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FAAH inhibition as a pharmacological strategy to promote stress resilience: insights from rodent studies

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“I love science, and it pains me to think that so many are terrified of the subject or feel that choosing science means you cannot also choose compassion, or the arts, or be awed by nature.”

Robert Sapolsky

Contents

Abstract (English version)

Our view of the endocannabinoid system has evolved, encompassing not only its classical components like anandamide but also a plethora of related non-cannabinoid mediators and alternative signaling routes. A noteworthy illustration of this versatility is exemplified by Fatty Acid Amide Hydrolase (FAAH), a key enzyme responsible for the degradation of the endocannabinoid anandamide and its bioactive congeners within the fatty acid ethanolamide (FAE) family, including the "anti-inflammatory" N-palmitoylethanolamide and the "satiety factor" N-oleylethanolamide. Pharmacological inhibition of FAAH activity has exhibited promise in enhancing stress resilience, ameliorating or preventing symptoms associated with a variety of stress-related disorders, such as depression and post-traumatic stress disorder. This thesis investigates putative “non-conventional” pathways (both central and peripheral) by which increased FAE tone promotes stress resilience, such as the gut microbiota and lipidome or inflammatory processes. Significantly, all these diverse pathways have been extensively implicated in stress-related psychopathologies, yet the impact of FAAH inhibitors on them is often overlooked. In the first study, male rats were subjected to chronic social stress and administered with a systemic FAAH inhibitor (URB694) to evaluate its effects on the gut microbial and lipidic profiles. Notably, this research reveals that systemic FAAH inhibition not only normalized stress-induced behavioral and neuroendocrine changes but also stabilized gut microbiota and lipidome. In the second investigation, a peripherally restricted FAAH inhibitor (URB937) was administered in male rats to assess its effects on behavioral responses and pro-inflammatory cytokines levels following acute social stress. The results demonstrate the potential of peripheral FAAH inhibition in mitigating stress-related consequences, including the normalization of behavior and pro-inflammatory cytokine response. These studies challenge the traditional brain-centric view of psychopathologies by highlighting the intricate bidirectional communication between the brain and periphery. The multifaceted impact of FAAH inhibition, particularly on non-cannabinoid FAEs, like palmitoylethanolamide and oleoylethanolamide, underscores the complexity of the endocannabinoid system and its interconnected pathways.

Abstract (Italian version)

La nostra visione del sistema endocannabinoide si è recentemente evoluta, includendo non solo i suoi canonici componenti, come l'anandamide, ma anche una varietà di mediatori non cannabinoidi ad esso collegati, e pathways di segnalazione alternativi. Un esempio rappresentativo di tale complessità deriva dall'enzima *Fatty Acid Amide Hydrolase* (FAAH), responsabile sia della degradazione dell'endocannabinoide anandamide che dei suoi congeneri bioattivi appartenenti alla famiglia delle etanolamidi degli acidi grassi (FAEs), che include composti come la palmitoiletanolamide (PEA), con note proprietà antiinfiammatorie, ed il cosiddetto "fattore della sazietà" oleiletanolamide (OEA). L'inibizione farmacologica di FAAH si è dimostrata una strategia promettente per l'aumento della resilienza allo stress, migliorando e prevenendo vari sintomi associati a diverse patologie stress-correlate, come depressione e disturbo post-traumatico da stress. Questa tesi ha lo scopo di esplorare potenziali pathways (sia periferici che centrali), come il microbiota e il lipidoma intestinale o i processi infiammatori, che risentano degli aumentati livelli di FAEs, attraverso i quali possono promuovere la resilienza allo stress a livello preclinico. Nel primo studio, ratti di sesso maschile sono stati sottoposti a stress sociale cronico e trattati con un inibitore sistemico dell'enzima FAAH (URB694) per valutare i suoi effetti sui profili microbici e lipidici intestinali. Il trattamento con URB694 è stato sia in grado di normalizzare i cambiamenti comportamentali e neuroendocrini indotti dallo stress che promuovere la stabilità dei profili microbici e lipidici intestinali dei ratti esposti a stress sociale ripetuto. Nel secondo studio, è stato utilizzato un inibitore periferico dell'enzima FAAH (URB937) per valutare i suoi effetti sulle risposte comportamentali e pro-infiammatorie in ratti maschi esposti ad un singolo episodio di stress sociale. I risultati di questa ricerca dimostrano il potenziale dell'inibizione periferica di FAAH nella mitigazione delle conseguenze legate all'esposizione allo stress, sia da un punto di vista comportamentale che nella risposta delle citochine pro-infiammatorie. Il focus di questa tesi è rappresentato dai processi che, partendo dalla periferia, possono contribuire alle conseguenze negative derivanti dall'esposizione allo stress, che sono in grado di influenzare sia il comportamento che altri processi centrali complessi. Gli studi qui riportati possono essere considerati un tentativo di ampliamento della tradizionale visione, la quale considera il cervello come un'entità isolata che governa il funzionamento del resto dell'organismo, evidenziando invece la comunicazione bidirezionale esistente tra sistema nervoso centrale e periferia. Infine, i numerosi effetti

dell'inibizione FAAH, con particolare attenzione alle FAEs non-cannabinoidi, come palmitoiletanolamide e oleiletanolamide, sottolineano la complessità del sistema endocannabinoide e i pathways ad esso interconnessi.

Chapter 1

General Introduction

1.1 The endocannabinoid system at a glance: from discovery to therapeutic potential

The discovery of the endocannabinoid system (ECS) as an endogenous neuromodulatory system is relatively recent. The historical and scientific journey that ultimately led to the birth of the endocannabinoid research field began in Israel in 1964, when Mechoulam and Gaoni established the structure of the major psychoactive phytocannabinoid, tetrahydrocannabinol (THC) (Gaoni & Mechoulam, 1964). Following the discovery of cannabinoid receptors and their endogenous ligands, the ECS emerged as a multifaceted and widespread system that plays a role in affective and cognitive functions, as well as psychophysiological and metabolic disorders, and may be the target of various therapeutic interventions. Today, the ECS is known to be made up of cannabinoid receptors, their endogenous ligands (the endocannabinoids) and the associated synthetizing and catabolic enzymes. Unlike other neurotransmitters, endocannabinoids, the arachidonoyl ethanolamide (anandamide- AEA) and 2-arachidonoyl glycerol (2-AG), are released on demand from membrane phospholipidic precursors after one or two rapid enzymatic steps, in response to a depolarization-induced increase in intracellular calcium or activation of various metabotropic receptors (Pacher et al., 2006). Although both 2-AG and AEA derive from arachidonic acid, their synthetic pathways are very different from one another. Multiple routes of synthesis have been described for AEA, but the most studied pathways are the hydrolysis of the N-acyl-phosphatidylethanolamine (NAPE) precursor through the N-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD) enzyme or the cleavage of the NAPE phosphodiester bond by a phospholipase C (PLC) (Lu & MacKie, 2016). 2-AG synthesis mainly occurs after sequential hydrolysis of an inositol bisphosphate with an arachidonoyl group, followed by the hydrolysis of the resultant diacylglycerol by a diacylglycerol lipase (DAGL) enzyme (Murataeva et al., 2014).

Endocannabinoids primarily exert their effects by engaging CB1 and CB2 G protein-coupled receptors. Cannabinoid receptors, once activated, block adenyl cyclase and activate voltage-dependent calcium channels or potassium channels (Howlett et al., 2002). CB1 are mainly located in the central nervous system (CNS), particularly in the hippocampus and basal ganglia, suggesting a crucial role in conditions related to mood disorders and altered brain reward mechanisms (Kendall & Yudowski, 2017). This receptor subtype is present in several type of neurons, especially in the presynaptic compartment, but their glial expression has

also been described (Stella, 2010). The signalling mediated by CB1 receptor has been implied in cognition (Bilkei-Gorzo et al., 2005), motor function (Polissidis et al., 2013), memory (Akirav, 2011) and nociception in the CNS (Milligan et al., 2020). Nevertheless, CB1 receptors are also abundantly expressed in peripheral nerves as well as in peripheral organs, although at lower concentrations than the brain (O'Sullivan et al., 2021). CB2 receptors were first identified in macrophages of the spleen (Munro et al., 1993). They are mainly located in immune and microglial cells (with a moderate expression in other peripheral tissues) and several pieces of evidence suggest that one of the major functions of CB2 receptors is immunomodulation (Buckley et al., 2000). The activation of this subtype has been linked to anti-inflammatory effects and CB2 agonists have been studied as therapeutic tools for the treatment of some autoimmune diseases (Zhu et al., 2019). CB2 was formerly referred to as the "peripheral CB receptor" since its presence in the brain had not been detected. However, subsequent research has shown that this subtype is also expressed in the CNS, where it plays an important role in nociception and neuroinflammation (Jhaveri et al., 2007; Komorowska-Müller & Schmöle, 2021).

Multiple lines of evidence point to endocannabinoids mainly acting as retrograde messengers in the CNS, mediating a variety of inhibition phenomena (e.g., long-term inhibition or depolarization-induced suppression of excitation/inhibition) and/or synaptic plasticity (metabotropic-induced suppression of excitation/inhibition) (Lu & MacKie, 2016). These effects are primarily coordinated by CB1 receptors, while the contribution of CB2-mediated signalling in synaptic transmission remains largely undefined. Several studies have suggested a role of CB2 in the modulation of neuronal activity, through the activation of K^+ (Ma et al., 2019) or Cl^- (Den Boon et al., 2012) channels. Anyway, the modulatory activity of endocannabinoids is not restricted to the central or peripheral nervous system, as evidenced by the widespread expression of the CB receptors in the rest of the body. For instance, CB receptors are highly expressed in enterocytes, enteroendocrine and immune cells of the intestinal mucosa (Mackie, 2008) and endocannabinoid signalling contributes to the regulation of gut mobility and permeability, as well as to the control of food intake and energy balance (Dipatrizio, 2016).

CB receptors are also present in the cardiovascular system and there are several lines of evidence demonstrating the involvement of ECS in cardiovascular function (O'Sullivan, 2015). Specifically, CB1 and CB2 activation exert opposing effects, which are also context dependent. For example, under pathological conditions, CB2 activation decreases the pro-inflammatory and fibrotic cardiac response (Steffens & Pacher, 2012), while CB1 seems to promote cardiovascular injury (Maccarrone et al., 2015). Another example of the peripheral endocannabinoid activity comes from the muscular tissue, where the ECS was found to play a fundamental role in skeletal muscle formation (Iannotti et al., 2014).

AEA and 2-AG signals are mainly terminated by fatty acid amide hydrolase (FAAH) and monoacyl glycerol lipase (MAGL), respectively (Fowler, 2012; Labar & Michaux, 2007). FAAH catalyses the hydrolysis of AEA in arachidonic acid and ethanolamine, while MAGL catalyses the hydrolysis of 2-AG in arachidonic acid and glycerol (Basavarajappa, 2007). This thesis will specifically focus on FAAH, which, as its name suggests, is capable of catabolizing various fatty acid amides, with crucial implications for possible treatment techniques. In fact, FAAH can boost the levels of numerous bioactive fatty acid ethanolamides (FAEs), including N-palmitoylethanolamide (PEA) and N-oleoylethanolamide (OEA).

1.2 Beyond the basics: the expanding endocannabinoid system and the rise of fatty acid ethanolamides

Numerous investigations have demonstrated that the ECS is confounded by the promiscuity of its mediators as well as the overlap with other pathways and alternative metabolic processes (Cristino et al., 2019). This led to the expansion of the ECS, which now includes not only the "canonical" members (outlined in the previous paragraph) but also a wider endocannabinoid-related network (Figures 1 and 2). Starting with the targets, endocannabinoids can activate some non-cannabinoid receptors, such as the transient receptor potential vanilloid 1 (TRPV1), which was shown to mediate the anti-nociceptive effect of AEA at the spinal level (Horvath et al., 2008) and can be also activated by 2-AG (Zygmunt et al., 2013), or receptors belonging to the peroxisome proliferator-activated receptors (PPAR) family (Iannotti & Vitale, 2021). Several works have demonstrated the role of TRPV1 in higher functions, such as the regulation of fear (Iglesias et al., 2023) and memory (Cui et al., 2018). PPAR receptors are expressed both in neurons and glial cells (astrocytes and microglia) and they have an immunomodulatory (Youssef & Badr, 2004) and neuroprotective (Bordet et al., 2006) function. The complexity of the expanded ECS also derives from the redundancy of multiple endocannabinoid biosynthetic and catabolic routes (Figures 1 and 2). For example, AEA and 2-AG can both be degraded via oxidation by cyclooxygenase-2 (COX-2) or lipoxygenases (LOXs) or cytochrome P450 (Fezza et al., 2014). Lastly, the main catabolic enzymes, such as FAAH and MAGL, can also metabolize other bioactive lipids belonging to the same chemical families of AEA and 2-AG. Consequently, inhibiting the primary catabolic enzymes may not necessarily increase, in a selective and/or effective way, only tissue levels of AEA or 2-AG, but may also influence the levels of other bioactive congeners of endocannabinoids. In fact, AEA is a member of the Fatty Acid Ethanolamides (FAE) family of endogenous lipids, which include some other bioactive molecules. Among the most intriguing ones, there are the “satiety factor” OEA and the “immunomodulatory” PEA. Their activity often depends on engagement of PPARs and TRPV1 receptors (Ambrosino et al., 2013; Fu et al., 2003).

OEA is widely known for its modulatory role in feeding behaviour. The biosynthesis of this lipid was first shown to be markedly reduced in the small intestine of rats after food-deprivation (Fu et al., 2003). Administration of OEA in rodents causes a potent and persistent anorexic effect and is associated with the

activation of several brain regions, such as the paraventricular hypothalamic nucleus, which is involved in the control of satiety (Fu et al., 2003; Rodríguez De Fonseca et al., 2001). OEA can be produced from oleic acid in the enterocytes of the gut (Igarashi et al., 2023), where it also regulates lipid metabolism (Pan et al., 2018), intestinal motility (Cluny et al., 2009) and inflammation (Lama et al., 2020). Recently, a clinical trial on obese patients found that OEA supplementation reduced serum levels of inflammatory markers (IL-6 and TNF-α) and fat mass and was associated with a significant increase of *Akkermansia muciniphila*, a commensal bacterium known for its beneficial role in maintaining gut homeostasis, strongly suggesting a link between OEA and gut microbiota (Payahoo et al., 2019). Accordingly, Di Paola et al. (2018) demonstrated that OEA sub-chronic treatment in mice was associated with the modulation of the gut microbiota. Furthermore, a recent work has demonstrated that OEA can be generated by gut commensal bacteria and can activate TRPV1 receptors on sensory neurons, increasing dopamine levels in the ventral striatum during exercise (Dohnalová et al., 2022). According to this study, the interplay between FAEs and microbiota in the gut may play a role in the modulation of motivation, a complex cognitive and behavioral phenomenon.

Within FAEs, great attention has been also paid to PEA, due to its multifaceted effects: among them, the most studied and recognized is immunomodulation. Indeed, more than 3500 peer-reviewed articles have already reported the beneficial effects of this FAE on immune function (Clayton et al., 2021). The immunomodulatory actions of PEA take place via numerous mechanisms, including regulation of mast cells degranulation, cytokine production (Cerrato et al., 2010) and macrophage migration (Guida et al., 2017b). Thanks to this, PEA also exerts a neuroprotective function, regulating microglia (D'aloia et al., 2021) and astrocytes activation (Beggiato et al., 2018). Other neuroprotective mechanisms mediated by PEA include reduction of apoptosis (D'Agostino et al., 2012), via Bax/Bcl-2 (Caltagirone et al., 2016) or Akt/mTOR/P70S6k (Cordaro et al., 2016) pathways, and increase of neurogenesis (Cristiano et al., 2018). All these actions can underlie PEA's beneficial effects in neurological disorders such as Alzheimer's disease (Bronzuoli et al., 2018; Scuderi et al., 2018), Parkinson's disease (Esposito et al., 2012) and stroke (Caltagirone et al., 2016; Kong et al., 2021). Similar to OEA, PEA plays a regulatory role in the gut, maintaining gut barrier and microbiota "homeostasis" (Cristiano et al., 2018; D'Antongiovanni et al., 2021). These abilities may have an impact on

CNS physiology, making PEA a putative modulator of the so-called “gut-brain axis”. Two other intriguing yet relatively lesser-known FAEs are N-stearoylethanolamine (SEA) and N-linoleoylethanolamine (LEA). SEA is a cannabimimetic compound (even if it does not bind to CB receptor) with anti-inflammatory properties (Berdyshev et al., 2015; Lykhmus et al., 2017), which was also shown to restore pancreatic lipid composition in an insulin-resistant rat model (Onopchenko et al., 2014). In rats with ischemic stroke, LEA has been proved to have some favorable effects, such as reduced cortical infarct volume and improved functional outcome (Garg et al., 2011).

Considering the abundant evidence pointing to the multifaceted biological functions of FAEs, it becomes evident that research endeavours should not solely focus on unravelling the actions of canonical endocannabinoids (i.e., AEA and 2-AG). Rather, it is essential to extend investigations to encompass the study of the entire FAE family, as these bioactive molecules have remarkable potential.

As research on the ECS continues to expand, it is crucial to broaden our perspective beyond the traditional focus on endocannabinoids and acknowledge the emerging significance of endocannabinoid-like compounds. While investigations in this field are still in their nascent stages, it is essential to delve deeper into understanding the intricacies of this complex system to fully grasp its potential for therapeutic interventions.

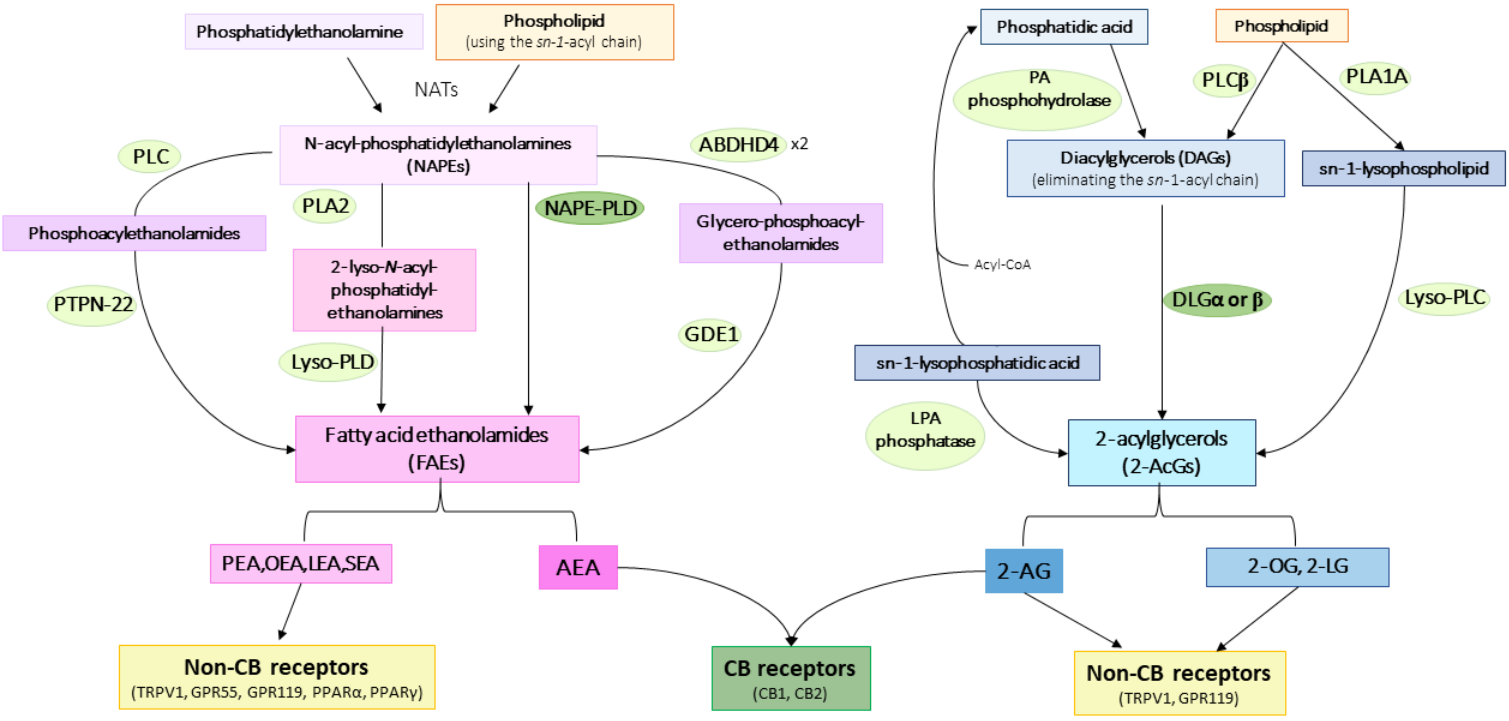

Figure 1. Biosynthetic routes of the expanded ECS. N-acyl-phosphatidylethanolamine-phospholipase D (NAPE-PLD) and diacylglycerol lipase α (DGLα) or DGLβ are considered the main biosynthetic enzymes for fatty acid ethanolamides (FAEs) and 2-acylglycerols (2-AcGs), respectively. However, numerous other potential anabolic pathways exist. Although FAEs, such as palmitoyl-ethanolamide (PEA), oleyl-ethanolamide (OEA), linoleyl-ethanolamide (LEA) and stearoyl-ethanolamide (SEA), share the same biosynthetic pathways of anandamide (AEA), they do not bind to cannabinoid (CB) receptors, but they exert interesting biological functions through the activation of other receptors. Similarly, 2-oleyl-glycerol (2-OG) and 2-linoleoylglycerol (2-LG) do not bind to CB receptors as 2-arachidonoylglycerol (2-AG). ABHD4= α/β hydrolase 4; GDE1=glycerophosphodiester phosphodiesterase 1; Lyso-PLC= Lyso-phospholipase C; Lyso-PLD= Lyso-phospholipase D; NATs=N-acyltransferases; PA=Phosphatidic Acid; PLA1A= phospholipase A1, member A; PLA2= phospholipase A2; PLC= phospholipase C; PTPN22= protein tyrosine phosphatase non-receptor type 22.

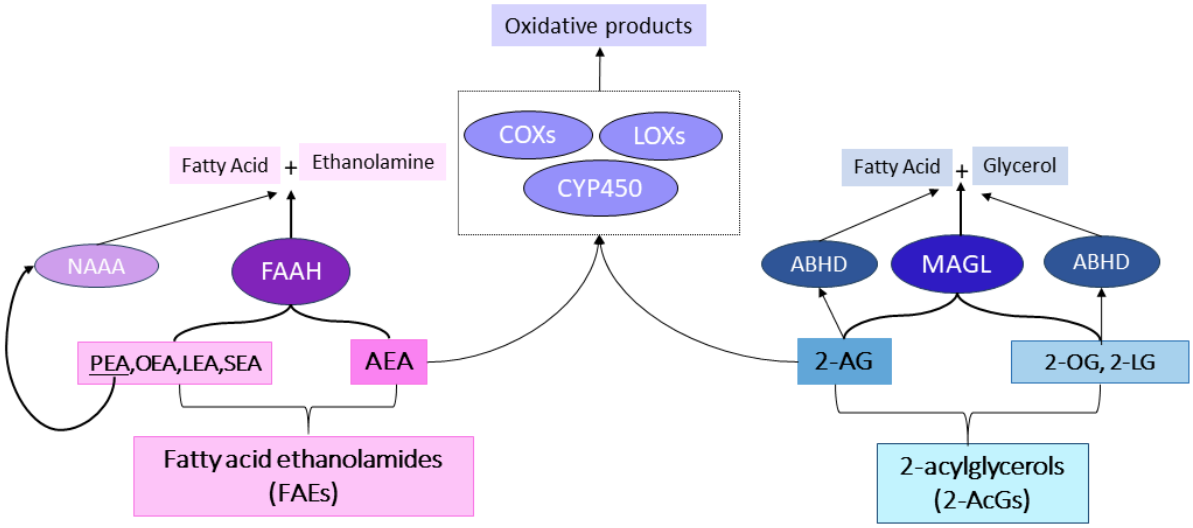

Figure 2. Catabolic routes of the expanded endocannabinoid system. Fatty acid amid hydrolase (FAAH) and monoacylglycerol lipase (MAGL) are considered the primary degrading enzymes for fatty acid ethanolamides (FAEs) and 2-acylglycerols (2-AcGs), respectively. However, FAEs and 2-AcGs can be degraded via alternative pathways. Both endocannabinoids (AEA and 2-AG) share many catabolic routes with the non-CB member of their chemical families (FAEs and 2-AcGs respectively). ABHD= α/β hydrolase; COX= cyclo-oxygenase; CYP450=cytochrome P450, LOX= lipo-oxygenase; NAAA= N-acylethanolamine-hydrolyzing acid amidase.

1.3 Implication of the ECS in stress-related psychopathologies and the emergent significance of non-cannabinoid fatty acid ethanolamides

The ECS plays a key role in the modulation of the stress response, which has evolved in order to maintain homeostasis, threatened by intrinsic or extrinsic adverse forces (the so called “stressors”). The stress response is a complex interplay of various mechanisms, akin to an orchestra, involving the activation of the hypothalamus-pituitary-adrenal (HPA) axis and the sympathetic-adreno-medullary (SAM) system, which interact with several areas of the CNS and tissues/organs in the periphery to mobilize a successful adaptive response against stressor(s) (Koolhaas et al., 2011). Dysregulation of the stress response in association with potent and/or chronic stress exposure can markedly disrupt body homeostasis, increasing the risk of developing different kind of pathologies. Among psychopathologies, stress is a major risk factor for the development and/or exacerbation of depression, anxiety and post-traumatic stress disorder (PTSD) (Daviu et al., 2019; Tafet & Nemeroff, 2016; Vinkers et al., 2014).

Different lines of evidence have demonstrated a link between stress response and the ECS, suggesting a regulatory function of the latter. In fact, the ECS plays a pivotal role in attenuating HPA axis activation during stress exposure and facilitating the negative feedback of glucocorticoids on the axis (Hillard et al., 2016). This is crucial for the effective deactivation of the stress response, which is often compromised in pathological conditions (Mikulska et al., 2021). Accordingly, blockade of CB1 has been reported to increase both basal and stress-induced levels of plasmatic corticosterone in rodents (Barna et al., 2004; Steiner & Wotjak, 2008; Wade et al., 2006). Furthermore, CB1 receptors are expressed on the terminals of sympathetic neurons and adrenal medullary cells, and CB1 agonists limit the release of norepinephrine and epinephrine from these cells, respectively (Niederhoffer et al., 2001; Pfitzer et al., 2005). Interestingly, the signalling mediated by CB1 in these cells is essential for the modulation of memory consolidation after exposure to a stressful condition (Busquets-Garcia et al., 2016).

Several studies have shown that endocannabinoids levels are altered in several animal models of stress. Generally, AEA levels have been shown to decrease after stress exposure in several limbic structures, such as the hippocampus and amygdala (Dubreucq et al., 2012; Hill et al., 2008; Rademacher et al., 2008), while the majority of studies describes a stress-induced increase in 2-AG brain levels (Hill et al., 2004; Wang et al., 2012). Decrease in AEA levels are partly justified by studies showing a significant increase of FAAH activity in animals exposed to different stress paradigms (Navarria et al., 2014; Patel et al., 2009).

