at room temperature with the reaction product being intensified with nickel. Sections were washed with distilled water,

mounted on gelatin-coated slides, cover-slipped, allowed to dry overnight, and photographed with a digital camera.

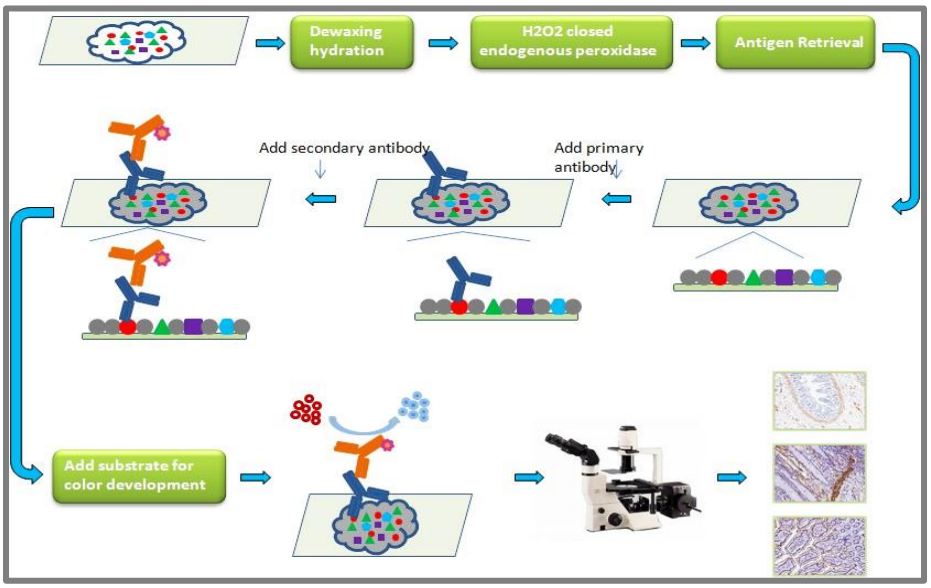


Figure 8. Schematic representation of immunohistochemistry assays

5.7. Western blot analysis

One day after the completion of all treatments, animals of each group were euthanized by decapitation, and adrenal glands were removed and frozen with liquid nitrogen; a CTRL animal group was included for comparison. Both adrenals were then homogenized in 400 µL of 0.1 M Tris buffer (pH 7.4) and 16 µL of 25X complete protease-free inhibitor cocktail (complete mini EDTA-free; Roche Applied Science, Penzberg, Germany), and 10 µL of 0.2 M sodium orthovanadate (pH 10; Sigma-Aldrich Inc., St. Louis, MO, USA) using a Polytron (Kinematica AG, Luzern, Switzerland). Adrenal glands samples from each group were pulled together and the assays were performed in quadruplicate. The adrenal gland homogenates were centrifuged at 13000 rpm for 15 min at 4°C, and the supernatants were collected. Protein samples were sonicated, and 10 µL aliquots were used for protein determination using the Lowry method

Identical amounts of protein (35 μg) of adrenal gland samples from each group were prepared in 1X laemmli buffer and loaded; samples were then separated electrophoretically in a 10% SDS-PAGE gel. Next, the gels were transferred on to polyvinylidene difluoride membranes (PVDF; Immobilon transfer membranes, Millipore Corporation, Billerica, USA). PVDF membranes were then blocked with 5% non-fat milk in Tris-buffered saline (TBS)-Tween-20 (0.1%) (pH=7.4) for 1.5 h at room temperature and then incubated with a primary antibody-based on the interest of protein present in the sample, membrane incubated for overnight at 4°C. Next, membranes were rinsed three times with TBS-Tween-20 (0.1%) for 30 min (10 min each) and then incubated with HRP-conjugated secondary antibody for 2 h at room temperature, respectively. Membranes were rinsed three times again with TBS-Tween-20 (0.1%) for 30 min (10 min each) and developed afterward by incubation with chemiluminescent reagent (SuperSignal West Femto Maximum Sensitivity Substrate, Thermo Scientific, Rockford, USA) for subsequent image capturing with a ChemiDoc Imaging System (Bio-Rad Laboratories Inc.). To estimate the amount of protein relative to GAPDH for each sample, densitometric analysis was performed using the Quantity One® Software (Bio-Rad Laboratories Inc.).

Primary antibodies	Dilution	Secondary antibodies	Dilution
5-HT7 (Goat sc-19160)	1 :200	Rabbit anti-goat (Invitrogen)	1:10000
TPH1 (rabbit AB-15570)	1 :300	Goat anti-rabbit (Jackson)	1:5000
TPH2 (Rabbit ABN-602452473)	1:2500	Goat anti-rabbit (Jackson)	1:5000
GAPDH (GeneTex, GTX100118)	1:50,000	Goat anti-rabbit (Jackson)	1:5000

5.8. Reverse transcription polymerase chain reaction assays

The adrenal glands from treated animals were excised, and both sides of adrenal were used for the assays; a home cage CTRL group was also included for comparison. Expression of the mRNA encoding for the 5HT-7 receptor and TPH -1, TPH -2, was measured by reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted using the Trizol reagent Invitrogen, Carlsbad, CA, USA). Reverse transcription was conducted in a reaction volume of 50 μL using 5 μg of total RNA and Superscript II One-Step RT-PCR system Invitrogen, Carlsbad, CA, USA), following the manufacturer´s protocol in an end-point thermal cycler (Gene Cycler; Bio-Rad) for amplification of 5-HT7 receptor, TPH1, TPH2, CREB and GR cDNA the primer sequences shown below were used.

Gene	Forward Primer	Reverse Primer
TPH1	AGCATAACCAGCGCCATGAA	GGAATCATTGACGACATCGAG
TPH2	TGAGAACCCCAAATCCTGCA	CCCAGCCAACAGACCTAACTGA
5-HT7	AGGATTTTGGCTACACGATC	GAGGAAAAACGGCAGCCA GCA
GAPDH	ACCACAGTCCATGCC TCAG	TCCACCACCCTGTTGCTG TA
Creb	CAGCCACAGATTGCCACATTAG	CTTATGGAGACTGGATAACTGATG
GR	ATCCCACAGACCAAAGCACCTT	TCCAGTTTTCAGAACCAACAGG

Negative controls were made with total RNA to ensure the avoidance of genomic contamination. Positive controls were carried out with total RNA from the frontal cortex of the rat brain (McNamee et al., 2016). The amplification profile involved a cDNA synthesis cycle at 60ºC for 30 min, a denaturation cycle at 94ºC for 2 min, and 35 cycles involving denaturation at 92ºC for 1 min, annealing at 60ºC for 1 min, and extension at 74ºC for 2 min. After amplification, PCR products were electrophoresed on a 1.5 % agarose gel for 1 h at 94 volts. After agarose gel

electrophoresis bands were visualized with ethidium bromide under a UV light lamp and digitalized; then, their intensities were measured by densitometry using the Quantity One 1-D Image Analysis Software (Bio-Rad Laboratories Inc.). Since GAPDH mRNA expression did not vary among treatment groups, all samples were normalized against this.

5.9. Neuroendocrine studies

One day after the end of varies treatments concerning control, veh, corticosterone, and chronic stress groups with pCPA (i.e., on day 15), animals from each group were submitted (0 min, 10 min; and 30 min) for acute restraint stress respectively. After the acute stress periods, animals were killed by decapitation and trunk blood samples collected and centrifuged; animals. Plasma was stored at -80ºC until used. Commercially available Elisa kits for ACTH (cat. EK-001-21, Phoenix Pharmaceuticals Inc., Burlingame, CA, USA) and CORT (cat.ADI-900-097, Enzo, Farmingdale, NY, USA) were employed for hormone level measurements. The minimum detection concentration for each assay was 0.1 ng/mL and 27 pg/mL, respectively, with an intra-assay precision of <10% (each group).

5.10. Measurement of 5-HT, 5-HIAA and L-tryptophan levels by HPLC

Concentrations of 5-HT, 5-HIAA and L-tryptophan in the DRN and adrenal glands were determined by HPLC as previously described (Peat and Gibb, 1983). Briefly, all tissues were homogenized with a solution of 0.1 M HClO4 with 4 mM sodium metabisulfite. The homogenate was centrifuged at 15,000 g for 15 min at 4°C. Subsequently, 20 µl of the supernatant was injected into the HPLC (Waters Corporation, Milford, MA, USA) using a C18 reversed-phase column (5 µM particle size, 3.9 x 150 mm in length). A binary solution system of 2 mM

KH2PO4 (pH 3.4) and acid solution (1 g/L) and a mixture of methanol/water at a ratio of 3:2 v/v at the rate of 1 mL/min were used. Determinations of 5-HT, 5-HIAA and L-tryptophan were performed using a fluorometric detector (Model 474, Waters Corporation). Concentration of 5-HT, 5-HIAA and L-tryptophan was considered as the maximum signal height according to standards of known quantities of the substances. Results were expressed as nmol/mg of tissue.

5.11. Tryptophan hydroxylase enzyme activity measurements

TPH activity in the dorsal raphe nucleus (DRN) and both adrenals were analysed by HPLC measurement of 5-HT with a fluorometric detector (Model 474, Waters Corporation). Briefly, 500 µg of protein was incubated in a buffer solution of Tris-HCl 50 mM, pH 7.4, 1.0 mM EGTA, 15 µg catalase, and 200 µM 2-amino-4-hydroxy-1-methyl tetrahydrobiopterin. The reaction was incubated at 37°C for 30 min and then stopped with the addition of HClO4 6 M plus 5 mM EDTA and 0.1% ascorbic acid. Twenty µL of reagent medium was injected into the HPLC. C18 symmetry column (5-µm particle size, 3.9 x 150 mm) was used. The mobile phase was prepared with 40 mM sodium acetate, pH 3.30, and acetonitrile at a ratio of 95:5, respectively, and run at 1 mL/min. Lengths of excitation and emission used for detection of 5-hydroxytryptophan were 280 nm and 340 nm, respectively. The retention time for 5-hydroxytryptophan was 2.3 min. Results were expressed as 5-HTP nmol/mg protein of tissue/30min.

5.12. Experimental design

The study was designed to determine the impact of CRS exposure, chronic CORT treatment and pCPA pretreatment on CRS-induced changes in a number of variables depicted in Figure 9.

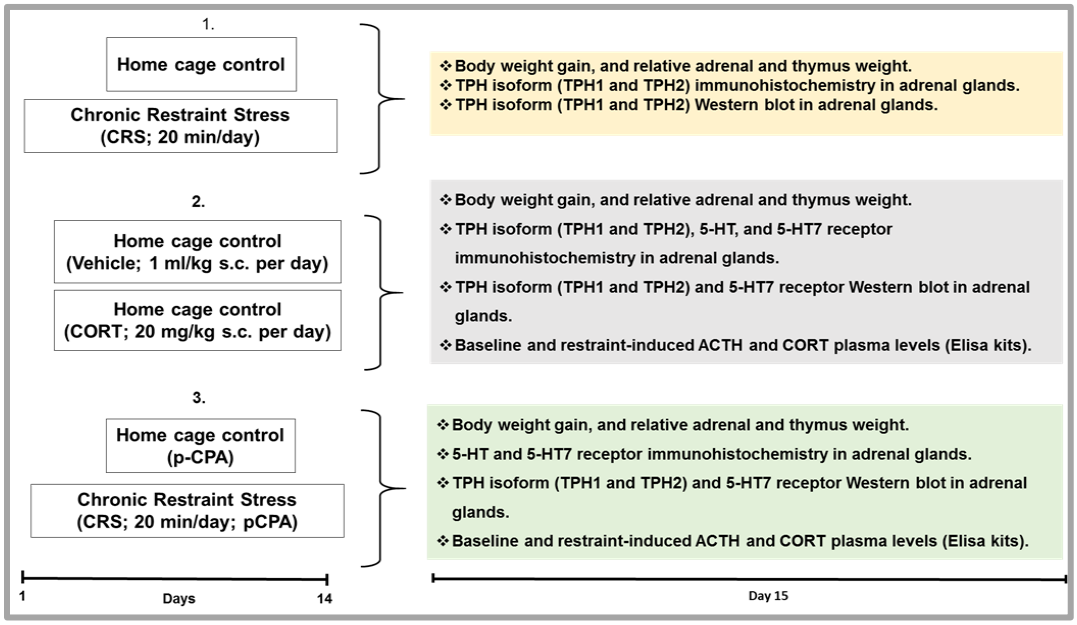


Figure 9. Experimental design of the study

5.13. Data presentation and statistical evaluation

All data in the text, table, and figures are presented as the mean ± SEM of at least four determinations. The differences in body weight gain and relative organ weight, as well as related tissue content of 5-HT, 5-HT7 receptor TPH1 and TPH2 protein and mRNA between CTRL, Veh, and CORT-treated animals as well CRS were all compared by one-way ANOVA. Both one-way and two-way ANOVA compared the effects of chronic VEH and CORT treatments and the effect of CRS with p-CPA on acute restraint-induced ACTH and CORT secretion. The ANOVA tests were followed by Bonferroni post-hoc tests to determine differences. In all cases, the level of significance was set at $P < 0.05$. Statistical analyses were performed using GraphPad Prism version 6.00 (GraphPad Software, La Jolla, California, USA).

6. RESULTS

6.1 Effect of chronic restraint stress on somatometric variables

The physiological parameters known to be altered after exposure to chronic stress were measured. Animals that received CRS exhibited a significantly lower total body weight gain as compared to CTRL animals (Fig. 10, panel A) and this change was correlated to a reduction in food intake which was statistically significant from the sixth day of the 14 day chronic stress treatment (Fig. 10, panel B). Likewise, exposure to CRS induced a significant increase of relative AG weight (Fig. 10, panel C) while promoting a significant decrease of relative thymus weight (Fig. 10, panel D) as compared to tissues from CTRL animals.

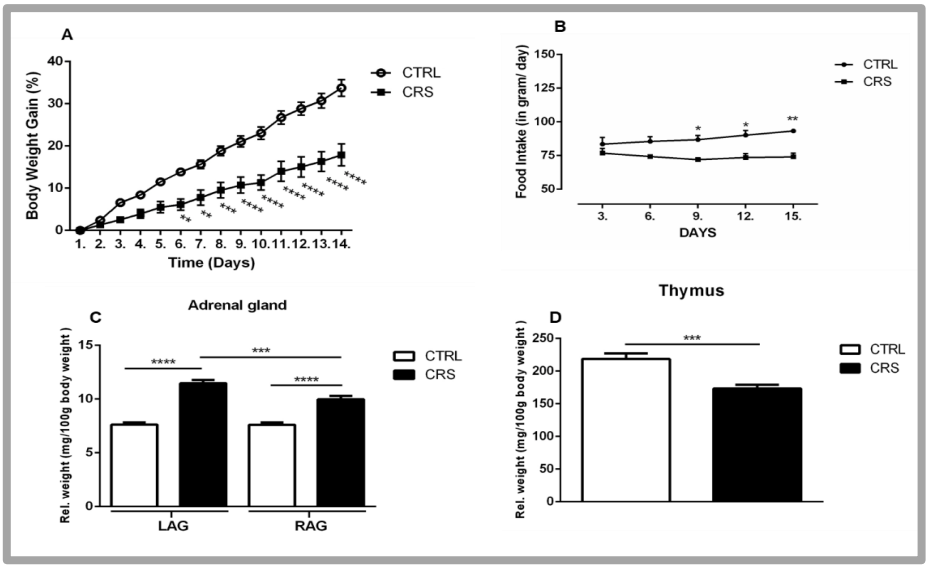


Figure 10. Effect of chronic restraint stress (CRS) on somatometric variables. The effects of CRS as compated to control (CTRL) conditions on body weight gain (A), food intake (B), relative adrenal gland weight (C) and relative thymus weight (D) are shown. *** $P < 0.001$; ** $P < 0.01$

6.2. Effect of chronic restraint stress on TPH-like immunoreactivity in adrenal glands

As shown in Figure 11, exposure to CRS had no effect on TPH1-like immunoreactivity (TPH1-LI) in adrenal glands (Fig. 11; upper set of panels) but it induced strong TPH2-like

immunoreactivity (TPH2-LI) in the cortex of left adrenal glands (LAG) while having modest or no effect on TPH2-LI in right adrenal glands (RAG) (Fig. 11; lower set of panels) thus suggesting asymmetry as to the impact of CRS on adrenocortical expression of TPH.

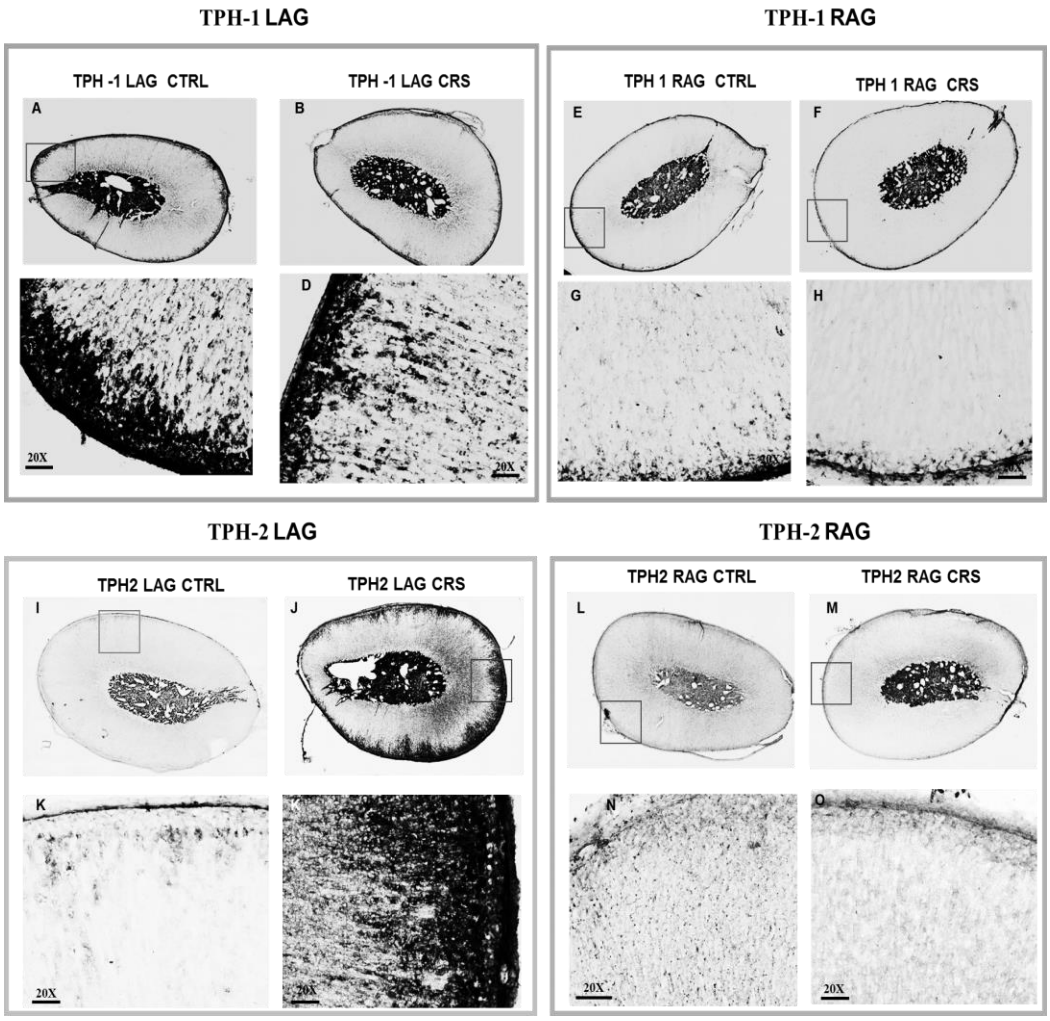


Figure 11. The effect of chronic restraint stress (CRS) as compared to control (CTRL) conditions on TPH1 and TPH2 immunohistochemistry in adrenal glands. The upper and lower sets of panels show results of TPH1- and TPH2-like immunoreactivity, respectively.

