Universidade do Minho

Escola de Ciências

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The therapeutical potential of TUDCA against stress-related brain pathologies

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The therapeutical potential of TUDCA against stress-related brain pathologies

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STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

RESUMO

O potencial terapêutico do TUDCA em patologias cerebrais associadas ao stress

O ácido tauroursodesoxicólico (TUDCA) é um ácido biliar hidrofílico endógeno que demonstrou ter ação neuroprotetora em diferentes modelos *in vitro* e *in vivo* de diversas doenças neurodegenerativas, incluindo a doença de Alzheimer e a doença de Parkinson. Contudo, os seus mecanismos de ação ainda não são totalmente compreendidos. A neuroproteção é atribuída principalmente à capacidade do TUDCA de diminuir o stress oxidativo, suprimir a apoptose, atenuar a inflamação e aliviar o stress do retículo endoplasmático. Considerando os efeitos descritos do TUDCA em muitas moléculas e vias afetadas pelo stress crónico e/ou níveis elevados de glucocorticóides (GCs), bem como a sua capacidade de se ligar ao seu recetor (GR), há necessidade de estudar os efeitos deste ácido biliar contra défices relacionados ao stress no contexto da depressão. Além disso, tendo em consideração que níveis elevados de GCs estão fortemente associados à hiperfosforilação e acumulação da Tau, e consequente atrofia e disfunção neuronal, surge também a necessidade de estudar os efeitos do TUDCA nessas vias, bem como contra patologias cerebrais relacionadas ao stress crónico.

Estudos *in vivo* foram conduzidos em murganhos Wild-Type com 3 meses de idade submetidos a 5 semanas de protocolo de Stress Crónico Imprevisível (CUS). Nos últimos 15 dias de CUS, os animais foram injetados por via intraperitoneal com TUDCA (50 mg/kg) ou Fluoxetina (10 mg/kg), um conhecido antidepressivo. Depois de 10 dias de tratamento, vários testes de comportamento foram realizados para avaliar ansiedade, depressão e cognição, seguidos de análises moleculares e neuroestruturais.

Como esperado, o CUS induziu um comportamento depressivo, bem como défices na memória de longo prazo, enquanto o tratamento com TUDCA evitou estes resultados negativos, apresentando resultados positivos semelhantes aos do tratamento com Fluoxetina. A nível molecular e celular, o TUDCA modulou vias relacionadas com a inflamação, plasticidade sináptica, fosforilação da Tau e sinalização do GR, revertendo os efeitos induzidos pelo CUS no córtex pré-frontal (PFC). É importante ressalvar que o sexo dos animais desempenhou um papel nos efeitos comportamentais, celulares e moleculares do TUDCA e da Fluoxetina observados.

Embora ainda seja necessário analisar mais detalhadamente os mecanismos moleculares envolvidos nos efeitos terapêuticos benéficos do TUDCA contra patologias cerebrais relacionadas com o stress e o seu potencial como modulador da função neuronal e sináptica, o seu potencial terapêutico contra estas patologias é impressionante.

Palavras-chave: Ácido tauroursodeoxicólico (TUDCA); Depressão; Glucocorticóides; Inflamação; Stress crónico

ABSTRACT

The therapeutical potential of TUDCA against stress-related brain pathologies

Tauroursodeoxycholic acid (TUDCA) is an endogenous hydrophilic bile acid that has demonstrated a neuroprotector action in different *in vitro* and *in vivo* models of various neurodegenerative diseases including Alzheimer's (AD) and Parkinson's (PD) disease. However, its mechanisms of action are not yet fully understood. The neuroprotection is mostly attributed to TUDCA's ability to decrease oxidative stress, suppress apoptosis, attenuate inflammation, and alleviate endoplasmic reticulum (ER) stress. Considering TUDCA's described effects on many molecules and pathways affected by chronic stress and/or high level of glucocorticoids (GCs), as well as its ability to bind to the Glucocorticoid receptor (GR), there is a need to study the effects of this bile acid against stress-related deficits in the context of depression. Furthermore, considering that high levels of glucocorticoids are strongly associated with Tau accumulation, hyperphosphorylation and missorting, and consequential neuronal atrophy and dysfunction, the need to study the effects of TUDCA on these pathways as well as against stress-related brain pathologies also arises.

In vivo studies were conducted on 3 months-old Wild-Type mice subjected to 5 weeks of Chronic Unpredictable Stress protocol (CUS). In the last 15 days of CUS, mice were intraperitoneally injected with TUDCA (50 mg/kg) or Fluoxetine (10 mg/kg), a known anti-depressant. Later, mice were subjected to several behaviour tests to access anxiety, depression, and cognition, followed by molecular and neurostructural analysis.

As expected, CUS induced a depressive-like behaviour as well as an impairment on long-term memory, while treatment with TUDCA prevented these negative outcomes, displaying similar positive outcomes to treatment with Fluoxetine. At a molecular and cellular level, TUDCA modulated pathways related to inflammation, synaptic plasticity, Tau phosphorylation and GR signalling, reverting CUS-induced effects in the prefrontal cortex (PFC). Importantly, sex played a role in the behavioural, cellular, and molecular effects of TUDCA and Fluoxetine.

While we still need to analyse further in detail the molecular mechanisms involved in the beneficial therapeutic effects of TUDCA against stress-related brain pathologies and its potential as a modulator of neuronal and synaptic function, the therapeutical potential of the drug against these pathologies is striking.

Keywords: Chronic Stress; Depression; Glucocorticoids; Inflammation; Tauroursodeoxycholic acid (TUDCA)

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LIST OF ABBREVIATIONS AND ACRONYMS

A	Aβ	Amyloid-β
	ACTH	Adrenocorticotropic Hormone
	AD	Alzheimer's Disease
	Akt	Protein kinase B
	ANOVA	Analysis of variance
	AP-1	Activator Protein-1
	APP	Amyloid Precursor Protein
	ATF4	Activating Transcription Factor 4
	ATF6α	Activating Transcription Factor 6 α
	AVP	Arginine vasopressin
B	B2M	Beta-2-Microglobulin
	BDNF	Brain-Derived Neurotrophic Factor
	BAG1	Bcl-2-associated athanogene 1
C	CA1	Cornu ammonis 1
	CA3	Cornu ammonis 3
	cdk5	Cyclin-dependent kinase 5
	cDNA	Complementary DNA
	CHOP	C/EBP homologous protein
	CNS	Central Nervous System
	CORT	Corticosterone
	CREB	cAMP Response Element-binding Protein
	CRH	Corticotropin-releasing hormone
	CYP27A1	Sterol 27-hydroxylase
D	DAPI	4',6-diamidino-2-phenylindole
	DG	Dentate gyrus
	DMSO	Dimethyl sulfoxide
	DNMT	DNA methyltransferase
	DTT	Dithiothreitol
E	ECL	Enhanced Chemiluminescence
	EDTA	Ethylenediaminetetraacetic acid
	EPM	Elevated Plus Maze
	ER	Endoplasmatic Reticulum
F	FDA	Food and Drug Admnistration
	FBS	Fetal bovine sérum
	FKBP5	FK506-binding protein 5
	FST	Forced Swimming Test
	Fwd	Forward
	FXR	Farnesoid X Receptor
G	GABA	Gamma-aminobutyric acid
	GC	Glucocorticoid
	GFAP	Glial fibrillary acidic protein
	GR	Glucocorticoid Receptor
	GRE	Glucocorticoid Response Element
	GRP78	Glucose-regulated protein 78
	GSH	Glutathione
	GSK3β	Glycogen synthase kinase 3 β
H	HAT	Histone acetyltransferase
	HDM	Histone demethylase
	HMT	Histone methyltransferase
	HPA	Hypothalamo-pituitary-adrenal
	Hsp70	Heat shock protein 70

	Hsp90	Heat shock protein 90
I	IBA-1	Ionized calcium-binding adapter molecule 1
	IKKβ	IκB kinase β
	IL1β	Interleukin 1 β
	IL18	Interleukin 18
	IL6	Interleukin 6
	i.p.	Intraperitoneal
	IRE1α	inositol-requiring enzyme 1α
L	LC3	Microtubule-associated protein 1A/1B-light chain 3
	LDH	Lactate dehydrogenase
	LTD	Long-Term Depression
	LTP	Long-Term Potentiation
	LPS	Lipopolysaccharide
M	MAPK	Mitogen-Activated Protein Kinase
	MDA	Malondialdehyde
	MDD	Major depressive disorder
	MR	Mineralocorticoid receptor
	MWM	Morris Water Maze
N	NCAM	Neural Cell Adhesion Molecule
	NFκB	Nuclear Factor kappa-light chain-enhancer
	nGRE	Negative GRE
	NLRP3	Nod-like receptor protein 3
	NOR	Novel Object Recognition
	NSF	Novelty Supressed Feeding
O	OF	Open Field
P	PBS	Phosphate-Buffered Saline
	PBS-T	PBS 1x supplemented with tween
	PCR	Polymerase Chain Reaction
	PD	Parkinson's Disease
	PERK	PKR-like ER kinase
	PFA	Paraformaldehyde
	PFC	Prefrontal cortex
	PI3K	Phosphatidylinositol 3-kinase
	PrL	Prelimbic
	PSD-95	Postsynaptic density-95 protein
	PVN	Paraventricular nucleus
	qRT-PCR	quantitative real-time polymerase chain reaction
	Rev	Reverse
R	ROS	Reactive oxygen species
	RT	Room Temperature
S	SEM	Standard Error of Mean
	SPT	Sucrose Preference Test
	SSRIs	Selective serotonin reuptake inhibitors

T	Tau	Tubulin associated unit
	TBS	Tris-Buffered Saline
	TBS-T	Tris-Buffered Saline with Tween
	TF	Transcription Factor
	TGFβ	Transforming Growth Factor β
	TGR5	Takeda G Protein-Coupled Receptor 5
	TNFα	Tumor necrosis factor α
	TST	Tail Suspension Test
	TUDCA	Tauroursodeoxycholic acid
U	uCMS	unpredictable Chronic Mild Stress
	UDCA	ursodeoxycholic acid
	UPR	Unfolded Protein Response
W	WB	Western Blot
	WT	Wild-type
X	XBP1	X-box binding protein 1
Others	5-HT	Serotonin
	5-HTT	Serotonin transporter

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CHAPTER 1

INTRODUCTION

1.1. Stress: an everyday problem

Commonly, stress is associated with an overwhelming state accompanied by negative emotions, triggered by adversities or events viewed as challenges or threats. Scientifically, stress may be broadly defined as a natural adaptative response to a stimulus (stressor) that disrupts homeostasis. It's a fundamental physiological phenomenon to survival as it allows adaptation and coping to the experiences that are constantly happening to the organism. An individual's ability to cope with stress is essential to the maintenance of a physical and mental health. The development of adaptative or maladaptive consequences is dependent on neuroendocrine, neurochemical, and genetic factors as well as on exterior variables, such as the duration, intensity, and predictability of the stress exposure (de Kloet et al., 2005; Franklin et al., 2012). When these variables of stress exposure reach a certain threshold, the stress response switches from adaptative and physiological to harmful and toxic to the organism, impairing physical and psychiatric health. Chronic stress, normally designated as a repeated and/or prolonged exposure to stressful events, is associated with maladaptive responses and long-term negative effects on the organism, impairing various systems such as the nervous system. It has been proven that the brain is a fundamental key player in stress response, as it reacts to psychological or physical stressors with emotional, behavioural, cellular, and molecular adaptations, in human (Dai et al., 2020; de Kloet et al., 2005; McEwen & Akil, 2020) and in rodent studies (Maniam & Morris, 2012; McEwen, 2007; McEwen et al., 2015; Tran & Gellner, 2023). On the other hand, it has been shown that the brain is also a major target of stress, with several studies demonstrating chronic stress' association with structural, molecular, and cellular changes in limbic brain regions, linked with several Central Nervous System (CNS) stress-related disorders (McEwen, 1998; McEwen & Akil, 2020; Popoli et al., 2012; Radley et al., 2015; Tran & Gellner, 2023).

1.2. Stress Response: HPA axis and Glucocorticoids

The first physiological response to stress consists of the activation of the sympathetic nervous system and the consequent secretion of epinephrine and norepinephrine from the adrenal gland. These two hormones act very quickly by increasing the basal metabolic rate, blood pressure and respiration, as well as the blood flow to vital organs (Joëls et al., 2018).

The second phase of the stress response involves the activation of the hypothalamo-pituitary-adrenal (HPA) axis and the synthesis and release of corticosteroids (cortisol in humans and corticosterone in rodents) from the adrenal cortex. The HPA axis consists of the hypothalamus, pituitary, and adrenal glands, and has as a primary function the regulation of the adaptative response to psychogenic (e.g.,

fear) and physical (e.g., cellular lesion or pathogen invasion) stressors (McEwen, 2007; Vyas et al., 2016). The exposure to a stressful stimulus induces the release of the corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) in the paraventricular nucleus of the hypothalamus (PVN). In turn, these neuropeptides stimulate the anterior pituitary to secrete adrenocorticotropic hormone (ACTH), leading to the synthesis and secretion of glucocorticoids (GCs) from the adrenal gland (de Kloet et al., 2005; McEwen, 2007) (**Figure 1**). GCs are steroid hormones that act through two main corticosteroid hormone receptors: the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR). As these two receptors are expressed in several tissues, GCs can reach and initiate responses throughout the body, from peripheral organs to the brain (McEwen et al., 1986). As GR and MR are ligand-activated transcription factors mostly present in the cytoplasm, the binding of GCs induces the receptors' nuclear translocation and the modulation of their transcriptional activity, resulting in the regulation of the expression of their target genes and, consequently, in the modulation of several physiological (e.g., metabolic, cardiovascular, and immune) as well as behavioural (e.g., emotion, cognition, and motor) processes (Vyas et al., 2016). GCs effects, especially in the brain, range from these transcriptional effects to non-transcriptional actions to epigenetic modifications (Bartlett et al., 2019; Gray et al., 2017; Provençal, 2019; Seifuddin et al., 2017; Zannas, 2021).

Due to MRs' higher affinity for GCs than GRs (which possess a 10-fold lower affinity), at basal levels, GCs bind to MRs, occupying and activating these receptors. GR's activation only happens when GC levels increase to a certain level, for example, in the circadian peak or during stress (de Kloet et al., 1998; Gray et al., 2017). In the brain, MRs and GRs are expressed differentially and function coordinatively, through genomic and non-genomic mechanisms, modulating multiple neurochemical systems, including neurotransmitters (e.g. GABA and glutamate), neuromodulators (e.g. dopamine and 5-hydroxytryptamine), neurotrophic factors (e.g. brain-derived neurotrophic factor (BDNF)), and other extracellular signalling systems, like endocannabinoids (de Kloet et al., 2009; Gray et al., 2017; Joëls et al., 2007; McEwen, 1999, 2007). Moreover, both receptors have an impact on the control of the stress response, with MR being implicated in the modulation of basal glucocorticoid circadian rhythm and pulsative secretion of ACTH into the blood, while GR has an essential role in stress negative feedback (Bradbury et al., 1991; Gjerstad et al., 2018).

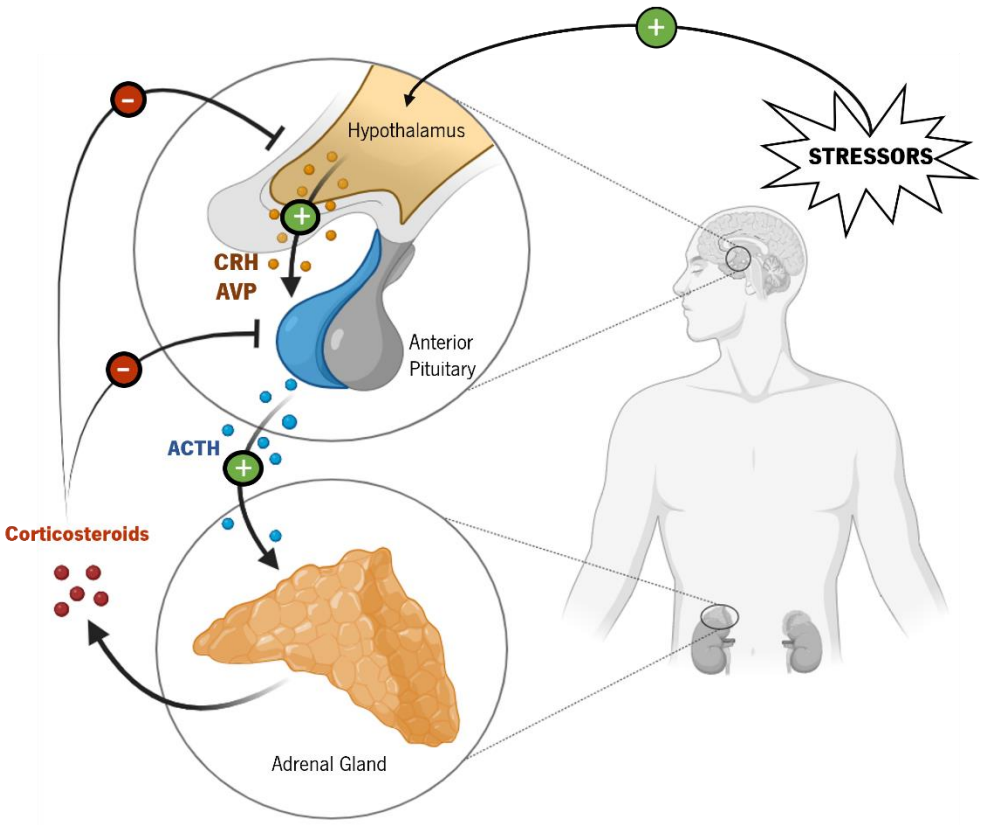


Figure 1. Schematic representation of the HPA axis and its regulation. Exposure to stressful stimuli (stressors) triggers the release of the corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) in the paraventricular nucleus of the hypothalamus (PVN), activating the synthesis and secretion of the adrenocorticotropic hormone (ACTH) at the anterior pituitary. ACTH stimulates the adrenal gland to synthesize and secrete glucocorticoids (GCs), which serve as a feedback inhibitory signal to hypothalamus and pituitary.

1.3. Glucocorticoids Receptor (GR)

The Glucocorticoids Receptor (GR) is expressed ubiquitously in tissue-specific and condition-dependent manner, which accounts for the tissue and cell-specific effects of the receptor. Although GR has a ubiquitously distribution in the brain, it has an enriched expression in the PVN and limbic areas. In fact, it has been shown that GR expression is high in the hippocampus, which as lead to its association with stress response and regulation, emotion, memory, and cognition (de Kloet et al., 2005; Joëls et al., 2018; Sotiropoulos et al., 2015).

As mentioned above, the GR is a ligand-activated transcription factor encoded by the *NR3C1* gene and expressed in most mammalian tissues, in cytosol, at the cell membrane, and on mitochondria (Gray et al., 2017) (**Figure 2**). It's mostly found in the cytoplasm in association with chaperone proteins such as the heat shock proteins 90 and 70 (Hsp90 and Hsp70), the immunophilins FKBP51, FKBP52, and BAG1, that mask the nuclear localization signal (Grad & Picard, 2007; Gray et al., 2017; Oakley & Cidlowski, 2013).

When GCs bind to the cytoplasmatic GR, there is a conformational change to the GR-chaperones complex that results in the exposure of two nuclear localization signals and, consequent, nuclear translocation (Gray et al., 2017) (**Figure 2**).

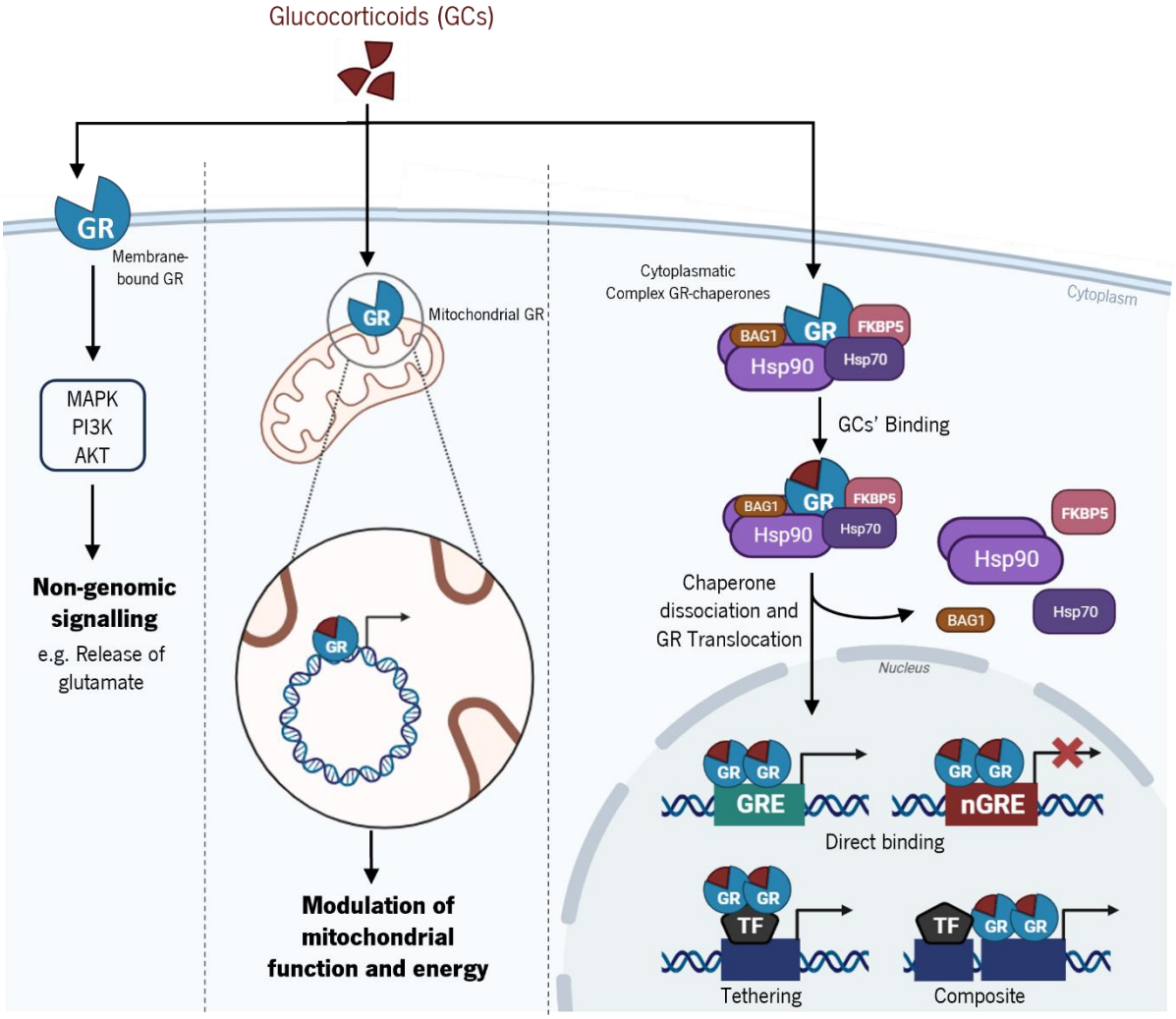


Figure 2. GR signalling pathway. Glucocorticoid Receptor (GR) is a ligand-activated transcription factor expressed in most mammalian tissues at the cell membrane, on mitochondria and in the cytoplasm. Most of non-genomic effects of GR are due to the action of membrane-bound GR (left side of the figure), which, upon binding to Glucocorticoids (GCs), can lead to rapid alteration of neuronal function by modulating processes such as excitatory amino acid release (e.g. glutamate). As lipid-soluble molecules, GCs can freely cross the cellular membrane and bind to the mitochondrial GR or to the cytoplasmatic GR. Binding of GCs to mitochondrial GR is associated with modulation of mitochondrial function and energy. When GCs bind to the cytoplasmatic GR (right part of the figure), conformational changes are induced, causing the dissociation of the GR chaperones (e.g. Hsp70, Hsp90, FKBP5 and BAG1), GR dimerization and translocation to the nucleus. In the nucleus, dimerized GRs can act as a transcriptional factor by directly binding to specific regions of DNA called glucocorticoid response elements (GREs), stimulating transcription, or to negative GREs, stopping transcription. Additionally, GRs can also affect gene expression via tethering to other transcription factors (TFs), such as nuclear factor NF-kappa-B (NF-κB) or AP1, or through binding to "composite" GREs, that possess binding sites for GR and other TFs.

In the nucleus, GR exerts tissue- and cell type-specific genomic effects, by regulating the transcription of its target genes in three main ways: (I) acting as a transcriptional factor via direct binding to specific regions of DNA called glucocorticoid response elements (GREs); (II) interacting with other

transcription factors (TFs), or (III) by a conjugation of these two mechanisms (Gray et al., 2017; B. Liu et al., 2019; Oakley & Cidlowski, 2013; Schoneveld et al., 2004) (**Figure 2**).

GREs are located within the regulatory regions of target genes, and usually part of combined regulatory elements or possess binding sites for transcription factors. The classical GRE sequence (5'-AGAACAnnnTGTTCT-3') is a palindromic sequence that consists of two hexamers that are separated by three base pairs (Hudson et al., 2013; B. Liu et al., 2019). As GR binds to GREs as a homodimer, where each half site is occupied by one receptor subunit, the separation of the half sites by three nucleotides is essential for the dimerization of the GR. This induces GR conformational changes that allow the recruitment of cofactors that change the chromatin state facilitating gene expression (Scheschowitsch et al., 2017). On another hand, through binding to negative GREs (nGREs), GR can repress negatively regulated target genes. The sequence and function of nGREs are different from the classic GREs, as nGREs' sequence (5'-CTCC(n)0-2GGAGA-3') is occupied by two GR monomers that do not homodimerize, thus repressing the transcription of that gene. nGREs are abundant throughout the genome and contribute to the regulation of the HPA axis, inflammation, and angiogenesis (Hudson et al., 2013; B. Liu et al., 2019).

Besides these direct approaches to gene regulation, GR can indirectly regulate gene transcription through protein-protein interactions between GR and TFs. These interactions may take place on promoters that do not contain GREs (tethering mechanism) or on those that have "composite" GREs (Scheschowitsch et al., 2017). The tethering mechanism does not require the binding of GR to DNA as the receptor interacts with TFs that will modulate DNA transcription. While the "composite" GREs possess binding sites for GR as well as other TFs, whose combined action will result in the modulation of the target gene transcription. GR's interaction with TFs like Nuclear Factor-kB (NF-kB), Activator Protein-1 (AP-1), cAMP Response Element-binding Protein (CREB) or Transforming Growth Factor β (TGF β) explain a substantial part of the effects of GCs on the immune system and stress response, for example (Kassel & Herrlich, 2007; Scheschowitsch et al., 2017).

These GR's mechanisms of transcriptional regulation have been described throughout the organism and contribute to a series of tissue-specific and context-dependent changes. This might be attributed to several factor such as: GR ubiquitous expression; several conditions and mechanism that alter GR's function; and to the various GR-binding sites and their availability. For example, the availability of binding sites as well as the occupancy of said binding sites by GR are dependent on chromatin accessibility (tissue- and cell-specific) and GC concentration (context-dependent), respectively (Gray et al., 2017; Oakley & Cidlowski, 2013; Scheschowitsch et al., 2017).