Collectively, all these findings strongly support the functional role of the ECS as a "stress management system" (Deroon-Cassini et al., 2020). In fact, the ECS has been proposed to be critical for stress adaptation: such response is required to avoid all the adverse physiological and behavioral implications of chronic activation of the stress response, making the ECS an appealing target for therapeutic interventions (Deroon-Cassini et al., 2020; Carnevali et al. 2017). In fact, given the significant impact of stress in the development of psychopathologies, extensive evidence has consistently shown the involvement of the ECS in depression, anxiety and PTSD.

ECS and Depression

Depression is a complex and varied psychological condition that encompasses a wide range of clinical manifestations (Gorzalka & Hill, 2011). This pathology is characterized by a great heterogeneity, with numerous distinct phenotypes observed among individuals experiencing depressive symptoms. In fact, the Diagnostic and Statistical Manual of Mental Disorders (DSM-5®), a widely accepted classification system, acknowledges the existence of at least 256 different symptom presentations that can qualify as Major Depressive Disorder (MDD) (Buch & Liston, 2021). The symptoms of depression can differ significantly from person to person, making it challenging to pinpoint a universally shared experience. However, some common symptoms often experienced by patients with depression include persistent feelings of sadness, fatigue and loss of interest or pleasure in activities they once enjoyed (anhedonia), leading to a general lack of motivation (American Psychiatric Association, 2013).

Multiple lines of evidence have suggested that perturbations of ECS-mediated signalling are associated with depressive phenotypes, both at a preclinical and clinical level. For example, mice with a genetic deletion of CB1 displayed an increased response to stress, associated with depressive-like behavioral symptoms, reduced adult neurogenesis and BDNF levels (Aso et al., 2008; Jin et al., 2004; Steiner et al., 2008). Moreover, pharmacological blockade of CB1 in rodents was able to provoke alterations of reward sensitivity (Sanchis-Segura et al., 2004) and increased perseverance of emotionally aversive memories (Plendl & Wotjak, 2010). Interestingly, chronic stress paradigms – widely used to generate a depressive phenotype in preclinical models - induce reductions of brain levels of AEA and CB1 receptors in subcortical structures involved in the stress response, such as the hippocampus and amygdala (Gray et al., 2015; Hill et al., 2005).

The involvement of the ECS in depression was inadvertently suggested at a clinical level by Rimonabant, a CB1 antagonist, which was initially intended as an anti-obesity drug, but was withdrawn from the market after 2 years from its release because of adverse psychiatric effects. Indeed, the use of this drug was associated to a high incidence of depressive symptoms and suicidal ideation in patients taking Rimonabant compared to those taking placebo (Hill et al., 2009a; Nissen et al., 2008).

Accordingly, single nucleotide polymorphisms in CB1 receptor are associated to an increased vulnerability to develop a depressive episode after stress exposure (Juhasz et al., 2009) and to increased risk of antidepressants resistance (Domschke et al., 2008). Moreover, two independent populations of patients with Major Depressive Disorder have shown reduced plasmatic levels of endocannabinoids (Hill et al., 2008, 2009b). Lastly, CB1-mediated signalling has been shown to be involved in serotonergic neurotransmission (Bambico et al., 2007; Tzavara et al., 2003) and in the modulation of HPA axis activity, which is dysregulated in depressive disorders (Cota et al., 2007; Newsom et al., 2020; Patel et al., 2004). Specifically, these studies indicate a negative modulatory role on HPA axis activity by the ECS, suggesting once again its therapeutic potential for treating depression.

Several studies in rodent models have demonstrated that stress-induced depression is associated with altered FAAH expression. For instance, rats of both sexes exposed to chronic mild stress displayed increased FAAH protein levels in the hippocampus (Marco et al., 2014), while a different study has reported an increase in FAAH gene expression in the frontal cortex of male rats and in the hippocampus of female rats exposed to maternal deprivation (Reich et al., 2009).

This body of evidence points to a putative role of the ECS in the pathogenesis of depression. Consequently, a potentiation of ECS-mediated neurotransmission may be a useful strategy to produce antidepressant effects. Indeed, several studies have demonstrated the positive effects of the “endocannabinoid tone” potentiation. For instance, pharmacological enhancement of endocannabinoid signalling has been shown to have an antidepressant-like effect in several rodent studies. CB1 activation has been demonstrated to induce a reduction of passive coping behaviours in the forced swim test (Adamczyk et al., 2008; Hill et al., 2005) and the same result has been obtained with inhibition of endocannabinoids catabolism in both female (Carnevali et al., 2020) and male rodents (Tejeda-Martínez et al., 2021). In addition, facilitation of endocannabinoid neurotransmission dampens stress-induced activation of the HPA axis (Patel et al., 2004) and increases neurogenesis (Tejeda-Martínez et al., 2021).

This antidepressant capacity is supported by the fact that AEA inhibits monoamine oxidase enzymatic activity, thus increasing the levels of monoamines (Fišar, 2010), which represent a "biochemical signature" of

antidepressant efficacy. AEA was also shown to inhibit the synaptic reuptake of serotonin, mimicking the mechanism of action of selective serotonin reuptake inhibitors (SSRIs) (Velenovská & Fišar, 2007).

Moreover, the close interplay between the ECS and antidepressant activity is further proved by the fact that some conventional antidepressant drugs enhance endocannabinoid signalling in specific brain regions. For example, chronic administration of desipramine (a tricyclic antidepressant, TCA) has been reported to increase CB1 receptor binding in the hippocampus (Hill et al., 2006) of treated rats, while chronic treatment of imipramine, tianeptine (TCA) and escitalopram (SSRI) increased endocannabinoids levels in several limbic regions (Smaga et al., 2014).

ECS and Anxiety

Anxiety is considered an adaptive element of the acute stress response when faced with circumstances that pose a threat to the homeostasis of the body (Viveros et al., 2005). Anxiety-related responses, such as tachycardia, avoidance and hypervigilance are thus considered important for survival (Lutz et al., 2015). Nevertheless, if these responses become excessively intense or prolonged, or if they occur without an actual threat, they can signify a maladaptive reaction or even a psychopathological condition (Viveros et al., 2005). Multiple lines of evidence have demonstrated the involvement of the ECS in the modulation of anxiety-related responses, suggesting this system as a new pharmacological target to treat anxiety-related disorders. Pharmacological studies have described the biphasic regulation of anxiety circuits and related behaviors by CB1 receptors. Indeed, it is known that administration of exogenous cannabinoids, through CB1-mediated mechanisms, exhibits an anxiogenic effect at high doses and an anxiolytic effect at low doses (Rubino et al., 2008). The proposed underlying mechanism implicates CB1-expressing glutamatergic cortical neurons in anxiogenesis and CB1 receptors on forebrain GABAergic neurons in the anxiolytic effect (Rey et al., 2012). Furthermore, CB2 receptors have been also implied in anxiety, even if the subjacent mechanisms are still elusive. In fact, Garcia-Gutierrez and colleagues have demonstrated that chronic blockade of CB2 in mice exerts an anxiolytic effect through modulation of GABAa receptors (García-Gutiérrez et al., 2012). The same

group has also shown that mice overexpressing CB2 receptors are more vulnerable to anxiety and resistant to anxiolytic effects of benzodiazepines (García-Gutiérrez & Manzanares, 2011).

Another line of evidence confirming the important role of ECS in anxiety comes from studies demonstrating that genetic/pharmacological manipulation of endocannabinoids synthesis or degradation can alter anxiety-related behaviors in preclinical tests. For instance, genetic deletion or pharmacological inhibition of FAAH resulted in the reduction of anxiety-like behaviors in the light-dark box test and elevated plus maze test (Kathuria et al., 2003; Marco et al., 2015; Scherma et al., 2008). Similar results were obtained for 2-AG signalling: reduced brain levels of 2-AG were reported to increase anxiety-like behaviors in mice, which were rescued with the pharmacological inhibition of the MAGL enzyme (Shonesy et al., 2014).

In concert, the presented evidence strongly supports the notion that the ECS holds significant potential as a captivating target for the treatment of anxiety-like behaviors. However, it is crucial to acknowledge that further research is warranted to deepen our understanding and establish more conclusive evidence regarding the beneficial effects of targeting the expanded ECS for the treatment of anxiety-related disorders.

ECS and PTSD

Post-Traumatic Stress Disorder (PTSD) is an anxiety-related disorder that can develop after experiencing a traumatic event (Steardo et al., 2021). When confronted with a traumatic stressor, individuals may experience a maladaptive response characterized by specific symptoms. These symptoms encompass persistent intrusive thoughts associated with the traumatic event (caused by impairments in fear extinction processes), ongoing avoidance of stimuli linked to the trauma, as well as long-term changes in arousal and reactivity (Mayo et al., 2022; Steardo et al., 2021). These deficits are associated with decreased activation of the ventromedial prefrontal cortex (vmPFC) and hippocampus, which are involved in fear extinction processes, and with hyperactivity of amygdala, a brain region that is crucial for the activation of the threat response (Mayo et al., 2022). As for depression, the role of the ECS in PTSD has been studied in several preclinical and clinical studies.

In preclinical research, exposure to a single severe traumatic event (during childhood or adulthood) is a form of trauma frequently used as a potential model for PTSD in rodents (Nia et al., 2019).

Early life trauma, such as maternal separation, has been demonstrated to alter endocannabinoids and CB receptors hippocampal levels and behavioral responses (including depressive-like symptoms and alcool-drinking behavior) in rodents (Llorente et al., 2008; Portero-Tresserra et al., 2018; Suárez et al., 2009). Accordingly, adult rats exposed to foot-shock displayed decreased accumbal levels of AEA (Korem et al., 2017).

Some clinical studies have demonstrated alterations in the ECS in subjects diagnosed with PTSD, even if results are sometimes inconsistent. However, many confounding factors and/or different methodologies applied in these studies may in part explain these inconsistencies (Nia et al., 2019). For example, Hill and colleagues found reduced circulating levels of 2-AG in individuals exposed to the World Trade Center attack four to six years after the terroristic event (Hill et al., 2013), while a different study reported that patients with PTSD showed higher peripheral levels of AEA and 2-AG compared to healthy controls (Hauer et al., 2013). However, the population of the second study was characterized by high heterogeneity (different ethnicities and comorbidities with other pathologies) and a smaller sample size compared to the former.

Despite these divergences in the direction of endocannabinoids alterations in PTSD, preclinical and clinical studies agree on the therapeutic potential of ECS-mediated signaling enhancement for the treatment of PTSD symptomatology. Indeed, augmentation of AEA levels prevented the development of post-traumatic anxiety-like behavior in rats exposed to a predator odour (Danandeh et al., 2018) and inhibition of AEA catabolism or reuptake has been shown to facilitate fear extinction processes in rats exposed to contextual fear conditioning (Ahmad et al., 2020; Chhatwal et al., 2004). The same results have been obtained in CB1-deficient mice or mice treated with a CB1 antagonist, which have been reported to display impaired short-term and long-term extinction in auditory fear-conditioning tests (Marsicano et al., 2002; Soria-Gómez et al., 2015). The involvement of the ECS in modulation of acquisition and storage of aversive memories is also confirmed by genetic studies in humans, where individuals with the loss-of-function *FAAH-385A* allele displayed increased levels of AEA and enhanced fear extinction (Mayo et al., 2020a).

All these pieces of evidence unequivocally support the idea that the ECS may be a fascinating target for the treatment of stress-related psychopathologies. However, further research is still needed to deepen our understanding of the precise mechanisms and to establish the clinical efficacy and safety of ECS-targeted interventions for the disorders discussed in this paragraph.

Moreover, recent investigations have begun shedding light on the role of two other promising compounds, OEA and PEA, in stress-related psychopathologies. While research on these molecules in the context of psychopathologies is still in its early stages, preliminary findings have shown their beneficial effects. Thus, exploring the therapeutic potential of OEA and PEA in stress-related psychopathologies represents a novel and exciting avenue of investigation. For instance, some preclinical studies have demonstrated the anti-depressant capacity of PEA. Specifically, oral administration of PEA was reported to reduce typical depressive-like behaviors in the forced swim test and tail-suspension test in mice (Yu et al., 2011). Furthermore, in another murine model of depression (induced by chronic corticosterone treatment), PEA was able to reverse the depressive-like phenotype (Crupi et al., 2013). Other rodent studies have demonstrated the anti-depressant and anxiolytic effects of PEA in other pathological models, such as neuropathic pain or traumatic brain injury (Guida et al., 2017a; Guida et al., 2015). However, it is important to acknowledge that in these studies, distinguishing the antidepressant/anxiolytic effect of PEA from its well-known analgesic and anti-inflammatory properties can be challenging. Given the complex interplay between pain, inflammation and behavior, further investigations - specifically designed to disentangle these effects - are warranted.

In humans, a first clinical trial has shown the anti-depressant potential of PEA. Despite its evident limits (small sample size, predominant male sex and short follow-up period), this study has demonstrated the efficacy of PEA adjunctive therapy to the SSRI citalopram in improving symptoms in patients diagnosed with Major Depressive Disorder (Ghazizadeh-Hashemi et al., 2018). Furthermore, some studies have suggested the putative role of PEA as a “stress biomarker” (DeGregorio et al., 2019). Notably, Hill and colleagues discovered that women with Major Depressive Disorder exhibited significantly decreased serum levels of PEA compared to age-matched healthy controls following exposure to psychosocial stress, despite having similar basal

levels. Interestingly, aerobic exercise, which is known to improve mood (Chan et al., 2019), has been reported to considerably boost circulating levels of PEA (Brellenthi et al., 2017)

These promising findings strongly support further investigations on PEA potential role in depression and other psychopathologies. PEA may exert antidepressant/anxiolytic actions through different pathways, such as anti-inflammatory mechanisms - according to recent studies that suggest an inflammatory component in the pathophysiology of depression and PTSD (Eswarappa et al., 2019) - or through enhancement of neurogenesis (Bronzuoli et al., 2018; Facchinetti et al., 2020) and synaptic plasticity (Caltagirone et al., 2016). By elucidating the distinct contributions of PEA's various mechanisms of action, we could gain a more comprehensive understanding of its therapeutic potential in treating stress-related psychopathologies, while also addressing the intricacies of its multifaceted pharmacological profile.

Similar to PEA, OEA has also been reported to improve anhedonia, enhance reward sensitivity, and increase BDNF levels in a mouse model of chronic stress-induced depression (Jin et al., 2015). In a separate study, OEA oral administration in mice for 7 days resulted in reduced immobility time in the forced swim test and tail suspension tests, along with increased levels of brain monoamines (serotonin and norepinephrine) (Yu et al., 2015). Given its regulatory role in feeding behaviour and appetite (Fu et al., 2003; Laleh et al., 2018), which can often be dysregulated in stress-related psychopathologies (Coccurello, 2019; Simmons et al., 2016), OEA holds the potential to address these specific symptoms as well.

Furthermore, the implication of both PEA and OEA in stress-related psychopathologies, such as depression, is suggested by the fact that antidepressant treatment (e.g., imipramine) was reported to increase levels of these FAEs in several limbic regions of the rat brain, as also described for AEA (Smaga et al., 2014).

Lastly, but not less important, the involvement of both OEA and PEA in the regulation of microbiota homeostasis and gut integrity (Cristiano et al., 2018; D'Antongiovanni et al., 2021; De Filippo et al., 2023) may also suggest that the positive effects reported in the context of psychopathologies may derive from the modulation of the gut-brain axis, which is increasingly recognized as a key player in the pathophysiology of these types of diseases.

In conclusion, given the currently limited evidence, it is imperative that we delve deeper into understanding the implications of FAEs in psychopathologies. Importantly, peripheral mechanisms which can be influenced by FAE-mediated signalling (e.g., inflammatory processes and gut-brain axis), should be taken into consideration in this context. This is especially crucial if we aim to safely utilize ECS-targeted pharmacological tools, including FAAH inhibitors.

1.4 The promise of FAAH inhibitors in the treatment of stress-related psychopathologies: revitalizing hope

With the crucial role of the ECS in several pathological conditions becoming clearer, research has begun investigating different pharmacological strategies to promote the enhancement of its neuromodulation. AEA and 2-AG levels can be manipulated through the inhibition of their main catabolic enzymes, namely FAAH and MAGL. This thesis and the following paragraph will specifically focus on FAAH inhibition.

FAAH is a membrane-bound enzyme, belonging to the serine-hydrolase family, which is widely distributed throughout the body. In the rat, it was found in large quantities in the liver, followed by the small intestine, testes, uterus, kidneys and spleen, while skeletal muscles and the heart lack this enzyme (Papa et al., 2022; Tripathi, 2020). In fact, any activity reported in the heart probably derived from FAAH located in the endothelial cells lining the blood vessels (Maccarrone et al., 2000; Tripathi, 2020). In the brain, FAAH is mainly expressed in neurons, such as Purkinje cells in the cerebral cortex or pyramidal cells of the hippocampus (Papa et al., 2022). This enzyme is responsible for the degradation of different FAEs, such as AEA, PEA and OEA (Tuo et al., 2017). Consequently, FAAH inhibition increases endogenous concentration of all its substrates, prolonging and potentiating all their biological effects (Seierstad & Breitenbucher, 2008).

FAAH inhibitors development has made significant progress in recent decades. Numerous selective FAAH inhibitors belonging to various chemical classes have been created and synthesized, demonstrating substantial progress in this field (Tuo et al., 2017). Some examples include:

- α-ketoheterocycle derivatives (e.g., OL-135,(Boger et al., 2005))

- sulfonylfluoride inhibitors (e.g., AM3506, (Alapafuja et al., 2012))
- urea-based inhibitors (e.g., JNJ-40355003,(Keith et al., 2012))
- carbamates derivatives (e.g., URB597,(Kathuria et al., 2003))

Focusing on carbamates derivatives, the first class of aryl carbamate derivatives as FAAH inhibitors was developed 20 years ago, when URB597 was shown to block FAAH and boost brain AEA levels after a 0.3 mg/kg intraperitoneal injection in rats (Kathuria et al., 2003; Mor et al., 2004). This specific group of compounds was jointly developed by three universities (University of California at Irvine, University of Parma and University of Urbino "Carlo Bo") in collaboration with Kadmus Pharmaceuticals (Lodola et al., 2015). URB597 is a potent inhibitor of FAAH, able to block its activity by irreversible carbamoylation of the catalytic nucleophile Ser_{241} (Alexander & Cravatt, 2005). Despite its excellent selectivity for FAAH in the brain, without affecting other serine hydrolases, URB597 was found to interfere with the function of several liver carboxylesterases and had a short *in vivo* half-life, limiting its applicability in chronic studies (Lodola et al., 2015; Zhang et al., 2007). Therefore, a second-generation aryl carbamate based FAAH inhibitor, URB694, was developed to address these limitations. URB694 exhibited weaker affinity for hepatic carboxylesterases and demonstrated an increased *in vivo* half-life compared to URB597, while retaining the same potency in rats (Clapper et al., 2009). More recently, FAAH inhibitors with restricted access to the CNS were developed. Specifically, Clapper and colleagues modified the chemical structure of URB597 to create URB937, a CNS-impenetrant FAAH inhibitor designed to exclusively inhibit FAAH activity in peripheral tissues (Clapper et al., 2010). While URB937 was initially developed as an anti-nociceptive agent, its potential extends far beyond that application. In fact, this compound could represent a valuable tool for neuroscientific research, particularly in the context of stress-related disorders. The complex and not fully understood physiopathology of these disorders often involves dysfunction across multiple peripheral pathways, such as inflammation and the gut microbiota, which in turn can affect brain physiology (Haapakoski et al., 2016; Lee & Kim, 2021). By selectively targeting peripheral FAAH activity, URB937 offers researchers a means to investigate and unravel

the intricate interplay between these peripheral pathways and the CNS, as well as the peripheral contribution of the ECS.

Given the pleiotropic effects of the expanded ECS and the protective impact of its upregulation in various pathological conditions (Toczek & Malinowska, 2018), FAAH inhibitors have shown promise in alleviating symptoms of various diseases in animal models. These pathologies include inflammatory bowel disease (Shamran et al., 2017), hypertension (Godlewski et al., 2010), Parkinson's disease (Celorrio et al., 2016) and depression (Tejeda-Martínez et al., 2021). Indeed, pharmacological blockade of FAAH has been found to induce analgesic effects (Jayamanne et al., 2006), reduce anxiety (Duan et al., 2017) and exhibit anti-depressant properties (Bortolato et al., 2007).

In the context of stress-related psychiatric disorders, initial evidence from Kathuria and co-workers revealed that systemic FAAH inhibition (with URB597) exhibits anxiolytic effects in rats (Kathuria et al., 2003). Subsequently, an increasing number of studies explored the effects of FAAH inhibitors in animal models of stress-related psychopathologies, particularly depression. Inhibition of FAAH activity with URB597 was reported to increase active coping behaviors (Gobbi et al., 2005), which are generally associated with improved resilience to stress. URB597 also mitigated some chronic stress-induced depressive-like symptoms, such as reduction in sucrose solution intake (an indicator of anhedonia) (Bortolato et al., 2007). Additionally, URB597 treatment has been demonstrated to prevent impairments in social behavior in both male and female rats exposed to early life stress, such as maternal deprivation (Alteba et al., 2020). Similarly, prolonged treatment with the second-generation carbamate derivative URB694 was demonstrated to normalize body weight, sucrose preference and immobility time in the forced swim test in male Wistar Kyoto rats (which are predisposed to stress-related psychopathologies - (Redei et al., 2023)) exposed to chronic social defeat stress (Carnevali et al., 2015). These results were replicated in female rats of a different strain (Wild Type Groningen) exposed to six weeks of social isolation (Carnevali et al., 2020). Moreover, systemic FAAH inhibition has been shown to increase the firing rate of serotonin neurons in the dorsal raphe (Gobbi et al., 2005), as well as dopamine and serotonin levels in the hippocampal dentate gyrus (Kędziora et al., 2023) and promoted hippocampal neurogenesis, contributing to development of an "anti-depressant signature". These

beneficial effects induced by FAAH inhibitors are often accompanied by increased AEA levels in several brain regions, such as hippocampus (Carnevali et al., 2020; Gobbi et al., 2005), prefrontal cortex (Gobbi et al., 2005) and/or striatum (Bortolato et al., 2007). Based on the majority of preclinical findings in the current literature, the prefrontal cortex, hippocampus and striatum, which are highly implicated in stress-related psychiatric disorders (Liu et al., 2017) are the brain regions most influenced by FAAH inhibitors (Rafiei & Kolla, 2021). Dysregulated FAAH activity in these brain areas may explain certain depressive symptoms, such as loss of motivation, anhedonia and cognitive dysfunction. For example, altered ECS signalling can be linked to some typical impairments observed in patients with depression, such as sad mood, attention deficit and difficulties in decision-making (Lai, 2019).

FAAH inhibitors can be also exploited for the treatment of PTSD. Indeed, inhibition of FAAH has been shown to enhance fear extinction and improve long-term consolidation of extinction memories in rodents (Fidelman et al., 2018; Morena et al., 2018). The pharmacological blockade of FAAH can also dampen the stress-induced HPA axis activation (Kathuria et al., 2003; Morena et al., 2016), suppress the behavioural expression of learned fear (Llorente-Berzal et al., 2015) and reduce anxiety-like behaviour in response to a stressor (i.e., a predator odour) in rats (Fotio et al., 2023). Collectively, evidence from animal research strongly supports the utility of FAAH inhibitors in managing other stress-related psychopathologies, including PTSD, alongside depression.

Importantly, the beneficial effects of such indirect pharmacological strategy are not accompanied by the negative effects of an indiscriminate CB1 activation. Indeed, the use of CB1 exogenous agonists has been demonstrated to provoke some unwanted effects, such as hypothermia, catalepsy or hyperphagia (Anderson-Baker et al., 1979; Scopinho et al., 2011), as well as receptor desensitisation phenomena (Kouznetsova et al., 2002; Martin et al., 2004), physical and psychological dependence (Pertwee, 2014). On the other hand, repeated treatment with a FAAH inhibitor (PF3845) has been reported to not induce signs of physical dependence and desensitization of CB receptors in mice, while these effects have been described after chronic treatment with the MAGL inhibitor JZL184 (Schlosburg et al., 2010). Moreover, a different FAAH inhibitor (URB597) was shown to lack reinforcing properties in monkeys (Justinova et al., 2008). In addition,

URB597 did not promote reinstatement of extinguished drug-seeking behavior previously maintained by THC, AEA, or cocaine, which distinguishes FAAH inhibitors from direct-acting cannabinoid agonists (Justinova et al., 2008).

Positive findings from preclinical research encouraged the start of some clinical trials for the treatment of depression. So far, few clinical trials have been conducted to assess the effectiveness of FAAH inhitors in treating depression (Rafiei & Kolla, 2021). For instance, a phase II trial (NCT00822744) evaluated the efficacy of the FAAH inhibitor SSR411298 in elderly individuals diagnosed with Major Depressive Disorder. However, the results indicated that the compound used in this study did not lead to an improvement of depressive symptoms, which were assessed using the Hamilton Depression Rating Scale (Hamilton, 1960; Rafiei & Kolla, 2021). It is worth noting that FAAH inhibition may have shown more effectiveness in a younger population, as the nature of this psychopathology varies considerably between younger and older patients (Gottfries, 1998; Rice et al., 2019). As for the second clinical trial (NCT02498392), its aim was to assess the efficacy of the FAAH inhibitor JNJ-42165279 as adjunctive treatment compared to adjunctive placebo, in participants with Major Depressive Disorder accompanied by anxious distress and who had a suboptimal response to current treatment with a standard antidepressant, but results haven't been published yet.

Besides depression and other psychopathologies, several FAAH inhibitors are being tested in phase II clinical trials for several pathological conditions in order to treat some specific symptoms, such as osteoarthritic pain (PF04457845, Pfizer, USA; NCT00981357 (Huggins et al., 2012)), neuropathic pain induced by spinal cord injury (V158866, Vernalis, UK; NCT01748695) or diabetic peripheral neuropathy (ASP8477, Astellas Pharma Europe; NCT02065349).

However, despite the promising data from animal research, enthusiasm for FAAH inhibitors has somewhat waned after the disappointing results from some of these clinical studies, where FAAH inhibition showed lack of analgesic or antidepressant effects (Bradford et al., 2017; Huggins et al., 2012; NCT00822744). In addition, the tragic accident involving the FAAH inhibitor BIA10274 has raised suspicion on the safety of this pharmacological strategy. In fact, during a Phase I clinical trial for BIA102474 (January 2016), mild to severe neurologic adverse effects (e.g., headache, memory impairment, diplopia and sedation to coma) appeared

in five healthy participants and one person died three days after the onset of symptoms (Chaikin, 2017). Anomalies in the hippocampus, pons, thalamus and cerebral cortex were detected by magnetic resonance imaging in the deceased volunteer (Chaikin, 2017). These toxic effects were probably due to off-target effects, which are not linked to FAAH inhibition, as suggested by several hints. First, BIA102 474 shows a low specificity for FAAH and it is able to inhibit other serine hydrolases in the brain (Mallet et al., 2016; Toczek & Malinowska, 2018). Moreover, such severe side effects have not been observed with different FAAH inhibitors developed by other pharmaceuticals companies, such as Pfizer, Vernalis and Merck (Tuo et al., 2017). Indeed, a later study has shown that BIA102474 induced significant changes in the lipidic profile of human cortical neurons (Van Esbroeck et al., 2017). Anyway, excluding BIA10274, FAAH inhibitors are generally well tolerated in humans, without serious adverse events (Huggins et al., 2012; Mayo et al., 2020b; Postnov et al., 2018). Thus, FAAH can be still considered as a safe target for drug development. Moreover, recent results have provided preliminary human evidence that FAAH inhibition can improve the recall of fear extinction memories and attenuate the anxiogenic effects of stress in healthy participants (Mayo et al., 2020b); while in other recent study, treatment of subjects with social anxiety disorder with the FAAH inhibitor JNJ-42165279 was associated with moderate anxiolytic effects (Schmidt et al., 2021), revitalizing hope for this class of drugs.