6.3. Effect of chronic restraint stress on TPH activity in the dorsal raphe nucleus and adrenal glands

Figure 12 depicts the effects of CRS exposure on TPH activity in the dorsal raphe nucleus (DRN; Fig. 12, panel A) and the left (LAG; Fig. 12, panel B) and righ adrenal glands (RAG; Fig. 12, panel C), as determined by HPLC measurements. Interestingly, whereas CRS exposure evoked a significant decrease in TPH activity in the DRN, it induced a significant increase of it in the LAG only thus supporting the seemingly asymmetrical effect of chronic stress on TPH in the adrenal cortex.

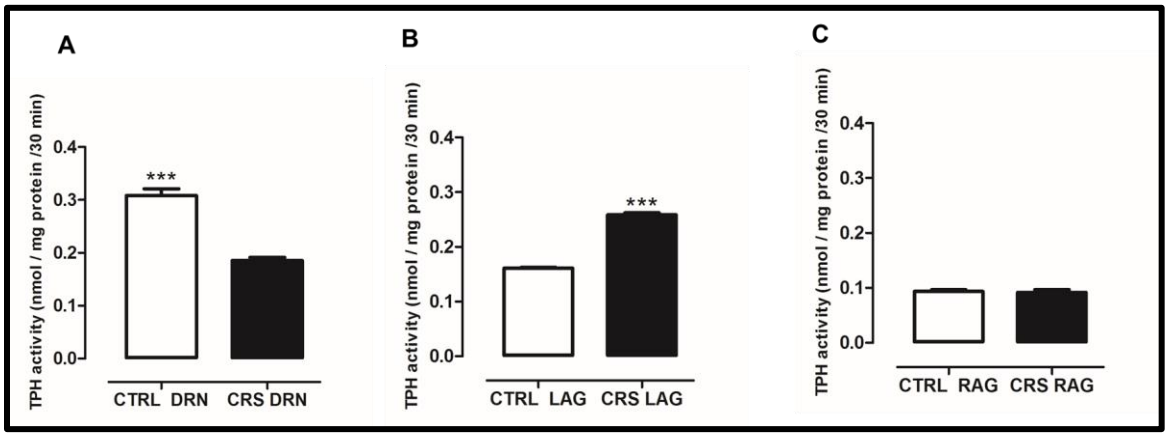


Figure 12. The effect of chronic restraint stress (CRS) as compared to control conditions (CTRL) on TPH activity in the dorsal raphe nucleus (DRN) and adrenal glands. Panels show the results in DRN (A), and the left (LAG; B) and right adrenal glands (RAG; C). *** $P < 0.001$

6.4. Effect of chronic restraint stress on TPH isoform protein levels in adrenal glands

In agreement with our immunohistochemical observations in adrenal glands, exposure of the animals to CRS had no effect on TPH1 protein levels in the adrenals from both sides (Fig. 13, panel A), but it produced however a significant increase of TPH2 protein levels in the left (LAG) but not in the right adrenal glands (RAG), as compared to CTRL tissues (Fig. 13, panel B).

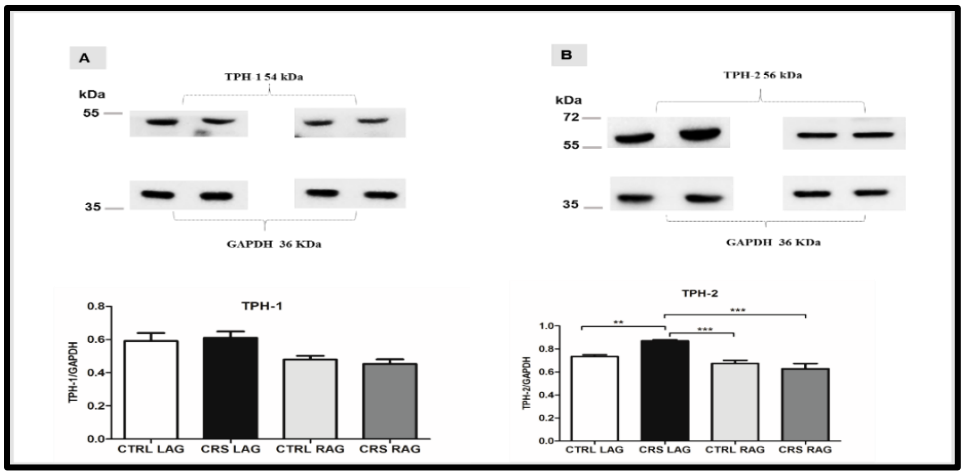


Figure 13. The effect of chronic restraint stress (CRS) as compared to control conditions (CTRL) on TPH protein levels in adrenal glands. Panels show protein content of TPH1 (A) and TPH2 (B) in the left (LAG) and right adrenal glands (RAG). ** $P < 0.01$; *** $P < 0.001$

6.5. Effect of chronic restraint stress on TPH protein levels in the dorsal raphe nucleus

Consistent with the aforementioned results of TPH activity in the DRN (Fig. 12), exposure to CRS evoked a significant decrease in the protein levels of TPH2, but not of TPH1, in the DRN (Fig. 14). These findings provide support to the notion that chronic stress exposure may lead to decreased central levels of 5-HT through a reduction of TPH2 expression and activity in the DRN, which is the major source of 5-HT in the brain.

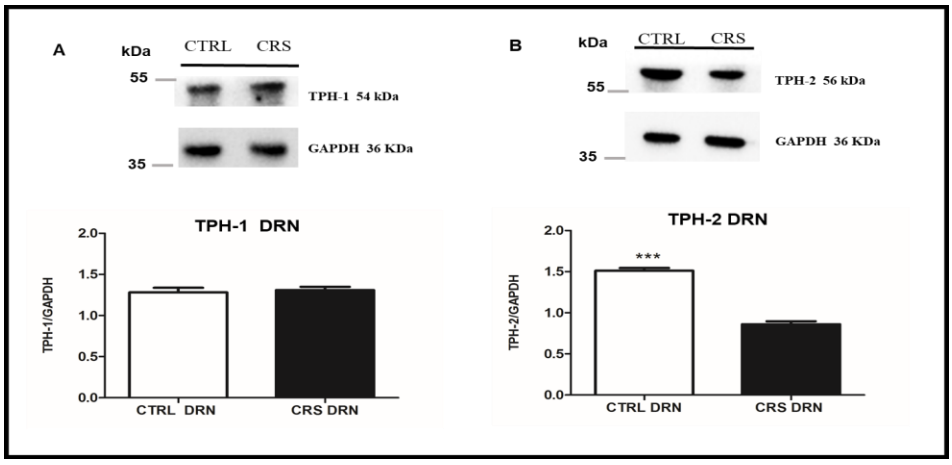


Figure 14. The effect of chronic restraint stress (CRS) as compared to control conditions (CTRL) on TPH protein levels in the dorsal raphe nucleus (DRN). Panels show protein content of TPH1 (A) and TPH2 (B). *** $P < 0.001$

6.6. Effect of chronic restraint stress on TPH isoform mRNA expression in adrenal glands

In keeping with the TPH activity results in adrenal glands (Fig. 12), exposure to CRS also induced a significant change in adrenal TPH mRNA levels. The effect of chronic stress exposure was limited to the TPH2 isoform in adrenals of the left side (LAG), with no change of it in adrenals of the right side (RAG) (Fig. 15). Again, these results support the notion that chronic stress-induced upregulation of TPH expression and activity is asymmetrical in nature (i,e, occurs in the LAG only) and corresponds to the TPH2 -but not the TPH1- isoform.

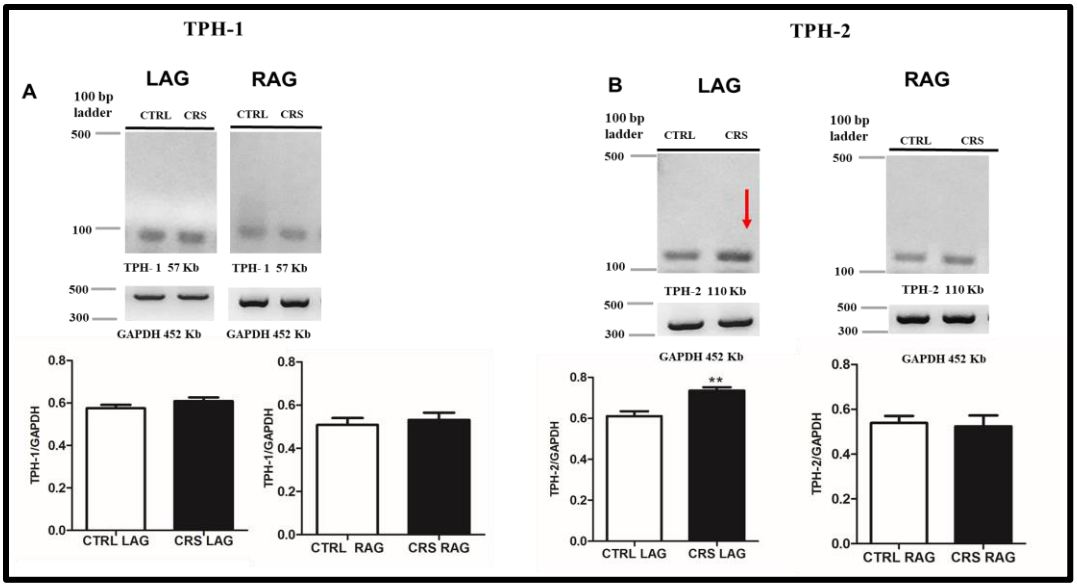


Figure 15. The effect of chronic restraint stress (CRS) as compared to control conditions (CTRL) on TPH isoform mRNA expression in adrenal glands. Panels show mRNA levels of TPH1 (A) and TPH2 (B) in the left (LAG) and right adrenal glands (RAG). ** $P < 0.01$

6.7. Effect of chronic corticosterone treatment on somatometric variables

In order to determine the involvement of glucocorticoids in the effects of chronic stress on the classical stress-sensitive somatometric variables, the effect of a 14-day chronic CORT treatment was tested. Thus as depicted in Figure 16, and similar to the impact of CRS exposure (García-Iglesias et al., 2013) (present results; Fig. 10), chronic CORT administration induced a decrease

in body weight gain (Fig. 16, panel A), which correlated with a significant decrease in food intake (Fig. 16, panel B). Also resembling the effects of chronic stress exposure (García-Iglesias et al., 2013) (present results; Fig. 10), chronic CORT treatment decreased relative thymus weight (Fig. 16, panel C). In contrast to CRS exposure however, chronic CORT administration produced a significant reduction of relative adrenal gland weight (Fig. 16, panels D and E) thereby suggesting that adrenal gland hypertrophy and hyperplasia reported in chronically stressed animals (Ulrich-Lai et al., 2006b) might result from chronic stimulation of the physiological stress response and not from the increase of CORT levels itself.

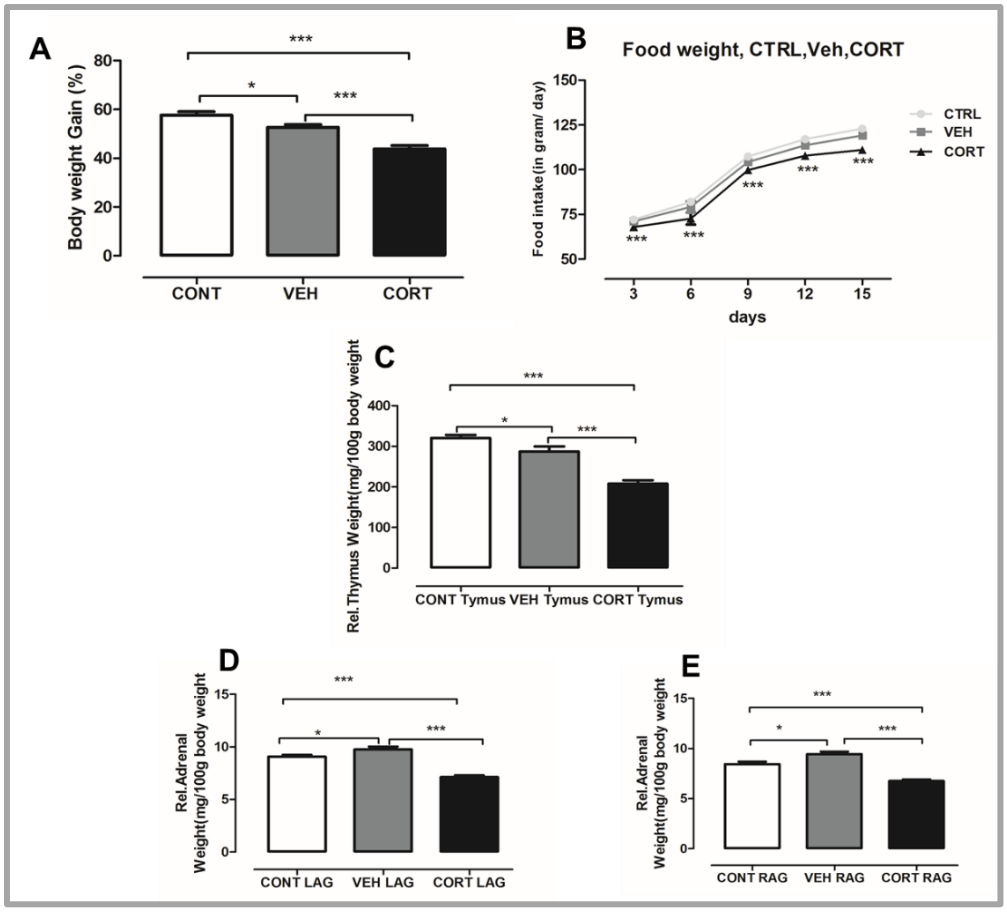


Figure 16. The effect of chronic glucocorticoid treatment on somatometric variables. The effects of chronic corticosterone (CORT) administration as compared to vehicle (VEH) and control (CTRL) treatments on body weight gain (A), food intake (B), relative thymus weight (C), relative left (LAG; D) and right adrenal gland weight (RAG; E) are shown. * $P < 0.05$; *** $P < 0.001$

6.8. Effect of chronic corticosterone treatment on adrenal TPH-like immunoreactivity

In support of the idea that chronic stress exposure might induce TPH2-LI in the adrenal cortex via a glucocorticoid-dependent mechanism (Fig. 11), chronic administration of CORT had no effect on TPH1-LI (Fig. 17), but it did mimick the effects of CRS exposure on TPH2-LI in adrenocortical cells as compared to CTRL and VEH treatments (Fig. 18). Similar to our observations in chronically stressed animals (Fig. 11), chronic CORT-induced TPH2 immunostaining occurred in the LAG only, with no visible changes in the RAG (Fig. 18).

