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CIRCADIAN RHYTHMS AND SEX DIFFERENCES SET ENDOCANNABINOIDS TO INFLUENCE MEMORY UNDER STRESS

Alessia Santori

Director of the PhD Program
Prof. Silvana Gaetani

Supervisor
Prof. Patrizia Campolongo

Thesis Committee
Prof.ssa Beatrice Maria Passani
Prof. Giuseppe Battaglia
Prof. Carlo Polidori

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"Time present and time past,
Are both perhaps present in time future,
And time future contained in time past."

T.S. Eliot, Burnt Norton (1935)

CONTENTS

GENERAL INTRODUCTION

Although forgetting is the common fate of most of our experiences, the ability to learn and remember is essential for our survival. Remembering what has happened enables us to predict what is likely to happen and alter our behavior accordingly (McGaugh, 2013). The neurocircuitry underlying emotional memory involves brain regions that belong to the corticolimbic system, including the medial prefrontal cortex (mPFC), the amygdala, and the hippocampus (Campolongo et al., 2009; Roozendaal et al., 2009; Barsegyan et al., 2010; Atsak et al., 2012a; Fastenrath et al., 2014).

The finding that emotion influences memory at multiple levels has been so far consistent in both human and animal studies, with emotional arousal enhancing long-term memory consolidation, when experienced during or after learning, while impairing memory retrieval, when stress exposure occurs before memory retention testing, through a modulation that involves both norepinephrine and glucocorticoids (de Quervain et al., 1998, 2017; Roozendaal, 2002). Despite most studies have focused on the effects of stress before or after learning, or eventually before memory testing, extensive evidence demonstrated that stress can influence subsequent memory also if it is presented after retrieval, thus revealing its additional role in the modulation of extinction and/or reconsolidation processes (Morena et al., 2018; Morena and Campolongo, 2014).

How stress influences memory depends on when and by what an individual is stressed (Schwabe et al., 2012). Stress intensity and memory performance are known to follow an inverted U-shaped relationship, with maximal memory strength at an intermediate level of stress (Yerkes and Dodson, 1908). Furthermore, whereas memory performance associated with complex cognitive tasks is sensitive to stress in an inverted-U fashion, simple forms of emotional memory induced by traumatic experiences can be strong and persistent (Finsterwald and Alberini, 2014). In this regard, literature evidence revealed

that the noradrenergic system might be responsible for the persistence of traumatic memories in stress-related disorders, such as post-traumatic stress disorder (PTSD) (Liu et al., 2019).

To date, many studies demonstrated that drugs of abuse influence the physiological modulation of mnemonic functions of the hippocampus, dorsal striatum and amygdala through the activation of the noradrenergic and dopaminergic neurotransmissions (LaLumiere et al., 2005; McGaugh and Roozendaal, 2009), by indirectly regulating other memory systems (Goodman and Packard, 2016).

Among the most known psychostimulants, amphetamine and the "bath salt" 3,4-methylenedioxypyrovalerone (MDPV) have been shown to have an impact on memory retention processing in rodents by differentially modulating the noradrenergic and dopaminergic systems (Roozendaal et al., 2008; Atehortua-Martinez et al., 2019). However, their role in memory generalization was poorly investigated.

Dysfunctional information processing is a common feature of stress-related disorders like PTSD, which is characterized by abnormal consolidation and retrieval, overgeneralization, and insufficient extinction of traumatic memories (Sherin and Nemeroff, 2011; Bian et al., 2019). Hereinbefore, the study of the influence of drugs of abuse on the quality of memory has increasingly acquired attention, thus making demanding to determine which mechanisms can alter the perception of reality, with severe consequences on both the consolidation and generalization of memories.

In humans, time-of-day influences mental activities, such as mood (McClung, 2013), vigilance (Maire et al., 2018) and cognitive throughput (Chellappa et al., 2018), so it may not be surprising that memory also falls under circadian control, with different cognitive functions showing optimal performance at different times of the day, including memory retrieval, which usually declines in the late afternoon-early evening (Ebbinghaus, 1985). While this effect has been recognized for decades, the underlying neurobiological mechanisms are currently not completely understood.

The suprachiasmatic nucleus (SCN) in the anterior hypothalamus serves as the master pacemaker that sets the timing of rhythms by regulating neuronal activity, body

temperature and hormonal signals (Colwell, 2011). Disruption of circadian rhythms is associated with altered stress response (Koch et al., 2017) and higher risk of several neurodevelopmental disorders such as attention-deficit hyperactivity disorder (ADHD) and autism spectrum disorders (ASDs) (Logan and McClung, 2019). Moreover, disrupted circadian timekeeping and altered daily hormone release profiles were reported in conditions involving memory impairment, including old age (Krishnan and Lyons, 2015; Reinhart and Nguyen, 2019), Alzheimer's disease (Videnovic et al., 2014), major depression, bipolar disorder and psychosis (Jones and Benca, 2015). Much of the currently available evidence linking brain disorders to circadian dysfunction is correlational. Less clear is the exact nature of time-of-day effects on the modulation of memory under stress: both enhancing and impairing effects have been reported (Hauber and Bareiß, 2001; Gritton et al., 2012; Shahmoradi et al., 2015; Snider and Obrietan, 2018; Hasegawa et al., 2019; Flyer-Adams et al., 2020; Meseguer Henarejos et al., 2020; Poveda et al., 2020).

Considerable evidence indicates that the endocannabinoid signaling plays a key role in many fundamental physiological processes that are altered in a circadian manner (Vaughn et al., 2010), simultaneously regulating both the activation of the hypothalamus-pituitary-adrenal (HPA) axis (Patel et al., 2004) and the termination of the stress response (Di et al., 2003). The endocannabinoid system consists in a neuromodulatory lipid signaling that is widely distributed throughout the corticolimbic circuits that are linked to stress response (Hill et al., 2010b) and represents one of the main systems modulating both the norepinephrine- and the glucocorticoid-dependent modulation of emotional memories (Campolongo et al., 2009; Atsak et al., 2012b; Morena et al., 2014; Atsak et al., 2015; Morena et al., 2015, 2016; Wyrofsky et al., 2019).

The two major endocannabinoids, N-arachidonylethanolamide (anandamide, AEA; Devane et al., 1992) and 2-arachidonoyl glycerol (2-AG; Sugiura et al., 1995) are retrograde messengers that are synthesized "on demand" in the postsynaptic membrane by Ca^{2+}-dependent and -independent mechanisms (Kano et al., 2009) and feedback onto

presynaptic terminals, thus modulating both excitatory and inhibitory signaling within specific neuronal circuits that are implicated in learning and memory processes for emotionally arousing experiences via cannabinoid type 1 and 2 (CB1 and CB2) receptor activation (Akirav, 2013; Morena et al., 2014; Tasker et al., 2015; Ratano et al., 2017). After being released into the synaptic cleft, AEA and 2-AG are primarily degraded by distinct hydrolytic enzymes, the fatty acid amide hydrolase (FAAH; Cravatt et al., 2001) and monoacylglycerol lipase (MAGL; Dinh et al., 2002), respectively.

Compelling evidence indicates that drugs that target the endocannabinoid system induce biphasic effects on cognitive and emotional behavior depending on the level of stress and emotional arousal at the time of encoding and drug consumption (Morena and Campolongo, 2014). Stress influences the endocannabinoid system with effects that are complex, regionally specific, and time-dependent relative to stress exposure and its chronicity (Morena et al., 2016). However, the interaction between stress and the endocannabinoid system has typically been investigated in the same time window (Campolongo et al., 2013; Gunduz-Cinar et al., 2013; Morena et al., 2015, 2019; Hartley et al., 2016), thus leaving unexplored the influence of time-of-day on the glucocorticoid-endocannabinoid crosstalk modulation of stress effects on memory (Balsevich et al., 2017).

Chronic stress exposure might lead to circadian rhythm dysfunctions, which in turn alter HPA axis activity and glucocorticoid concentrations, possibly causing severe brain disorders like major depression and PTSD (Koch et al., 2017; Steinach and Gunga, 2020). In PTSD, hippocampal-dependent memory is compromised while amygdala-dependent memory is abnormally strengthened (Segev et al., 2018). The discovery of stress hormone receptors in the hippocampus has fostered research showing that this brain structure is crucially involved in the negative feedback regulation of the HPA axis (McEwen, 2013). Recent evidence demonstrated that optogenetic stimulation of the BLA projections to the ventral hippocampus regulates anxiety-mediated behavior (Felix-Ortiz et al., 2013) and emotional, but not contextual, memory consolidation (Huff et al., 2016). Simultaneously, manipulations of the GABAergic interneurons

within the ventral hippocampus ↔ BLA neurocircuitry were reported to be effective to modulate memory function in different protocols of fear conditioning (Herry et al., 2008; Müller et al., 2012). Hereinbefore, only an important study reported that the connectivity between the dorsal hippocampus and the BLA is crucial to induce bidirectional switch of memory valence (Redondo et al., 2014), thus opening the avenue to further investigate the role of different components of the amygdalohippocampal circuits in fear memory processes.

Stress – in its many forms – is generally perceived as an excessive demand on human psychological and/or physiological adaptive capabilities, and can have a direct influence on different physiological, biological and behavioral processes due to the integration of neuronal and hormonal pathways of the stress reaction and the circadian regulation (Steinach and Gunga, 2020).

Experiments investigating the circadian aspect of acute or chronic stress have frequently suggested that sex differences in the circadian timing system are critical to unveil core mechanisms regulating the response to both endogenous and exogenous stress factors (Bailey and Silver, 2014), with potential important implications for understanding behavior and physiology (McCarthy et al., 2012). Morphological sex differences in the SCN are well established in both animals and humans (Bailey and Silver, 2014). Therefore, circadian rhythms have been suggested to differentially influence both acute stress response (Verma et al., 2010; Bangasser and Wiersielis, 2018) and anxiety (Verma et al., 2010; Meseguer Henarejos et al., 2020) in rodent males and females that were tested in different behavioral paradigms.

Recent evidence has shown that the endocannabinoid signaling regulates plasticity within the amygdala-mPFC circuit under stressful experiences (Marcus et al., 2020), and fundamental sex differences have been identified within this amygdala-mPFC fear circuit, underlying differences in fear expression (Gruene et al., 2015). To date, little is known about whether endocannabinoids regulate fear extinction in females.

Outline

A large amount of evidence indicates that stress exposure triggers the brain processing through different specific pathways that converge in both norepinephrine- and glucocorticoids-dependent regulation of memory processes by influencing central noradrenergic mechanisms (de Quervain et al., 1998; McGaugh and Roozendaal, 2002). The amygdala has long been known to be the hub of fear memory, which is usually remembered over time (Fanselow and LeDoux, 1999; Roozendaal et al., 2009). However, when an aversive stimulus occurs, it might happen that the accuracy of such emotional memory could be distorted progressively, leading to memory generalization (Asok et al., 2019). Drugs of abuse were identified to alter the experience of reality, thus affecting memory processes (Goodman and Packard, 2016).

Chapter 1 explores more in deep the role of the psychostimulants amphetamine and MDPV in the modulation of memory strength and accuracy in a previously validated model exploiting the inhibitory avoidance discrimination task, in order to assess fear memory generalization for a novel/safe, yet not identical, context that was not used to induce shocks (Atucha and Roozendaal, 2015). Previous studies indicated that both amphetamine and MDPV, through different mechanisms of action, increase brain monoamines release, particularly norepinephrine and dopamine, two neurotransmitters extensively involved in the modulation of memory (LaLumiere et al., 2005; McGaugh and Roozendaal, 2009). Therefore, Chapter 1 investigates the involvement of the noradrenergic and dopaminergic systems in mediating the amphetamine effects on memory strength and both amphetamine and MDPV effects on fear memory generalization.

Extensive evidence demonstrates that norepinephrine is crucially involved in the regulation of long-term memory consolidation for emotionally arousing experiences (Ferry et al., 1999; McGaugh and Roozendaal, 2002; Roozendaal et al., 2008; Lalumiere et al., 2017; Chen et al., 2018). It is widely recognized that amphetamine

enhances the consolidation of memory processing in both humans and rodents (Soetens et al., 1993; Sanday et al., 2013). **Chapter 2** evaluates the influence of different intensities of stress on the amphetamine modulation of long-term memory consolidation, further characterizing the involvement of any stress-induced activation of the peripheral adrenergic response in such process.

The endocannabinoid system plays a key role in the control of emotional responses to environmental challenges (Morena and Campolongo, 2014). CB1 receptors are abundantly expressed within corticolimbic regions, including the basolateral complex of the amygdala (BLA), hippocampus and mPFC (Hill et al., 2011).
Glucocorticoids are stress response mediators which interact with the endocannabinoid system in the regulation of memory function (Campolongo et al., 2009; Hill et al., 2010a; Atsak et al., 2012a; Morena et al., 2016; Balsevich et al., 2017), with an emotional buffer outcome in such interaction (Morena and Campolongo, 2014). Their synthesis is characterized by a circadian release pattern, with peak levels linked to the start of the activity phase and diurnal regulation under control of the circadian clock (Dickmeis, 2009). Literature evidence indicated that the endocannabinoid signaling exhibits a circadian rhythm with variations reported in CB1 receptor expression (Rueda-Orozco et al., 2008), endocannabinoids tissue contents and in the enzymes controlling their synthesis and degradation (Valenti et al., 2004). **Chapter 3** investigates how different stress intensities, soon after encoding, influence rat short-term memory in an object recognition task, whether the effects depend on circadian rhythm and if exogenous augmentation of AEA levels restores any memory impairment provoked by stress exposure.

Exposure to stress alters both hippocampal anatomy and functionality (McEwen, 1999), with negative consequences on memory processes (de Kloet et al., 2018). Indeed, the hippocampus represents a key forebrain structure highly associated with emotional and recognition memory processes (Broadbent et al., 2010). According to the timing of

stress exposure, stress-mediated secretion of glucocorticoids alters hippocampal functions and plasticity (Kim et al., 2015), thus affecting hippocampal-dependent memories in rodents and humans (Donley et al., 2005). Furthermore, previous findings from our laboratory have demonstrated the involvement of the 2-AG signaling in counteracting the stress-mediated impairments on memory function (Morena et al., 2014, 2015; Ratano et al., 2018). By adding on Chapter 3 findings, **Chapter 4** highlights that stress impairing effects on short-term recognition memory depend on time-of-day in a stress intensity-dependent fashion and examines if different stress intensities affect the hippocampal endocannabinoid system components, whether the effects are time-of-day-dependent, and if boosting 2-AG signaling ameliorates memory performance.

Excessive fear and anxiety are hallmarks of a variety of disabling psychiatric disorders (Myers and Davis, 2007). The neurocircuitry of fear memory involves the BLA as the key region modulating the acquisition, retrieval and extinction of fear response (Johansen et al., 2011; Adolphs, 2013; Herry and Johansen, 2014; Zelikowsky et al., 2014), by receiving inputs from somatosensory cortex, thalamus, and hippocampus that encodes contextual information and compares current contextual cues to previously encoded memories (Maren and Quirk, 2004). **Chapter 5** evaluates whether the endocannabinoids AEA and 2-AG, in the BLA or the CA1 region of the dorsal hippocampus, differentially regulate fear memory retrieval depending on the environment-associated emotional arousal, if these outcomes are mediated by indirect activation of CB1 and/or CB2 receptors, and whether the BLA-dorsal CA1 interplay plays any role in such effects.

Women are twice as likely as men to develop PTSD making the search for biological mechanisms underlying these gender disparities especially crucial (Breslau, 2009). One striking feature of PTSD is the alteration in the ability to extinguish fear responses to trauma-associated cues (Yehuda et al., 2015). In male rodents, the endocannabinoid system can modulate fear extinction and has been suggested as a therapeutic target for

PTSD (Morena et al., 2018; Segev et al., 2018). **Chapter 6** investigates whether exogenous augmentation of the endocannabinoids AEA and 2-AG in male and female rats affect fear expression and extinction, which is the role of CB1 and transient potential receptor of vanilloid type-1 channel (TRPV1) receptors in such mediation, and how the endocannabinoid machinery within the amygdala, PFC and periaqueductal grey (PAG) is influenced post-extinction.

Chapter 7 provides a review of the existing literature regarding the effects of time-of-day on memory function, shedding light on the underlying mechanisms of contrasting results by portraying how stress-dependent modulation of memory is influenced by circadian rhythms. Chapter 7 also focuses on the interaction between the endocannabinoid system and the level of stress associated to the experimental context / previous aversive experiences and capitalizes on our recent findings that a manipulation of the endocannabinoid system might be capable to effectively modulate the circadian-dependent effects of stress on memory and to prevent its detrimental effects on memory function.

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CHAPTER 1

AMPHETAMINE AND THE SMART DRUG 3,4-METHYLENEDIOXYPYROVALERONE (MDPV) INDUCE GENERALIZATION OF FEAR MEMORY IN RATS

Paola Colucci[1,2], Giulia Federica Mancini[1,2], Alessia Santori[1,2], Clemens Zwergel[3,4], Antonello Mai[3], Viviana Trezza[5], Benno Roozendaal[6,7], Patrizia Campolongo[1,2]

[1]Dept. of Physiology and Pharmacology, Sapienza University of Rome, Rome, Italy; [2]Neurobiology of Behavior Laboratory, Santa Lucia Foundation, Rome, Italy; [3]Dept. of Drug Chemistry & Technologies, Sapienza University of Rome, Rome, Italy; [4]Dept. of Medicine of Precision, University of Campania Luigi Vanvitelli, Naples, Italy; [5]Dept. of Science, Section of Biomedical Sciences and Technologies, University Roma Tre, Rome, Italy; [6]Dept. of Cognitive Neuroscience, Radboud University Medical Center, Nijmegen, The Netherlands; [7]Donders Institute for Brain, Cognition and Behavior, Radboud University, Nijmegen, The Netherlands

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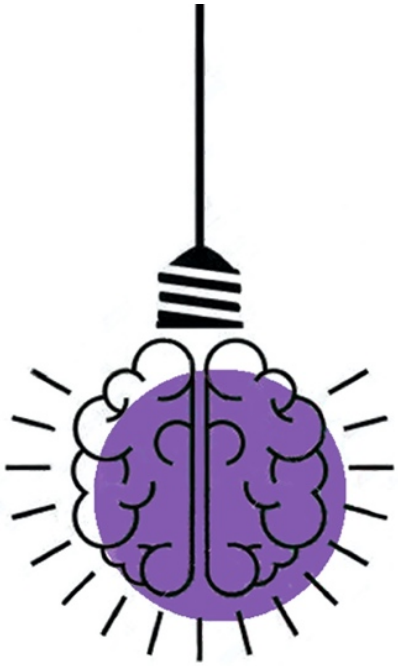

Abstract

Human studies have consistently shown that drugs of abuse affect memory function. The psychostimulants amphetamine and the "bath salt" 3,4-methylenedioxypyrovalerone (MDPV) increase brain monoamine levels through a similar, yet not identical, mechanism of action. Findings indicate that amphetamine enhances the consolidation of memory for emotional experiences, but still MDPV effects on memory function are under investigated. Here, we tested the effects induced by these two drugs on generalization of fear memory and their relative neurobiological underpinnings. To this aim, we used a modified version of the classical inhibitory avoidance task, termed *inhibitory avoidance discrimination* task. According to such procedure, adult male Sprague–Dawley rats were first exposed to one inhibitory avoidance apparatus and, with a 1-min delay, to a second apparatus where they received an inescapable footshock. Forty-eight hours later, retention latencies were tested, in a randomized order, in the two training apparatuses as well as in a novel contextually modified apparatus to assess both strength and generalization of memory. Our results indicated that both amphetamine and MDPV induced generalization of fear memory, whereas only amphetamine enhanced memory strength. Co-administration of the β-adrenoceptor antagonist propranolol prevented the effects of both amphetamine and MDPV on the strength and generalization of memory. The dopaminergic receptor blocker cis-flupenthixol selectively reversed the amphetamine effect on memory generalization. These findings indicate that amphetamine and MDPV induce generalization of fear memory through different modulations of noradrenergic and dopaminergic neurotransmission.