Considering the great complexity of the expanded ECS, the effects of compounds enhancing "endocannabinoidome" tone may be multidirectional and not fully predictable (Di Marzo, 2020). Thus, the therapeutic potential of agents modulating the ECS, such as FAAH inhibitors, must be supported by profound preclinical evidence.

In the context of stress-related psychopathologies, research should investigate putative "non-conventional" pathways (both central and peripheral), that can be impacted by enhanced endocannabinoidome tone, such as the gut-brain axis, peripheral lipidomic profiles or inflammatory processes. Indeed, all these diverse pathways have been extensively implicated in psychopathologies, but the effects of FAAH inhibitors on them are not often considered.

Furthermore, FAAH inhibition's beneficial effects are generally mainly ascribed to increased AEA levels, but FAAH also degrades other bioactive FAEs (e.g., PEA and OEA), whose beneficial properties have been outlined in the previous paragraphs. Due to this promiscuity, the effects of FAAH inhibitors can be unpredictable at times and further preclinical research is needed, for a more conscious clinical use. Anyway, the lack of specificity of FAAH inhibitors is not necessarily negative, as bioactive FAEs exerts several functions that may be useful to treat some specific symptoms of psychopathologies. In fact, depressive or anxious disorders are characterized by a great clinical variability, and they can manifest as diverse phenotypes.

1.5 Aims and outlines of the thesis

As extensively discussed in the previous paragraphs, the potentiation of FAE-mediated signalling through FAAH inhibition holds promise within the context of stress-related psychopathologies. Beyond its impact on AEA levels, FAAH also modulates the levels of other beneficial FAEs, such as PEA and OEA. Consequently, the effects of FAAH inhibition may be intricate and warrant further preclinical scrutiny to ensure its informed clinical application. Moreover, given the broad impact of the enhancement of the expanded ECS on various pathways, including microbiota, lipidome and inflammation, this pharmacological strategy presents novel research avenues for stress-related psychopathologies. This approach acknowledges the complexity of depressive and anxious disorders, encompassing their varied clinical manifestations.

The core objective of this thesis is to test the effects of both systemic and peripheral FAAH inhibition in social stress preclinical models. The focus lies on diverse pathways that can be impacted by FAAH inhibitors, such as inflammatory processes, gut microbiota and lipidome.

Specifically, **Chapter 2** offers a thorough analysis of fecal samples' lipidomic and microbial profiles from male rats subjected to repeated social defeat stress and chronically treated with URB694, a systemic FAAH inhibitor. **Chapter 3** describes a study in which a peripherally restricted FAAH inhibitor (URB937) was used to explore the impact of potentiation of peripheral FAEs on behavioral and peripheral biochemical responses (with a focus on pro-inflammatory cytokines) in rats exposed to acute social stress. Chapter 3 also includes

an appendix (**Appendix A**), examining the impact of URB937 on heart rate variability, another crucial peripheral mediator, which has been implicated in stress resilience or vulnerability.

In both studies, a social defeat model was employed due to the profound ethological significance and strong translational relevance of this paradigm. Social defeat stress is based on a naturalistic stressor, involving agonistic interactions between two male counterparts, which inherently holds a social dimension. Notably, social stress constitutes one of the most prevalent stressors encountered in human daily life, exerting a more potent impact on individuals compared to other stressor types.

Finally, Chapter 4 provides a synthesis and brief discussion of the main findings.

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Chapter 2

Social stress-induced depressive-like symptoms and changes in gut microbial and lipidomic profiles are prevented by pharmacological inhibition of FAAH activity in male rats*

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Abstract

Accumulating preclinical evidence demonstrates that pharmacological inhibition of fatty acid amide hydrolase (FAAH) activity promotes behavioral resilience in various stress models. FAAH terminates signals of bioactive fatty acid ethanolamides (FAEs), including anandamide, oleoylethanolamide and palmitoylethanolamide. These FAEs have been implicated in the interplay between the central nervous system and both gut microbiota and lipidome. In this study, we investigated the effects of the FAAH inhibitor URB694 on the gut microbial and lipidic profiles of male rats exposed to chronic social defeat stress. Rats experienced five weeks of either social defeat or control procedures with URB694 (0.3 mg/kg/day, i.p.) or vehicle treatment starting from the third week. URB694 administration ameliorated stress-induced behavioral changes, including sucrose preference reduction and emergence of passive coping behaviors in the forced swim test, and prevented the increase in plasmatic corticosterone levels. Social stress was associated with significant modifications of gut microbial and lipidic profiles and URB694 attenuated these stress-induced changes. Our findings indicate that chronic social defeat stress induces depressive-like behavioral and neuroendocrine alterations in male rats, which can be effectively mitigated by URB694 treatment. Importantly, we provide initial evidence that URB694 may promote stability in gut microbial and lipidic profiles under conditions of social stress, suggesting that the gut microbial-lipidic crosstalk might contribute to the development of stress-related disorders and offering a novel biological target for FAAH inhibitors.

1. Introduction

Social stress is a common precipitating factor for the development and progression of several psychiatric disorders, such as anxiety and depression. Recent stressful events such as the COVID-19 pandemic, which caused a 25% increase in the prevalence of anxiety and depression during the first year (World Health Organization (WHO), 2022), have exacerbated the social and economic burden of these stress-related psychiatric disorders. Despite the increased availability of pharmacological treatments, we are witnessing a "treatment-prevalence paradox" which further highlights the need for a broader identification of biological targets for more effective interventions (Ormel et al., 2022).

In this context, accumulating preclinical evidence has shown that pharmacological inhibition of fatty acid amide hydrolase (FAAH) activity promotes behavioral resilience in acute and chronic stress models (Carnevali et al., 2020; Carnevali et al., 2015a,b; Wang & Zhang, 2017). FAAH is an integral membrane serine-hydrolase that terminates the signalling of the fatty acid ethanolamide (FAE) family of bioactive lipid mediators (Tripathi, 2020), which include endogenous agonists of cannabinoid receptors (e.g., anandamide, AEA) and of other signalling systems comprising orphan GPCRs, transient receptor potential channels and peroxisome proliferator-activated receptor-α (e.g., palmitoylethanolamide (PEA) and oleoylethanolamide (OEA)) (Fu et al., 2003; Lo Verme et al., 2005). Relatedly, pharmacological inhibition of FAAH activity has been found to enhance monoaminergic neurotransmission, to suppress stress-induced activation of the hypothalamic-pituitary-adrenal axis and to increase hippocampal neurogenesis in rodent models (Gobbi et al., 2005; Hill et al., 2009; Tejeda-Martínez et al., 2021). Through these mechanisms, FAAH inhibition may promote active coping strategies and adaptation to stress, dampening anxiety and regulating mood (Carnevali et al., 2017). Yet, the picture may be more complex and a greater understanding of the biological mechanisms through which FAAH inhibitors (e.g., URB597, URB694) promote behavioral stress adaptations is key for stimulating a more informed exploitation of this pharmacological strategy for the treatment of stress-related psychiatric disorders (Carnevali et al., 2017).

Several pieces of evidence have indicated that the FAE family may be involved in the crosstalk between the central nervous system and the gut microbiota (Caesar et al., 2015; Cristiano et al., 2018; Di Paola et al., 2018;

Geurts et al., 2015; Russo et al., 2018). Notably, the gut microbiota is thought to play a significant role in the pathophysiology of central nervous system-related diseases and recent studies report correlations between altered composition of the gut microbiota and depression. For example, the gut microbiota was found to be significantly altered in rodent models of stress-induced depression (Wu et al., 2021; Yang et al., 2021) and animals with antibiotic-induced dysbiosis were shown to exhibit depressive-like behaviors (Fan et al., 2022). Moreover, depressive-like behavioral symptoms have recently been associated with alterations in both the gut microbiota compostion and lipidomic profile in mice exposed to chronic social defeat stress (Gong et al., 2021). This preliminary finding supports the hypothesis that conditions of dysbiosis associated with lipid imbalance in the gut can lead to altered gut-brain axis signalling, which may contribute to the onset of central nervous system-related diseases (Russo et al., 2018). Contextually, the crosstalk between microbiota, lipids, and gut-brain axis may emerge as the basis of therapeutic strategies aimed at enhancing FAE-mediated signalling to counteract the development of stress-related psychiatric disorders (Baptista et al., 2020).

In the current study, we adopted a well-validated model of repeated social defeat stress in male rats and conducted a thorough analysis of the lipidomic and microbial profiles from fecal samples. Anticipating behavioral and neuroendocrine changes that are reminiscent of a depressive-like state in socially stressed rats, we tested the hypotheses that (i) depressive-like symptoms would be associated with changes in gut microbial and lipid composition, and that (ii) inhibition of FAAH activity with URB694 would promote behavioral stress resilience and preserve gut microbial and lipidomic profiles.

2. Materials and methods

2.1 Animals

Experiments were conducted on 3-month-old Wistar male rats (n=32), weighing 367±7 g at the beginning of the study, bred in the animal facility of the University of Parma. Experimental rats were kept in individual cages in climate-controlled rooms (temperature: 22±2°C, humidity: 50 to 60%), on a reversed 12:12 light-dark cycle (light on at 7pm), with *ad libitum* food and water. Additional older Wild-type Groningen male rats, weighing 500–600 g, were housed in a separate room with an oviduct-ligated female partner and used as residents in the resident-intruder paradigm as outlined below. Experimental procedures were performed in accordance with the European Community Council Directive 2010/63/UE and approved by the Italian legislation on animal experimentation (D.L. 04/04/2014, n. 26, authorization n. 276/2022-PR). Analyses were performed under blinded conditions, and sample size was not predetermined. The study report follows ARRIVE guidelines (Kilkenny et al., 2010).

2.2 Chemicals

URB694 (N-cyclohexyl-carbamic acid, 6-hydroxy[1,1'-biphenyl]-3-yl ester) was synthesized as previously described (Tarzia et al., 2006). The analytical standards of fatty acid ethanolamides, anandamide (AEA), oleoylethanolamide (OEA) and palmitoylethanolamide (PEA) and their deuterated analogs AEA-d4, OEA-d4 and PEA-d4 were purchased from Cayman Chemical (Ann Arbor, MI, USA) as stock solutions in ethanol. Corticosterone and dexamethasone were provided by Sigma-Aldrich (Milan, Italy). LC-MS grade solvents acetonitrile (MeCN), isopropanol (IPA) and methanol (MeOH) were purchased from Scharlab (Barcelona, Spain). All other reagents were employed in the highest available grade and purchased from Sigma-Aldrich (Milan, Italy).

2.3 General experimental protocol

The experimental protocol was similar to that adopted in a previous experiment conducted by our group (Carnevali et al., 2015a). Briefly, after baseline determinations, rats were randomly assigned to a 5-week social stress (STR) protocol or a control (CTR) procedure. Starting from the beginning of the third week, STR and CTR rats were given daily injections of the globally active FAAH inhibitor URB694 (URB, 0.3 mg/kg) or vehicle (VEH) (Figure 1). Specific experimental procedures are outlined below.

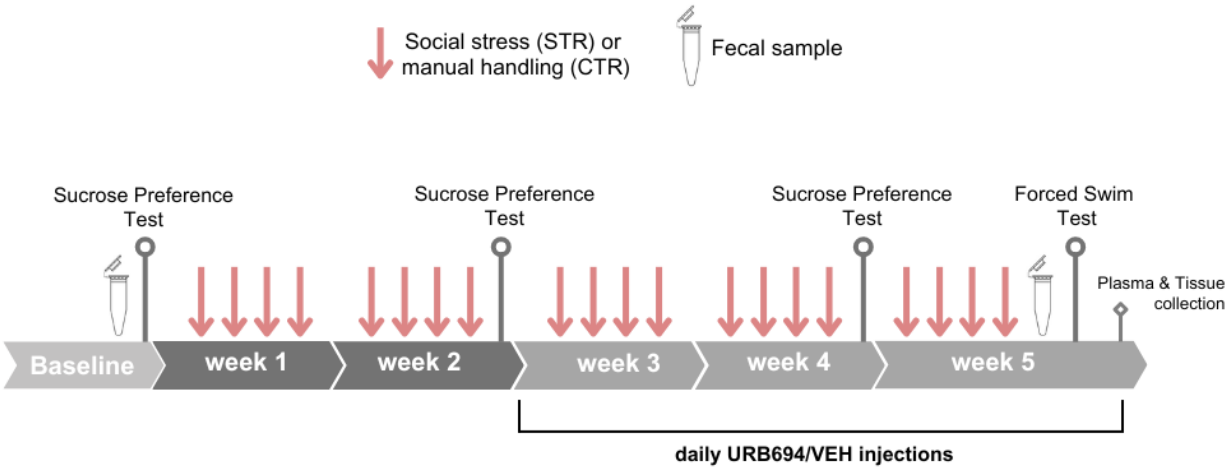

Figure 1. Timeline of experimental procedures

2.4 Social stress

The social stress protocol consisted of repeated exposures to social defeat, which was based on the traditional resident-intruder paradigm (Koolhaas et al., 2013). The resident Wild-type Groningen male rats were screened for their aggressive behavior before the beginning of the procedure. Initially, the female partners of the resident rats were removed from the cages. Each rat from STR group ("intruder") was then introduced into the cage of a resident rat, with a wire-mesh partition separating the two animals. After 20 minutes, the partition was removed allowing physical interaction for the remaining 10 minutes of the paradigm. During this phase, the resident rats repeatedly threatened and attacked the intruders, which typically exhibited behavioral signs (e.g., supine posture) of social defeat and subordination. STR rats were exposed to 4 daily sessions of social defeat stress every week (from week 1 to week 5), for a total of 20 social

defeat stress exposures. On the same days, but in a different room, CTR rats were handled briefly (2 min) by the same experimenter. After the STR/CTR procedure, which took place between 9am and 11am, all rats were returned to their home cages. STR rats were closely inspected after each social defeat episode and none of them reported any improper injury or wound.

2.5 Drug preparation and treatment

URB694 is a second-generation FAAH inhibitor with improved metabolic stability and selectivity for FAAH. More specifically, it is a carbamate that irreversibly carbamoylates the nucleophile catalytic serine in FAAH active site (Clapper et al., 2009). Each day, URB694 was freshly dissolved in vehicle (VEH) containing 5% Tween 80, 5% PEG and 90% saline. The mixture was vigorously vortexed and sonicated for 10 min in a warm bath to obtain a clear solution. URB694 and VEH were administered by intraperitoneal (IP) injection in a volume of 1 ml/kg. Starting from the beginning of the third week of the STR/CTR procedure, rats were injected daily with URB694 (0.3 mg/kg) or VEH, between 2pm and 3pm. The dose of URB694 was determined based on available data in the literature (Clapper et al., 2009) and our experience with acute and chronic treatments with URB694 in rats (Carnevali et al., 2020; Carnevali et al., 2015a,b).

2.6 Fecal sample collection

Fresh fecal samples were collected from the home cage of STR and CTR rats and stored at -20°C until analysis. Fecal samples were collected in baseline conditions and at the end of the experimental protocol (week 5). All samples were collected between 8h30am and 9h30am. Cage was changed the evening before fecal collection, between 6 and 7pm.

2.7 Sucrose Preference Test

The sucrose preference test was based on a two-bottle testing paradigm and was performed in baseline conditions and at the end of the second and fourth week of the STR/CTR procedure. Rats were food and water deprived for 15 hours prior to each test. Subsequently, they were given access to two drinking bottles (one containing 2% sucrose in tap water and the other only tap water) for an hour. Bottles were weighed immediately before and after the test to evaluate water and sucrose solution intakes. Sucrose preference was calculated as the percentage of sucrose solution intake over the total amount of liquid intake.

2.8 Forced Swim Test

Twenty-four hours after the last STR/CTR procedure, rats were tested in an adapted version of the forced swim test, originally described by Porsolt (Porsolt et al., 1978). Experimental rats were individually placed in a Plexiglas cylinder (height: 40 cm, diameter: 30 cm) filled with water (temperature: 24 ± 2 °C; depth: 30 cm) for 5 minutes and the rat's behaviour was video-recorded. A trained experimenter blind to animals' condition and treatment scored the overall time spent in immobility (floating and making only those movements necessary to keep the head above water) and the latency to the display of the first immobility.

2.9 Measurements at sacrifice

Rats were sacrificed by decapitation under isoflurane anaesthesia (2% in 100% oxygen) seventy-two hours after the last STR/CTR procedure and twenty-four hours after the last injection of URB694 or VEH. Trunk blood was collected in EDTA tubes (Sarsted AG, Numbrecht, Germany) and plasma was separated by centrifugation (4°C, 2600 g, 15 min). Brains were removed and hippocampus, striate nucleus and prefrontal cortex (PFC) were dissected. All samples were then stored at -80 °C until further analyses, as described below. Finally, adrenal glands were harvested and weighted.

2.10 Analysis of plasmatic corticosterone

Corticosterone was extracted from rat plasma following previously published experimental methods (Carnevali et al., 2015a) by adding two volumes of ice-cold acetonitrile (2:1 ratio) containing 100 nM dexamethasone as internal standard. After centrifugation (13,000 rpm, 4°C, 10 min), the supernatant was collected and analyzed by liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS) to quantify corticosterone levels.

2.11 Analysis of fatty acid ethanolamides

AEA, OEA, and PEA were measured in 10% w/v rat brain homogenates and rat plasma. Brain tissue was homogenized in 10 volumes of 50 mM TRIS buffer pH 7.4, at 4°C. Fatty acid ethanolamides (FAEs) were extracted by adding a double volume of ice-cold acetonitrile containing 100 nM AEA-d4, OEA-d4 and PEA-d4 as internal standard mix to 50 µL of 10% w/v rat brain homogenate or 50 µl of rat plasma. FAE levels, expressed as pmol, were normalized for tissue wet weight (pmol/g) (Carnevali et al., 2020b; Carnevali, Vacondio, Rossi, Callegari, et al., 2015). Preparation of fecal samples for FAEs quantification was performed according to the procedure described in the following paragraph, related to lipidomic analysis.

2.12 UHPLC-HR-MS lipidomic analysis of stool samples

50-100 mg of rat feces were accurately weighed in 2 mL screw cap tubes pre-filled with 1.4 mm ceramic beads, then added with equal volumes of MeOH and TRIS buffer (Manca et al., 2020) to a final concentration of 100 mg/mL. Fecal material was mechanically homogenized by a 4-place Mini Bead Mill (VWR International, Milan, Italy) to obtain a fecal slurry, 200 µL of which were transferred into a glass centrifuge tube, added with 400 µL of TRIS, 400 µL of MeOH and 1 mL of chloroform. Samples were thoroughly vortexed, to promote lipid extraction and centrifuged (6000 g, rt, 5 min) to allow phase separation. Extracting procedure was repeated on the aqueous layer, then the organic aliquots from the two extraction steps were pooled. 500 µL of the lipid enriched organic phase were evaporated under nitrogen and kept at -80°C until analysis. Dried samples

were reconstituted to half-volume by adding IPA:MeCN:water = 2:1:1 and centrifuged to separate insoluble pellet (13000 rpm, 4°C, 5 min) before undergoing lipidomic analysis.

A Waters Acquity UHPLC I-Class (Waters, Milford, MA, USA) coupled to a Vion ion mobility hybrid Quadrupole-Time of Flight Mass Spectrometer (IMS-Q-ToF MS) (Waters, Manchester, UK) was employed for lipidomic data acquisition.

Chromatographic separation was achieved by using an ACQUITY UPLC HSS T3 (2.1 x 100 mm, 1.8 µm; Waters, USA) column, maintained at 55 °C. Mobile phases A and B were MeCN:water=40:60 and IPA:MeCN:wat=90:5:5, respectively, both added with 5 mM ammonium acetate and 0.1% acetic acid. The linear gradient was as follows: 0 min: 60%A; 0-14 min: 60-0%A; 14-17 min: 0%A; 17-17.5 min: 0-60%A; 17.5-20 min: 60%A; total run time was 20 min; flow rate was equal to 0.4 mL/min and the injection volume set to 5 µL. The autosampler was cooled at 5 °C.

Acquisition was performed in High Definition MS^E positive ion mode ($HDMS^E$, ESI^+), by which a low and a high energy mass spectrum are recorded in parallel for precursor ions and relative fragments, respectively. Mass range spanned between 50-1200 amu with a scan time of 0.2 s and interscan delay time of 0.02 s. Instrumental parameters were set as follows: source temperature: 120°C; desolvation temperature: 500°C; source gas flow: 50 L/h; desolvation gas flow: 1000 L/h; capillary voltage: 2.5 kV (ESI+); sample cone voltage: 40 V; collision energy: low energy: 5 V; high energy: 20-40 V. Before the analysis, external calibration of mass and drift time was performed by infusing the MajorMix IMS/TOF calibration kit (Waters, Manchester, UK), prepared following manufacturer's instructions, at a flow rate of 20 µL/min. Internal lock mass calibration was used to ensure accuracy in mass measurement during the analyses: reference mass leucine enkephalin ($[M+H]^+$ *m/z* = 556.27658; 50 ng/mL) was thus injected every 3 min.

System performance was checked during the analytical session for accurate mass and retention time accuracy and precision by injecting a mixture of synthetic lipids (Lipidomix, Avanti Polar, Alabaster, AL, USA), prepared according to vendor's instructions.

Three technical replicates for each biological sample were randomly injected to minimize the impact of time-dependent system changes during the analysis. Quality Control (QC) samples, made by pooling an aliquot of

each extract, were repeatedly injected to equilibrate the system before running samples and run throughout the entire analytical batch to allow alignment of chromatographic traces (see below). UNIFI v.1.8.2 (Waters, Manchester, UK) software was employed for system control and data acquisition in profile mode.

2.13 Lipidomic data processing

Progenesis QI software v.2.6 (Nonlinear Dynamics, UK) was used for data pre-processing of UNIFI raw data in *.uep* format: it performed automatic alignment of chromatographic runs selecting the most suitable QC sample in the batch as alignment reference. Sensitivity of the peak picking algorithm was set at default value, minimum peak width at 0.025 min, retention time window between 1.5 and 17 min and fragment sensitivity at 5% of the base peak of the spectrum. To account for experimental and technical variations within the analytical batch, Progenesis QI "normalize to all compounds" algorithm was applied for data normalization, consisting in the selection of a reference QC run and the picking of common peaks between it and each sample all over the chromatographic run-time. Quantitative abundance ratio (i.e., ion intensity in the sample/intensity in the reference) was determined for each extracted ion and distribution of log-transformed ratios was calculated for each sample: only those log ratios that fell within a confidence interval around 0 were averaged to calculate a scalar multiplier (i.e., scaling factor) for each run in the batch. According to these settings, Progenesis QI returned 7724 ion features, which were subsequently filtered according to the coefficient of variation in QC samples (%CV≤25) and mass-over-charge ratio (m/z > 250). 3750 ion features were thus selected, then technical replicates were averaged and Log_{10}-transformed. Finally, differences between Log_{10} value at week five and baseline (ΔLogw5,b) were calculated and used as independent variables for data analyses.

2.14 Multivariate analysis of lipidomic data

Partial Least Square (PLS) analyses were performed using SIMCA v.16 (Sartorius Stedim Biotech, Sweden). ΔLogw5,b values (3750 variables) were employed as independent variables (X), while condition (stress or

control, parameterized with 1 and 0, respectively) and treatment (URB694 or vehicle, 1 and 0, respectively) as discrete response variables (Y). Data were centered to zero-mean prior to modelling.

A Principal Component Analysis (PCA) was performed separately on each experimental group considering the 46 lipids significantly correlated with at least one bacterial genus, considering their signal intensity at baseline and week 5. Data were centered to zero-mean prior to modelling.

2.15 Identification of lipid markers

LC-HRMS analytical conditions allowed detection of lipids belonging to the five lipid categories of: fatty acyls (FA), glycerolipids (GL), glycerophospholipids (GPL), sphingolipids (SP) and sterol lipids (ST) according to the LMSD Classification System (Fahy et al., 2005). Possible adducts (i.e., $[M+H]^+$, $[M-H_2O+H]^+$, $[M+Na]^+$, $[M+NH_4]^+$ and $[M+K]^+$) were assigned to each ion feature by Progenesis QI.

Putative identities were selected by elemental composition analysis with calculated mass, mass tolerance (ppm), isotopic similarity (calculated isotopic pattern vs. experimental one) and mass fragmentation by searching into ChemSpider database (www.chemspider.com) and Lipid Maps Database (www.lipidmaps.com). Only identities assigned with a matching score ≥ 40 were considered reliable. Retention times of putatively identified features were compared to the ones of external standards (i.e., Lipidomix, containing representative lipids for each lipid class) to corroborate identity attribution. This procedure confidently assigned identity to 363 ion features. In reported Figures (Figure 8 and Supplementary Figures S2) and Table 2 lipids were annotated as follows: ceramides (Cer), phosphosphingolipids (PSL), glycosphingolipids (GlycoSP), other SL (sphingolipids), sphingoid bases (SPB), fatty acids (FA), fatty esters (FE), fatty amides (NA), amino fatty acids (NAA), esters of hydroxy fatty acids (FAHFA), neutral glycosphingolipids (HexCer and Hex2Cer), mono-, di- and tri-acylglycerols (MG, DG, TG) monoglycosyldiacylglycerols (MGDG), sterol lipids (ST), secosteroids (SECOST), phosphatidic acids (PA), (lyso)phosphatydylcholines (PC), (lyso)phosphatidylethanolamines (PE), phosphatidylserine (PS), glycerophosphates (PG), glycerophosphoinositols (PI). Individual annotations returned the total number of carbons in fatty acyl chains, the total number of double bonds and the number of oxygen atoms, when present (e.g., DG 30:0; Cer

44:1;O3). Determination of the length of individual acyl chains and of the position of double bonds exceeded the capability of the analytical system, thus they were not annotated. Individual lipids were grouped into main classes, made of at least n=3 exponents, according to LMSD, leading to a total of 21 lipid classes. Changes in the lipidomic profiles were evaluated as class trends (i.e., comparison of ΔLogw5,b of all the lipids belonging to the same class between experimental groups).

2.16 DNA extraction and sequencing of rat stool samples

The 64 rat stool samples collected at different time points were subjected to DNA extraction using the QIAmp DNA Stool Mini Kit collected kit following the manufacturer's instructions (Qiagen, Hilden, Germany). Subsequently, partial 16S rRNA gene sequences were amplified from extracted DNA using the primer pair Probio_Uni/Probio_Rev, targeting the V3 region of the 16S rRNA gene sequence (Milani et al., 2013). 16S rRNA gene amplification and amplicon checks were carried out as previously described (Milani et al. 2013). 16S rRNA gene sequencing was performed using an Illumina MiSeq sequencer with MiSeq Reagent Kit v3 chemicals, according to the protocol previously reported (Milani et al., 2013).