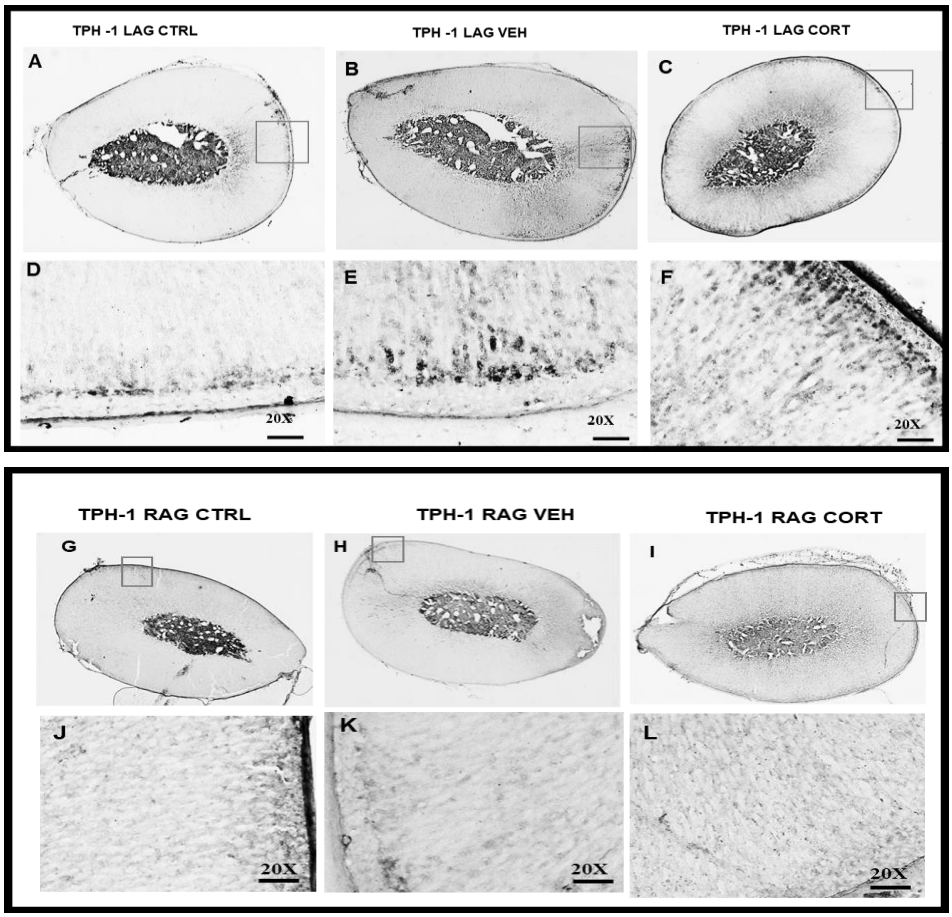


Figure 17. The effect of chronic corticosterone (CORT) treatment on TPH1-like immunoreactivity in adrenal glands. Panels show TPH1 immunostaining in the left (LAG; A-F) and the right adrenal glands (RAG; G-L) adrenal glands. VEH: chronic vehicle treatment; CTRL: control conditions

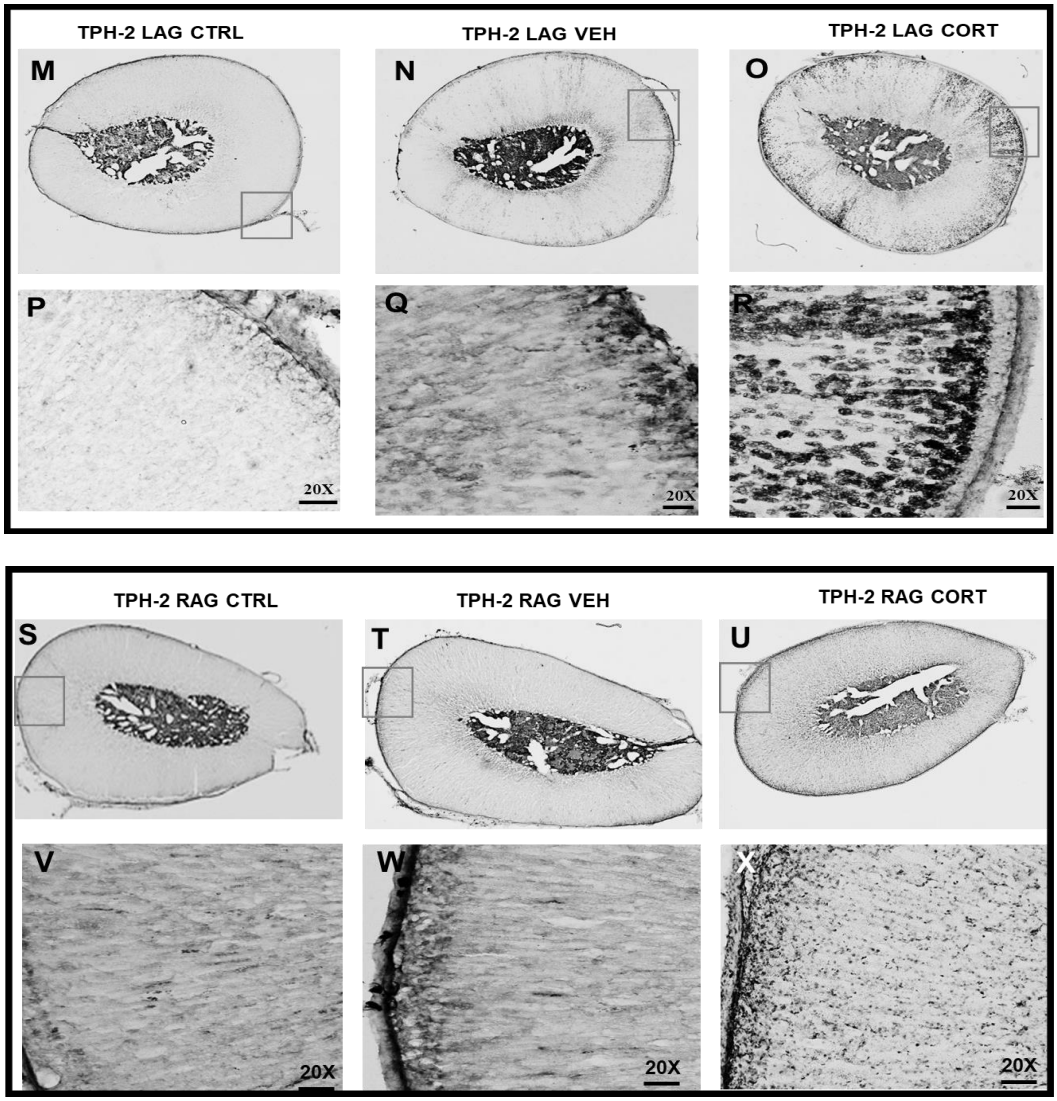


Figure 18. The effect of chronic corticosterone (CORT) treatment on TPH2-like immunoreactivity in adrenal glands. Panels show TPH2 immunostaining in the left (LAG; M-R) and the right adrenal glands (RAG; S-X). VEH: chronic vehicle treatment; CTRL: control conditions

6.9. Effect of chronic corticosterone treatment on TPH protein levels in adrenal glands

As shown in Figure 19, chronic CORT administration closely resembled the effects of CRS exposure on TPH protein levels in the adrenal glands as compared to VEH and CTRL treatments (Fig. 13). Indeed, whereas no change in adrenal TPH1 protein was detected in CORT treated animals (Fig. 19, panels A and B), a significant increase of TPH2 protein levels was observed in

LAG (LAG; Fig. 19, panel C) but not in RAG (Fig. 19, panel D). These findings again reveal asymmetry as to the impact of chronic CORT exposure on TPH2 expression and suggest that CRS-induced TPH2 upregulation is a glucocorticoid-dependent phenomenon.

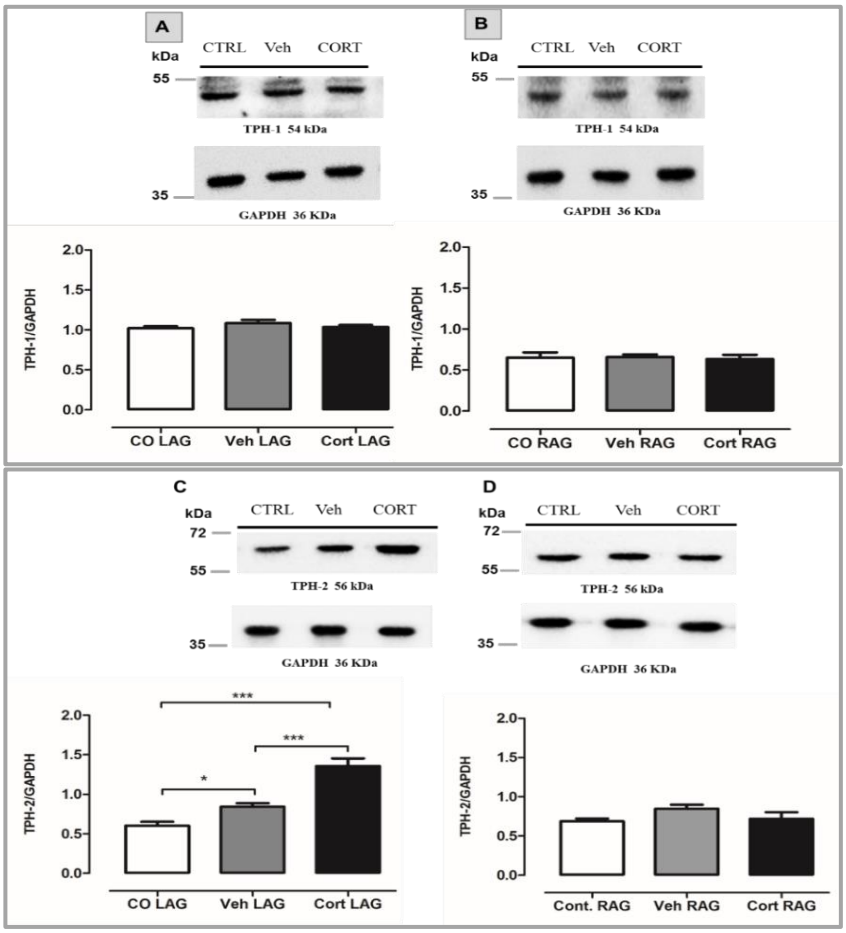


Figure 19. The effect of chronic corticosterone (CORT) treatment on TPH isoform protein levels in adrenal glands. Panels show TPH1 (A and B) and TPH2 (C and D) protein levels in the left (LAG) and right adrenal glands (RAG). VEH: chronic vehicle treatment; CTRL: control conditions * $P < 0.05$; *** $P < 0.001$

6.10. Effect of chronic corticosterone treatment on TPH activity in the dorsal raphe nucleus and adrenal glands

We evaluated the effect of chronic CORT treatment as compared to VEH and CTRL conditions on TPH activity in the DRN and adrenal glands. Thus as depicted in Figure 20, chronic exposure

to high circulating levels of CORT induced a strong increase of TPH activity in the DRN (panel A), while inducing a significant increase of TPH activity in the left (LAG; panel B) but not in the right adrenal glands (RAG; panel C). These data support the notion that CRS-induced increase of TPH activity in both the DRN and the LAG occurs via a glucocorticoid-dependent mechanism, with the effect of the CORT treatment being asymmetrical.

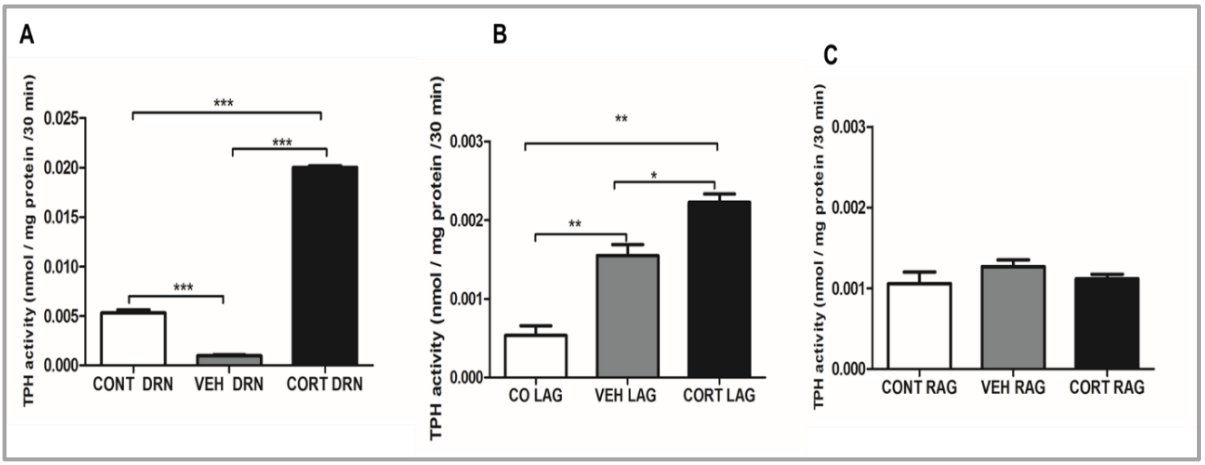


Figure 20. The effect of chronic corticosterone (CORT) treatment on TPH activity in the dorsal raphe nucleus (DRN) and adrenal glands. Panels show TPH activity in the DRN (A), and the left (LAG; B) and right adrenal glands (RAG; C). VEH: chronic vehicle treatment; CTRL: control conditions * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

6.11. Effect of chronic corticosterone treatment on TPH isoform gene expression in adrenal glands

In accordance with our results regarding the effect of chronic CORT treatment on TPH activity, we found that chronic CORT administration induced no change in TPH1 mRNA levels in adrenals from both sides (Fig. 21, A and B), but it caused a significant increase in TPH2 mRNA expression levels in LAG only (Fig. 21, C and D) as compared to (CTRL) and vehicle treatments.

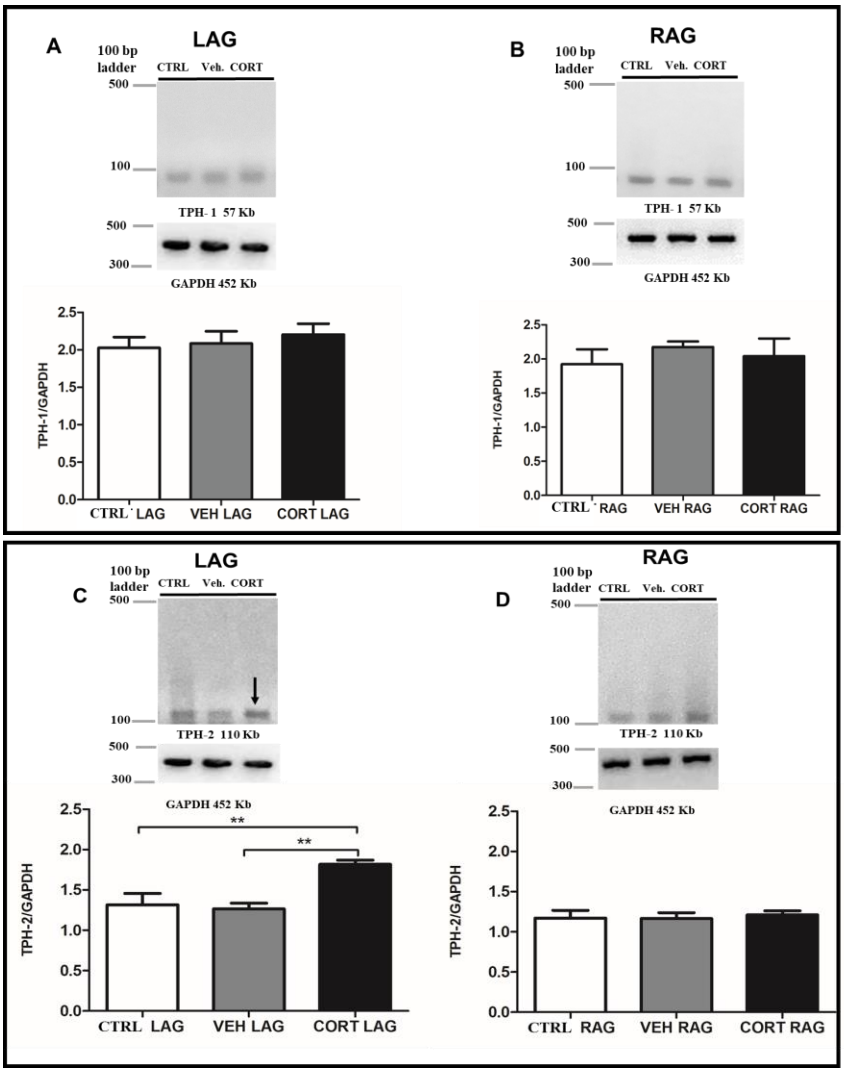


Figure 21. The effect of chronic corticosterone (CORT) treatment on TPH mRNA expression in adrenal glands. Panels show TPH1 (A and B) and TPH2 (C and D) mRNA levels in the left (LAG) and right adrenal glands (RAG). VEH: chronic vehicle treatment; CTRL: control conditions. ** $P < 0.01$

6.12. Effect of chronic corticosterone treatment on 5-HT-like immunoreactivity in adrenal glands

Sensitized stress-induced CORT secretion in chronically stressed rats is paralleled by strong 5-HT-like immunoreactivity (5-HT-LI) in the adrenal cortex together with increased adrenal content and turnover of 5-HT (García-Iglesias et al., 2013). On this basis, we asked the question whether these chronic stress-induced changes in adrenal 5-HT might be glucocorticoid-

dependent. Thus, chronic CORT treatment induced a marked increase of 5-HT-LI in the cortex of the LAG (Fig. 22, C and F) as compared to CTRL (A and D) and VEH (B and E) treatments. Chronic CORT treatment however had no effect on 5-HT-LI in RAG (Fig. 22, I and K) as compared to CTRL (Fig. 22, G and J) and VEH (Fig. 22, H and K) treatments.

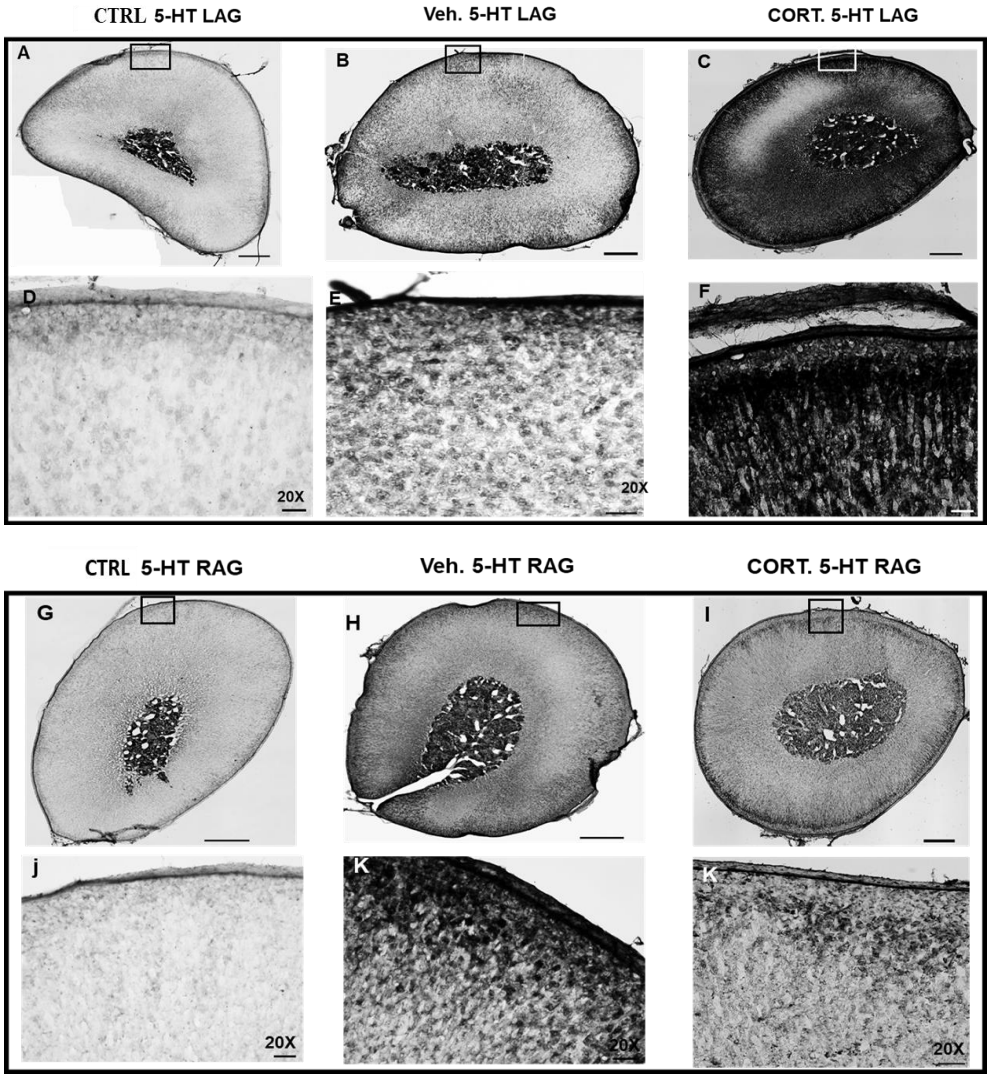


Figure 22. The effect of chronic corticosterone (CORT) treatment on 5-HT-like immunoreactivity in adrenal glands. Panels show 5-HT immunostaining in the left (LAG; A-F) and right adrenal glands (RAG; G-K). VEH: chronic vehicle treatment; CTRL: control conditions.