Introduction

Drugs of abuse are characterized by rewarding effects induced by the engagement of specific pathways in the brain (McHugh and Kneeland, 2019). Such rewarding effects are the principal reason that moves people to a compulsive use of these substances, which frequently ends with drug dependence (Koob, 2017). It has long been observed in humans that the intake of drugs of abuse affects memory processes (Kutlu and Gould, 2016; Goodman and Packard, 2016). More specific studies conducted in laboratory animals have been focused on which neurobiological and biochemical pathways are exploited by drugs of abuse to influence memory. Amphetamine, one of the most well-known psychostimulants, has been shown to enhance the consolidation of memory processing in rodents (McGaugh, 1973; Martinez et al., 1980a; Martinez et al., 1980b; Roozendaal et al., 1996; McGaugh and Roozendaal, 2009). We recently demonstrated that the 3,4-methylenedioxypyrovalerone (MDPV), a newer synthetic cathinone also known as "bath salt", enhances short-term spatial and recognition memory performance (Atehortua-Martinez et al., 2019). Moreover, it has been shown that MDPV induces a disruption of functional connectivity networks (i.e., striatum) involved in cognitive processes (Colon-Perez et al., 2016). This new psychostimulant has recently emerged in the illegal market as a smart drug and it rapidly became highly popular (Prosser and Nelson, 2012; Baumann et al., 2017). However, its fame is also associated with several important adverse effects, and among these, long-term cognitive impairments in humans have been documented (Karila et al., 2015). One in vitro study on MDPV activity demonstrated that it has a similar, yet not identical, mechanism of action compared to amphetamine. Indeed, both drugs of abuse have the same molecular targets represented by the norepinephrine (NE), dopamine (DA) and serotonin re-uptake transporters (NET, DAT and SERT, respectively), but MDPV displays greater potency than amphetamine with regard to DA re-uptake transport (Baumann et al., 2013). Amphetamine effects on memory consolidation are dependent on its pharmacological action which increases NE and DA release (Martinez et al., 1983; Fleckenstein et al.,

2007; LaLumiere et al., 2005; Roozendaal et al., 2008). Very recently, it has been shown that the effect on short-term memory induced by MDPV is linked to D1 dopaminergic receptor activation (Atehortua-Martinez et al., 2019). The role of noradrenergic and dopaminergic neurotransmission on memory, especially for the consolidation phase, is well established (LaLumiere et al., 2005; Roozendaal et al., 2008; Wideman et al., 2018; Quaedflieg and Schwabe, 2018; Schwabe, 2017). Although it has been demonstrated that both amphetamine and MDPV can affect memory retention, no evidence exists on whether such drugs can also affect the quality of memory. The study about the influence of drugs of abuse on the quality of memory increasingly acquired attention during last century and is just nowadays growingly becoming an intriguing issue, even if up to date there are only sparse studies (Koriat et al., 2000; Oeberst and Blank, 2012; Hoscheidt et al., 2014; Loftus, 2005, Horry et al., 2014; Carter et al., 2013; Easton and Bauer, 1997; Ballard et al., 2012). However, the study of the mechanisms through which drugs of abuse affect memory quality could be a riveting topic, mainly in the light of increasing evidence that drugs of abuse (e.g. psychedelic drugs, hallucinogens) can alter the experience of reality (Bohling, 2017). Such altered perception might be one of the causes why some people are prompted to a recreational use of such substances (Moro et al., 2011; Kjellgren and Soussan, 2011), thus making it an important and urgent issue to be investigated. Emotions have a considerable impact on memory (Tyng et al., 2017), for example, when an aversive stimulus occ urs, the associated fear leads to remembering the information over time (Rogan et al., 1997), but sometimes the accuracy of such emotional memory can be altered and distorted over time, eventually leading to memory generalization (Asok et al., 2018). This emotional/fear generalization effect has been studied for many decades through the contextual fear conditioning paradigm (Rohrbaugh and Riccio, 1968; Ruediger et al., 2011). Recently, a novel experimental model suitable to investigate both strength and accuracy of memory has been validated for rodents (Atucha and Roozendaal, 2015, Atucha et al., 2017): the inhibitory avoidance discrimination task. This task allows to evaluate whether fear memory associated with footshock can be generalized to a novel and safe,

yet similar. context. Hence, the aim of the present study was to investigate whether the two psychostimulants amphetamine and MDPV affect generalization of fear memory to a novel and safe yet similar context using an inhibitory avoidance discrimination task. Since both amphetamine and MDPV modulate NE and DA tone, we also aimed at evaluating the involvement of the noradrenergic and dopaminergic systems in mediating the effects of amphetamine and MDPV on fear memory generalization.

Materials and Methods

Animals and procedures

Male adult Sprague-Dawley rats (320–370 g at the time of behavioral experiments) from Charles River Laboratories (Calco, Italy) were housed individually in a temperature-controlled (21 ± 1°C) vivarium room and maintained under a 12 h/12 h light/dark cycle (7:00 A.M. to 7:00 P.M. lights on). Food and water were available ad libitum. Rats were handled for 1 min for 3 consecutive days prior to training. Training and testing were performed during the light phase of the cycle between 11:00 A.M. and 2:00 P.M. All procedures involving animal care or treatments were performed in compliance with the ARRIVE guidelines, Directive 2010/63/EU of the European Parliament, the D. L. 26/2014 of the Italian Ministry of Health, the Declaration of Helsinki and the Guide for the Care and Use of Mammals in Neuroscience and Behavioral Research (National Research Council, 2004).

Inhibitory avoidance discrimination task

For all experiments, rats were trained and tested on a modified version of the classic inhibitory avoidance task, termed inhibitory avoidance discrimination task, that allows to investigate strength and accuracy of memory (Atucha and Roozendaal, 2015, Atucha et al., 2017). Rats were subsequently trained in two contextually distinct inhibitory avoidance apparatuses within a single training session, but footshock was delivered only

in the latter context. On the retention test, they were tested in both training contexts as well as in a novel context. These training and test procedures, as previously demonstrated by Atucha and Roozendaal (2015), allow to investigate whether rats remember the two contexts they visited during the training trial, as well as if they display a specific episodic-like memory of the association between footshock and the correct training context. Each apparatus had the same geometry and consisted of a trough-shaped alley (91 cm long, 15 cm deep, 20 cm wide at the top, and 6.4 cm wide at the bottom) divided into two compartments, separated by a sliding door that opened by retracting into the floor. The starting compartment (60 cm) was made of two dark, electrifiable metal plates and was not illuminated. The training context in which footshock was given (Shock box) did not have any contextual modifications. The safe training context (Non-Shock box) had four vertical white stripes (2 cm wide) taped in the dark compartment together with tape placed on the floor, closing the gap between the two plates. The Novel box (used on the retention test only) had two white circles (3.5 cm diameter) taped on each wall of the dark compartment, and the gap between the plates was closed with tape. All three inhibitory avoidance apparatuses were located next to one another in a sound- and light-attenuated room.

For training, rats were initially placed in the starting compartment of the Non-Shock box and their latency to enter the dark compartment with all four paws (maximum latency of 30 s) was recorded. No footshock was delivered in this box. Afterward, the rats were removed from the apparatus and, after a delay of 1-min, placed in the starting compartment of the second inhibitory avoidance apparatus (Shock box). We selected a 1-min delay because, as previously demonstrated (Atucha and Roozendaal, 2015), although animals do not discriminate between the two training contexts with such short interval between the two training episodes, the fear does not generalize to a novel context. After the rat stepped completely into the dark compartment, the sliding door was closed and a single inescapable footshock (0.30 mA; 1 s) was delivered. Rats were removed from the apparatus 20 s after termination of footshock and, after drug treatment, returned to their home cages. On the retention test, two days after training,

they were tested, in a randomized order and without delay, in the two training contexts (i.e., Shock box and Non-Shock box) and in a Novel box they had not visited before. No footshock was delivered on the retention test trial, and for all three boxes, the rats were placed in the starting compartment and their latency to enter the dark compartment with all four paws (maximum latency of 600 s) was recorded. Longer latencies in the Shock box compared with the Non-Shock or Novel box were interpreted as indicating accurate memory of the shock–context association. Moreover, long retention latencies in all the three boxes were considered as an index of memory generalization across contexts. Immediately after training or testing of each animal, each apparatus was wiped clean with a 70% ethanol solution. The experimental design is illustrated in Fig. 1.

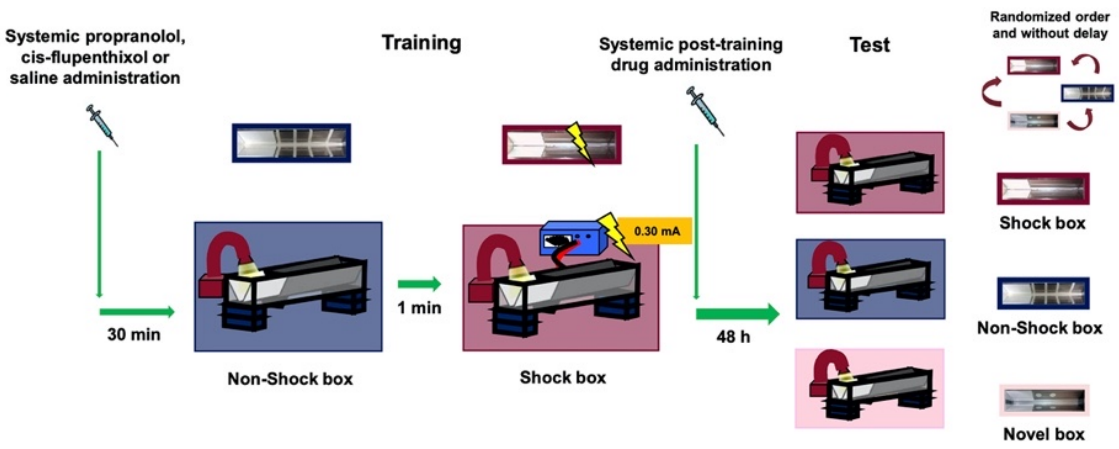

Figure 1 – Schematic representation of the experimental design.

Drug administration

Amphetamine ((RS)-1-phenylpropan-2-amine) (1 and 3 mg/kg) and MDPV (3,4-methylenedioxypyrovalerone) (0.5 and 1 mg/kg) were dissolved in saline (vehicle) and administered intraperitoneally, at the volume of 1 ml/kg, immediately after the training session (Fig. 1). In the second experiment, to examine whether the amphetamine and MDPV effects on memory involve the noradrenergic system, the β-adrenoceptor antagonist propranolol (1-naphthalen-1-yloxy-3-propan-2- ylaminopropan-2-ol) (1

mg/kg) or saline (vehicle) was administered intraperitoneally 30 min prior to training, followed by amphetamine (3 mg/kg), MDPV (1 mg/kg) or saline immediately after training (Fig. 1). In the third experiment, to investigate the involvement of the dopaminergic system in mediating amphetamine and MDPV effects on memory, the non-selective D1/D2 dopaminergic receptor antagonist cis-flupenthixol (2-[4-[(3Z)-3-[2-(trifluoromethyl)thioxanthen- 9-ylidene]propyl]piperazin-1-yl]ethanol) (0.25 mg/kg) or saline (vehicle) was administered intraperitoneally 30 min prior to training, followed by an immediate post-training intraperitoneal injection of amphetamine (3 mg/kg), MDPV (1 mg/kg) or saline (Fig. 1). Drug doses were chosen on the basis of literature data (Roozendaal et al., 2004, Trost and Hauber, 2014) also showing that MPDV has a greater pharmacological potency than amphetamine (Bauman et al, 2013). All drugs were dissolved in sterile 0.9% saline. Drug solutions were freshly prepared before each experiment.

Statistical analysis

Data are expressed as mean ± SEM. All data were analyzed with ANOVA for Repeated Measures (RM ANOVA) with drug treatment as between-group factor and retention latencies of individual animals in the different test contexts (Shock, Non- Shock, and Novel boxes) as repeated measure. Two-way ANOVAs were used to analyze retention latencies of rats treated with propranolol vs saline alone and cis- flupenthixol vs saline alone. The source of the detected significances was determined by Tukey–Kramer post hoc tests for between and within-group differences. P values of less than 0.05 were considered statistically significant. The number of rats per group is indicated in the figure legends.

Results

Amphetamine and MDPV induce memory generalization in an inhibitory avoidance discrimination task

Rats were trained on the inhibitory avoidance discrimination task and given an immediate post-training intraperitoneal injection of amphetamine, MDPV or saline. With regard to amphetamine effects, as shown in Fig. 2a, RM ANOVA for retention latencies indicated significant effects for treatment ($F_{(2,29)} = 10.23$, $P < 0.01$) as well as context ($F_{(2,29)} = 4.08$, $P = 0.02$), but no significant interaction between these two factors ($F_{(4,58)} = 0.48$, $P = 0.75$). Post-hoc analysis, in accordance to what it has been previously demonstrated (Atucha and Roozendaal, 2015), revealed that saline-treated animals showed longer retention latencies in the Shock box ($P < 0.01$) and Non-Shock box ($P < 0.01$) compared to those in the Novel box, indicating that saline- treated rats were able to discriminate the two training contexts from the new one they had visited only during the test trial (Fig. 2a). Retention latencies in the Shock box of rats treated with amphetamine (3 mg/kg) were significantly longer than those of animals treated with saline ($P < 0.05$), indicating that amphetamine, at the higher dose tested, enhanced the strength of memory. Furthermore, amphetamine (3 mg/kg)-treated rats showed longer retention latencies in both the Non-Shock box ($P < 0.05$) and Novel box ($P < 0.01$) compared to saline-treated animals. Thus, these results revealed that amphetamine induced memory generalization across contexts. With regard to MDPV effects, as shown in Fig. 2b, RM ANOVA for retention latencies indicated no significant effect for treatment ($F_{(2,30)} = 1.83$, $P = 0.18$), a significant context effect ($F_{(2,30)} = 3.37$, $P = 0.04$), and no significant interaction between these two factors ($F_{(2,60)} = 1.04$, $P = 0.39$). Post-hoc analysis confirmed that the performance of control animals was the same as for the amphetamine experiments (Fig. 2b). Retention latencies of animals treated with MDPV (1 mg/kg) did not differ from those of saline-treated controls in both Shock and Non-Shock boxes but were significantly longer than those of saline-treated animals ($P < 0.05$) in the Novel box. These results show that rats that were treated with MDPV (1

mg/kg) had similar retention latencies in all three boxes, indicating that MDPV induced generalization across contexts. Taken together, these findings indicate that amphetamine and MDPV have differential effects on memory strength, but that both drugs increase generalization of fear memory to a novel safe context. All training latencies are shown in Supplementary Table S1.

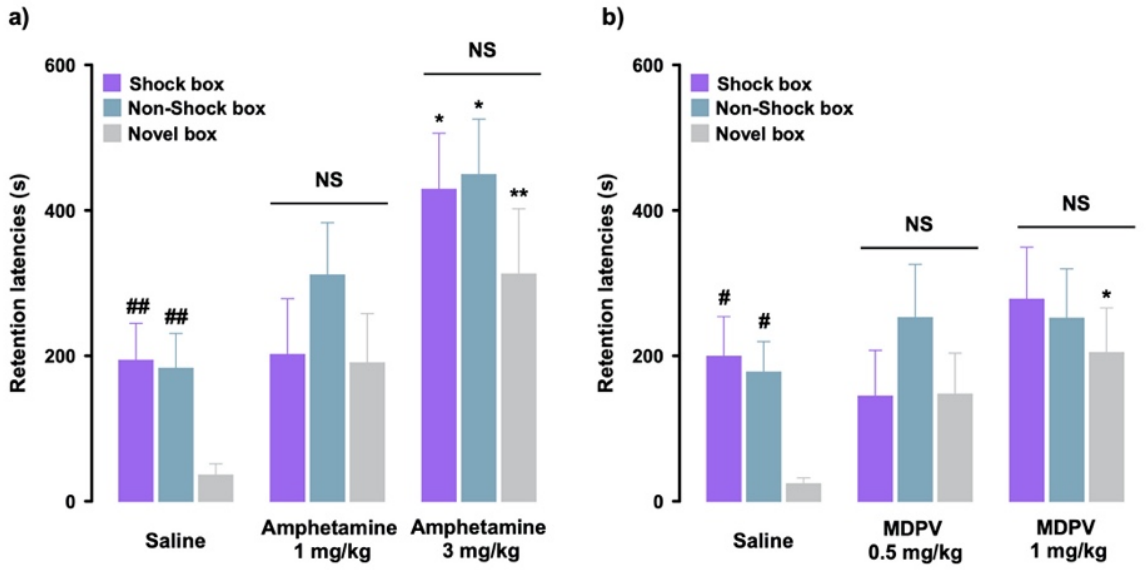

Figure 2: Amphetamine and MDPV induce memory generalization of inhibitory avoidance discrimination task. On the 48-h retention test, rats were sequentially tested in all three contextually modified inhibitory avoidance apparatuses in a random order and their retention latencies were analyzed. **a)** Retention latencies of amphetamine and saline-treated rats. Saline-treated animals showed longer retention latencies in the Shock box and Non-Shock box compared to those induced in the Novel box. In all three boxes, amphetamine 3 mg/kg induced higher retention latencies than saline-treated rats. ##, $P < 0.01$ saline group latencies in the Shock box or Non-Shock box vs saline group latencies in the Novel box; *, $P < 0.05$, **, $P < 0.01$ amphetamine 3 mg/kg latencies in the Shock box, Non-Shock box or Novel box vs saline group in the Shock box, Non-Shock box or Novel box; NS, no significant differences (n = 9-13 rats). **b)** Retention latencies of MDPV and saline-treated rats. Saline- treated animals showed longer retention latencies in the Shock box and Non-Shock box compared to those induced in

the Novel box. In the Novel box retention latencies induced by MDPV 1 mg/kg were significantly longer than those induced by saline-treated rats in the same box. #, $P < 0.05$ saline group latencies in the Shock box or Non-Shock box vs saline group latencies in the Novel box; *, $P < 0.05$ MDPV 1 mg/kg treated group latencies in the Novel box vs saline group latencies in the Novel box; NS, no significant differences (n = 10-12 rats).

Noradrenergic system activation mediates the effects of amphetamine and MDPV on memory generalization

We sought to test whether the amphetamine- and MDPV-mediated effects on strength and generalization of memory involved activation of the noradrenergic system. Here in, rats were given intraperitoneal injections of the β-adrenoceptor antagonist propranolol or saline 30 min prior to training, followed by post-training administrations of the effective doses of amphetamine (3 mg/kg), MDPV (1 mg/kg), or their corresponding vehicles.

To investigate whether the noradrenergic system influences on amphetamine- mediated effects on memory generalization, we first analyzed retention latencies of saline- and propranolol alone-treated animals in the three contexts (Fig. 3a). RM ANOVA for retention latencies of the saline-treated animals showed a significant effect of context ($F_{(2,36)} = 4.80$, $P = 0.01$). Similar to the control rats described above, post-hoc analysis confirmed that saline-treated animals showed longer retention latencies in the Shock box ($P < 0.05$) and Non-Shock box ($P < 0.05$) as compared to those in the Novel box, thus indicating that control rats were able to discriminate the two training contexts from the new one that they visited only during the test trial. The same results were obtained with the RM ANOVA analysis for retention latencies of propranolol alone-treated animals ($F_{(2,35)} = 4.52$, $P = 0.02$). Post-hoc analysis revealed that propranolol alone-treated rats showed longer retention latencies in the Shock box ($P < 0.05$) and Non-Shock box ($P < 0.05$) as compared to those in the Novel box. These findings indicate that also rats that were treated with propranolol accurately remembered the two training

contexts, even if they were not able to discriminate in which training context they received the footshock. Moreover, two-way ANOVA for retention latencies of rats treated with saline and propranolol did not reveal a significant treatment effect ($F_{(1,69)}$ = 0.59, P = 0.44) or treatment x context interaction effect ($F_{(2,69)}$ = 0.03, P = 0.97), but revealed a significant effect of the context ($F_{(2,69)}$ = 9.23, P < 0.0001), suggesting that treatment does not affect animals memory retention for different apparatuses (Fig. 3a). As shown in Fig. 3a, as for the noradrenergic influences in the amphetamine effects on memory function, RM-ANOVA for retention latencies revealed significant effects of treatment ($F_{(3,42)}$ = 11.70, P < 0.01) as well as context ($F_{(2,42)}$ = 6.01, P < 0.01), and no significant differences for the interaction between both factors ($F_{(6,84)}$ = 0.50, P = 0.80). Retention latencies of rats treated with amphetamine alone in the Shock box (P < 0.05), Non-Shock box (P < 0.05) and Novel box (P < 0.01) were all significantly longer than those displayed by saline-treated animals in the same boxes. Retention latencies of rats that were treated with propranolol together with amphetamine in the Shock box (P < 0.05), Non-Shock box (P < 0.01) and Novel box (P < 0.01) were significantly shorter compared to those of animals treated with amphetamine alone in the same boxes. Moreover, retention latencies of rats treated with amphetamine alone in the Shock box (P < 0.05), Non-Shock box (P < 0.01) and Novel box (P < 0.01) were significantly longer than those of rats treated with propranolol alone in the same boxes.

To evaluate whether noradrenergic activity is also involved in the modulation of the MDPV effects on memory generalization, we analyzed retention latencies of both saline and propranolol alone-treated animals and confirmed the results that we described above for the experiments involving amphetamine (Fig. 3b). Furthermore, as previously described, also in this experiment no significant differences were found between saline and propranolol alone-treated rats (Fig. 3b).

As shown in Fig. 3b, RM ANOVA for retention latencies indicated no significant effect of treatment ($F_{(3,32)}$ = 1.70, P = 0.19) or treatment x context interaction effect ($F_{(6,64)}$ = 1.12, P = 0.36), but revealed a significant effect of the context ($F_{(2,32)}$ = 7.32, P < 0.01). Rats treated with MDPV alone showed longer retention latencies in the Novel box than

those of saline alone- ($P < 0.01$) or propranolol alone-treated rats ($P < 0.05$) exposed to the same box. Moreover, retention latencies of animals treated with propranolol together with MDPV in the Shock-box were significantly longer compared to the Novel box ($P < 0.05$) and in the Non-Shock box compared to the Novel box ($P < 0.05$). Particularly in the Novel box, retention latencies of animals treated with propranolol together with MDPV were significantly shorter compared to those of MDPV alone-treated animals in the same box.