2.17 16S rRNA microbial profiling analysis

The *fastq* files obtained from the sequencing were processed using QIIME2 software (Bokulich et al., 2018). Paired-end reads were merged, and quality control retained sequences with a length between 140 and 400 bp and mean sequence quality score of >20, while sequences with homopolymers of >7 bp and mismatched primers were omitted. In order to calculate downstream diversity measures (alpha and beta diversity indices, Unifrac analysis), 16S rRNA amplicon sequence variants (ASV) were defined at ≥ 99% sequence homology using DADA2 (Callahan et al., 2016) and ASVs with less than 2 sequences in at least one sample were removed. All reads were classified to the lowest possible taxonomic rank using QIIME2 (Caporaso et al., 2010) and a reference dataset from the SILVA database (Quast et al., 2013). Biodiversity of the samples (alpha-diversity) was calculated with Chao1 index, while similarity between samples (beta-diversity) was calculated by weighted uniFrac (Lozupone & Knight, 2005). The similarity range is calculated

between the values 0 and 1. PCoA representations of beta-diversity were performed using QIIME2 (Caporaso et al., 2010).

2.18 Statistical Analyses

Statistical analyses for behavioral and biochemical descriptors were conducted using IBM SPSS Statistics for Windows, version 28 (IBM Corp., Armonk, N.Y., USA). The normal distribution of variables was assessed using the Kolmogorov-Smirnov test. Data were analysed using 2 ("group": STR or CTR) x 2 ("treatment": URB694 or VEH) factorial design ANOVAs or repeated measures ANOVAs with "time" as the within-subject factor and "group" and "treatment" as the between-subject factors. Post-hoc analyses were conducted using unpaired t-tests, with a Bonferroni correction for multiple comparisons. Statistical significance was set at $p<0.05$.

In lipidomic experiments, multiple Student's t tests followed by Bonferroni correction were applied on ΔLogw5,b to test the statistical differences in lipid classes among the four experimental groups.

Microbiota analysis differences in biodiversity between groups were assessed by t-test analyses. Furthermore, PERMANOVA analyses were performed using 1000 permutations to estimate possible significant differences among populations in PCoA analyses. Moreover, the taxonomic relative percentage difference (Δ%) evaluated bacterial differences at the genus level. In detail, the relative percent difference between time week 5 and Baseline (Δ%) was calculated. Moreover, based on Spearman's rank correlation coefficient, a specific correlation analysis was performed between bacterial genera and lipid compounds identified for each sample of the four groups and time points. The False Discovery Rate (FDR) correction applied to correlation analyses was based on Benjamin and Hochberg correction.

3. Results

3.1 Effects of repeated social stress exposure and FAAH inhibition on behavioral and neuroendocrine parameters

First, we exposed male rats to 5 weeks of repeated social defeat to induce depressive-like behavioral and neuroendocrine symptoms and verify the antidepressant-like effects of FAAH inhibition with URB694.

The top panel of Figure 2 reports sucrose solution preference during the sucrose preference test. Repeated measure ANOVA yielded a significant effect of "time" ($F_{(2,54)}$ = 8.76, p < 0.001) and a significant interaction between "time" and "group" ($F_{(2,54)}$ = 8.76, p < 0.001). Specifically, stressed rats treated with vehicle (STR+VEH) displayed a lower preference for the sucrose solution compared to their respective basal level (p < 0.01) and CTR group at week 2 (p = 0.039) and week 4 (p < 0.01), suggesting the onset of an anhedonic behavior. Similar changes were described in the STR+URB group at week 2 (i.e., before the beginning of the treatment). Importantly, the anhedonic effects of social stress exposure were corrected by daily treatment with URB694, with STR+URB rats showing a return of sucrose preference to baseline levels at week 4. Consequently, sucrose preference at week 4 was significantly higher in STR+URB than STR+VEH rats (p = 0.001).

The middle panel of Figure 2 illustrates rats' behavior during the forced swim test, which was performed at the end of the stress protocol (week 5). Two-way ANOVA yielded a significant effect of "group" ($F_{(1,26)}$ = 5.32, p = 0.03) and "treatment" ($F_{(1,26)}$ = 26.02, p<0.001) and a significant interaction between "group" and "treatment" ($F_{(1,26)}$ = 8.32, p = 0.008) on immobility time, a behavior that is suggestive of passive stress coping. Specifically, STR+VEH rats spent significantly more time in immobility compared to their respective CTRs (p= 0.001). Notably, this behavioral effect was prevented by URB694 treatment (STR+URB vs STR+VEH, p < 0.001). Accordingly, the latency to the first display of immobility was shorter in STR+VEH rats compared with STR+URB rats (p = 0.002).

The bottom panel of Figure 2 reports plasma corticosterone levels and adrenal gland weights 24h after the last STR/CTR procedure. ANOVA yielded a significant effect of "group" ($F_{(1,28)}$ =7.32, p=0.011) and "treatment" ($F_{(1,28)}$ = 17.41, p < 0.001), and a significant interaction between "group" and "treatment" ($F_{(1,28)}$ = 7.27, p =

0.012) for corticosterone levels. Specifically, STR+VEH rats had significantly higher levels of corticosterone compared to both CTR+VEH ($p < 0.001$) and STR+URB rats ($p < 0.001$). Likewise, ANOVA yielded a significant effect of "group" ($F_{(1,28)} = 15.37$, $p < 0.001$) and "treatment" ($F_{(1,28)} = 5.52$, $p = 0.026$) on adrenal gland weight corrected for body weight. As expected, adrenal glands of STR+VEH rats were significantly heavier compared to both CTR+VEH ($p < 0.001$) and STR+URB groups ($p = 0.016$).

In sum, repeated social defeat induced behavioral (anhedonia, passive coping) and neuroendocrine changes that are reminiscent of a depressive-like state and were prevented by FAAH inhibition with URB694 in male rats.

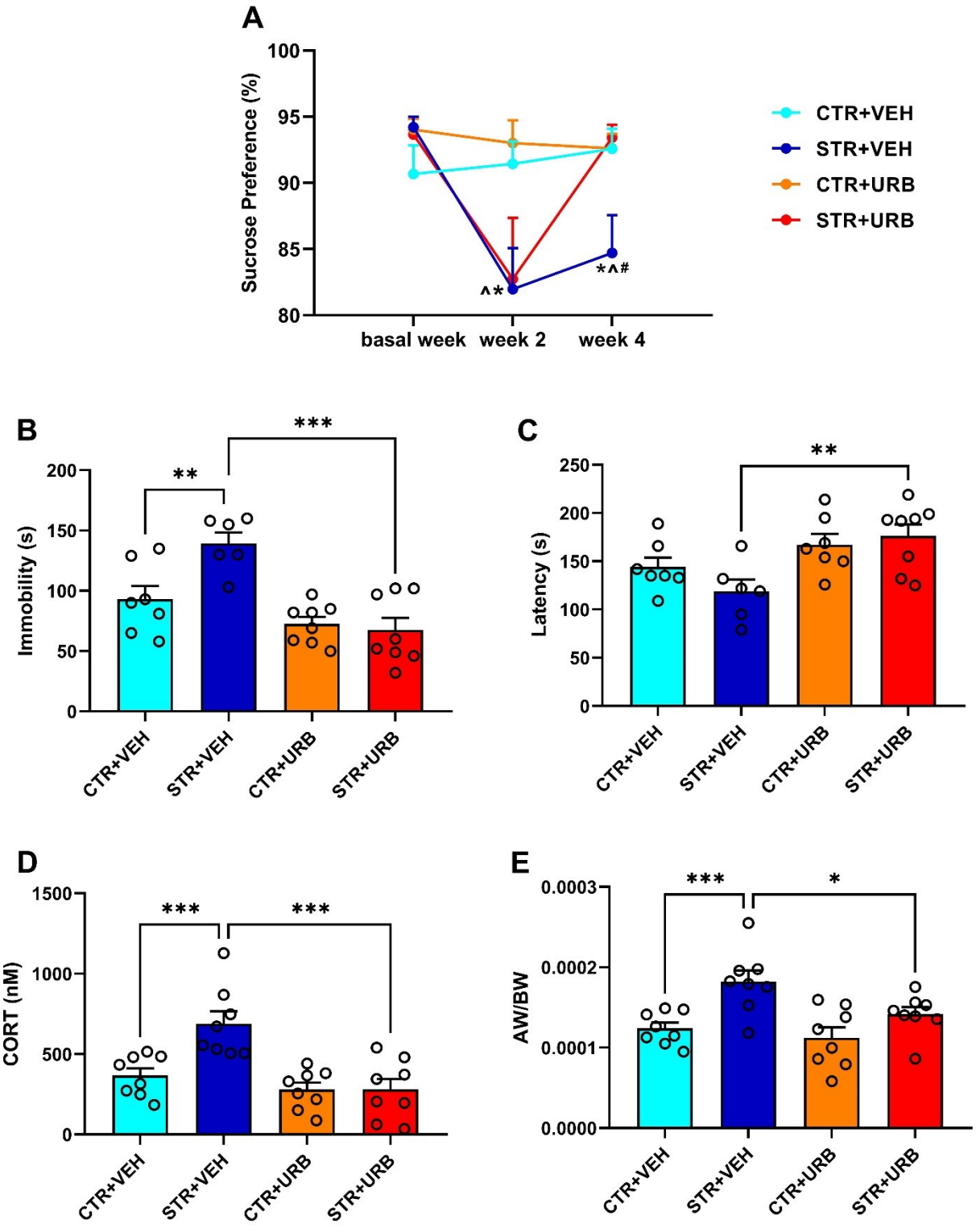

Figure 2. Behavioral and neuroendocrine parameters in control (CTR) and socially stressed (STR) rats treated with vehicle (VEH) or URB694 (URB). (A) Sucrose preference during the sucrose preference test at each assessment point: * = p<0.05 vs CTRs; # = p<0.05 vs STR+URB; ^ = p<0.05 vs basal levels. (B) Time spent in immobility and (C) latency to the first immobility during the forced swim test: **= p<0.01; *** = p<0.001. (D) Plasmatic levels of corticosterone (CORT) and (E) adrenal gland weight (AW) corrected per body weight (BW): * = p<0.05. *** = p<0.001. All data are expressed as means±SEM.

3.2 Effects of repeated social stress exposure and FAAH inhibition on fatty acid ethanolamide levels in brain, plasma, and fecal samples

Next, we quantified brain AEA, OEA, and PEA levels in the prefrontal cortex, hippocampus, and striatum. As shown in the top panel of Figure 3, while there was no significant effect of stress exposure, treatment with URB694 produced a significant increase in OEA levels in the hippocampus ($F_{(1,28)} = 19.86$, $p < 0.001$) and striatum ($F_{(1,28)} = 18.04$, $p < 0.001$), but not in the prefrontal cortex (PFC), in both STR and CTR rats. Similarly, two-way ANOVA yielded a significant effect of URB694 treatment on PEA levels in the hippocampus ($F_{(1,28)} = 18.88$, $p < 0.001$) and striatum ($F_{(1,28)} = 39.19$, $p < 0.001$) of STR and CTR groups, and in the PFC ($F_{(1,25)} = 6.19$, $p = 0.020$) of STR rats. On the opposite, brain AEA levels were not influenced by URB694 administration.

Likewise, URB694 treatment, but not stress exposure, affected plasma FAE levels (Figure 3D) (AEA: $F_{(1,28)} = 5.63$, $p = 0.025$; OEA: $F_{(1,28)} = 22.58$, $p < 0.001$; PEA: $F_{(1,28)} = 39.45$, $p < 0.001$). AEA, OEA and PEA levels were significantly increased in CTR+URB compared with CTR+VEH rats ($p = 0.008$ for AEA; $p = 0.035$ for OEA; $p < 0.001$ for PEA), and OEA and PEA levels were augmented in STR+URB compared with STR+VEH rats ($p<0.001$ for OEA; $p<0.0001$ for PEA). Interestingly, OEA and PEA levels were significantly higher in STR+URB compared to CTR+URB rats ($p = 0.024$ for OEA and $p = 0.032$ for PEA).

As for FAEs levels in fecal samples (Figure 3E), AEA was under the limit of quantification. Two-way ANOVA reported a significant effect of URB694 treatment, but not stress exposure on levels in both groups OEA ($F_{(1,27)} = 11.813$, $p = 0.002$) . PEA fecal levels were not affected by URB694 or stress exposure.

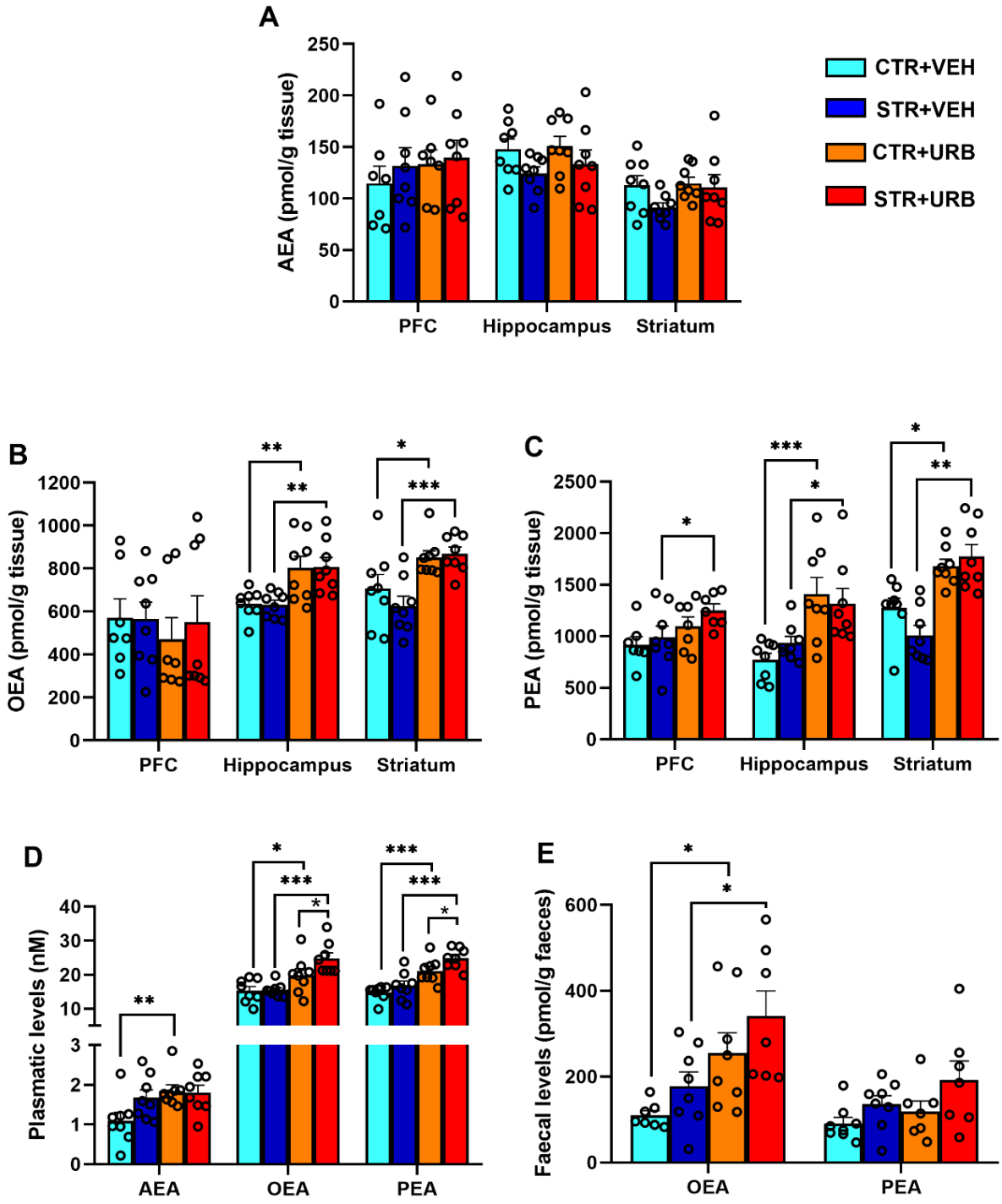

Figure 3. Brain, plasma, and fecal levels of fatty acid ethanolamides in control (CTR) and socially stressed (STR) rats treated with vehicle (VEH) or URB694 (URB). Brain levels of (A) anandamide (AEA), (B) oleoylethanolamide (OEA), and (C) palmitoylethanolamide (PEA) in the prefrontal cortex (PFC), hippocampus and striatum. (D) Plasma levels of AEA, OEA and PEA. (E) Fecal levels of OEA and PEA Data are expressed as mean±SEM. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

3.3 Effects of repeated social stress exposure and FAAH inhibition on gut lipidomic profile

To verify the effects of repeated social stress exposure and FAAH inhibition on gut lipidomic profile, we performed LC-HRMS untargeted lipidomic analysis on fecal samples obtained at baseline and at week 5. To focus on changes in lipid content over time, the difference between Log_{10} value of peak intensity at week 5 and baseline was calculated for each ion feature. Partial Least Squares (PLS) analysis performed on 3750 variables clearly distinguished STR+VEH from CTR+VEH rats. Such difference was not observed in rats treated with URB694, as represented in the three latent variables (LV)-PLS score plot of Figure 4 in which STR+URB and CTR+URB rats occupy an intermediate position between CTR+VEH and STR+VEH groups. Analysis of the information content of the three LVs highlights that the first two LVs are related to changes in fecal lipid profiles induced by URB694 treatment, whereas the third LV mostly discriminates between CTR+VEH and STR+VEH groups (Supplementary Figure S1).

Putative identities were assigned to 363 ion features which were grouped into 21 lipid classes according to the LMSD Classification System. Changes in the levels of each lipid class following the social stress protocol and URB694 treatment are reported in Supplementary Figure S2. For certain lipid classes, social stress-induced changes were prevented by URB694 treatment. Specifically, social stress induced a positive modulation of glycerophospholipids, as shown by the significant increase of phosphatidylethanolamine- (PE), phosphatidylcholine- (PC) and phosphatidylserine (PS)-related signals in STR+VEH compared to CTR+VEH ($p<0.001$), which was not significant in STR+URB rats (Figure 5).

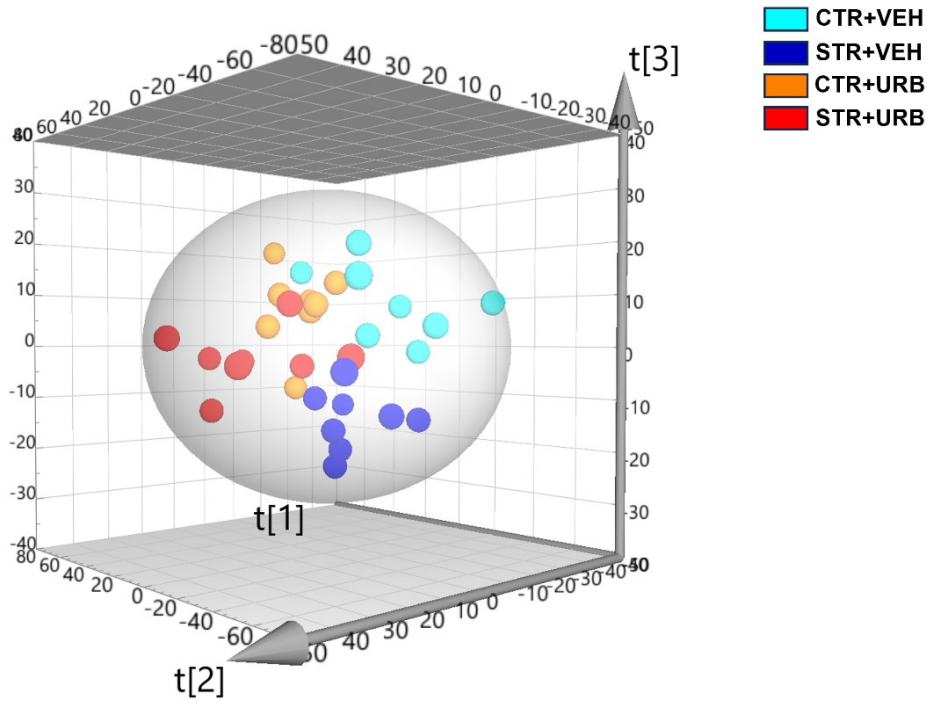

Figure 5. Score plot of Partial Least Squares Analysis based on differences between Log_{10} values of ion intensities at week 5 and at baseline (n=3750 LC-HRMS ion features) in control (CTR) and socially stressed (STR) rats treated with vehicle (VEH) or URB694 (URB). Explained (R2Y) was 63% and the predictive ability (Q2) was 16%.

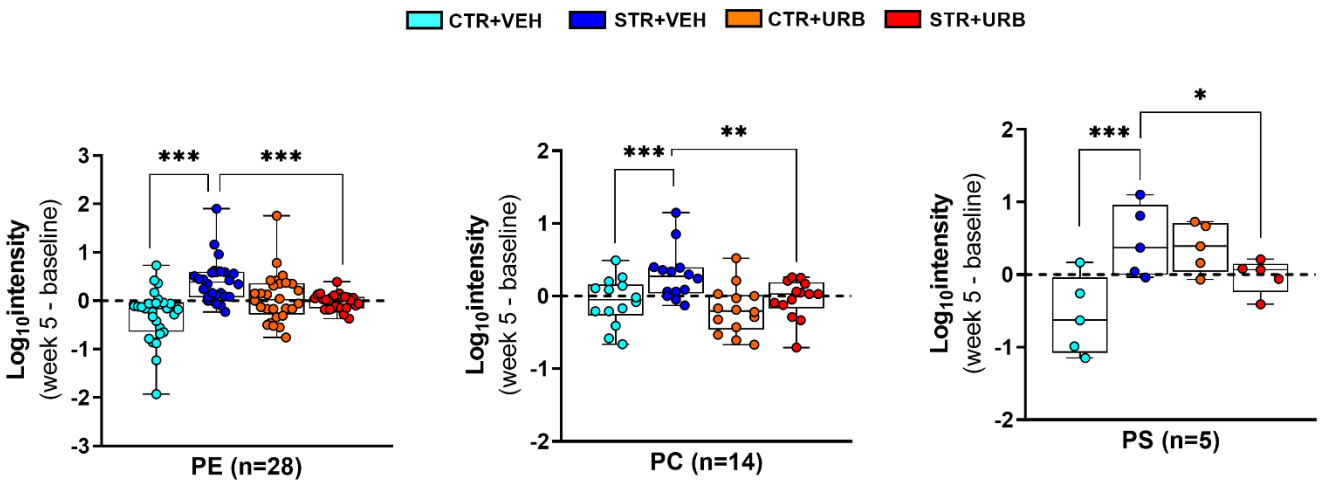

Figure 4. Whiskers plot representing changes in glycerophospholipid levels between week 5 and baseline in fecal samples of control (CTR) and socially stressed (STR) rats treated with vehicle (VEH) or URB694 (URB). The boxes are determined by the 25th and 75th percentiles. The whiskers are determined by 1.5 interquartile range (IQR).* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$. PE = phosphatidylethanolamine ; PC = phosphatidylcholine; PS = phosphatidylserine. Every dot represents the average value of a single lipidic compound (belonging to PE, PC or PS respectively) for each group of rats.

3.4 Effects of repeated social stress exposure and FAAH inhibition on gut microbial profile

To investigate the effects of repeated social stress exposure and FAAH inhibition on gut microbial profile, we first calculated the alpha-diversity (i.e., species richness) as delta difference between the number of identified species at week 5 compared to baseline within each group. As shown in Figure 6, stress exposure and URB694 had no effects on species richness. Then, 3D PCoA were performed independently for each group to identify possible variations in the composition of microbiota (i.e., beta-diversity) over time (i.e., week 5 vs. baseline). Interestingly, PERMANOVA analyses revealed a significant variation in microbiota composition over time in STR+VEH rats (PERMANOVA $p = 0.044$), but not in the other groups (Figure 7). Subsequent analyses performed at genus level revealed that 14 taxa differed significantly in STR+VEH rats between week 5 and baseline (Table 1).

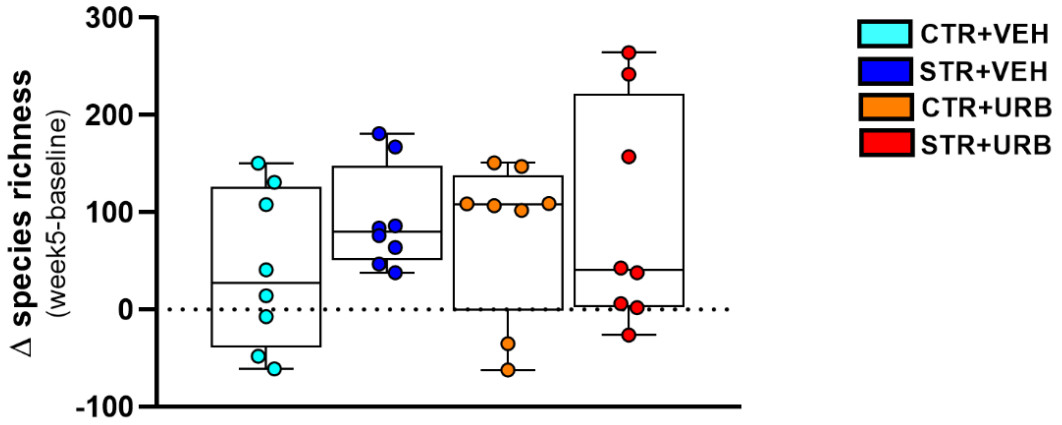

Figure 6. Whiskers plot representing changes in species richness (i.e. alpha diversity) between week 5 and baseline in fecal samples of control (CTR) and socially stressed (STR) rats treated with vehicle (VEH) or URB694 (URB). The 25th and 75th percentiles determine the boxes. The whiskers are determined by 1.5 interquartile range (IQR).