In addition to modulating gene expression, it has been shown that GR can exert effects via epigenetic mechanisms. Epigenetics refers to stable modifications on gene and protein expression that do not require alternations on the DNA sequence. These modifications can persist through cell division and, in some cases, be inherited through the generations (Cavalli & Heard, 2019). Epigenetic mechanisms are sensitive to environmental factors, such as stress, aging or diet. Two well-known and common epigenetic marks influenced by environmental stimuli (e.g. stress) are histone modification and DNA methylation, which consists in the addition of a methyl group at a CpG site in DNA . In fact, one of the lasting epigenetic modifications caused by GR activation is DNA methylation via recruitment of DNA methyltransferases (DNMTs) (Gray et al., 2017; Provençal, 2019; Seifuddin et al., 2017; Zannas, 2021). Additionally, it has been reported that this receptor can regulate the expression and recruit multiple histone-modifying enzymes, like histone methyltransferases (HMTs), histone demethylases (HDMs) and histone acetyltransferases (HATs). Reciprocally, GR expression can be regulated by DNMTs and histone-modifying enzymes (Bartlett et al., 2019; Gray et al., 2017).

Apart from the described genomic effects of GR on gene transcription and protein synthesis, this receptor can exert non-genomic effects. Part of these effects are due to the actions of GRs present in the mitochondria or at the cell membrane (**Figure 2**). These receptors can cause, upon ligand binding and independently of translocation to the nucleus, rapid alteration of neuronal function by modulating processes such as excitatory amino acid release or energy production by increasing calcium buffering capacity and resistance to apoptosis (Hunter et al., 2016; Popoli et al., 2012). Furthermore, after the dissociation of the complex of proteins bound to cytoplasmatic GR, some of the dissociated chaperones like Src or the heat shock proteins can exert effects of their own (e.g. influencing cellular signalling) (B. Liu et al., 2019).

1.4. Chronic Stress

The mechanisms of stress response mentioned above are fundamental for maintaining homeostasis and overall mental and physical health. However, when the balance between stress and the organism's response is disturbed or the exposure becomes persistent, maladaptive responses can be developed. A healthy response to stress requires the activation of the HPA axis and an effective mean to terminate this response, which is ensured by GCs that serve as a negative feedback signal to the hypothalamus and pituitary. A failure or malfunction of this feedback mechanism leads to exposure to high levels of GCs for extended periods of time. The quality, duration, and intensity of stress exposure influence the ability to maintain the negative feedback loops and consequent GC-shut off. In fact, exposure

to chronic stress and its detrimental effects have been associated with elevated GC levels and GR activation (de Kloet et al., 2005; Sotiropoulos et al., 2015; Tran & Gellner, 2023; Vyas et al., 2016).

As GR is expressed ubiquitously, prolonged exposure to elevated levels of GCs has several implications throughout the organism. For example, it has been casually implicated in neuropsychiatric disorders such as depression, or even dementia. Elevated GC levels and/or exposure to chronic stress have a profound impact on the brain, triggering structural, molecular, and cellular changes connected to neuronal atrophy and dysfunction, impaired cognition, as well as mood and affective disorders (McEwen & Akil, 2020; Sotiropoulos et al., 2008) (**Figure 3**).

In a context of chronic stress and/or high GC levels, human and rodent studies have reported structural changes, namely in the hippocampus and prefrontal cortex (PFC), the most studied areas in the context of chronic stress and/or high GC levels. In fact, it was shown that, in a context of chronic stress, there is a volumetric reduction in these brain areas as well as an impaired connectivity between them, associated with dendritic reorganization, neuronal atrophy, synaptic loss, and changes in astroglia and microglial cells (Banasr et al., 2021; Cerqueira et al., 2005, 2007; Rothman & Mattson, 2010). At a molecular and cellular level, exposure to chronic stress and/or high GC levels has numerous effects on the brain. These effects include the induction of apoptotic pathways (e.g. by modulating the expression of Bcl-2), proto-oncogenes and cell cycle-related proteins (e.g. p53 and p21), which can trigger cell cycle arrest and cell death (Sotiropoulos et al., 2008; Yu et al., 2008). The impairment of cellular functioning may also be explained by the increased production of reactive oxygen species (ROS), mitochondrial fragmentation, and impairment of mitochondrial function caused by chronic stress and increased levels of GCs (Bassil et al., 2023).

Furthermore, chronic stress and/or high GC levels can cause changes in the expression pattern of several proteins, such as BDNF, CREB, Mitogen-Activated Protein Kinases (MAPKs), Neural Cell Adhesion Molecules (NCAMs) and Fos protein. These proteins, either individually or collaboratively, play pivotal roles in modulating cellular activity and survival, thereby exerting a significant influence on neuroplasticity. An influence that can also be explained by epigenetic changes capable of shaping neuronal structure and function. These modifications can also alter the expression of genes involved in various aspects of brain function, including synaptic plasticity, neuroprotection, and the brain's response to stress (Joëls et al., 2018; McEwen et al., 2015; McEwen & Akil, 2020; Radley et al., 2015; Sotiropoulos et al., 2008; Tran & Gellner, 2023).

Chronic stress also exerts a significant impact on the immune response and system. Within the CNS, microglia are the resident immune cells and express receptors for catecholamines and GCs. These

receptors play a role in regulating microglial activity and their response to stress-induced signals. In the context of chronic stress, studies have reported cytokine dysregulation, sensitization (or priming) of microglia to inflammatory stimuli, microglia activation accompanied by morphological changes, and an increase in microglia numbers, notably within regions like the PFC (Sugama & Kakinuma, 2020; Vyas et al., 2016).

Furthermore, chronic stress and elevated GC levels are implicated in significant alterations in neural structure. This includes both the loss of dendrites and structural remodelling of synapses, as well as the degradation of essential synaptic proteins and receptors, leading to impaired synaptic plasticity. Synaptic plasticity represents the adaptability of synapses in response to various factors. Two well-known types of synaptic plasticity are Long-Term Potentiation (LTP), which involves the strengthening of synapses, and Long-Term Depression (LTD), which weakens them. Both processes are vital to memory formation and information processing. While physiological levels of GCs promote LTP, stress-induced levels of GCs induce LTD, furthering the disruption of the synaptic plasticity (Cerqueira et al., 2007; Joëls & Krugers, 2007; Sotiropoulos et al., 2008).

This interference of elevated levels of GCs is potentialized by the production of amyloid-beta (Aβ). Studies in both humans and rodents have uncovered an association between high GC levels and Aβ production. Chronic stress leads to the improper processing of Amyloid Precursor Protein (APP) along the amyloidogenic pathway, resulting in Aβ production (Catania et al., 2009). While the soluble, non-aggregated Aβ has been suggested to play a role in adapting to stress, the stress-induced misprocessing of APP and consequent Aβ production are associated with neurotoxic effects and disruption of synaptic remodelling (Catania et al., 2009; Green et al., 2006). Moreover, chronic stress and/or elevated corticosteroid levels have been shown to lead to hyperphosphorylation, accumulation and missorting of Tau protein, modifications that are observed in neurodegenerative conditions such as Alzheimer's disease (AD) and other Tauopathies (Green et al., 2006; Pinheiro et al., 2016; Sotiropoulos et al., 2011, 2015; Sotiropoulos & Sousa, 2016).

Altogether, the described structural, molecular, and cellular changes caused by stress and high GC levels (**Figure 3**) are widely believed to be the factors driving the stress-induced behavioural changes. Numerous studies have consistently demonstrated that chronic stress exerts substantial impacts on behaviour, both in humans and rodents. These behavioural shifts manifest as alterations in mood, emotional disturbances, depressive-like symptoms, heightened anxiety states, and cognitive deficits. These behavioural changes can be largely attributed to the effects of chronic stress and/or GC elevated

levels in the hippocampus and PFC (Cerqueira et al., 2007; McEwen & Akil, 2020; Sotiropoulos et al., 2011, 2015).

The hippocampus is the most vulnerable to chronic stress due to its neurons having the highest expression of GC receptors. This region not only plays a role in regulating physiological mechanisms of the stress response, as it is essential for emotional behaviour, learning and memory formation (e.g. declarative, spatial and contextual memory). The PFC is implicated in the processing of emotional stimuli, and in complex cognitive tasks, such as temporal organization of behaviour, decision-making, rule learning, and behaviour flexibility. Connections between these two brain regions contribute to the synergistic regulation of the behavioural and endocrine responses to stress (Godsil et al., 2013; Jay et al., 1995).

The PFC and hippocampus are targets to GCs and seem to be affected by stress in similar ways. In these areas, chronic stress and high GC levels often result in significant modulation of neurogenesis, dendritic atrophy, and synaptic loss, accompanied by a reduction in the volume and size of these brain regions (Banasr et al., 2021; Jay et al., 1995; Joëls et al., 2018; Joëls & Krugers, 2007; Sotiropoulos et al., 2008). The neuromorphological changes in the PFC are strongly correlated to disruption of the networks regulating mood and cognition as well as impairments in PFC-dependent behavioural adaptability (Cerqueira et al., 2005, 2007). Concurrently, hippocampal neuromorphological changes are linked to deficits in processes essential to learning and memory, like the hippocampal-dependent declarative, spatial and contextual memory (Rothman & Mattson, 2010; Sotiropoulos et al., 2015).

Interestingly, similar neuromorphological changes (e.g. reduction of volume and size) in these two brain areas have been identified in brains of Major Depressive Disorder (MDD) patients. Moreover, disturbances in the mechanisms that control the GC negative feedback can result in hypersecretion of these hormones, thought to contribute to at least 50 % of MDD cases. These findings, coupled with the observation that depressive-like behaviours are a consequence of chronic stress, provide substantial support for a plausible correlation between chronic stress and depression. This intricate interplay between chronic stress, neuromorphological changes in critical brain regions, and mood disorders underscores the profound impact of stress on mental health (Banasr et al., 2021; Radley et al., 2015; Sotiropoulos et al., 2008).

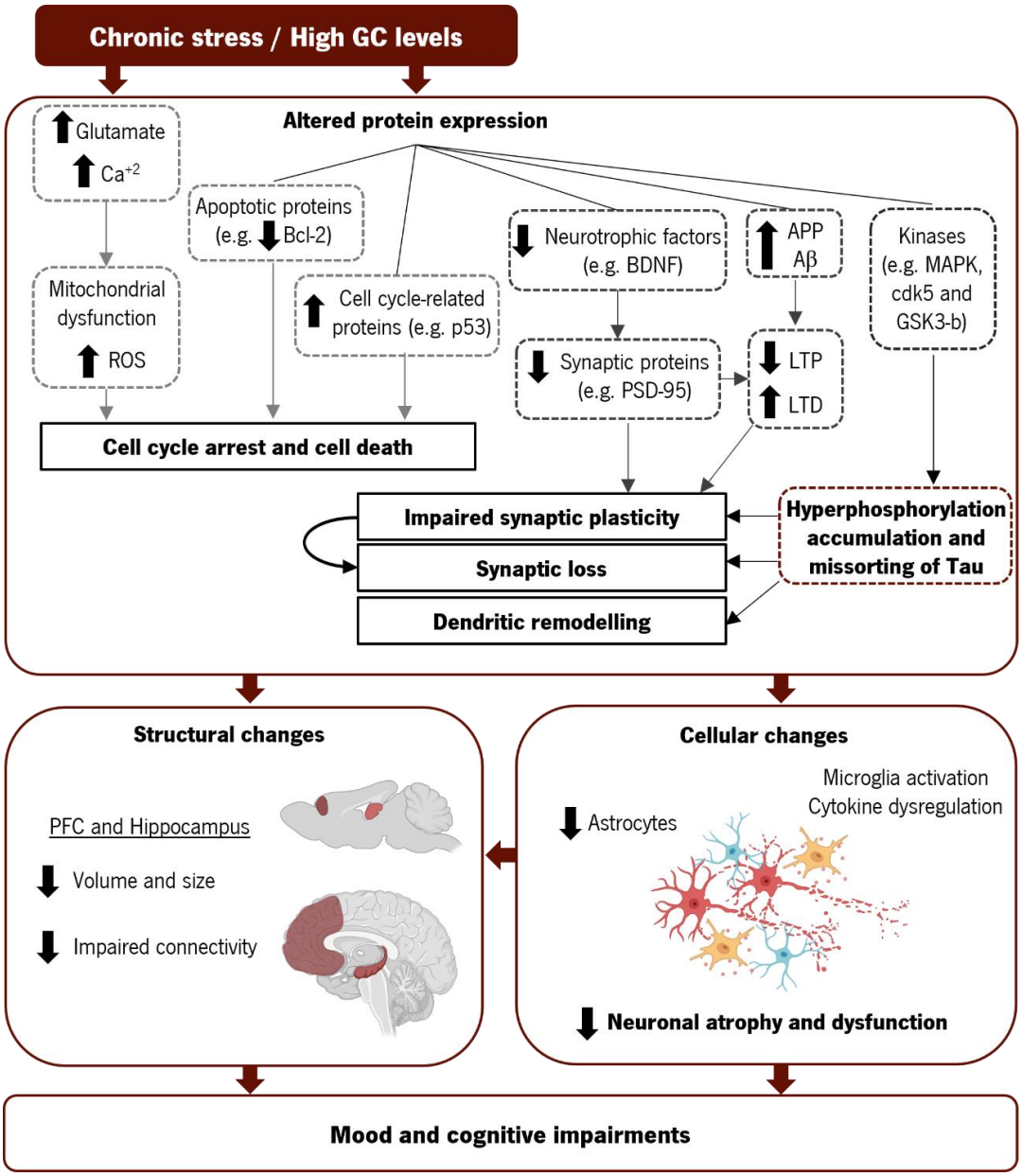


Figure 3. Molecular, cellular, structural, and behavioural changes associated with Chronic Stress and/or high levels of glucocorticoids (GCs). Chronic stress and elevated GC levels may trigger changes in the expression pattern of several proteins, such as apoptotic proteins (e.g. proteins from the Bcl-2 family) and/or cell-cycle related proteins (e.g. p53) that lead to cell cycle arrest and cell death. In addition, stress/GC can cause increased production of reactive oxygen species (ROS), mitochondrial fragmentation, and impairment of mitochondrial function, further enhancing the impairment of cellular functioning. Other proteins whose expression is affected by chronic stress/high GC levels are kinases (e.g. MAPK, cdk5 and GSK3-b), leading to aberrant phosphorylation, accumulation and missorting of Tau, leading to various consequences such as dendritic remodelling and synaptic loss. Negative changes enhanced by the inhibition of production of neurotrophic factors, such as brain derived nerve growth factor (BDNF), or the improper processing of Amyloid Precursor Protein (APP) and Aβ production. These numerous molecular changes will eventually lead to dendritic reorganization, neuronal atrophy, synaptic loss, and changes in astroglia and microglial cells, associated with structural changes, such as volumetric changes in brain areas (e.g. PFC and Hippocampus) and impaired connectivity between them. All these molecular, cellular and structural changes contribute to behavioural shifts, preceding, or similar to, those found in major depressive disorder and Alzheimer's Disease. Behavioural changes can manifest as alterations in mood, emotional disturbances, depressive-like symptoms, heightened anxiety states, and cognitive deficits.

1.4. Chronic Stress and Tau pathology

Tau protein belongs to the family of natively unfolded microtubule-associated proteins that bind to microtubules, the structural scaffolds within neurons that facilitate the transportation of cellular materials (e.g. nutrients) (Grundke-Iqbal et al., 1986; Weingarten et al., 1975). Tau plays a crucial role in the assembly and stabilization of microtubules, facilitating several processes as axonal transport and neurite outgrowth, with Tau phosphorylation being critical for proper function. Tau is also present at dendrites and synapses, where it acts as a scaffold protein and interacts with several proteins/receptors that modulate synaptic signalling and plasticity (Rodríguez-Martín et al., 2013; Ye et al., 2022).

As mentioned, chronic stress and/or high GC levels can lead to hyperphosphorylation, accumulation and missorting of Tau protein (**Figure 3**). Chronic stress and/or elevated levels of GCs induce the recruitment and activation of various kinases (e.g. MAPK, cdk5 and GSK3-b) in the PFC and hippocampus. These kinases, when overactivated, contribute to the aberrant hyperphosphorylation of Tau. As Tau function and distribution depend on its phosphorylation status, this excessive phosphorylation causes a disruption in Tau's interaction with microtubules, leading to its detachment and, consequent, somatodendritic accumulation. Moreover, the hyperphosphorylation leads to synaptic missorting, which consists of Tau migrating to the dendritic shafts from where it may diffuse to spines, and eventually to synapses. Tau accumulation and missorting trigger dendritic remodelling and synaptic loss, resulting in neuronal malfunction and, ultimately, cognitive impairment (Pinheiro et al., 2016; Sierra-Fonseca & Gosselink, 2018; Silva et al., 2019; Sotiropoulos et al., 2008; Sotiropoulos & Sousa, 2016; Vyas et al., 2016).

The impact of GCs and chronic stress extend further, interfering with the turnover of Tau, suggesting that GCs may reduce Tau degradation by dysregulation of molecular chaperones responsible for tau proteostasis, such as Hsp90 and Hsp70, two GR chaperones. Additionally, other members of the cytosolic GR-chaperones complex, FKBP51 and FKBP52, can also interact with Tau and cause its hyperphosphorylation and aggregation (Hoover et al., 2010; Mietelska-Porowska et al., 2014). Besides stress and GCs having an impact on the chaperones and proteases that regulate Tau levels, autophagy may also be an important player in this non-proteostasis pathway (Silva et al., 2019; Sotiropoulos et al., 2008). Altogether, these findings suggest an intersection between GR signalling and Tauopathies.

Tauopathies is the term used to describe a group of neurodegenerative diseases characterized by the aberrant accumulation of Tau within neurons in the brain. A major example of a Tauopathy is AD, which has as a hallmark the formation of neurofibrillary tangles (formed by Tau aggregates) (Sierra-Fonseca & Gosselink, 2018). Studies in AD models have shown that Tau has a significant role in the

neurodegenerative potential of chronic stress and GC towards the development of AD (Sotiropoulos & Sousa, 2016). It has been demonstrated that chronic stress and/or GC treatment can trigger Tau hyperphosphorylation in neuron cell bodies in several animal models of AD. This hyperphosphorylation of Tau epitopes (e.g. pSer262; pThr231) has been associated with cytoskeletal pathology, synaptic loss and hippocampal atrophy as well as cognitive impairment (Sierra-Fonseca & Gosselink, 2018).

In summary, chronic stress has been identified as a significant contributor to the exacerbation of Tau pathology, ultimately resulting in neurodegeneration and cognitive decline. Given its association with several detrimental lasting effects of exposure to elevated levels of GCs, as well as its link to Tau-related pathology, chronic stress has emerged as a risk factor for the development of neurodegenerative diseases, like AD and neuropsychiatric disorders in humans. These conditions include MDD, post-traumatic stress disorder and co-morbid anxiety (McEwen & Akil, 2020; Popoli et al., 2012; Radley et al., 2015; Sotiropoulos & Sousa, 2016).

1.5. Chronic Stress and the neuropathology of Depression

Chronic stress has emerged as a major contributing factor in the development of several psychiatric disorders, particularly Major Depressive Disorder (MDD) and Alzheimer's Disease (AD) (Kessler, 1997; McEwen & Akil, 2020; Popoli et al., 2012; Radley et al., 2015; Sotiropoulos & Sousa, 2016)

MDD is an affective disorder characterized by a persistent depressed mood, pessimistic thoughts, reduced pleasure or interest (also known as anhedonia), and diminished energy levels. Additionally, cognitive deficits, such as poor concentration and memory problems, are frequently observed in individuals experiencing depression (Hasler et al., 2004).

Depression is a complex condition with a heterogeneous nature, featuring varying courses and inconsistent responses to treatment. It is now widely accepted that depression's onset and progression involve a complex interplay of genetic, psychological, and environmental factors, including recurring episodes of stress (Villas Boas et al., 2019). Genetic risk factors for major depression exist in varying degrees across all individuals, however how these genetic influences play out depends on the individual's response and adaptation to stress (Wray et al., 2018). Chronic stress is recognized as one of the most common risk factors associated with the onset of depression, with GC playing a pivotal role in the disorder's pathophysiology (Krishnan & Nestler, 2008; Villas Boas et al., 2019).

Due to its complexity, the molecular neuropathology of depression remains largely elusive, existing two primary hypotheses for the development of MDD: the "Monoamine Hypothesis" and the "Neurotrophic Hypothesis" (Krishnan & Nestler, 2008; Villas Boas et al., 2019).

The monoamine hypothesis of depression suggests that reduced levels of neurotransmitters like serotonin (5-HT), noradrenaline and dopamine in the brain contribute to the disorder. Serotonin is critical for mood regulation, and alterations in its levels and receptors have been implicated in depression. In fact, many antidepressant drugs are design to increase monoamine transmission acutely either by inhibiting its uptake like the selective serotonin reuptake inhibitors (SSRIs), or by inhibiting degradation such as monoamine oxidase inhibitors (e.g. tranylcypromine). The SSRIs (e.g. Fluoxetine) are a common class of antidepressants that target the serotonin transporter (5-HTT), which plays a crucial role in serotonin clearance from synapsis (Krishnan & Nestler, 2008; Villas Boas et al., 2019).

The neurotrophic hypothesis of depression involves stress hormone (GC) and neurotrophic factors signalling. Emerging evidence indicates that impaired neuroplasticity is a central element in the pathophysiology of depression. This includes reduced neurogenesis in the hippocampus, dendritic atrophy, and decreased hippocampal volume, all of which have been implicated in the onset, progression, and remission of depressive symptoms. Dysregulation of adult hippocampal neurogenesis has also been linked to both the pathophysiology of depression and the action of certain antidepressant drugs, as some exert their effects through upregulation of neurotrophic factors expression, such as BDNF, one of the factors linking chronic stress and high GC levels to decreased neurogenesis (Mateus-Pinheiro et al., 2013; Villas Boas et al., 2019)

These two hypotheses are not mutually exclusive, the pathophysiology of depression is most likely a combination of the two, with the immune system, HPA axis and GCs, and monoamines levels contributing to altered neurotransmission, reduced plasticity and decreased neurogenesis.

These molecular and cellular changes are examples of common alterations present in a context of chronic stress and of depression. Additionally, clinical studies have reported that a substantial proportion of individuals with depression exhibit high baseline cortisol levels and a dysfunction in the HPA axis (Holsboer, 2000; Modell et al., 1997; Pfohl et al., 1985). Moreover, imaging studies have identified similar structural and functional abnormalities in the PFC and Hippocampus in individuals with depression and in models of chronic stress. Elevates GC levels are known to cause neuronal atrophy and synaptic loss in the PFC and Hippocampus, as well as reduced neurogenesis in the hippocampal dentate gyrus (DG), thereby eliciting structural changes, such as volumetric decreases in the PFC and hippocampus and altered functional connectivity to and from these limbic areas to other structures implicated in emotion regulation (Banasr et al., 2021; Mateus-Pinheiro et al., 2013; Villas Boas et al., 2019).

In conclusion, depression is a complex neuropsychiatric disorder influenced by genetic, psychological, and environmental factors, including chronic stress. It involves alterations in brain regions

associated with emotion regulation, learning, and memory. Dysregulated HPA axis activity and impaired neuroplasticity, particularly in the hippocampus, are key elements in its pathophysiology.

1.6. Animal Models and Therapies for Chronic Stress

The development of animal models of chronic stress has been instrumental in uncovering the mechanisms and detrimental effects linked to chronic stress. These models, while not perfect, can partially mimic the physiological and behavioural changes observed in humans under stress conditions. Consequently, these models have facilitated the study of the underlying pathological mechanisms and potential treatments for stress-related brain pathologies (Jaggi et al., 2011).

Stress models can be classified based on the type of stressor employed: physical (e.g. electric foot shocks) or psychological stressors (e.g. exposure to loud noises or immobilization). Notably, these different stressors tend to evoke distinct behavioural and physiological responses, emphasizing the importance of selecting models that can resonate across various mammalian species, including humans (Atrooz et al., 2021). Stress models can be further categorized based on the duration of stress exposure: acute and chronic models. In the acute model, stressors are applied for a short period of time (e.g. lipopolysaccharide (LPS) injection), whereas chronic stress entails the repeated application of stressful stimuli over an extended period (Atrooz et al., 2021).

There are multiple protocols to induce chronic stress in rodent models, differing in duration of the protocol, stressor type and research objectives. Examples include administration of GCs, sleep deprivation, separation of neonatal rodents from their mothers, social isolation, and exposure to repeated social stressors, such as social defeat by a dominant conspecific (Atrooz et al., 2021). Two frequently used protocols are the unpredictable Chronic Mild Stress (uCMS) and the Chronic Unpredictable Stress (CUS) paradigms. In the uCMS paradigm, rodents are exposed to a variety of mild stressors, such as alteration of light/dark cycle, social isolation, and food or water deprivation, in a random and unpredictable manner over a period of at least four weeks. The unpredictability of stressors mimics the chronic, low-grade stress experienced by humans in everyday life. This model is used frequently as a model for depression and anxiety (Monteiro et al., 2015).

Similarly, the CUS protocol consists of the intermittent exposure to daily stressful stimuli, randomly and unpredictably administered over a period of at least four weeks. Stressors used in CUS include immobilization (through restraint), light/dark cycle alterations, removal of bedding, and exposure to loud noises. While both protocols share similarities, differences regarding nature and unpredictability of the stressors applied exist. Notably, the main difference is the avoidance of stressors related to water and/or

food deprivation in the CUS protocol, rendering it more relevant to psychological stressors and offering a potentially more accurate representation of everyday life stressors in humans (Jaggi et al., 2011; Monteiro et al., 2015). The CUS model was developed to study the mechanisms underlying the stress response and its impact on various physiological and behavioural parameters. It has been shown to induce depressive- and anxiety-like behaviours, anhedonia, cognitive impairment, and alterations in neurochemistry and endocrine function in rodents (Atrooz et al., 2021; Jaggi et al., 2011; Monteiro et al., 2015).

Prolonged exposure to stressors or elevated GC levels in rodents has been associated with reactive glial cell proliferation, dendritic atrophy in the CA3 area of the hippocampus, and reductions in hippocampal volume, ultimately inducing depressive- and anxiety-like behaviours, anhedonia, and cognitive impairments (Atrooz et al., 2021; Jaggi et al., 2011; Monteiro et al., 2015). These neuronal and behavioural deficits are reversed with antidepressant treatment, which restores GCs levels to normal (Mateus-Pinheiro et al., 2013). Hence, these animal models have further elucidated the link between elevated glucocorticoid levels and depressive behaviours, supporting stress as, at the very least, a precipitating factor in development of depression. Thus, these models are known as Rodent Depression models. This recognition has spurred the adaptation of stress paradigms in models related to this disorder.