In summary, these findings indicate that the amphetamine effect on enhancing memory strength is mediated by the noradrenergic system. Moreover, our findings indicate that the amphetamine effect on memory generalization appears to be only partially due to a modulation of the noradrenergic system, whereas the memory generalization effect induced by MDPV is entirely dependent on noradrenergic activity. All training latencies are indicated in Supplementary Table S2.

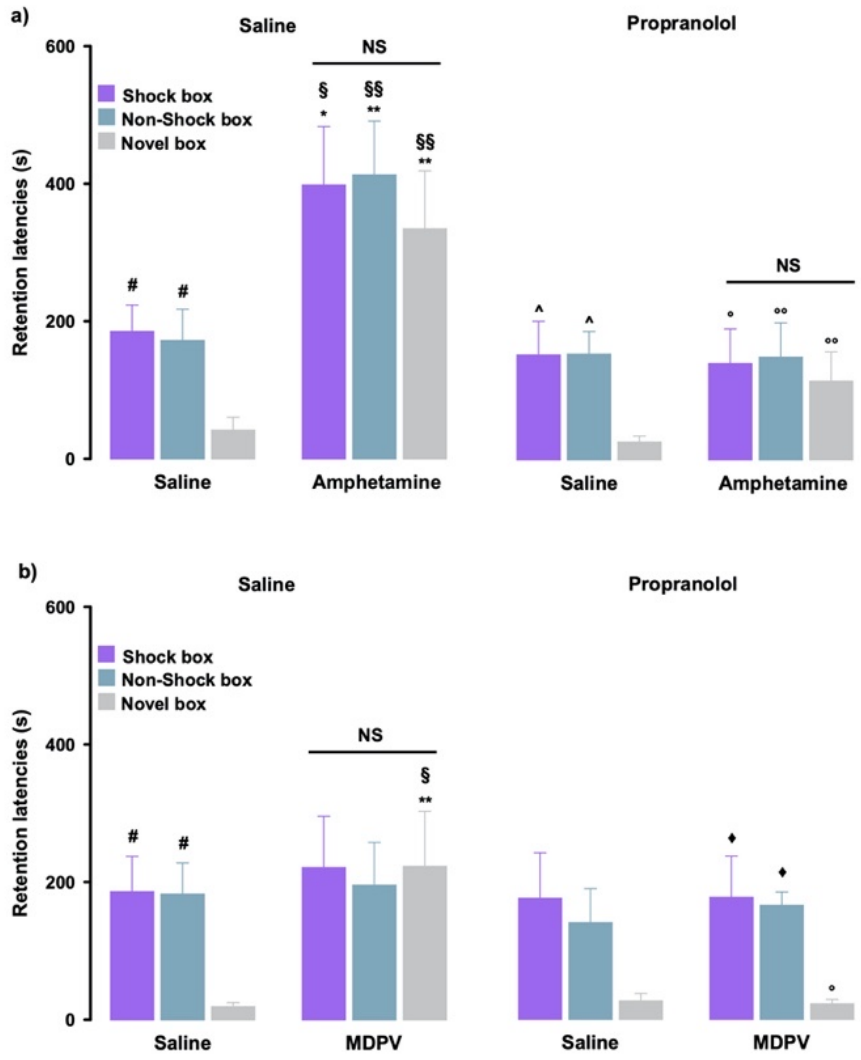

Figure 3: Noradrenergic activation mediates amphetamine and MDPV effects on memory generalization. On the 48-h retention test, rats were sequentially tested in all three contextually modified inhibitory avoidance apparatuses in a random order and their retention latencies were analyzed. **a)** Retention latencies of rats treated with propranolol or saline 30 min prior to training together with amphetamine or saline administered immediately after training. Saline alone-treated animals showed longer retention latencies in the Shock box and Non-Shock box compared to those induced in the Novel box, the same happens for the propranolol alone-treated animals. In all three boxes, amphetamine alone-treated rats showed higher retention latencies than saline alone-treated rats and then those exerted by rats given propranolol alone. Retention

latencies of group treated with propranolol together with amphetamine in all three boxes were significantly lower compared to those of amphetamine alone-treated rats. #, $P < 0.05$ saline group latencies in the Shock box or Non-Shock box vs saline group latencies in the Novel box; ^, $P < 0.05$ propranolol alone latencies in the Shock box or Non-Shock box vs propranolol alone latencies in the Novel box; *, $P < 0.05$, **, $P < 0.01$ amphetamine alone-treated group latencies in the Shock box, Non-Shock box or Novel box vs saline group latencies in the Shock box, Non-Shock box or Novel box; §, $P < 0.05$, §§, $P < 0.01$ amphetamine alone-treated group latencies in the Shock box, Non-Shock box or Novel box vs propranolol alone group latencies in the Shock box, Non-Shock box or Novel box; °, $P < 0.05$, °°, $P < 0.01$ propranolol and amphetamine-treated group latencies in the Shock box, Non-Shock box or Novel box vs amphetamine alone-treated group latencies in the Shock box, Non-Shock box or Novel box; NS, no significant differences (n = 9-13 rats). **b)** Retention latencies of rats treated with propranolol or saline 30 min prior to training together with MDPV or saline administered immediately after training. Saline alone-treated animals showed longer retention latencies in the Shock box and Non-Shock box compared to those induced in the Novel box, the same happens for the propranolol together with MDPV-treated animals. In the Novel box retention latencies induced by MDPV alone treatment were significantly longer than those exerted by rats treated with saline alone and propranolol alone. Retention latencies of group treated with propranolol together with MDPV in the Novel box were significantly lower compared to those of MDPV alone-treated rats. #, $P < 0.05$ saline group latencies in the Shock box or Non-Shock box vs saline group latencies in the Novel box; ♦, $P < 0.05$ propranolol together with MDPV latencies in the Shock box or Non-Shock box vs propranolol together with MDPV latencies in the Novel box; **, $P < 0.01$, MDPV alone-treated group latencies in the Novel box vs saline group latencies in the Novel box; §, $P < 0.05$, MDPV alone-treated group latencies in the Novel box vs propranolol alone-treated group latencies in the Novel box; °, $P < 0.05$, propranolol and MDPV-treated group latencies in the Novel box vs MDPV alone-treated group in the Novel box; NS, no significant differences (n = 8-11 rats).

Dopaminergic system activation mediates the effects of amphetamine, but not MDPV, on memory generalization

In this set of experiments, we tested whether dopaminergic activity is involved in the effects induced by amphetamine and MDPV on memory generalization. To this aim, rats were intraperitoneally treated with the dopamine receptors antagonist cis-flupenthixol or saline 30 min before the training trial and subjected to post-training administration of the effective doses of amphetamine (3 mg/kg), MDPV (1 mg/kg), or their corresponding vehicle solutions.

As previously done in the experiments involving the noradrenergic system, we first analyzed the retention latencies of saline- and of cis-flupenthixol alone-treated animals in the three experimental contexts. Animals that were treated with saline showed comparable latencies to control groups that were discussed above (Fig. 4a). Moreover, in line with the previous set of experiments, no significant differences between saline- and cis-flupenthixol alone-treated animals (Fig. 4a) were detected. As for the involvement of the dopaminergic system in the amphetamine effects on memory function, as shown in Fig. 4a, RM ANOVA for retention latencies indicated significant effects of treatment ($F_{(3,34)} = 10.87$, $P < 0.01$) and context ($F_{(2,34)} = 17.62$, $P < 0.01$), but not significant interaction between both factors ($F_{(6,68)} = 0.47$, $P = 0.83$) effect. Post-hoc analysis revealed that retention latencies of amphetamine alone- treated rats were significantly longer than those of rats that were given saline alone in the Shock box ($P < 0.05$), Non-Shock box ($P < 0.05$) and Novel box ($P < 0.01$). Retention latencies of rats that were treated with amphetamine alone were significantly longer than those of cis-flupenthixol alone-treated rats in the Shock box ($P < 0.05$), Non-Shock box ($P < 0.01$) and Novel box ($P < 0.01$). Retention latencies in the Novel box of rats treated with cis-flupenthixol together with amphetamine were significantly shorter with respect to rats given amphetamine alone ($P < 0.01$) in the same box. Moreover, they showed longer latencies in the Shock box and in the Non-Shock box compared to the Novel box ($P < 0.05$).

Concerning the dopaminergic role on MDPV-mediated generalization effects on memory, for the retention latencies of both saline- and cis-flupenthixol alone-treated rats we confirmed the same results of above described (Fig. 4b); again, no significant differences were found between the two treatment groups (Fig. 4b). As shown in Fig. 4b, RM ANOVA for retention latencies indicated no significant treatment effect ($F_{(3,38)}$ = 1.71, P = 0.18), a significant effect of the context ($F_{(2,38)}$ = 5.06, P < 0.01) and no significant interaction between these two factors ($F_{(6,76)}$ = 0.81, P = 0.56) effect. Post-hoc analysis revealed that retention latencies of rats treated with MDPV alone were significantly longer than those of rats given saline alone and cis- flupenthixol alone in the Novel box (P < 0.01), and that the retention latencies of rats treated with cis-flupenthixol together with MDPV were significantly longer than those of rats given saline alone and cis-flupenthixol alone in the Novel box (P < 0.05).

In conclusion, these results demonstrated that the dopaminergic system is involved in modulating the effects of amphetamine on memory generalization as well with only a partial interference on its effects on memory strength. However, the blockade of dopamine receptors does not influence MDPV effects on memory generalization. All training latencies are shown in Supplementary Table S3.

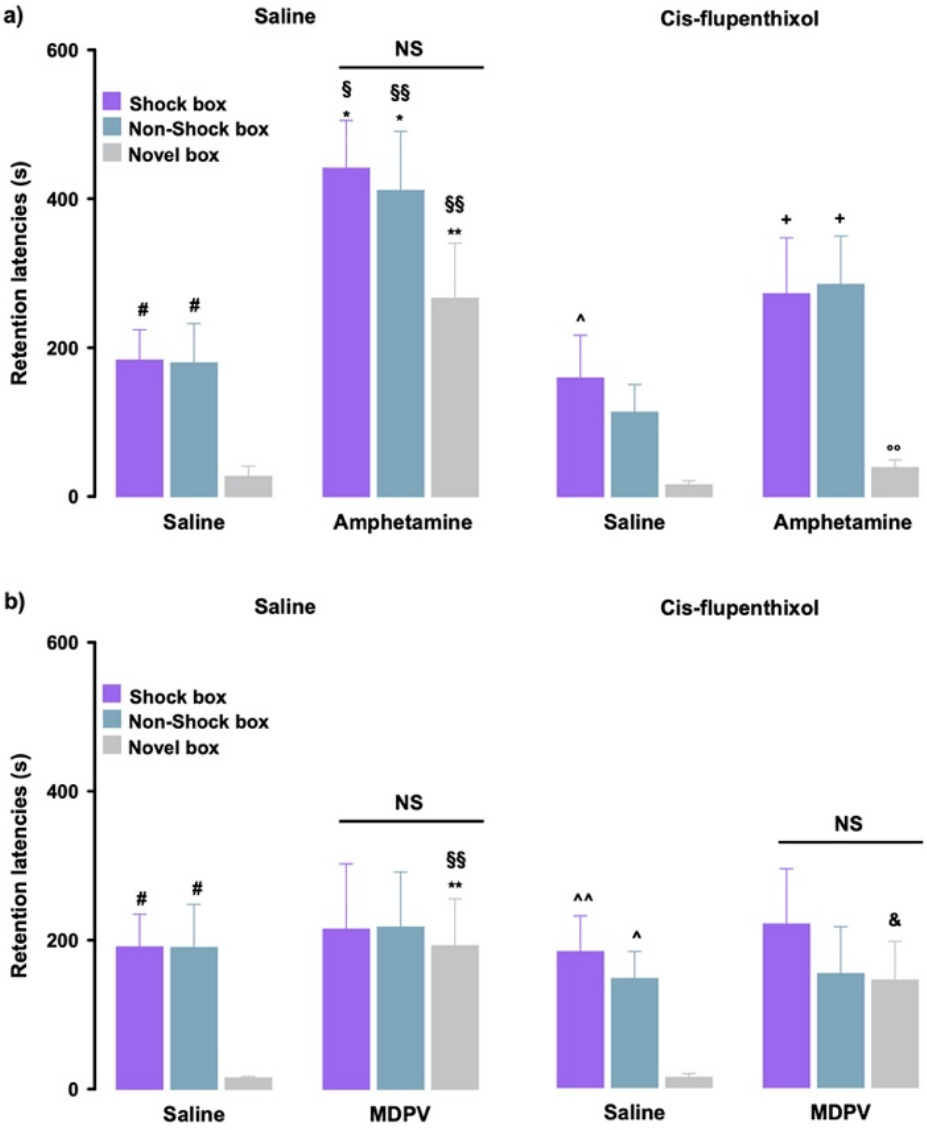

Figure 4: Dopaminergic activation mediates the effects induced by amphetamine, but not MDPV, on memory generalization. On the 48-h retention test, rats were sequentially tested in all three contextually modified inhibitory avoidance apparatuses in a random order and their retention latencies were analyzed. **a)** Retention latencies of rats treated with cis-flupenthixol or saline 30 min prior to training together with amphetamine or saline administered immediately after training. Saline alone-treated animals showed longer retention latencies in the Shock box and Non-Shock box compared to those induced in the Novel box. Cis-flupenthixol alone-treated animals showed higher retention latencies in Shock box compared only to those showed in the

Novel box. Cis-flupenthixol together with amphetamine treated-rats showed longer retention latencies in the Shock box and Non-Shock box compared to those induced in the Novel box. In all three boxes, amphetamine alone-treated rats showed higher retention latencies than saline alone-treated rats and cis-flupenthixol alone-treated rats. Retention latencies of rats treated with cis-flupenthixol together with amphetamine were significantly lower than those of amphetamine alone-treated rats, only in the Novel box. #, $P < 0.05$ saline group latencies in the Shock box or Non-Shock box vs saline group latencies in the Novel box; ^, $P < 0.05$ cis-flupenthixol alone latencies in the Shock box vs cis-flupenthixol alone latencies in the Novel box; +, $P < 0.05$, cis-flupenthixol together with amphetamine latencies in the Shock or Non-Shock box vs cis-flupenthixol together with amphetamine latencies in the Novel box; *, $P < 0.05$, ** $P < 0.01$, amphetamine alone-treated group latencies in the Shock box, Non-Shock box or Novel box vs saline group latencies in the Shock box, Non-Shock box or Novel box; §, $P < 0.05$, §§, $P < 0.01$, amphetamine alone group latencies in the Shock box, Non-Shock box or Novel box vs cis-flupenthixol alone-treated group latencies in the Shock box, Non-Shock box or Novel box; °°, $P < 0.01$, cis-flupenthixol and amphetamine-treated group latencies in the Novel box vs amphetamine alone-treated group in the Novel box; NS, no significant differences (n= 9-10 rats). **b)** Retention latencies of rats treated with cis-flupenthixol or saline 30 min prior to training together with MDPV or saline administered immediately after training. Saline alone-treated animals showed longer retention latencies in the Shock box and Non-Shock box compared to those induced in the Novel box, the same happens to cis-flupenthixol alone-treated animals. In the Novel box, MDPV alone- treated rats showed higher latencies with respect to saline-treated rats and cis-flupenthixol alone-treated rats; cis-flupenthixol and MDPV-treated rats showed higher latencies with respect to cis-flupenthixol alone-treated rats and with respect to cis-flupenthixol alone-treated. #, $P < 0.05$ saline group latencies in the Shock box or Non-Shock box vs saline group latencies in the Novel box; ^, $P < 0.05$, ^^, $P < 0.01$, cis-flupenthixol alone latencies in the Shock box or Non-shock box vs cis-flupenthixol alone latencies in the Novel box; **, $P < 0.01$, MDPV alone-treated group

latencies in the Novel box vs saline group latencies in the Novel box; §§, $P < 0.01$, MDPV alone-treated group latencies in the Novel box vs cis-flupenthixol alone- treated group in the Novel box; &, $P < 0.05$, cis-flupenthixol together with MDPV retention latencies in the Novel box vs cis-flupenthixol alone latencies in the Novel box; NS, no significant differences (n = 8-11 rats).

Discussion

The present findings indicate that amphetamine and MDPV have different effects on memory strength, but both drugs increase generalization of fear memory to a novel safe context. We further show that noradrenergic and dopaminergic neurotransmission is differentially involved in the effects mediated by amphetamine and MDPV on memory. As previously showed, saline-treated animals trained in the inhibitory avoidance discrimination task, with a 1-min interval between the two training apparatuses, were able to discriminate the two training contexts from the new one visited only during the test trial (Atucha and Roozendaal, 2015), indicating that fear memory associated with footshock did not generalize to the novel safe box. Here, we specifically selected this short time delay to evaluate whether amphetamine and MDPV could induce fear memory generalization of footshock to the novel safe context. Our findings first demonstrate, in accordance to previous reports (McGaugh, 1973; Martinez et al., 1980a; Martinez et al., 1980b; Roozendaal et al., 1996, McGaugh and Roozendaal, 2009), that amphetamine increases memory strength as indicated by the longer retention latencies in the Shock box. Of more interest, we also found that amphetamine induces fear memory generalization by enhancing retention latencies in all three boxes, including the box never visited before. MDPV did not directly affect memory strength, but induced generalization of memory, as well as demonstrated by the finding that MDPV-treated animals exerted similar retention latencies in all three boxes. Such evidence that both psychostimulants induce fear memory generalization to a context to which animals

were never exposed before is a truly novel and important finding. Previous studies have indicated that both amphetamine and MDPV, through a similar, yet not identical, mechanism of action increase brain monoamines release, particularly NE and DA, two neurotransmitters extensively involved in the modulation of memory (LaLumiere et al., 2005; McGaugh and Roozendaal, 2009). In fact, amphetamine acts as a substrate of NET, DAT and SERT inducing a 'reverse transport' of neurotransmitters (Robertson et al., 2009), whereas MDPV, like cocaine, is an inhibitor of NET, DAT and SERT (Simmler et al., 2013; Marusich et al., 2014; Baumann et al., 2017). Amphetamine also interacts with the vesicular monoamine transporter (VMAT), in particular VMAT2, depleting synaptic vesicles of their neurotransmitter content (Teng et al., 1998; Eiden and Weihe, 2011), and inhibits monoaminooxidase (MAO), which is a family of enzymes that catalyzes monoamine oxidation (Miller et al., 1980; Liu et al., 2016). The affinity between MDPV and MAO has not yet been investigated. Literature data indicate that other two synthetic cathinones, mephedrone and methylone, have a similar mechanism of action of amphetamine but present a lower affinity for VMAT2 and probably decrease activity on MAO with respect to amphetamine (Baumann et al., 2017). There is evidence that MDPV is more powerful as an uptake blocker of DAT than of NET and SERT (Baumann et al., 2007). Therefore, although this remains purely speculative, it is possible that the different effects induced by amphetamine and MDPV on memory strength may be related to variation of the specific expressions of these monoamine transporters in different brain regions. Notwithstanding the different mechanism of action through which these two psychostimulants enhance NE and DA levels, both drugs of abuse enhance noradrenergic and dopaminergic neurotransmission (Baumann et al., 2013; Robertson et al., 2009) and the involvement of these two systems on the effects induced by drugs of abuse on memory strength and generalization had not been previously investigated. Here, we found that noradrenergic influences, mediated by an action on β-adrenoceptors, were responsible for the enhancing effects of amphetamine on memory consolidation. Extensive evidence indicates that noradrenergic activation is crucially

involved in regulating memory consolidation for emotional experiences (Gold et al., 1975; Gold and van Buskirk, 1978; Gallagher et al., 1977; Liang et al., 1986; McIntyre et al., 2003; Ferry et al., 2015; LaLumiere et al., 2017). Hence, it is possible that amphetamine effects on memory strength could be due to an indirect activation of central β-adrenoceptors. Of more novel interest, we demonstrated that the noradrenergic system also modulates the generalization effects induced by both amphetamine and MDPV. In particular, our findings indicate that amphetamine effects on generalization are partially blocked by preventive administration of the β-adrenoceptor antagonist propranolol, while MDPV effects are totally blocked. Previous findings demonstrated that the administration of the physiological noradrenergic stimulant yohimbine, a selective α2-adrenoceptor antagonist, ameliorates the accuracy of memory in the inhibitory avoidance discrimination task (Atucha and Roozendaal, 2015) and that NA infusion into the basolateral amygdala maintains accuracy of episodic-like memory of the two distinct training contexts, preventing the generalization effect induced by a memory reorganization over time (Atucha et al., 2017). However, our results unexpectedly suggest that if the noradrenergic system is activated by a drug of abuse it alters memory accuracy, inducing generalization. This effect could be explained considering the activation of the noradrenergic system in brain areas particularly involved in memory generalization, such as medial prefrontal cortex, nucleus reunions, and hippocampus (Xu and Sudhof, 2013). Conversely, no data are available with regard to the potential role of dopaminergic modulation on memory accuracy. Herein, we demonstrate that the dopaminergic system is involved in modulating the effects of amphetamine on memory generalization as well with only a partial interference on memory strength. However, the blockade of dopamine receptors does not influence MDPV effects on memory generalization. Together these findings indicate that the generalization effect induced by amphetamine is strongly regulated by the dopaminergic system, whereas the MDPV effects on memory generalization seem to be due to a selective activation of the noradrenergic system. Although these results require further investigation, it can be hypothesized that there is a differential recruitment

induced by amphetamine and MDPV on the monoamine systems in different brain areas.