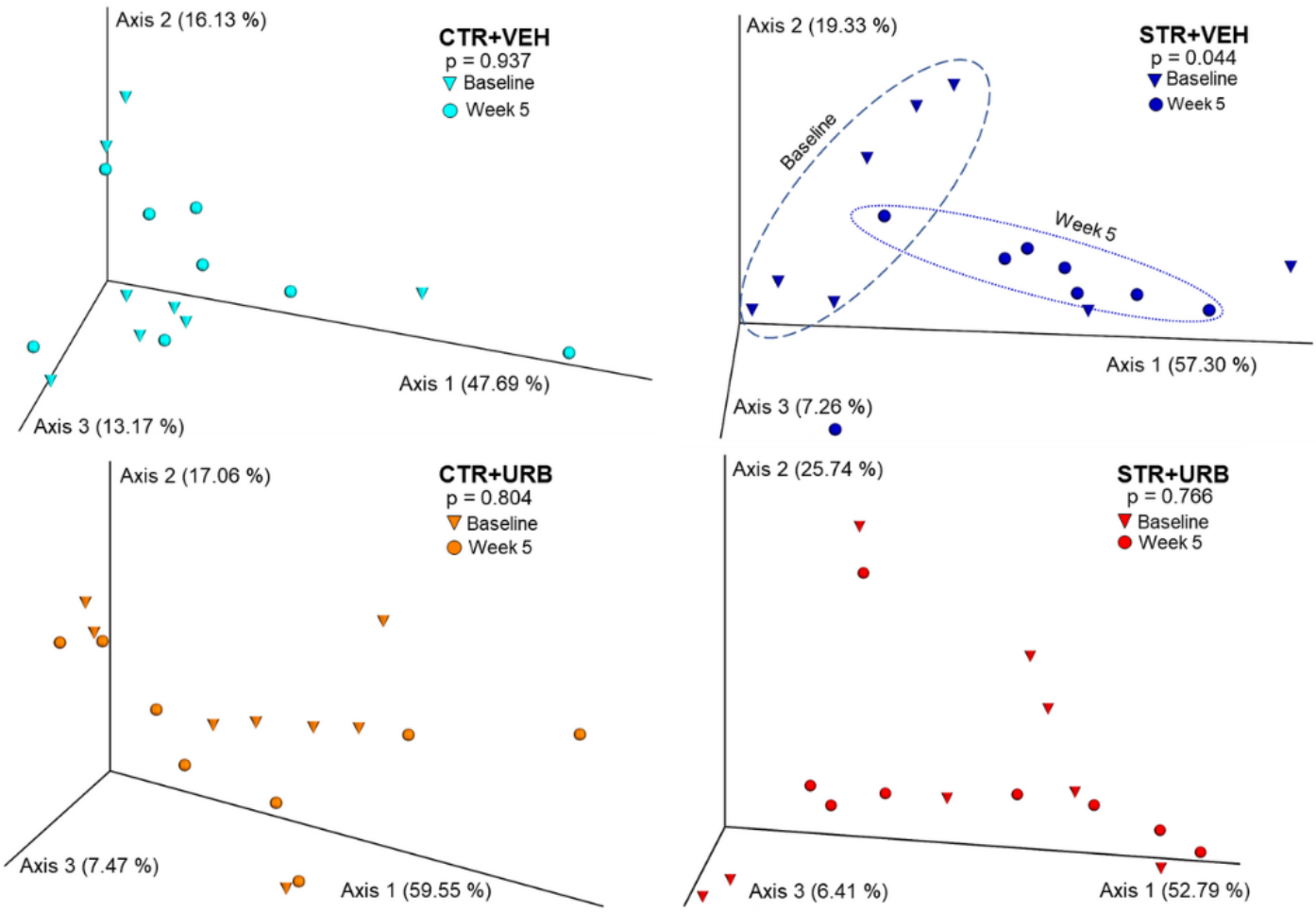

Figure 7. Principal coordinate analyses (PCoA) of microbiota composition (beta-diversity) conducted on fecal samples of control (CTR) and socially stressed (STR) rats treated with vehicle (VEH) or URB694 (URB) collected at week 5 and baseline.

Table 1. Bacterial taxa that significantly varied over time (baseline vs week5) in STR+VEH rats (N=8)

	Relative abundance (%)			Prevalence (%)	
Taxonomy	**Baseline**	**Week5**	**p**	**Baseline**	**Week5**
GCA-900066225 (Ruminococcaceae family)	0.09 ± 0.03	0.02 ± 0.01	.048	75%	38%
Azospirillum	0.16 ± 0.09	0.00 ± 0.00	.011	63%	0%
Lachnoanaerobaculum	2.73 ± 1.30	0.41 ± 0.14	.035	88%	75%
Rikenellaceae RC9 gut group	0.29 ± 0.22	2.44 ± 0.45	.001	25%	100%
Prevotella	0.31 ± 0.22	1.40 ± 0.36	.033	25%	75%
Odoribacter	0.05 ± 0.03	0.17 ± 0.04	.035	38%	88%
U. m. of Mollicutes RF39 order	0.74 ± 0.26	2.21 ± 0.59	.046	100%	100%
Lachnospira	0.00 ± 0.00	0.05 ± 0.02	.011	0%	63%
U. m. of Bacteroidales order	0.01 ± 0.01	0.10 ± 0.04	.021	13%	75%
Faecalibacterium	0.00 ± 0.00	0.05 ± 0.02	.027	0%	50%
Ruminococcaceae NK4A214 group	0.13 ± 0.06	0.30 ± 0.07	.027	88%	100%
U. m. of Desulfovibrionaceae family	0.16 ± 0.14	0.24 ± 0.06	.028	25%	88%
U. m. of Bacteroidales RF16 group family	0.38 ± 0.25	2.03 ± 0.89	.037	25%	88%
U. m. of Erysipelotrichaceae family	0.01 ± 0.01	0.06 ± 0.02	.043	50%	88%

Note. Data related to the relative abundance are reported as means±SEM and were analyzed with the Mann-Whitney U test.

3.5 Relationship between gut microbiota composition and lipid content

Finally, we attempted to correlate bacterial taxa and lipid compounds considering all animals, and highlighted those taxa and compounds that were found to be altered by stress exposure (Figure 8 and 9).

The results of Spearman's rank correlation coefficient analysis revealed a total of 35 bacterial taxa with at least one significant ($p < 0.05$) correlation with a lipid compound. The greatest number of correlations were found for the genera *Azospirillum* (32 significant correlations), Prevotellaceae UCG-001 (32 significant correlations) and *Alloprevotella* (31 significant correlations). Similarly, 46 lipid compounds showed a

significant ($p < 0.05$) correlation with at least one bacterial genus. The greatest number of correlations were found for the lipid classes Cer and LPE (7 and 6 significant correlations, respectively) (Table 2). PCA analyses, performed separately for each group of rats, on the 46 lipids correlating with bacterial taxa highlighted a clear distinction between STR+VEH animals at baseline and week 5 (Figure 10). A similar separation between the two time points was not observed in the other groups.

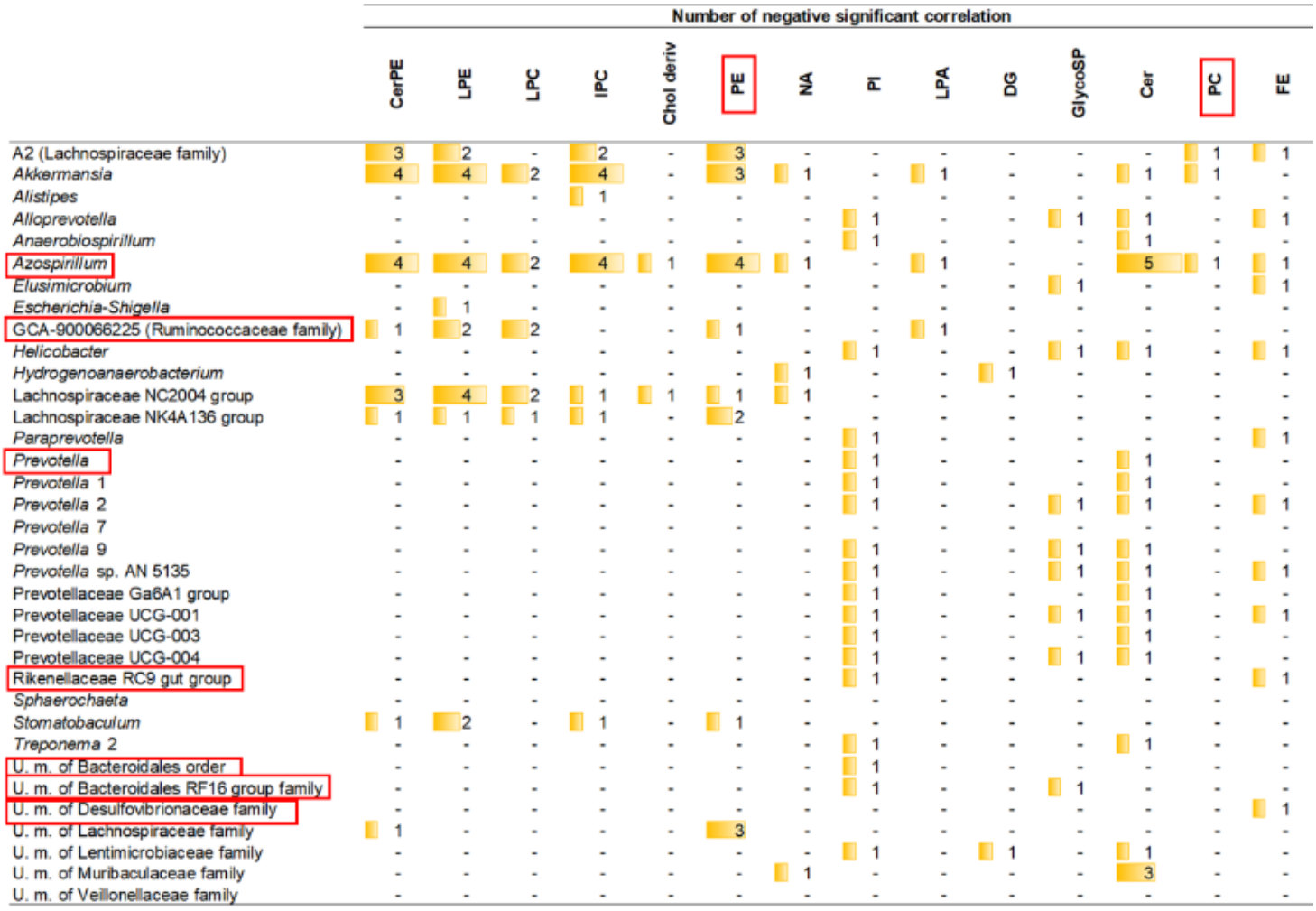

Figure 8. Negative correlations between bacterial taxa and lipid compounds. The barplots report bacterial taxa with at least one significant correlation with a lipid compound ($p < 0.05$) considering all animals. The length of the yellow bar is proportional to the number of significant correlations. Bacterial taxa and lipid compounds showing a significant variation in STR+VEH rats between baseline and week 5 are highlighted with a red rectangle.

Figure 9. Positive correlations between bacterial taxa and lipid compounds. The barplots report bacterial taxa with at least one significant correlation with a lipid compound ($p < 0.05$) considering all animals. The length of the yellow bar is proportional to the number of significant correlations. Bacterial taxa and lipid compounds showing a significant variation in STR+VEH rats between baseline and week 5 are highlighted with a red rectangle.

Table 2. Lipidic compounds with at least one significant correlation with a bacterial taxon (p-value < 0.05) considering all rats. Compounds with a significant variation in STR+VEH rats between baseline and week 5 are reported in bold.

Compound	Identity	Lipid class	N of significant correlations
8.11_554.5147m/z	Cer 34:1;O3	Cer	20
10.02_762.7344m/z	Cer 49:2;O3		17
7.80_540.4988m/z	Cer 33:1;O3		14
7.97_534.4881m/z	Cer 34:2;O3		13
7.17_538.5178m/z	Cer 34:1;O2		9
9.51_554.5509m/z	Cer 35:0;O2		7
6.37_552.5349m/z	Cer 35:1;O2		2
8.02_706.5636n	CerPE 36:0;O3	CerPE	18
7.45_690.5313n	CerPE 35:1;O3		17
7.75_705.5536m/z	CerPE 36:1;O3		12
7.73_661.5294m/z	CerPE 34:1;O2		9
8.18_707.5711m/z	CerPE 36:0;O3		5
10.43_758.6068n	14:0-Glc-Cholesterol	Chol deriv	10
11.01_786.6385n	16:0-Glc-Cholesterol or 16:0-Gal-Cholesterol		1
7.57_523.4729m/z	DG 30:0	DG	1
4.58_665.5511m/z	DG 41:6		1
7.18_554.4783m/z	DG 30:2		1
7.60_556.5300m/z	FAHFA 34:0;O	FE	10
8.89_584.5619m/z	FAHFA 36:0;O		10
7.85_561.4880m/z	FAHFA 36:4;O		1
7.59_717.5760n	HexCer 34:0;O3	GlycoSP	11
6.55_824.5661m/z	IPC 36:0;O4	IPC	18
6.95_825.5748n	IPC 36:0;O3		14
6.76_826.5820m/z	IPC 36:0;O3		13
5.93_797.5434n	IPC 34:0;O3		7
2.47_393.2398m/z	LPA 16:0	LPA	7
2.47_453.2866n	LPC 13:0	LPC	21
2.05_439.2708n	LPC 12:0		6
1.83_543.3333n	LPC 20:4		2
1.85_519.3333n	LPC 18:2		1
2.80_479.3021n	LPE 18:1	LPE	22
2.95_467.3020n	LPE O-17:1;O		22
2.65_479.3019n	LPE 18:1		14
2.09_477.2860n	LPE 18:2		6
1.96_477.2861n	LPE 18:2		3
5.16_496.3767m/z	LPE O-20:0		1
7.05_654.5195n	Flavolipin	NA	10
7.69_540.4986m/z	NA 33:1;O3		1
3.69_282.2796m/z	Fatty amide 18:1		1
8.32_721.5848m/z	**PC O-31:1**	**PC**	**10**
6.85_665.5238m/z	**PE O-30:1**	**PE**	**16**
7.19_679.5395m/z	**PE O-31:1**		**15**
7.30_679.5399m/z	**PE O-31:1**		**13**
8.78_717.5328n	**PE 34:1**		**12**
8.68_730.5396m/z	**PE 35:2**		**1**
9.50_994.7334m/z	PI 44:1	PI	21

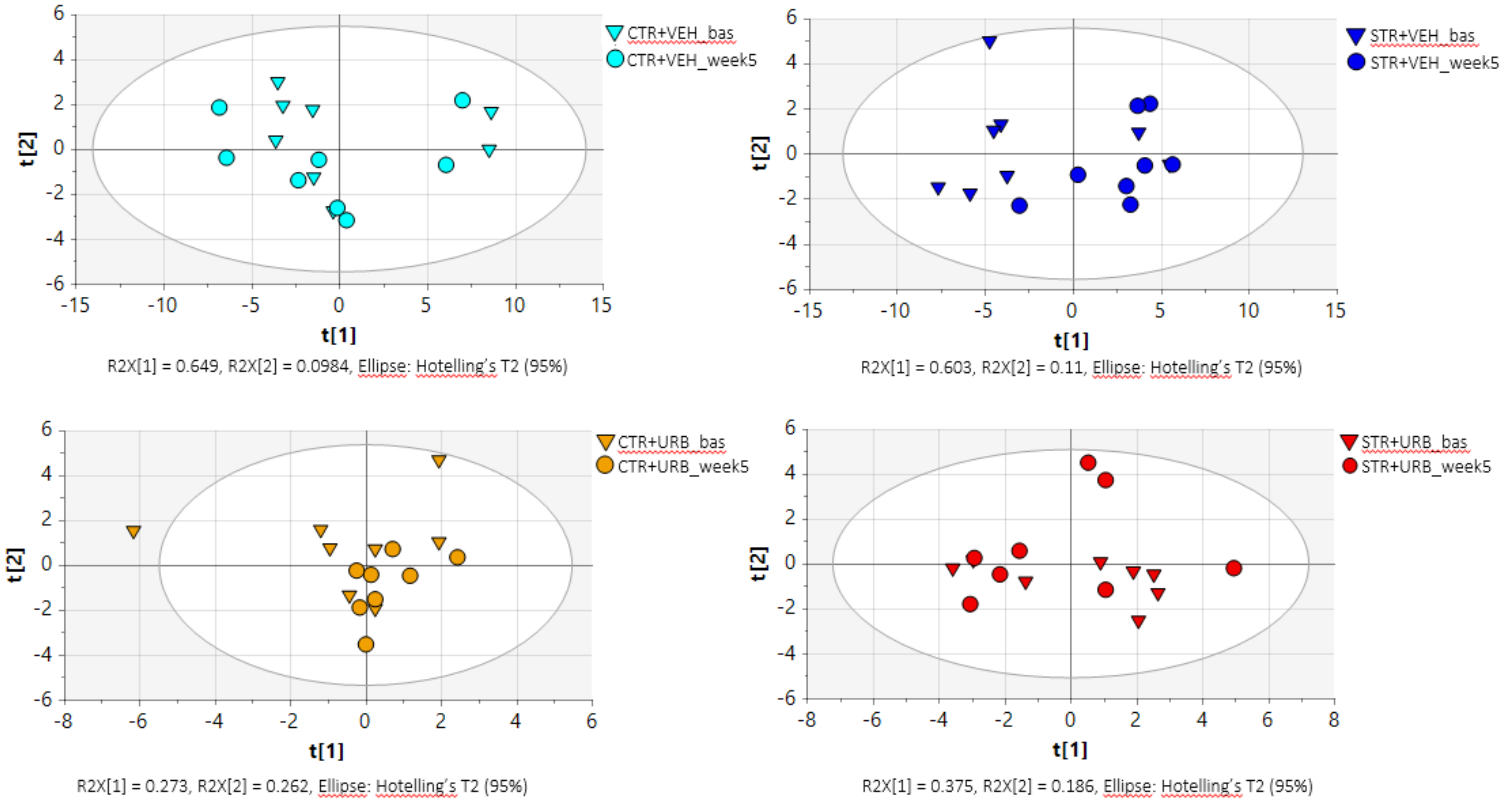

Figure 10. Principal component analyses (PCA) of the 46 lipids correlating with bacterial taxa conducted on fecal samples of control (CTR) and socially stressed (STR) rats treated with vehicle (VEH) or URB694 (URB) collected at week 5 and baseline (bas).

4. Discussion

The major findings of the present study are that (i) depressive-like behavioral and neuroendocrine changes induced by repeated social stress exposure are associated with alterations in both the gut microbial and lipidomic profiles in male Wistar rats, and that (ii) systemic inhibition of FAAH activity with URB694 promotes behavioral stress resilience and favors the maintenance of gut microbial and lipidomic stability under social stress conditions.

Psychosocial stress has been recognized as a risk factor for the development of depressive syndromes (Gilman et al., 2013; Siegrist, 2008). In animal studies, various stress paradigms are employed to explore the mechanisms underlying the development of depressive-like phenotypes and to evaluate the effectiveness of new therapeutic interventions (Planchez et al., 2019). Among these paradigms, repeated social defeat in rodents is known to have a strong and long-lasting effect on brain physiology and behavior which bears similarities with human depression (Hollis & Kabbaj, 2014). Confirming this, in the current study repeated social defeat stress over a 5-week period induced both behavioral and neuroendocrine changes in male rats that resemble a depressive-like phenotype. Specifically, stressed rats exhibited a reduced preference for the consumption of a sucrose solution, which is indicative of the emergence of an anhedonic state, one of the key symptoms of depression (Coccurello, 2019; Planchez et al., 2019). Additionally, stressed rats displayed prolonged immobility during the forced swim test, which may be interpreted as a passive stress coping strategy commonly observed in patients with depression (Atrooz et al., 2021; Chou et al., 2011; Commons et al., 2017; Orzechowska et al., 2013). Furthermore, stressed rats exhibited greater plasma corticosterone levels and heavier adrenal gland weights. Importantly, these alterations were observed four days after the last social defeat episode, suggesting a persistent hyperactivity of the hypothalamic-pituitary-adrenal axis, a well-established neuroendocrine feature of depression (Mikulska et al., 2021b; Pariante & Lightman, 2008). Collectively, these findings confirm the utility of the repeated social stress model for inducing depressive-like phenotypes in male rats.

Emerging evidence has suggested that microbiota and lipid interactions within the gut may influence central nervous system functioning and therefore may contribute to the onset of stress-related psychopathologies,

including depression (Tian et al., 2022; Zheng et al., 2021). For example, at the preclinical level, alterations in gut microbiota have been described in stressed mice (Chevalier et al., 2020; Yu et al., 2017), and probiotic treatments have shown promise in ameliorating symptoms associated with depression and/or stress (Gao et al., 2022; Mindus et al., 2021). Therefore, one of the major objectives of the current study was to analyze gut lipidomic and microbial profiles in socially stressed rats with depressive-like symptoms. Our findings revealed that stressed rats displayed significant changes in their gut lipidomic profile. For instance, the levels of glycerophospholipids (i.e., phosphatidylethanolamines, phosphatidylcholines, and phosphatidylserine) were significantly increased after repeated social stress exposure. Interestingly, similar lipidic changes were found in the colon of mice exposed to chronic social defeat stress (Gong et al., 2021). Glycerophospholipids are the major lipid constituents of eukaryote cellular membranes and can directly affect cell physiology. Perturbations in glycerophospholipid metabolism have been associated with irritable bowel syndrome (Guan et al., 2020; Murgia et al., 2018; Tefas et al., 2020; Wu et al., 2022), a pathological condition characterized by dysbiosis (Frank et al., 2007) and by a high comorbidity with depressive syndromes (Barberio et al., 2021). Relatedly, in our rats with stress-induced depressive-like symptoms, gut lipidic changes were accompanied by significant alterations in microbiota composition, but not in species richness. This is in line with previous studies in rodents showing an effect of stress exposure on microbiota taxonomical composition, without altering the number of species (Chevalier et al., 2020; Gong et al., 2021; Zhang et al., 2021). In detail, we found that the abundance of 11 bacterial taxa, such as *Prevotella* and *Odoribacter* genera, increased, while the abundance of three other taxa, such as *Azospiriullum*, decreased after 5 weeks of repeated social stress. Interestingly, increased *Odoribacter* abundance was also previously described in stressed mice (Bangsgaard Bendtsen et al., 2012), and increased *Prevotella* levels were found in a genetic murine model of depression (Shoji et al., 2023) as well as in patients with depression (Lin et al., 2017). In the present study, the identification of many significant correlations between lipidic compounds and bacterial taxa further supports the intimate relationship between gut lipids and microbiota (Russo et al., 2018). Remarkably, we found positive and negative correlations, respectively, between bacterial taxa (e.g., *Prevotella* and *Azospirillum)* and lipid classes (eg., PE) affected by stress exposure, suggesting that social stress may have independent

effects on gut microbial and lipidic composition but also influence their mutual relationships. Collectively, these findings indicate that gut lipids and microbiota may be intricately linked, and that perturbations in their crosstalk may contribute to the development of central nervous system-related diseases, including depression (Liu et al., 2023). However, we cannot exclude the possibility that changes in gut microbial and lipidic profiles represent a manifestation of a specific endophenotype that have relevance to stress-related psychiatric disorders.

The efficacy of FAAH inhibitors to promote behavioral resilience has been documented in several rodent models of acute and chronic stress (for a review see Carnevali et al., 2017). Likewise, in the current study, the FAAH inhibitor URB694 corrected/prevented the effects of repeated social stress on both behavioral and neuroendocrine readouts. The antidepressant-like effects of FAAH inhibitors have generally been ascribed to an enhancement of FAE-mediated signaling (particularly AEA-) within the central nervous system. It is interesting to note that in this study brain levels of FAEs were not affected by stress exposure, similar to previous findings in Wistar Kyoto rats (Carnevali et al., 2015a). However, FAAH inhibition resulted in a significant increase of OEA and PEA, but not AEA, in the selected brain areas, in contrast with the same study in Wistar Kyoto rats (Carnevali et al., 2015a). Several hypotheses may be put forward to explain this discrepancy: (i) Wistar Kyoto rats, compared to the Wistar rat strain used in the current study, show lower brain levels of AEA and greater FAAH activity (Vinod et al., 2012) and therefore may be more sensible to its pharmacological inhibition; (ii) the regulation of AEA levels is complex and enzymes other than FAAH, such as N-Acylethanolamine Acid Amidase (Ueda et al., 2010) or COX-2, (Glaser & Kaczocha, 2010; Kozak et al., 2004) can contribute to its degradation; (iii) analyses were conducted 24h after the last URB694 administration and therefore we cannot exclude the presence of an effect on brain AEA levels in the preceding hours. Similar results were found in plasma samples, suggesting that the stress resilience effects of FAAH inhibition might not exclusively be attributed to an enhancement of AEA-mediated signaling. Also, AEA was not detectable in fecal samples, while, to the best of our knowledge, this is the first study to describe a significant increase in fecal OEA levels after inhibition of FAAH activity with URB694, without concurrent changes in PEA levels. Nevertheless, these results provide evidence that inhibition of FAAH activity with

URB694 favours the maintenance of gut microbial and lipidomic stability in rats exposed to repeated social defeat. However, they do not allow to clarify the predominant FAE-mediated signalling responsible for such effects.

5. Conclusions

This study investigated the association between social stress-induced depressive-like phenotypes and gut microbiota and lipidomic composition in male rats and tested the efficacy of the FAAH inhibitor URB694 in counteracting the effects of stress on behavioral, neuroendocrine, and intestinal readouts. We utilized a “multi-omic” approach, which is useful for describing complex physiopathological mechanisms involving several key actors, such as lipids and microbiota, in a more comprehensive way. "Omics" techniques are particularly useful when assessing the efficacy of novel pharmacological interventions, as they offer crucial insights into the underlying mechanisms of actions. Here, we describe the co-occurrence of depressive-like behavioral and neuroendocrine changes and alterations in the gut microbial and lipidomic profile in rats exposed to repeated social stress. Notably, pharmacological inhibition of FAAH activity not only promoted behavioral stress resilience, corroborating previous rodent findings (Carnevali et al., 2017), but also favoured the maintenance of gut microbial and lipidomic stability under conditions of social stress. Even though the cause-effect relationship cannot be established in this descriptive study, the current findings suggest that the gut microbial-lipidic crosstalk might contribute to the development of stress-related disorders and represent a novel biological target for FAAH inhibitors.

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Supplementary Figures

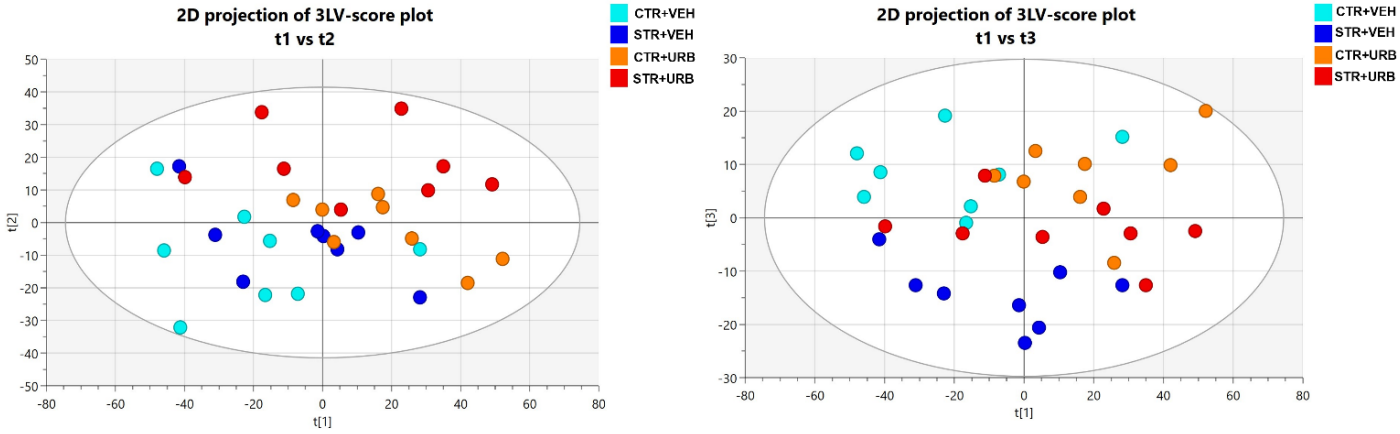

Figure S1. 2D projections of the 3-LV PLS analysis performed on changes in the fecal lipid composition between week 5 of the experimental protocol and basal condition (Δweek5-basal).