Unfortunately, there are currently no approved drugs specifically targeting chronic stress as a pathology. Existing therapeutical approaches focus on managing/relieving symptoms linked with chronic stress and related brain pathologies, such as anxiety disorders and depression. Consequently, chronic stress models have been used as preclinical models for the research of antidepressant drugs. Several studies have reported that antidepressant treatments can effectively revert the behavioural changes induced by the CUS protocol (Atrooz et al., 2021).

One of the most widely used antidepressant drugs is Fluoxetine, a classical SSRI that blocks serotonin transporters, thereby prolonging the action of serotonin. However, apart from its effects on serotonin, Fluoxetine has been shown to also have neuroprotector effects, mainly through its anti-inflammatory and antioxidant effects, inhibition of inflammation-induced neuronal apoptosis or promotion of neurogenesis (Khodanovich et al., 2018; Malberg et al., 2000; Zhao et al., 2020).

One challenge posed by these therapeutic approaches is that they primarily tackle the symptoms or outcomes of chronic stress, rather than directly addressing the root cause itself. The development and validation of a treatment approach capable of directly influencing multiple aspects of the stress paradigm would indeed represent a groundbreaking advance.

1.7. Tauroursodeoxycholic acid (TUDCA)

Bile acids are amphipathic molecules derived from cholesterol that play a crucial role in the digestion and absorption of lipids from the diet. These molecules aid in the emulsification, solubilization, and absorption of lipids, primarily in the small intestine. Bile acids are mostly known for their traditional role in lipid metabolism and digestion (Schonewille et al., 2016). However, their relevance to the nervous system remains unclear. Ever since it was suggested that bile acids have a role as endogenous neurosteroids and their systematic application provides neuroprotection, there has been an exponential rise in the number of studies involving bile acids as potential therapeutic approaches in neurodegenerative diseases (Ackerman & Gerhard, 2016; Dionísio et al., 2015; Keene et al., 2001; Khalaf et al., 2022; Rosa et al., 2018).

An example of a hydrophilic bile acid that has garnered significant attention in the field of neurodegenerative diseases is Tauroursodeoxycholic acid (TUDCA). TUDCA is produced, in low concentrations, in the liver by conjugation of taurine to ursodeoxycholic acid (UDCA), resulting in its distinctive chemical structure (Khalaf et al., 2022). UDCA is a primary bile acid approved by FDA (US Food and Drug Administration) for treatment of primary biliary cholangitis (Ackerman & Gerhard, 2016; Vang et al., 2014). While TUDCA is yet not approved by the FDA, it's already used in the treatment of cholestatic liver disease and cholelithiasis (Ma et al., 2016). Hydrophilic bile acids, such as UDCA and TUDCA, present a low toxicity profile and the ability to cross the blood brain barrier, advantageous properties that lead to their study as potential therapeutic agents in various models of diseases (Vang et al., 2014). This bile acid has displayed a remarkable range of actions, particularly in the context of neuroprotection and alleviating the development of neurodegenerative disorders including AD, Parkinson's Disease (PD) and Huntington's (Ackerman & Gerhard, 2016; Khalaf et al., 2022).

TUDCA's neuroprotection effects are mostly attributed to its ability to reduce oxidative stress, protect the mitochondria, inhibit apoptosis, trigger an anti-neuroinflammatory response, alleviate endoplasmic reticulum (ER) stress, and act as a chemical chaperone (**Figure 4**) (Huang et al., 2022; Khalaf et al., 2022; Zangerolamo et al., 2021). TUDCA orchestrates an anti-inflammatory response by modulating glial activation and mRNA expression of cytokines. Animal studies have shown that TUDCA can inhibit pro-inflammatory mediators' expression, such as TNF-α and IL-1β, and induce the expression of an anti-inflammatory mediators, such as arginase 1 and Annexin-A1, thereby dampening neuroinflammation (Kim et al., 2019; Mendes et al., 2019; Zangerolamo et al., 2021). It can also act through the activation of the transforming growth factor beta (TGFβ) pathway (Yanguas-Casás et al.,

2017). A major effect of TUDCA on the immune response is the decrease of astrogliosis and microgliosis, cellular reactions that occur in the CNS in response to injury or disease, characterized by proliferation and activation of astrocytes and microglia, respectively. This reduction in proliferation and activation of glial cells (Yanguas-Casás et al., 2014), may be attributed to the inhibition of the activation of NFκB (Kim et al., 2019), the decreased production of nitrites in astrocytes and microglia, and to the activation of the receptor GPBARI/TGR5, which causes an increase in intracellular cAMP levels in microglia to induce anti-inflammatory markers, while reducing pro-inflammatory ones (Yanguas-Casás et al., 2017).

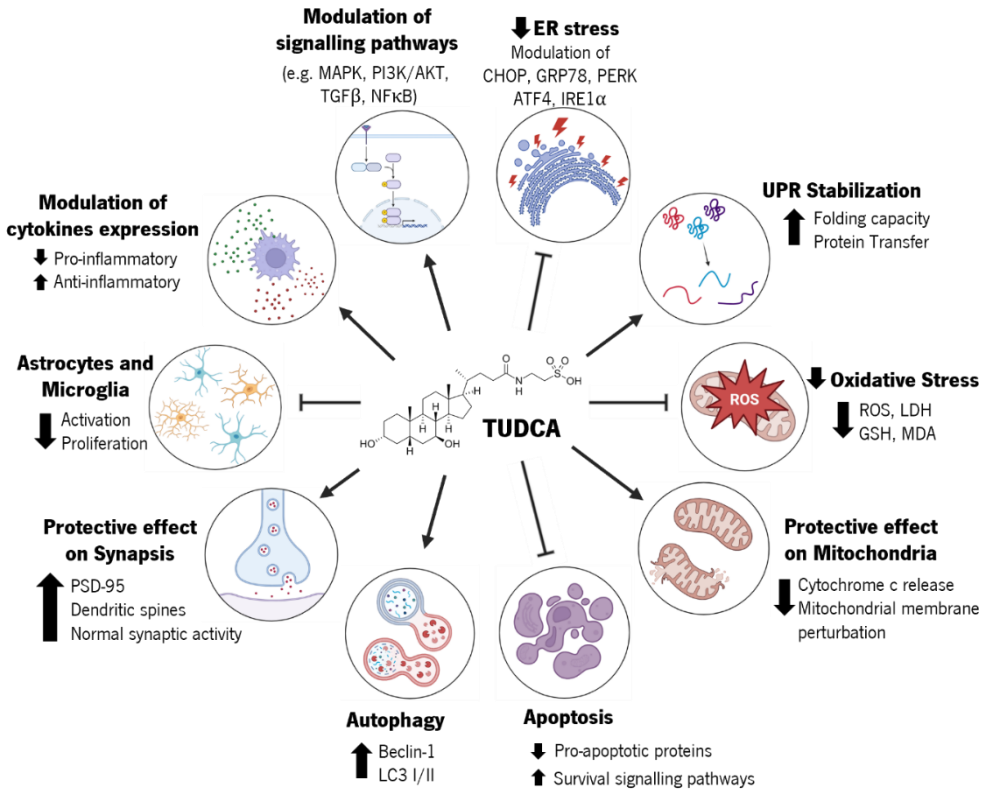


Figure 4. TUDCA's described in vitro and in vivo effects. *In vitro* and *in vivo* studies have reported several effects of TUDCA that contribute to its neuroprotective effects. It has been described that TUDCA can modulate signalling pathways (e.g. MAPK, PI3K/AKT, TGFβ, NFκB) as well as the expression of several proteins. By promoting calcium homeostasis, protein-folding, and the trafficking of mutant proteins, as well as inhibiting key mediators of Unfolded Protein Response (UPR) and endoplasmic reticulum (ER) stress, TUDCA can suppress the ER stress-induced cell death. Additionally, TUDCA can decrease oxidative stress through antioxidant effects and by protecting the mitochondria. These effects combined with TUDCA's modulation of the expression of proteins involved in apoptosis cascades and survival signalling pathways contribute to TUDCA anti-apoptotic effects. In addition, it has been reported that TUDCA may activate autophagy, through the up-regulation of autophagic related proteins, such as Beclin-1 and LC3II/I. At the synapsis level, TUDCA has been shown to increase the expression of PSD-95 and the number of dendritic spines, leading to a normal synaptic activity. Furthermore, TUDCA orchestrates an anti-inflammatory response by modulating mRNA expression of cytokines, as well as glial activation and proliferation. In summary, TUDCA's neuroprotective effects are mainly attributed to its ability to reduce oxidative stress, protect the mitochondria, inhibit apoptosis, trigger an anti-neuroinflammatory response, alleviate ER stress, and act as a chemical chaperone.

In addition to its anti-inflammatory effects, TUDCA exhibits *in vitro* and *in vivo* actions on ER stress, a cellular response triggered by various stressors (e.g. oxidative stress) that can culminate in cell death. ER stress is characterized by the accumulation of misfolded proteins, that initiate an adaptative response

called the unfolded protein response (UPR). Acting as a chemical chaperone, TUDCA stabilizes UPR by aiding in the correct folding and stability of proteins. Across diverse cell types, TUDCA enhances the removal of mutant proteins through the activation of the transcription factor 6α (ATF6α), a major UPR sensor and initiator (Kusaczuk, 2019; Yoon et al., 2016). Additionally, *in vitro* studies have showcased TUDCA's ability to repress the activation of several key-mediators of ER stress and UPR (Chen et al., 2022; Kusaczuk, 2019; Yoon et al., 2016). These include C/EBP homologous protein (CHOP), glucose-regulated protein 78 (GRP78), PKR-like ER kinase (PERK), activating transcription factor 4 (ATF4), inositol-requiring enzyme 1α (IRE1α) and caspase-12. Through the inhibition of caspase-12 activation in an ER stress-induced model of apoptosis, this bile acid stopped calcium efflux from the ER, preventing cell ER stress-induced cell death (Xie, 2002). Thus, by promoting calcium homeostasis, protein-folding, and the trafficking of mutant proteins, as well as inhibiting key mediators of UPR and ER stress, TUDCA can suppress the ER stress-induced cell death (Ożcan et al., 2006).

Besides suppressing apoptosis induced by ER stress, TUDCA acts in the apoptotic cascade, protecting neurons from cell death pathways. Two main pathways have been described in apoptosis: the extrinsic and the intrinsic apoptotic pathways. The extrinsic pathway is characterized by the activation of death receptors on the cell surface, leading to the activation of caspase 8, which activates caspase 3 and other downstream caspases. The intrinsic pathway, also known as the mitochondrial pathway, is initiated from within the cell, often in response to stressors like DNA damage, and involves the release of cytochrome c from the mitochondria, activating caspase 9, which then activates caspase 3 and other downstream caspases (O'Brien & Kirby, 2008). *In vitro* and *in vivo* studies have shown that TUDCA can interfere with the apoptotic pathways by inhibiting the death receptors, and by modulating mitochondrial membrane perturbation, inhibit cytochrome c release and caspase activation in several cell types, including neuronal cells (Ramalho et al., 2004; Rodrigues et al., 2000; Rodrigues, Solá, Sharpe, et al., 2003; Solá et al., 2003). TUDCA has been reported to influence the expression of pro- and anti-apoptotic factors, such as Bax, caspase 3 and Bcl-2, further reinforcing its anti-apoptotic profile(Ramalho et al., 2004; Rodrigues, Solá, Nan, et al., 2003). Additionally, this bile acid can promote neuronal survival and resilience, by modulating survival signalling pathways, such as the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) pathway (PI3K-Akt) and MAPK pathways (Qiao et al., 2002; Rust et al., 2000; Solá et al., 2003). Furthermore, it has been reported that TUDCA may activate autophagy, through the up-regulation of autophagic related proteins, such as Beclin-1 and LC3II/I (Chang et al., 2022; Rosa et al., 2017). Overall, TUDCA exhibits multifaceted anti-apoptotic effects through its actions on various apoptotic pathways, mitochondrial function, ER stress, and survival signalling.

In addition, TUDCA demonstrates a potent protective effect against oxidative stress, a cellular response to various stimuli, including reactive oxygen species (ROS), that may lead to cell damage and death. *In vivo* studies have reported that TUDCA can mitigate the changes in oxidative stress-related factors, such as the levels of ROS, lactate dehydrogenase (LDH), glutathione (GSH) and malondialdehyde (MDA) (Chen et al., 2022; Rosa et al., 2018). This reversion helps protecting cells against oxidative stress, maintaining cellular integrity and functionality.

Besides TUDCA's effects on neuronal survival, it seems to also have a protective effect at the synaptic level. In a model of AD, TUDCA counteracted the downregulation of postsynaptic density-95 protein (PSD-95), a fundamental synaptic scaffolding protein that plays a role in synaptic integrity and function. Simultaneously, the bile acid increased the number of dendritic spines, and reduced spontaneous miniature excitatory synaptic activity (Ramalho et al., 2013). Thus, TUDCA appears to have positive effects on synapses by preserving synaptic proteins, reducing abnormal synaptic activity, and having a protective action against detrimental effects of factors like Aβ.

In conclusion, TUDCA exhibits a multifaceted and wide range of neuroprotective effects (**Figure 4**), which collectively support its potential as a therapeutic agent in the context of neurodegenerative disorders. However, further research is warranted to fully understand TUDCA's mechanisms of action and its true therapeutic potential in the clinic.

1.8. TUDCA as a potential therapeutical drug against stress-related brain pathologies

Although several studies have researched and reported the effects of TUDCA, the mechanisms of action of this bile acid are still not fully understood. As a signalling molecule, TUDCA can activate membrane receptors, such as Takeda G Protein-Coupled Receptor 5 (TGR5) or α5β1-Integrin, and can act through activation of nuclear receptors, mostly common through the Farnesoid X Receptor (FXR) (JUŘICA et al., 2016; Yoon et al., 2016). Additionally, it has been demonstrated that TUDCA can interact with the GR and requires this receptor for some of its cellular effects (Cai et al., 2005; Khalaf et al., 2022; Solá et al., 2006). While these receptors are present in the brain and have been implicated in TUDCA' neuroprotective actions, whether TUDCA directly exerts its effects through these receptors, as well as the mechanisms and interactions involved, are still unclear.

Comparing the described cellular and molecular changes induced by the chronic stress and/or elevated levels of GCs with the described effects of TUDCA in various *in vitro* and *in vivo* models, there is a noticeable overlap in various pathways (**Figure 5**), raising the question if TUDCA may revert stress-induced effects.

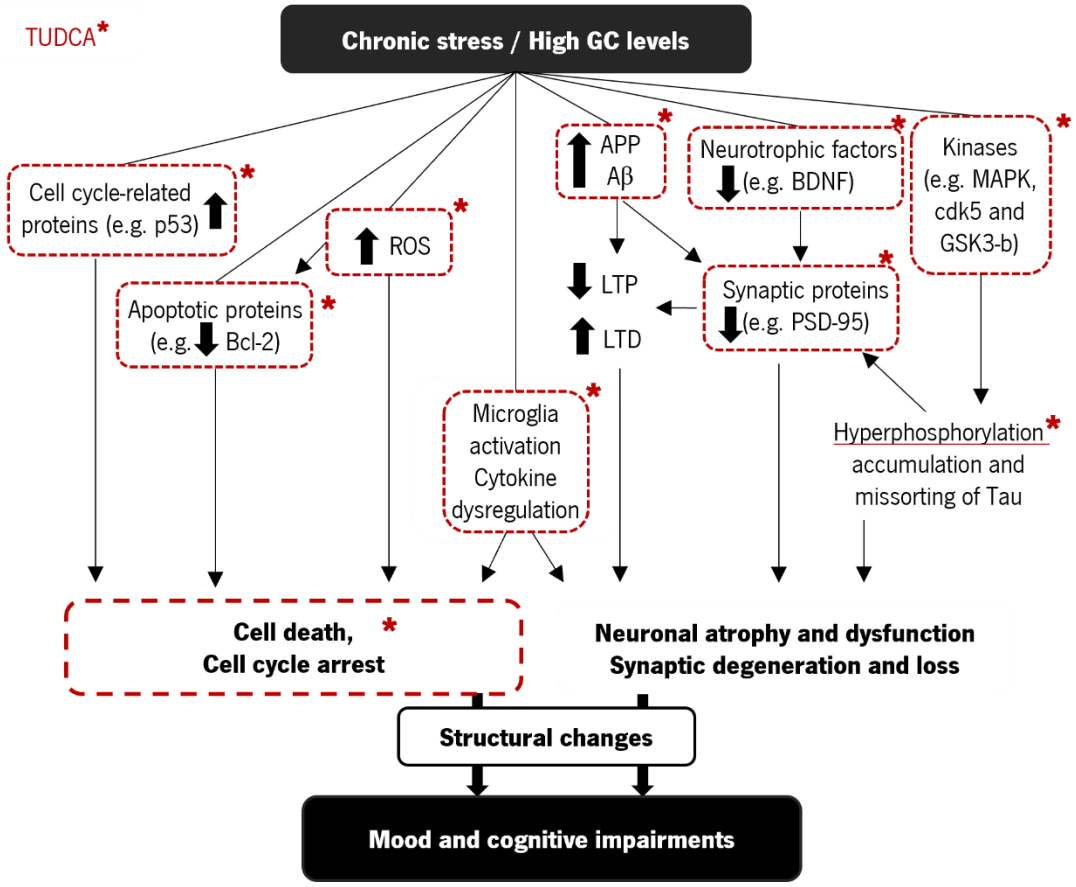


Figure 5. Overlapping effects of TUDCA on chronic stress-related pathways. Chronic stress and elevated GC levels may trigger changes in the expression pattern of several proteins, such as apoptotic proteins and/or cell-cycle related proteins that lead to cell cycle arrest and cell death. In addition, stress/GC can cause increased production of reactive oxygen species (ROS) and impairment of mitochondrial function, as well as inhibition of production of neurotrophic factors, and Aβ production. Other proteins whose expression is affected by chronic stress/high GC levels is associated with aberrant phosphorylation, accumulation and missorting of Tau. All these changes lead to dendritic reorganization, neuronal atrophy, synaptic loss, and ultimately to mood and cognitive impairments. TUDCA has been shown to modulate molecules and pathways involved in the chronic stress effects. *In vitro* and *in vivo* studies have reported that TUDCA can modulate signalling pathways (e.g. MAPK, NFκB) as well as the expression of several proteins such as those involved in apoptotic cascades and cell cycle, preventing cell death. Moreover, TUDCA has an anti-inflammatory effect by modulating mRNA expression of cytokines, as well as glial activation and proliferation. Additionally, TUDCA can decrease oxidative stress through antioxidant effects and by protecting the mitochondria. At the synapsis level, TUDCA appears to have positive effects by preserving synaptic proteins, reducing abnormal synaptic activity, and having a protective action against detrimental effects of factors like Aβ. In summary, TUDCA's neuroprotective effects are mainly attributed to its ability to reduce oxidative stress, protect the mitochondria, inhibit apoptosis, trigger an anti-neuroinflammatory response, alleviate ER stress, and act as a chemical chaperone. The overlapping effects of TUDCA on chronic Stress-related pathways are highlighted by a red box and a *.

So far only three studies have indicated TUDCA as a potential modulator of depression due to exhibiting antidepressant-like activities in depression mice models induced by CUS and LPS via attenuation of neuroinflammation, ER stress and oxido-nitrosative stress (Cheng et al., 2019; Lu et al.,

2018; Wu et al., 2019). TUDCA was shown to revert the CUS-induced negative effects on behaviour, such as increased immobile time in the Forced Swimming (FST) and Tail Suspension tests (TST) and decrease in sucrose intake in the Sucrose Preference Test (SPT), three frequently used tests to assess depressive-like behaviours (Cheng et al., 2019). Additionally, treatment with TUDCA was shown to alleviate LPS-induced cognitive deficits in the Morris Water Maze (MWM) and Y-maze avoidance (Wu et al., 2019). At a molecular level, TUDCA normalized the levels of pro-inflammatory cytokines, such as tumor necrosis factor α (TNFα) and interleukin-6 (IL6), in the PFC and hippocampus. It reduced the CUS-induced increase of inflammasome and microglial activation markers, including nod-like receptor protein 3 (NLRP3) and IBA-1(Cheng et al., 2019; Lu et al., 2018; Wu et al., 2019). Changes in oxidative–nitrosative and ER stress markers induced by CUS, such as nitric oxide, reduced GSH, MDA, CREB and GRP78, were also reverted by treatment with TUDCA (Cheng et al., 2019; Lu et al., 2018). Moreover, TUDCA was also shown to alleviate the LPS-induced synaptic dysfunction in the hippocampus by inhibiting the LPS-induced decrease in the expression of BDNF, PSD-95 and synaptophysin (Wu et al., 2019).

As chronic stress, high levels of GCs and Tau pathology are intertwined, it is also important to test TUDCA's potential action on Tau pathology. Two *in vitro* studies have briefly reported that TUDCA can prevent Tau hyperphosphorylation by inhibiting UPR and ER stress in human neuroblastoma cell line, previously treated with 2-deoxy glucose, a UPR inducer (van der Harg et al., 2014), and prevent the cleavage of Tau by caspase 3 through inhibition of apoptosis and caspase 3 activation in primary cortical neurons incubated with fibrillar $A\beta_{1-42}$ (Ramalho et al., 2008). A *in vivo* study has reported that TUDCA supplementation alters Akt/GSK3β activities and prevents tau phosphorylation in APP/PS1 mouse brains, well-established models of AD (Dionísio et al., 2015).

Taken together, all these findings suggest that TUDCA may be a promising therapeutical strategy against stress and stress-related brain pathologies like depression and Tau pathology. Hence, this project aims to study the therapeutic effects of TUDCA on stress-related brain pathologies as Tau pathology and depression induced by CUS, and to explore the intracellular pathways that mediate the effects of TUDCA, using an *in vivo* model of chronic stress.

CHAPTER 2

AIMS

Previous studies have suggested TUDCA as a potential therapeutic strategy for depression and stress-related brain pathologies, due to its neuroprotective effects. However, there is a lack of studies regarding TUDCA' molecular, cellular, and behavioural effects on *in vivo* models of Chronic Stress. Therefore, this project's goal is to study TUDCA's therapeutical effects against stress and related brain pathologies, in particular depression and Tau pathology, understanding the role of GR for the modulation of the previously proposed mechanisms of action (on neuroinflammation, ER stress and UPR). In the light of our premises, the current project aims to:

I. **Study the potential therapeutic effects of TUDCA against chronic stress** through the use of an *in vivo* model to study the potential therapeutic effects of TUDCA against stress-related anxiety- and depressive-like behaviours and cognitive deficits;

II. **Analyse TUDCA effects in a cell specific manner, in the context of chronic stress** through analysis of different cell populations of the brain, astrocytes, microglia and neurons;

III. **Understand the molecular effects of TUDCA in a chronic stress context** through analysis of protein and mRNA levels of proteins involved in GR signalling, inflammation, ER stress, synaptic plasticity, and tau hyperphosphorylation/accumulation and missorting.

With this project, we expect to clarify the therapeutic potential of TUDCA against stress-related brain pathologies. In addition, we will use this experimental design to clarify the molecular mechanisms modulated by TUDCA and the importance of GR for its mechanisms of action, focusing on neuroinflammation, ER stress and UPR.

CHAPTER 3

MATERIAL AND METHODS

3.1. Animals and Experimental Design

Wild-type mice (3-month-old C57BL/6J) were used in this study. Mice were housed in groups of 4–5 per cage under standard environmental conditions (8 a.m.– 8 p.m. light cycle; 22 °C; 55 % humidity, ad libitum access to food and water). Animals were kept and handled following the guidelines for the care and handling of laboratory animals in Directive 2010/63/EU of the European Parliament and Council. All experiments were conducted following the Portuguese national authority for animal experimentation, Direção Geral de Alimentação e Veterinária (ID: DGAV9457). Animals were divided into 4 groups: control (CON, n=28, 13 males and 15 females), stressed group (STR, n=27, 11 males and 16 females), stressed treated with TUDCA (STR + TUDCA, n=28, 13 males and 15 females) and stressed treated with Fluoxetine (STR + FLUOX, n=28, 11 males and 17 females). Animals were divided into two sets, to assure validity of the data, and later joint for complex behaviour analysis and sex separation.

3.2. Chronic Unpredictable Stress (CUS) protocol

Stressed animals were subjected to a Chronic Unpredictable Stress (CUS) protocol over a period of 5 weeks before the treatments and behavioural testing as shown in **Figure 6**. The CUS protocol was done during the daily period of light and consisted of four different stressors: restraint, vibrating platform, overcrowding, and exposure to a hot air stream (hairdryer). Animals were exposed to one stressor per day for 2-3 hours (restraint, vibrating platform, overcrowding) or 30 minutes (hairdryer). The order of stressors and the time of the day at which the stressor was applied was randomly chosen and varied from week to week to promote unpredictability. During the stress period, control (non-stressed; CON) mice remained undisturbed in their home cages. Body weights were assessed every week throughout the study as an indication of CUS protocol efficacy.

After these 5 weeks, the CUS protocol was maintained until the end of the experiment to avoid regression of the stress-induced effects. During behavioural testing, stressors, as well as injections, were applied only after the behaviour test was done to not affect the mice's performance.

3.3. Drugs and Treatments

Tauroursodeoxycholic Acid (TUDCA; Sigma) and Fluoxetine (FLUOX; Kemprotec, Middlesbrough, UK) were used as potential treatments for stress-related brain effects. TUDCA (50mg/Kg) and Fluoxetine (10 mg/Kg) solutions were prepared weekly by dissolving in sterile 0.9 % (v/v) saline for intraperitoneal (i.p.) injections. Sterile 0.9 % saline was used as the vehicle control and administered to animals from the control and stress groups at the same time points as TUDCA and Fluoxetine. After being subjected to 5

weeks of CUS protocol, animals from STR+TUDCA and STR+FLUOX started receiving daily i.p. injections of TUDCA and Fluoxetine, respectively, for 10 sequential days. After, behaviour tests were performed and treatment was given every other day until the end of the experiment as represented in **Figure 6**.