Brain regions with high density of DAT and dopaminergic receptors, such as the striatum and nucleus accumbens (Efimova et al., 2016) may be responsible for regulating amphetamine effects on memory generalization. Conversely, it is possible that the effects of MDPV on memory generalization are linked to brain areas with high levels of NET and β-adrenoceptors such as the dentate gyrus of the hippocampus and the perirhinal cortex, which are known to play a critical role in the regulation of memory discrimination (Miranda et al., 2017; van Dijk and Fenton, 2018). In agreement with these results, it could be hypothesized that the generalization induced by MDPV is mediated by β-adrenoceptors in such brain areas. Thus, our findings demonstrate that both amphetamine and MDPV induce generalization of fear memory via a different involvement of NE and DA neurotransmission. These results pave the way for future studies aimed at investigating the role of specific brain areas in mediating the differential effects of both psychostimulant drugs on strength and quality of memory, thus ultimately leading to reveal the neurobiological underpinnings of memory alterations induced by drugs of abuse.

Supplementary Information

Amphetamine and MDPV induce memory generalization of inhibitory avoidance discrimination task

During the training trial rats were first exposed to the Non-Shock box and, with a 1-min delay, to the Shock one. Approach latencies to enter the dark compartment, before footshock, were evaluated. Immediately after training, rats received an intraperitoneal injection of amphetamine (1 and 3 mg/kg), MDPV (0.5 and 1 mg/kg) or saline. In the group given post-training injection of amphetamine or saline, RM ANOVA for approach latencies did not reveal significant effects of post-training treatment ($F_{(2,29)}$ =

0.17, P = 0.85), context ($F_{(1,29)}$ = 1.47, P = 0.24) or interaction between these two factors ($F_{(2,29)}$ = 0.47, P = 0.63; Table 1). In the group given post-training injection of MDPV or saline, RM ANOVA for approach latencies revealed no significant effects of post-training treatment ($F_{(2,30)}$ = 0.05, P = 0.95), context ($F_{(1,30)}$ = 0.32, P = 0.58) or interaction between these two factors ($F_{(2,30)}$ = 0.17, P = 0.84; Table 1).

	Approach latencies in the Non-Shock box	**Approach latencies in the Shock box**
saline amphetamine 1 mg/kg amphetamine 3 mg/kg	14.6 ± 1.0 14.2 ± 1.4 13.4 ± 1.5	14.5 ± 1.5 16.0 ± 0.9 15.8 ± 1.1
saline MDPV 0.5 mg/kg MDPV 1 mg/kg/kg	14.3 ± 2.1 14.5 ± 2.3 14.7 ± 3.4	14.7 ± 1.3 12.7 ± 1.2 13.5 ± 2.7
Time spent to enter the dark compartment during the training (in seconds) of all groups. Data are expressed as mean ± SEM (n = 9-13 per group).		

Table 1 – Approach latencies in the Non-Shock and Shock boxes of rats post-training treated with amphetamine, MDPV or saline.

Noradrenergic activation mediates amphetamine and MDPV effects on memory generalization

Thirty minutes prior to training rats were given an intraperitoneal injection of the β-adrenoceptors antagonist propranolol (1 mg/kg) or saline together with post-training administration of the effective doses of amphetamine (3 mg/kg), MDPV (1 mg/kg), or their corresponding vehicles. Approach latencies to enter the dark compartment during the training, before footshock, were evaluated. In the group given pre-training injection of propranolol or saline followed by a post-training administration of amphetamine or

saline, RM ANOVA for approach latencies did not show significant effects of post-training treatment ($F_{(3,42)} = 0.16$, $P = 0.92$), context ($F_{(1,42)} = 0.01$, $P = 0.91$) or interaction between these two factors ($F_{(3,42)} = 0.54$, $P = 0.66$; Table 2). In the group treated with a pre-training injection of propranolol or saline together with post-training administration of MDPV or saline, RM ANOVA for approach latencies indicated no significant effects of post-training treatment ($F_{(3,32)} = 0.13$, $P = 0.94$), context ($F_{(1,32)} = 0.94$, $P = 0.34$) or interaction between these two factors ($F_{(3,32)} = 0.36$, $P = 0.79$; Table 2).

	Approach latencies in the Non-Shock box	**Approach latencies in the Shock box**
saline-saline	16.1 ± 2.1	13.6 ± 1.1
propranolol-saline	14.1 ± 0.9	14.7 ± 1.6
saline-amphetamine	14.7 ± 1.1	16.9 ± 2.1
propranolol-amphetamine	15.4 ± 2.7	15.8 ± 3.2
saline-saline	13.8 ± 2.1	13.9 ± 1.0
propranolol-saline	13.9 ± 1.0	13.9 ± 1.3
saline-MDPV	15.8 ± 3.4	12.3 ± 1.4
propranolol-MDPV	14.0 ± 4.9	11.3 ± 1.7
Time spent to enter the dark compartment during the training (in seconds) of all groups. Data are expressed as mean ± SEM (n = 8-13 per group).		

Table 2 - Approach latencies in the Non-Shock and Shock boxes of rats treated with propranolol or saline 30 min prior to training together with amphetamine, MDPV or saline administered immediately after training.

Dopaminergic activation mediates the effects induced by amphetamine, but not MDPV, on memory generalization

Rats were treated with an intraperitoneal injection of the dopamine receptors antagonist cis-flupenthixol (0.25 mg/kg) or saline 30 min prior to training, followed by a post-training administration of the effective doses of amphetamine (3 mg/kg), MDPV (1

mg/kg), or their corresponding vehicles. Approach latencies to enter the dark compartment during the training, before footshock, were evaluated. In the group given pre-training injection of cis-flupenthixol or saline together with post-training administration of amphetamine or saline, RM ANOVA for approach latencies indicated no significant effects of post-training treatment ($F_{(3,34)} = 0.05$, $P = 0.99$), context ($F_{(1,34)} = 0.003$, $P = 0.96$) or interaction between these two factors ($F_{(3,34)} = 0.28$, $P = 0.84$; Table 3). In the group given pre-training injection of cis-flupenthixol or saline together with post-training administration of MDPV or saline, RM ANOVA for approach latencies did not indicate significant effects of post-training treatment ($F_{(3,38)} = 0.65$, $P = 0.59$), context ($F_{(1,38)} = 0.07$, $P = 0.79$) or interaction between these two factors ($F_{(3,38)} = 0.17$, $P = 0.92$; Table 3).

	Approach latencies in the Non-Shock box	**Approach latencies in the Shock box**
saline-saline	14.6 ± 2.9	13.9 ± 1.3
cis-flupethixol-saline	13.8 ± 1.9	13.5 ± 3.0
saline-amphetamine	12.5 ± 1.6	15.1 ± 2.4
cis-flupethixol-amphetamine	15.8 ± 5.9	13.8 ± 1.8
saline-saline	11.8 ± 2.8	12.5 ± 1.5
cis-flupethixol-saline	14.4 ± 3.7	13.5 ± 2.3
saline-MDPV	12.4 ± 3.1	15.7 ± 3.0
cis-flupethixol-MDPV	17.2 ± 5.1	16.6 ± 3.6

Time spent to enter the dark compartment during the training (in seconds) of all groups. Data are expressed as mean ± SEM (n = 8-11 per group).

Table 3 - Approach latencies in the Non-Shock and Shock boxes of rats treated 30 min prior to training with cis-flupenthixol or saline together with amphetamine, MDPV or saline administered immediately after training.

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CHAPTER 2

AMPHETAMINE MODULATION OF LONG-TERM OBJECT RECOGNITION MEMORY IN RATS: INFLUENCE OF STRESS

Paola Colucci[1,2], Alessia Santori[1,2], Luca Romanelli[1], Clemens Zwergel[3], Antonello Mai[3], Sergio Scaccianoce[1], Patrizia Campolongo[1,2]

[1]Dept. of Physiology and Pharmacology, Sapienza University of Rome, Rome, Italy; [2]Neurobiology of Behavior Laboratory, Santa Lucia Foundation, Rome, Italy; [3]Dept. of Drug Chemistry & Technologies, Sapienza University of Rome, Rome, Italy

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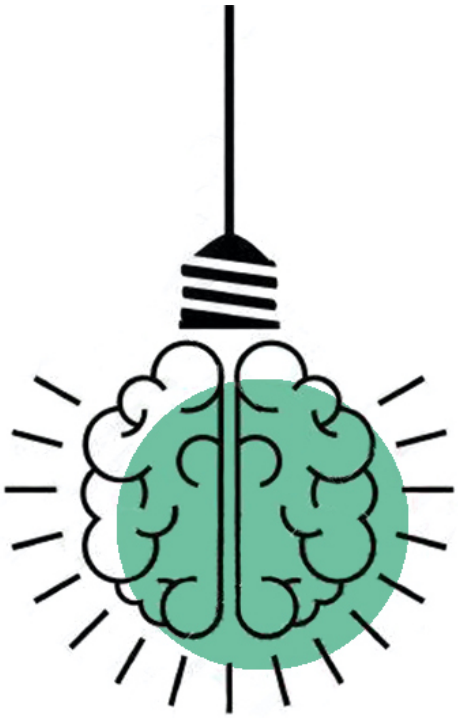

Abstract

Amphetamine is a potent psychostimulant that increases brain monoamine levels. Extensive evidence demonstrated that norepinephrine is crucially involved in the regulation of memory consolidation for stressful experiences. Here, we investigated amphetamine effects on the consolidation of long-term recognition memory in rats exposed to different intensities of forced swim stress immediately after training. Furthermore, we evaluated whether such effects are dependent on the activation of the peripheral adrenergic system. To this aim, male adult Sprague Dawley rats were subjected to an object recognition task and intraperitoneally administered soon after training with amphetamine (0.5 or 1 mg/kg), or its corresponding vehicle. Rats were thereafter exposed to a mild (1 min, 25 ± 1 °C) or strong (5 min, 19 ± 1 °C) forced swim stress procedure. Recognition memory retention was assessed 24-h after training. Our results showed that amphetamine enhances the consolidation of memory in rats subjected to mild stress condition, while it impairs long-term memory performance in rats exposed to strong stress. These dichotomic effects appear to be dependent on the stress-induced activation of the peripheral adrenergic response.

Introduction

The psychostimulant amphetamine was discovered more than a century ago (see Heal et al., 2013 for recent review). Chemical structure analogies among amphetamine and other monoamine neurotransmitters, such as norepinephrine, dopamine and serotonin, are crucial not only for amphetamine's mechanism of action, but also for its pharmacological properties (Ferris and Tang, 1979). It is well known that amphetamine acts as a competitive substrate of the norepinephrine, dopamine and serotonin re-uptake transporters (NET, DAT and SERT, respectively) (Sulzer et al., 2005). Once entered in the presynaptic neuron, amphetamine disrupts the monoamine storage vesicles and, consequently, increases the monoamine levels in the neural cytosolic pool (Teng et al.,

1998). Such enhanced cytosolic concentration of monoamines reverts the transport direction of NET, DAT and SERT, thus increasing the amount of norepinephrine, dopamine and serotonin in the synaptic cleft (Robertson et al., 2009). It has been demonstrated that augmented levels of monoamines, in particular norepinephrine and dopamine, at the synaptic terminal, are responsible for euphoria, mood improvements and the general sense of wellbeing induced by amphetamine intakes (de Wit et al., 2002, Pester et al., 2018). Over the years, literature data demonstrated that amphetamine induces profound effects on learning and memory processes (Ballard et al., 2014, Bardgett et al., 2019, Martinez et al., 1980a). Interestingly, it has been shown that these effects are dependent on the amphetamine-induced activation of the noradrenergic system (Lee and Ma, 1995, Colucci et al., 2019).

The noradrenergic system activation is critically involved in the modulation of long-term memory consolidation (Ferry et al., 1999, LaLumiere et al., 2017, Roozendaal and McGaugh, 2011). We recently demonstrated that the dissociative drug ketamine enhances memory performance through a mechanism that activates both the central and peripheral noradrenergic signaling (Morena et al., 2017, Morena et al., 2020). It is widely recognized that emotionally arousing experiences, which activate the endogenous stress systems, are well remembered over time (McGaugh, 2006). The activation of the hypothalamic–pituitary–adrenal (HPA) axis, mediated by the stress response, culminates with the release, by the adrenal glands, of the stress hormones: particularly, epinephrine, from the adrenal medulla, and glucocorticoids, from the adrenal cortex (Biddie et al., 2012). Such stress hormones finely tune the noradrenergic tone in the central nervous system (Smith and Vale, 2006) and modulate cognitive function, by promoting an inverted U-shaped dose-effect curve (Schilling et al., 2013), by which optimal levels of stress hormones are responsible for memory performance potentiation, whereas their maladaptive expression leads to memory impairment (McEwen, 2013, Salehi et al., 2010).

Hence, considering amphetamine modulation of noradrenergic system, which in turn influences memory processes, and taking into account that different stress intensities

distinctly prompt stress hormone levels with divergent effects on cognitive functions, here we first aimed at investigating amphetamine effects on the consolidation of long-term recognition memory in rats that were exposed to different levels of stress. In a second set of experiments, we further evaluated whether the effects of amphetamine on long-term recognition memory consolidation were dependent on the activation of the peripheral adrenergic system, soon after exposure to different stress conditions.

Materials and Methods

Animal Care and Use

Male adult Sprague-Dawley rats (350–450 g at the time of training and testing, Charles River Laboratories, Calco, Italy) were kept individually in an air-conditioned colony room (temperature: 21 ± 1°C; lights on from 07:00 AM to 7:00 PM) with pellet food and water available ad libitum. Training and testing were performed during the light trial of the cycle between 11:00 AM and 2:00 PM. All procedures involving animal care or treatments were performed in compliance with the ARRIVE guidelines, the Directive 2010/63/EU of the European Parliament, and the D.L. 26/2014 of the Italian Ministry of Health.

Drug Treatment

Amphetamine ((RS)-1-phenylpropan-2-amine) (0.5 and 1 mg/kg) was dissolved in saline 0.9% (vehicle) and administered intraperitoneally (i.p.) in a volume of 1 ml/kg, immediately after the training trial. Doses were chosen on the basis of pilot experiments performed in our laboratory and on literature data (Roozendaal et al., 1996, Colucci et al., 2019). The solutions were freshly prepared on the day of the experiment and protected from exposure to light.

Behavioral Procedures

Object recognition task. A previously validated object recognition (OR) procedure described by Campolongo et al. (2013) was used. The experimental apparatus consisted of a grey open-field box (in cm, 40 wide x 40 deep x 40 high) with the floor covered with sawdust, positioned in a dimly illuminated room. The objects to be discriminated were transparent glass vials (5.5 cm diameter and 5 cm height) and white glass light bulbs (6 cm diameter and 11 cm length). All rats were handled twice per day for 1 min each and extensively habituated to the experimental context twice per day for 3 min each for 7 days preceding the training day. During habituation, rats were allowed to explore the apparatus in the absence of objects freely. The animals were randomly assigned to two different groups: mild and strong stress conditions. On the training trial, each rat was individually placed in the experimental apparatus at the opposite end from the objects. Rats were allowed to explore two identical objects (A1 and A2) for 6 min, then they were removed from the apparatus and, after drug treatment, according to the stress condition group, were subjected to a mild or strong swim stress procedure; subsequently, each rat was returned to the home cage. To avoid the presence of olfactory trails, sawdust was stirred, fecal boli were removed and the objects were cleaned with 70% ethanol after each trial. Rat's behavior was recorded by a video camera positioned above the experimental apparatus and videos were analyzed with Observer XT 12 (Noldus Information Technology BV, Wageningen, The Netherlands) by a trained observer who was unaware of treatment condition. Exploration of an object was defined as pointing the nose to the object at a distance of < 1 cm and/or touching it with the nose. Turning around or sitting on an object was not considered as exploration. During the training trial, the time spent exploring the two objects (total object exploration time, s) was taken as a measure of object exploration, and the exploratory behavior of the experimental apparatus was analyzed by measuring the total number of crossings and rearings. For crossings, the floor of the apparatus was divided into four imaginary squares and the total number of crossings between squares was determined. Long-term memory retention was tested 24-h after the training trial. On the testing trial, one copy

of the familiar object (A3) and a new object (B) were placed in the same location as stimuli during the training trial. All combinations and locations of objects were used to reduce potential biases due to preference for particular locations or objects. Each rat was placed in the apparatus for 6 min, and its behavior was recorded. To analyze cognitive performance, during the retention test, a discrimination index (DI) was calculated as the difference in time exploring the novel (B) and the familiar object (A3), expressed as the percentage ratio of the total time spent exploring both objects (B+A3).

Forced swim stress procedure. This procedure was carried out accordingly to Santori and colleagues (Santori et al., 2019, 2020). Immediately after the training trial of the OR task rats were forced to swim in a tank (50 cm in height x 20 cm in diameter), filled to a depth of 30 cm with water, in a separate room from that where the OR task was performed. Thereafter, rats were removed from the water and carefully wiped to dryness with absorbent paper before returning to the home cage. Mild and strong stress condition rat groups were subjected to a 1- or 5-min forced swim stress procedure at different water temperatures of 25 ± 1°C or 19 ± 1°C, respectively, known to elicit different plasma corticosterone levels (Santori et al., 2019, Morena et al., 2015).

Surgical Procedures

Adrenal medullectomy. In a second set of experiments, rats were subjected to adrenal medullectomy, which was performed as previously reported in literature (Martinez et al., 1980b, Shin et al., 2017, Wilkinson et al., 1981). Summarily, each rat was anesthetized with a mixture of Zoletil and Domitor (40 mg/kg and 35 μg/kg respectively), given i.p. Animals were placed on a flat surface with their limbs in the extended position and their dorsal area was trichotomized. An incision of 2 cm was made on the right and left dorsal lateral surface of the animal just over each kidney. The overlying adipose tissue was removed, and it was possible to identify the adrenal glands. Small incisions were made on the adrenal capsule and the medulla was gently squeezed out. The wound was closed with an autoclip. Sham surgery was performed in the same

manner, except for the removal of adrenal medullae. Consistently with literature data, rats were provided with drinkable 0.45% saline and allowed to recover from surgery for at least 7 days before experimental procedures (Khasar et al., 2009).

Data and Statistical Analysis

One-sample t-tests were used to determine whether the DI was different from zero. OR data were analyzed by one- or two-way ANOVAs. When appropriate, Tukey-Kramer *post hoc* tests were used to determine the source of the detected significances. P values of < 0.05 were considered statistically significant. To be included in the statistical analysis rats had to reach a minimum criterion of total object exploration time > 10 s on either training or testing. Prior findings indicate that such rats adequately acquire the task (Campolongo et al., 2013, Okuda et al., 2004). All data are expressed as mean ± standard error of the mean (SEM) and each group's n is indicated in the corresponding figure legend.

Results

Amphetamine enhances long-term memory consolidation in rats subjected to the mild stress condition

This experiment investigated whether amphetamine administration, immediately after the training trial, modulates long-term memory consolidation in an OR task, when animals were subjected to a mild forced swim stress condition.

Training trial. One-way ANOVA for total exploration time of the two identical objects on the training trial, before drug administration and stress exposure, revealed no significant effect of post-training treatment ($F_{(2,23)} = 1.074$, $P = 0.358$; Table 1). Examination of rats' exploratory behavior of the experimental apparatus during the training trial indicated that there were no significant differences among groups for the number of crossings or rearings before drug treatment and stress exposure (Table 1). In

fact, one-way ANOVA for the number of crossings or rearings on the training trial revealed no significant post-training treatment effects ($F_{(2,23)}$ = 0.675, P = 0.519 and $F_{(2,23)}$ = 0.289, P = 0.752, respectively).

Testing trial. As expected, vehicle-treated rats did not express long-term memory retention for the familiar object. One sample t-test revealed that the DIs were not significantly different form zero for vehicle ($t_{(8)}$ = 0.028, P = 0.978) and amphetamine 0.5 mg/kg ($t_{(7)}$ = 1.378, P = 0.211) treated animals. In contrast, rats that were administered with amphetamine at the dose of 1 mg/kg discriminated the new object ($t_{(8)}$ = 5.078, P = 0.010). Consistently, one-way ANOVA for the DI reported a significant treatment effect ($F_{(2,23)}$ = 4.341, P = 0.025). *Post hoc* analysis indicated that the DI of rats treated with 1 mg/kg of amphetamine was significantly higher with respect to that of vehicle-treated rats (P < 0.05) (Fig 1). One-way ANOVA for the total object exploration time on the testing trial, the number of crossings and rearings revealed no significant treatment effect ($F_{(2,23)}$ = 1.310, P = 0.289; $F_{(2,23)}$ = 0.425, P = 0.659 and $F_{(2,23)}$ = 0.246, P = 0.784, respectively; Table 2).