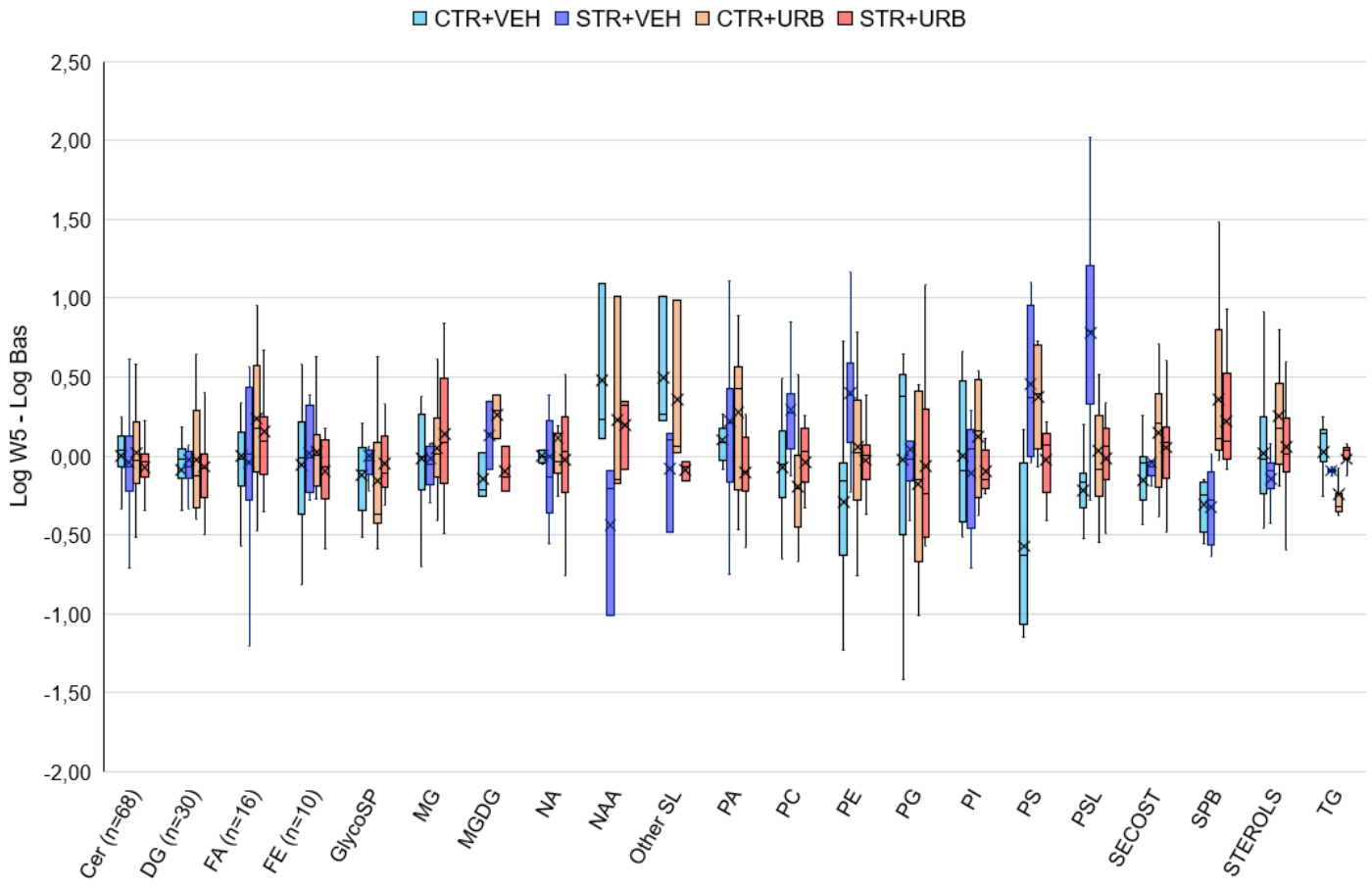

Figure S2. Whisker's plot representing changes in lipid levels belonging to the 21 lipid classes identified between week 5 and baseline in fecal samples of control (CTR) and socially stressed (STR) rats treated with vehicle (VEH) or URB694 (URB). The boxes are determined by the 25th and 75th percentiles. The whiskers are determined by 1.5 interquartile range (IQR). CER= ceramides; DG, MG, TG = mono-, di- and tri-acylglycerols; FA= fatty acids; FE= fatty esters; GlycoSP= glycosphingolipids; MGDG= monoglycosyldiacylglycerols; NA= fatty amides; NAA= amino fatty acids; other SL= sphingolipids; PA= phosphatidic acids; PC= (lyso)phosphatydylcholines; PE= (lyso)phosphatidylethanolamines; PG= glycerophosphates; PI= glycerophosphoinositols; PS= phosphatidylserine; PSL= phosphosphingolipids; SECOST=secosteroids; SPB= sphingoid bases; STEROLS= sterol lipids.

Chapter 3

Enhancement of peripheral fatty acy ethanolamide signaling prevents stress-induced social avoidance and anxiety-like behaviors in male rats*

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Abstract

Exposure to stressful events can result in changes in social and anxiety-related behaviors. Emerging evidence suggests that peripheral inflammatory processes are implicated in the expression of stress-induced behavioral responses and may be targeted to mitigate the negative consequences of stress exposure. In this study, we used the peripherally restricted FAAH inhibitor URB937 to explore the effects of potentiation of the fatty acid ethanolamide (FAE) family of lipid mediators – which include the endocannabinoid anandamide and the non-cannabinoid oleoylethanolamide and palmitoylethanolamide – on behavioral and peripheral biochemical responses in rats exposed to acute social stress.

Adult male rats were exposed to acute social defeat (or control condition) and subsequently treated with URB937 (3 mg/kg, intraperitoneal) or vehicle. Behavioral assessment was conducted 24h after social stress or control procedure exposure. URB937 administration prevented the development of social avoidance behavior after social defeat stress. Further, URB937 administration blocked social defeat-induced transient increase in plasma concentrations of pro-inflammatory cytokines and the rise in plasma corticosterone levels observed 24 h after social defeat. Enhancement of peripheral FAAH-regulated FAE signalling prevents stress-induced social avoidance behaviour and increase in corticosterone levels in male rats through mechanisms that may involve an attenuation of peripheral cytokine release induced by stress exposure.

1. Introduction

The experience of traumatic or stressful events sets off a series of neural reactions that, in vulnerable individuals, can lead to the development of post-traumatic stress disorder (PTSD), anxiety, and other psychopathologies (Fekadu et al., 2019; Shepherd & Wild, 2014). In addition to central processes, peripheral mechanisms have emerged as an important component of the physiopathological reaction to stress. Such mechanisms could be targeted to mitigate the negative sequelae of stress exposure. A possible pharmacological strategy is to enhance the protective effects of the fatty acid ethanolamide (FAE) family of bioactive lipid mediators. The FAEs include endogenous agonists of cannabinoid receptors (e.g., anandamide, (AEA)) and peroxisome proliferator-activated receptor-α (e.g., palmitoylethanolamide (PEA) and oleoylethanolamide (OEA)) (Fezza et al., 2014; Iannotti & Vitale, 2021). FAE-mediated signalling is terminated by the intracellular serine hydrolase, fatty acid amide hydrolase (FAAH) (McKinney & Cravatt, 2005). Relatedly, preclinical studies have shown that globally active FAAH inhibitors such as URB597 (Kathuria et al., 2003) increase availability of endogenous FAEs and promote behavioral resilience to stress through mechanisms that have generally been ascribed to central processes, including enhanced monoaminergic neurotransmission and increase neurogenesis in the hippocampus (Carnevali et al., 2017a; Danandeh et al., 2018; Gobbi et al., 2005). Yet, FAEs-mediated signaling also impacts peripheral processes as shown, for example, by the analgesic and anti-inflammatory properties of peripherally restricted FAAH inhibitors such as URB937 (Mabou Tagne et al., 2022; Piomelli & Sasso, 2014). Significantly, the release of pro-inflammatory cytokines is emerging as an important peripheral mediator contributing to the pathological consequences of psychosocial stress (Hodes et al., 2014; Niraula et al., 2019). For example, increases in circulating concentrations of pro-inflammatory cytokines and chemokines have been observed in patients with PTSD or anxiety as well as in rats and mice that exhibit anxiety-like behaviors following stress exposure (Cheng et al., 2015; Kalinichenko et al., 2014; Passos et al., 2015). Preclinical studies have also found that stress-induced peripheral expression of the pro-inflammatory cytokine interleukin-6 (IL-6) plays a significant role in the recruitment of monocytes to the brain and development of behavioral abnormalities in mice exposed to repeated social defeat stress (Hodes et al., 2014; Niraula et al., 2019). Supporting a role for peripheral IL-6,

the administration of a monoclonal IL-6 antibody, which does not enter the brain, effectively prevented the emergence of stress-induced social avoidance behavior (Hodes et al., 2014). Importantly, the concept that peripheral cytokine release may play a role in the development of stress-related behavioral changes gains support from the finding that "sickness behavior" – a term used to describe the abnormal behavior (i.e., social withdrawal) that characterizes physically ill individuals and animals – may be triggered by pro-inflammatory cytokines produced at the site of injury and/or infection (Dantzer et al., 2008). Collectively, these studies suggest that the expression of abnormal behavioral responses to stress may also be mediated by peripheral cytokine release, which could be targeted by enhancing peripheral FAE-mediated signalling through peripheral FAAH inhibition. In the present study, we exposed male rats to acute social defeat, a paradigm that captures key aspects of the human response to psychosocial stress (Huhman, 2006; Koolhaas et al., 2013), and we inhibited peripheral FAAH activity after exposure to the stressor, using the selective, brain-impermeant FAAH inhibitor URB937 (Clapper et al., 2010; Moreno-Sanz et al., 2011). We tested the hypothesis that enhancement of peripheral FAE-mediated signalling promotes behavioral resilience after an acute social stress episode and explored potential associated changes in peripheral pro-inflammatory cytokine release.

2. Materials and Methods

2.1 General experimental design

Male Wistar rats were randomly assigned to social defeat stress (SDS) or control (CTR) procedure, as outlined below. Thirty min later, they received a single injection of vehicle or URB937. We administered the peripherally restricted FAAH inhibitor URB937 at a dose (3 mg/kg) known to cause marked FAAH inhibition in peripheral organs but no detectable effect in the brain (Clapper et al., 2010; Vozella et al., 2019).

On the following day, behavior was evaluated using either the social avoidance/approach (SAA) test or the elevated plus maze (EPM) test (Figure 1A). A separate group of SDS and CTR rats was euthanized 6 and 24 h after the procedure, and blood and brains were collected to measure plasma and brain concentrations of proinflammatory cytokines and FAEs, and plasma concentrations of corticosterone (Figure 1B). Experimental

procedures were approved by the Italian legislation on animal experimentation (D.L. 04/04/2014 n. 26, authorization n. 449/2017-PR). Analyses were performed under blinded conditions, and sample size was not predetermined. Behavioral tests were conducted between 9am and 10am. The study report follows ARRIVE guidelines (Kilkenny et al., 2010).

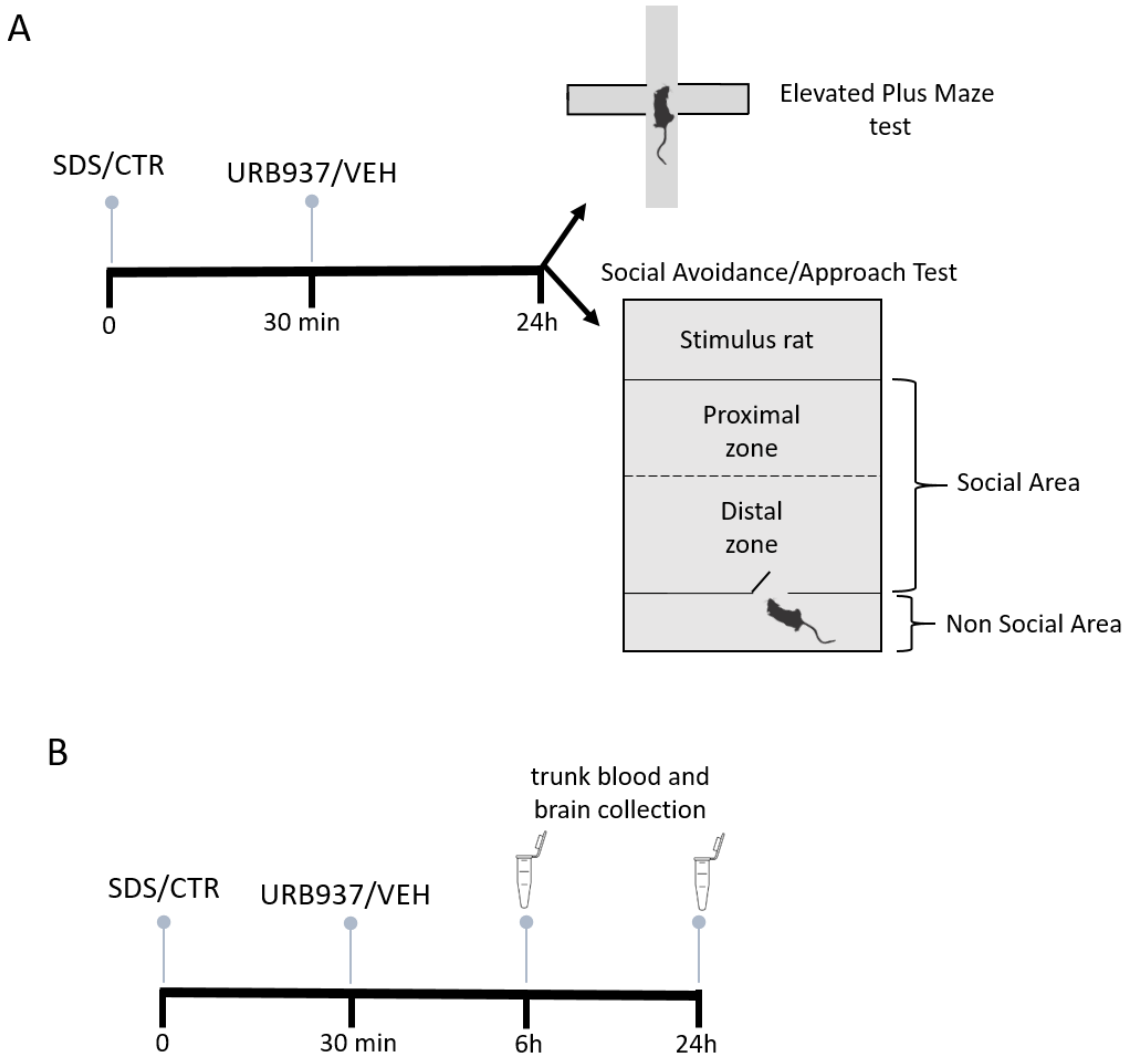

Figure 1. Timelines of experimental procedures. Abbreviations: SDS = social defeat stress; CTR= control; VEH = vehicle.

2.2 Chemicals

URB937 (N-cyclohexyl-carbamic acid, 3'-(aminocarbonyl)-6-hydroxy[1,1'-biphenyl]-3-yl ester) was synthesized as described in (Moreno-Sanz et al., 2014). All other reagents and solvents were of the highest available grade and were purchased from Sigma-Aldrich (St. Louis, USA).

2.3 Drug preparation

URB937 was freshly prepared by suspension into polyethylene glycol (PEG-400) and addition of an equal volume of Tween-80, as previously described in (Vozella et al., 2019). The mixture was vortexed and sonicated to obtain a clear solution. Sterile saline was added, and the final solution (10% PEG-400, 10% Tween-80 and 80% saline) was sonicated for 5 min at 37°C. URB937 and vehicle (VEH) were administered by intraperitoneal (IP) injection in a volume of 1 ml/kg.

2.4 Animals

3-month-old male Wistar rats (n=120; bred at the University of Parma) were singly housed under a 12-h reverse light/dark cycle (lights on at 7pm) and used as experimental animals. Additional 6-month-old male wild-type Groningen rats, each housed with an oviduct-ligated female partner, were used as aggressive residents in the resident-intruder paradigm. All animals were kept in climate-controlled rooms (temperature: 22°C and humidity: 50-60%).

2.5 Acute social defeat stress

The acute social defeat stress (SDS) model is based on a modified version of the classical “resident– intruder” paradigm (Koolhaas et al., 2013). Initially, resident rats were screened for their aggressive behavior before the beginning of the procedure. After the removal of the resident’s female partner, each rat from the SDS group was placed in the cage of a resident rat, separated from the latter by a wire mesh partition. Fifteen min later, the partition was removed allowing physical interaction – consisting of repeated attacks by the aggressive resident and subordination of the intruder – for additional 15 min. On the same day but in a separate room, CTR rats were placed in a novel and empty cage for 30 min. After the procedure, all rats were

returned to their home cages. Rats were closely inspected after SDS and none of them reported any improper injury or wound.

2.6 Behavioral tests

Both EPM and SAA were conducted 24 h after SDS or CTR procedure. The apparatus used in the EPM test consisted of a plus-shaped maze elevated (100 cm) above the floor, with two oppositely positioned open arms (25x10 cm) and closed arms (25x10 cm with 40 cm high walls) and a center area (10x10 cm). Each rat was placed on the central platform of the maze, facing an open arm, and behavior was video recorded for 5 min. A blinded observer measured the amount of time spent in and the number of entries into the open and closed arms. The anxiety index was calculated as previously described (Fotio et al., 2021): 1-[(time spent in open arms/total time) + (open arm entries/total entries)]/2. Between tests, the apparatus was cleaned with an ethanol solution (20% in water). The SAA test allows the evaluation of anxiety-like behaviors in rats exposed to a new social context (Haller & Bakos, 2002). The test was conducted as described in Carnevali et al., 2014). The experimental apparatus consisted of two chambers – a "non-social" (40×40×40 cm) and a "social" (20×40×40 cm) compartment – connected by a sliding door. The social chamber contained an enclosure (15×40×40 cm) delimited by a wire mesh partition in which a male stimulus-unfamiliar rat - of the same wild-type Groningen strain of the resident rats - was confined (Fig. 1A). On the test day, the rats were individually placed in the non-social chamber for a 2-min habituation period. The sliding door was then opened, and the animals were allowed to move freely for 10 min. Behavior was recorded using a video camera positioned above the apparatus. At the end of each test, the apparatus was thoroughly cleaned.

Behavior was scored by trained operators blinded to the experimental condition. For analyses, the social chamber was divided into two zones of equal size – proximal to and distal from the stimulus rat – as described (Carnevali et al., 2014). The following parameters were measured: (i) time spent in the non-social compartment, in the proximal zone and in the distal zone of the social compartment (expressed as % of total time), and (ii) latency (expressed as seconds) to the first access to the social compartment.

2.7 Biochemical measurements

Rats were euthanized 6 or 24 h after the SDS/CTR procedure, brains were removed, and trunk blood was collected into EDTA-coated test tubes (Sarsted AG, Numbrecht, Germany). Plasma was immediately prepared by centrifugation (2600 g; 4°C; 10 min) and stored at -20°C until analysis. Brains were stored at -80°C until analysis. Brain levels of IL-6 and IL-1β were measured by solid-phase sandwich ELISA kits for rat tissue lysates following the manufacturer's procedural guidelines (IL-6: catalog Reference: ERA32RB; IL-1β: catalog reference: ERIL1B; Thermo Fisher Scientific, Waltham, MA, USA). Plate absorbance was read at the wavelength of 450 nm employing a Tecan Spark 10M Multimode Plate Reader (Tecan, Wien, Austria). Plasma concentrations of IL-6, IL-1β and TNF-α were measured using the ProcartaPlex™ Multiplex Immunoassay kit (Thermo Fisher Scientific, USA).

Corticosterone was quantified in rat plasma by high performance liquid chromatography (HPLC) coupled to tandem mass spectrometry (MS/MS) employing a previously published bioanalytical method (Carnevali et al., 2015). A detailed description of the method and instrumental configuration is reported under Supplementary Material. A previously reported HPLC-MS/MS method was employed to quantify anandamide, OEA and PEA in brain tissue homogenates (10% w/v) and plasma (Carnevali et al., 2015; 2020). A detailed description of the HPLC-MS/MS method is reported in the Supplementary Material.

2.8 Statistical analyses

Statistical analyses were conducted using IBM SPSS Statistics, version 28 (IBM Corp., Armonk, N.Y., USA). The normal distribution of variables was determined using the Kolmogorov–Smirnov test. Data were analyzed using 2 ("group": stress or control condition) x 2 ("treatment": URB937 or VEH injection) factorial design analysis of variance, followed by pre-planned analyses using unpaired Student's t test with a Bonferroni correction for multiple comparisons for each outcome variable separately. Statistical significance was set at $p < 0.05$.

3. Results

3.1 URB937 prevents avoidance behavior evoked by SDS

SDS produced an evident social avoidance behaviour in the SAA test, which was prevented by administration of URB937 (3 mg/kg) 30 min after SDS exposure (Fig. 2). ANOVA showed a significant stress x treatment interaction for the time spent in the non-social compartment ($F_{(1,36)}$=3.9, $p < 0.05$) and in the proximal zone of the social compartment ($F_{(1,36)}$=4.12, $p < 0.05$). Specifically, SDS+VEH rats spent more time in the non-social area and less time in the proximal area of the social compartment ($p<0.05$) compared to CTR+VEH rats (Fig. 2A). Importantly, no such effects were observed in stressed rats treated with URB937 (SDS+URB). Subsequent analysis of the latency to first access to the social compartment (Fig. 2B) yielded a significant stress x treatment interaction ($F_{(1,36)}$=7.72, $p<0.01$). SDS+VEH rats showed a longer latency to the first access to the social area compared to CTR+VEH and SDS+URB animals ($p < 0.01$).

On the other hand, SDS did not produce any significant effect on anxiety-like behaviors in the EPM (Table 1).

The results suggest that inhibition of peripheral FAAH activity after exposure to acute SDS blocks the emergence of social avoidance behavior in male rats.

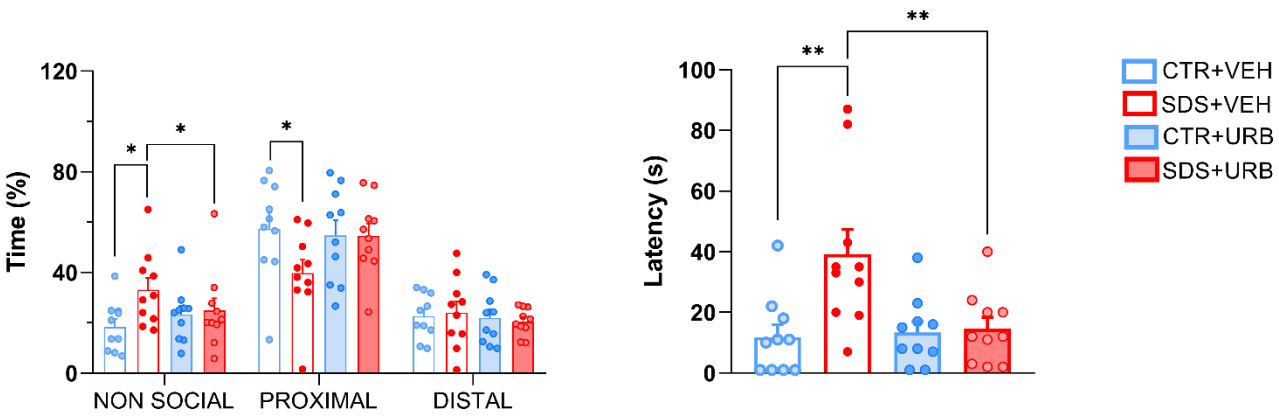

Figure 2. Behavior in the Social Approach/Avoidance test 24 h after social defeat stress/control procedure. (A) Time spent by experimental rats in the non-social and social (i.e., proximal to and distal to the stimulus rat, respectively) compartments of the social approach–avoidance apparatus. (B) Latency to the first access to the social compartment. Values are expressed as means (±SEM). Abbreviations: SDS = social defeat stress; CTR = control; VEH = vehicle; URB = URB937. * and ** = $p < 0.05$ and $p < 0.01$, respectively.

	Time in open arms (s)	Time in closed arms (s)	Open arms entries (n)	Closed arms entries (n)	Anxiety index
CTR+VEH	41.7 ± 12.7	258.3 ± 12.7	2.0 ± 0.5	5.5 ± 0.9	0.79 ± 0.05
SDS+VEH	38.5 ± 10.1	261.5 ± 10.1	2.3 ± 0.6	8.2 ± 0.8	0.83 ± 0.03
CTR+URB	46.7 ± 6.8	253.3 ± 6.8	3.3 ± 0.2	6.3 ± 0.6	0.76 ± 0.03
SDS+URB	34.5 ± 5.5	265.5 ± 5.5	2.2 ± 0.3	5.5 ± 0.6	0.80 ± 0.03

Table 3. Behavior in the Elevated Plus Maze test 24 h after social defeat stress/control procedure

Note. The anxiety index was calculated as 1-[(time in the open arms/total time) + (open arm entries/total entries)]/2 and is reported as mean ± SEM (n = 7/group). Abbreviations: SDS = social defeat stress; CTR = control; VEH = vehicle; URB = URB937.

3.2 URB937 prevents the increase in plasma corticosterone evoked by SDS

Next, we investigated whether peripheral FAAH inhibition might also prevent the rise in circulating corticosterone evoked by acute SDS. Male rats were exposed to SDS/CTR procedure and were euthanized 6 h or 24 h later for analysis. ANOVA revealed a significant effect of pharmacological treatment ($F_{(1,23)}$=4.18, $p<0.05$) and a marginally significant effect of stress ($F_{(1,23)}$=3.77, $p=0.06$) at the 24 h time point. Specifically, plasma corticosterone levels were significantly higher in SDS+VEH rats compared with CTR+VEH rats ($p<0.05$) and the effect of SDS was blocked by post-stress URB937 administration (Fig. 3B). Neither stress nor URB937 affected plasma corticosterone at 6 h (Fig. 3A). We interpret these findings as indicating that inhibition of peripheral FAAH activity after acute SDS attenuates the delayed hormonal change caused by this procedure in male rats.

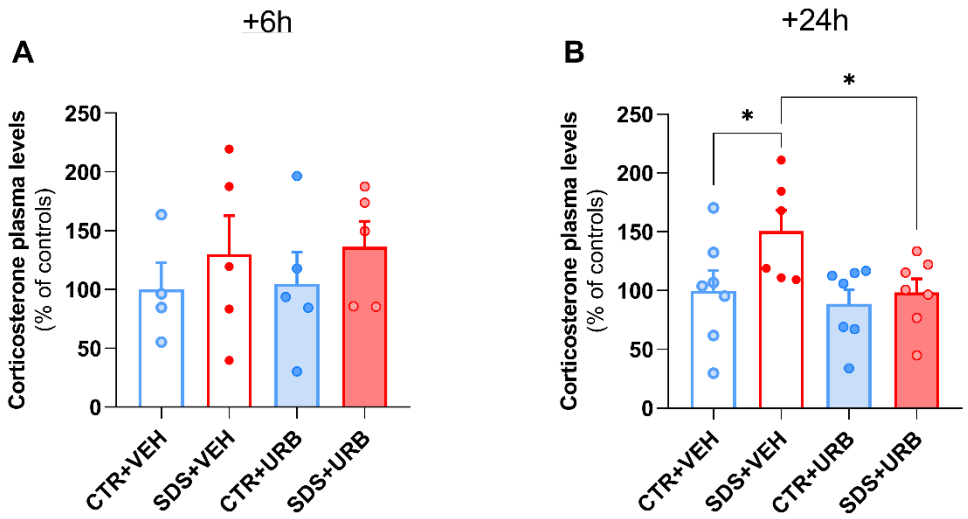

Figure 3. Plasma levels of corticosterone measured 6 h (A) and 24 h (B) after social defeat stress/control procedure. Values are expressed as means (±SEM). Abbreviations: SDS =social defeat stress; CTR = control; VEH = vehicle; URB = URB937. * = $p < 0.05$.