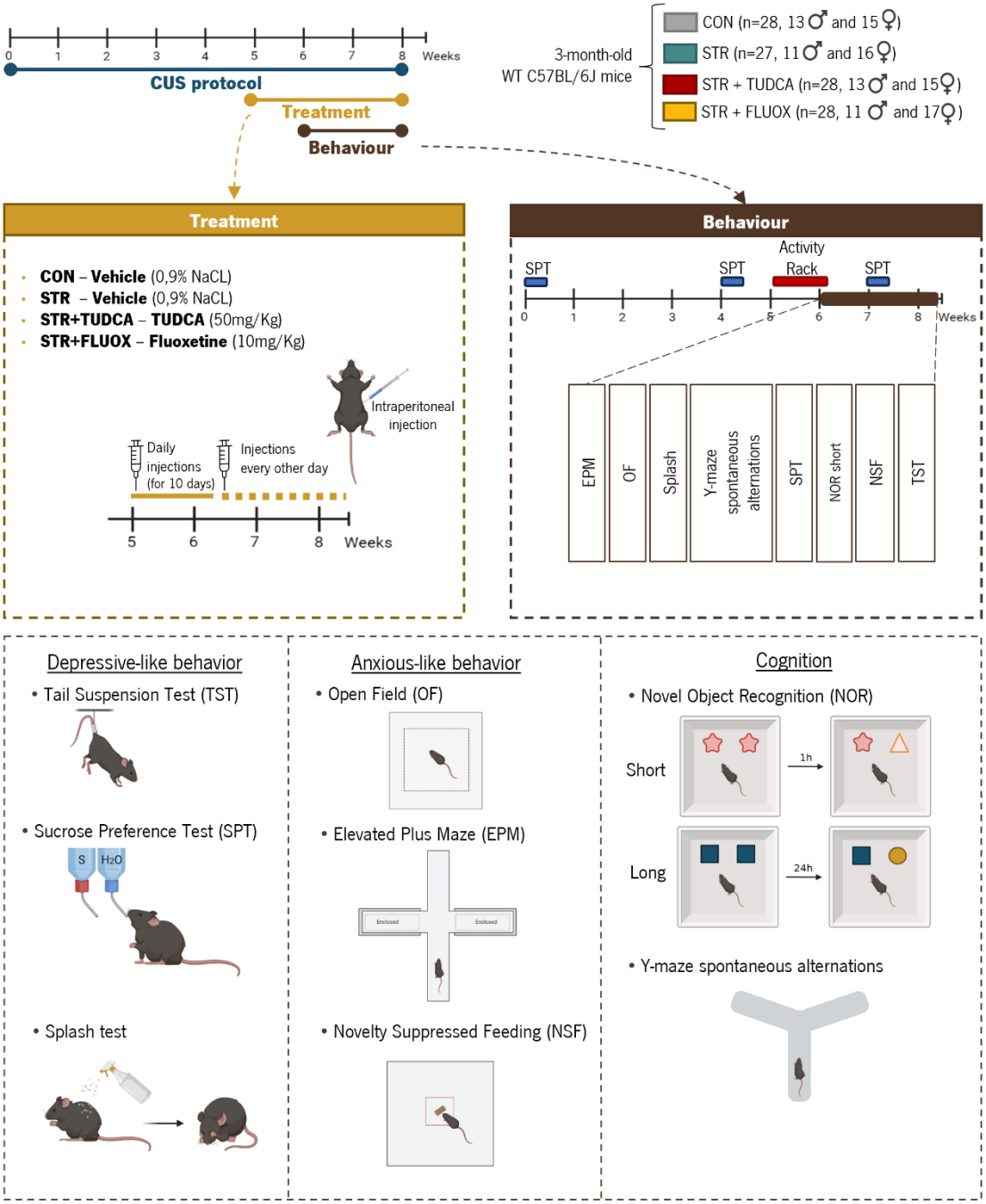


Figure 6. Experimental Design. 3-month-old Wild-type male and female mice were subjected to a Chronic Unpredictable Stress (CUS) protocol over a period of 5 weeks before the treatments and behavioural testing. After this 5-weeks period, the CUS protocol was maintained until the end of the experiment to avoid regression of the stress-induced effects. Treatments started at the 5th week and consisted of daily intraperitoneal injection for 10 sequential days. After this period, behaviour tests started being performed and animals received the treatment every other day until the end of the experiment. Sterile 0.9 % (v/v) saline was used as the vehicle control and was administered to animals from the control and stress groups at the same time points as TUDCA (50 mg/Kg) and Fluoxetine (10 mg/Kg). For behaviour assessment a battery of tests was performed to assess depressive-like behaviour (Tail Suspension Test and Sucrose Preference), anxiety-like behaviour (Open Field, Elevated Plus Maze, Novelty Supressed Feeding and Splash test) and cognition (Novel Object Recognition short and long, and Y-maze spontaneous alternations).

3.4. Behaviour analysis

Open Field (OF)

The Open Field (OF) test was conducted to assess the anxiety-like behaviour of the animals. It was performed in a square arena (43.2 cm x 43.2 cm) surrounded by transparent acrylic walls and with a white floor (Med Associates Inc., St. Albans City, VT). Mice were placed in the centre and allowed to explore the area for 5 minutes. After each trial, the arena was cleaned with a 10 % (v/v) ethanol solution. Two 16-beam infrared arrays and the manufacturer's software (Activity Monitor Software) were used to automatically register animals' movements. Time spent and distance travelled in the centre of the arena were used as indexes of anxiety. Total distance travelled was used as an indicator of locomotor activity.

Elevated Plus Maze (EPM)

EPM was used to evaluate anxiety-like behaviour. Animals were placed in the centre of the EPM apparatus consisting of two open arms (50.8 cm × 10.2 cm) and two closed arms (50.8 cm × 10.2 cm × 40.6 cm), and entries, as well as time spent in open and closed arms, were measured for 5 minutes. After each trial, the maze was cleaned with a 10 % (v/v) ethanol solution to prevent movement based on olfactory cues. Data were collected with a charge-coupled device (CCD) camera and analysed with EthoVision®XT software using the NIH Image program (Noldus).

Novelty Supressed Feeding (NSF)

Novelty Supressed Feeding (NSF) is a conflict-based test to assess anxiety-like behaviour. After being deprived of food for 24 hours, animals face the choice of approaching and consuming a piece of food in the centre of an open arena or staying to the side and avoiding the centre. This test was performed in the arena used for the OF test and a pre-weighted pellet was placed in the centre defined by a square (7 cm x 7 cm) drawn with a red marker. Animals were placed in the right corner of the arena and their latency to go to the centre, as well as to start eating the pellet, were measured manually. Immediately after the animal started eating the pellet, both were placed in an individual box and allowed to stay undisturbed for 30 minutes. A limit of 10 minutes was given to every animal, mice that did not start feeding within this time were excluded from the analysis. After the 30 minutes, mice were returned to the home cage and the pellet was weighted to calculate the amount consumed by each mouse in the 30 minutes.

Splash test

The Sucrose Splash test consisted of spraying a sucrose solution (10 % (w/v)) on the dorsal coat of mice in cages identical to their home cages. The sucrose solution was chosen as a releaser and a persisting factor of grooming due to its viscosity. The latency to initiate a grooming behaviour, as well as the duration of grooming, was recorded for 5 minutes and manually scored using Kinoscope software (http://sourceforge.net/projects/kinoscope/).

Sucrose Preference Test (SPT)

The Sucrose Preference Test (SPT) was performed to evaluate anhedonia, which is a depressive-like behaviour. Baseline sucrose preference values were established during a test session before the beginning of the CUS protocol. The SPT was performed again, on the same conditions, on weeks 4 and 7 of the CUS protocol, before and after the treatments were given, respectively (**Figure 6**). For this test, mice were moved to an experimental room and housed individually in cages identical to their home cages for 48 hours. The animals were presented with two pre-weighted bottles (water and sucrose) which were re-weighed at the end of the test. The sucrose (2 % (w/v)) and water bottles' positions were switched 24 hours after the test began, to prevent any confound produced by a side bias. The following parameters were assessed: sucrose preference and total intake. Animals that presented no intake were excluded from the analysis. The sucrose preference was calculated as follows: Sucrose preference index (%) = (sucrose solution consumed / (water + sucrose) solution consumed) × 100

Tail Suspension Test (TST)

Mice were suspended by the tail for 5 minutes. The activity was recorded and, subsequently, immobility time was scored by EthoVision®XT software using the NIH Image program (Noldus) and used as a depressive-like behaviour indicator. Animals that managed to climb and rest in the platform where their tails were suspended with tape were excluded from the analysis.

Y-maze spontaneous alternations

The Y-maze test was used to assess PFC-dependent working memory based on the spontaneous alternations task. Animals were placed in the centre of the Y-maze apparatus (33 cm × 7 cm × 15 cm) and allowed to freely move for 7 minutes. The number and order of arm entries were recorded. Spontaneous alternations were calculated as the ratio of the number of triads (sequence of three

consecutive arm entries) and arm entries. The trials were recorded with a camera and analysed with EthoVision®XT software using the NIH Image program (Noldus).

Novel Object Recognition (NOR)

The Novel Object Recognition (NOR) test was performed to evaluate cognition, particularly recognition memory. The experiment takes advantage of rodents' predisposition to investigate unfamiliar objects more than familiar ones. The choice to investigate the novel object shows how learning and recognition memory are used. For 3 days, animals were habituated individually to an open arena (33 cm × 33 cm × 33 cm) for 20 minutes. To assess short-term memory, on the fourth day, each animal was allowed to explore 2 identical objects for 10 minutes in the open arena previously explored. One hour later, mice were returned to the arena, with one of the objects being replaced by a different object in the same position as the previous one. For the assessment of long-term memory, on the fifth day, each animal was allowed to explore a pair of new identical objects for 10 minutes. Approximately 24 hours later, mice were returned to the arena, with one of the objects being replaced by a different object. The chamber was cleaned with 10 % (v/v) ethanol and dried with paper towels to avoid odour impregnation after each trial. In these tests, exploration time was defined as the time the mouse's nose was no more than 1 cm away from the object. The discrimination ratio was calculated by dividing the difference between the time spent exploring the novel object and the time in the old object by the total time spent exploring the two objects. Sessions were videotaped and evaluated using ANY-maze Video Tracking System 7.16.

The Activity Rack

This is a test with an extended length that enables a more complete investigation of the circadian rhythm, activity patterns, and behavioural changes in mice over time. Mice are placed in an activity rack (DCV rack) that is outfitted with sensors to continually monitor their activities throughout the day. As placement in the DCV rack requires housing only two animals per cage, only a part of the animals was used in this assessment (CON: 6 males and 6 females; STR: 6 males and 8 females; STR+TUDCA: 8 males and 8 females; STR+FLUOX: 4 males and 12 females). In this experiment, animals were placed in the activity rack for 9 days which coincided with the last days of daily injections and the first two behaviour tests.

3.5. Brains preparation for Neuronal structure analysis

Mice were deeply anesthetized [ketamine hydrochloride (150 mg/kg) plus medetomidine (0.3 mg/kg)] and transcardially perfused with saline. Each brain (n=5-6 per group) was immersed in Golgi-Cox solution for 14 days and then transferred to 30 % (w/v) sucrose solution. These brains will be cut in vibratome-cut coronal brain sections (thickness of 200 µm) and used to perform three-dimensional morphometric analysis (**Figure 7**). Dendritic arborization and spines will be analysed in dorsal hippocampus (DG area) and prefrontal cortex (Prelimbic area).

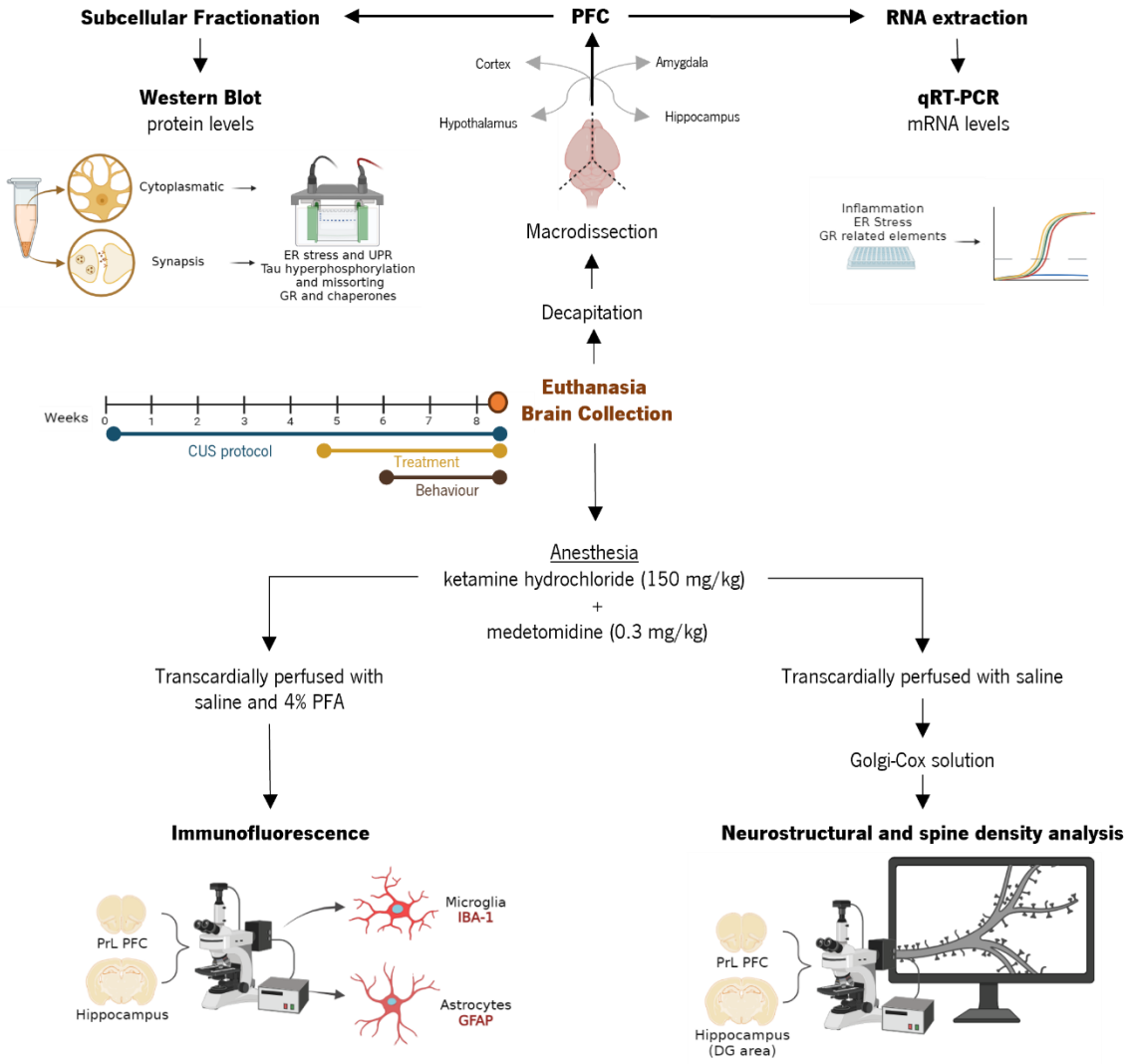


Figure 7. Brain collection for cellular and molecular analysis. After behavioural assessment, some animals were euthanized by decapitation, their brains were collected and macrodissected for posterior molecular analysis. RNA was extracted for qRT-PCR analysis of mRNA levels in the PFC, while, in other PFC samples, a subcellular fractionation protocol was performed to study protein levels by Western Blot. Other animals were deeply anaesthetized, transcardially perfused with either saline solution or saline followed by 4 % (v/v) PFA and their brains collected. Brains collected after perfusion with saline were put in a Golgi-Cox solution for posterior neurostructural and spine density analysis in subregions of the PFC and hippocampus. The remaining brains collected after perfusion with saline and PFA were used to perform cellular analysis by immunostaining hippocampal and PFC coronal slices with antibodies for microglia and astrocyte markers.

3.6. Immunofluorescence

Mice (n=4-6 per group) were deeply anesthetized [ketamine hydrochloride (150 mg/kg) plus medetomidine (0.3 mg/kg)] and transcardially perfused with saline followed by ice-cold 4 % (v/v) paraformaldehyde, as represented in **Figure 7**. Brains were removed, post-fixed in 4% paraformaldehyde for 4 h, and then transferred to a 30 % (w/v) sucrose solution until they sunk. After impregnation with sucrose, the brains were immersed in a 2 % (w/v) agarose solution and sectioned coronally in slices with 30 μm of thickness using a vibratome (Leica VT1000S, Germany). Brain slices were maintained in phosphate-buffered saline (PBS) solution until the immunolabeling protocol or stored in cryopreserving solution for long-term storage at -20 °C.

IBA-1 staining

Brain slices were washed twice with PBS solution and then submitted to an antigen retrieval step with citrate buffer (10 mM, pH 6.0, Sigma-Aldrich). Briefly, slices were placed in a preheated citrate solution and incubated for 20 minutes at 80 °C then left to cool for a few minutes. The antigen retrieval step was followed by permeabilization with 0.5 % (v/v) Triton-X100 (Sigma Aldrich, United States) in PBS solution (0.5 % (v/v) PBS-T) for 20 minutes. Then the slices were incubated with the blocking solution of foetal bovine serum (FBS at 5% (v/v)) in 0.3 % (v/v) PBS-T for 30 minutes at RT. This blocking step was followed by washes with 0.3 % (v/v) PBS-T (RT; 3 × 5 min) and an overnight incubation, at 4 °C, with the primary antibody rabbit polyclonal anti-IBA-1 (1:2000; FujiFilm 019-19741) in a 0.3 % (v/v) PBS-T solution with 1 % (v/v) FBS. On the next day, the slices were washed in 0.3 % (v/v) PBS-T (RT; 3 × 5 min) and incubated with the secondary antibody Alexa Fluor® 594 donkey anti-rabbit (1:1000; Invitrogen, 2066086) in a 0.3 % (v/v) PBS-T solution with 1 % (v/v) FBS, for 2 hours at RT. After incubation, the slices were washed with 0.3 % (v/v) PBS-T (RT; 3 × 10 min), and the nuclei were labelled for 10 minutes at RT with 4′,6-diamidino-2-phenylindole (DAPI, 1:1000; Invitrogen, United States) in PBS solution. Finally, slices were washed with PBS (RT; 3 × 3 min) and mounted using an aqueous mounting medium (Epredia™ Lab Vision™ PermaFluor™ Aqueous Mounting Medium).

GFAP staining

Brain slices containing the Hippocampus were washed with PBS and then submitted to the antigen retrieval step with citrate buffer (10 mM, pH 6.0, Sigma-Aldrich). Briefly, slices were placed in a preheated citrate solution and incubated for 20 minutes at 80 °C then placed immediately in water to cool. After, slices were washed with PBS (RT; 2 × 3 min) and then 0.1 % (v/v) PBS-T (RT; 5 min), followed by

permeabilization with 0.5 % (v/v) PBS-T for 15 min at RT. After permeabilization, the slices were incubated with the blocking solution of FBS (10 % (v/v)) in 0.5 % (v/v) PBS-T and DMSO (1 % (v/v)) for 30 minutes at RT. This blocking step was followed by an overnight incubation, at 4° C, with the primary antibody rabbit polyclonal anti-GFAP (1:2000; Proteintech, 16825-1-AP) in a 0.5 % (v/v) PBS-T solution. On the next day, the slices were washed in 0.1 % (v/v) PBS-T (RT; 3 × 3 min) and incubated with the secondary antibody Alexa Fluor® 594 donkey anti-rabbit (1:1000; Invitrogen, 2066086) in a 0.5 % (v/v) PBS-T solution for 2 hours at RT. After incubation, the slices were washed with 0.1 % (v/v) PBS-T (RT; 2 × 5 min) and PBS (RT; 10 min). The nuclei were labelled for 10 minutes at RT with 4′,6-diamidino-2-phenylindole (DAPI, 1:1000; Invitrogen, United States) in PBS solution. Finally, slices were washed with PBS (RT; 3 × 3 min) and mounted using a Mounting Medium (Epredia™ Lab Vision™ PermaFluor™ Aqueous Mounting Medium).

3.7. Microglial and Astrocyte Density Analysis

For the analysis of microglial and astrocyte density, three coronal brain sections per animal (n=5-6 per group) were imaged twice (in both hemispheres) for each region of interest (PrL PFC and dorsal Hippocampus). The inverted fluorescence microscopy (Olympus Widefield Inverted Microscope IX81) with a resolution of 1376 × 1038 px and a 40× objective was used to obtain all images, which include two distinct channels (red for IBA-1 or GFAP; blue for DAPI). ImageJ software was used to count the total number of IBA-1-positive cells and to measure the fluorescence intensity in IBA-1 and GFAP.

3.8. Subcellular Fractionation and Western Blot Analysis

Part of the animals (N=6-7 per group) were killed by decapitation and their hippocampus and PFC tissue were macrodissected and immediately stored at –80 °C (**Figure 7**). For fractionation, a well-established fractionation protocol was used. PFC and hippocampi were homogenized with 6 volumes of homogenization buffer [sucrose 9 % (w/v); 5 mM DTT; 2 mM EDTA; 25 mM Tris pH 7.4; complete protease inhibitor (Roche), and phosphatase inhibitor cocktails II and III (Sigma)] using a glass-Teflon homogenizer. Post-nuclear supernatant was subjected to centrifugation (12,500 g) and divided into the crude synaptossomal fraction (P2) and synaptosome-depleted fraction (S2).

The P2 fraction was further lysed hypoosmotically and centrifuged (25,000 g) for 20 minutes to obtain the synaptossomal fraction (LP1) and soluble synaptic proteins (LS1). S2 and LP1 fractions were used for Western blot analysis.

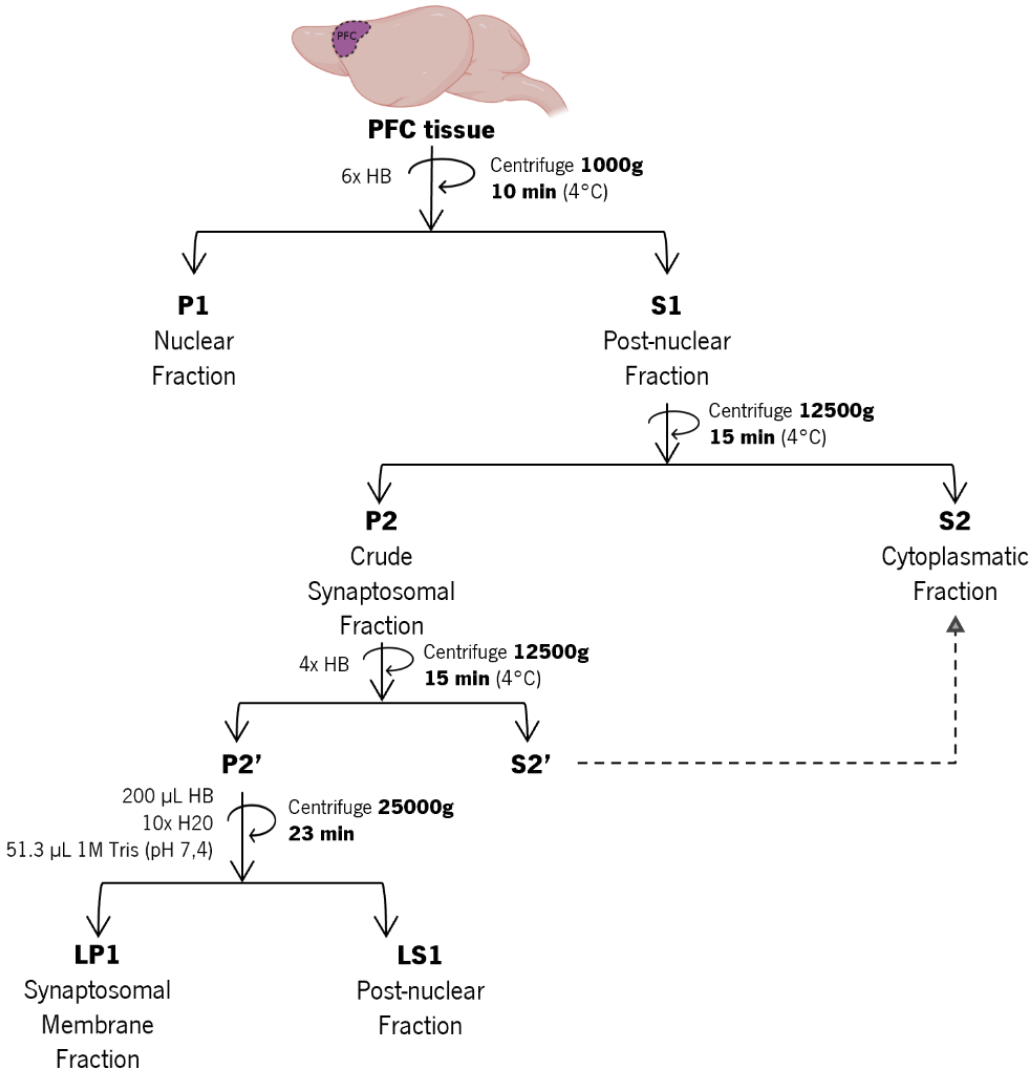


Figure 8. Subcellular fractionation protocol. Briefly, Prefrontal cortex (PFC) tissue was homogenised and sequentially centrifuged to obtain post-nuclear fraction (S1) which was subsequently centrifuged to yield crude synaptossomal (P2) and synaptosome-depleted fractions (S2). The crude synaptossomal fraction (P2) was lysed in a hypo-osmotic solution and centrifuged to obtain the synaptossomal membrane (LP1) and post-nuclear fraction (LS1).

Samples were electrophoresed on 10 % (v/v) acrylamide gels and transferred onto nitrocellulose membranes (Trans-Blot® Turbo™ Blotting System, BioRad). Membranes were blocked in 5 % (w/v) non-fat milk in TBS-T buffer before incubation with the primary antibodies. After incubation with the appropriate secondary antibody listed, antigens were revealed by ECL (Clarity, Bio-Rad), and signal quantification was achieved using a ChemiDoc instrument and ImageLab software (Bio-Rad). All values were normalized and expressed as percentages of controls. All antibodies and their characteristics are listed in **Table 1**.

Table 1. Antibodies used for the Western Blot analysis.

	Antibody	Molecular Weight (kDa)	Dilution	Incubation	Species	Company	Company's Code
Primary antibodies	**GR 5**	90-95	1:500	Overnight	Mouse	Santa Cruz	sc-393232
	Hsp90α	90	1:1000	Overnight	Mouse	Enzo	ADI-SPA-840
	IKKβ (D30C6)	87	1:1000	Overnight	Rabbit	Cell Signaling	8943S
	Tau5	50-70	1:2000	Overnight	Mouse	ABCAM	ab80579
	pThr231	50-70	1:2000	Overnight	Rabbit	ABCAM	ab151559
	pSer396	50-70	1:2000	Overnight	Rabbit	ABCAM	ab109390
	Actin	42	1:5000	2h at room temperature	Mouse	ABCAM	ab8226
Secondary Antibodies	**Anti-mouse**	-	1:5000	2h at room temperature	Goat	BioRad	170-6516
	Anti-rabbit	-	1:5000	2h at room temperature	Goat	BioRad	170-6515

3.9. mRNA expression levels

3.9.1. RNA extraction

RNA was extracted from prefrontal tissue previously macrodissected and stored at -80 °C. The extraction was made using TRIzol (Life technologies, 15596018), the homogenization in 1 mL of TRIzol was performed by cutting the tissue into small pieces, followed by transferring the resulting mixture through a 1 mL syringe with a 20 G needle until a homogeneous mixture was obtained. For phase separation, the mixture was incubated for 5 minutes at RT. After chloroform addition and an incubation of 2 minutes at RT, a centrifugation at 8000 rpm for 15 minutes (4° C) was performed. Then, the aqueous phase was transferred to a new RNase-free tube with isopropanol, incubated for 10 minutes at RT and centrifuged at 9000 rpm for 10 minutes at 4 °C. The pellet was washed with 75 % (v/v) ethanol and centrifuged at 5000 rpm for 5 minutes at 4 °C. The supernatant was discarded, and, for 5 minutes, the pellet was let to dry. After, the pellet was eluted with RNase-free water (DEPC-treated water) and incubated at 65 °C for 10 minutes. The total RNA was extracted and quantified on a NanoDrop ND100 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

3.9.2. cDNA synthesis

For gene expression, the total RNA was reverse transcribed into cDNA using a NZY First-Strand cDNA Synthesis Kit (NZYTech, MB12502). Thus, 1 μg of RNA was added to 10 μL of NZYRT 2× Master Mix and 2 μL of NZYRT Enzyme Mix, making up with nuclease-free water until 20 μL. The complete mix was incubated in a thermal cycler using the following protocol: 10 minutes at 25 °C (priming); 30 minutes

at 50 °C (reverse transcription); 5 minutes at 85 °C (enzyme inactivation). After, 1 µL of NZY RNase H was added, to remove RNA bond to cDNA, and the samples were incubated at 37 °C for 20 minutes. The cDNA was stored at -20 °C.