	Total object exploration time (s)	Number of crossings	Number of rearings
MILD STRESS			
Vehicle	76.8 ± 5.7	32.7 ± 2.8	42.9 ± 4.7
Amphetamine 0.5	80.4 ± 13.2	30.0 ± 3.1	38.1 ± 3.7
Amphetamine 1	63.6 ± 5.9	27.4 ± 3.8	63.6 ± 5.9
STRONG STRESS			
Vehicle	79.5 ± 6.5	38.2 ± 3.8	44.6 ± 3.5
Amphetamine 0.5	79.9 ± 7.1	32.9 ± 2.4	38.4 ± 1.3
Amphetamine 1	74.6 ± 8.8	32.5 ± 3.5	35.2 ± 3.9
	SHAM		
MILD STRESS			
Vehicle	72.5 ± 4.9	33.3 ± 3.1	40.6 ± 5.8
Amphetamine 1	61.1 ± 6.8	29.9 ± 4.0	40.1 ± 3.7
STRONG STRESS			
Vehicle	73.2 ± 6.8	38.5 ± 3.6	42.8 ± 1.3
Amphetamine 1	83.5 ± 9.7	33.3 ± 3.1	35.7 ± 3.7
	MEDULLECTOMY		
MILD STRESS			
Vehicle	82.5 ± 8.5	28.8 ± 5.0	39.5 ± 2.5
Amphetamine 1	88.2 ± 7.6	30.3 ± 3.4	37.2 ± 2.9
STRONG STRESS			
Vehicle	69.7 ± 5.6	26.9 ± 2.9	35.7 ± 3.9
Amphetamine 1	82.8 ± 7.7	26.6 ± 3.1	37.7 ± 4.3

Table 1. Exploratory behavior on the training trial for post-training vehicle- and amphetamine-treated rats that were subjected to mild or strong stress conditions immediately after training.

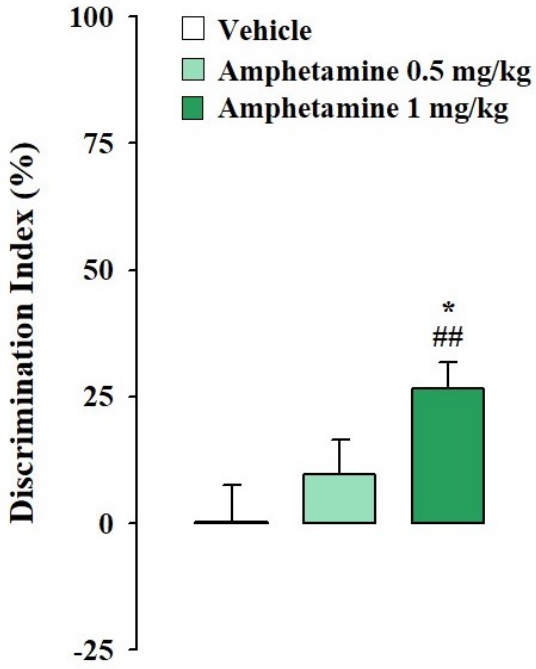

Figure 1. Amphetamine effects on the consolidation of long-term OR memory in rats exposed to the mild stress condition immediately after training. DI on the testing trial for vehicle- and amphetamine-treated rats that were subjected to the mild stress condition immediately after training. *Post hoc* comparisons reported significant differences between groups as follows: * $P < 0.05$ vs the corresponding vehicle group. ## $P < 0.01$, one-sample t-test significantly different from zero. Data are expressed as mean ± SEM (n = 8-9 per group).

	Total object exploration time (s)	Number of crossings	Number of rearings
MILD STRESS			
Vehicle	26.9 ± 4.6	19.2 ± 2.5	45.6 ± 5.7
Amphetamine 0.5	37.2 ± 7.6	20.4 ± 2.7	42.0 ± 4.1
Amphetamine 1	26.5 ± 3.1	17.1 ± 2.4	40.7 ± 5.4
STRONG STRESS			
Vehicle	40.9 ± 5.4	30.5 ± 4.0	51.9 ± 5.0
Amphetamine 0.5	39.8 ± 4.8	22.1 ± 2.2	42.5 ± 4.3
Amphetamine 1	36.1 ± 6.3	23.7 ± 3.0	41.2 ± 5.0
		SHAM	
MILD STRESS			
Vehicle	36.0 ± 5.7	19.7 ± 2.7	46.0 ± 5.9
Amphetamine 1	34.6 ± 4.7	17.1 ± 2.7	36.6 ± 4.3
STRONG STRESS			
Vehicle	44.4 ± 4.7	14.7 ± 1.8	23.4 ± 1.9
Amphetamine 1	36.9 ± 5.2	14.1 ± 1.9	21.8 ± 2.3
		MEDULLECTOMY	
MILD STRESS			
Vehicle	49.2 ± 10.9	18.0 ± 3.5	58.5 ± 11.5
Amphetamine 1	38.0 ± 4.6	15.3 ± 2.5	43.2 ± 6.6
STRONG STRESS			
Vehicle	49.9 ± 7.9	15.0 ± 1.9	24.0 ± 3.3
Amphetamine 1	48.5 ± 6.0	19.2 ± 2.3	27.5 ± 3.4

Table 2. Exploratory behavior on the testing trial for vehicle- and amphetamine-treated rats that were subjected to mild or strong stress conditions immediately after training.

Amphetamine impairs long-term memory consolidation in rats subjected to the strong stress condition

This experiment investigated whether immediate post-training administration of amphetamine alters the consolidation of rat long-term recognition memory when animals are exposed to a strong forced swim stress condition.

Training trial. One-way ANOVA for total exploration time of the two identical objects on the training trial, before drug administration and stress exposure, revealed no significant effect of post-training treatment ($F_{(2,28)}$ = 0.144, P = 0.866; Table 1). Concerning rats' exploratory behavior of the experimental apparatus during the training trial, one-way ANOVA for the number of crossings ($F_{(2,28)}$ = 0.932, P = 0.406) and rearings ($F_{(2,28)}$ = 2.406, P = 0.109) indicated no significant differences among groups before drug treatment and stress exposure (Table 1).

Testing trial. In accordance to our previous findings (Campolongo et al., 2013), one sample t-test revealed that the DI of vehicle-treated rats was significantly different from zero ($t_{(9)}$ = 3.007, P = 0.015), thus indicating that these animals discriminated the novel object with respect to the familiar one. Conversely, the DI of rats treated with amphetamine at 0.5 and 1 mg/kg were not significantly different from zero ($t_{(10)}$ = 1.930, P = 0.082; $t_{(9)}$ = -0.765, P = 0.464; respectively), demonstrating that such rats were not able to express memory retention for the familiar object. One-way ANOVA for the DI revealed a significant treatment effect ($F_{(2,28)}$ = 3.889, P =0.032). *Post hoc* analysis indicated that the DI of rats treated with 1 mg/kg of amphetamine was significantly lower than that of vehicle-treated rats ($P < 0.05$) (Fig 2). Finally, rats' exploratory behavior of the apparatus during the testing trial did not differ among the different experimental groups. One-way ANOVA reported no significant effects for the total object exploration time ($F_{(2,28)}$ = 0.206, P = 0.815), the number of crossings ($F_{(2,28)}$ = 2.079, P = 0.144) or rearings ($F_{(2,28)}$ = 1.478, P = 0.245) (Table 2).

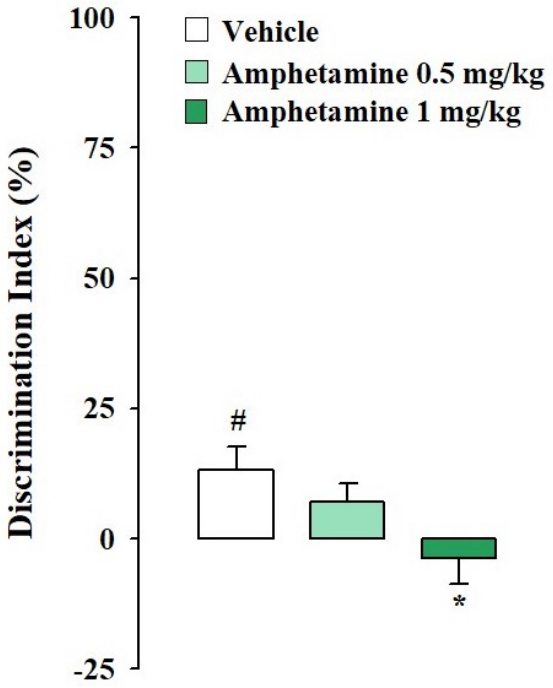

Figure 2. Amphetamine effects on the consolidation of long term OR memory in rats exposed to the strong stress condition immediately after training. DI on the testing trial for vehicle- and amphetamine-treated rats that were subjected to the strong stress condition immediately after training. *Post hoc* comparisons reported significant differences between groups as follows: * $P < 0.05$ vs the corresponding vehicle group. # $P < 0.05$, one-sample t-test significantly different from zero. Data are expressed as mean ± SEM (n = 10-11 per group).

Amphetamine impairs long-term memory consolidation in adrenal medullectomized rats subject to the mild stress condition

In this experiment we sought to determine whether amphetamine enhancing effects on long-term memory consolidation in rats exposed to the mild forced swim stress condition were dependent on the activation of the peripheral adrenergic system.

Training trial. Two-way ANOVA for total exploration time of the two identical objects on the training trial revealed a significant adrenal medullectomy effect ($F_{(1,33)} = 5.947$, $P = 0.020$), but no significant treatment ($F_{(1,33)} = 0.136$, $P = 0.715$) or the interaction

between these two factors ($F_{(1,33)}$ = 1.252, P = 0.271) effects (Table 1). Two-way ANOVA for the number of crossings and rearings revealed no significant effects of post-training drug treatment (crossings: $F_{(1,33)}$ = 0.052, P = 0.821; rearings: $F_{(1,33)}$ = 0.137, P = 0.714), adrenal medullectomy (crossings: $F_{(1,33)}$ = 0.261, P = 0.613; rearings: $F_{(1,33)}$ = 0.284, P = 0.598) or the interaction between these two factors (crossings: $F_{(1,33)}$ = 0.384, P =0.540; rearings: $F_{(1,33)}$ = 0.058, P = 0.812) (Table 1).

Testing trial. One sample t-test revealed that the DIs of both sham and medullectomized rats that were treated with vehicle were no significantly different from zero (sham: $t_{(7)}$ = 0.774, P = 0.464; medullectomized: $t_{(7)}$ = 2.007, P = 0.085), thus indicating that both experimental groups were not able to express long-term retention for the familiar object. On the contrary, the DIs of both sham and medullectomized animals treated with amphetamine were significantly different from zero (sham: $t_{(7)}$ = 8.423, P < 0.0001; medullectomized: $t_{(12)}$ = 4.519, P = 0.0007), thus suggesting that both experimental groups were able to discriminate the two objects. Two-way ANOVA for the DI revealed significant effects for treatment ($F_{(1,33)}$ = 11.329, P = 0.002), adrenal medullectomy ($F_{(1,33)}$ = 4.538, P = 0.041) and the interaction between both factors ($F_{(1,33)}$ = 6.081, P = 0.019). As expected, *post hoc* analysis revealed that sham rats treated with amphetamine showed higher DI with respect to sham rats treated with vehicle (P < 0.01). Surprisingly, *post hoc* analysis indicated that medullectomized rats treated with amphetamine showed lower DI than the respective sham group (P < 0.01) (Fig. 3). Finally, rats' exploratory behavior of the apparatus during the testing trial did not differ among the different experimental groups. Indeed, two-way ANOVA did not express any significant effects for total object exploration time (treatment: $F_{(1,33)}$ = 0.941, P = 0.339; adrenal medullectomy: $F_{(1,33)}$ = 1.674, P = 0.205; treatment x adrenal medullectomy: $F_{(1,33)}$ = 0.582, P = 0.451), the number of crossings (treatment: $F_{(1,33)}$ = 0.838, P = 0.367; adrenal medullectomy: $F_{(1,33)}$ = 0.377, P = 0.543; treatment x adrenal medullectomy: $F_{(1,33)}$ = 0.001, P = 0.991) and rearings (treatment: $F_{(1,33)}$ = 2.590, P = 0.117; adrenal medullectomy: $F_{(1,33)}$ = 1.557, P = 0.221; treatment x adrenal medullectomy: $F_{(1,33)}$ = 0.148, P = 0.703) (Table 2).

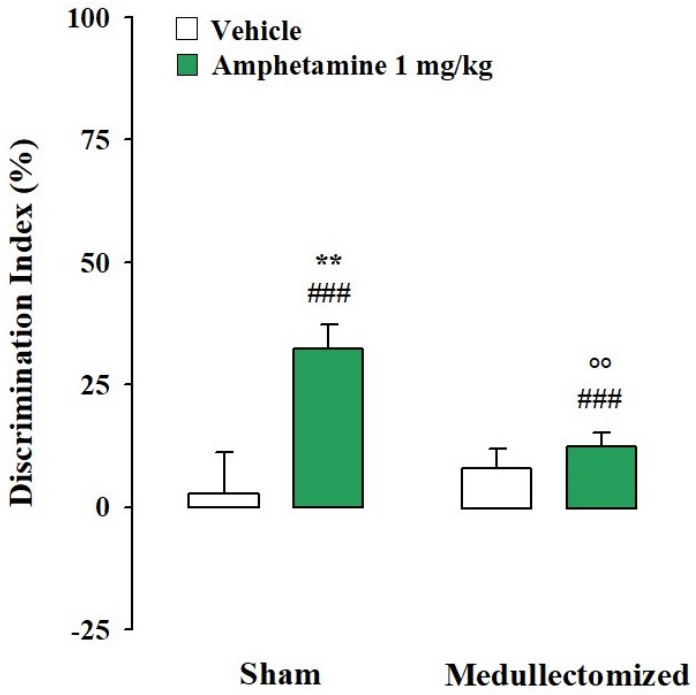

Figure 3. Influence of the peripheral adrenergic system on amphetamine effects on the long-term OR memory consolidation in rats exposed to the mild stress condition immediately after training. DI on the testing trial for sham and medullectomized rats that were treated with vehicle or amphetamine and subjected to the mild stress condition immediately after training. *Post hoc* comparisons reported significant differences between groups as follows: ** $P < 0.01$ vs the corresponding vehicle group. °° $P < 0.01$ vs the corresponding sham group. ### $P < 0.001$, one-sample t-test significantly different from zero. Data are expressed as mean ± SEM (n = 8-13 per group).

Amphetamine enhanced long term memory consolidation in adrenal medullectomized rats subject to the strong stress condition

In this experiment we sought to determine whether amphetamine impairing effects on long-term memory consolidation in rats exposed to the strong forced swim stress condition were dependent on the activation of the adrenergic system.

Training trial. Two-way ANOVA for total object exploration time on the training trial revealed no significant post-training treatment ($F_{(1,40)}$ = 2.243, P = 0.142), adrenal medullectomy ($F_{(1,40)}$ = 0.073, P = 0.788), or treatment x adrenal medullectomy ($F_{(1,40)}$ = 0.029, P = 0.865) effects. Two-way ANOVA for the number of crossings on the training trial revealed a significant adrenal medullectomy effect ($F_{(1,40)}$ = 8.158, P = 0.007), but no significant treatment ($F_{(1,40)}$ = 0.751, P = 0.391), or adrenal medullectomy x treatment ($F_{(1,40)}$ = 0.610, P = 0.440) effects. *Post hoc* analysis revealed that, among the medullectomized rats, post-training vehicle-treated animals showed a lower number of crossings with respect to the corresponding sham group (P < 0.05) (Table 1). Concerning the number of rearings, two-way ANOVA reported no significant effects of treatment ($F_{(1,40)}$ = 0.532, P = 0.470), adrenal medullectomy ($F_{(1,40)}$ = 0.507, P = 0.481) or interaction between these two factors ($F_{(1,40)}$ =1.683, P = 0.202) (Table 1).

Testing trial. One sample t-test revealed that in the sham group, only vehicle-treated animals were able to express long-term memory retention for the familiar object (sham: $t_{(9)}$ = 2.275, P = 0.049; amphetamine: $t_{(11)}$ = -0.127, P = 0.901). In the medullectomized groups, rats treated with vehicle or with amphetamine significantly discriminated the two objects (sham: $t_{(10)}$ = 7.003, P < 0.0001; amphetamine: $t_{(10)}$ = 2.775, P = 0.020). Two-way ANOVA for the DI revealed significant effects of treatment and adrenal medullectomy ($F_{(1,40)}$ = 5.662, P = 0.022; $F_{(1,40)}$ = 17.932, P = 0.0001, respectively), but no significant effect for the interaction between both factors ($F_{(1,40)}$ = 0.264, P = 0.610). *Post hoc* analysis indicated that medullectomized animals treated with vehicle or amphetamine showed higher DIs with respect to the corresponding sham groups (P < 0.01; P < 0.05, respectively) (Fig 4). Concerning rats' exploratory behavior of the experimental apparatus during the testing trial, two-way ANOVA indicated no significant effects for total object exploration time (treatment: $F_{(1,40)}$ = 0.453, P = 0.505; adrenal medullectomy: $F_{(1,40)}$ = 1.777, P = 0.190; treatment x adrenal medullectomy: $F_{(1,40)}$ = 0.319, P = 0.575), number of crossings (treatment: $F_{(1,40)}$ = 0.753, P = 0.391; adrenal medullectomy: $F_{(1,40)}$ = 1.743, P = 0.194; treatment x adrenal medullectomy: $F_{(1,40)}$ = 1.432, P = 0.238) and of the number of rearings (treatment: $F_{(1,40)}$ = 0.118, P =

0.733; adrenal medullectomy: $F_{(1,40)}$ = 1.232, P = 0.274; treatment x adrenal medullectomy: $F_{(1,40)}$ = 0.774, P = 0.384) (Table 2).

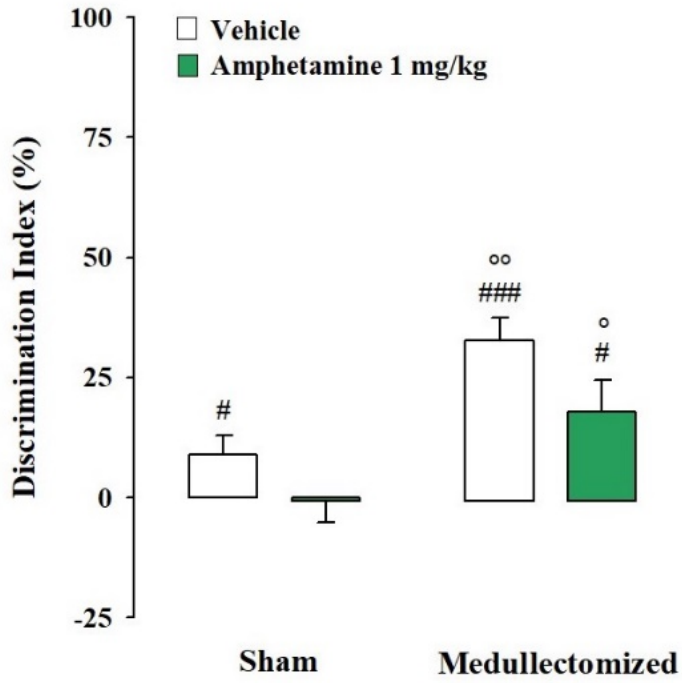

Figure 4. Influence of the peripheral adrenergic system on amphetamine effects on the long-term OR memory consolidation in rats exposed to the strong stress condition immediately after training. DI on the testing trial for sham and medullectomized rats that were treated with vehicle or amphetamine and subjected to the strong stress condition immediately after training. *Post hoc* comparisons reported significant differences between groups as follows: ° P < 0.05; °° P < 0.01 vs the corresponding sham group. # P < 0.05; ### P < 0.001, one-sample t-test significantly different from zero. Data are expressed as mean ± SEM (n = 10-12 per group).

Discussion

The present findings show that the psychostimulant amphetamine exerts dichotomic effects on long-term recognition memory, which are strictly dependent on the level of stress experienced during the early phase of memory consolidation. Specifically, our results indicate that amphetamine enhances long-term consolidation of recognition memory when rats are exposed to a mild stress condition immediately after training, yet impairing memory performance in case of strong stress exposure.

Over time, amphetamine has become greatly famous for its powerful central nervous system stimulation properties (Heal et al., 2013). Unfortunately, nowadays it is consequently considered one of the most commonly abused drugs (Berman et al., 2008). The psychostimulant properties of amphetamine depend on its modulation of both the noradrenergic and dopaminergic system (Fleckenstein et al., 2007). Amphetamine regulation of memory processes has been studied for many decades (Bardgett et al., 2019, Martinez et al., 1983, Oscos et al., 1988). It has been shown that amphetamine-dependent enhancement of memory consolidation depends on the noradrenergic system stimulation properties (Lee and Ma, 1995). Many studies demonstrated that the noradrenergic signaling activation finely regulates cognitive functions (Ferry and McGaugh, 2008, McIntyre et al., 2002, Wichmann et al., 2012), including memory consolidation for emotional experiences (McIntyre et al., 2012, Roozendaal and McGaugh, 2011, McGaugh, 2013, Campolongo et al., 2009a). In this regard, we very recently demonstrated that post-training administration of amphetamine is capable to improve long-term memory consolidation of an inhibitory avoidance discrimination task, and that such effect is totally attributable to the modulation of the noradrenergic tone rather than any alteration of the dopaminergic system (Colucci et al., 2019).