6.13. Effect of chronic corticosterone treatment on 5-HT levels in adrenal glands

In agreement with the above immunohistochemical observations (Fig. 22), chronic CORT treatment produced, as compared to CTRL and VEH treatments, a significant increase of 5-HT levels both in the LAG and RAG, with the effect being more prominent in the former (Fig. 23).

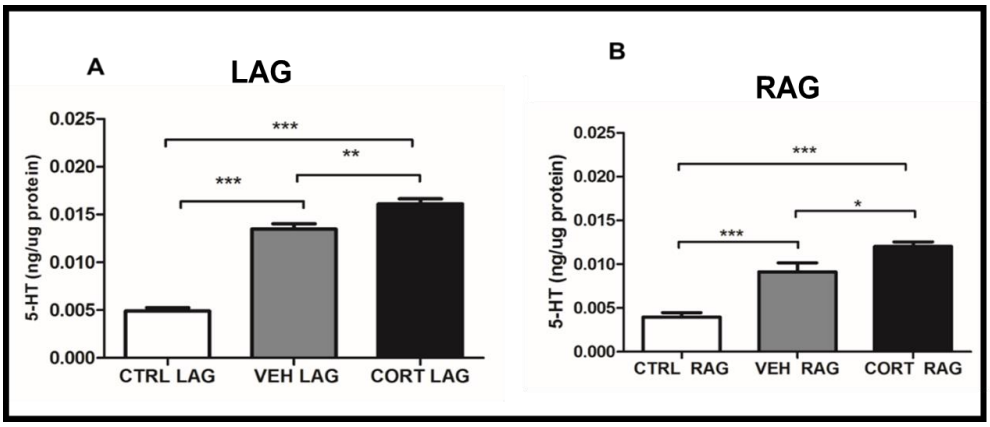


Figure 23. The effect of chronic corticosterone (CORT) treatment on 5-HT levels in adrenal glands. Panels show 5-HT content in the left (LAG; A) and right adrenal glands (RAG; B). VEH: chronic vehicle treatment; CTRL: control conditions. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

6.14. Effect of chronic corticosterone treatment on 5-HT7 receptor-like immunoreactivity in adrenal glands

Sensitized restraint-induced CORT responses in animals with a history of CRS involve activation of 5-HT7 receptors, most likely those which are overexpressed in the adrenal cortex of these animals (García-Iglesias et al., 2013). To determine whether the effect of CRS on 5-HT7 receptor expression in adrenocortical cells might be glucocorticoid-dependent we evaluated the effect of chronic CORT treatment on 5-HT7 receptor-like immunoreactivity (5-HT7R-LI) in adrenal gland sections and found that the treatment strongly increased it in the cortex of the LAG (Fig. 24, C and F) as compared to CTRL (Fig. 24, A and D) and VEH (Fig. 24, B and E) treatments, whereas no clear changes were observed in the RAG (Fig. 24, G to L).

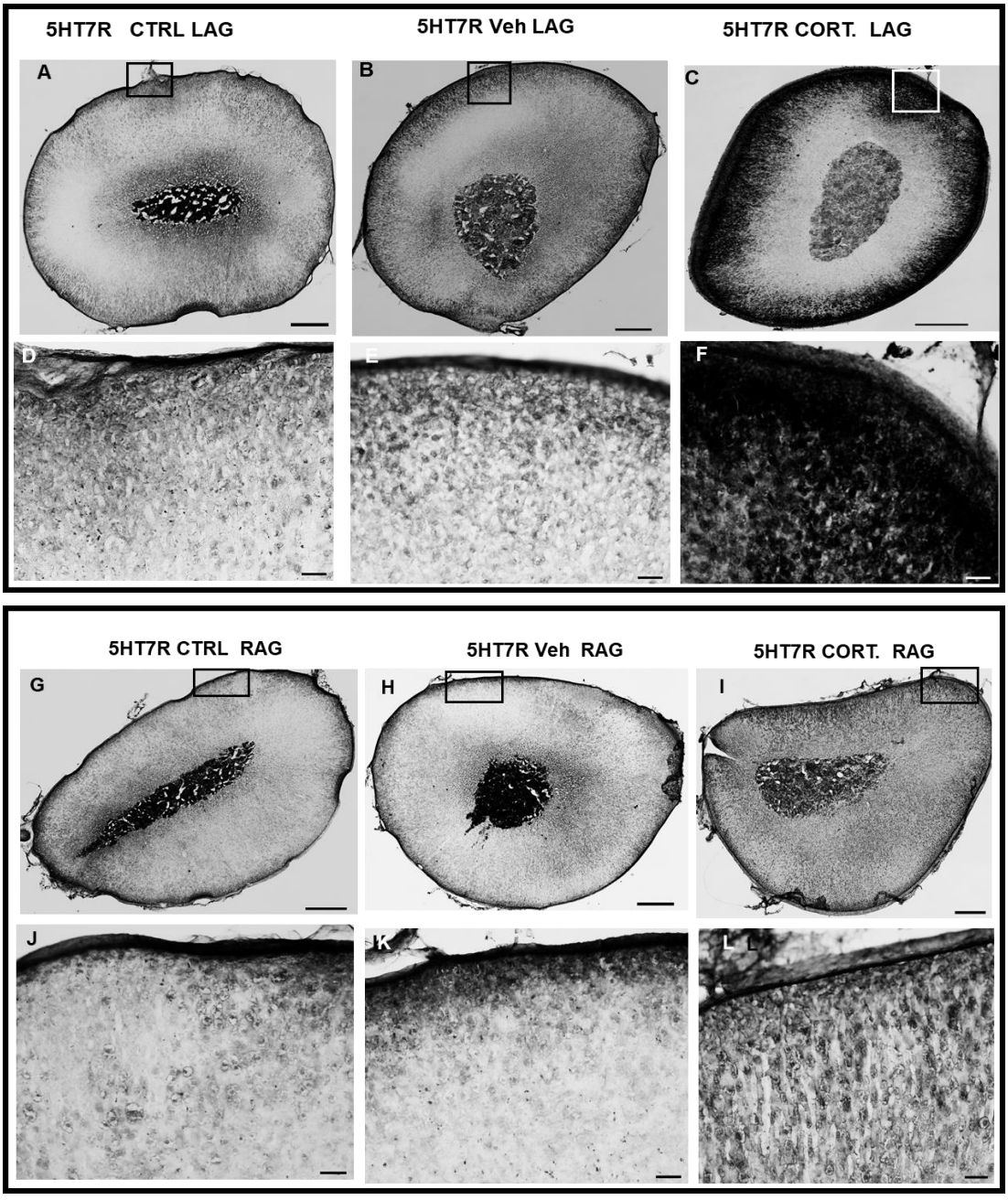


Figure 24. The effect of chronic corticosterone (CORT) treatment on 5-HT7 receptor-like immunoreactivity in adrenal glands. Panels show 5-HT7 receptor immunostaining in the left (LAG; A to F) and right adrenal glands (RAG; G to L). VEH: chronic vehicle treatment; CTRL: control conditions.

6.15. Effect of chronic corticosterone treatment on 5-HT7 receptor protein levels in adrenal glands

In keeping with the above immunohistochemical observations in adrenal glands, chronic CORT induced a significant increase of 5-HT7 receptor protein content in the LAG (Fig. 25, panel A), but not in the RAG (Fig. 25, panel B).

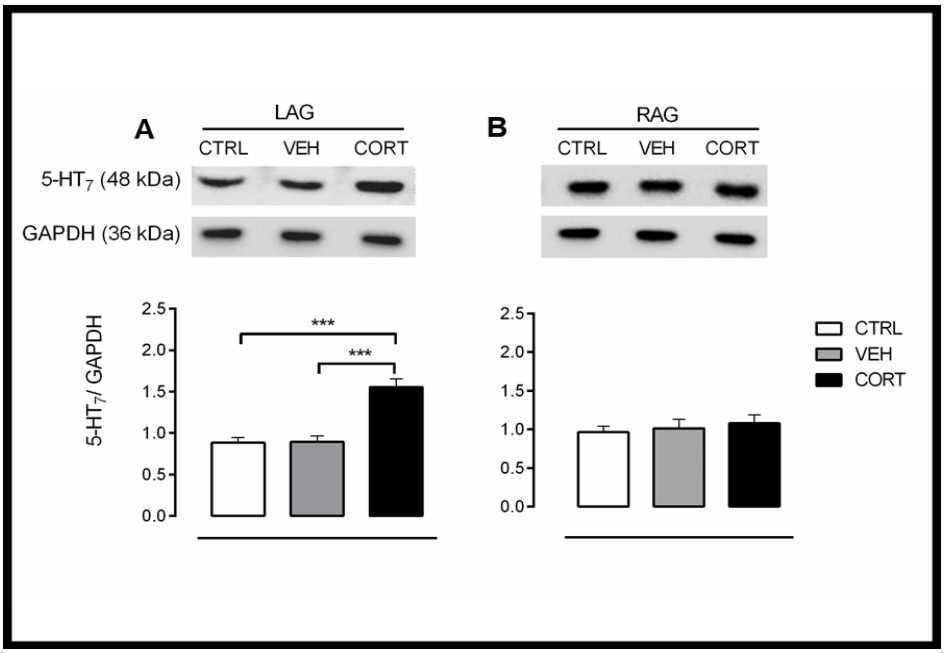


Figure 25. The effect of chronic corticosterone (CORT) treatment on 5-HT7 receptor protein levels in adrenal glands. Panels show 5-HT7 receptor protein content in the left (LAG; A) and right adrenal glands (RAG; B). VEH: chronic vehicle treatment; CTRL: control conditions. *** $P < 0.001$

6.16. Effect of chronic corticosterone treatment on 5-HT7 receptor mRNA expression in adrenal glands

In order to determine whether the above changes of 5-HT7-RLI and 5-HT7 receptor protein levels in left adrenals from animals receiving chronic CORT administration might be accounted for by increased expression, the 5-HT7 receptor mRNA levels were measured by RT-PCR

assays. As expected, chronic CORT treatment induced a significant increase of 5-HT7 receptor mRNA expression levels in the LAG (Fig. 26, panel A), but not in the RAG (Fig. 26, panel B), as compared to CTRL and VEH treatments.

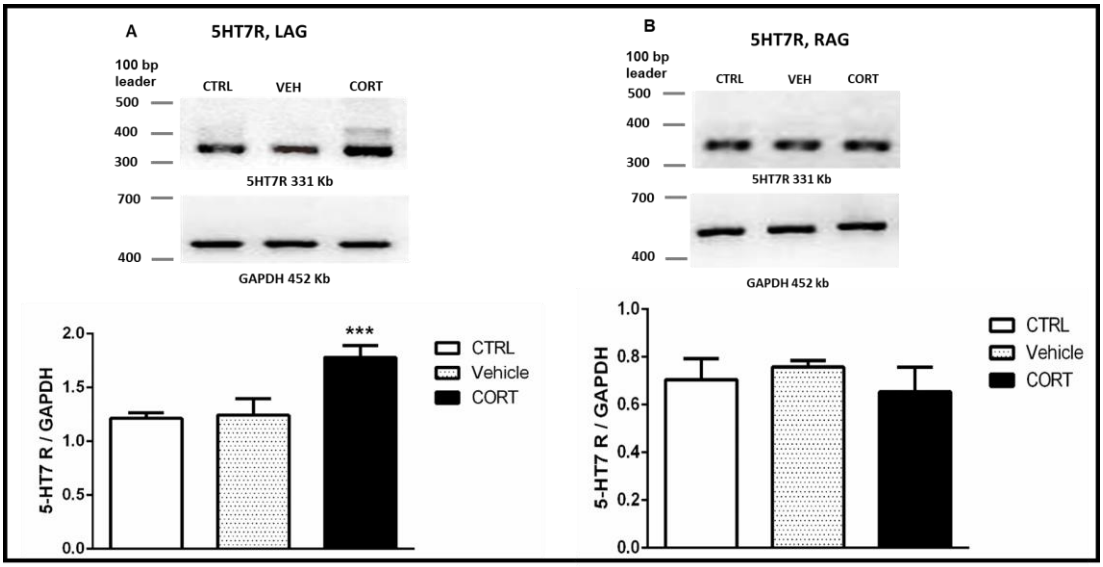


Figure 26. The effect of chronic corticosterone (CORT) treatment on 5-HT7 receptor mRNA levels in adrenal glands. Panels show 5-HT7 receptor mRNA expression in the left (LAG, A) and right adrenal glands (RAG, B). VEH: chronic vehicle treatment; CTRL: control conditions. *** $P < 0.001$

6.17. Effect of chronic corticosterone treatment on baseline and restraint-induced ACTH and corticosterone secretion

It has been reported that exposure to CRS induces sensitization of restraint-induced CORT secretion in parallel with blunted restraint-induced ACTH responses (García-Iglesias et al., 2013). In contrast to this effect of CRS exposure however, chronic CORT administration resulted in a significant decrease of restraint-induced ACTH (Fig. 27, A) and CORT (Fig. 27, B) responses at 30 min of restraint only as compared to chronic VEH administration.

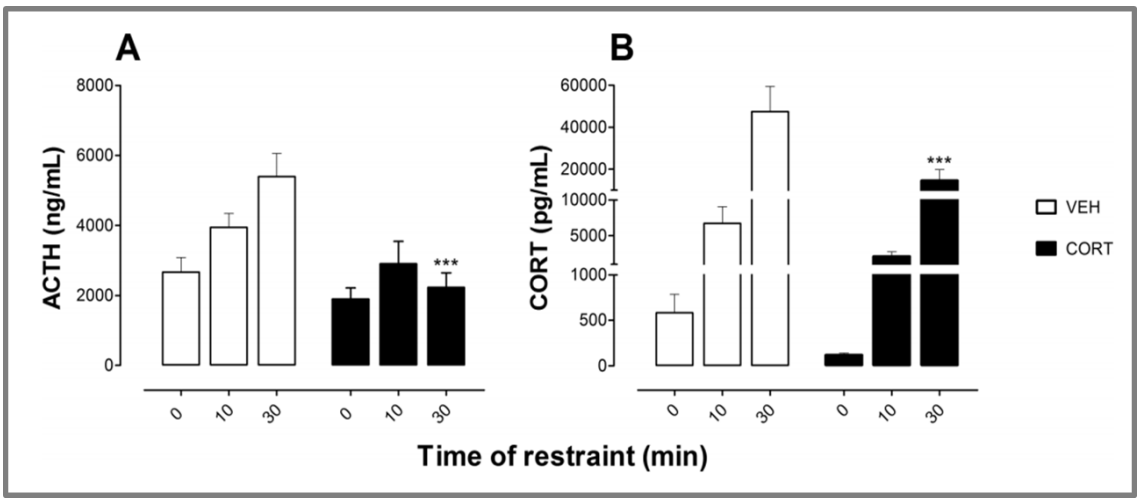


Figure 27. The effect of chronic glucocorticoid treatment on ACTH and CORT secretion levels. Panels show baseline (0 min) and restraint-induced ACTH (A) and CORT (B) secretion. CORT: chronic corticosterone treatment; VEH: chronic vehicle treatment. *** $P < 0.001$

6.18. Effect of p-chlorophenylalanine pretreatment on chronic stress-induced alterations

The rationale underlying the use of the TPH inhibitor, pCPA, in the present study is that chronic stress-induced endocrine dysregulation, which is paralleled by a number of CRS-induced adrenocortical alterations, seems to involve the development of a stimulatory serotonergic loop in the adrenal cortex. These adrenocortical alterations resulting from CRS exposure include increased adrenal 5-HT levels and turnover, and ectopic expression of 5-HT7 receptors. On these bases, we hypothesized that increased adrenal CRS-induced 5-HT content might be due to increased 5-HT synthesis via TPH expression and activity. Thus, we tested the effects of pCPA pretreatment (given from days 9^{th} to 12^{th} of the 14-day treatments), on the number of alterations induced by CRS exposure as compared to CTRL conditions.

6.18.1. Effect of p-chlorophenylalanine pretreatment on chronic restraint stress-induced somatometric changes

In support of a role of the 5-HT system in modulating feeding behavior (Medeiros et al., 2005), pretreatment with pCPA itself caused a decrease of body weight gain in CTRL animals as compared to CTRL rats that received VEH (Fig. 29, A). Furthermore, pCPA pretreatment prevented the decrease of body weight gain induced by CRS exposure (i.e. no significant change between VEH- and pCPA-pretreated CRS animals was observed) (Fig. 29, A). In addition, pCPA pretreatment produced a partial but significant reversal of CRS-induced decrease of food intake so that no significant difference between VEH- and pCPA-pretreated chronically stressed animals was detected at the end of the chronic stress treatment (Fig. 29, B). Interestingly, whereas pCPA pretreatment had no effect on CRS-induced thymus involution (Fig. 29, C), it did prevent CRS-induced increase of relative adrenal gland weight (Fig. 29, D and E).