3.9.3. Quantitative Reverse-Transcription PCR (qRT-PCR)

RT-qPCR was performed using a SsoFast™ EvaGreen Supermix (BioRad, #172-5202) and the primer sequences listed in **Table 2**. Amplification conditions were the following: initial denaturing at 95 °C for 30 seconds, followed by 40 amplification cycles at 95 °C for 5 seconds and 60 °C for 30 seconds. The specificity of the amplified product was verified by a melting curve. The Beta-2-Microglobulin (*b2m*) was used as the endogenous control to normalize each gene expression level and mRNA levels were expressed as $2^{-\Delta\Delta_{CT}}$. Each sample was measured in triplicate. The cDNA synthesis was performed in a thermocycler (T100™ Thermal Cycler, Bio-Rad), and the RT-qPCR was run on a 7500 Fast Real-Time PCR System (Applied Biosystems).

Table 2. Primer sequences used for the analysis of gene expression (qRT-PCR).

Primer	Sequences
mb2m	Fwd.: 5′-CCTTCAGCAAGGACTGGTCT-3′
	Rev: 5′-TCTCGATCCCAGTAGACGGT-3′
il-1b	Fwd.: 5′-GGGCTGCTTCCAAACCTTTG-3′
	Rev.: 5′-AAGACACAGGTAGCTGCCAC-3′
nlrp3	Fwd.: 5′-TCTGCACCCGGACTGTAAAC-3′
	Rev.: 5′-CATTGTTGCCCAGGTTCAGC-3′
tnfα	Fwd.: 5′-TGCCTATGTCTCAGCCTCTTC-3′
	Rev.: 5′-GAGGCCATTTGGGAACTTCT-3′
il18	Fwd.: 5′-TGGTTCCATGCTTTCTGGACTCCT-3′
	Rev.: 5′-TTCCTGGGCCAAGAGGAAGTGATT-3′
mxbp1	Fwd.: 5′-CAGCACTCAGACTATGTGCA-3′
	Rev.: 5′-GTCCATGGGAAGATGTTCTGG-3′
creb	Fwd.: 5′-AAGCTGAAAGTCAACAAATGACAGTT-3′
	Rev.: 5′-TGGACTGTCTGCCCATTGG-3′
psd95	Fwd.: 5′-TTCTCAGCACCTGGACCATGA-3′
	Rev.: 5′-ATTGCGGAGGTCAACACCAT-3′
gr	Fwd.: 5'-ATGGCGTGAGTACCTCTGGA-3'
	Rev.: 5′-AATTGTGCTGTCCTTCCACTG-3'
hsp90	Fwd.: 5′-GCTTTCAGAGCTGTTGCGGTAC-3′
	Rev.: 5′- AAAGGCGGAGTTAGCAACCTGG-3′
fkbp5	Fwd.: 5′-GGCCAAGGCCAGGTTATCAA-3'
	Rev.: 5′-TTCCCCAACAACGAACACCA-3'

3.10. Statistical Analysis

All statistical analysis and graphical representations were performed with GraphPad Prism 9.4.1 software for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com). Outliers were removed when applicable using the ROUT method (Q = 1 %). Normality was assessed using the Kolmogorov-Smirnov and Shapiro-Wilks statistical tests. When normality was verified, molecular analysis and behaviour data were analysed using one-way ANOVA and/or t student's-test. When the one-way ANOVA test led to a significant result, multiple comparisons between groups were accomplished through Tukey's test for *post-hoc* comparisons. When data was not normally distributed, Kruskal-Wallis and Mann-Whitney tests were performed to test differences between the groups. All data is presented as mean ± standard error of the mean (SEM). Differences between groups were deemed statistically significant when $p \leq 0.05$.

CHAPTER 4
RESULTS

4.1. TUDCA and Fluoxetine treatment did not revert stress-induced decrease in body-weight gain or daily activity

As chronic stress is characterized by loss of body weight and hyperactivity of the HPA axis, body weight and serum corticosterone levels were monitored. The body weight of the animals was recorded weekly throughout the entire experiment and used as an indicator of the effectiveness of the stress protocol. As shown in **Figure 9B**, the weights of animals subjected to the CUS protocol drooped in the first week of the protocol and were always lower than the mean weight of the control group, whose weight kept increasing throughout the weeks, however a Mixed effects analysis revealed no effect of the group on weight [$F_{(3, 107)}$= 1,318]. To further evaluate the impact of the CUS protocol and treatments, weight variation between the beginning and end of the experiment was calculated (Δweight = last weight – first weight) (**Figure 9C**). One-way ANOVA showed significant differences between the Δ weight of the groups [$F_{(3, 107)}$=20,45; p<0,0001], suggesting CUS protocol's effectiveness. *Post-hoc* analysis revealed a significant decrease in the weight variation of each group subjected to the CUS protocol when compared with the control group [p<0.0001], while the treated groups showed no significant differences when compared to the untreated stress group [STR vs STR+TUDCA: p >0,9999; STR vs STR+FLUOX: p=0,5477] or between themselves [STR+TUDCA vs STR+FLUOX: p=0,5778].

Additionally, body weight was used to monitor mice's health to ensure animal welfare. A weight loss equal to or greater than 20 % was considered an endpoint. No animals registered this body weight loss along the experimental protocol, and all mice completed the experiment.

To monitor animal activity throughout the day, some animals from each group were placed on the DCV rack – Activity rack. Animals were placed in the activity rack before the start of treatments administration and remained there for 9 days. Placement in the DCV rack requires housing only two animals per cage in a different room from where the animals were housed. Therefore, to ensure that the analysis of animal activity wasn't affected by these changes, the first three days were considered an adjustment period and were not considered in the analysis of the activity. When compared to the controls, all stressed groups tended to show increased activity during active and resting periods, while the treatments displayed no effect on this [$F_{(3, 25)}$=1,003; p=4077] (**Figure 9D**).

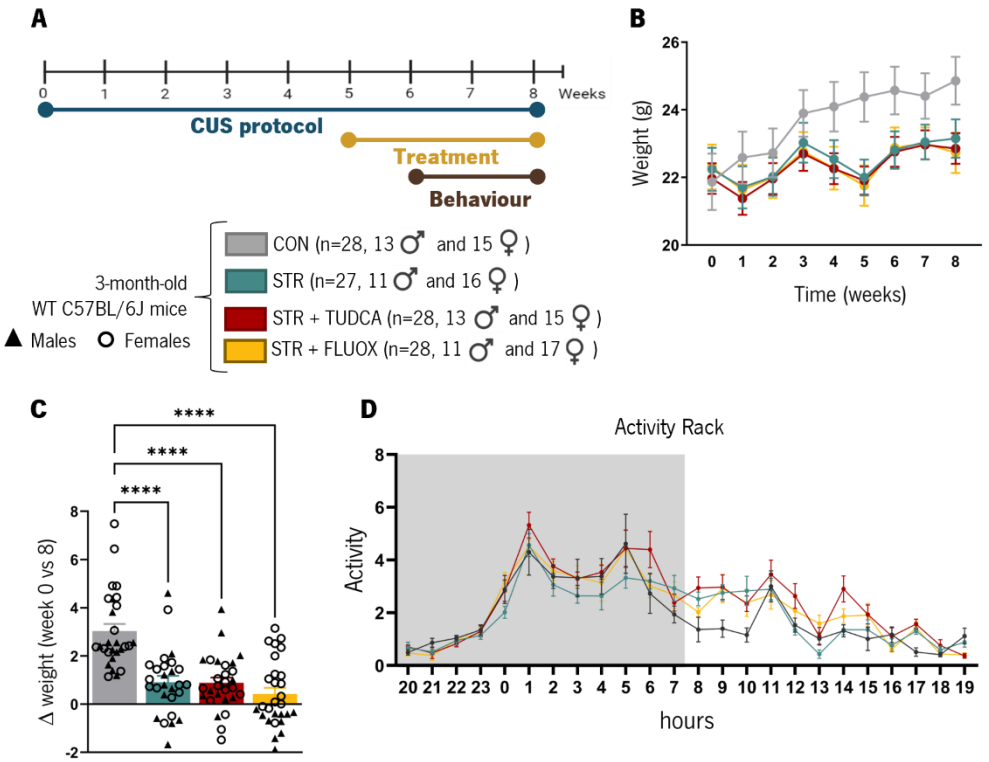


Figure 9. Treatments with TUDCA and Fluoxetine did not interfere with biomarkers of the stress response. **(A)** Schematic representation of the experimental design: briefly, 3-month-old Wild-type mice were subjected to a Chronic Unpredictable Stress (CUS) protocol, and 5 weeks later were treated with vehicle, TUDCA and Fluoxetine. In the 6th week, behaviour was assessed. **(B)** Variation of the mice's body weight throughout the weeks of the experiment was used as an indicator of the effectiveness of the stress protocol. The weights of animals subjected to the CUS were always lower than the mean weight of the control group, whose weight kept increasing throughout the weeks. **(C)** One-way ANOVA showed significant group differences between the variation of animals' weight between the beginning and end of the experiment (Δweight = last weight – first weight) [$F_{(3, 107)}$=20,45; p<0,0001], suggesting the CUS protocol's effectiveness. **(D)** Activity assessment in an activity rack revealed that all groups subjected to CUS tend to present increased activity during the active and resting periods of the day. All data are presented as mean±SEM. Significance is represented as: **** p<0,0001.

4.2. Chronic unpredictable stress induced anxiety and depressive like behaviour, that was partially reverted by TUDCA

To understand whether treatment with TUDCA or Fluoxetine could modulate the chronic stress-related effects on anxiety-related behaviours, the Open Filed (OF), Elevated Plus-Maze (EPM), Novelty Supressed Feeding (NSF) and the Splash tests were performed (**Figure 10A-D**).

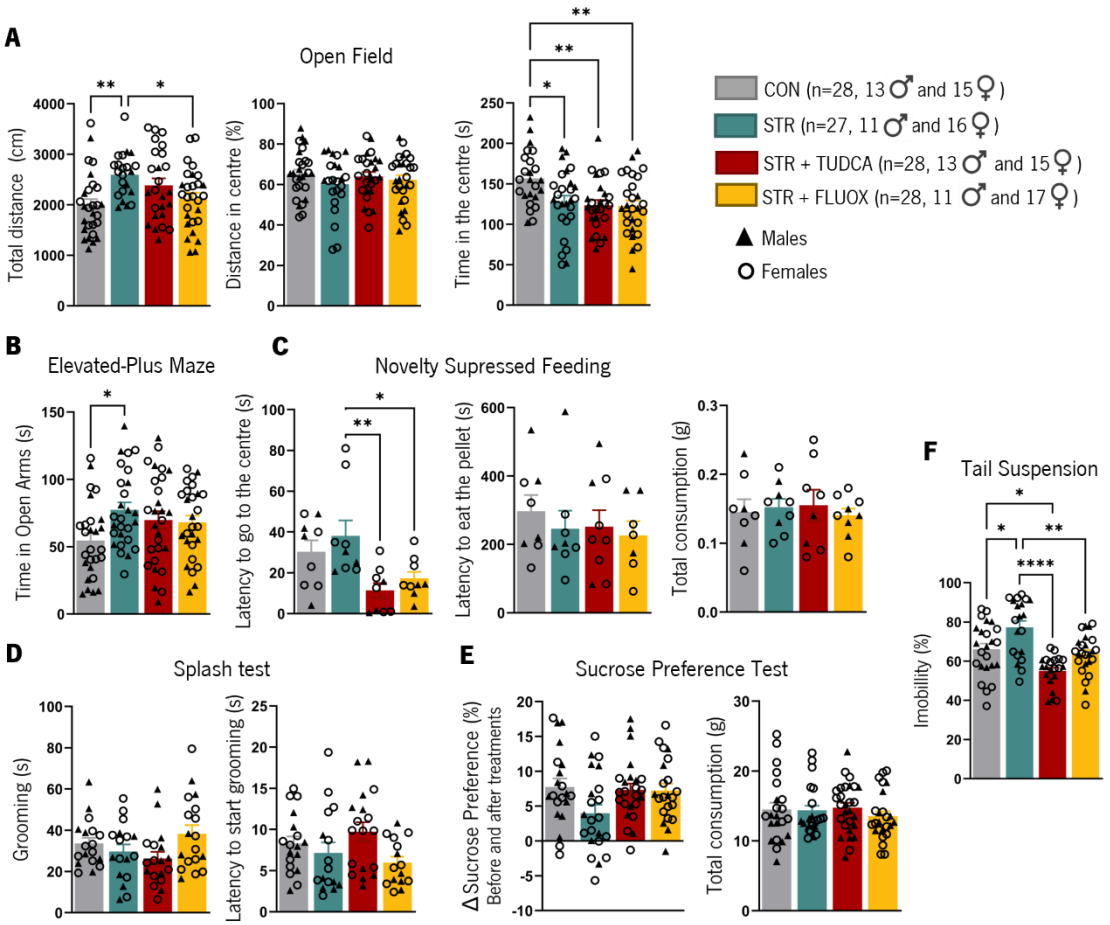


Figure 10. Treatments with TUDCA and Fluoxetine did not interfere with anxiety-related behaviours but reverted CUS-induced depressive-like behaviours in the TST. (A) CUS protocol elicited anxiety-related behaviours in the Open Field test, increasing the total distance travelled [p=0,0042] and lowering the amount of time spent in the centre [p=0,0254]. Neither of these anxiety-related behaviours were reverted by treatment with TUDCA or Fluoxetine. **(B)** In the Elevated Plus Maze test, controls spent significantly less time in the open arms than mice from the Stress group [$F_{(3, 105)}$ =2,775; p=0,0450]. **(C)** Regarding the Novelty Supressed Feeding, TUDCA [p=0,0061] and Fluoxetine [p=0,0424] significantly decreased the latency to go to the centre, compared to the Stress group. No differences were found in the latency to start eating the pellet or in its total consumption. **(D)** In the Splash test, no significant differences were found in the time spent grooming or in the latency to start grooming. **(E)** In the Sucrose Preference Test, no statistically significant differences between the groups were found in the variation of the sucrose preference before and after the treatments or in the total consumption. **(F)** In the Tail Suspension Test, CUS elicited a significant increase in mice's immobility time [p=0,0148], which was reverted by TUDCA [p<0,0001] and Fluoxetine [p=0,0015]. All data are presented as mean±SEM. Significance is represented as: * p<0,05; ** p<0,01; *** p<0,001; **** p<0,0001.

The OF and EPM tests involve balancing natural curiosity to explore new things against avoidance of threatening situations, such as open and exposed areas, or elevated positions. Testing in the OF, showed an overall anxiogenic effect of the CUS protocol (**Figure 10A**). CUS elicited a significant increase in the total distance travelled in the arena [One-way ANOVA: $F_{(3, 99)}$=4,837; p=0,0035] compared to controls [CON vs STR: p=0,0042]. Treatment with Fluoxetine reverted the effect of stress on the total distance travelled [STR vs STR+FLUOX: p=0,038], while TUDCA treatment showed a tendency to travel more distance than controls [p=0,0964] and less than animals from the Stress group [p=0,8343]. In the time spent and the percentage of distance travelled in the centre of the arena was used as indexes of

anxious behaviour, with a typical anxious-like behaviour consisting of animals staying in the periphery of the arena, where they feel more secure. No significant difference was found between the groups in the percentage of distance travelled in the centre of the arena. However, one-way ANOVA revealed significant differences between the groups in the time spent in the centre of the arena [One-way ANOVA: $F_{(3, 101)}$=5,068; p=0,0026]. CUS provoked a significant decrease in the time spent in the centre of the arena [CON vs STR: p=0,0254] with either treatment not reverting the stress-related effects [STR vs STR+TUDCA: p=0,9705; STR vs STR+FLUOX: p=0,9454].

We should note the variability in the distribution of the data in the OF, and that is possibly due to the use of both sexes in all experimental sets. Given previous findings suggesting potential sex-specific responses to chronic stress (Sotiropoulos et al., 2008), a secondary phase of analysis was conducted to investigate any sex-related differences in treatment outcomes. When comparing female groups and their male counterparts, females from the STR group displayed more anxiety-like behaviours such as a higher distance travelled, a lower percentage of this distance in the centre, as well as shorter periods of time spent in the centre. In both sexes, treatment with Fluoxetine and TUDCA did not have a significant effect on these behaviours, but they appeared to elicit different tendencies. In males, treated animals did not show a reversal of the effects of stress, while in females, although not statistically significant, TUDCA and Fluoxetine treatments caused a slight reversal of the effects of CUS (**Supplementary Figure 1A**).

In the EPM test, anxiety-related behaviour is defined as a decrease in the time spent in the open arms of the maze. Unexpectedly, controls spent significantly less time in the open arms than mice in the Stress group [One-way ANOVA: $F_{(3, 105)}$=2,775; p=0,0450; *post-hoc* CON vs STR: p=0,0283] (**Figure 10B**). While EPM is the first behaviour test to be performed due to its high sensitivity to external factors, sometimes is perceived by the animals has a highly anxious experience, and leads to extreme effects, like the ones observed.

The NSF is a variation of the OF test that assesses whether animals' natural drive to eat is supressed by anxiety when placed in a new and potentially threatening environment. When analysing the time animals take to go to the centre of the arena where the pellet is located (Latency to go to the centre (s)) (**Figure 10C**), one-way ANOVA [$F_{(3, 32)}$=5,254; p=0,0046] and *post-hoc* analysis showed that TUDCA and Fluoxetine significantly decreased the latency, compared to the Stress group [STR vs STR+TUDCA: p=0,0061; STR vs STR+FLUOX: p=0,0424]. However, no significant differences were found in the latency to start eating the pellet or in the total pellet consumption (**Figure 10C**). Analysis of the latency of animals to go to the centre in the NSF test revealed that males and females from every group behaved differently (**Supplementary Figure 1B**). The male controls tend to take more time than the other

groups, a tendency not followed by the female controls. In females, a One-way ANOVA revealed significant differences between the groups [$F_{(3, 18)}$=3,262; p=0,0456], with TUDCA-treated females displaying a significant lower latency time than females from the Stress group [p=0,0323].

The sucrose splash test assesses self-care in mice. It consists of spraying a viscous sucrose solution on mice's fur eliciting grooming, the act of cleaning the fur, which is a natural and very important behaviour for rodents. In this test, the latency to initiate grooming behaviour, as well as the duration of this activity, was measured (**Figure 10D**). Even though no statistically significant differences were found in animal performance [Latency: $F_{(3, 63)}$=2,386; p=0,0774; Grooming: $F_{(3, 67)}$=2,270; p=0,0884], there was a trend towards an increase in the time spent grooming in Fluoxetine-treated animals when compared to untreated CUS animals. On the other hand, TUDCA-treated mice displayed a tendency to increase the latency to start grooming other stressed animals and to decrease grooming time, suggesting that the drug had no positive effect.

Altogether, these data suggest that, while the CUS protocol elicited anxiety-related behaviours in the OF test, it did not have the same anxiogenic effect in other tests. Treatment with TUDCA and Fluoxetine did not significantly revert any stress-related effects on anxiety.

Regarding depressive-like behaviour, we used two tests: the Sucrose Preference Test (SPT) and Tail Suspension Test (TST) (**Figure 10E-F**). These tests focus on anhedonia, a common symptom of depression in humans, and learned helplessness, an index of depressive-like behaviour in rodents, respectively. For the SPT, the factors analysed were the variation in sucrose preference before and after the treatments and total consumption (**Figure 10E**). Analysis of the total consumption revealed no differences between the groups. For the variation in sucrose preference, even though the analysis revealed no statistically significant difference [$F_{(3, 85)}$=2,612; p=0,0566], there was a tendency of the Stress group to have a lower increase in variation than other groups. Overall, anhedonia was not observed in these rodents. One-way ANOVA revealed significant difference between the groups [$F_{(3, 86)}$=12,14; p<0,0001] (**Figure 10F**). Accordingly, a *post-hoc* analysis revealed a significant increase in the immobility time of mice from the Stress group compared to the controls [p=0,0148], while both treatments with TUDCA and Fluoxetine significantly reverted the stress-related increase in the time spent immobile [STR vs STR+TUDCA: p<0,0001; STR vs STR+FLUOX: p=0,0015]. Mice treated with TUDCA displayed a significantly shorter period of immobility than controls [p=0,0123]. Hence, CUS protocol elicited a depressive-like behaviour in the TST, which was reverted by treatment with TUDCA and with Fluoxetine. When analysing separately the sexes, it is noticeable a difference in the strength of CUS-induced effects between the sexes (**Supplementary Figure 1D**). In males, there is a significant difference between the

groups [$F_{(3, 39)}$=9,462; p<0,0001], corroborated by a *post-hoc* analysis that shows a significant decrease of immobility time in TUDCA- [p<0,0001] and Fluoxetine-treated males [p=0,0237], compared to untreated stressed males. On the other hand, there are no significant differences between the female groups [$F_{(3, 49)}$=0,9114; p=0,4424]. Females from the Stress group did not display a significant increase in the immobility time and even though the treated groups appear to a shorter time, it's not statistically significant.

4.3. TUDCA and Fluoxetine had a big impact in the reversion of cognitive impairment induced by CUS, reverting deficits in long-term memory

Cognition and different forms of memory were evaluated using a battery of tests that included the Y-maze spontaneous alternations and novel object recognition (NOR) short-term and long-term tests (**Figure 11**).

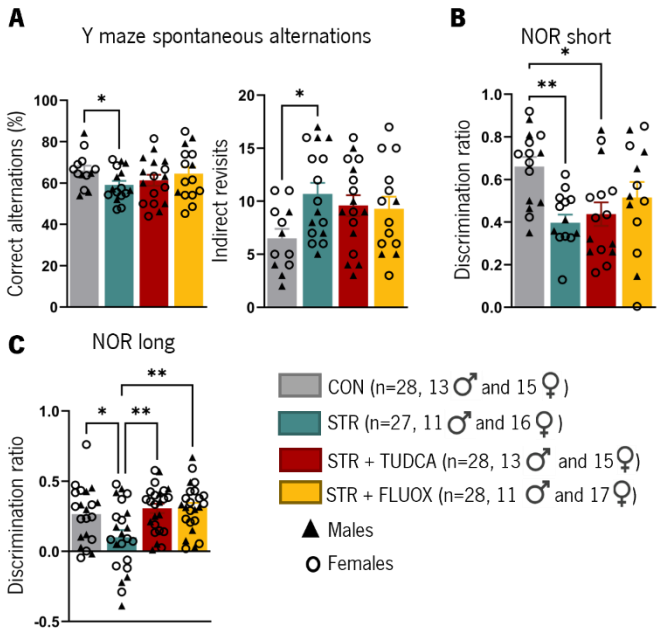


Figure 11. TUDCA and Fluoxetine reverted CUS-induced deficits in long-term memory, but not in the short-term. (A) In the Y-maze spontaneous alternations, CUS elicited a lower percentage of correct alternations [p=0,0309] and a higher number of indirect revisits when compared to controls [p=0.0401]. Fluoxetine and TUDCA didn't display a significant reverting effect on this test, even though treated animals tend to have a better performance than the untreated stressed ones. **(B)** In NOR short, there was an overall negative effect of the CUS protocol in short-term recognition memory, with animals from the Stress [p=0,005] and STR+TUDCA [p=0,0199] groups displaying a lower discrimination ratio than controls. Discrimination ratio = (time spent exploring novel object – time exploring old object) / total exploration time) **(C)** In NOR long, CUS caused a significant decrease of the discrimination ratio [p=0,0323], which was reverted by treatment with TUDCA [p=0,0031] and Fluoxetine [p=0,0022]. All data are presented as mean±SEM. Significance is represented as: * p<0,05; ** p<0,01.

The Y-maze spontaneous alternations test was used to assess spatial working memory, focusing on short-term memory. As mice tend to naturally alternate between the arms of the Y-shaped maze, a correct alternation was recorded each time the mouse chose different arms on consecutive entries,

following a sequence. An indirect revisit was defined as the animal did not follow the sequence, entering completely or not in a repeated arm. In this test, animals from the Stress group displayed a lower percentage of correct alternations [unpaired t-test: p=0,0309] and a higher number of indirect revisits compared to controls [One-way ANOVA: $F_{(3, 55)}$=2,611; p=0,0605; *post-hoc* analysis of CON vs STR: p=0,0401] (**Figure 11A**). Fluoxetine and TUDCA didn't display a significant reverting effect on this test, even though treated animals tend to have a better performance than the untreated stressed ones.

Recognition memory was evaluated using the NOR test, both at short- and long-term memory (with NOR short and NOR long, respectively) (**Figure 11B-C**). The discrimination ratio was used as the comparison measure between the groups. In the short-term memory test (NOR-short), control animals showed a significantly higher discrimination ratio than animals from the Stress [p=0,005] and STR+TUDCA groups [p= 0,0199], demonstrating an overall negative effect of the CUS protocol in short-term recognition memory [$F_{(3, 53)}$=4,854; p=0,0047] (**Figure 11B**). In contrast, in long-term memory (NOR-long), CUS caused a significant decrease in the discrimination ratio [$F_{(3, 88)}$=5,914; p=0,0010; *post-hoc* CON vs STR: p=0,0323], which was reverted by treatment with TUDCA [p=0,0031] and Fluoxetine [p=0,0022] (**Figure 11C**). When analysis was performed by sexes, NOR short test showed a stronger effect of CUS in males than in females, with a reversion of stress-induced effects with TUDCA and Fluoxetine (**Supplementary Figure 1C**). In both sexes, one-way ANOVAs uncovered significant differences between the groups [Males: $F_{(3, 22)}$= 6,114; p=0,0035; Females: $F_{(3, 31)}$= 4,093; p=0,0147]. A *post-hoc* analysis confirmed the negative effects of CUS [CON vs STR: p=0,0044] and their reversion with Fluoxetine [STR vs STR+FLUOX: p=0,0118] and TUDCA, even if not statistically significant [STR vs STR+TUDCA: p= 0,0559], in male animals. In females, treatments did not have any effect on the stress-induced deficits [CON vs STR: p=0,0228; CON vs STR+TUDCA: p=0,0264].