During stress response, the HPA axis is activated and different stress mediators and modulators, such as epinephrine, glucocorticoids (i.e. cortisol in humans and corticosterone in rodents) and endocannabinoids are released and act as endogenous modulators of memory consolidation (McIntyre and Roozendaal, 2007, Campolongo et

al., 2009b, Campolongo et al., 2012, Morena and Campolongo, 2014, Morena et al., 2014, Atsak et al., 2015, Morena et al., 2015). It is well known that the relationship between stress exposure and memory function follows an inverted U-shaped curve in which memory performance increases with optimal levels of stress (Salehi et al., 2010). An inverted U-shaped dose-response curve has also been documented for amphetamine effects on memory processes, similarly to several other adrenergic agents (Krivanek and McGaugh, 1969, Baldi and Bucherelli, 2005). Our results that amphetamine influences rat long-term recognition memory consolidation in a stress intensity-dependent fashion reinforce this evidence and highlight the existence of a modulatory interaction between amphetamine and different stress intensities in the modulation of long-term memory consolidation. This dichotomic effect could be explained in view of the noradrenergic modulation of memory, which is influenced by both amphetamine administration and stress experience and considering the inverted U-shape dose-response curve existing between norepinephrine and memory performance (Baldi and Bucherelli, 2005). Our results demonstrate that exposure to mild stress, immediately after the training trial of an OR task, prevents rats from expressing long-term memory retention for the familiar object. However, this effect is counteracted by post-training administration of amphetamine (1 mg/kg), which enhances long-term recognition memory retention. Accordingly, previous findings indicated that both amphetamine and stress are able to enhance norepinephrine brain levels (Ferrucci et al., 2019, Valentino et al., 1993). Hence, it is tentative to speculate that the norepinephrine levels elicited by exposure to a mild stress condition are not sufficient to enhance memory consolidation processes, but that treatment with amphetamine, specifically at the higher dose of 1 mg/kg, raises the norepinephrine concentration to a critical level able to enhance long term-memory consolidation. If a mild stress experience is not per se sufficient to create a long-term memory trace of the training trial, a more intensive stress, experienced immediately after training, is able to induce long-term memory retention of the training experience (Santori et al., 2019). Conversely, the concurrent treatment with amphetamine 1 mg/kg leads to a long-term memory consolidation impairment.

Therefore, it can be hypothesized that if the strong stress condition enhances the norepinephrine concentration a critical level eligible to create a long-term trace of the training experience, the treatment with amphetamine, combined with a strong stress experience, induces a norepinephrine release which is strongly exceeding this level, leading to an impairment of long-term memory consolidation.

Previous evidence demonstrated that amphetamine administration completely blocked the forced swim stress-induced expression of the corticotropin-releasing hormone (hnCRH) and it partially reduced c-fos expression in the paraventricular nucleus of the hypothalamus (PVN), indicating that a negative synergy between amphetamine and stress occurs dampening the characteristic peripheral physiological response to stress and activation of the PVN (Gomez-Roman et al., 2016). However, it has also been shown that amphetamine administration augmented the plasma adrenocorticotropin (ACTH) levels and HPA hormone concentrations, such as epinephrine and glucocorticoids (Gomez-Roman et al., 2016). Early studies suggested a key role of epinephrine in the modulation of norepinephrine release in the brain (Gold and van Buskirk, 1978). Epinephrine is not able to cross the blood-brain barrier and its central effects are due to the stimulation of β-adrenoceptors on vagal afferents terminating in the nucleus of the solitary tract (NTS) (Roozendaal and McGaugh, 2011). NTS innervate the Nucleus Paragigantocellularis (PGi) and other brain regions; PGi sends excitatory fibers, to the Locus Coeruleus (LC); in turn, LC sends noradrenergic projections to many brain areas involved in the modulation of memory consolidation (Roozendaal and McGaugh, 2011).

Previous findings have demonstrated that surgical removal of adrenal medulla abolishes the amphetamine enhancing effects on memory consolidation in rats not exposed to any stressful condition (Martinez et al., 1980b), thus demonstrating that amphetamine effects on memory consolidation are mediated by the peripheral adrenergic tone. In the second set of experiments, we therefore aimed at examining the potential role of the peripheral adrenergic tone in the modulation of long-term memory consolidation exerted by amphetamine administration and different stress intensities experienced soon

after learning. Our results clearly indicate that the peripheral adrenergic system plays a key role in the amphetamine modulatory effects on memory. Particularly, here we found that in medullectomized rats, which were unable to synthesize and release epinephrine, exposure to the mild stress condition immediately after training and amphetamine treatment, not only was sufficient to block the amphetamine enhancing effects on memory consolidation, but it also impaired memory performance; on the contrary, exposure to strong stress alone immediately after training ameliorated long-term memory retention. There is thus tentative to speculate that the stress intensity-dependent epinephrine release alters, through the vagal nerve-NTS-PGi-LC pathway, the norepinephrine transmission in the brain. Such influence, together with the amphetamine-mediated modulation of the noradrenergic system, finely tunes norepinephrine release in specific brain areas crucially involved in memory consolidation (e.g. hippocampus, amygdala), determining, according to the norepinephrine dose-response U-shaped curve, either impairing or enhancing effects on long-term memory consolidation.

Disruption of memory function is seen in a number of stress-associated disorder such as post-traumatic stress disorder (PTSD) (Berardi et al., 2014, Morena et al., 2018, Watson, 2019). Many studies indicated that the noradrenergic system might be responsible for the persistence of traumatic memories in PTSD (Liu et al., 2019, Gazarini et al., 2014, Debiec et al., 2011). A hallmark feature of such psychiatric condition is the over-consolidation of the traumatic experience, which in turn leads to maladaptive behavior (Desmedt et al., 2015). Exaggerated memories are generally potentiated by drug of abuse consumption (Gisquet-Verrier and Le Dorze, 2019, Colucci et al., 2019). Increases of norepinephrine contents were detected in response to both amphetamine administration, known to stimulate the noradrenergic system, and after exposure to trauma and its relative reminders (Le Dorze et al., 2019). Growing evidence supports a crucial link between psychostimulant abuse and PTSD development (Crum-Cianflone et al., 2015, Ruglass et al., 2014). Our findings highlight that amphetamine induces dichotomic effects on long-term memory consolidation, by

activating the peripheral adrenergic system, which in turns finely tunes memory performance according to the level of stress experienced immediately after learning. Further investigations of a possible amphetamine contribution to the modulation of the mechanisms underlying stress-related disorders development will be thus necessary.

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CHAPTER 3

ANANDAMIDE MODULATION OF CIRCADIAN- AND STRESS-DEPENDENT EFFECTS ON RAT SHORT-TERM MEMORY

Alessia Santori[1], Paola Colucci[1], Giulia Federica Mancini[1], Maria Morena[2], Maura Palmery[1], Viviana Trezza[3], Stefano Puglisi-Allegra[4], Matthew N. Hill[2], Patrizia Campolongo[1,4]

[1]Dept. of Physiology and Pharmacology, Sapienza University of Rome, 00185, Rome, Italy; [2]Hotchkiss Brain Institute, Depts. of Cell Biology and Anatomy & Psychiatry, University of Calgary, T2N 4N1, Calgary, Canada; [3]Dept. of Science, Section of Biomedical Sciences and Technologies, University Roma Tre, 00146, Rome, Italy; [4]Neurobiology of Behavior Laboratory, Santa Lucia Foundation, 00143, Rome, Italy

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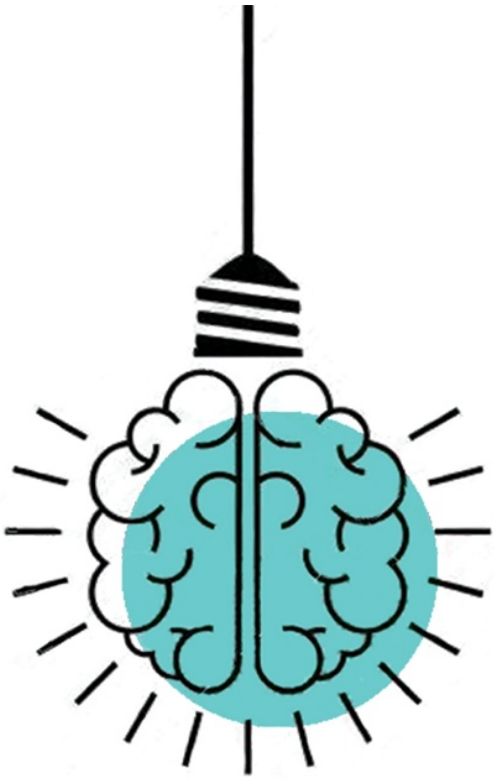

Abstract

The endocannabinoid system plays a key role in the control of emotional responses to environmental challenges. CB1 receptors are highly expressed within cortico-limbic brain areas, where they modulate stress effects on memory processes. Glucocorticoid and endocannabinoid release is influenced by circadian rhythm. Here, we investigated how different stress intensities immediately after encoding influence rat short-term memory in an object recognition task, whether the effects depend on circadian rhythm and if exogenous augmentation of anandamide levels could restore any observed impairment. Two separate cohorts of male adult Sprague-Dawley rats were tested at two different times of the day, morning (inactivity phase) or afternoon (before the onset of the activity phase) in an object recognition task. The anandamide hydrolysis inhibitor URB597 was intraperitoneally administered immediately after the training trial. Rats were thereafter subjected to a forced swim stress under low or high stress conditions and tested 1-h after training. Control rats underwent the same experimental procedure except for the forced swim stress (no stress). We further investigated whether URB597 administration might modulate corticosterone release in rats subjected to the different stress conditions, both in the morning or afternoon. The low stressor elevated plasma corticosterone levels and impaired 1-h recognition memory performance when animals were tested in the morning. Exposure to the higher stress condition elevated plasma corticosterone levels and impaired memory performance, independently of the testing time. These findings show that stress impairing effects on short-term recognition memory are dependent on the intensity of stress and circadian rhythm. URB597 (0.3 mg kg^{-1}) rescued the altered memory performance and decreased corticosterone levels in all the impaired groups yet leaving memory unaltered in the non-impaired groups.

Introduction

The endocannabinoid system plays a key regulatory role in many fundamental physiological processes, such as sleep/wake cycles (Lovinger, 2008, Murillo-Rodríguez et al., 2017; Pava, et al., 2016), learning and memory (Akirav, 2011; Atsak et al., 2015; Morena and Campolongo, 2014) and central nervous system (CNS) regulation of endocrine functions (Hillard, 2015; Balsevich et al., 2017). The two major endocannabinoids, N-arachidonylethanolamide (anandamide, AEA; Devane et al., 1992) and 2-arachidonoyl glycerol (2-AG; Sugiura et al., 1995) are synthesized on demand and travel retrogradely to presynaptic sites to bind cannabinoid type-1 (CB1) receptors (Kano et al., 2009). After being released into the synaptic cleft, AEA and 2-AG are primarily degraded by distinct hydrolytic enzymes, the fatty acid amide hydrolase (FAAH; Cravatt et al., 2001) and monoacylglycerol lipase (MAGL; Dinh et al., 2002), respectively.

Emotion influences memory at multiple levels (McGaugh, 2000), from perceptual recognition and identification (Zeelenberg et al., 2006) to explicit recognition and recall of emotional stimuli (Kensinger and Schacter, 2008). Compelling evidence indicates that drugs that target the endocannabinoid system induce biphasic effects on cognitive and emotional behavior depending on the level of stress and emotional arousal at the time of encoding and drug consumption (Campolongo et al., 2013; Manduca et al., 2014; Morena et al., 2014, 2015, 2016a). Glucocorticoids are stress response mediators which interact with the endocannabinoid system in the regulation of memory function (Campolongo et al., 2009; Hill et al., 2018; Morena and Campolongo, 2014). Their synthesis is characterized by a circadian release pattern, with peak levels linked to the start of the activity phase and diurnal regulation under control of the circadian clock (Dickmeis, 2009). Literature evidence indicates that the endocannabinoid signaling exhibits a circadian rhythm with variations reported in CB1 receptor expression (Rueda-Orozco et al., 2008), endocannabinoids tissue contents and in the enzymes controlling their synthesis and degradation (Valenti et al.,

2004). Extensive research has identified glucocorticoid-endocannabinoid crosstalk as crucial mediator of the glucocorticoid dependent modulation of emotional memories (Atsak et al., 2015; Campolongo et al., 2009), but still it remains uncertain the influence of circadian rhythm on this mediation. Moreover, far less well understood is the relationship between circadian rhythm biology and memory formation (Gerstner and Yin, 2010). Therefore, the main purpose of the present study was to evaluate how different stress intensities may influence short-term recognition memory in rats, investigating whether their action is regulated by circadian rhythm and if AEA has any role on this process. To this aim we investigated the effects of post-training systemic administration of the FAAH inhibitor, URB597, which increases AEA levels at active synapses, on short-term retention of object recognition memory under three different stress conditions (no, low or high forced swim stress), at two different times of the day, morning (inactivity phase) or afternoon (before the onset of the activity phase). Behavioral experiments were paralleled by biochemical measurement aimed at measuring plasma corticosterone levels in all the experimental groups.

Materials and Methods

Animal Care and Use

Male adult Sprague-Dawley rats (350–450g at the time of training and testing, Charles River Laboratories, Calco, Italy) were kept individually in an air-conditioned colony room (temperature: 21 ± 1°C; lights on from 07:00 AM to 7:00 PM) with pellet food and water available *ad libitum*. Training and testing were performed during the light phase of the cycle between 10:00 AM and 6:00 PM. All procedures involving animal care or treatments were performed in compliance with the ARRIVE guidelines, the Directive 2010/63/EU of the European Parliament, and the D. L. 26/2014 of Italian Ministry of Health.

Drug Treatment

The anandamide hydrolysis inhibitor URB597 [(3′-(aminocarbonyl)[1,1′-biphenyl]-3-yl)-cyclohexylcarbamate] (0.1 or 0.3 mg kg^{-1}; Tocris Bioscience, Bristol UK) was administered intraperitoneally (i.p.) in a volume of 1 ml kg^{-1} immediately after the training trial. Doses were chosen on the basis of pilot experiments performed in our laboratory and on literature data (Kathuria et al., 2003; Campolongo et al., 2013; Morena and Campolongo, 2014), in order to have a maximum augmentation of AEA release in the synaptic cleft. The solutions were freshly prepared on the day of the experiment and dissolved in 5% polyethylene glycol, 5% Tween-80 and 90% saline (vol/vol). The vehicle solution contained 5% polyethylene glycol and 5% Tween-80 in saline only.

Behavioral Procedures

Object recognition task. A slightly modified procedure of that described by Campolongo et al. (2013) was used. The experimental apparatus was a gray open-field box (in cm, 40 wide × 40 deep × 40 high) with the floor covered with sawdust, positioned in a dimly illuminated room. The objects to be discriminated were transparent glass vials (5.5 cm diameter and 5 cm height) and white glass light bulbs (6 cm diameter and 11 cm length). All rats were handled twice per day for 1 min each and extensively habituated to the experimental context twice per day for 3 min each for 7 days preceding the training day. During habituation, rats were allowed to freely explore the apparatus in the absence of objects. The animals were randomly assigned to three different groups: no stress, low stress and high stress conditions and tested either in the morning (rats' inactive phase, 10:00 AM - 12:30 PM) or in the afternoon (before the onset of the activity phase, 3:30 PM - 6:00 PM). On the training trial, each rat was individually placed in the experimental apparatus at the opposite end from the objects. The rat was allowed to explore two identical objects (A1 and A2) for 6 min, then it was removed from the apparatus and, after drug treatment, if belonging to the low or high stress condition group, it was subjected to a forced swim stress; then, he was returned

to his home cage. The no stress group was placed back to its home cage immediately after drug injection. To avoid the presence of olfactory trails, sawdust was stirred, foecal boli were removed and the objects were cleaned with 70% ethanol after each trial. Rat's behavior was recorded by using a video camera positioned above the experimental apparatus and videos were analyzed with Observer XT 12 (Noldus Information Technology BV, Wageningen, The Netherlands) by a trained observer who was unaware of treatment condition. Exploration of an object was defined as pointing the nose to the object at a distance of < 1 cm and/or touching it with the nose. Turning around or sitting on an object was not considered as exploration. During the training trial, the time spent exploring the two objects (total object exploration time, s) was taken as a measure of object exploration, and exploratory behavior of the experimental apparatus was analyzed by the measuring total number of crossings and rearings. For crossings, the floor of the apparatus was divided into four imaginary squares and the total number of crossings between squares was determined. Memory retention was tested 1 h after the training trial. On the retention test trial, one copy of the familiar object (A3) and a new object (B) were placed in the same location as stimuli during the training trial (Fig. 1). All combinations and locations of objects were used to reduce potential biases due to preference for particular locations or objects. Each rat was placed in the apparatus for 6 min, and its behavior was recorded. To analyze cognitive performance, during the retention test, a discrimination index (DI) was calculated as the difference in time exploring the novel and the familiar object, expressed as the percentage ratio of the total time spent exploring both objects.

Forced swim stress procedure. Forced swimming was used as the stressor because its neurochemical and hormonal effects are well defined and meet the criteria of a stress-inducing agent (Schneider and Simson, 2007). Immediately after the training trial of the object recognition task rats were forced to swim in a tank (50 cm in height × 20 cm in diameter), filled to a depth of 30 cm with water. At the end of the swimming period, the rats were removed from the water and were immediately and gently wiped to dryness

with absorbent paper before they were returned to the home cage. Rats in the low and high stress condition groups were subjected to a low or high intensity stressor by using a 1- or 5-min forced swim stress procedure at different water temperatures of 25 ± 1°C or 19 ± 1°C, respectively, known to elicit different plasma corticosterone levels (Morena et al., 2015).

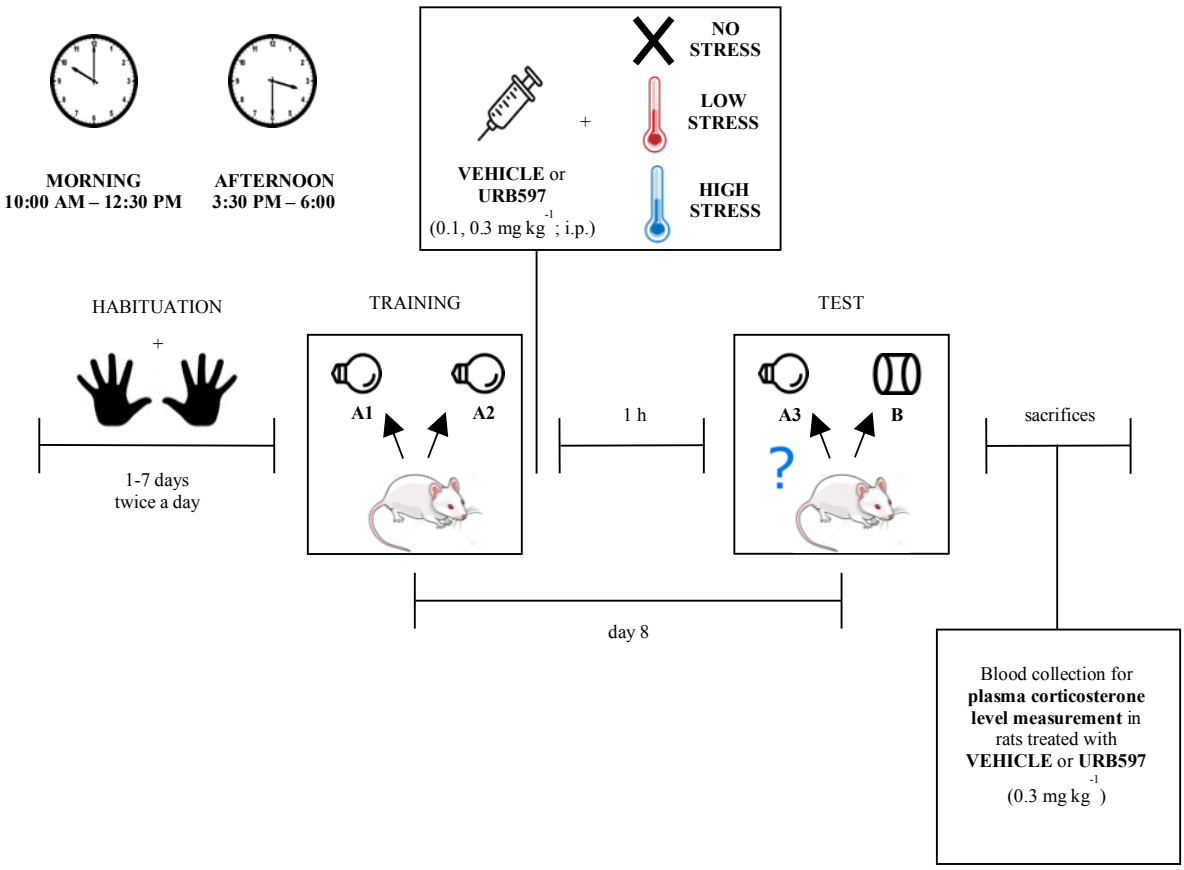

Figure 1 – Diagram of the experimental procedure.