3.3 URB937 prevents the increase in circulating cytokines evoked by SDS

The release of pro-inflammatory cytokines may contribute to the pathological consequences of psychosocial stress (Hodes et al., 2014; Niraula et al., 2019). We examined therefore whether post-stress administration of URB937 might affect the plasma concentrations of three representative cytokines – IL-1β, IL-6, and TNF-α – 6 and 24 h following acute SDS. Six hours after the challenge, plasma levels of IL-6 ($p<0.01$) and TNF-α ($p<0.05$) were significantly higher in SDS+VEH than CTR+VEH rats (Fig. 4A). Likewise, plasma levels of IL-1β trended higher in SDS+VEH than CTR+VEH rats, although this difference did not reach statistical significance ($p=0.07$) (Fig. 4a). Cytokine levels were normalized by treatment with URB937 (Fig. 4A) and receded at the 24h time point (Fig. 4B). SDS did not alter cytokines production in brain tissue (Table 2).

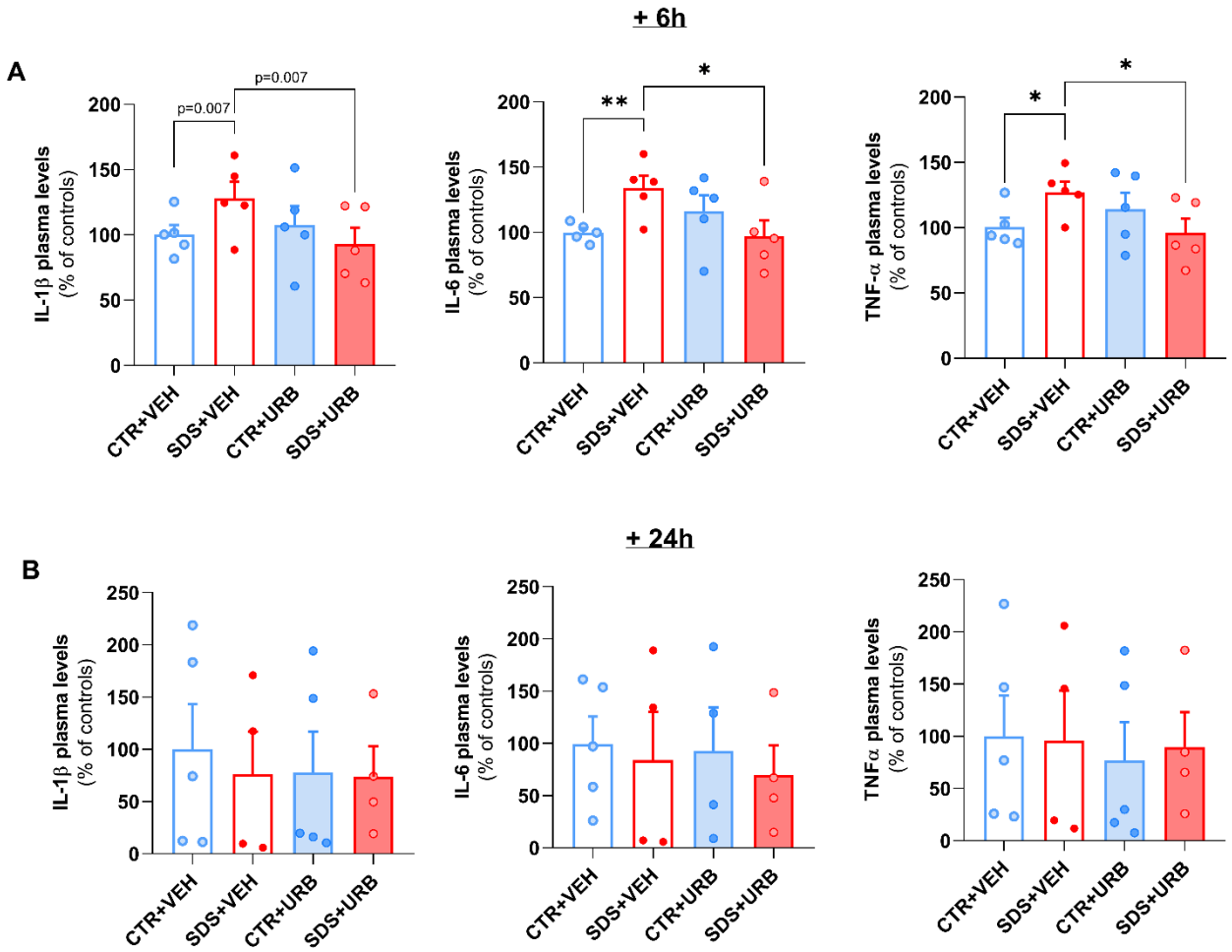

Figure 4. Plasma levels of IL-6, TNF-α and IL-1β measured 6 h (A) and 24 h (B) after social defeat stress /control procedure. Values are expressed as means (±SEM). Abbreviations: SDS = social defeat stress; CTR = control; VEH = vehicle; URB = URB937. * and ** = $p < 0.05$ and $p < 0.01$, respectively.

Table 2. Brain levels of pro-inflammatory cytokines after social defeat stress/control procedure

		+ 6h	+ 24h
IL-1β (% of controls)	CTR+VEH	100.0 ± 9.3	100.0 ± 8.1
	SDS+VEH	95.4 ± 6.3	92.4 ± 9.7
	CTR+URB	113.0 ± 12.0	88.4 ± 7.2
	SDS+URB	110.0 ± 22.7	86.1 ± 10.0
IL-6 (% of controls)	CTR+VEH	100.0 ± 5.9	100.0 ± 5.3
	SDS+VEH	97.6 ± 4.3	82.2 ± 14.7
	CTR+URB	108.3 ± 10.9	82.7 ± 7.4
	SDS+URB	111.7 ± 16.4	107.9 ± 3.4

Note. Brain levels of IL-6 and IL-1β measured 6 h and 24 h after social defeat stress or control procedure (n=5 per group). Values are expressed as means (±SEM). Abbreviations: SDS = social defeat stress; CTR = control; VEH = vehicle; URB = URB937.

3.4 URB937 inhibits peripheral, not central FAAH activity

Stress may affect the permeability of the blood-brain barrier (Menard et al., 2017) and might thus allow URB937, a substrate for the multi-drug transporters Abcg1 and Abcg2 (Moreno-Sanz et al., 2011, 2014), to access the CNS. To test this, we quantified AEA, OEA and PEA in plasma and brain of SDS and CTR rats 6 h and 24 h after the SDS/CTR procedure. The results indicate that post-stress administration of URB937 selectively increased concentrations of the three analytes in plasma (Fig. 5) but not in brain (Table 3). Two-way ANOVA showed a significant effect of treatment on circulating anandamide ($F_{(1,15)}$=17.1, $p < 0.01$), OEA ($F_{(1,15)}$= 82.1, $p < 0.01$), and PEA ($F_{(1,15)}$=307.9, $p<0.01$) 6 h after SDS (Fig. 5A), whereas no such change was seen in the brain (Table 3). As expected for this covalently acting agent, the effect of URB937 was still detectable 24 h after

administration (AEA: $F_{(1,23)}$=12.5, p<0.01; OEA: $F_{(1,24)}$=25.9, p<0.01; PEA: $F_{(1,24)}$ =38.1, p<0.01) (Fig. 5B). Of note, exposure to acute SDS did not significantly affect FAE levels in plasma (Fig. 5) or brain tissue (Table 3). *Ex vivo* enzyme activity measurements in brain and liver homogenates confirmed that URB937 selectively inhibited peripheral but not central FAAH activity (Table 4).

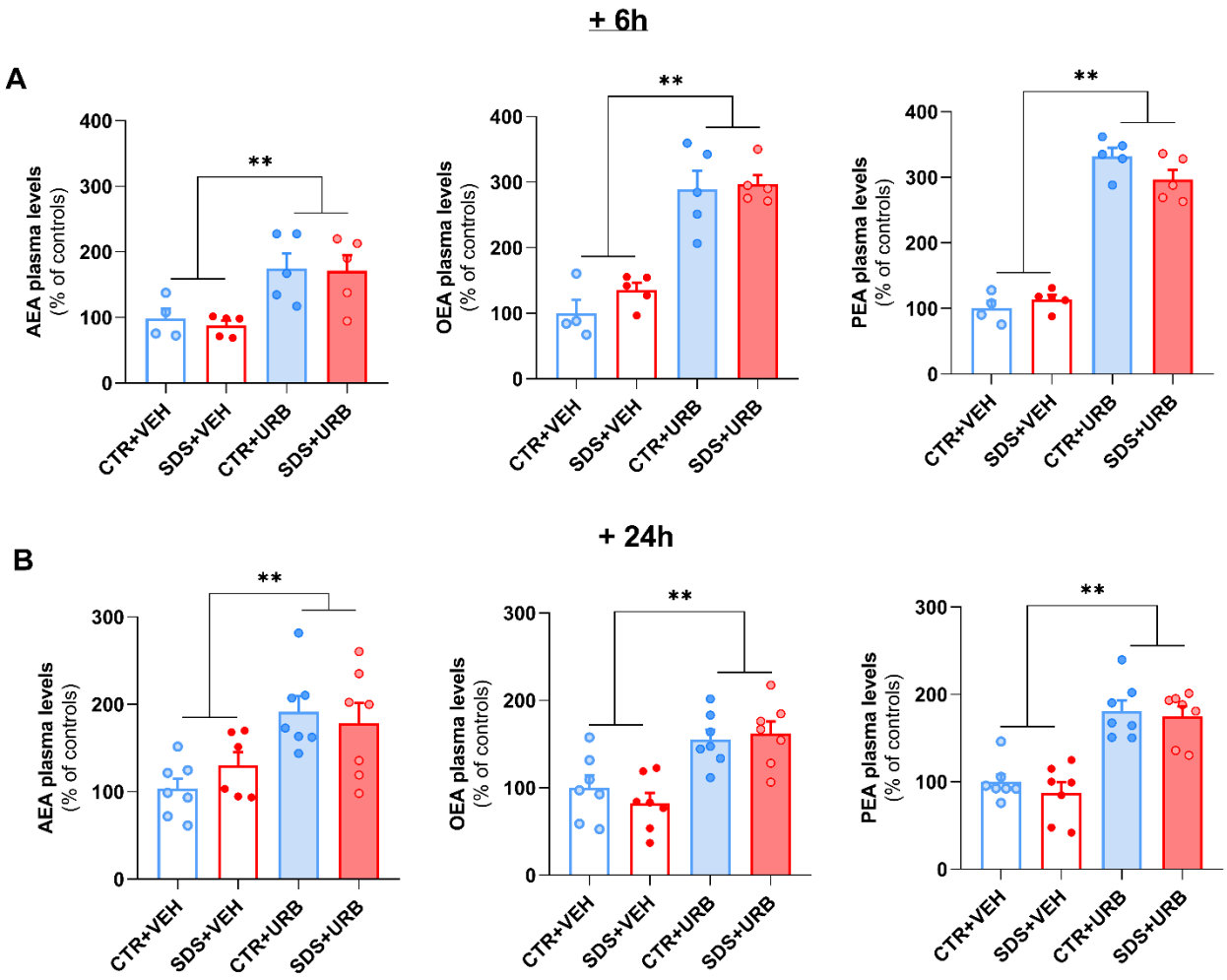

Figure 5. Plasma levels of fatty acid ethanolamide 6h (A) and 24 h (B) after social defeat stress/control procedure. Abbreviations: AEA = anandamide; OEA = oleoylethanolamide; PEA = palmitoylethanolamide. SDS = social defeat stress; CTR = control; VEH = vehicle; URB = URB937. Values are expressed as means (±SEM). ** = p < 0.01.

Table 3. Brain fatty acyd ethanolamide levels after social defeat stress/control procedure

		+ 6h	+ 24h
AEA (% of controls)	CTR+VEH	100.0 ± 8.8	100.0 ± 8.1
	SDS+VEH	88.1 ± 8.1	100.5 ± 7.3
	CTR+URB	112.4 ± 11.6	97.6 ± 10.7
	SDS+URB	105.3 ± 12.4	112.6 ± 8.2
OEA (% of controls)	CTR+VEH	100.0 ± 11.6	100.0 ± 4.5
	SDS+VEH	98.4 ± 9.1	116.7 ± 7.1
	CTR+URB	105.4 ± 8.5	98.5 ± 7.1
	SDS+URB	94.0 ± 6.2	123.3 ± 8.4
PEA (% of controls)	CTR+VEH	100.0 ± 7.0	100.0 ± 8.2
	SDS+VEH	94.9 ± 11.7	118.7 ± 11.9
	CTR+URB	98.9 ± 4.5	97.7 ± 9.2
	SDS+URB	105.3 ± 8.0	127.5 ± 11.7

Note. Brain levels of AEA, OEA and PEA measured 6 h and 24 h after social defeat stress or control procedure (n = 6-8 per group). Values are expressed as means (±SEM). Abbreviations: PEA = palmitoylethanolamide; AEA = anandamide; OEA = oleoylethanolamide; SDS = social defeat stress; CTR = control; VEH = vehicle; URB = URB937.

Table 4. FAAH activity in brain and liver homogenates after social defeat stress/control procedure

		Brain		Liver
		+ 6h	+ 24h	+ 24h
FAAH activity (% of controls)	CTR+VEH	100.0 ± 1.0	100.0 ± 8.8	100.0 ± 8.0
	SDS+VEH	99.7 ± 1.0	89.0 ± 6.5	82.9 ± 4.8
	CTR+URB	99.0 ± 1.8	71.9 ± 3.5	17.2* ± 4.3
	SDS+URB	98.8 ± 1.7	89.6 ± 9.1	22.5* ± 5.4

Note. Values are reported as means ± SEM (n = 6/8 per group). Abbreviations: FAAH = fatty acid amide hydrolase; SDS = social defeat stress; CTR = control; VEH = vehicle; URB = URB937. * = $p < 0.05$ versus corresponding VEH value.

4. Discussion

In the present study, we investigated the contribution of peripheral FAAH-regulated FAE signalling to the response to social defeat stress, a rodent model known to induce behavioral and biological phenotypes related to trauma exposure (Verbitsky et al., 2020). Peripheral FAAH activity was inhibited after exposure to the stressor by administering the brain-impermeant inhibitor URB937 (Clapper et al., 2010; Vozella et al., 2019), whose selectivity for FAAH outside the CNS was verified by LC/MS-MS. The results show that post-stress treatment with URB937 normalized both social behavior and circulating levels of corticosterone and pro-inflammatory cytokines following social defeat. These findings suggest that peripheral FAAH-regulated FAE signalling may exert a previously unrecognized modulatory effect on behavioral responses to acute traumatic/stressful events, suggesting an alternative pharmacological approach outside the CNS to enhance behavioral stress resilience.

4.1 Behavioral and biochemical effects of social defeat stress exposure

The development of social avoidance 24 h after social defeat stress was not paralleled by signs of generalized anxiety-like behavior as assessed by the EPM test. This finding is in line with previous work showing that repeated social defeat disrupts social behavior in mice but does not affect performance in the EPM test (Hodes et al., 2014). This likely reflects the fact that the EPM test is more suitable for capturing general anxiety-like behavior than the SAA test (Walf & Frye, 2007), which is designed to model anxiety-like behavior in an environmental and social context that reevokes the previous exposure to social defeat (Haller & Bakos, 2002). Importantly, one of the most recognized symptoms of PTSD in humans is the avoidance of stimuli associated with traumatic event exposure (American Psychiatric Association, 2013). Therefore, signs of social avoidance behavior observed in male rats previously exposed to a social defeat episode may recapitulate the behavioral changes that predispose an individual to the development of PTSD. Furthermore, rats exposed to acute social defeat stress exhibited a transient increase in plasma concentrations of pro-inflammatory cytokines (IL-6, TNF-α, IL-1β), which was found to be significant 6 h after social defeat. The 6-h interval was chosen based on previous work in mice showing that several cytokines (including those measured in the

current study) reach peak levels between 6 and 12 h after a stressful event (Cheng et al., 2015). These findings are consistent with other studies documenting an increase in peripheral pro-inflammatory cytokines after acute and chronic stress exposure in rodents, as well as after acute psychological stress in healthy humans (Ando et al., 1998; Marsland et al., 2017). On the other hand, elevated plasma corticosterone levels were found in stressed rats 24 h but not 6 h after social defeat, which may be indicative of prolonged activation of the hypothalamic-pituitary-adrenal (HPA) axis after social defeat stress. The reason for the lack of a significant difference in corticosterone levels at 6h between SDS-exposed and CTR rats is not fully understood. However, this may be attributed to the stress experienced also by CTR animals – albeit to a lesser extent - after exposure to a novel cage and i.p. injection. Of note, other studies have reported a temporal dissociation between general HPA axis stress responses and stress-induced increases in proinflammatory cytokines as in the present investigation (e.g., Hodes et al. 2014). Notably, elevated peripheral markers of inflammation have been described in patients with PTSD and anxiety disorders (Passos et al., 2015) and rodent studies have strongly suggested that peripheral inflammatory cells play a causative role in the establishment of anxiety- and depressive-like symptoms (Hodes et al., 2014; Niraula et al., 2019; Wohleb et al., 2015a). The CNS has historically been viewed as an immune-privileged organ, in which adaptive immunity and inflammation are tightly controlled. However, peripheral mediators can influence CNS function, and potentially behavior, in several ways: for example, cytokines can cross the blood-brain barrier (Banks et al., 1991, 1994), activate primary afferent nerves (e.g., vagal nerve; (Bluthé et al., 1994)) or indirectly promote both microglia activation and myeloid cells recruitment to the brain (Engler et al., 2004; Wohleb et al., 2014). In the present study, brain cytokines levels did not differ between CTR and socially stressed rats. However, evidence from various rodent models suggests that peripheral monocyte trafficking to the brain may promote the development of anxiety-like behaviors following stress exposure (Wohleb et al., 2014). It is plausibile to speculate that elevated cytokine levels might have played a similar role also in our experiment. Although we don't present direct evidence to either support or refuse this hypothesis, several preclinical studies have established a causal link between monocyte trafficking to the brain and anxiety-like behavior. For example, inhibition of monocyte egress from bone marrow prevented the development of anxiety-like behavior in

mice exposed to repeated social defeat (Engler et al., 2004; Wohleb et al., 2011). Similarly, monocyte trafficking to the brain was not observed in stressed mice lacking the IL-1 receptor type-1 and showing a behavioral resilient phenotype (Wohleb et al., 2014).

4.2 Effects of peripheral FAAH inhibition with URB937 after exposure to a traumatic/stressful event

Previous studies have shown that systemic FAAH inhibitors such as URB597 enhance behavioral resilience in rodent models of acute and chronic stress and exhibit profound anxiolytic- and antidepressant-like properties, which have been attributed – based on pharmacological, genetic, and biochemical data – to increased AEA availability and heightened activation of CB1 receptors in stress-controlling circuits of the CNS (Bortolato et al., 2007; Carnevali et al., 2017b; Gobbi et al., 2005; Kathuria et al., 2003). Notably, the current study provides first evidence that administration of the brain-impermeant FAAH inhibitor URB937 immediately after social defeat normalizes social behavior and blunts the stress-dependent rise in circulating levels of corticosterone and pro-inflammatory cytokines. Further, confirming prior work (Clapper et al., 2010; Moreno-Sanz et al., 2011), URB937 increased the levels of three functionally significant FAEs – the endocannabinoid AEA and the endogenous PPAR-α agonists OEA and PEA – in plasma and not in the brain. These results have three important implications: first, they suggest that peripheral mechanisms contribute to the behavioral response to stress; second, they suggest that the widely documented effects of systemic FAAH inhibitors on behavioral adaptations to stress may, at least partly, be ascribed to their peripheral action; third, they warrant further investigation on the utility of enhancing peripheral FAE levels as a strategy to counteract the negative behavioral sequelae of stress.

An important question raised by the data obtained in the present study pertains to the specific mechanism through which peripheral FAE signalling modulates the response to stressful events. Two non-exclusive scenarios seem particularly plausible. Enhanced AEA-mediated activation of CB1 receptors on peripheral noradrenergic nerve endings might dampen sympathetic outflow (Martínez-Torres et al., 2023; Pakdeechote et al., 2007), which is expected to impact the release of corticosterone and pro-inflammatory cytokines

(Jänig, 2014). Alternatively, or additionally, enhanced PEA/OEA-mediated stimulation of PPAR-α – which are highly expressed in monocytes, macrophages, and other inflammatory cells (Grabacka et al., 2021)– might suppress the peripheral reaction to stress. Contextually, it is important to point out that (i) circulating PEA levels are decreased in persons with PTSD (Hauer et al., 2013b), as well as in healthy subjects experiencing a short-term depressed mood (Darmani et al., 2005); (ii) PEA adjunctive therapy to citalopram improves symptoms in patients with depression (Ghazizadeh-Hashemi et al., 2018); and (iii) intense physical activity improves depression and PTSD symptoms while concomitantly elevating plasma PEA levels (Heyman et al., 2012).

4.3 Conclusions

The present report provides the first evidence that peripheral FAAH-regulated lipid signalling protects male rats from the behavioral and biochemical consequences of acute social stress, thus suggesting that pharmacological inhibition of FAAH activity outside the CNS may offer a new approach to the prevention of PTSD and other trauma-related diseases. These results are novel and relevant to our understanding of the potential contribution of peripheral processes in the normal and abnormal reactions to social challenges and to the discovery of innovative pharmacological strategies to foster behavioral resilience to stress.

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Supplementary Materials

FAAH activity in rat brain and liver homogenates

Ex vivo determination of FAAH activity in brain and liver tissue was carried out as previously reported (Clapper et al., 2009). [^{3}H]-AEA (specific activity: 60 Ci/mmol), employed as substrate for ex vivo FAAH assay, was supplied by American Radiolabeled Chemicals (ARC Inc., St. Louis, MI, USA).

Briefly, rat brain and liver tissue was weighted and homogenized in 10 volumes of 50 mM ice-cold Tris buffer, pH 7.4, containing 0.32 M sucrose. Resulting homogenates were centrifuged (1,000 X g; 10 min; 4°C) and total protein content was quantified in the supernatant by the bicinchoninic acid (BCA) protein kit (Pierce Biotechnology, Rockford, IL, USA).

FAAH activity was measured in Tris buffer (0.5 ml, 50 mM, pH 7.5) at 37°C in the presence of 0.05% w/v fatty acid-free bovine serum albumin (BSA), 50 μg of protein from brain or 10 μg of protein from liver homogenates, 10 μM AEA and [^{3}H]-AEA (10000 disintegrations per minute). After 30 min, enzymatic reaction was quenched by the addition of 1 ml chloroform:methanol (1:1 v/v). After centrifugation (2,000 X g; 10 min; 4°C), [^{3}H]-ethanolamine was measured in the aqueous phase by liquid scintillation counting.

HPLC-MS/MS quantification of corticosterone in rat plasma

Corticosterone (CORT) was quantified in rat plasma employing a previously developed HPLC-MS/MS bioanalytical method. Calibration curves were built in the concentration range 1500-10 nM by spiking charchoal treated rat plasma, pooled from control animals, with serially diluted stock solutions of CORT in DMSO (final DMSO concentration = 1%). Both calibration and unknown plasma samples were processed by addition of a double volume of ice-cold acetonitrile containing the structural analogue dexamethasone (DEXA) as internal standard (IS) at the concentration of 75 nM, centrifuged (16,000 X g, 10 min, 4 °C) and the supernatant directly analyzed by HPLC-MS/MS. The LOQ was equal to 10 nM for CORT. Calibration curves showed good linearity with coefficients of correlation (r^2) > 0.99. Compound-dependent parameters were optimized by flow injection analysis of 5 μM standard solutions of CORT and IS DEXA in methanol. HPLC-MS/MS analysis was carried out in positive ion (ESI+) and in multiple-reaction monitoring (MRM) mode. The

following parent ion → product ion transitions were monitored: CORT: m/z 347.1 $[M+H]^+$ → m/z 105.1 + m/z 121.1 + m/z 329.2 (TL: 106 V; CE: 32; 24; 13 eV); DEXA: m/z 435.2 $[M+H]^+$→ m/z 291.1 + m/z 309.2 + m/z 319.1 (TL: 95 V; CE: 18; 10; 11 eV). A Phenomenex Synergi Fusion Reverse Phase column (100x2.0 mm, 4 m particle size; Phenomenex Srl, Italy) was employed for gradient separation at a flow rate of 0.35 ml/min. Eluent A: acetonitrile + 0.1% v/v formic acid; eluent B: water + 0.1% v/v formic acid. Gradient conditions: t(0 min): 5%A:95%B; t(1 min): 5%A:95%B; t(6 min): 100%A:0%B; t(11 min): 100%A:0%B; t(12 min): 5%A:95%B, with a 3-min equilibration time; total run time: 15 min. Injected volume: 10µl.

HPLC-MS/MS quantification of fatty acid ethanolamides in rat brain and plasma

The fatty acid ethanolamides AEA, OEA and PEA were extracted from 10% w/v brain tissue homogenates by protein precipitation via acetonitrile addition, as previously reported (Carnevali et al., 2020b; Carnevali et al., 2015). Briefly, brain tissue was weighted and homogenized in Tris buffer (50 mM, pH 7.5, 0.32 M sucrose) to get a 10% w/v homogenate. For quantitative analysis, calibration curves were built in the 500-0.5 nM concentration range by spiking charcoal-treated rat plasma (pooled, from control animals) with freshly prepared stock solutions of AEA, OEA and PEA, serially diluted in DMSO (final DMSO concentration = 1%). Both calibration standards and unknown brain and plasma samples were processed by adding two volumes of ice-cold acetonitrile, containing 100 nM AEA-d_4, OEA-d_4 and PEA-d_4 as internal standards, and, after a centrifugation step (16,000 X g; 10 min; 4°C), levels of fatty acid ethanolamides were quantified by HPLC–MS/MS. Calibration curves showed good linearity with coefficients of correlation (r^2) > 0.99. The LOQ was equal to 0.5 nM for AEA, 2.5 nM for OEA and PEA. Compound-dependent parameters were optimized by flow injection analysis of 5 µM standard solutions in methanol. Acquisition occurred in positive ion (ESI+) and in multiple-reaction monitoring (MRM) mode. For quantitative analysis, the following parent ion → product ion transitions were selected: AEA: m/z 348.2 $[M+H]^+$→ m/z 62.1 + m/z 90.9 Tube Lens (TL): 54 V; Collision Energy (CE): 14; 42 eV; AEA-d_4: m/z 352.2 $[M+H]^+$→ m/z 66.1 + m/z 202.8 + m/z 269.9 (TL: 76 V; CE: 17; 12; 19 eV); OEA: m/z 326.3 $[M+H]^+$ → m/z 309.3 + m/z 93.3 + m/z 62.3 (TL: 115 V; CE: 10, 19 and 28 eV); OEA-d_4: m/z 330.1 $[M+H]^+$ → m/z 313.4 + m/z 66.2 (TL: 67 V; CE:14 and 15 eV); PEA: m/z 300.3 $[M+H]^+$ → m/z 62.3 (TL: 54

V; CE:14 and 42 eV); PEA-d_4: m/z 304.2 $[M+H]^+$ → m/z 287.1 + m/z 66.3 (TL: 72 V; CE: 12 and 15 eV). A Waters XSelect HSS T3 column (100x2.1 mm, 3.5 µm particle size; Waters Corp, USA) was employed for separation employing a linear gradient at a flow rate of 0.22 ml/min. Eluent A: acetonitrile+0.1% v/v formic acid; eluent B: water+0.1% v/v formic acid; t(0 min): 5% A:95% B; t(1 min): 5% A:95% B; t(6 min): 100%A:0% B; t(11 min): 100% A:0% B; t(12 min): 5% A:95% B, followed by a 3 min equilibration time; total run time: 15 min). Injected volume: 10µl.