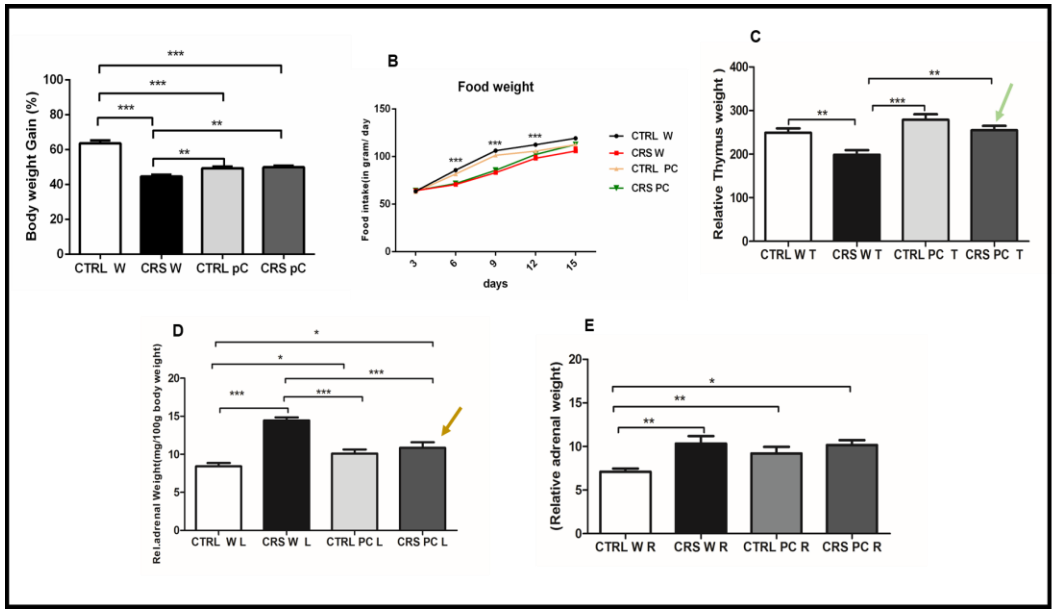


Figure 28. Effect of TPH inhibition on chronic restraint stress (CRS)-induced somatometric changes. Panels show the effect of CRS on body weight gain (A), food intake (B), relative thymus weight (C) and relative left (LAG; D) and right RAG; E) adrenal gland weight. CTRL: control conditions; pCPA: *p*-clorophenylalanine. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

6.18.2. Effect of p-chlorophenylalanine pretreatment on chronic restraint stress-induced TPH2-like immunoreactivity in adrenal glands

On the basis of our findings showing the ability of CRS exposure to induce strong TPH2-LI -but not TPH1-LI- in the adrenal cortex as compared to CTRL tissues (Fig. 11), we analysed the effect of TPH inhibition with pCPA pretreatment. Thus, CRS-induced TPH2-LI in the cortex of the LAG was actually prevented by pCPA pretreatment (Fig. 29, D and H) as compared to Veh (Fig. 29, B and F). Pretreatment with pCPA produced a noticeable decrease of TPH2-LI in the LAG from CTRL animals (Fig. 29, C and G) as compared to Veh (Fig. 29, A and E) while having no effect on TPH2-LI in the RAG and on TPHI-LI in LAG and RAG from CTRL and CRS-exposed animals (results not shown).

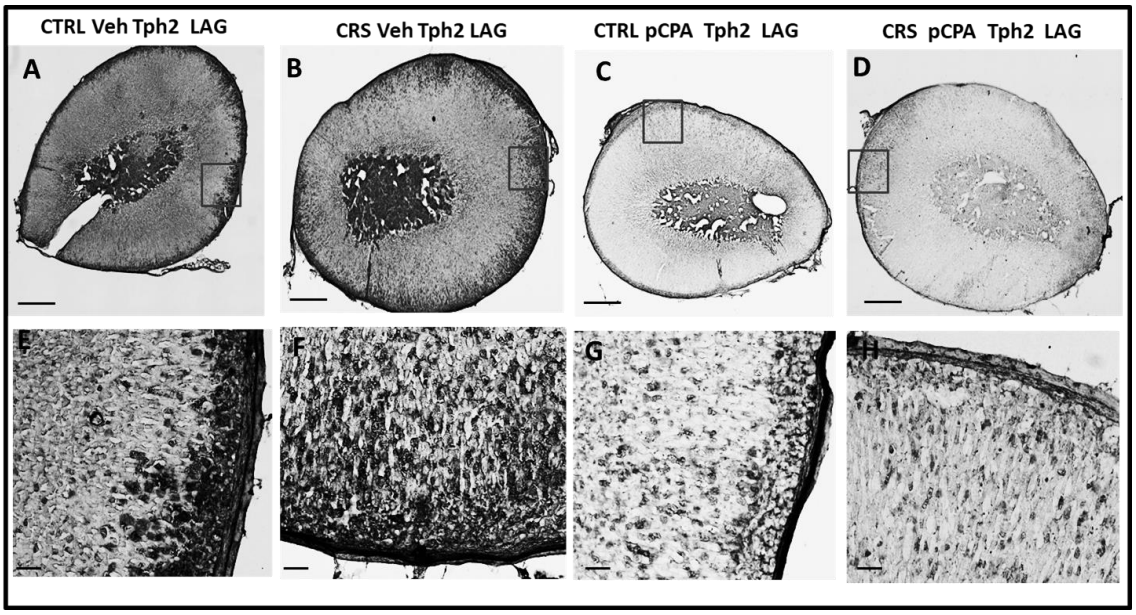


Figure 29. The effect of p-chlorophenylalanine (pCPA) pretreatment on TPH2-like immunoreactivity (TPH2-LI). Panels show TPH2 immunostaining in left adrenal glands (LAG) taken from animals exposed to chronic restraint stress (CRS; B and F) or control conditions (CTRL; A and E). Strong adrenocortical TPH2-LI induced by CRS exposure was abolished by pCPA (D and H).

6.18.3. Effect of p-chlorophenylalanine pretreatment on chronic restraint stress-induced increase of TPH protein levels in adrenal glands

In support of a potential role of TPH2 expression and activity in CRS-induced increase of 5-HT levels in the adrenal glands (see Fig. 12), specifically in the cortex (García-Iglesias et al., 2013; present results), pCPA pretreatment prevented the increase of TPH2 protein levels in LAG (Fig. 30, A), but not in RAG (Fig. 30, B) as a result of CRS exposure. Interestingly, pretreatment with pCPA also induced a significant decrease of TPH2 levels in adrenals from CTRL animals (Fig. 30, A and B) thus suggesting that the enzyme has baseline activity under CTRL conditions. Pretreatment with pCPA had no effect on adrenal TPH1 protein levels (results not shown), which is indeed not sensitive to CRS exposure (see Figs 13 and 15)

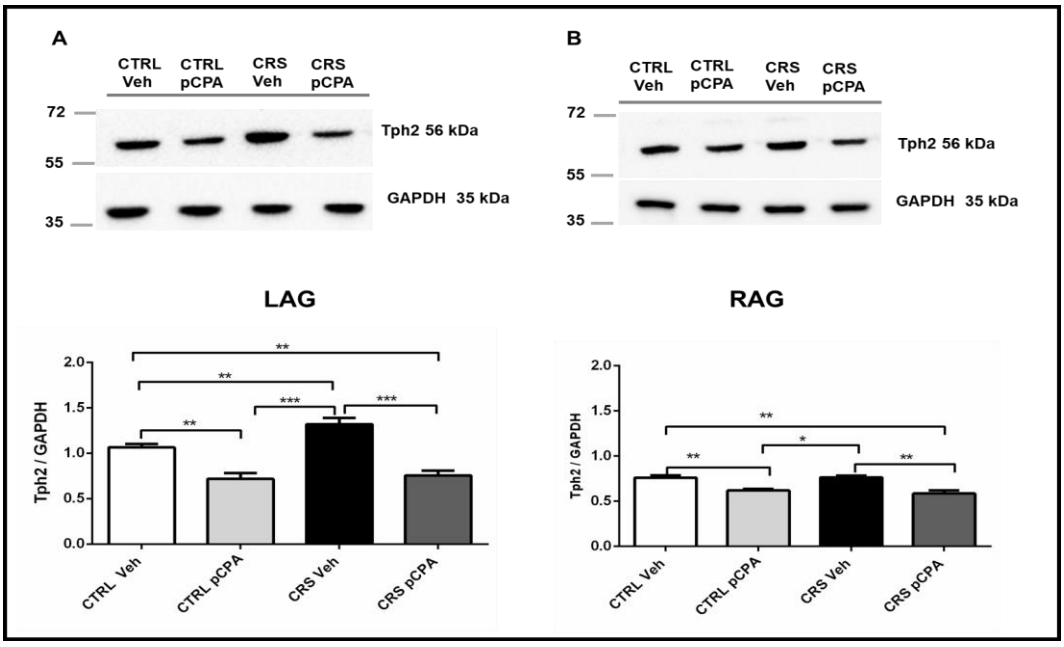


Figure 30. The effect of p-chlorophenylalanine (pCPA) pretreatment on TPH2 protein levels in adrenal glands. Panels show the effect of chronic restraint stress (CRS) as compared to control conditions (CTRL) in the left (LAG) and right adrenal glands (RAG). Exposure to CRS increased TPH2 protein levels in the LAG (A) but not in the RAG (B), and this effect was prevented by pCPA pretreatment. TPH2 protein levels in LAG and RAG from CTRL animals were also decreased by pCPA pretreatment. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

6.18.4. Effect of p-chlorophenylalanine pretreatment on chronic restraint stress-induced changes in TPH mRNA expression levels in adrenal glands

In close agreement with the effect of pCPA pretreatment on CRS-induced changes in TPH2-LI (Fig. 29) and TPH2 protein levels (Fig. 30) in the LAG, but not in the RAG, pCPA pretreatment similarly induced a significant decrease of TPH2 mRNA levels in the LAG (Fig. 31, A) but not in the RAG (Fig. 31, B) thus suggesting that the inhibitor's effect may occur at gen level. Pretreatment with pCPA had no effect on adrenal TPH1 mRNA levels (results not shown), which are actually not sensitive to CRS exposure (see Fig. 15)

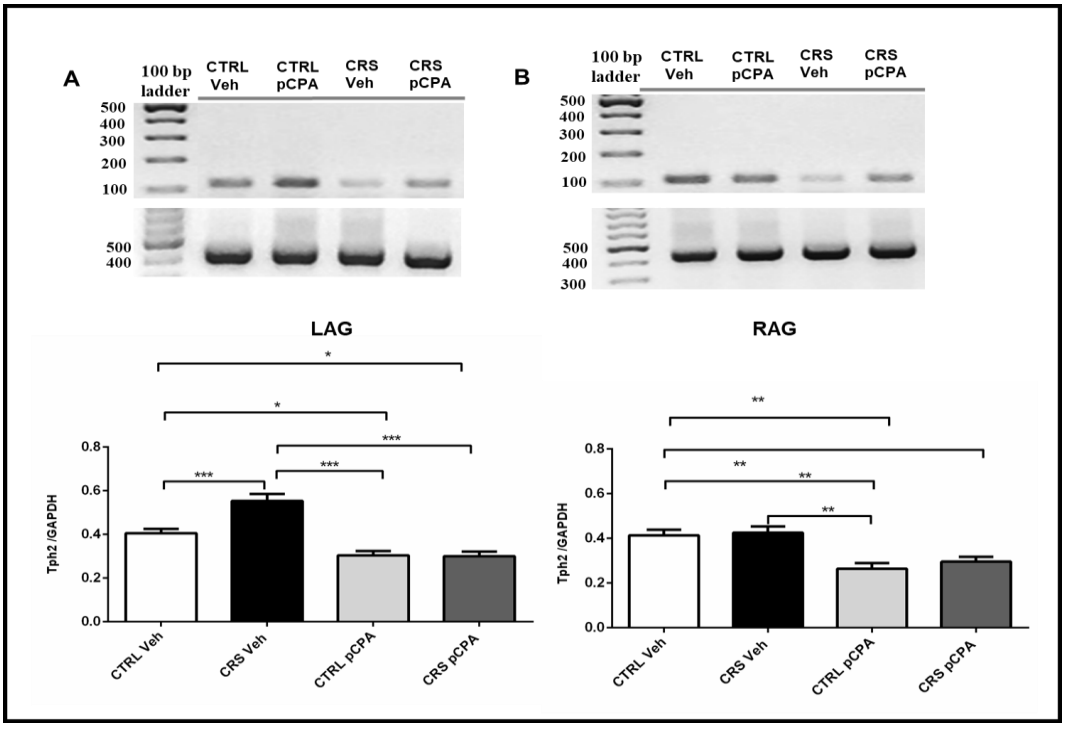


Figure 31. The effect of p-chlorophenylalanine (pCPA) pretreatment on TPH2 mRNA levels in adrenal glands. Panels show the effect of chronic restraint stress (CRS) as compared to control conditions (CTRL) on TPH2 mRNA expression in the left (LAG) and right adrenal glands (RAG). Exposure to CRS increased TPH2 mRNA levels in the LAG (A) but not in the RAG (B), and this effect was prevented by pCPA pretreatment (A). TPH2 mRNA levels in LAG and RAG from CTRL animals were also decreased by pCPA pretreatment. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

6.18.5. Effect of p-chlorophenylalanine pretreatment on chronic restraint stress-induced changes of TPH activity in adrenal glands

Further to the ability of CRS exposure to induce TPH2-LI as well as increased TPH2 protein and mRNA levels in LAG, and the ability of pCPA pretreatment to inhibit such CRS-induced changes (see Figs 29-31), we asked the question whether CRS exposure could also induce an increase in TPH activity, and this effect could be antagonized by TPH inhibition with pCPA. Hence, exposure to CRS resulted in a significant increase of TPH activity in the LAG (Fig. 32, B) but not in the RAG (Fig. 32, C). On the other hand, CRS exposure induced a significant decrease of TPH activity in the DRN (Fig. 32, A). Pretreatment with pCPA significantly inhibited the above CRS-induced changes in the LAG (Fig. 32, B) and DRN (Fig. 32, A). Further, TPH activity was decreased by pCPA in CTRL tissues (Fig. 32).

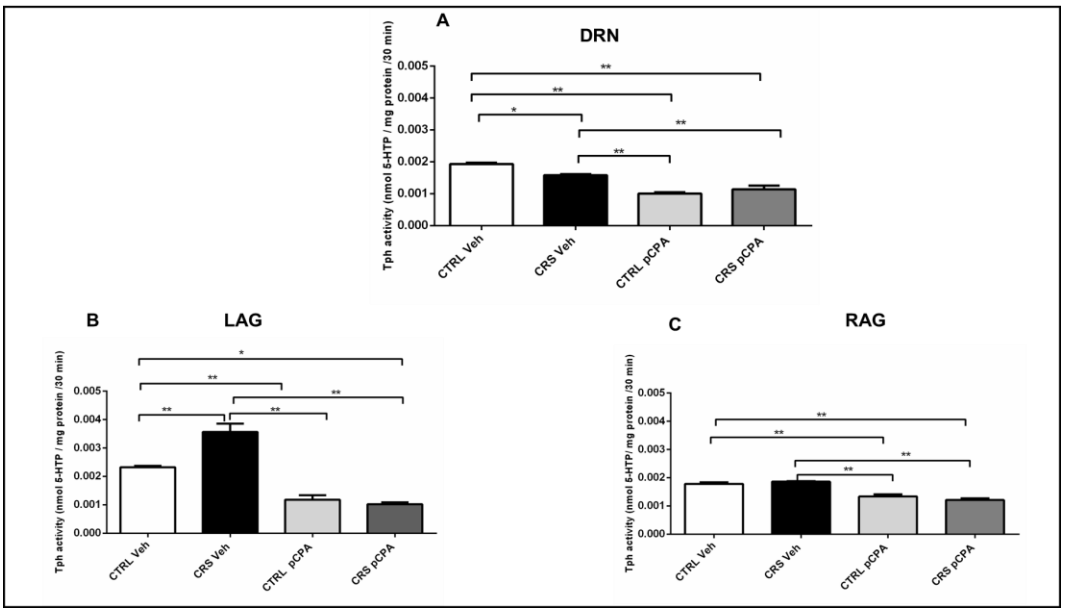
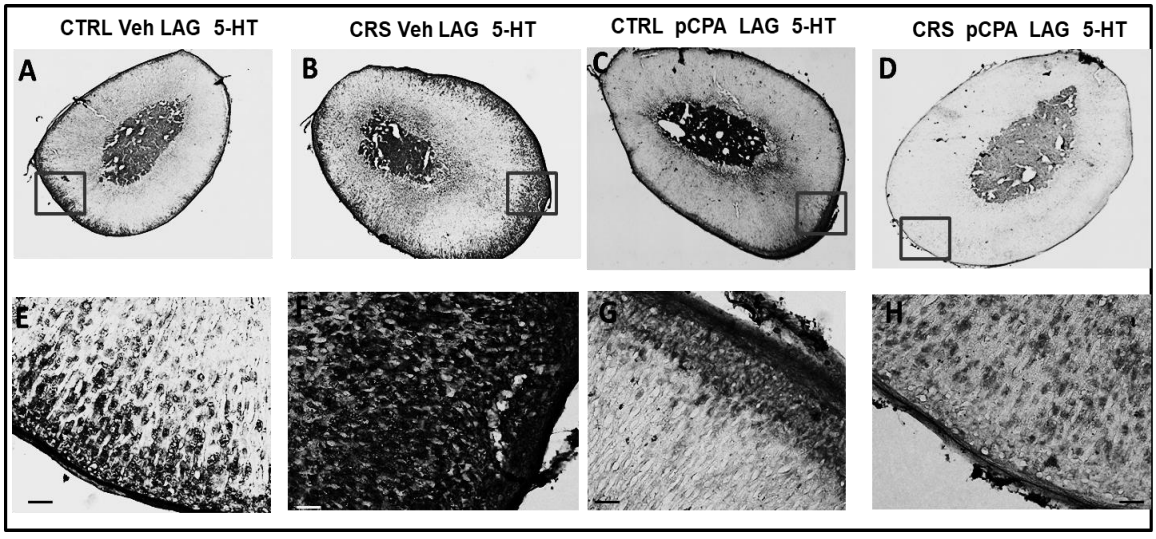


Figure 32. The effect of p-chlorophenylalanine (pCPA) pretreatment on TPH activity. Panels show the effect of chronic restraint stress (CRS) as compared to control conditions (CTRL) in the dorsal raphe nucleus (DRN; A) and the left (LAG; B) and right adrenal glands (RAG; C). Pretreatment with pCPA reversed CRS-induced changes in the DRN (A) and LAG (B) and decreased TPH activity in tissues from CTRL animals. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

6.18.6. Effect of p-chlorophenylalanine pretreatment on 5-HT-like immunoreactivity in adrenal glands

Since hydroxylation of L-tryptophan by TPH is the rate-limiting step in the biosynthesis of 5-HT, it was essential to test whether or not inhibition of TPH activity by pCPA pretreatment would prevent CRS-induced increase of 5-HT in adrenal glands, as determined by immunohistochemistry, and HPLC measurements. Then, as expected from our previous findings (García-Iglesias et al., 2013), exposure to CRS induced a remarkable increase of 5-HT-like immunoreactivity (5-HT-LI) in the adrenal cortex, and this effect was much more prominent in the LAG (Fig. 33, B and F) than in the RAG (Fig. 33, J and N) as compared to CTRL tissues (Fig. 33, A and E, and I and M, respectively). These CRS -induced changes were completely abolished by pCPA pretreatment both in the LAG (Fig. 33, D and H) and RAG (Fig. 33, L and P) thus suggesting the involvement of adrenocortical TPH activity.