Plasma Corticosterone Levels

Corticosterone levels were determined in rats in the no stress, low stress and high stress conditions that were tested in the morning or afternoon and in rats that were handled (twice per day for 7 days) but not trained (home cage), at the two different times of the day. As novelty stimulation triggers an HPA-axis response that leads to a corticosterone plasma peak at 30 min and normalizes within 90 min after stress exposure (de Kloet et al., 2005), rats were killed immediately after the test trial, 60 min after the URB597

administration. Trunk blood was collected after decapitation in tubes containing 200 µl of 0.1 M EDTA and samples were centrifuged at 1000 × g for 15 min at 4°C. Plasma was stored at –20°C and analyzed for corticosterone levels using a DetectX ELISA kit (Arbor Assays, Ann Arbor, MI, USA) according to the manufacturer's instructions as previously described (Fletcher et al., 2018). In compliance with EU animal legislation (3R principle: reduction) corticosterone levels were measured in vehicle-treated and in URB 0.3 mg kg^{-1} (effective dose in rescuing stress-dependent memory impairments) treated rats.

Data and Statistical Analysis

One-sample t-tests were used to determine whether the discrimination index was different from zero. Object recognition data and plasma corticosterone levels were analyzed by two-way ANOVAs. Tukey-Kramer *post hoc* tests were used to determine the source of the detected significances. P values of < 0.05 were considered statistically significant. To be included in the statistical analysis rats had to reach a minimum criterion of total object exploration time > 10 s on either training or testing. Prior findings indicate that such rats adequately acquire the task (Okuda et al., 2004; Roozendaal et al., 2008; Winters et al., 2009; Campolongo et al., 2013; Barsegyan et al., 2019). All data are expressed as mean ± standard error of the mean (SEM).

Results

Effects of different stress intensities and circadian rhythm on short-term recognition memory retention performance and plasma corticosterone levels

To determine whether different stress intensities modulate short-term memory retention performance and whether these effects are dependent on circadian rhythm, we first analyzed the behavioral performance of all vehicle-treated rats, used in the subsequent URB597 experiments, at different times of the day (e.g. morning and afternoon), in

order to unveil any possible influence of stress or time on memory and corticosterone levels.

One-sample t-tests revealed that the discrimination indexes of vehicle-treated rats were significantly different from zero for both the no stress groups tested either in the morning or in the afternoon ($t_{(7)}$ = 4.654, P = 0.002 and $t_{(9)}$ = 4.384, P = 0.002, respectively; Fig. 2a) and for the low stress condition group tested in the afternoon ($t_{(10)}$ = 3.715, P = 0.004; Fig. 2a), thus indicating that these three animal groups discriminated the novel object. In contrast, rats in the remaining low and high stress conditions morning groups and the high stress condition afternoon group did not express memory retention for the familiar object. Two-way ANOVA for discrimination index revealed a significant stress condition effect ($F_{(2.50)}$ = 4.313, P = 0.019) and a tendency toward significance for the time of the testing ($F_{(1.50)}$ =3.082, P = 0.085) and for the interaction between these two factors ($F_{(2.50)}$ = 2.493, P = 0.093). *Post hoc* analysis showed that the low stress condition significantly decreased the discrimination index of rats tested in the morning as compared to the no stress group tested at the same time of the day and the corresponding low stress condition group tested in the afternoon (P < 0.05 for both comparisons; Fig. 2a).

Regarding the total object exploration time on the testing trial, two-way ANOVA revealed a significant stress condition effect ($F_{(2.50)}$ = 12.693, P < 0.0001), but no significant time of testing or stress condition x time of testing interaction effects. Finally, rats' exploratory behavior of the apparatus during the test trial did not differ among the different experimental groups. Two-way ANOVAs for number of crossings or rearings revealed no significant stress condition, no time of testing or stress condition x time of testing interaction effects (Table 1).

Furthermore, we evaluated whether plasma corticosterone levels were differentially modulated by the different stress conditions, at two times of the day.

Two-way ANOVA for plasma corticosterone levels immediately after test, revealed a significant stress condition effect ($F_{(3.54)}$ = 17.836, P < 0.0001), but no significant time or stress condition x time effects. *Post hoc* analysis showed that rats that were subjected

to low stress condition had higher corticosterone levels than home cage control rats only in the morning ($P < 0.01$; Fig. 2b). Moreover, rats subjected to the high stress condition presented significant higher corticosterone levels than home cage control rats and no stress groups both in the morning ($P < 0.01$, for both comparisons; Fig. 2b) and in the afternoon ($P < 0.01$ and $P < 0.05$; for home cage and no stress groups, respectively; Fig. 2b).

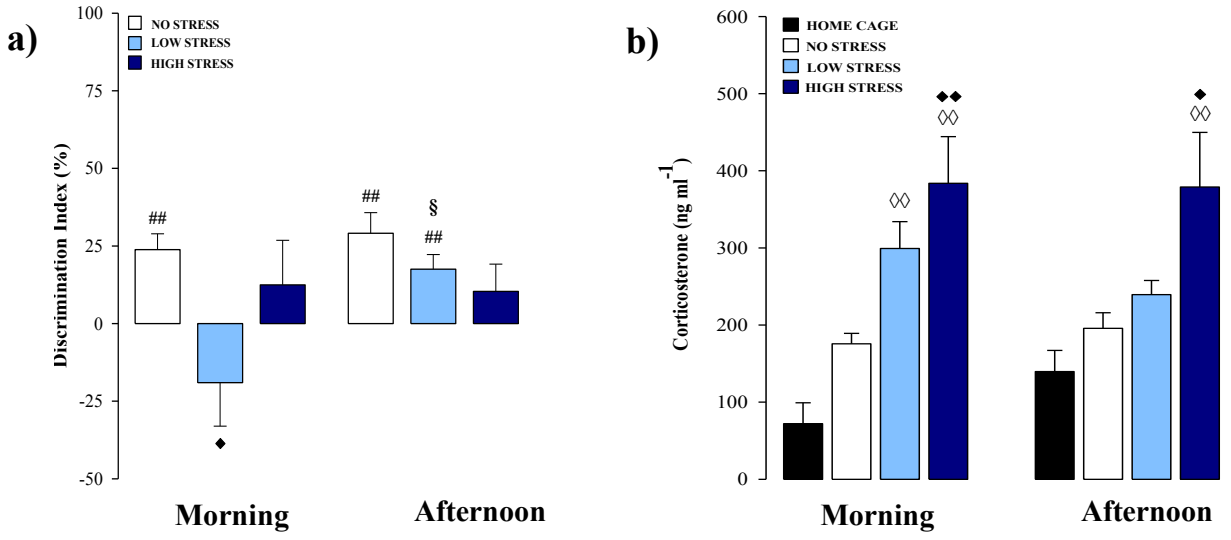

Figure 2 – Circadian-dependent effects of different stress conditions on short-term memory. **a)** Discrimination index on the testing trial for vehicle-treated rats that were subjected to no, low or high stress conditions immediately after the training trial performed in the morning or afternoon. *Post hoc* comparisons reported significant differences between groups as follows: ♦ $P < 0.05$ vs the corresponding no stress group. § $P < 0.05$ vs the corresponding low stress group trained in the morning. ## $P < 0.01$, one-sample t-tests significantly different from zero. Data are expressed as mean ± SEM (n = 8-11 per group). **b)** Plasma corticosterone levels of home cage and vehicle-treated rats subjected to no, low or high stress condition immediately after the training trial that were euthanized, in the morning or in the afternoon, 60 min after stress exposure, immediately after test. *Post hoc* comparisons reported significant differences between groups as follows: ◊◊ $P < 0.01$ vs the corresponding home cage group. ♦ $P < 0.05$; ♦♦ P

< 0.01 vs the corresponding no stress group. Data are expressed as mean ± SEM (n = 7-9 per group).

	Morning			Afternoon		
	Total object exploration time	**Number of crossings**	**Number of rearings**	**Total object exploration time**	**Number of crossings**	**Number of rearings**
NO STRESS						
VEHICLE	58.2 ± 8.2	21.4 ± 4.2	26.4 ± 5.8	46.3 ± 7.5	17.6 ± 4.1	26.6 ± 4.8
URB 0.1	50.6 ± 9.4	20.8 ± 4.1	34.6 ± 6.7	68.5 ± 18.3	24.0 ± 4.9	31.1 ± 5.4
URB 0.3	47.0 ± 5.7	23.6 ± 3.6	30.5 ± 4.9	48.4 ± 7.0	20.0 ± 4.0	26.4 ± 4.4
LOW STRESS						
VEHICLE	33.4 ± 5.9 *	23.0 ± 3.7	39.1 ± 4.6	32.8 ± 4.0	16.9 ± 2.3	28.7 ± 3.7
URB 0.1	32.4 ± 6.4	16.8 ± 2.1	32.0 ± 4.3	31.5 ± 5.7 *	13.8 ± 3.1	24.6 ± 4.3
URB 0.3	20.9 ± 3.9 **	16.9 ± 2.3	31.8 ± 5.8	35.6 ± 5.0	19.8 ± 2.5	26.1 ± 3.8
HIGH STRESS						
VEHICLE	17.0 ± 4.8 **	15.6 ± 3.4	43.0 ± 6.8	30.9 ± 3.0	15.9 ± 2.0	34.6 ± 8.6
URB 0.1	17.3 ± 4.0 *	13.6 ± 3.5	33.6 ± 9.0	29.9 ± 3.4 *	10.9 ± 1.1	39.1 ± 4.7
URB 0.3	15.5 ± 3.6 **	17.3 ± 2.5	37.8 ± 4.0	28.1 ± 4.8 *	10.5 ± 1.3	30.0 ± 7.6

Total time spent exploring the two objects (in seconds) and the number of crossings and rearings of all groups tested in the morning and in the afternoon. * P < 0.05; ** P < 0.01 vs the corresponding no stress group. Data are expressed as mean ± SEM (n = 8-12 per group).

Table 1 – Exploratory behavior on the testing trial for vehicle- and URB597-treated rats that were subjected to no, low or high stress conditions immediately after the training trial, in the morning and in the afternoon sessions.

Effects of the AEA hydrolysis inhibitor URB597 on short-term object recognition memory performance and plasma corticosterone levels in the no, low and high stress condition groups tested in the morning

This experiment investigated whether immediate post-training injection of the AEA hydrolysis inhibitor URB597 modulates short-term performance on an object recognition task and plasma corticosterone levels and whether these effects are influenced by different stress conditions in animals tested in the morning.

As shown in figure 3a, one-sample t-tests revealed that the discrimination indexes were significantly different from zero for all no stress treatment groups ($t_{(7)}$ = 4.654, P = 0.002; $t_{(7)}$ = 2.741, P = 0.029 and $t_{(7)}$ = 4.745, P = 0.002; vehicle, URB597 0.1 and

URB597 0.3 mg kg^{-1}, respectively), while, for the low and high stress groups, only URB597 0.3 mg kg^{-1}treated rats discriminated the new object ($t_{(7)}$ = 3.206, P = 0.015, $t_{(7)}$ = 5.533, P = 0.001, for the low and high stress conditions URB597 0.3 mg kg^{-1}groups, respectively). In contrast, low and high stressed rats in the remaining vehicle and URB597 0.1 mg kg^{-1} groups did not express memory retention for the familiar object. Two-way ANOVA for the discrimination index revealed significant stress condition ($F_{(2.63)}$ = 3.838, P = 0.027) and treatment ($F_{(2.63)}$ = 7.257, P = 0.002) effects as well as a tendency toward significance for the interaction between these two factors ($F_{(4.63)}$ = 2.112, P = 0.090). *Post hoc* analysis showed that URB597 0.3 mg kg^{-1} treated rats subjected to low or high stress presented a better discrimination index relative to their corresponding vehicle groups (P < 0.05, for both comparisons; Fig. 3a). Moreover, rats that were treated with URB597 0.3 mg $kg^{-1}$and then subjected to the high stress condition showed a high discrimination index as compared to those administered the same dose of URB597 but subjected to the no or low stress procedure (P < 0.05, for both comparisons; Fig. 3a). Concerning the total exploration time of the two objects on the testing trial, two-way ANOVA revealed a significant stress condition effect ($F_{(2.63)}$ = 24.885, P < 0.0001), but no significant treatment or stress condition x treatment effects. Finally, rats' exploratory behavior of the apparatus during the test trial did not differ among the different experimental groups. Two-way ANOVAs for number of crossings and rearings revealed no significant stress condition, treatment or stress condition x treatment interaction effects (Table 1).

Two-way ANOVA for plasma corticosterone levels revealed significant stress condition ($F_{(2.41)}$ = 6.969, P = 0.003) and treatment ($F_{(1.41)}$ = 10.634, P = 0.002) effects, but no significant interaction between these two factors. *Post hoc* analysis showed that URB597 0.3 mg kg^{-1} treated rats subjected to low or high stress presented lower corticosterone levels than their corresponding vehicle groups (P < 0.05, for both comparisons; Fig. 3b), suggesting that URB597 0.3 mg kg^{-1} counteracted the stress-induced increase on plasma corticosterone levels, in both the low and high stress conditions.

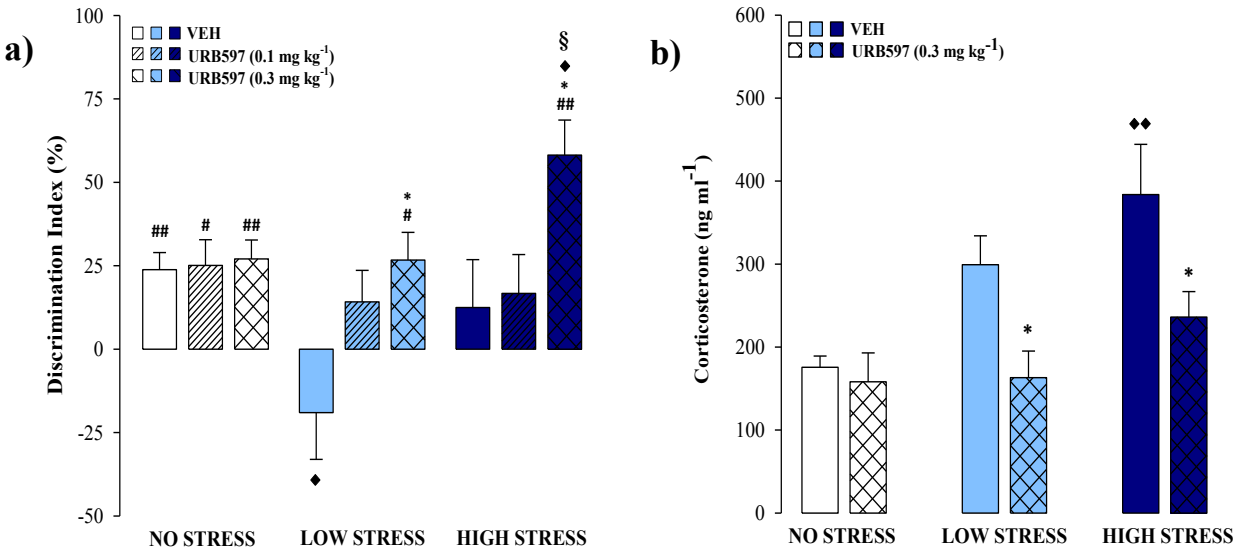

Figure 3 – URB597 modulation of stress-dependent effects on short-term memory in the morning. a) Discrimination index on the testing trial for vehicle- and URB597-treated rats that were subjected to no, low or high stress conditions immediately after the training trial performed in the morning. *Post hoc* comparisons reported significant differences between groups as follows: * $P < 0.05$ vs the corresponding vehicle group. ♦ $P < 0.05$ vs the corresponding no stress group. § $P < 0.05$ vs the corresponding low stress group. # $P < 0.05$; ## $P < 0.01$, one-sample t-tests significantly different from zero. Data are expressed as mean ± SEM (n = 8-9 per group). **b)** Plasma corticosterone levels of vehicle and URB597 0.3 mg kg^{-1} treated rats subjected to no, low or high stress condition immediately after the training trial that were euthanized in the morning, 60 min after stress exposure, immediately after test. *Post hoc* comparisons reported significant differences between groups as follows: * $P < 0.05$ vs the corresponding vehicle group. ♦♦ $P < 0.01$ vs the corresponding no stress group. Data are expressed as mean ± SEM (n = 6-9 per group).

Effects of the AEA hydrolysis inhibitor URB597 on short-term object recognition memory performance and plasma corticosterone levels in the no, low and high stress condition groups tested in the afternoon

This experiment investigated whether immediate post-training injection of the AEA hydrolysis inhibitor URB597 altered short-term performance on an object recognition task and plasma corticosterone levels and whether these effects were influenced by different stress conditions (no, low and high stress) when animals were tested in the afternoon.

As shown in figure 4a, one-sample t-tests revealed that the discrimination indexes were significantly different from zero for the no stress and low stress vehicle, URB597 0.1 mg kg^{-1} and URB597 0.3 mg kg^{-1} groups ($t_{(9)} = 4.384$, $P = 0.002$; $t_{(8)} = 2.658$, $P = 0.029$ and $t_{(7)} = 2.805$, $P = 0.026$, respectively for no stress groups; $t_{(10)} = 3.715$, $P = 0.004$; $t_{(10)} = 2.435$, $P = 0.035$ and $t_{(10)} = 4.412$, $P = 0.001$, respectively for low stress condition groups) and the high stress condition URB597 (0.1 and 0.3 mg kg^{-1}) groups ($t_{(11)} = 3.266$, $P = 0.008$; $t_{(11)} = 7.987$, $P < 0.0001$), thus indicating that these animals discriminated the novel object with respect to the familiar one. Rats in the remaining high stress vehicle group did not express memory retention for the familiar object (Fig. 4a). Two-way ANOVA for discrimination index revealed no significant stress condition or treatment effects, but a significant interaction between these two factors ($F_{(4.86)} = 2.593$, $P = 0.042$). *Post hoc* comparisons showed that, among rats tested under the high stress condition, URB597 0.3 mg kg^{-1} significantly increased the discrimination index as compared to vehicle treated rats ($P < 0.01$; Fig. 4a). Moreover, rats treated with the high dose of URB597 and subjected to the high stress condition presented a significant high discrimination index as compared to their corresponding low stress group ($P < 0.05$; Fig. 4a). Concerning the total exploration time of the two objects on the testing trial, two-way ANOVA revealed a significant stress condition effect ($F_{(2.86)} = 9.794$, $P = 0.0001$), but no significant treatment or stress condition x treatment effect (Table 1). Two-way ANOVA for number of crossings revealed a significant stress condition effect ($F_{(2.86)} = 5.902$, $P = 0.004$), but no significant treatment or stress condition x treatment

interaction effects (Table 1). Concerning the number of rearings, two-way ANOVA revealed no significant stress condition effect, no treatment effect or any interaction between these two factors (Table 1).

Two-way ANOVA for plasma corticosterone levels revealed significant treatment ($F_{(1.37)} = 6.169$, $P = 0.018$) and stress condition x treatment interaction ($F_{(2.37)} = 6.289$, $P = 0.005$) effects, but no significant effect of the stress condition. *Post hoc* analysis showed that only URB597 0.3 mg kg^{-1} treated rats subjected to high stress presented lower corticosterone levels than their corresponding vehicle group ($P < 0.01$; Fig. 4b), suggesting that URB597 0.3 mg kg^{-1} counteracted the stress-induced increase on plasma corticosterone levels in the high stress condition.

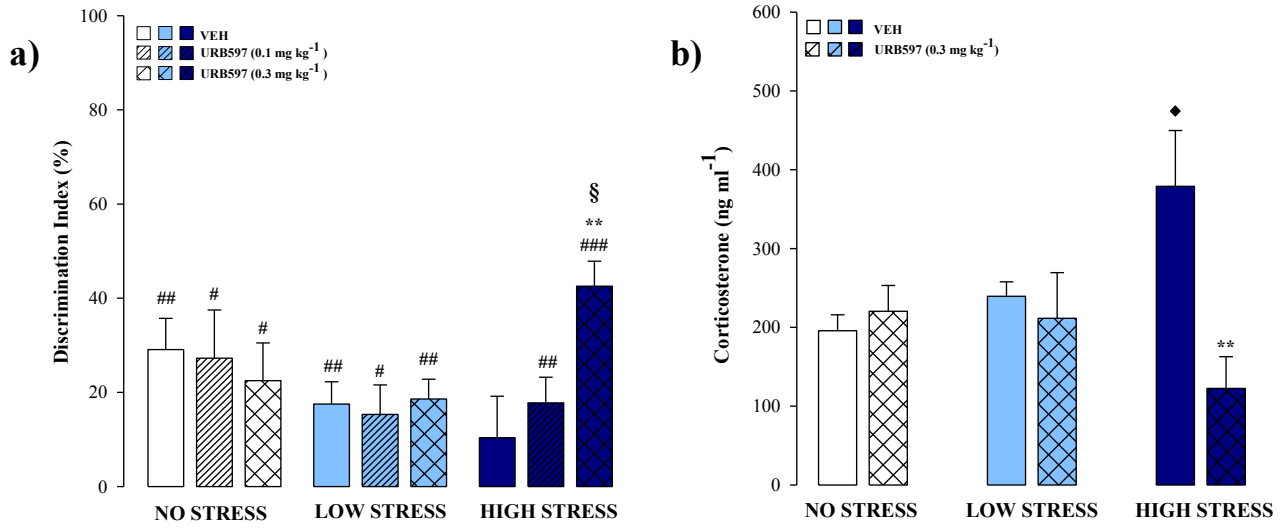

Figure 4 – URB597 modulation of stress-dependent effects on short-term memory in the afternoon. a) Discrimination index on the testing trial for vehicle- and URB597-treated rats that were subjected to no, low or high stress conditions immediately after the training trial, in the afternoon session. *Post hoc* comparisons reported significant differences between groups as follows: ** $P < 0.01$ vs the corresponding vehicle group. § $P < 0.05$ vs the corresponding low stress group. # $P < 0.05$; ## $P < 0.01$; ### $P < 0.0001$, one-sample t-tests significantly different from zero. Data are expressed as mean ± SEM (n = 8-12 per group). **b)** Plasma corticosterone levels of vehicle and URB597 0.3 mg kg^{-1} treated rats subjected to no, low or high stress condition immediately after

the training trial that were euthanized in the afternoon, 60 min after stress exposure, immediately after test. *Post hoc* comparisons reported significant differences between groups as follows: ** $P < 0.01$ vs the corresponding vehicle group. ♦ $P < 0.05$ vs the corresponding no stress group. Data are expressed as mean ± SEM (n = 6-8 per group).