HPLC-MS/MS system configuration

A Thermo Accela UHPLC gradient system coupled to a Thermo TSQ Quantum Max triple quadrupole mass spectrometer (Thermo Fisher, USA) equipped with a heated electrospray ionization (H-ESI) ion source was employed for the analysis of corticosterone in rat plasma samples and fatty acid ethanolamides in brain and plasma samples. H-ESI source tune parameters were set as follows: probe middle (D) position; capillary temperature: 270 °C; spray voltage: 4.0 kV. Nitrogen was the nebulizing gas at the following pressures: sheath gas: 35 psi; auxiliary gas: 15 arbitrary units (a.u.). Argon was used as the collision gas at a pressure of approximately 1.5 mtorr. The software Xcalibur version 2.2 (Thermo, Madison, WI, USA) was employed for HPLC-MS/MS data acquisition and processing.

Chapter 4

Brief Summary and Discussion

This thesis reports two studies focusing on FAAH inhibition (and the consequent enhancement of FAE-mediated signals) as a pharmacological strategy to foster stress resilience. These investigations delve into the impact of this approach on different potential physiopathological pathways that have garnered significant attention in stress-related psychopathologies, such as depression and PTSD.

Specifically, in **Chapter 2**, the pathway under investigation is the intricate crosstalk between gut microbial and lipidic environments. The disruption of this interplay can detrimentally affect gut physiology, triggering inappropriate brain-gut axis signalling and subsequent consequences for CNS function, potentially leading to psychopathological conditions, such as depressive syndromes (Russo et al., 2018). In this context, FAEs are emerging as pivotal players in regulating the crosstalk between gut microbiota and lipids, opening a new avenue for therapeutic exploration. Therefore, a chronic social defeat stress model in male rats was employed to evaluate the effects of the systemic FAAH inhibitor URB694 on the gut microbial and lipidic profiles. This stress model effectively mirrored human depressive symptoms and simultaneously led to significant alterations in both gut microbiota and lipidic composition. Importantly, we found mutual correlations between gut lipids and microbiota, suggesting that social stress affects the complex relationship between the microbial and lipidic intestinal environment. Systemic FAAH inhibition via URB694 normalized stress-induced behavioral and neuroendocrine changes, corroborating previous findings available in literature (Carnevali et al., 2015). Most notably, URB694 promoted stability within the gut microbiota and lipidome, pointing to a potential mechanism underlying the beneficial effects of FAE-mediated signalling enhancement achieved through FAAH inhibition. Previous research has implicated FAEs in the modulation of the gut lipidome and microbiota. Notably, a study by (Geurts et al., 2015) revealed that the genetic deletion of NAPE-PLD, a key enzyme involved in FAE synthesis, induce alterations in lipid metabolism and gut microbiota in mice. Building upon these insights, our study marks the first demonstration that the favorable outcomes resulting from the augmentation of FAE signaling (obtained through FAAH inhibition) are closely linked to the protective effects on both the gut microbiota and lipidome. Some limitations of this study should be acknowledged: firstly, our findings do not pinpoint the specific FAE-mediated signal(s) responsible for these beneficial effects, which requires clarification through future investigations; secondly, we did not

perform a lipidomic analysis on brain tissue. It would be intriguing to assess the impact of FAAH inhibition on the brain's lipidomic profile, particularly in regions susceptible to stress exposure (e.g., prefrontal cortex, hippocampus, and striatum). In fact, rats exposed to chronic unpredictable stress exhibited changes in brain lipid composition, such as alterations in glycerophospholipids (Faria et al., 2014) . This parallels our findings in the gut, prompting curiosity about whether FAAH inhibition could also promote stability of lipidic profiles in specific brain areas.

However, our primary focus centered on the crosstalk between lipids and microbiota in the intestinal environment – a critical peripheral pathway deserving further scrutiny - in a preclinical model of chronic social stress.

Looking ahead, the advent of peripherally restricted FAAH inhibitors presents an intriguing prospect. The availability of compounds that do not permeate the brain challenges us to explore whether their peripheral action can elicit comparable positive effects on gut microbiota and lipidomic and ultimately behaviour in preclinical stress models.

A "peripheral" approach was adopted in the study described in **Chapter 3**, where male rats were treated with a brain-impermeant FAAH inhibitor (URB937) after exposure to a single episode of social defeat stress. The acute stress experienced by animals elicited signs of social avoidance, which were accompanied by heightened levels of specific pro-inflammatory cytokines in the periphery. It is important to note that our findings do not establish a causal association between behavioral changes and cytokine response. Nonetheless, these results lend support to the growing body of evidence suggesting that peripherally derived cytokines can influence behavioral responses, challenging the conventional notion of the brain as immune privileged. Notably, the research work of Kipnis and colleagues in the recent years has suggested that the brain is surrounded by several anatomical sites in which neuro-immune crosstalk can occur (Rustenhoven & Kipnis, 2019, 2022). For instance, unlike the specialized vasculature of the blood-brain barrier (which highly expresses tight junctions proteins), meningeal vasculature enables immune trafficking (Rustenhoven et al., 2021). In our study, we did not detect any differences in brain levels of cytokines between stressed and

control rats, but several studies have suggested that monocyte trafficking to the brain may promote the establishment of anxiety-like behaviors (Wohleb et al., 2015b). In future investigations, it would be intriguing to explore whether a single episode of social defeat stress could prime such a peripheral monocyte response and if these immune cells could access the CNS via a "meningeal route".

Interestingly, the behavioural response witnessed in the Social Avoidance/Approach test may resemble the avoidance of the stimuli linked to the traumatic experience (i.e., social defeat), akin to patterns often seen in patients diagnosed with PTSD. Moreover, we found a transient significant stress-induced increase in pro-inflammatory cytokines levels, consistent with previous preclinical and clinical findings (Cheng et al., 2015; Renner et al., 2022). Notably, we provided first evidence indicating that enhancement of peripheral FAE-mediated signalling, through peripheral FAAH inhibition, was able to normalize behavior and the pro-inflammatory cytokine response to acute stress. Even if we did not elucidate the molecular mechanism(s) through which peripheral FAE-dependent signalling may modulate the response to stress and we did not establish a causative link between cytokine levels and behavioral readouts, these findings pave the way for the implementation of peripheral interventions as a viable approach to mitigate the negative repercussions of stress exposure. Peripheral pharmacological strategies present the exciting possibility of bypassing the undesirable side effects often associated with psychotropic drugs.

Both studies were based on the idea that beneficial effects of FAAH inhibition comes from the augmentation of the signaling of the endocannabinoid AEA. However, in both cases, we found a greater impact of FAAH inhibition on non-cannabinoid FAEs, namely OEA and PEA. These findings further fortify the emerging "expanded" perspective of the endocannabinoid system, including several mediators biochemically related to endocannabinoids, their receptors and metabolic enzyme (Cristino et al., 2019). In the context of FAAH inhibition, this novel perspective is undeniably more complex, yet it better captures the impact of this pharmacological strategy, which cannot disregard other intriguing FAE-mediated pathways, such as those related to PEA and OEA. Indeed, it would be reductionist to think that the favorable effects resulting from FAAH inhibition in the context of stress-related pathologies are solely attributed to the signaling mediated by a single compound, namely AEA. This perspective disregards the fact that in mammalian tissues, AEA is

accompanied by its congeners, which are normally present at higher concentrations and exhibit intriguing biological activities (Waluk et al., 2014).

The impact of FAAH-inhibitors on different pathways should not be considered as a limitation. Instead, this is particularly helpful when it comes to stress-related psychopathologies. Indeed, pathologies such as depression and PTSD are characterized by high heterogeneity, which make their treatment very difficult. In this context, pharmacological multifaceted approaches may become very useful.

This thesis is mainly focused on peripheral processes that, starting from periphery, may contribute to the negative consequences of stress exposure and ultimately affecting behavior and complex central processes.

In the current dissertation, FAAH inhibition has been demonstrated to affect two important peripheral pathways, which have been shown to play a crucial role in stress-related psychopathologies: gut microbiota and peripheral inflammation. The findings presented herein challenge the conventional view of the brain as an isolated entity governing the body's development and functioning. Instead, these studies highlight the intricate bidirectional communication between the brain and the periphery, suggesting that the brain is not merely the conductor, but also a highly responsive participant in this dynamic interplay.

By embracing this paradigm shift, we open new avenues for therapeutic interventions and a deeper understanding of the mechanisms underlying stress-related disorders. The evidence presented underscores the potential of targeting peripheral processes as means to modulate central functions. The outcomes of FAAH inhibition on non-cannabinoid FAEs, such as OEA and PEA, further emphasize the complexity of the endocannabinoid system and its interconnected pathways.

As we move forward, it is imperative to extend these insights to the realm of clinical applications. The multifaceted nature of stress-related psychopathologies calls for innovative and comprehensive approaches. Integrating peripheral interventions alongside central strategies could pave the way for personalized and more effective treatments, addressing the heterogeneity inherent in conditions like depression and PTSD.

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APPENDIX A (of Chapter 3)

Peripheral FAAH inhibition and Heart Rate Variability

1. Introduction

Heart Rate Variability (HRV) is a measure of beat-to-beat variability in heart rate over time and it is recognized as a non-invasive indicator of autonomic nervous system activity (Jung et al., 2019). This metric underscores the intricate connection between the heart and the brain. Variability within the heart rate is regulated by the sympathetic nerves, which accelerate heart rate, and the parasympathetic (vagus) nerves, which slow it down. Optimal cardiac function is characterized by high parasympathetic modulatory activity - and thus increased variability - protecting the heart against adverse cardiac events such as heart failure and myocardial infarction (Wood, 2014). The modulatory activity of the autonomic nervous system on the heart is in turn regulated by various cortical and subcortical pathways, involving the PFC, the anterior cingulate cortex, the insula, the hypothalamus and the brainstem (Shaffer et al., 2014; Thayer et al., 2009). Alterations in HRV have been noted in various mental disorders, including depression, anxiety and PTSD (Jung et al., 2019). Notably, there is high comorbidity between depression and cardiovascular disease (Carnevali et al., 2017a) and a large body of evidence indicate that reduced HRV is often associated with depressive symptoms (Hartmann et al., 2019; Kemp et al., 2010), while elevated sympathetic tone and reduced parasympathetic tone have been suggested as psychophysiological signs of PTSD (Jung et al., 2019). In fact, multiple investigations have documented lower resting HRV in patients diagnosed with PTSD compared to healthy individuals (Blechert et al., 2007; Ge et al., 2020; Meyer et al., 2016). In line with this, a meta-analysis by Chalmers and colleagues based on 36 articles - including 2086 patients with an anxiety disorder and 2294 controls - reported that patients with anxiety disorders (PTSD, generalized anxiety disorder or social anxiety disorder) exhibit lower HRV (i.e., reduced vagal modulation) in comparison to their respective controls (Chalmers et al., 2014).

Moreover, PTSD is known to have a strong inflammatory component, as evidenced by elevated levels of pro-inflammatory markers in affected individuals (Hori & Kim, 2019). Intriguingly, a comprehensive meta-analysis involving more than 150 studies reported strong negative associations between HRV parameters and inflammatory markers (Williams et al., 2019), confirming the theory that the autonomic nervous system plays a substantial role in the regulation of the so-called "inflammatory reflex" (Tracey, 2002).

The connection between HRV and psychopathologies is further confirmed by preclinical studies. Rodents exposed to several stress paradigms often display anxiety-like and depressive-like symptoms in association with reduced HRV (Grippo et al., 2012; Sévoz-Couche et al., 2013; Wood, 2014).

As extensively reported in this thesis, the potentiation of the endocannabinoid AEA and other FAE-mediated signals through FAAH inhibition represents a promising therapeutic tool to treat stress-related psychopathologies (Carnevali et al., 2017b).

Considerable evidence suggests the involvement of the ECS in cardiovascular functions, including blood pressure regulation, cardiac contractility and heart rate (Sierra et al., 2018). CB1 and CB2 receptors are both expressed in myocardium (Bonz et al., 2003; Defer et al., 2009), vascular endothelium (J. Liu et al., 2000; Ramirez et al., 2012) and vascular smooth muscle cells (Rajesh et al., 2008a,b). The well documented bradycardic and hypotensive effects of AEA are mainly mediated by CB1, even though alternative non-CB pathways have been described (Kunos et al., 2000), confirming once again in intricate complexity of the expanded ECS. AEA cardiovascular effects involve the parasympathetic and sympathetic system, both of which express CB receptors. For instance, CB1 activation via AEA has been found to inhibit noradrenaline release by sympathetic nerve terminals (Ishac et al., 1996), suggesting a potential contribution of ECS in the autonomic modulation of cardiac activity.

Involvement of ECS in cardiovascular physiology is further underscored by the fact that chronic marijuana use leads to sustained reductions in blood pressure and heart rate, while acute use raises heart rate without affecting blood pressure (Gorelick et al., 2006; Pacher et al., 2005). Cannabis-induced cardiovascular effects are largely related to its biphasic effect on the autonomic nervous system, depending on the dose absorbed (Fisher, 2005).

The cardiovascular implications of the ECS involve a range of factors, potentially encompassing modulation of autonomic outflow, through inhibition of sympathetic neurotransmission or stimulation of parasympathetic pathways in the heart (Ishac et al., 1996; Szabo et al., 1998), as well as direct effect on the myocardium and vasculature (Pacher et al., 2008).

Preclinical studies suggest that systemic pharmacological inhibition of FAAH yields anxiolytic and antidepressive-like effects, improves cardiac autonomic function and the electrical stability of the myocardium in rodent models mirroring human psychological and cardiac comorbidity (Carnevali et al., 2015a,b; 2017). Therefore, the analyses proposed below aimed to investigate whether the favorable effects of the peripheral FAAH inhibitor URB937 on behavioral and biochemical parameters in rats exposed to acute stress (as detailed in **Chapter 3**) extend to HRV modulation.

2. Methods

2.1 Animals

Twenty 3-month-old male Wistar rats were used for this experimental protocol. Animals were housed in individual cages and kept at ambient temperature of 22±2 °C on a reversed 12:12 light-dark cycle (light on at 7:00 pm), with food and water available *ad libitum*. Ten additional 8-month-old Wild-type Groningen rats were housed in a separate room with an oviduct-ligated female partner and they served as residents in the resident-intruder paradigm.

2.2 Experimental protocol

This experiment was designed in order to investigate the effect of peripheral FAAH inhibition with URB937 on HRV in rats exposed to an acute episode of social defeat stress (SDS). The timeline of the experimental protocol is illustrated in Figure A1.

Briefly, all Wistar rats were implanted with radio-telemetric transmitters for ECG recordings. After two weeks of recovery from the surgery, ECG signals were recorded for 48 hours prior the start of the experiment. After basal recordings, rats were exposed to a single episode of SDS. Thirty minutes after SDS, rats were injected either with vehicle (VEH, 1 ml/kg i.p.; N=10) or URB937, (3 mg/kg i.p.; N=10). ECG signals were recorded after SDS for 5 hours. Details of specific experimental procedures are described in the next sections.

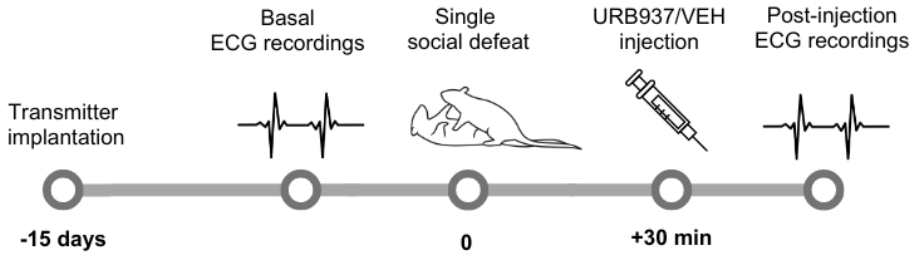

Figure A11. General experimental protocol

2.3 Transmitter implantation and radio-telemetry system

Animals were implanted under isoflurane anaesthesia (2% in 100% oxygen) with radio-telemetric transmitters (TA11CTA-F40, DataScience International, St. Paul, MN, USA) following a surgical procedure previously described by (Sgoifo et al., 1996), in order to record ECG signals (sampling frequency: 100 Hz). The transmitter body was placed in the abdominal cavity, with one electrode fixed to the dorsal surface of the xyphoid process and the other placed in the anterior mediastinum near the right atrium. This electrode location ensures high-quality ECG recordings, even during intense physical activity. After the surgery, rats were given a 15-day recovery period.

2.4 Acute Social defeat stress

The acute SDS model is based on a modified version of the classical "resident– intruder" paradigm (Koolhaas et al. 2013) and was performed as described in the **Paragraph 2.5 of Chapter 3**.

2.5 Chemicals and drug preparation

The chemicals used in this experimental protocol, as well as the procedure used to dissolve the drug (URB937) are the same of those described in **Paragraphs 2.2 and 2.3 of Chapter 3**.

2.6 ECG: data acquisition and analysis

Continuous ECG recordings were performed for 48 consecutive hours in baseline conditions (with the rats in their home cages), immediately after SDS, and after URB937/VEH injection.

Abnormal heart beats and artifacts were removed from ECG signals. HRV is quantified by analysis of variations of the intervals between consecutive R wave peaks (i.e., heart beats). The time-course of the RR intervals is plotted in a graph called "tachogram" and further quantitative analyses of this curve allows the determination of HRV parameters (Shaffer & Ginsberg, 2017). Tachograms are corrected for ectopic events and missed beats before processing.

HRV quantifications methods can be divided in two main categories: time and frequency domains. Time domains calculate the amount of variability present in a specific time in a continuous ECG recording. Among time-domain parameters, in this study we employed the root mean square of successive differences between adjacent RR intervals (RMSSD, ms). To obtain this parameter, each difference between successive RR intervals is squared, summed, the result averaged and then the square root obtained. RMSSD is commonly recognized as a vagal index because it quantifies the short- term fluctuations of the RR interval, which are due to parasympathetic activity (Stein et al., 1994). The "frequency domain" method is based on the spectral analysis of the tachogram. The oscillatory components of the spectrum are obtained using a non-parametric algorithm, the fast Fourier transform. In the present study, we focused on the high frequency (HF, ms^2) component (0.75-2.5 Hz), which reflects parasympathetic activity and is highly correlated with the RMSSD time-domain measure (Shaffer & Ginsberg, 2017).

ECG recordings were acquired on a computer with ART-Gold data acquisition system (DataSciences Int., St. Paul, MN, USA) and analyzed using ChartPro 5.0 software (ADInstrument, Sydney, Australia). Each ECG recording was divided in 3-minutes temporal segments. Specifically, we examined HRV parameters of 5 hours (from 10 am to 3pm) of the successive 48 hours in baseline condition, 30 minutes post-SDS and 5 hours (from 10 am to 3 pm) post-injection, to record the animals during their dark/active phase.

2.7 Statistical analysis

Statistical analyses were performed using the IBM SPSS statistical package (International Business Machines Corporation, Armonk, NY, USA, version 28). Normal distribution of variables was checked by means of the Kolmogorov–Smirnov test. ANOVA for repeated measures was used to analyze heart rate (HR) and HRV data (expressed as the variations from basal values, Δ) after VEH/URB937 injection with "time" as within-subject factor (9 levels: 30,60,90,120,150,270 minutes after injection) and "treatment" as between-subject factor (two levels: VEH and URB937). Follow-up analyses were conducted using a Bonferroni correction for multiple comparisons.

A separate ANOVA for repeated measures was applied to analyze HR and HRV data before and after SDS with "time" as within-subject factor (2 levels: basal and post-SDS) and "treatment" as between-subject factor (two levels: VEH and URB937). Follow-up analyses were conducted using a Bonferroni correction for multiple comparisons.

The statistical significance was set at $p < 0.05$. Data are reported as means ± standard error of the mean (SEM).

3. Results

3.1 Heart Rate and Heart Rate Variability after URB937/VEH injection

Basal levels of HR, RMSSD and HF was similar in the two groups (table A1). When comparing baseline HR, RMSSD and HF values with those recorded during the thirty minutes after SDS (before the injection) in both groups, repeated measures ANOVA yielded a significant effect of time for HR ($F_{(1,18)}$=37,53, $p<0.001$), RMSSD ($F_{(1,18)}$=28.51, $p<0.001$) and HF ($F_{(1,18)}$=21.15, $p<0.001$). Specifically, there was a significant increase in HR in both groups after SDS compared to baseline condition ($p<0.01$ for both groups) and a stress-induced significant reduction in RMSSD ($p<0.01$ for both groups) and HF ($p<0.01$ for both groups).

Delta values of HR, RMSSD and HF after URB937/VEH injection are represented in Fig. A2 (panel A). ANOVA for repeated measures yielded a significant effect of time ($F_{(8,144)}$=3.07, p=0.02), but not a significant

interaction between treatment (URB937 or VEH) and time for delta HR values. Consequently, the variations of HR compared to basal levels was similar between SDS+URB and SDS+VEH at every time-point considered. Similar results were obtained for vagally-mediated HRV indexes, namely RMSSD and HF (Fig. A2, panel B and C). ANOVA for repeated measures yielded a significant effect of time (RMSSD: $F_{(8,144)}$=6.77, p=0.001; HF: $F_{(8,144)}$=4.39, p=0.004), but not a significant interaction between treatment (URB937 or VEH) and time for delta RMSSD and HF values. Again, delta values of both vagal indexes were similar between SDS+URB and SDS+VEH at every time-point analyzed.

Basal levels	**SDS+VEH (n=10)**	**SDS+URB (n=10)**
HR (bpm)	401.12±8.68 bpm	395.98±7.17 bpm
RMSSD (ms)	3.11±0.22 ms	3.68±0.20 ms
HF (ms^2)	3.44±0.46 ms^2	3.77±0.29 ms^2

Table A5. Basal levels of HR, RMSSD and HF. Baseline values represented the average values of two consecutive dark phases (from 10 am to 3 pm).

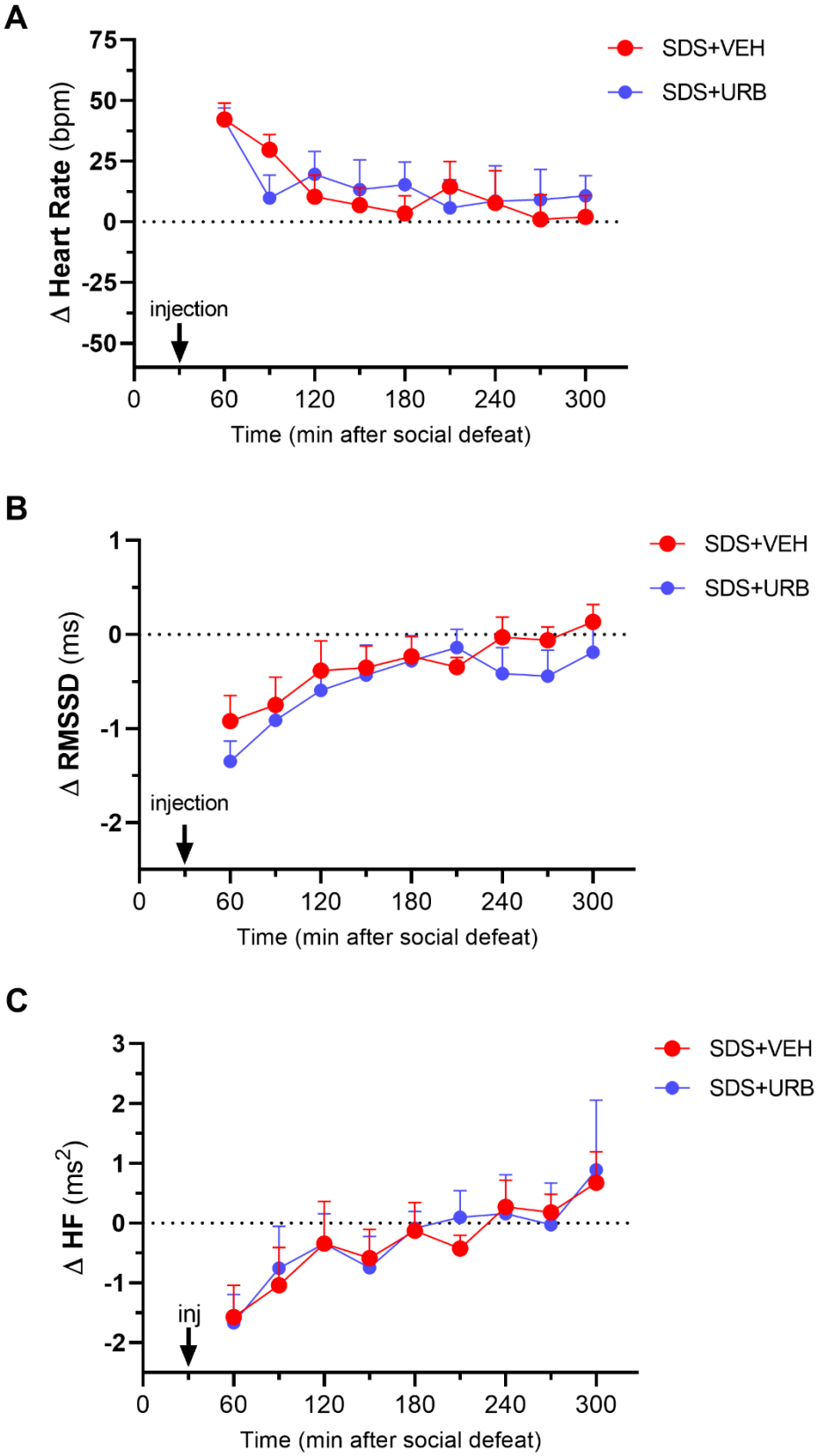

Figure A12. **Delta Heart Rate (HR) and HRV after URB937/VEH injection**. Time course of changes in heart rate (A), RMSSD (B) and HF (C) (compared to basal levels) after URB937 (n=10) or VEH (n=10) injection. Every point in the graph represents the mean value of 30-minutes recordings. Data are reported as means ± SEM.

4. Summary of Results

These findings show that SDS produces an enduring positive chronotropic effect on the heart, as evidenced by increased HR and reduced RMSSD values (compared to baseline) in the first hours after SDS exposure, confirming previous works (Barbetti et al., 2022; Park et al., 2017). We did not detect any modulatory effects of acute peripheral FAAH inhibition on heart rate and vagal indexes. These supplementary results suggest that the beneficial effects described in stressed rats treated with URB937 (outlined in **Chapter 3**), might not be ascribed to an impact on vagal activity, and thus to a modulation of the stress-induced cardiac autonomic activation.

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raggiungerla sia il pensiero costante.

Soprattutto, non affrettare il viaggio;

fa che duri a lungo, per anni, e che da vecchio

metta piede sull'isola, tu, ricco

dei tesori accumulati per strada

senza aspettarti ricchezze da Itaca.

Itaca ti ha dato il bel viaggio;

senza di lei, mai ti saresti messo sulla via."

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