Overall, CUS elicited deficits in both short- and long-term recognition memory as well as in referential and spatial working memory. Treatment with TUDCA and with Fluoxetine did not revert the stress-induced impairments in short-term memory tasks; however, both treatments reverted the stress-induced decline in long-term recognition memory.

4.4. TUDCA and Fluoxetine reverted the effect of chronic stress on microglia in the PrL subregion of PFC

Microglia are the predominant resident immune cells in the brain, essential for the maintenance of homeostasis, responsible for the immune surveillance and inflammatory response as well as a modulator of synaptic plasticity. It has been shown that not only stress induces morphological and

functional changes in microglia, as, following chronic stress and/or elevated GC levels, microglia mediate neuronal remodelling and behavioural impairments (Nair & Bonneau, 2006; Hinwood et al., 2012; Horchar & Wohleb, 2019; Sanguino-Gómez et al., 2022). As previous works have described TUDCA' modulatory effect on microglia number and activation in various models of neuroinflammation and neurodegenerative diseases, we assessed TUDCA's effects in this immune brain cells (Lo et al., 2013; Noailles et al., 2014; Yanguas-Casás et al., 2014; Yanguas-Casás et al., 2017; Cuevas et al., 2022).

To evaluate the effects of TUDCA and Fluoxetine on microglia's number in mice brains, slices were immunostained with an antibody for IBA-1, a known marker of microglia. The number of IBA-1 positive cells and the intensity of fluorescence were assessed in the PrL area of the PFC and three sub-regions of the hippocampus (CA1, CA3 and DG) (**Supplementary Figure 2**; **Figure 12**). The fluorescence intensity was divided by the number of IBA-1 positive cells to obtain the average fluorescence intensity per cell, enhancing the accuracy and reliability of the data, and allowing comparison between groups and areas. Generally, throughout the three analysed sub-regions of the hippocampus, significant differences between the groups were found [CA1: $F_{(3, 76)}$=52,02; p<0,0001; CA3: $F_{(3, 78)}$=15,24; p<0,0001; DG: $F_{(3, 77)}$=15,36; p<0,0001] (**Figure 12 D-F**), and the same was observed in the PrL PFC, a One-way ANOVA analysis reported significant differences between the groups [$F_{(3, 72)}$= 5,828; p=0,0013] (**Figure 12B**).

When the data from IBA-1 staining was analysed according to the animals' sex, divergences in the effects of the CUS protocol and the treatments were found (**Figure 12B-F**). In the PrL subregion of the PFC, significant differences were found between the groups of males [$F_{(3, 28)}$=23,11; p<0,0001] (**Figure 12C**), with CUS eliciting a higher average fluorescence intensity per cell [CON vs STR: p<0.0001], which was reverted by treatment with TUDCA [p<0.0001] and with Fluoxetine [p<0.0001]. In contrast, females from the Stress group did not display a significantly higher levels than controls or the treated animals. TUDCA-treated females displayed a higher average fluorescence per cell than any other group [CON vs STR+TUDCA: p= 0,0122; STR vs STR+TUDCA: p=0,0387; STR+TUDCA vs STR+FLUOX: p=0,0001], suggesting a different modulation of microglia by TUDCA in males and females. We should note that in TUDCA treated group, there are 2 groups of females, ones with higher intensity then CUS, and others with lower. Although differences were found between the groups in the total number of IBA-1+ cells in the PrL [$F_{(3, 86)}$=4,454; p=0,0059], there is no difference between the Stress and STR+TUDCA groups [p=0,9995] (**Supplementary Figure 2 A**).

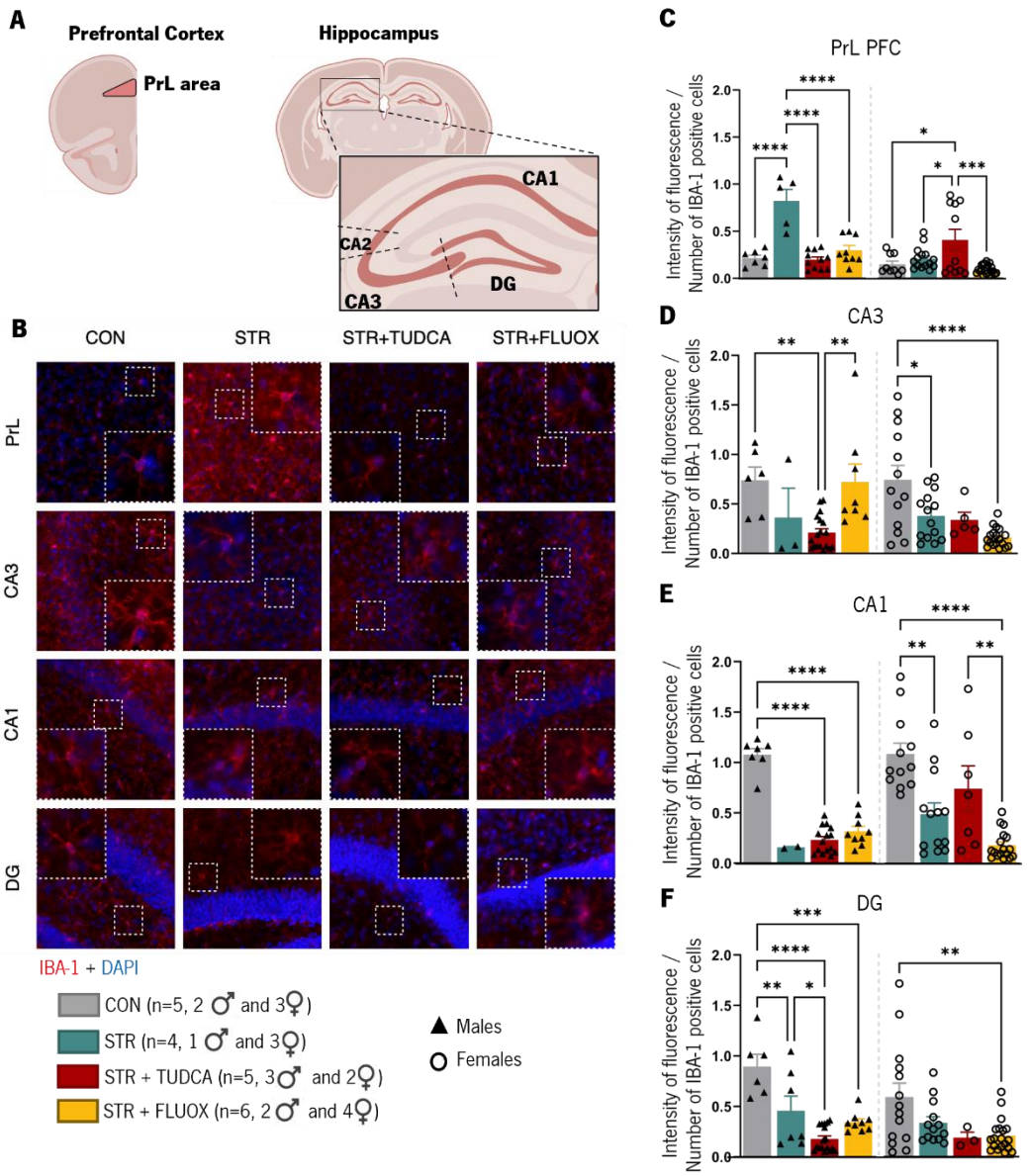


Figure 12. TUDCA and Fluoxetine reverted the effect of chronic stress on Microglia in the PrL PFC of males. **(A)** Schematic representation of the areas assessed with IBA-1 immunostaining: Prelimbic area of the Prefrontal Cortex (PrL PFC) and three subregions of the Hippocampus: CA1 - Cornu ammonis 1; CA3 - Cornu ammonis 3; DG - Dentate Gyrus. **(B)** Immunofluorescence staining of IBA-1 in the PrL PFC, CA1, CA3 and DG in Control, Stress, STR+TUDCA and STR+FLUOX animals with representative microglia cells highlighted. **(C)** In the PrL PFC, CUS elicited a higher average fluorescence intensity per cell in males (left part of the graph), which was reverted by treatment with TUDCA and with Fluoxetine. In females (right part of the graph), TUDCA elicited an increase in the average fluorescence per cell. **(D)** In the CA3 subregion, Fluoxetine caused different tendencies in males (left part of the graph) and females (right part of the graph). Fluoxetine-treated males displayed an average fluorescence per cell similar to controls, while in females it had a significantly lower average fluorescence per cell than controls. **(E)** In CA1, TUDCA and Fluoxetine elicited different tendencies in males (left part of the graph) and females (right part of the graph). **(F)** In the DG area, CUS elicited a decrease in the average fluorescence per cell, which was not reverted by TUDCA or Fluoxetine. Both treatments had similar effects on males (left part of the graph) and females (right part of the graph). All data are presented as mean±SEM. Significance is represented as: * p<0,05; ** p<0,01; *** p<0,001; **** p<0,0001.

In hippocampal subregions, one-way ANOVA analysis revealed significant differences between the groups in both sexes, in CA1 [Males: $F_{(3, 29)}$ =63,67; p<0,0001; Females: $F_{(3, 45)}$=15,12; p<0,0001], CA3

[Males: $F_{(3, 31)}$=6,650; p=0,0013; Females: $F_{(3, 46)}$ =9,413; p<0,0001] and DG [Males: $F_{(3, 35)}$=16,66; p<0,0001; Females: $F_{(3, 45)}$=4,311; p=0,0093] (**Figure 12D-F**). The major differences between sexes were found in CA3 and CA1 (**Figure 12D-E**). In hippocampal subregion CA3, males treated with Fluoxetine displayed an average fluorescence per cell similar to controls, with TUDCA-treated groups presenting a lower average to both groups [CON vs STR+TUDCA: p=0,009; STR+TUDCA vs STR+FLUOX: p=0,0046]. In contrast, in females, Fluoxetine did not display this effect, having a significantly lower average fluorescence per cell than controls [CON vs STR+FLUOX: p= <0,0001]. In the CA1, the two treatments did not revert the CUS-induced decrease of the average fluorescence per cell in males [CON vs STR+TUDCA and CON vs STR+FLUOX: p<0,0001] (**Figure 12E**); while in female mice, even though neither treatment caused a significant reversion of the CUS-induced decrease in average fluorescence per cell [CON vs STR: p=0,001], it's possible to observe different tendencies [STR+TUDCA vs STR+FLUOX: p=0,0071]. Treatment with TUDCA caused a slight increase in the fluorescence compared to untreated stressed females. The female group treated with Fluoxetine displayed a decrease in the average fluorescence per cell compared to the Control group [CON vs STR+FLUOX: p<0,0001], and a tendency to have a lower fluorescence per cell than the Stress group.

In the DG, CUS induced a decrease in the average IBA-1 fluorescence per cell in males [CON vs STR: p= 0,0047] (**Figure 12F**), with TUDCA causing an even stronger decrease in the average fluorescence per cell [CON vs STR+TUDCA: p<0,0001; STR vs STR+TUDCA: p=0,0327]. Similarly, in female mice neither treatment tended to provoke a reversion of the CUS-induced effects on the average fluorescence per cell. We should note that, in all subregions of the hippocampus, TUDCA exerts a decrease in total cell numbers of IBA-1+ cells when comparing to controls [$F_{(3, 77)}$=6,810; p=0,0004; *post-hoc*: p=0,0329 (CA1); $F_{(3, 84)}$=5,060; p=0,0029; *post-hoc*: p=0,0047 (CA3); $F_{(3, 84)}$=4,660; p=0,0046; *post-hoc*: p= 0,0457 (DG)] (**Supplementary Figure 2 B-D**).

Altogether, the data suggests that CUS induced different effects on the PFC and Hippocampus. The stress protocol caused an overall decrease in the average fluorescence of IBA-1 staining per cell in the Hippocampus, while it caused an increase in the PrL. Additionally, data suggest that sex may play a role in the effects of treatments in the PFC. In the PrL area, the treatments were able to revert the stress-induced effects on microglia in males, while in females TUDCA appeared to have a variable effect, without changing cell numbers. In the hippocampal slices, both sexes displayed similar tendencies, with neither treatment causing a significant reversion of CUS-induced decrease in the average fluorescence of IBA-1 per cell, with TUDCA significantly decreasing the number of IBA+ cells in all hippocampal sub-regions. However, regarding Fluoxetine, it's interesting to notice possible sex-related tendencies displayed in

different areas. In the CA3, when compared to the Stress group, Fluoxetine tends to induce an increase in average fluorescence per cell in males, but a decrease in females. In CA1, the same tendencies are shown, even though it's less noticeable, while in the DG, Fluoxetine appears to not revert stress-related effects in both sexes.

4.5. Chronic stress effects on astrocytes is sex dependent, while TUDCA and Fluoxetine may be able to revert it in males

For assessment of the treatments' effects on astrocyte number, brain slices were stained with an antibody for a well-known marker of these cells, GFAP. Due to the difficulty of counting astrocytes with images taken in the fluorescence microscope, only the fluorescence intensity was measured in three areas of the dorsal hippocampus: CA1, CA3 and DG (**Figure 13A**).

Interestingly, differences between males and females in the tendencies displayed by the CUS protocol and treatments are noticeable. In females, statistical analysis by One-way ANOVA test revealed significant differences between the groups in all hippocampal subregions [CA1: $F_{(3, 46)}$=43,01; p<0,0001; CA3: $F_{(3, 44)}$=41,01; p<0,0001; DG: $F_{(3, 46)}$=66,42; p<0,0001] (**Figure 13B-D**). Stress leads to an increase in the intensity of GFA staining in comparison to controls, in CA1 [p=0.0076] and DG [p=0.0022]; with treatments leading to a even higher GFAP staining throughout all hippocampal subregions [STR vs STR+TUDCA: p<0,0001 in all areas; STR vs STR+FLUOX: p<0,0001 in all areas] (**Figure 13B-D**). These differences indicate that, in females, CUS elicited an increase in fluorescence intensity, and that treatments with TUDCA and Fluoxetine enhanced this increase.

On the other hand, in males, tendencies displayed by treated animals were different (**Figure 13B-D**). In all hippocampal subregions, compared to the Control group, TUDCA and Fluoxetine tended to induce a decrease in the intensity of GFAP immunostaining to control levels. In CA1, TUDCA elicited a significant decrease in the fluorescence intensity compared to Fluoxetine-treated males [Mann-Whitney test: p<0,0001] and to controls [Mann-Whitney test: p=0,0036] (**Figure 13B**). This decrease of TUDCA-treated males compared to controls was also found in the other two hippocampal subregions [Mann-Whitney test: p=0,0060 (CA3); Mann-Whitney test: p=0,0017 (DG)] (**Figure 13C-D**). Although males from the Stress group appear to show a tendency for much higher fluorescence intensity, due to the low number of samples, no statistical test was applied for this group. Further analysis and repetitions of the GFAP immunostaining should be performed to increase the sample number and confirm these results.

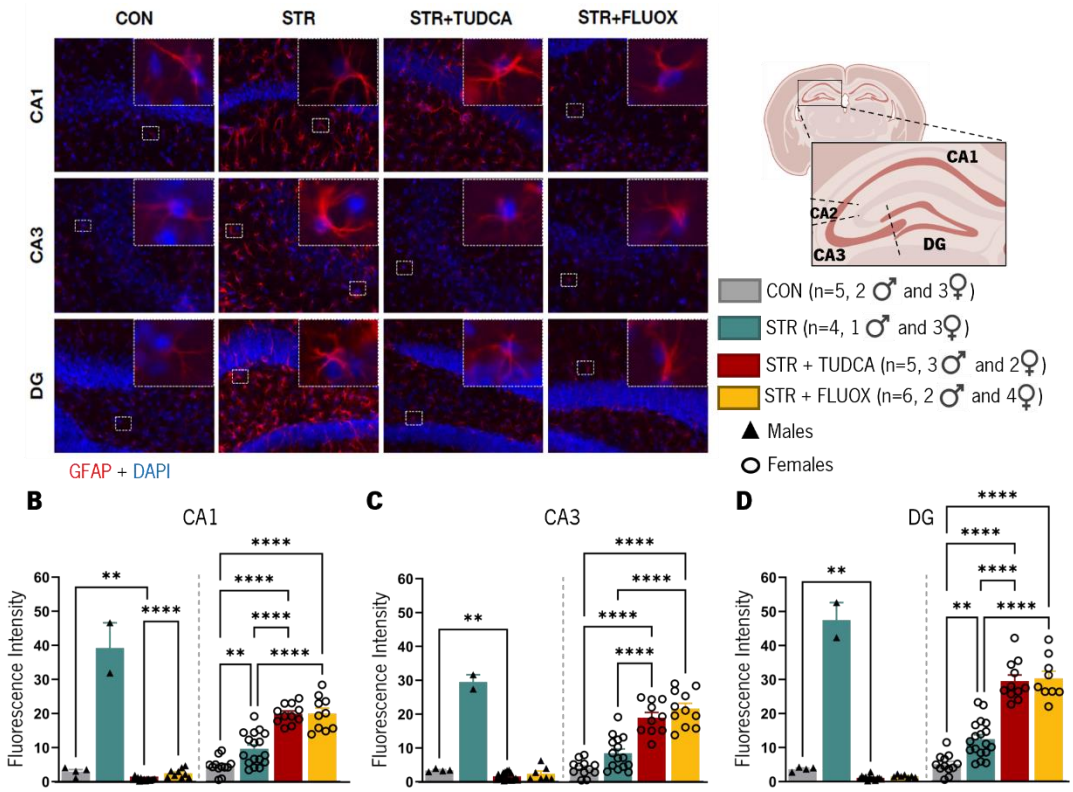


Figure 13. TUDCA and Fluoxetine caused sex-related effects on Astrocytes in the Hippocampus. (A) Schematic representation of the hippocampal areas assessed with GFAP immunostaining: CA1 - Cornu ammonis 1; CA3 - Cornu ammonis 3; DG - Dentate Gyrus. Immunofluorescence staining of GFAP in the CA1, CA3 and DG in Control, Stress, STR+TUDCA and STR+FLUOX animals with representative astrocytic cells highlighted. **(B)** In the CA1 subregion, TUDCA elicited a significant decrease in fluorescence intensity compared to Fluoxetine-treated and control males (left part of the graph). In females (right part of the graph), CUS elicited an increase in fluorescence intensity, and that treatments with TUDCA and Fluoxetine enhanced this increase. **(C)** In the hippocampal subregion CA3, TUDCA-treated males displayed lower fluorescence intensity than control animals (left part of the graph). In females (right part of the graph), CUS elicited an increase in fluorescence intensity, and that treatments with TUDCA and Fluoxetine enhanced this increase. **(D)** In the DG, TUDCA elicited a significant decrease in fluorescence intensity compared to control males (left part of the graph). In females (right part of the graph), CUS elicited an increase in fluorescence intensity, and that treatments with TUDCA and Fluoxetine enhanced this increase. All data are presented as mean±SEM. Significance is represented as: * p<0,05; ** p<0,01; *** p<0,001; **** p<0,0001.

4.6. Treatment with TUDCA modulated pathways related to GR signalling, synaptic plasticity and inflammation, with sex playing an important role

With the major changes of TUDCA in behaviour being related with depression, but also long-term memory recognition, we decided to understand the potential beneficial effects of TUDCA against CUS in the PFC, a region that is necessary for the regulation of both behaviour dimensions, exploring molecular pathways, known to be classically modulated by TUDCA in disease context and other related with Stress and stress-induced pathology.

Regarding inflammation, qRT-PCR analysis revealed that, in males, TUDCA increased the mRNA levels of two molecules involved in the regulation of inflammation and the inflammasome pathway, IL1β [$F_{(3, 26)}$=11,36; p<0,0001] and NLRP3 [$F_{(3, 29)}$= 6,292; p=0,0020] (**Figure 14A-B**), even further then stress did. *Post-hoc* analysis reported a significant increase of IL1β and NLRP3 levels in the STR+TUDCA

group when compared to controls and Stress mice [CON vs STR+TUDCA: p=0,0001 (IL1β) and p=0,0021 (NLRP3); STR vs STR+TUDCA: p=0,0024 (IL1β) and p=0,0131 (NLRP3)]. In contrast, females presented a decrease in all stress groups (treated and non-treated) in IL1β [$F_{(3, 32)}$=3,146; p=0,0385; CON vs STR+TUDCA: p=0,0422; CON vs STR+FLUOX: p=0,0185], and in NLRP3 [$F_{(3, 32)}$=4,184; p=0,0132] only significant in fluoxetine [CON vs STR+FLUOX: p=0,0487]. The analysis of TNFα and IL18, two pro-inflammatory cytokines, did not reveal significant differences between groups in males [$F_{(3, 26)}$=1,013; p=0,4030 (TNFα); $F_{(3, 29)}$=0,7986; p=0,5047 (IL18)) (**Figure 14C-D**). One-way ANOVA analysis showed female group differences in TNFα [$F_{(3, 37)}$=4,245; p=0,0113], but not in IL18 [$F_{(3, 32)}$=1,955; p=0,1406] mRNA levels, with Fluoxetine decreasing significantly TNFα levels when comparing to controls [p=0,0409], while TUDCA only showed a tendency [p= 0,1074]. This data indicates that TUDCA can modulate the NLRP3 inflammasome pathway, causing the activation of IL1β, but does not have the same modulating effect on other pro-inflammatory cytokines such as TNFα and IL18.

In addition, we measured cytoplasmatic protein levels of IKKβ, involved in neuro-inflammation due to IKKβ's role in the regulation of the NF-κB pathway (**Figure 15D**). In neither males or females, there was no statistically significant difference between groups [$F_{(3, 38)}$=2,449; p=0,0785 (males); Kruskal-Wallis test: p=0,1510 (females)]. We should further explore this pathway, analysing other proteins involved.

For the assessment of ER stress and UPR, classical pathways known to be modulated by stress and TUDCA, the mRNA levels of a crucial transcription factor in UPR (XBP1) were evaluated (**Figure 14E**). One-way ANOVA analysis exposed significant differences between groups in males [$F_{(3, 26)}$=3,123; p=0,0431], but not in females [$F_{(3, 30)}$=2,243; p=0,1037]. In males, CUS elicited an increase in XBP1 levels [p=0,0291], and, although not statistically significant, Fluoxetine and TUDCA tend to revert this effect of stress. In females, even without statistical significance, it's possible to see a tendency of stressed untreated females to have a higher expression of XBP1 and a tendency of Fluoxetine to cause a reversion of this effect. This should be further explored with an increase in animal samples.

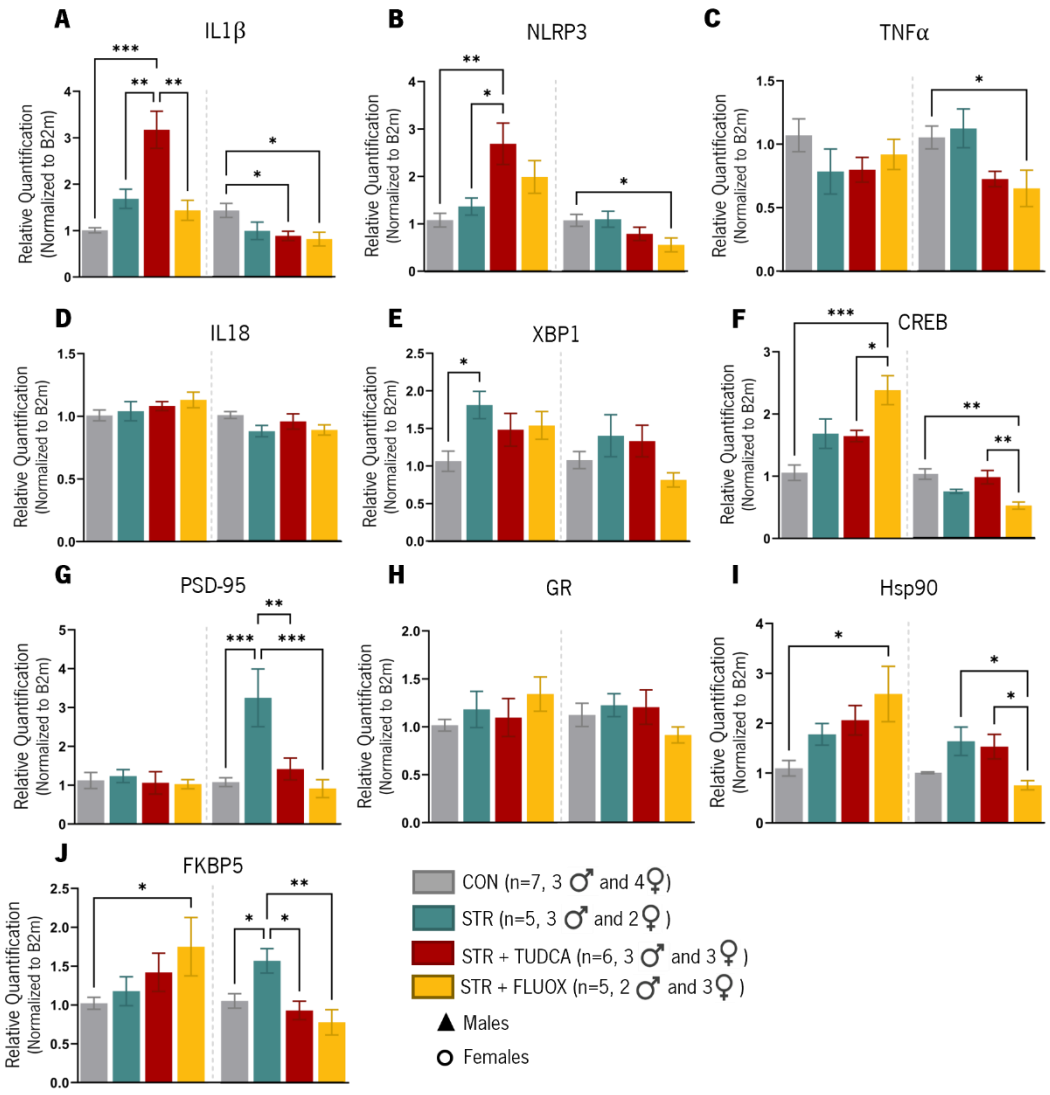


Figure 14. TUDCA and Fluoxetine modulated in a sex-related way pathways related to GR signalling, the inflammasome and synaptic plasticity in the PFC. (A) TUDCA caused an increase of IL1β mRNA levels in males (right part of the graph) [$F_{(3, 26)}$=11,36; p<0,0001], but a decrease in females (left part) [$F_{(3, 32)}$=3,146; p=0,0385]. **(B)** The same effects of TUDCA were found in mRNA levels of NLRP3 in males [$F_{(3, 29)}$=6,292; p=0,0020] and females [$F_{(3, 32)}$=4,184; p=0,0132]. In females, Fluoxetine caused a decrease in NLRP3 mRNA levels [p=0,0487]. **(C)** Between male groups (right part of the graph), no significant differences were found in TNFα mRNA levels [F(3, 26)=1,013; p=0,4030]. However, in females, significant differences were found [$F_{(3, 37)}$=4,245; p=0,0113], with a decrease of mRNA levels of TNFα in Fluoxetine-treated females [p=0,0409]. **(D)** Regarding IL18 mRNA levels, no differences were found between the groups in males [$F_{(3, 29)}$=0,7986; p=0,5047] or females [$F_{(3, 32)}$=1,955; p=0,1406]. **(E)** Significant differences in XBP1 mRNA levels were found in males [$F_{(3, 26)}$=3,123; p=0,0431], but not in females [$F_{(3, 30)}$=2,243; p=0,1037]. In males (left part of the graph), CUS elicited an increase of XBP1 mRNA levels [p=0,0291], with treatments with TUDCA and Fluoxetine showing a tendency to lower this increase. **(F)** Fluoxetine provoked an increase in CREB mRNA levels compared to control [p=0,0001] and TUDCA-treated males [p=0,0446]. In females, Fluoxetine had the opposite effect, causing a decrease in CREB mRNA levels compared to control [p=0,0011] and TUDCA-treated females [p=0,0054]. **(G)** qRT-PCR analysis did not reveal differences of mRNA levels of PSD-95 between male groups [$F_{(3, 17)}$=0,1503; p=0,9281] In females, CUS induced an increase of PSD-95 mRNA levels in the PFC [p=0,0003], reverted by treatments with TUDCA [p=0,068] and Fluoxetine [p=0,0005]. **(H)** No statistically significant difference was found in GR mRNA levels in either sex [Males: F(3, 29)=0,6043; p=0,6175; Females: F(3, 41)=1,056; p=0,3782]. **(I)** In males, Fluoxetine caused a significant increase in mRNA levels of Hsp90 [p=0,0103], while in females, it caused a significant decrease of Hsp90 mRNA levels, compared to the Stress [p= 0,0152] and STR+TUDCA groups [p= 0,0193]. **(J)** Regarding the mRNA levels of FKBP5, Fluoxetine elicited an increase in males [p=0,0384], while, in females, TUDCA [p=0,017] and Fluoxetine [p=0,0024] reverted the CUS-induced increase in FKBP5 mRNA levels [p=0,044]. All data are presented as mean±SEM. Significance is represented as: * p<0,05; ** p<0,01; *** p<0,001.