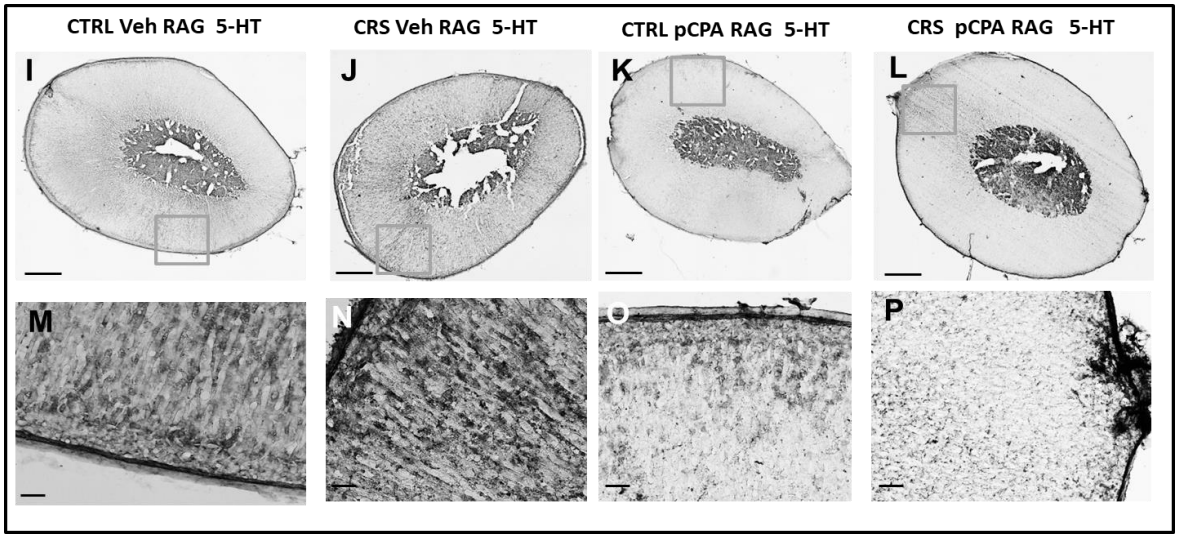


Figure 33. The effect of p-chlorophenylalanine (pCPA) pretreatment on 5-HT-like immunoreactivity (5-HT-LI) in adrenal glands. Panels show 5-HT immunostaining in the left (LAG; A-H) and right adrenal glands (RAG; I-P) from animals exposed to chronic restrain stress (CRS) or control conditions (CTRL). The strong CRS-induced 5-HT-LI in the LAG (B and F) was completely absent in LAG from animals that reveived pCPA pretreatment (D and H) as compared to vehicle (Veh). A similar effect was observed in the RAG taken from pCPA-pretreated rats (L and P).

6.18.7. Effect of p-chlorophenylalanine pretreatment on chronic restraint stress-induced changes in 5-HT levels and turnover, and L-trytophan levels in adrenal glands

Consistent with the notion that chronic stress-induced increase of adrenal 5-HT levels and turnover may involve *de novo* synthesis of the monoamine via TPH activity, pretreatment with pCPA completely inhibited CRS-induced magnification of adrenal levels of 5-HT (Fig. 34, A), 5-HIAA (Fig. 34, B), turnover (5-HIAA/5-HT; Fig. 34, C) and L-tryptophan (Fig. 34, D) in LAG. In contrast, CRS exposure had no effects on these variables in the RAG (Fig. 34, E, F, G and H). In addition, pCPA pretreatment induced a significant decrease of 5-HT and 5-HIAA levels, but not of 5-HIAA/5-HT ratio, in adrenals from CTRL animals, thereby suggesting that TPH might display intrinsic baseline activity in these tissues.

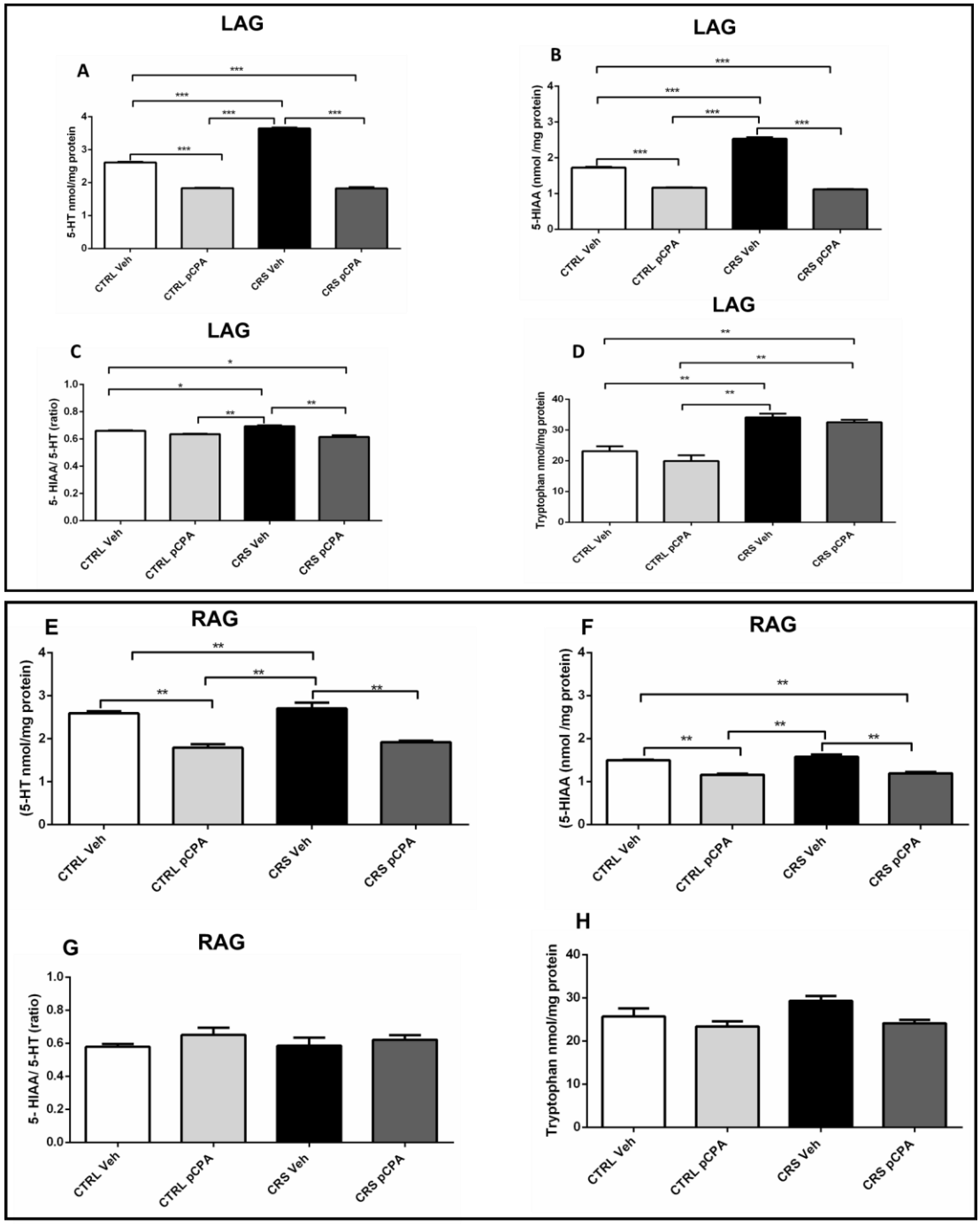


Figure 34. The effect of p-chlorophenylalanine (pCPA) pretreatment on 5-HT and 5-HIAA levels, 5-HT turnover and L-tryptophan levels in adrenal glands. Panels show observations in the left (LAG; A-D) and right adrenal glands (RAG; E-H) from animals submitted to chronic restraint stress (CRS) or control (CTRL) conditions. Pretreatment with pCPA abolished CRS-induced increase of 5-HT, 5-HIAA, and 5-HIAA/5-HT ratio but not that of L-tryptophan in LAG, and it significantly decreased 5-HT and 5-HIAA levels in LAG and RAG taken from CTRL animals. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

In order to exclude the possibility that the inhibitory effect of pCPA on 5-HT-LI as well as on adrenal 5-HT and 5-HIAA levels and turnover (5-HIAA/5-HT) might have been caused by a decreased availability of the TPH substrate, L-tryptophan, as a result of CRS exposure, the adrenal levels of this essential aminoacid were also measured by HPLC both in CTRL and CRS exposed animals. Thus, exposure to CRS induced a significant increase of L-tryptophan levels in the LAG (Fig. 35, D) but not in the RAG (Fig. 35, H) as compared to CTRL conditions. This change of L-tryptophan levels in the LAG was not modified by pCPA pretreatment (Fig. 35, D) thus suggesting that the effect of pCPA on 5-HT-LI (Fig. 34) and adrenal 5-HT levels and turnover (Fig. 35, A-C) was most likely due to a direct inhibitory interaction of pCPA with TPH.

6.18.8. Effect of p-chlorophenylalanine pretreatment on chronic restraint stress-induced 5-HT7 receptor expression in adrenal glands

The effect of pCPA pretreatment on CRS-induced expression of 5-HT7 receptors in adrenal glands, as determined by 5-HT7 receptor immunohistochemistry and 5-HT7 receptor protein measurements by Western blot assays, was analysed. Thus, exposure to CRS induced strong 5-HT7 receptor-like immunoreactivity (5-HT7-RLI) in the cortex of LAG (Fig. 35, B and F) as compared to CTRL conditions (Fig. 35, A and E), and also increased 5-HT7 receptor protein in the LAG (Fig. 36, A). Interestingly, these effects were prevented by pCPA pretreatment (Fig. 35, D and H; Fig. 36, A). Exposure to CRS had minor or no effects on 5-HT7-RLI in the RAG which were unaffected by pCPA pretreatment (results not shown). Similarly, CRS exposure had no significant effects on 5-HT7 receptor protein in the RAG (Fig. 36, B); pCPA pretreatment however did induce a significant decrease of 5-HT7 receptor protein in RAG taken from CRS-exposed animals (Fig. 36, B). Taken together, these observations support the contention that

CRS-induced 5-HT7 receptor expression in the cortex of LAG might involve *de novo* synthesized 5-HT via TPH2 activity.

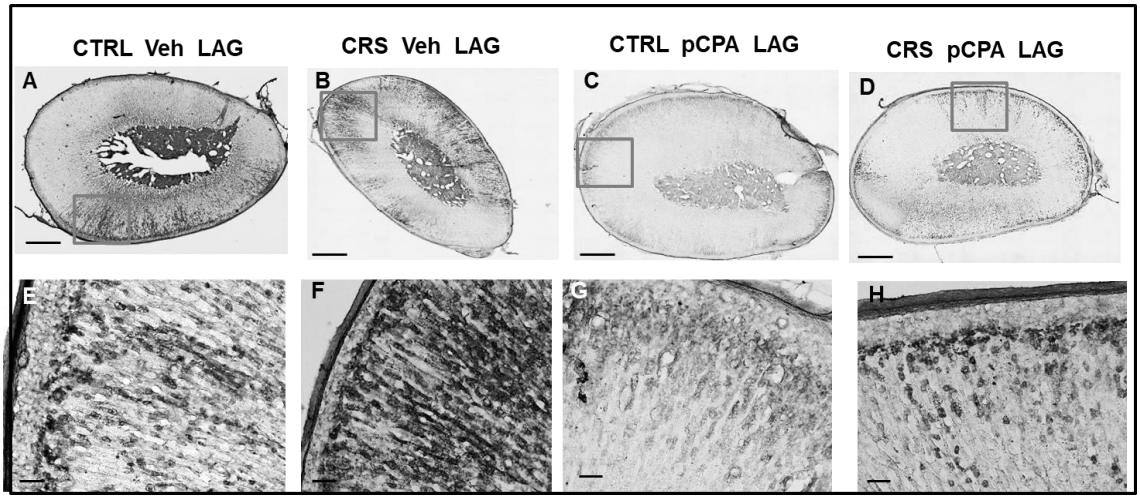


Figure 35. The effect of p-chlorophenylalanine (pCPA) on 5-HT7 receptor immunostaining in left adrenal glands (LAG). Exposure to chronic restraint stress (CRS) induced strong 5-HT7 receptor-like immunoreactivity (5-HT7-RLI; B and F) as compared to control conditions (CTRL; A and E); this effect was prevented by pCPA pretreatment (D and H). pCPA also decreased 5-HT7-RLI in CTRL tissues.

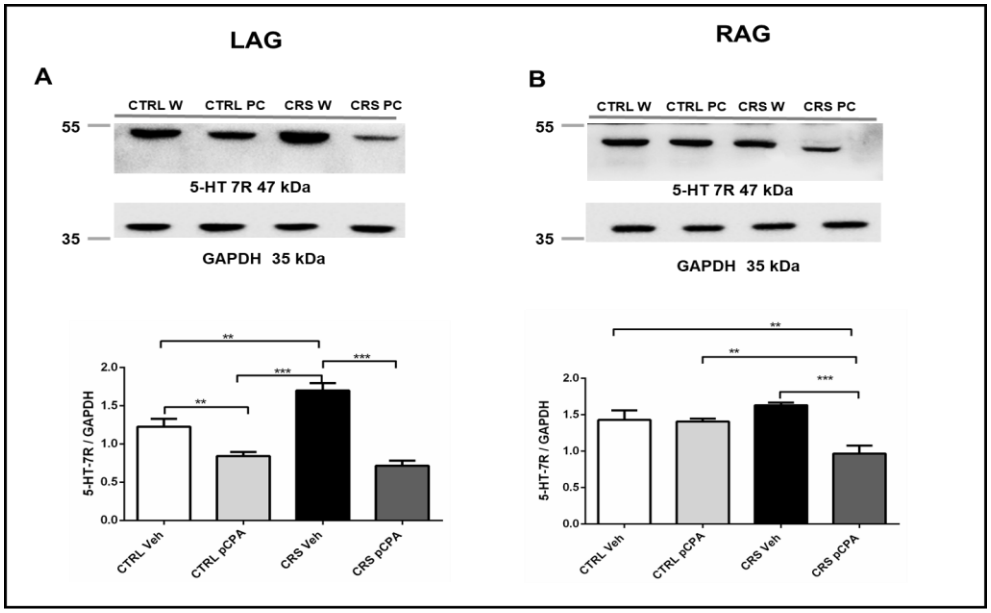


Figure 36. The effect of TPH inhibition on 5-HT7 receptor protein in adrenal glands. Exposure to chronic restraint stress (CRS) increased 5-HT7 receptor protein in the left (LAG; A) but not in the right adrenal glands (RAG; B) as compared to control conditions (CTRL). Pretreatment with p-chlorophenylalanine (pCPA) abolished CRS-induced increase of 5-HT7 receptor protein in LAG, and also decreased 5-HT7 receptor protein in RAG from CRS-exposed animals. ** $P < 0.01$; *** $P < 0.001$

6.18.9. Effect of p-chlorophenylalanine pretreatment on chronic restraint stress-induced changes in plasma ACTH and CORT

Exposure to CRS induced an increase of baseline secretion levels of CORT (Fig. 37, A) without altering baseline secretion of ACTH (Fig. 37, B). Interestingly, TPH inhibition with pCPA pretreatment significantly decreased baseline CORT secretion in CRS-exposed animals thus suggesting a role of TPH in CORT production in chronically stressed animals in the absence of acute stress.

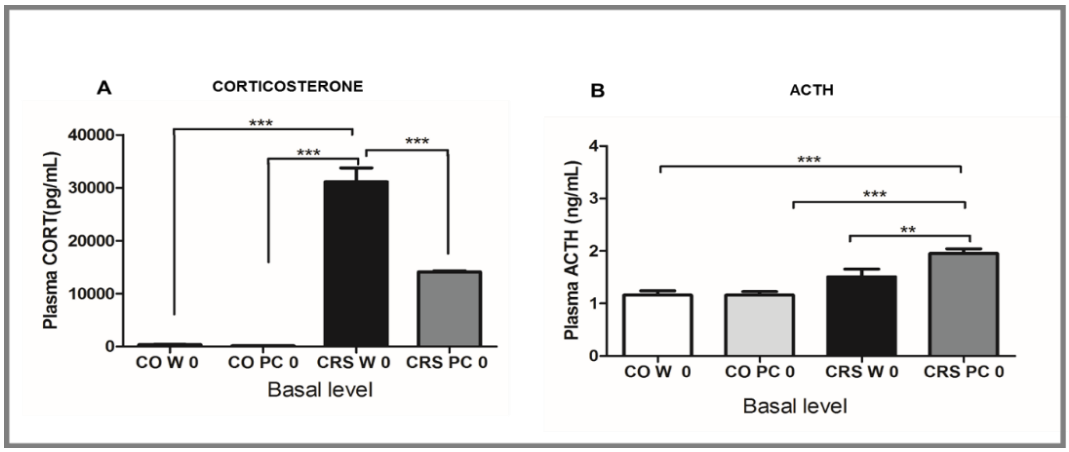


Figure 37. Effect of TPH inhibition on baseline hormone secretion. Baseline secretion of corticosterone (CORT; A) but not of ACTH (B) was increased by chronic restraint stress (CRS) as compared to control conditions (CTRL). Pretreatment with p-chlorophenylalanine (pCPA) significantly decreased baseline CORT secretion and increased that of ACTH in CRS-exposed animals. ** $P < 0.01$; *** $P < 0.001$

6.18.10. Effect of chronic restraint stress and chronic corticosterone treatment on glucocorticoid receptor mRNA levels in adrenal glands

In support of the notion that CRS-induced changes in the adrenal cortex may be a glucocorticoid-dependent phenomenon, we have recently reported that, similar to the effects of CRS exposure (García-Iglesias et al., 2013), chronic CORT treatment produced strong 5-HT7 receptor expression in the adrenal cortex and a significant increase of the adrenal 5-HT content as

compared to chronic VEH administration (Saroj et al., 2019). Since the above effects of CORT administration implicate activation of glucocorticoid receptors (GR), it was decided to analyse the expression levels of these in adrenals from CRS-exposed animals. Furthermore, in order determine whether potential CRS-induced changes in GR expression might be glucocorticoid-dependent, expression of GR was also evaluated in adrenals from animals that received a chronic CORT treatment as compared to VEH. Thus, CRS exposure resulted in a significant increase of GR mRNA levels in LAG (Fig. 38, A) but not in RAG (Fig. 38, B) as compared to CTRL conditions, thus further supporting the existence of asymmetry as to the impact of chronic stress exposure on adrenal gland function. These changes were mimicked by chronic CORT administration (Fig. 38, D) as compared to VEH treatment (Fig. 38, C).

6.18.11. Effect of p-chlorophenylalanine pretreatment on chronic restraint stress-induced changes in glucocorticoid receptor mRNA levels in adrenal glands

Available evidence suggests that GR expression in the rat hippocampus may be regulated by 5-HT via 5-HT7 receptor activation (Laplante et al., 2002). To determine whether a similar mechanism might underlie the effects of CRS exposure on GR expression in the adrenal glands, the GR mRNA levels were measured in adrenals from pCPA-pretreated animals. Thus, the increased levels of GR mRNA in LAG from CRS-exposed animals were completely blunted by pCPA pretreatment as compared to Veh (Fig. 39, A). Interestingly, significant decreases of GR mRNA levels were detected in the RAG from both CTRL and CRS-exposed animals (Fig. 39, B).

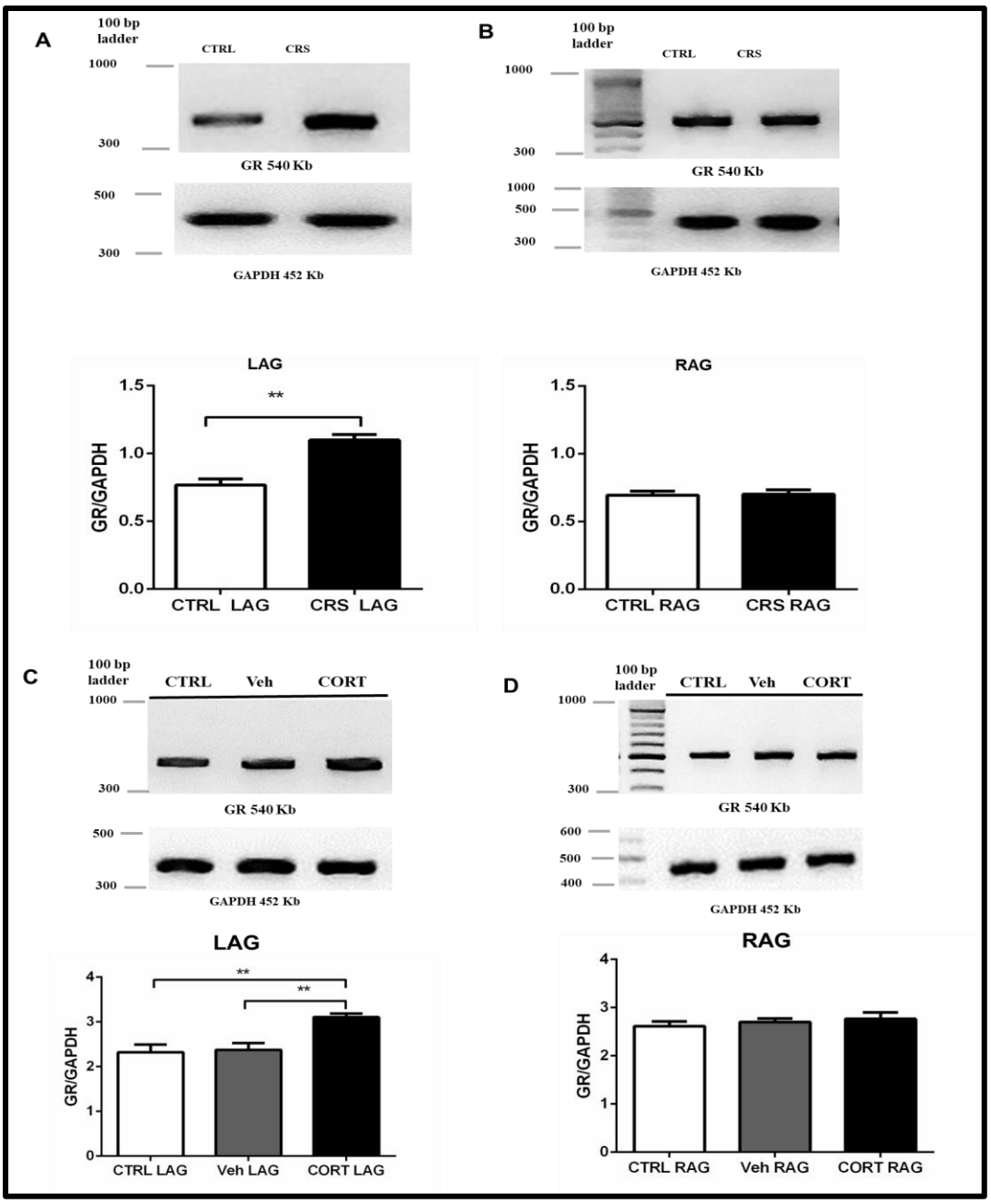


Figure 38. The effect of chronic restraint stress (CRS) and chronic corticosterone (CORT) treatment on glucocorticoid receptor (GR) mRNA expression in adrenal glands. CRS exposure increased GR mRNA levels in the left (LAG; A) but not in the right adrenal glands (RAG; B) as compared to control conditions (CTRL). These effects were mimicked by chronic CORT treatment (C) as compared to vehicle (VEH; D). ** $P < 0.01$

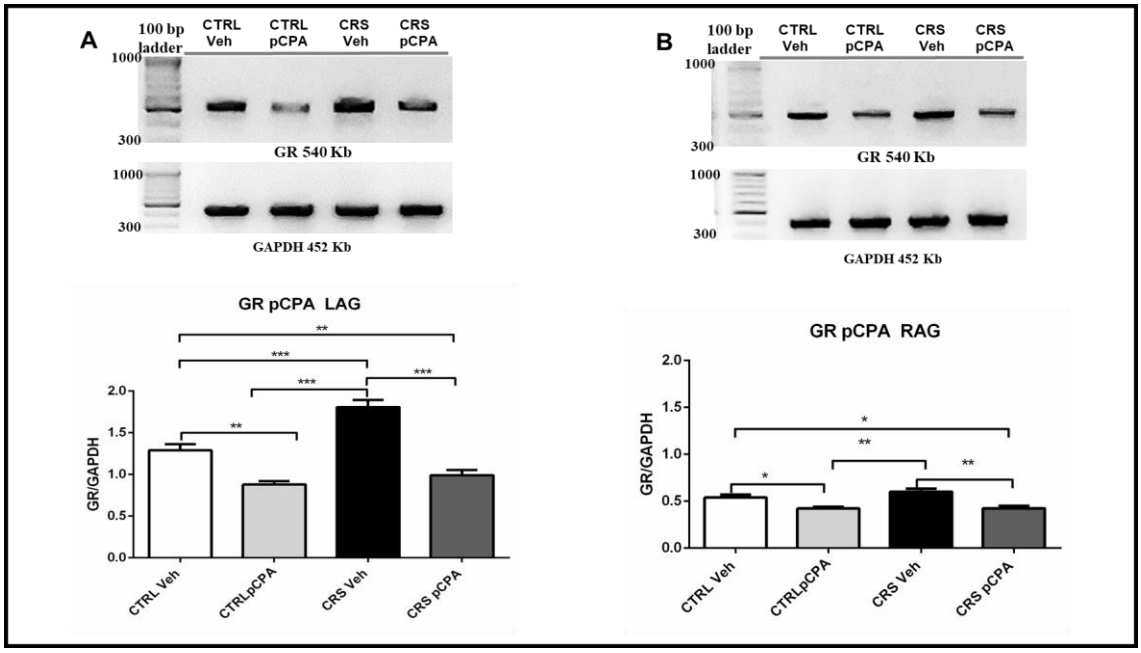


Figure 39. The effect of TPH inhibition on glucocorticoid receptor (GR) mRNA in adrenal glands. CRS exposure increased GR mRNA levels in the left (LAG; A) but not in the right adrenal glands (RAG; B) as compared to control conditions (CTRL). pCPA pretreatment decreased GR mRNA in LAG from CTRL animals and prevented CRS-induced increase of GR mRNA in the LAG (A); further, pCPA induced a significant decreased of GR mRNA in the RAG from both CTRL and CRS-exposed animals. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

6.18.12. Effect of chronic restraint stress and chronic corticosterone treatment on CREB mRNA expression levels in adrenal glands

The primary signalling pathway involved in ACTH-induced glucocorticoid production and secretion in the adrenal cortex comprises increased intracellular levels of cAMP and activation of PKA that in turn results in phosphorylation of cAMP response element-binding protein (CREB), which binds to cAMP response elements (CRE) and induces gene transcription and synthesis of steroidogenic proteins (Spiga et al., 2011; Walker et al., 2015). In an attempt to look at the activity of this transduction pathway subsequent to CRS exposure, the expression levels of CREB mRNA were measured by RT-PCR assays in whole adrenal glands. We also determined CREB mRNA levels in adrenals from animals that had received a chronic CORT treatment as

compared to VEH. Thus, exposure to CRS increased CREB mRNA levels in the LAG (Fig. 40, A) but not in the RAG (Fig. 40, B) as compared to CTRL conditions. These CRS-induced changes were closely mimicked by chronic CORT administration as a significant increase of CREB mRNA levels was observed in the LAG (Fig. 40, C) but not in the RAG (Fig. 40, D) from CORT-treated as compared to VEH-treated and CTRL animals thereby suggesting that CRS-induced CREB mRNA expression in the LAG is glucocorticoid-dependent.

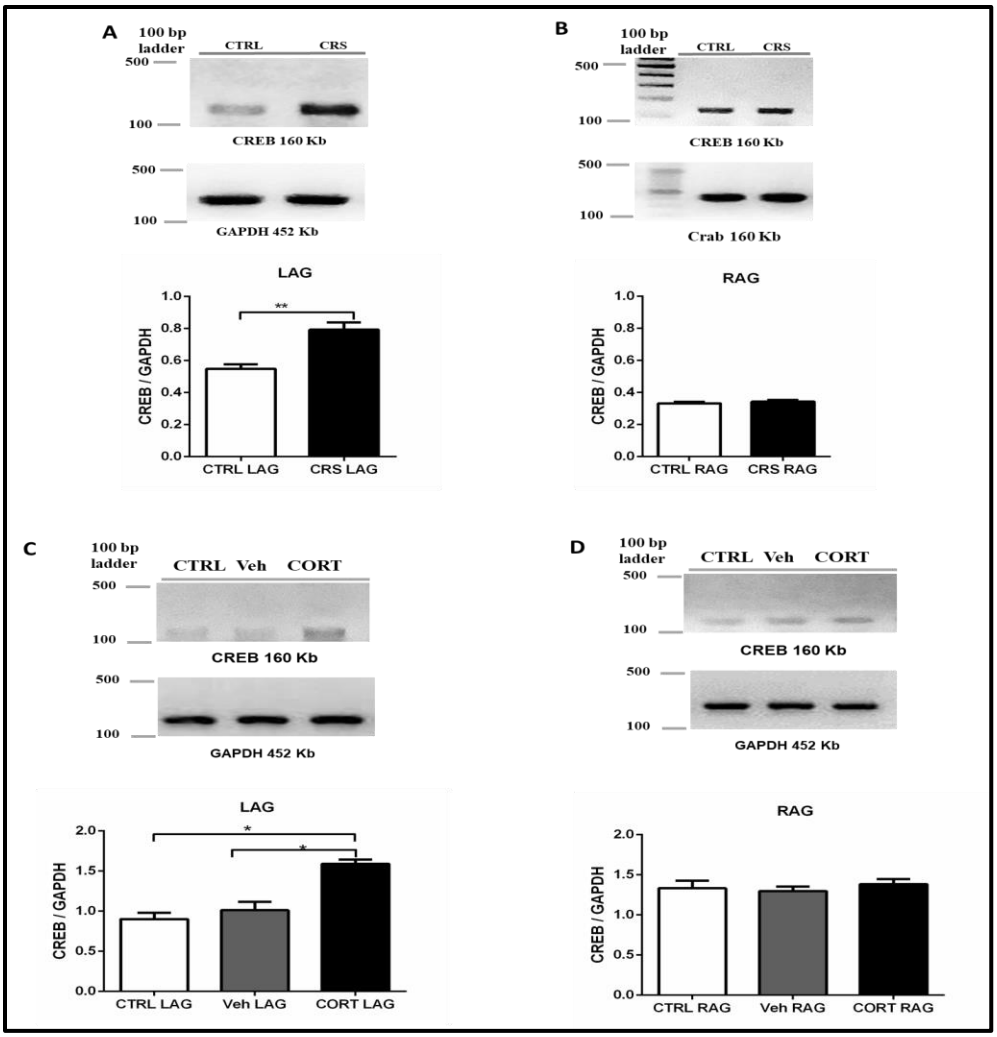


Figure 40. The effect of chronic restraint stress (CRS) and chronic corticosterone (CORT) treatment on cAMP response element-binding (CREB) mRNA in adrenal glands. CRS exposure increased CREB mRNA levels in the left (LAG; A) but not in the right adrenal glands (RAG; B) as compared to control conditions (CTRL). These effects were mimicked by chronic CORT treatment (C) as compared to vehicle (VEH; D). * $P < 0.05$; ** $P < 0.01$

6.18.13. Effect of p-chlorophenylalanine pretreatment on chronic restraint stress-induced changes in CREB mRNA levels in adrenal glands

Several lines of clinical evidence have unravelled the involvement of an ectopic serotonergic loop in mediating magnified glucocorticoid secretion in adrenocortical cells under conditions of high circulating levels of cortisol (Bram et al., 2016; Contesse et al., 2005; Lefebvre et al., 2015; Louiset et al., 2008). Interestingly, our own previous evidence (García-Iglesias et al., 2013; Saroj et al., 2019) together with the results of the present study suggest that a closely similar serotonergic loop seems to operate in mediating stress-induced glucocorticoid secretion in animals with a history of CRS. It has been shown that an important component of this serotonergic pathway is the activation of 5-HT7 receptors, which are known to be overexpressed in adrenocortical cells involved in steroidogenesis as a result of CRS exposure (García-Iglesias et al., 2013) or chronic CORT treatment (Saroj et al., 2019). It should be recalled that adrenal steroidogenesis is activated by the binding of ACTH to the specific melanocortin type-2 receptor (MC2R), a cell surface GPCR. Upon ACTH binding, MC2R undergoes conformational changes that activate adenylyl cyclase, leading to an increase in intracellular levels of cAMP and subsequent activation of pKA. Activation of PKA results in phosphorylation of cAMP response element-binding protein (CREB), which binds to cAMP response elements (CRE) and induces gene transcription and synthesis of steroidogenic proteins (Spiga et al., 2011). In view that the above signalling pathway of adrenocortical ACTH receptors (i.e. MC2R) is shared by 5-HT7 receptors (Ruat et al., 1993; Shen et al., 1993), it is likely that the functional role of the later in mediating sensitized restraint-induced CORT responses in chronically-stressed animals (García-Iglesias et al., 2013) might involve phosphorilation of CREB and subsequent binding of it to CRE to induce transcription of steroidogenic protein gens. Since activation of adrenocortical 5-

HT7 receptors requires the availability of 5-HT, most likely synthesized *de novo* via TPH2 activity (present results), it was decided to test whether inhibition of TPH with pCPA pretreatment -thus leading to decreased availability of 5-HT- would decrease the expression levels of CREB. Thus, CRS exposure increased CREB mRNA levels in the LAG (Fig. 41, A) but not in the RAG (Fig. 41, B) and this effect was prevented by pCPA pretreatment as compared to Veh thus suggesting that 5-HT-mediated activation of adrenocortical 5-HT receptors (i.e. 5-HT7) may actually involve CREB gen transcription.

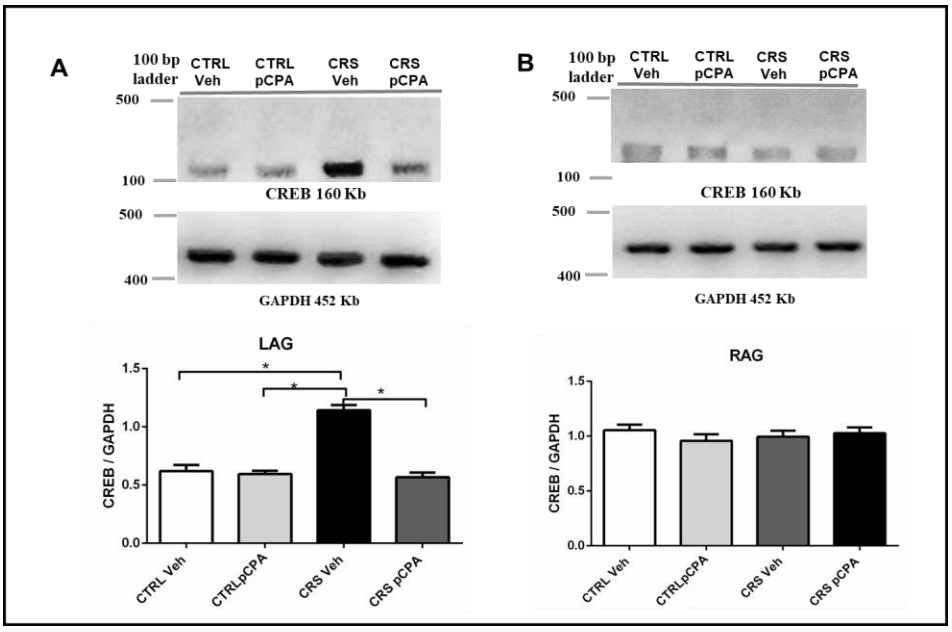


Figure 41. The effect of TPH inhibition on CREB mRNA expression in adrenal glands. Increased expression of CREB mRNA induced by chronic restraint stress (CRS) exposure in the left adrenal gland (LAG; A), as compared to control conditions (CTRL), was prevented by p-chlorophenylalanine (pCPA) pretreatment but not by vehicle (Veh). Pretreatment with pCPA had no effects in the right adrenal gland. * $P < 0.05$

7. DISCUSSION

7.1. General

The results of the present study provide further support to the notion that chronic stress exposure may result in the development of an adrenocortical serotonergic pathway as a primary mediator of exacerbated ACTH-independent corticosteroid secretion (García-Iglesias et al., 2013; Terrón, 2014). The operation of this mechanism most likely involves *de novo* synthesis of 5-HT via ectopic expression and function of TPH2 in adrenocortical cells involved in steroidogenesis, thus resulting in increased 5-HT levels and turnover in the adrenal cortex. Given the functional involvement of 5-HT7 receptors in magnified acute stress-induced CORT secretion in animals with a history of chronic stress (García-Iglesias et al., 2013), it seems obvious that stress-induced 5-HT release must take place for CORT to be released in an ACTH-independent manner. The core part of this study is related to TPH as another component of the stimulatory serotonergic loop involved in abnormal secretion of glucocorticoids under conditions of chronic stress (García-Iglesias et al., 2013; Terrón, 2014). Apart from the implications discussed below, the results of the present study with pCPA provide strong support to the notion that TPH may play an important functional role in promoting 5-HT-induced corticosteroid secretion in animals with a history of chronic stress thus suggesting that selective TPH2 inhibitors might have a potential therapeutic usefulness in SRD associated with hypercortisolemia.