To evaluate the effects at a synaptic level, mRNA levels of a transcription factor that plays a crucial role in processes related to learning, memory, and synaptic plasticity (CREB), and of a fundamental protein for the organization and functioning of excitatory synapses in the CNS (PSD-95) were measured. Concerning CREB levels, the qRT-PCR analysis revealed significant differences between groups in males [$F_{(3, 29)}$=8,367; p=0,0004] and females [$F_{(3, 20)}$=8,108; p=0,0010] (**Figure 14F**). CUS provoked a tendency to increase CREB mRNA levels [p=0,0608], then enhanced by the treatment with Fluoxetine [CON vs STR+FLUOX: p=0,0001], while no differences were observed in TUDCA. In females, CUS had a tendency for a decrease in CREB levels [p=0,1563], and the same was observed in Fluoxetine [p=0,0011]. TUDCA treated females present CREB levels has the controls. Regarding mRNA levels of PSD-95, no differences were observed between male groups [$F_{(3, 17)}$=0,1503; p=0,9281] (**Figure 14G**); whereas, in females, One-way ANOVA analysis reported significant differences between the groups [$F_{(3, 28)}$=8,986; p=0,0002], with CUS eliciting an increase of PSD-95 mRNA levels in the PFC [CON vs STR: p=0,0003], and TUDCA and Fluoxetine leading to a decrease in the levels of PSD-95 [STR vs STR+TUDCA: p=0,068; STR vs STR+FLUOX: p=0,0005], suggesting a reversion of the CUS-induced increase of PSD-95. This data should be further explored using WB analysis of synaptosomes for PSD-95 and other important structural synaptic proteins, in addition to spine density in Golgi-cox staining studies.

Since CUS is known to exert a dysregulation of the HPA axis and damage to GR response inside the cell, we accessed qPCR (**Figure 14 H-J**) and WB levels of GR and its cytoplasmatic chaperones (Hsp90 and FKBP5) were assessed (**Figure 15 C-D**). In both sexes, One-way ANOVA analysis did not show any differences between the groups regarding the mRNA levels of GR [Males: $F_{(3, 29)}$=0,6043; p=0,6175; Females: $F_{(3, 41)}$=1,056; p=0,3782]. But the analysis of the cytoplasmatic protein levels of GR suggest a similar effect of the treatments in both sexes (**Figure 15C**). CUS decreases GR levels in males [CON vs STR: p=0,0237] and, although not statistically significant, in females [Mann-Whitney test: p=0,2571], with both TUDCA and Fluoxetine leading to a reversion of this effect, with fluoxetine showing a significant increase of GR cytoplasmatic protein levels compared to untreated stressed females [Mann-Whitney test: p=0,0381]. We should note that these cytoplasmic levels of GR, should be completed by the nuclear levels of the receptor, since its movement to the nuclei, is highly relevant for its function.

Regarding GR chaperones, we observed that mRNA levels of Hsp90 present differences between males [$F_{(3, 28)}$=4,201; p=0,0142] and females [$F_{(3, 30)}$=5,467; p=0,0040] (**Figure 14I**). While stress seems to cause a tendency for an increase in Hsp90 mRNA levels in both sexes [Males: p=0,3242; Females: p=0,0694], only in females we observe a restoration to control levels in fluoxetine treated mice [p=0,0103], while TUDCA treatment doesn't seem to revert this increase by CUS in both sexes. In WB

analysis of cytosolic levels, no relevant changes were observed in the levels of Hsp90. However, we should do more technical replicates for WB protein levels assessment.

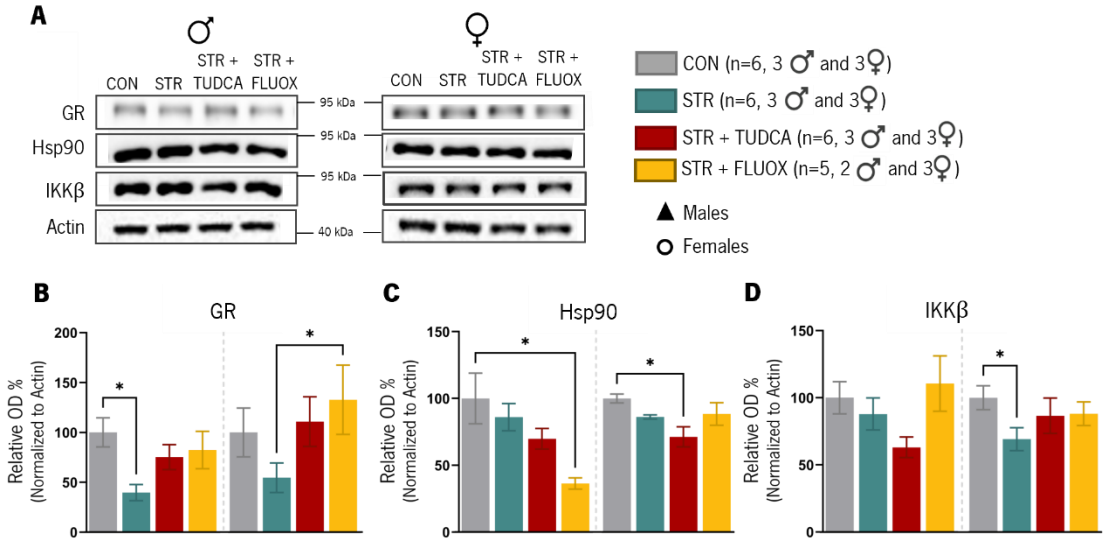


Figure 15. TUDCA and Fluoxetine modulated in a sex-related way pathways related to GR signalling and inflammation. (A) Representative images of Western Blot membranes of males and females from the cytoplasmatic fraction of PFC samples collected from Control, Stress, STR+TUDCA and STR+FLUOX groups. **(B)** WB analysis revealed that, in males, CUS induced a decrease in the cytoplasmatic GR protein levels [p=0,0237]. Although not statistically significant, TUDCA and Fluoxetine showed a tendency to increase CUS-induced levels of cytoplasmatic GR. In females, the same tendencies were shown, with Fluoxetine treatment provoking a significant increase in GR cytoplasmatic levels compared to females from the Stress group [p=0,0381]. **(C)** Compared to the Control group, males treated with Fluoxetine [p=0,0183] and females treated with TUDCA [p=0,0178] displayed lower cytoplasmic levels of the Hsp90 protein. **(D)** In males, there was no statistically significant difference in IKKβ cytoplasmatic protein levels between groups [$F_{(3, 38)}$=2,449; p=0,0785]. In females, CUS induced a significant decrease in IKKβ levels [p=0,0250]. All data are presented as mean±SEM. Significance is represented as: * p<0,05.

Regarding co-chaperone, FKBP5, the same tendencies observed in Hsp90 mRNA levels in a sex specific way, were also observed here (**Figure 14J**). In males, Fluoxetine caused an increase expression (by an unpaired t-test, CON vs STR+FLUOX: p=0,0384). In females, CUS elicited an increase in the levels of FKBP5 [p=0,044] that was reverted by treatment with TUDCA [p=0,017] and with Fluoxetine [p=0,0024].

In summary, qRT-PCR analysis suggests that sex plays a role in the effects of CUS, TUDCA and Fluoxetine in inflammation, ER stress, synaptic plasticity, and GR signalling. Moreover, data suggests that TUDCA and Fluoxetine can modulate GR signalling and synaptic plasticity, with TUDCA having an additional influence on the inflammasome pathway.

4.7. TUDCA reverts stress-induced increase in the phosphorylation levels of Tau and differently in males and females

For the study the effects of TUDCA and Fluoxetine on Tau hyperphosphorylation, accumulation and missorting, cytoplasmatic levels of total Tau (Tau5) and two phosphorylated epitopes of this protein (pSer396 and pThr231) were assessed (**Figure 16**). Kruskal-Wallis tests did not report a significant difference in the total cytoplasmatic Tau levels between the groups in either sex, suggesting no major effect of CUS in the cytoplasmatic levels of Tau (**Figure 16B**).

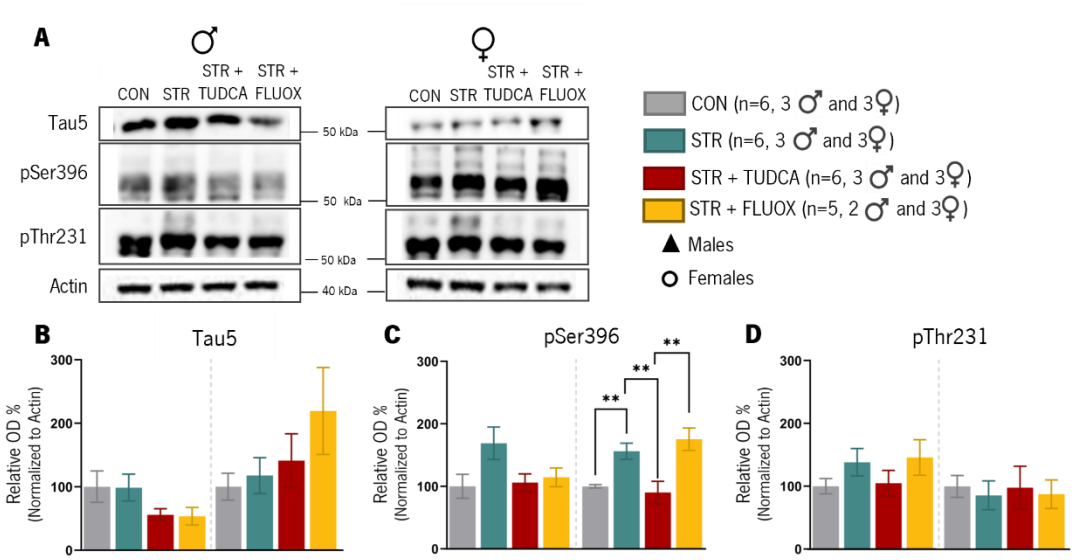


Figure 16. TUDCA modulated the protein levels of total Tau and its phosphorylated isoforms differently in males and females. (A) Regarding the total cytoplasmatic levels of Tau, no statistically significant difference was found in neither sex. However, TUDCA and Fluoxetine showed a tendency to decrease total cytoplasmatic Tau levels, in males, while tending to cause the opposite effect in females. **(B)** In males, no statistically significant differences in the cytoplasmatic levels of pSer396 were found between the groups. In females, CUS induced an increase of pSer396 cytoplasmatic levels [p=0,0012], reverted by TUDCA [p=0,0082], but further increased by Fluoxetine [p=0,0079]. **(C)** Analysis of the cytoplasmatic protein levels of pThr231 did not reveal a significant difference between groups in neither sex. However, it's possible to observe different tendencies in males and females. All data are presented as mean±SEM. Significance is represented as: ** p<0,01.

Interestingly, regarding the phosphorylated Tau isoform pSer396, a CUS elicited a Kruskal-Wallis test revealed differences between the female groups [p=0,0006], with CUS inducing an increase of its cytoplasmatic levels [Mann-Whitney: p=0,0012] (**Figure 16C**). Curiously, TUDCA reverted the CUS-induced increase in pSer396 levels [Mann-Whitney test: p=0,0082], while Fluoxetine caused a further increase [Mann-Whitney test: p=0,0079]. Although not statistically significant, in males the same tendencies of CUS and TUDCA were observed. Another p-Tau epitope known to be modulated by CUS, is pThr231. WB analysis of its cytoplasmic levels did not reveal a significant difference between groups in either sex (**Figure 16D**). Intriguingly, tendencies are observed in males with CUS tended to increase pThr231 protein levels in males, with TUDCA showing a tendency to revert it to normal levels.

In summary, possible sex-related differences were found in the effects and tendencies displayed by Stress and TUDCA in Tau phosphorylation in the PFC, where TUDCA appears to revert CUS-induced effects on cytoplasmatic levels of Stress-related p-Tau epitopes. However, we should note that part of stress-induced Tau pathology has to do with the toxic movement of these p-Tau epitopes to the synapses, and that will be later assessed by analysis the LP1 sub-cellular fractionation – synaptosomes.

CHAPTER 5
DISCUSSION

Chronic stress is normally associated with maladaptive responses connected to elevated levels of GCs and dysregulation of the HPA axis, leading to numerous drastic changes at a molecular, cellular, structural, and behavioural levels. High GC levels and chronic stress have been identified as, at the very least, risk factors for the development of several neurological diseases such as AD and MDD (McEwen & Akil, 2020; Popoli et al., 2012; Radley et al., 2015; Sotiropoulos & Sousa, 2016). The existing therapeutical approaches focus on managing/relieving symptoms linked with chronic stress, but not on the root cause itself, a huge barrier for the development of a treatment that targets various factors modulated by chronic stress and high levels of GCs. The main goal of this Master thesis was to test and study a novel therapeutical approach believed to be capable of directly influencing multiple aspects of the chronic stress paradigm.

TUDCA reverted CUS-induced effects on depressive-like behaviours in TST and on long-term memory in the NOR test

The first step of this study was to characterize the effects of TUDCA on the behaviour of a chronic stress model. For this, WT mice, from both sexes, were subjected to a CUS protocol to induce an anxious- and depressive-like behaviours, as well as cognitive impairments in these animals. After 5 weeks of CUS, mice were treated with i.p. injection of TUDCA and Fluoxetine, a widely used antidepressant whose effects on stress models have been demonstrated (Atrooz et al., 2021). Results from the Fluoxetine treatment was used as a comparative term for TUDCA effects, aiming to compare these drugs and see if they acted in a similar way.

Overall anxiety assessment revealed that the inefficacy of the CUS protocol to induce anxiety-like behaviours in all tests, except the OF. Contrasting to previous studies, in the NSF test, mice subjected to the CUS protocol did not display a statistically significant increase in the latencies to approach the centre or start feeding (Santarelli et al., 2003; Zhu et al., 2014). Additionally, in EPM, control animals spent small periods of time in the open arms of the EPM than previously described by other studies (David et al., 2009; Monteiro et al., 2015). A possible explanation for this unexpected result in the EPM can be the lack of handling of control animals before the start of injections and behavioural assessment. While animals subjected to the CUS protocol were used to being handled daily, controls were only manipulated during the weekly measure of body weight and during injections in the 10 days prior to the start of the behaviour, which might not have been sufficient for controls' habituation. As EPM was the first behaviour test performed, the lack of habituation to handling might be a cause for the higher anxious state of control animals displayed in the EPM results. Considering previous described effects of handling on the anxiety

of animals, the lack of habituation to handling in this experiment might have influenced the results (Novak et al., 2022). Moreover, as EPM is the first behaviour test to be performed due to its high sensitivity to external factors, it can also be perceived by the animals has a highly anxious experience, and leads to extreme effects, like the ones observed.

In the OF test, where CUS elicited anxiety-like behaviours, neither TUDCA or Fluoxetine had effects on the percentage of distance travelled in the centre or in the time spent in this more unprotected part of the arena, displaying similar results to the Stress group. These results suggest that TUDCA and Fluoxetine do not revert the CUS-induced effects on anxiety as has been previously described (Bessa et al., 2009; David et al., 2009; Farhan & Haleem, 2016). Possibly due to the use of males and females in all experiments, there is variability in the distribution of the data, which elicited a second behavioural analysis separated by sex. When comparing female groups and their male counterparts, females from the STR group displayed more anxiety-like behaviours such as a higher distance travelled, a lower percentage of this distance in the centre, as well as shorter periods of time spent in the centre, which is in line with previous findings that suggest a potential sex-specific responses to chronic stress (Sotiropoulos et al., 2008). In both sexes, treatment with Fluoxetine and TUDCA did not have a significant effect on these behaviours, but they appeared to elicit different tendencies. In males, treated animals did not show a reversal of the effects of stress, while in females TUDCA and Fluoxetine treatments caused a tendency for a reversal of the effects of CUS. Due to the CUS protocol inefficiency in inducing anxiety-related behaviours in the remaining tests, conclusions regarding TUDCA or Fluoxetine's effects on anxiety are very limited. The lack of an anxious phenotype nullifies possible anxiolytic effects of TUDCA and Fluoxetine as studies have reported that these drugs do not act under normal conditions (Cheng et al., 2019; David et al., 2009; Lu et al., 2018).

Regarding depressive-like behaviours, CUS had slightly different effects on two features of depression: anhedonia and learned-helplessness. In the SPT, no statistically significant differences were found in the sucrose preference, its variation with treatments administration or in the total consumption. Some studies have suggested that not every CUS protocol can induce anhedonia in mice, recommending the adjustment of the stress protocol to the wanted characteristics of behavioural stress response observed (Anisman & Matheson, 2005; Strekalova & Steinbusch, 2009). However, since there is a slight decrease in the variation of the sucrose preference between tests, in line to what was expected, there may not be a need of adjustment of the CUS protocol as variability of results within the groups may explain this lack of statistical differences. In contrast, the CUS protocol elicited a significant increase of the periods of time animals spent immobile during the TST, thus eliciting a clear depressive-like behaviour

in a test widely used in the testing of antidepressant compounds (Cryan et al., 2005). Furthermore, TUDCA and Fluoxetine display a strong reverting effect of the CUS-induced increased immobility time in the TST as expected (Cheng et al., 2019; Cryan et al., 2005; David et al., 2009; Lu et al., 2018; Manji et al., 2001). Curiously, when we separated data by sex, there was a noticeable difference in the strength of CUS-induced effects. In males, CUS induced a depressive-like behaviour with a reversion by TUDCA and Fluoxetine, while in females no significant differences between the groups were found. These differences have not been described in other studies; however, this may be due to the fact that most studies only work with male rodents and normally do not compare the data between the sexes.

Since the SPT and TST are associated with prefrontal cortical functions, these findings suggest that not only CUS had the expected negative effects on the PFC, as TUDCA is able to act upon this brain area and revert CUS-related detrimental effects, having similar effects to Fluoxetine. Negative changes in the function of this limbic brain area have also been identified in the Y-maze spontaneous alternations test. In this cognitive task, mice subjected to the CUS protocol showed a lower percentage of correct alternation, accompanied by a higher number of indirect revisits. As this test evaluates the spatial working memory associated with prefrontal cortical functions, these results support what was seen in the TST: the CUS protocol caused impairments in the functioning of the PFC.

Impairments in the hippocampus have also been shown by the other cognitive tests performed. In the NOR test, where hippocampal-dependent recognition memory is assessed, deficits in both the short- and long-term have been observed. In NOR short and long, mice from the Stress group displayed lower discrimination ratios than controls, indicating that CUS elicited impairments in short- and long-term recognition memory. Interestingly, in the NOR short, CUS affected males more strongly than females, but treatments with TUDCA and Fluoxetine were able to revert the stress-induced effects in both sexes. NOR relies heavily on the dorsal hippocampus, thereby suggesting that CUS might have caused impairment in the functioning of this brain area. However, it's important to say that the PFC and other cortical regions are also needed in these tests, therefore these behavioural outcomes may not be exclusively due to CUS-induced effects on dorsal hippocampal (Broadbent et al., 2010; Fanselow & Dong, 2010; Rocha et al., 2021; Rudy & Matus-Amat, 2005).

TUDCA had no significant effect on short-memory tasks such as the Y-maze and the NOR short, however it displays therapeutical effects on long-term memory assessed by NOR long. This positive effect of TUDCA on long-term recognition memory suggests that TUDCA can have therapeutical effects against the CUS-induced impairments in the dorsal hippocampus. This hypothesis is reinforced by previous studies that have demonstrated that TUDCA can prevent learning and memory impairments in the Morris

Water Maze (MWM) test in a well-established model of AD (APP/PS1 mice), as well as in an LPS-induced mice model of cognitive impairment (Lo et al., 2013; Wu et al., 2019). In addition, animals treated with TUDCA have displayed similar results to those treated with Fluoxetine in the Y-maze spontaneous alternations and NOR long. In the NOR short test, Fluoxetine appears to cause a slight increase in the discrimination ratio when compared to animals from the Stress and STR+TUDCA groups. The variability of female results in this test may explain why this increase is not statistically significant, leaving the question if Fluoxetine might have positive effects on short-term memory. Studies regarding effects of Fluoxetine in cognition are quite contradictory, some authors have reported that Fluoxetine improves performance in certain memory tasks, while others have described impairments on cognition caused by Fluoxetine's chronic administration (Carlini et al., 2012; El Hage et al., 2004; Sadegzadeh et al., 2020).

In summary, TUDCA reverted CUS-induced effects on depressive-like behaviours in TST and on long-term memory in the NOR test, suggesting its possible effects on the PFC and hippocampus, two brain areas crucial for depression and long-term memory. Moreover, animals treated with TUDCA displayed throughout the majority of the behaviours similar results to those treated with Fluoxetine. The similarity in behavioural outcomes may suggest that TUDCA and Fluoxetine may act through a similar mechanism. This hypothesis was already raised by an experiment that compared the effects of TUDCA and Fluoxetine in a co-treatment strategy and individually. In this *in vivo* study in a CUS model, it was found that Fluoxetine's effects were not enhanced by co-treatment with TUDCA, raising the hypothesis of a similar mechanism (Lu et al., 2018).

Moreover, when a secondary phase of analysis was conducted to investigate any sex-related differences in treatment outcomes, our findings suggest a possible sex-related effect of the stress protocols and of the treatments in specific parameters of anxiety, depression, and cognition assessment.

TUDCA's exerts a modulatory effect in inflammation in the PFC that is sex dependent

Neuroinflammation is a key factor in the detrimental effects of chronic stress and elevated GC levels. Chronic stress has been linked with the activation of several pro-inflammatory pathways that enhance the inflammation state in the brain, such as the NFκB and the inflammasome pathways (Kelley et al., 2019; Capece et al., 2022). This pro-inflammatory modulation has also been linked to changes in the morphology and proliferation of microglia, which then mediate some of the detrimental effects of stress (Hinwood et al., 2012; Horchar & Wohleb, 2019; Sanguino-Gómez et al., 2022). Several studies have reported TUDCA's ability to alleviate inflammation in disease context, modulating the expression of

several inflammatory mediators, and the activation and proliferation of microglia (Kim et al., 2019; Lu et al., 2018; Mendes et al., 2019).

In this experiment, the immunostaining of microglia with IBA-1 in the PFC of male mice revealed an increasing effect of the CUS protocol in the average fluorescence intensity per cell, suggesting a stress-induced increase in the number of microglia. However, CUS induced a decrease in the number of IBA$^+$ cells, implying that possible changes in the morphology or activation state may have occurred, causing the higher fluorescence intensity. TUDCA and Fluoxetine were able to revert this stress-induced increase in the average fluorescence. But, regarding microglia number, while TUDCA did not have an effect, Fluoxetine increased the number of IBA-1$^+$ cells. These data suggest that both treatments may modulate microgliosis in males, which is in line with previous studies (Lo et al., 2013; Lu et al., 2018; Yanguas-Casás et al., 2014). Further studies regarding TUDCA's effects on microglia need to be performed mainly regarding their morphology and activation, to conclude if TUDCA was able to modulate microglia activation state.

Contrary to previous studies, CUS was not able to elicit a classical inflammatory phenotype in males as PFC samples collected from males subjected to the CUS protocol did not display increased expression of pro-inflammatory mediators such as IL1β, NLRP3, IL18, TNFα and IKKβ. Moreover, the expression profile of these factors was very unexpected in TUDCA-treated males. TUDCA had no effect on the CUS-induced levels of TNFα and IL18, but it significantly increased the expression of NLRP3 and IL1β, suggesting a TUDCA-induced or enhanced activation of the inflammasome pathway and consequent activation of IL1β (Cheng et al., 2019; Lu et al., 2018). Even though WB data did not show a significant effect, there seems to be a slight decrease of IKKβ protein levels in the cytoplasmatic fraction of the PFC, which is rather intriguing. IKKβ is an essential kinase for the activation of NFκB signalling, thereby its decreased cytoplasmatic expression may suggest a decrease in NFκB activation. However, it has been demonstrated that IKKβ can also be present in the nucleus of the cell and still participate in NFκB activation, so it would be interesting to analyse the expression of this kinase in the nuclear fraction to better understand TUDCA's effects on its expression (Capece et al., 2022; Kelley et al., 2019; Tsuchiya et al., 2010). Our findings suggest that, in males, TUDCA elicited the activation of the inflammasome pathway and the reversion of the CUS-induced increase in the microglia number in the PFC.

In contrast, immunostaining of microglia in three subregions of the dorsal Hippocampus (CA1, CA3 and DG) revealed a decrease in the average fluorescence intensity per cell in TUDCA-treated males compared to other groups, even controls. Conclusions cannot be taken regarding the CUS effect on CA1 and CA3 because of the low number of samples in the Stress group, more replicates are needed to

increase the sample number and validate conclusions. In the DG, while the CUS protocol caused a decrease in the average fluorescence intensity of IBA-1 per cell, TUDCA seemed to enhance the decrease in this parameter. Since this significant decrease induced by TUDCA is present in all the three hippocampal areas, always lower than control animals, there seems to exist a general decrease of male hippocampal microglia numbers caused by TUDCA. While the microglia population appears to be affected by TUDCA in a similar way in all three areas of the dorsal Hippocampus, the same does not apply for Fluoxetine. The effect on microglia numbers seems to be similar between TUDCA and Fluoxetine in CA1 and DG, however, in CA3, the antidepressant caused an increase in fluorescence per cell, reaching controls' level. This differential effect on different hippocampal subregions is very interesting since it suggests a different effect and action of Fluoxetine in different subregions, while TUDCA appears to have a uniform effect throughout the dorsal hippocampus.