7.2 Chronic restraint as a model of chronic stress

Exposure to chronic stress can affect HPA axis activity and increase stress-induced glucocorticoid secretion (García-Iglesias et al., 2013; Keeney et al., 2006). In addition to inducing the above endocrine changes, chronic stress exposure is known to promote a marked

decrease in body weight gain (García-Iglesias et al., 2013; Keeney et al., 2006) and and caloric intake (Yau and Potenza, 2013). The adrenal gland is an essential stress-responsive organ that is part of both the HPA axis and the SAM systems and chronic stress exposure commonly increases adrenal weight. As expected, the present CRS paradigm effectively produced a number of chronic-stress related changes, including reduced body weight gain as well as increased relative adrenal and thymus weight, as previously observed in this stress model (García-Iglesias et al., 2013). This is consistent with the fact that high circulating levels of glucocorticoids during prolonged periods of stress lead to adrenal gland hypertrophy and hyperplasia (Ulrich-Lai et al., 2006b) along with decreased rate of body weight gain and thymus involution (Choi et al., 2017), all of which are used as reliable physiologic markers of chronic stress. In addition, we also observed that CRS exposed animals exhibited decreased food intake as compared to CTRL rats.

7.3. Regulation of TPH expression and activity by chronic restraint stress: involvement of glucocorticoids

Stress is known to influence 5-HT neurotransmission and to precipitate the onset of many psychiatric disorders including major depression (de Kloet et al., 2005; Lanfumey et al., 2008; Lupien et al., 2009). TPH is the rate-limiting enzyme responsible for the biosynthesis of 5-HT. To date, two isoforms of the enzyme have been reported, TPH1 and TPH2. Since the identification and characterisation of the second TPH isoform (Côté et al., 2003; Walther et al., 2003), TPH2 is regarded as the more influential isoform in terms of 5-HT production in the central nervous system, due in part to the almost exclusive distribution of TPH2 in the raphe nuclei (Patel et al., 2004), the primary locus of 5-HT production in the brain.

Accumulating evidence is consistent with the notion that high circulating levels of glucocorticoids may have a feedforward influence on transcription of the TPH2 gen (tph2) in the adrenal glands. Thus, 5-HT has been shown to participate in the pathophysiology of primary pigmented nodular adrenocortical disease (PPNAD), which is a rare cause of ACTH-independent hypercortisolism in humans. Interestingly, PPNAD tissues overexpress TPH2 and the 5-HT4, 5-HT6, and 5-HT7 receptors, leading to formation of an illicit stimulatory serotonergic loop whose pharmacological inhibition *in vitro* decreases cortisol production (Bram et al., 2016). This disease is primarily caused by germline mutations of the protein kinase A (PKA) regulatory subunit 1A (PRKAR1A) gene, which induces constitutive activation of PKA in adrenocortical cells. Hypercortisolism is thought to result from PKA hyperactivity, but PPNAD tissues exhibit features of neuroendocrine differentiation, which may lead to stimulation of steroidogenesis by abnormally expressed neurotransmitters, including 5-HT (Bram et al., 2016a). The results of the present study strongly suggest that, similar to the observations in PPNAD adrenocortical tissues (Bram et al., 2016), chronic stress exposure might also trigger tph2 transcription thus leading to ectopic expression of TPH2 in adrenocortical cells and *de novo* synthesis of 5-HT. Activity of TPH was determined by measuring the accumulation of 5-HTP through a fluorometric method. Interestingly, we observed contrasting changes of TPH activity in the DRN and adrenals glands (specifically in the LAG) subsequent to CRS exposure. Indeed, TPH activity decreased in the DRN but it increased in the LAG from CRS-exposed animals as compared to CTRL tissues, and this effect was inhibited by pCPA pretreatment (Fig. 32). In support of a heightened TPH2-like activity in the adrenal cortex from chronically stressed animals, the concentrations of 5-HT and 5-HIAA, as well as the corresponding 5-HIAA/5-HT ratio reflecting 5-HT turnover, were all significantly increased in LAG from CRS-treated animals, and this effect was prevented by

pharmacological inhibition of TPH with pCPA pretreatment (Fig. 34). Consistent with our observations in the DRN (Fig. 32), exposure to chronic mild stress decreased brain 5-HT levels together with a decreased expression of TPH2 through a mechanism involving increased methylation of the tph2 gen (Chen et al., 2017).

Further to the above mechanisms of TPH expression regulation, a hallmark of the functional control of enzymatic activity of the rate-limiting enzyme in a particular biosynthetic pathway is that activity is inhibited by the end-product(s), and that a negative feedback by the end product at the rate-limiting step has been observed for the 5-HT biosynthetic pathway. Thus, when a monoamine oxidase inhibitor was administered, 5-HT levels increased at the 5-HT nerve terminals and the conversion of [^{3}H]-tryptophan to [^{3}H]-5-HT was markedly decreased (Carlsson and Lindqvist, 1972; Macon et al., 1971). Although there are controversial reports indicating that high concentrations of 5-HT were unable to inhibit TPH activity *in vitro* (Jequier et al., 1969; Youdim et al., 1975), it is generally believed that 5-HT inhibits TPH activity *in vivo* (Hamon et al., 1972). In addition to end-product inhibition of TPH (i.e. by 5-HT), it has been reported that pharmacological inhibition of the enzyme by pCPA administration induced a significant increase in TPH mRNA levels at day 1 in the mid brain raphe nuclei when 5-HT could not be detected immunohistochemically, thus suggesting that a decrease in 5-HT concentrations may lead to up-regulation of TPH gene transcription (Park et al., 1994). Consistent with these findings, lesion of the serotonergic neurons with the neurotoxin 5,7-dihydroxytryptamine was found to significantly increase TPH mRNA levels in the raphe and dorsal hypothalamus (Bendotti et al., 1990; Ljubic-Thibal et al., 1999; Ljubić-Thibal et al., 1996). In the present study we found that pCPA adminsitration from days 9 to 12 induced a significant decrease not only in TPH activity in LAG from CRS-exposed animals (Fig. 32), but also in TPH mRNA levels in adrenals from both sides

(Fig. 31). This seemingly controversial observation regarding the upregulating effects of low 5-HT levels subsequent to acute TPH inhibition with pCPA (Park et al., 1994) may be explained by the fact that adrenal gland samples taken for RT-PCR measurements were collected on day 15, that is, 3 days after completion of pCPA administration, which is a time frame consistent with a significant recovery of 5-HT levels (Park et al., 1994). Although such potential recovery of adrenal 5-HT content after completion of pCPA pretreatment was not detected in our HPLC measurements (Fig. 34), some 5-HT immunostaining was nevertheless observed in the cortex of LAG from pCPA-pretreated CRS-exposed animals (Fig. 33), thereby suggesting that adrenocortical 5-HT levels did recover to some extent subsequent to TPH inhibition treatment. Alternatively, since the presence of 5-HT in adrenocortical cells does not occur as a result of a normal physiological biosynthetic pathway in this tissue (i.e. ectopic expression of TPH2 occurs as a result of CRS exposure), it is possible that TPH expression regulation by 5-HT (i.e. the end product of 5-HT biosynthetic pathway) might not take place in the adrenal cortex.

7.4 Chronic restraint stress-induced expression of TPH2 and 5-HT7 receptors in the adrenal cortex: are CRE/CREB and glucocorticoid receptors involved?

The transcription factor CREB (cAMP responsive element binding protein) is a member of a family of constitutive nuclear proteins that control transcription through a phosphorylation-dependent activation in response to signals carried by cAMP and also by Ca^{2+}. CREB modulates gene transcription by binding to the cAMP responsive element (CRE) in the promoter regions of a number of genes. Among the signals that trigger CRE/CREB-directed gene transcription is stress, as reported with chronic psychosocial stress which increased activation of this pathway, whereas antidepressant treatment with imipramine reversed the stress-induced effects on CREB

activation (Böer et al., 2007). Interestingly, in the present study we found that CRS exposure increased CREB mRNA expression levels in adrenals from the left side, and that this effect was mimicked by chronic CORT treatment (Fig. 40) thereby suggesting that transcriptional regulation of CREB is sensitive to glucocorticoids, perhaps due to the existence of glucocorticoid-responsive elements in the promoter region of the CREB gen. These observations nevertheless are at variance with previous findings in HT22 neuronal cells showing that chronic dexamethasone (1 μM) exposure had no effect on CREB protein levels but it induced however a decrease in the phosphorilation and dephosphorilation rates of CREB, as determined by the signal intensity of pCREB (Föcking et al., 2003).

Available evidence indicates on the other hand that glucocorticoids may regulate gene expression through their actions in a number of signalling pathways including those involving CRE/CREB (David and Kalb, 2005; Tan et al., 1996; Xie et al., 2018). Since a correlation between the effects of CRS exposure/chronic CORT treatment on CREB mRNA levels and TPH2 and 5-HT7 receptor expression levels was found (García-Iglesias et al., 2013; Saroj et al., 2019; present results), it is likely that the expression of these proteins in the LAG might be CRE/CREB-mediated. Moreover, since CRS exposure as well as chronic CORT treatment increased GR mRNA expression in adrenal glands (i.e. LAG), the present observations support the notion that a CRE/CREB-mediated feedforward pathway involving chronic stress-induced upregulation of GR, TPH2 and 5-HT7 receptors in the adrenal cortex might underlie magnified stress-induced ACTH-dissociated glucocorticoid secretion in animals with a history of CRS (García-Iglesias et al., 2013). This notion however is at variance with the fact that chronic glucocorticoid treatment altered the phosphorilation kinetics (i.e. decreased phosphorilation rates) of CREB in neurons of the HT22 hippocampal cell line, consistent with an impairment of the CRE/CREB pathway

(Föcking et al., 2003). This finding raises the possibility that TPH2 and 5-HT7 receptor expression in the adrenal cortex of animals undergoing CRS exposure and chronic CORT treatment might involve mechanisms other than GR receptor-mediated effects on the CRE/CREB pathway. Interestingly in this regard, we found that TPH inhibition with pCPA pretreatment strongly decreased CRS-induced expression of TPH2 (Figs. 29-31) and 5-HT7 receptors (Figs. 35 and 36) in the LAG, thus raising the possibility that 5-HT production itself, through the action of TPH2 in the adrenal cortex, might underlie, at least in part, TPH2 and/or 5-HT7 receptor expression. It is well know in this regard that CREB is phosphorylated in response to hormonal stimuli that increase intracellular cAMP production and can be phosphorylated in response to a wide variety of extracellular signals (Bonni et al., 1995; Dash et al., 1991), and that an increase in intracellular cAMP leads to activation of PKA which, in turn, phosphorylates CREB and other proteins (Carballosa-Gonzalez et al., 2014; Vega and Avila, 2010). Since positive coupling to adenylate cyclase and increased intracellular levels of cAMP (with subsequent activation of PKA-mediated effects) is the primary transduction pathway of 5-HT7 receptors (Ruat et al., 1993; Shen et al., 1993), it is then possible that 5-HT7 receptor PKA-mediated activation of CRE/CREB might induce tph2 and/or 5-ht7 receptor gen expression in the adrenal cortex from CRS-treated animals. In support of this possiblity, it has been reported that 5-HT7 receptor activation resulting from nucleus raphe magnus stimulation reverses the spreading deficits in cAMP, phosphorilated PKA and phosphorilated CREB (pCREB) in the cervical, thoracic and lumbar spinal cord subsequent to spinal cord injury (Carballosa-Gonzalez et al., 2014). Further studies aimed at elucidating the signalling pathways involved in CRS-induced expression of TPH2 and 5-HT7 receptors are warranted.

In the brain, GR modulate a broad range of neural functions, including stress-related activation of the HPA axis. In particular, GR in the hippocampus, the PVN and the anterior pituitary are thought to play a significant role in restraining the activity of the HPA axis. Furthermore, changes in GR function and expression are essential in the adaptation and maladaptation to chronic stress, and this is relevant to the pathophysiology of various SRD, such as depression. In the present study we found increased expression of GR in LAG as a result of CRS exposure and chronic CORT treatment. These findings would seem to suggest that most of the CRS-induced alterations in the HPA axis, particularly in the adrenal glands, are GR-mediated. Given the number of downstream signalling pathways associated with GR activation, the present results do not allow to reach a conclusion regarding the specific role of GR in CRS-induced adrenocortical and hormonal alterations.

7.5 The asymmetrical effect of chronic restraint stress and chronic corticosterone treatment in adrenal glands

The predominant expression of TPH2 in the LAG as compared to the RAG suggests asymmetry as to the impact of CRS exposure and chronic CORT treatment on adrenal function. Limited evidence nevertheless has been published on the morphological, physiological and/or pathophysiological asymmetric features of the adrenal glands and on the anatomical and functional laterality of brain structures controlling their function (Gerendai and Halász, 1997). Interestingly, previous transneuronal retrograde labelling studies have reported that the central innervation of rat adrenals is indeed asymmetrical, with a more prominent brain stem and hypothalamic (e.g. paraventricular nucleus) innervation to the left adrenal gland (Tóth et al., 2008), which is consistent with the possibility that predominant activation of the left adrenal

might take place under conditions of chronic stress or high circulating levels of glucocorticoids. Whether or not the asymmetrical pattern of adrenal gland innervation (Tóth et al., 2008) might involve higher brain centres implicated in the central regulation of the stress response remains to be elucidated. In support of this contention though, medial prefrontal cortex (mPFC) lesion studies in rats have shown that right or bilateral, but not left mPFC lesions, decreased baseline but not acute restraint-induced CORT secretion; by contrast, the same lesions significantly reduced restraint-induced CORT responses in chronically-restrained animals (Sullivan and Gratton, 1999). The mPFC is indeed believed to play a key role in the coordination of neuroendocrine and autonomic responses to stress through its interactions with a number of subcortical and hindbrain targets (see McKlveen et al., 2015 for review). Further investigation aimed at elucidating the role of endocrine asymmetry in the physiology and pathophysiology of the stress response is warranted.

8. CONCLUSION

The results of the present study further support the relationship between the HPA axis and the serotonergic system in the regulation of the stress response under chronic stress conditions. Indeed, we have shown that the increased 5-HT levels in whole adrenal glands and the strong 5-HT-LI in the adrenal cortex from CRS-exposed animals (García-Iglesias et al., 2013), are most likely due to *de novo* synthesis of 5-HT in adrenocortical cells involved in steroidogenesis. This seems to be accounted for by ectopic expression and activity of TPH2 in the adrenal cortex. Thus, TPH2 represents another component of the stimulatory serotonergic loop in the adrenal cortex mediating magnified stress-induced ACTH-independent glucocorticoid secretion in animals with a history of chronic stress (García-Iglesias et al., 2013; Terrón, 2014). Since these

findings closely resemble clinical pathophysiological observations in both cortisol-producing adrenal adenomas (Contesse et al., 2005; Lefebvre et al., 2015; Louiset et al., 2008, 2006) and PPNAD (Bram et al., 2016), they do suggest that the etiophysiopathology of the adrenocortical glucocorticoid-producing serotonergic mechanisms might actually be accounted for by chronic stress exposure. This study actually shows a straightforward relationship between CRS exposure and TPH2 expression in the adrenal cortex, with the later being glucocorticoid-dependent (i.e. CRS-induced effect were mimicked by chronic CORT treatment). Regarding the signaling pathways involved in the CRS-induced effects, our results showed that both CRS exposure and chronic CORT treatment evoked an increase both in GR and CREB expression in the adrenals. Interestingly however, the fact that pCPA pretreatment prevented CRS-induced adrenal GR and CREB expression suggests that 5-HT itself might play a role in GR and CREB gene transcription regulation, possibly through a 5-HT7 receptor mediated activation of the cAMP-PKA-CRE/CREB pathway. The effectiveness of the pharmacological inhibition of TPH through a pCPA pretreatment was confirmed by the ability of this drug to significantly decrease 5-HT, 5-HIAA and 5-HT turnover (5-HIAA/5-HT ratio) in the LAG without inducing any significant change in the levels of the 5-HT precursor, L-tryptophan. Interestingly, beyond the expected inhibitory effects of pCPA pretreatment on 5-HT and 5-HIAA levels (and 5-HT turnover as well), it was found that the drug decreased TPH2 gene expression, as suggested by its ability to blunt CRS-induced increases of TPH2 protein and mRNA levels in the LAG. Regarding the impact of pCPA pretreatment on hormone secretion, the present results suggest that baseline CORT secretion in chronically stressed animals requires serotonergic activity arising from TPH activity. Indeed, pCPA pretreatment significantly decreased baseline CORT secretion in CRS-exposed animals. Consistent with the notion that pCPA might induce a reversal of CRS-induced

endocrine dysregulation (i.e. magnified stress-induced CORT responses and blunted ACTH secretion), pCPA pretreatment increased baseline secretion of ACTH in chronically stressed animals. As pointed out above, the available evidence on the asymmetrical features of the stress endocrine system is scarce and limited. Our results however are in keeping with previous retrograde transneuronal labeling observations demonstrating more intense central innervation of the left adrenal glands, including that from the PVN (Tóth et al., 2008). Further studies aimed at elucidating the role of adrenal asymmetry in abnormal glucocorticoid secretion under chronic stress conditions are warranted. In summary, the results of the present investigation suggest a critical role of TPH2 expression and activity in mediating CRS-induced 5-HT-mediated secretion of glucocorticoids. Accordingly, they also suggest that selective TPH2 inhibitors might represent a novel therapeutic strategy for the treatment of SRD associated with hypercortisolemia.

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