Overall, further studies need to be performed regarding microglia morphology and activation to understand if TUDCA is modulating microglia's response and thus increasing the levels of pro-inflammatory mediators in the PFC. It would also be interesting to check the expression levels of cytokines in the hippocampus, to further study TUDCA's modulating effects on hippocampal neuroinflammation.

Interestingly, in our experiment, a different inflammatory profile was observed in female mice. Females subjected to the CUS protocol did not show any significant alteration in the expression levels of the pro-inflammatory mediators or in the number of microglia cells present in the PrL area of the PFC, even though they appear to have a tendency for a higher number of microglia than controls. Interestingly, treatment with TUDCA appears to have no significant effect on the expression of IL1β, NLRP3, IL18, TNFα and IKKβ, but it significantly increased the average fluorescence intensity per cell in the immunostaining of IBA-1, suggesting a modulation of microglia number in the PFC. In the female hippocampal subregions, CUS decreased the average fluorescence per IBA-1$^+$ cells, implying a decrease in microglia number in the Hippocampus. TUDCA-treated females did not display differences compared to the stressed ones, apart from the slight TUDCA-induced increase observed in CA3, which is different from Fluoxetine-induced decrease. In all hippocampal areas, Fluoxetine had a similar decreasing effect on the average fluorescence per cell, suggesting a similar effect throughout the dorsal hippocampus, contrasting to what was observed in males.

These sex differences in TUDCA's modulation of microglia and inflammatory response are not described in previous works, mainly due to most of them working only with males. Thereby, we were able to describe, for the first time, distinct sex-related inflammatory profiles of mice treated with TUDCA after a CUS protocol. However, for a better analysis and valid conclusions on the TUDCA's effects on

hippocampal and prefrontal cortical microglia, sample number needs to be increase and the microglial morphology and activation state must be evaluated.

TUDCA's effects on astrocytes in the Hippocampus

Astrocytes are brain cells that play a critical role in providing support and maintaining the normal functioning of neurons, participating in the regulation of synaptic activity. Studies have demonstrated anomalies in astrocytes in depression and chronic stress rodent models, reporting transcriptional and morphological changes, as well as impairments in astrocyte networks (Aten et al., 2023; Codeluppi et al., 2021). Effects of antidepressants like Fluoxetine in astrocytes in a context of stress have already been described. Studies indicate that Fluoxetine can act on astrocytes, reverting the stress-induced decrease in astrocyte number, regulating the expression of neurotrophic/growth factors, and reducing astrocyte reactivity in models of depression (Fang et al., 2022; Kinoshita et al., 2018). Described effects of TUDCA on astrocytes consist of its ability to attenuate proliferation and activation of astrocytes, decreasing GFAP protein levels in the hippocampus, and reducing the production of nitrites by astrocytes in models of AD, PD and acute neuroinflammation (Cuevas et al., 2022; Lo et al., 2013; Yanguas-Casás et al., 2014).

For assessment of the treatments' effects on astrocytes in this experiment, hippocampal brain slices were stained with an antibody for a well-known marker of these cells, GFAP. Although males from the Stress group appear to show a tendency for much higher fluorescence intensity, due to the low number of samples, no conclusions can be taken regarding this group. Compared to the male controls, TUDCA and Fluoxetine tended to induce a decrease in the intensity of GFAP immunostaining in all hippocampal subregions, suggesting a transversal effect of TUDCA and Fluoxetine in astrocytes throughout the dorsal hippocampus in males. Further analysis and repetitions of the GFAP immunostaining should be performed to increase the sample number, confirm these results, and allow comparison with the effect of stress.

In contrast, in females, CUS induced a higher fluorescence intensity of GFAP immunostaining, that might indicate a modulation of astrocytes population, which appears to be enhanced by treatment with Fluoxetine and TUDCA, contrasting findings from other studies. Compared to the Control and the Stress groups, Fluoxetine- and TUDCA-treated females present higher fluorescence intensity throughout all hippocampal subregions, contrasting with the results obtained in their male counterparts, suggesting once again a different effect of both drugs in males and females.

Working only with the fluorescence intensity of the GFAP immunostaining restricts the conclusions that can be taken regarding the modulation of astrocytes. Intensity fluorescence can be affected by many

factors such as slight variations during the immunostaining protocol or the variability between animals that can cause variations within the same group. To confirm the fluorescence results other assessments should be done such as morphology assessment through the drawing of astrocytes, and the evaluation of GFAP mRNA and protein levels to infer its expression.

Due to the differential expression and morphology of astrocytes in the PFC, the immunostaining protocol was not efficient in PFC slices, contrary to hippocampal slices. Several concentrations of the primary antibody against GFAP were used, but none was efficient in clearly staining astrocytes in the prefrontal cortical slices. Due to the importance of PFC in chronic stress and the already mentioned effects of the CUS protocol, TUDCA and Fluoxetine in this limbic brain area, it's important to obtain results regarding the prefrontal cortical astrocyte population. A possibility for bypassing this setback is trying to perform immunostaining of S100β, another widely used astrocytic marker. Additionally, as previously mentioned, assessment of astrocytic morphology and expression levels of astrocytic markers can be valuable in obtaining more information regarding the effects of TUDCA in astrocytes.

Molecular pathways modulation by TUDCA in the context of chronic stress

ER Pathway

TUDCA is widely recognized for its ability to attenuate ER stress, a characteristic that has made it a commonly used tool in numerous ER stress models (Khalaf et al., 2022; Lu et al., 2018; Zangerolamo et al., 2021). To connect our experiment with previous studies in neurodegenerative models and study one of TUDCA's classical effects in a model of CUS, XBP1 mRNA levels were assessed. XBP1 is a transcription factor that plays a role in the UPR, a cellular response activated by ER stress, thereby increased expression levels of this transcription factor are linked to ER stress. In this experiment, no significant differences between CUS' effect or TUDCA's were found in either sex. However, treatment with TUDCA tends to decrease the CUS-induced increase in the expression of XBP1, suggesting a possible reduction of ER stress. It's important to note that no definitive conclusion can be taken regarding ER stress as there is no statistical significance probably due to the variability of the expression data observed within the groups. This variability enforces the need for more replicates, as well as assessment of other established indicators of ER stress like protein levels of GRP78, caspase 12, CHOP, ATF4, ATF6α or the phosphorylation of PERK and IRE1 (Hetz & Saxena, 2017).

Synaptic function

It is well known that chronic stress has a negative impact on synaptic plasticity, a fundamental mechanism that underlies learning and memory. To evaluate TUDCA's effects on synaptic plasticity, the expression levels of two key players in the regulation of this mechanism were measured. CREB is a transcription factor whose activation is linked with the transcription of genes that regulate synthesis and trafficking of synaptic proteins such as PSD-95. This scaffolding protein is crucial for the organization of receptors and signalling complexes at the synapse, affecting synaptic strength and plasticity.

Intriguingly, males and females displayed different tendencies in the prefrontal cortical mRNA levels of CREB and PSD-95, suggesting again sex-dependent effects of Fluoxetine, which has already been described (Qi et al., 2008; Sadegzadeh et al., 2020), and of TUDCA. In males, CREB expression seems to be increased by the CUS protocol with a further increase by caused Fluoxetine, but no effect from TUDCA treatment, while PSD-95 levels presented no differences. In contrast, Fluoxetine-treated females present lower levels of CREB expression, with no significant effect of TUDCA or stress, yet CUS elicited an increase in PSD-95 expression, reverted by TUDCA and Fluoxetine. These influences of CUS, TUDCA and Fluoxetine appear to contrast with what has been shown before, however as CUS caused an increase of PSD-95, TUDCA and Fluoxetine show the ability to be able to revert the expression levels of PSD-95 to normal levels (Liu et al., 2015; Lo et al., 2013; Ramalho et al., 2013; Reinés et al., 2008).

Although it might have been expected to find a parallel increase of CREB and PSD-95 levels, we must keep in mind that the increase of mRNA levels of CREB may not translate to an increased activation of the transcriptional factor, thus not translating to higher mRNA levels of PSD-95. For this reason, nuclear protein levels of CREB should be assessed to check for the phosphorylation and activation of this transcription factor. Furthermore, WB analysis should be performed in the synaptossomal fraction (LP1) obtained from the subcellular fractionation for evaluation of the protein levels of PSD-95 and other synaptic proteins (e.g. synaptophysin, HOMER, SHANK). Analysis of synaptic density and morphology through Golgi-Cox would also provide a better understanding of what is happening at synapses.

Overall, our findings suggest that Fluoxetine and TUDCA can modulate synaptic plasticity in the PFC. Moreover, due to the contrast in Fluoxetine and TUDCA's influence on PSD-95 and CREB mRNA levels, it appears that the treatments may act through different mechanisms.

Glucocorticoid Receptor Modulation

Considering GR's major role in chronic stress and TUDCA's described ability to bind to this receptor, it was imperative that TUDCA's effects on GR signalling would be studied (Cai et al., 2005;

Khalaf et al., 2022; Solá et al., 2006). For this mRNA and protein levels of the receptor and two of its chaperones, Hsp90 and FKBP5, were evaluated. Regarding GR expression levels, no statistically significant differences in the GR mRNA levels were found between the four groups, although WB analysis has revealed a CUS-induced decrease in the cytoplasmatic levels of GR, which appears to be slightly reverted by TUDCA and Fluoxetine treatments. It would be important to assess GR's activation through WB analysis of the nuclear levels of GR in the nuclear fraction (P1), as well as to measure serum corticosterone levels to verify if there is a correlated increase of corticosterone in animals from the Stress group. Contrary to the differences observed in the effect of TUDCA in mRNA levels of the GR chaperones, the WB analysis did not reveal any sex-related difference in TUDCA's effects, suggesting that TUDCA's differential actions related to sex may be mainly associated with gene expression and transcription.

Interestingly, Fluoxetine displayed possible sex-related different tendencies and effects in mRNA levels of the GR, Hsp90 and FKBP5, which were also observed in the cytoplasmatic protein levels of Hsp90, but not in the cytoplasmatic GR. Surprisingly, the effects of Fluoxetine in mRNA and protein levels of Hsp90 are not parallel. While the antidepressant appears to increase the Hsp90 mRNA levels in males and a decrease in females, the opposite tendencies are shown in the protein levels. These findings suggests that Fluoxetine may affect different stages of gene expression and protein synthesis in males and females. Our findings suggest that TUDCA and Fluoxetine treatments modulate GR signalling, possibly in a sex-dependent way, reverting the effects of stress in the mRNA levels of the GR chaperones in females, and in cytoplasmatic GR levels in both sexes.

Tau hyperphosphorylation and accumulation

Tau pathology involving the accumulation, hyperphosphorylation and missorting of this important protein plays a significant role in the effects of chronic stress and/or GCs elevated levels. Not many studies have investigated TUDCA's effects on Tau pathology. Two studies have reported TUDCA's ability to prevent Tau hyperphosphorylation via inhibition of the UPR (van der Harg et al., 2014) and to abolish the caspase-3 cleavage of tau (Ramalho et al., 2008) in *in vitro* models. Regarding *in vivo* studies, only one has shown that TUDCA can prevent Tau phosphorylation at Ser396 by modulation of Akt/GSK3β activities in an AD model (Dionísio et al., 2015). Our findings are in line with what has been shown in this AD model, as TUDCA was able to revert CUS-induced increase of the cytoplasmatic levels of phosphorylated Tau isoform pSer396. Additionally, possible sex-related differences were found in the effects and tendencies displayed by TUDCA in Tau phosphorylation in the PFC, something not yet described.

In a context of a Tau pathology, not much has been described regarding Fluoxetine's effects on Tau phosphorylation. Two studies have demonstrated Fluoxetine's reverting effects in CUMS-induced Tau phosphorylation at Ser396, Thr231, Ser356 and Ser202 (Li et al., 2023; Wang et al., 2020). Interestingly, in our experiment, while, in females, Fluoxetine caused a further increase of the CUS-induced increase in pSer396 levels, in males, it tended to cause a decrease of the CUS-induced increase. Considering that the studies mentioned above only worked with male rodents, there might be a not yet described sex-related difference in the effects and tendencies displayed by Fluoxetine. Nevertheless, TUDCA and Fluoxetine's modulation effects in Tau phosphorylation must be analysed further in detail. More replicates of these WB data need to be done as well as analysis of Tau phosphorylation at other epitopes like Ser199/202 and Ser262 to confirm the results obtained. Moreover, considering that part of stress-induced Tau pathology has to do with the missorting of the phosphorylated Tau to the synapses, assessing the synaptosome fraction (LP1) will be a crucial step in uncovering the effects of TUDCA and Fluoxetine on Tau pathology.

Furthermore, it would be interesting to extend these same analyses to the hippocampus in order to see if the possible sex-related modulatory effects of TUDCA and Fluoxetine are also present in another brain area.

TUDCA's effects on hippocampus are still to be further clarified

Behaviour analysis has revealed an expected impact of the CUS protocol on dorsal Hippocampus-dependent cognitive tasks, implying CUS-induced detrimental effects on this brain area. In addition, the assessment of cellular changes in the microglia and astrocytes has demonstrated CUS impact on these cell populations in the hippocampus, as well as the effects of TUDCA and Fluoxetine. Furthermore, this cellular analysis also revealed a possible influence of sex in TUDCA's effects, an aspect not yet described.

These findings underline the necessity of future molecular analysis of the hippocampal tissue, crucial for a better understanding of TUDCA's mechanism of action and of the extent of the sex-related responses. Such analyses should include factors already evaluated in the PFC and parameters not described in TUDCA's papers such as: neuronal structural and spine density analysis; screening of expression levels of microglia and astrocytes markers, and of proteins related to inflammation, synaptic plasticity, ER stress and tau pathology; assessment of monoamines levels; and evaluation of neurogenesis.

CHAPTER 6

CONCLUSION AND FUTURE PERSPECTIVES

The present work shed light on TUDCA's therapeutic potential against chronic stress and stress-related brain pathologies, such as depression. To our knowledge, this is the first study that describes difference between sexes in the effects of TUDCA, suggesting an important role of sex in its modulatory effects. Here, we report that TUDCA has a modulatory effect on several pathways that ultimately lead to molecular, cellular, and behavioural changes, such as:

- **Potential as an antidepressant:** TUDCA reverted the depressive-like behaviour induced by Chronic Stress in the TST, implying a positive impact on the PFC.
- **Ameliorates impairments in long-term memory:** TUDCA reverted the Chronic Stress-induced detrimental effects on long-term memory, suggesting a potential of this drug for restoring normal hippocampal function.
- **Astrocytic modulation in the hippocampus:** TUDCA appeared to have modulatory effects on hippocampal astrocyte populations in a sex-dependent manner.
- **Sex-related modulatory effect in inflammation in the PFC:** after a CUS protocol, TUDCA elicited distinct sex- and brain area-related inflammatory profiles by differently modulating microglia and the expression of pro-inflammatory cytokines involved in the inflammasome pathway.
- **Modulation of synaptic plasticity in the PFC:** TUDCA appears to modulate the expression of key players in synaptic plasticity through different mechanisms than Fluoxetine.
- **Modulation of GR signalling:** TUDCA modulated GR signalling, possibly in a sex-dependent way, reverting the effects of stress in the mRNA levels of the GR chaperones in females, and in cytoplasmatic GR levels in both sexes.
- **Reversion of Tau phosphorylation:** TUDCA reverted CUS-induced increase of the cytoplasmatic levels of phosphorylated Tau isoform pSer396. Possible sex-related differences were found in the effects and tendencies displayed by TUDCA in the phosphorylation of cytoplasmatic Tau.

These findings collectively contribute to raise more questions regarding TUDCA's mechanisms of action, highlighting the need for further studies:

- **Nuclear Analysis:** WB analysis should be performed in the nuclear faction (P1) for the assessment of the activation of transcriptional factors such as CREB and GR.
- **Synaptossomal Analysis:** WB analysis should be performed in the synaptossomal fraction (LP1) for the evaluation of the protein levels of PSD-95 and other synaptic proteins (e.g. synaptophysin, HOMER, SHANK), as well as assessment of phosphorylated isoforms of Tau,

considering that the missorting of the phosphorylated Tau to the synapses plays a role in part of stress-induced Tau pathology.

- **Neurostructural and spine density analysis:** study TUDCA's effects on neuronal survival, dendritic remodelling, and synaptic density and morphology.
- **Characterization of microglia:** profiling microglia morphology and activation through drawing and reconstruction of these immune cells in the hippocampus and PFC.
- **Characterization of astrocytes in the PFC:** assessment of astrocytic morphology and expression levels of astrocytic markers in the hippocampus and PFC.
- **Measurement of established indicators of ER stress and UPR:** WB analysis of cytoplasmatic protein levels of GRP78, caspase 12, CHOP, ATF4, ATF6α or the phosphorylation of PERK and IRE1.
- **Molecular analysis of the hippocampal tissue:** To assess the broader effects of TUDCA and to study its sex-related differences, analyses of factors already evaluated in the PFC and parameters not described in TUDCA's papers should be conducted in the hippocampus, a region central to learning and memory.
- **Evaluation of monoamines:** To better understand the antidepressant properties of TUDCA, it would be important to do a comprehensive assessment of its influence on monoamines, a class of neurotransmitters and neuromodulators important in depression.
- **Sex-related differences:** further research is needed to investigate the role of sex in the effects of TUDCA, which might have implications for therapeutical strategies.

In conclusion, our findings offer a novel perspective on the sex-specific modulatory effects of TUDCA at a molecular, cellular, and behavioural levels, showing a high potential of this drug as an antidepressant. These insights pave the way for future studies regarding TUDCA's modulatory effects as well as its potentially therapeutical application in the context of neuropsychiatric disorders and cognitive deficits connected to chronic stress.

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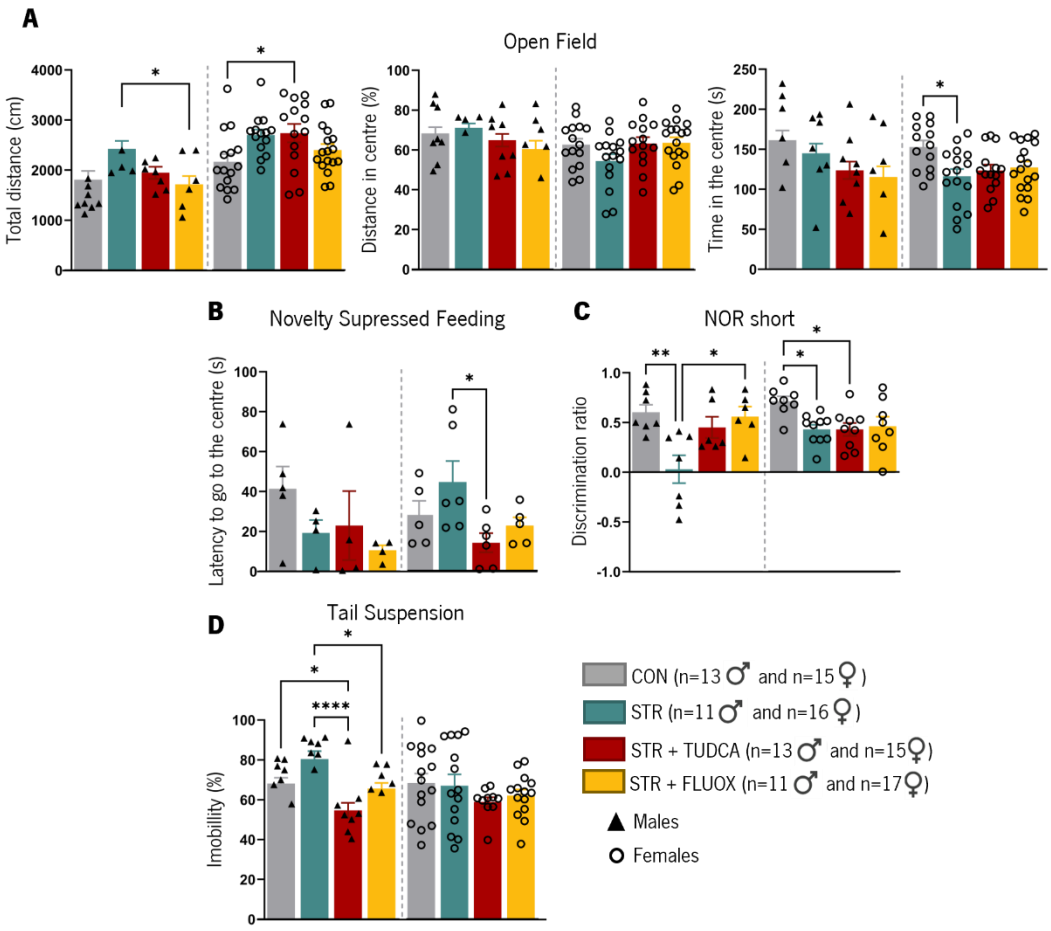
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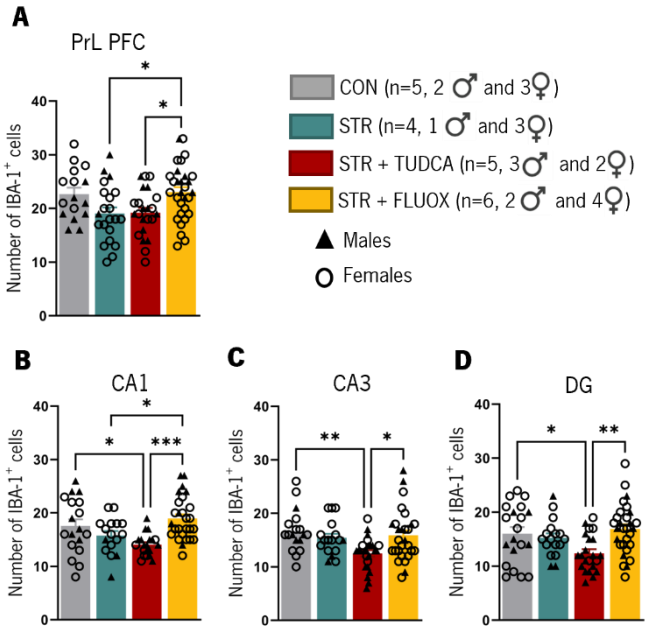
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SUPPLEMENTARY INFORMATION



Supplementary Figure 1. Sex may play a role in the effects of CUS, TUDCA and Fluoxetine. (A) In the Open Field, when compared to other female groups and to their male counterparts, females from the Stress group display more anxious-like behaviours such as a higher distance travelled, lower percentage of this distance being in the centre, as well as smaller periods of time spent in the centre. In both sexes, treatment with Fluoxetine and TUDCA did not have a significant effect on these behaviours, but they appeared to elicit different tendencies. **(B)** In the Novelty Supressed Feeding test, male controls tend to take more time than the other groups, a tendency not followed by female controls. In females, there are significant differences between the groups [F(3, 18)=3,262; p=0,0456], with CUS eliciting a higher latency time, decreased with the administration of TUDCA [p=0,0323]. **(C)** NOR short test showed a stronger effect of CUS in males [p=0,0044] than in females, with a reversion of stress-induced effects on short-term memory caused by treatments with TUDCA [p= 0,0559] and Fluoxetine [p=0,0118]. In females (left part of the graph), treatments did not have any effect on the stress-induced deficits. **(D)** In the Tail Suspension Test, TUDCA [p<0,0001] and Fluoxetine [p=0,0237] reverted CUS-induced increased of immobility time in males (left part of the graph). In females (right part of the graph), the Stress group did not display a significant increase in the immobility time and even though the treated groups appear to a shorter time, it's not statistically significant. All data are presented as mean±SEM. Significance is represented as: * p<0,05; ** p<0,01; *** p<0,001; **** p<0,0001.



Supplementary Figure 2. TUDCA decreased the number of IBA-1+ cells in the PrL PFC and dorsal hippocampus. (A) In the PrL area of the PFC, animals from the Stress group tended to have a lower number of microglia, a tendency reverted by Fluoxetine, but not by TUDCA [STR vs STR+FLUOX: p=0,0269; STR+TUDCA vs STR+FLUOX: p=0,0306]. **(B)** In the hippocampal subregion CA1, TUDCA and Fluoxetine displayed different effects on microglia number, with Fluoxetine-treated animals having a higher number of microglia [p=0,0004]. **(C)** In the CA3 subregion, TUDCA elicited a decrease of the number of microglia compared to controls and to Fluoxetine-treated animals [CON vs STR+TUDCA: p=0,0047; STR+TUDCA vs STR+FLUOX: p=0,0117]. **(D)** In the DG, TUDCA-treated animals displayed less microglia than animals from the Control and STR+FLUOX [CON vs STR+TUDCA: p=0,0457; STR+TUDCA vs STR+FLUOX: p=0,0027]. All data are presented as mean±SEM. Significance is represented as: * p<0,05; ** p<0,01; *** p<0,001.

ANNEX

ETHICAL POSITIVE EVALUATION FOR ANIMAL EXPERIMENTATION

Órgão Responsável pelo Bem-Estar Animal da EM/ICVS e do I3Bs

Referência do processo: ORBEA EM/ICVS-I3Bs_006/2018

Título do Projeto: A proteína Tau como efetora de mecanismos patológicos no cérebro

Investigador Principal: Ioannis Sotiropoulos

Estabelecimento: Unidade do Biotério do Instituto de Investigação em Ciências da Vida e da Saúde (ICVS) da Escola de Medicina da Universidade do Minho

PARECER

O Órgão Responsável pelo Bem-Estar Animal da Escola de Medicina e seu Instituto de Investigação em Ciências da Vida e Saúde, e do Instituto3Bs (Biomaterials, Biodegradables and Biomimetics) - ORBEA EM/ICVS-I3Bs - analisou o processo relativo ao seu projeto de investigação com recurso a modelos animais intitulado *A proteína Tau como efetora de mecanismos patológicos no cérebro*.
Os documentos apresentados revelam que o projeto obedece aos requisitos exigidos para as boas práticas na experimentação com recurso à utilização de modelos animais, considerando a aplicação dos 3 Rs de Russel e Burch e a aplicação de limites críticos de sofrimento - *humane endpoints*.

Face ao exposto, o ORBEA EM/ICVS-I3Bs não tem nada a opor à realização do projeto, emitindo o seu parecer favorável.
Salientamos, no entanto, que a autorização legal de projetos de investigação/ experimentação animal é feita pela Direção Geral de Alimentação e Veterinária (DGAV) a quem deverá ser submetido o respetivo formulário do projeto na sua versão final mais completa.

Braga,

MAGDA JOÃO CASTELHANO CARLOS

Assinado de forma digital por MAGDA JOÃO CASTELHANO CARLOS
Dados: 2019.02.13 10:44:46 Z

(Presidente do ORBEA EM/ICVS-I3Bs)