Discussion

The present findings show that systemic administration of the AEA hydrolysis inhibitor URB597 counteracts the stress impairing effects on short-term object recognition memory, in a stress intensity- and circadian-dependent fashion. We have previously shown that activation of CB1 receptors differentially modulates short-term recognition memory in rats depending on environmental aversiveness and on the level of stress the animal experienced at the time of drug administration and memory encoding (Campolongo et al., 2013, 2012). In particular, post-training administration of the CB1 receptor agonist WIN55,212-2 enhanced object recognition performance (tested 24 hour later) exclusively in animals training under a high arousal state (Campolongo et al., 2013). Literature data suggested that low versus high doses of THC and synthetic cannabinoid agonists provoke opposite stress-induced corticosterone release through CB1-mediated mechanisms (Mayer et al., 2014; Patel et al., 2004; Sano et al., 2009). Evidence has indicated that endocannabinoid augmentation approaches via FAAH or MAGL inhibitors generally produce dose-related decreases in the regulation of HPA-axis function and anxiety, whereas THC and exogenous cannabinoids produce biphasic effects with low doses mimicking endocannabinoid augmentation effects (Hill et al., 2018). Although there is one report showing that systemic administration of the FAAH inhibitor URB597 impairs the acquisition and early consolidation of contextual fear conditioning (Burman et al., 2016), other studies investigating the AEA signaling indicated that URB597 treatment enhanced consolidation (Morena et al., 2014) and impaired retrieval of aversive memories throughout indirect CB1 activation (Ratano et al., 2014). CB1 receptors are abundantly expressed in cortico-limbic regions, including

the basolateral complex of the amygdala (BLA), hippocampus and medial prefrontal cortex (mPFC), where they modulate emotional arousal effects on memory (Akirav, 2013; Morena et al., 2015, 2014; Tasker et al., 2015) and regulate hypothalamic–pituitary–adrenal (HPA) axis activity (Morena et al., 2016b). Extensive research has demonstrated that not only CB1 receptors, but also glucocorticoid receptors are located within this brain circuitry (Herkenham et al., 1990; Hill et al., 2010; Myers et al., 2014). Numerous evidence shows that glucocorticoids enhance memory consolidation of emotionally arousing experiences, but impair memory retrieval and working memory (de Quervain et al., 2017; McIntyre and Roozendaal, 2007). These different glucocorticoids effects are dependent on a non-genomically mediated interaction with noradrenergic transmission within the BLA and the hippocampus, wherein the endocannabinoid system has been shown to play an important role in mediating such effects (Atsak et al., 2015, 2012a; Jiang et al., 2014). Specifically, glucocorticoids or a stressor, administered shortly before or immediately after training, impair short-term memory performances in an object recognition task (Okuda et al., 2004; Roozendaal et al., 2006b), likely by negatively interfering with memory retrieval. Similarly, intrahippocampal infusions of the cannabinoid agonist WIN55,212–2 impair the retrieval of memory (Morena et al., 2015); however, antagonism of hippocampal β-adrenoceptor activity blocks the memory retrieval impairment induced by WIN55,212–2 (Atsak et al., 2012a), supporting the evidence that glucocorticoid and endocannabinoid signaling interact to impair the retrieval of emotional memory through their influence on downstream noradrenergic activity (Balsevich et al., 2017). The locus coeruleus (LC), the main source of norepinephrine in the mammalian forebrain, provides norepinephrine to different brain regions, including the BLA (McCall et al., 2017) and mPFC (Sara, 2009), wherein activation of CB1 receptors results in decreased cortical norepinephrine release (Reyes et al., 2012), when it is normally potentiated by acute swim stress exposure (Morilak et al., 2005). Evidence suggests that under high levels of stress the LC promotes fear learning by enhancing BLA function, while simultaneously blunting prefrontal function. Conversely, low levels of arousal are

sufficient for the LC to facilitate mPFC function and promote downstream inhibition of the amygdala (Giustino and Maren, 2018). Herein we demonstrated that exposure to a low stress immediately after the training trial selectively impairs short-term memory retention/retrieval when animals are tested in the morning while exposure to a high stress impairs short-term performance independently of the testing time. Interestingly, the stressed groups that were unable to discriminate between the 2 objects were those presenting increased levels of corticosterone. This is in accordance with extensive human and animal research showing that glucocorticoids impair memory retrieval (Roozendaal et al., 2006a; Wolf et al., 2016; de Quervain et al., 2019). Interestingly, our findings showed that post-training treatment with the AEA hydrolysis inhibitor URB597 counteracts these impairing effects of stress on memory performance, both in the morning and afternoon testing sessions. Specifically, systemic URB597 injection, at the dose of 0.3 mg kg^{-1}, enhances short-term memory retention in the low stress condition group tested in the morning, as well as in both the high stress groups tested either in the morning or in the afternoon, maintaining unaltered the performances of rats that did not show any cognitive impairment. Extensive evidence indicates that cannabinoids, either administered exogenously or released from endogenous sites, have pronounced effects on learning and memory (Hill et al., 2018; Marsicano and Lafenêtre, 2009; Morena and Campolongo, 2014; Ratano et al., 2017). Moreover, previous evidence has shown that AEA and 2-AG modulate emotional memory processes by interacting with glucocorticoids and other stress-activated neuromodulatory systems such as norepinephrine, in brain limbic regions (Atsak et al., 2015, 2012b; Campolongo et al., 2009; Morena et al., 2016a, 2015, 2014; Morena and Campolongo, 2014). Our finding that URB597 treatment has no effects in animals tested under no stress condition but selectively affects memory in the presence of a stressor, is in line with this evidence and has a high impact potential. On the light of this evidence it is tentative to speculate that stress of different intensities at two times of the day differentially regulated LC-NE action on the mPFC, since such interaction might be described by an inverted-U function such that it can either enhance or hinder learning depending on different

arousal states (Giustino and Maren, 2018). The exact mechanisms underlying cannabinoid modulation of norepinephrine has yet to be determined, but evidence indicated that it may involve direct influences of CB1 receptors that are localized to noradrenergic axon terminals in the mPFC (Oropeza et al., 2007), which contribute to regulating norepinephrine release. In particular, microdialysis data supported a mechanism whereby administration of WIN55,212–2 prior to swim stress exposure decreased cortical norepinephrine efflux by inhibiting presynaptic inhibitory α2-adrenergic autoreceptors (Reyes et al., 2012), and such evidence is supported by predominant presynaptic distribution of α2-adrenergic receptors in the mPFC (Cerrito and Preziosi, 1985; Dennis et al., 1987; Pudovkina et al., 2001).

It is well known that stress effects on memory performance follow an inverted U-shaped relationship; very low or very high levels of stress have detrimental effects, while intermediate levels lead to optimal memory performances (Baldi and Bucherelli, 2005). In mammals, an important feature of glucocorticoid regulation is a diurnal release pattern, with serum cortisol/corticosterone concentration peak in the morning and lowest at night (Dickmeis, 2009). Since rats are nocturnal animals, under laboratory circumstances of a regular light/dark cycle, the peak of HPA rhythm occurs in the afternoon, just before the onset of the activity phase; the nadir occurs during sleep, when corticosterone levels reach their lowest serum concentration, whereas in the morning (during the rats' inactive phase) the HPA axis activity begins to increase (Bertani et al., 2010; Gong et al., 2015). Although different studies have demonstrated that circadian clocks can influence learning and memory function (Tapp and Holloway, 1981; Gerstner and Yin, 2010; Smarr et al., 2014), no circadian effect has been documented on short-term memory recognition performances yet. Our results show that vehicle-treated animals tested in the morning session have impaired memory retention when exposed to both low or high stressors. These groups of rats also presented higher plasma corticosterone levels than no stress group. However, when vehicle-treated rats were tested in the afternoon, memory retention was only negatively affected by the exposure to the high stressor, which in parallel increased rats' plasma corticosterone levels. It is

tentative to speculate that when animals are tested during the low activity phase of the HPA axis (i.e. morning session), both low and high stressor exposures induce a severe deviation from homeostasis which negatively affects memory retention performance. Our finding that exposure to low and high stress conditions elevated plasma corticosterone levels in rats that were trained in the morning, is in line with this evidence. Conversely, when animals are tested at the beginning of their active phase (i.e. afternoon), at their plasma corticosterone concentration peak, the high, but not the low, stress exposure might induce a more robust deflection from homeostasis, thus only the high stress condition group presents impairments in memory retention performance and higher plasma corticosterone levels. Our results indicate that maximal memory strength requires an intermediate level of stress, thus are in line with the Yerkes-Dodson law. Of note, boosting AEA levels with systemic URB597 injections is capable to specifically counteract these stress detrimental effects on short-term memory performance, decreasing plasma corticosterone levels in impaired memory groups. Previous findings indicated that WIN55,212-2 inhibited stress-induced elevation in corticosterone levels (Campolongo et al., 2013; Ganon-Elazar and Akirav, 2012, 2009), ameliorating the detrimental effects of stress on memory. Nevertheless, evidence demonstrated that the effects of cannabinoid drugs such as WIN55,212-2 on plasma corticosterone levels strictly depend on the level of arousal at the moment of administration. Previous findings demonstrated that URB597 is capable to reduce plasma corticosterone levels in response to repeated stress exposures (Hill et al., 2010). Whether this URB597 effect is due to an interaction with the HPA axis activity or to a direct effect on memory performance, or both, needs to be further investigated, but the current data strongly indicate that URB597 is able to reduce plasma corticosterone levels in short-term memory impaired-groups. Taken together, our findings indicate that stress impairing effects on short-term recognition memory seem to be dependent on the intensity of stress and HPA axis circadian rhythm and that treatment with URB597 is capable of specifically counteracting these detrimental effects. These results suggest that FAAH inhibition may be a potential therapeutic target for stress-inducing memory

alterations highlighting the need for clinical studies to examine this possible cannabinoid mechanism of restoring memory impairments.

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CHAPTER 4

HIPPOCAMPAL 2-ARACHIDONOYL GLYCEROL SIGNALING REGULATES TIME-OF-DAY AND STRESS-DEPENDENT EFFECTS ON RAT SHORT-TERM MEMORY

Alessia Santori[1,2], Maria Morena[3,4], Matthew N. Hill[3,4], Patrizia Campolongo[1,2]

[1]Dept. of Physiology and Pharmacology, Sapienza University of Rome, 00185 Rome, Italy; [2]Neurobiology of Behavior Laboratory, Santa Lucia Foundation, 00143 Rome, Italy; [3]Hotchkiss Brain Institute, University of Calgary, Calgary, AB T2N 4N1 Canada; [4] Depts. of Cell Biology and Anatomy & Psychiatry, University of Calgary, Calgary, AB T2N 4N1 Canada

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Abstract

Background: Cannabinoids induce biphasic effects on memory depending on stress levels. We previously demonstrated that different stress intensities, experienced soon after encoding, impaired rat short-term recognition memory in a time-of-day-dependent manner, and that boosting endocannabinoid anandamide (AEA) levels restored memory performance. Here, we examined if two different stress intensities and time-of-day alter hippocampal endocannabinoid tone, and whether these changes modulate short-term memory. **Methods**: Male Sprague-Dawley rats were subjected to an object recognition task and exposed, at two different times of the day (i.e., morning or afternoon), to low or high stress conditions, immediately after encoding. Memory retention was assessed 1-h later. Hippocampal AEA and 2-arachidonoyl glycerol (2-AG) content and the activity of their primary degrading enzymes, fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL), were measured soon after testing.

Results: Consistent with our previous findings, low stress impaired 1-h memory performance only in the morning, whereas exposure to high stress impaired memory independently of testing time. Stress exposure decreased AEA levels independently of memory alterations. Interestingly, exposure to high stress decreased 2-AG content and, accordingly, increased MAGL activity, selectively in the afternoon. Thus, to further evaluate 2-AG's role in the modulation of short-term recognition memory, rats were given bilateral intra-hippocampal injections of the 2-AG hydrolysis inhibitor KML29 immediately after training, then subjected to low or high stress conditions and tested 1-h later. **Conclusions**: KML29 abolished the time-of-day-dependent impairing effects of stress on short-term memory, ameliorating short-term recognition memory performance.

Introduction

The impact of stress on learning and memory processes is a controversial topic that has largely been investigated to unveil its complex effects on cognition [1]. Whereas intense emotional events can generate vivid long-lasting memories [2], very strong emotional experiences can also induce amnesia [3]. The discovery of stress hormone receptors in the hippocampus has fostered research showing that this brain structure is crucially involved in the negative feedback regulation of the hypothalamic–pituitary–adrenal (HPA) axis [4]. Exposure to stress alters both hippocampal anatomy and functionality [5], with negative consequences on memory processes [6]. Indeed, the hippocampus represents a key forebrain structure highly associated with emotional and recognition memory processes [7].

Endocannabinoid signaling is widely distributed throughout corticolimbic circuits that are linked to stress response [8] and represents one of the main systems modulating hippocampal neuroplasticity [9]. The endocannabinoid system is a neuromodulatory lipid system, which consists of the cannabinoid type 1 and type 2 (CB1 and CB2) receptors [10] and two major endogenous ligands, 2-arachidonoyl glycerol (2-AG; [11])) and N-arachidonyl ethanolamide (anandamide, AEA; [12]). Endocannabinoids are retrograde messengers that are synthesized "on demand" in the postsynaptic membrane by Ca^{2+}-dependent and -independent mechanisms [13] and feedback onto presynaptic terminals, thus modulating afferent neurotransmitter release via activation of CB1 receptors [14]. 2-AG and AEA are primarily degraded by distinct hydrolytic enzymes, monoacylglycerol lipase (MAGL; [15]) and fatty acid amide hydrolase (FAAH; [16]), respectively. Considerable evidence indicates that endocannabinoid signaling plays a key role in fundamental physiological processes that are altered in a circadian manner [17], simultaneously regulating both the activation of the HPA axis [18] and the termination of stress response [19]. Stress exposure generally provokes alterations in endocannabinoid tone depending on the intensity, duration and nature of the stressor, but also the brain region investigated [20]. However, the interaction between stress and

the endocannabinoid system [21] has typically been investigated in the same time window [22–24]. We previously demonstrated that stress impairing effects on short-term recognition memory depend on the intensity of stress and time-of-day and that systemic augmentation of AEA levels restores memory performance in a stress intensity- and time-of-day-dependent fashion [25]. However, it still remains unexplored: i) if different stress intensities affect hippocampal endocannabinoid system components, ii) whether the effects are time-of-day-dependent, iii) how short-term memory is influenced, and iv) 2-AG's role in such regulation. Therefore, the present study aimed to determine how different stress intensities at two times of the day (i.e. morning or afternoon) influence hippocampal endocannabinoid modulation of short-term recognition memory, and how post-training bilateral intra-CA1 infusion of the 2-AG hydrolysis inhibitor KML29 influences short-term memory performance.

Results

Effects of different stress intensities on hippocampal 2-AG levels and MAGL hydrolytic activity in rats tested in the morning or afternoon

This experiment investigated whether different stress levels and times of the day (morning vs afternoon) associated to the test procedure induced any alteration in hippocampal 2-AG content and MAGL hydrolytic activity at the time of testing.

As shown in **Fig. 1A**, two-way ANOVA for hippocampal 2-AG levels showed a significant stress condition effect ($F_{(2.49)} = 9.727$, $P = 0.0003$), no significant effect of the time of testing, but a significant interaction between both factors ($F_{(2.49)} = 8.559$, $P = 0.0006$). *Post hoc* comparisons for hippocampal 2-AG content showed that among animals tested in the afternoon session, rats subjected to the high stress condition presented a significant decrease in 2-AG levels as compared with their corresponding no and low stress condition groups ($P < 0.01$ for both comparisons) and their morning counterpart ($P < 0.05$). Furthermore, within the low stress condition group, rats tested

in the afternoon showed increased 2-AG levels relative to rats tested in the morning session ($P < 0.01$).

Fig. 1B-C shows the effects of different stress intensities and times of the testing trial on hippocampal MAGL activity. Two-way ANOVA for V_{max} of MAGL reported a significant stress condition effect ($F_{(2.23)} = 3.956$, $P = 0.033$), no significant testing time effect and a significant interaction between both factors ($F_{(2.23)} = 6.519$, $P = 0.006$). *Post hoc* analysis showed a significant increase of MAGL V_{max} value in rats subjected to the high stress condition and tested in the afternoon as compared to no stress and low stress rats that were tested at the same time and to the high stress condition group tested in the morning ($P < 0.01$, for all comparisons; **Fig. 1B**). Two-way ANOVA for MAGL K_m revealed no significant stress condition, testing time, or stress condition × testing time interaction effects (**Fig. 1C**).

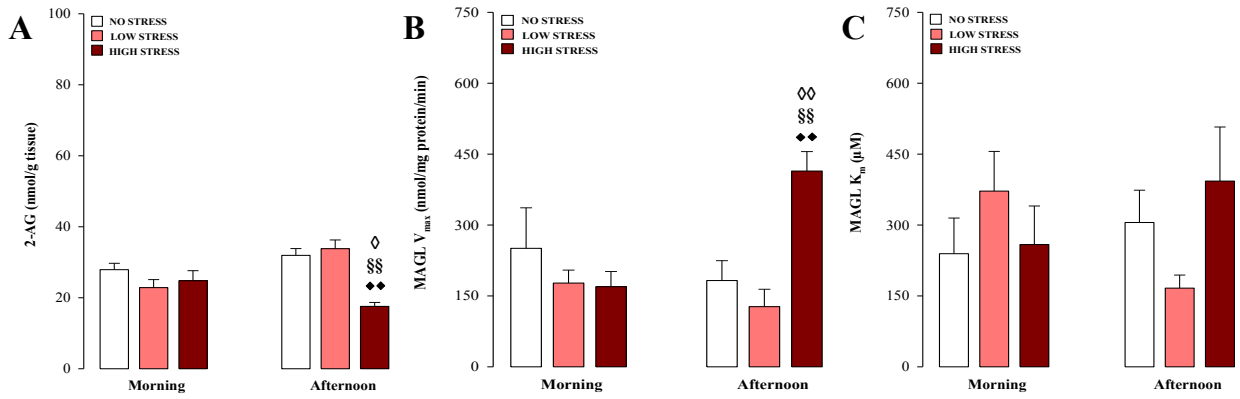

Fig. 1 – Time-of-day-dependent effects of stress on short-term recognition memory influence hippocampal 2-AG levels and its degradation. Hippocampal 2-AG levels (**A**), and MAGL V_{max} (**B**) and K_m (**C**) values, as assessed immediately after the testing trial in non-cannulated rats that were subjected to no, low or high stress conditions after the training trial performed in the morning or afternoon. *Post hoc* comparisons reported significant differences between groups as follows: ♦♦ $P < 0.01$ vs the corresponding no stress group. §§ $P < 0.01$ vs the corresponding low stress group. ◊ $P < 0.05$; ◊◊ $P < 0.01$ vs the corresponding stress condition groups trained in the morning. Data are expressed as mean ± SEM (n = 4-10 per group).

Effects of different stress intensities on hippocampal AEA levels and FAAH hydrolytic activity in rats tested in the morning or afternoon

This experiment investigated whether different stress levels and times of the day (morning vs afternoon) associated to the test procedures induced any alteration in hippocampal AEA content and FAAH hydrolytic activity at the time of testing.

As shown in **Fig. 2A**, two-way ANOVA for hippocampal AEA levels revealed that there was a significant stress condition effect ($F_{(2.49)}$ = 3.388, P = 0.042), but no significant effect of the time of testing or interaction between both factors.

The effects of different stress intensities and times of the testing trial on hippocampal FAAH activity are shown in **Fig. 2B-C**. Two-way ANOVAs for FAAH V_{max} or K_m did not reveal any significant stress condition, testing time, or stress condition × testing time interaction effects (**Fig. 2B-C**).

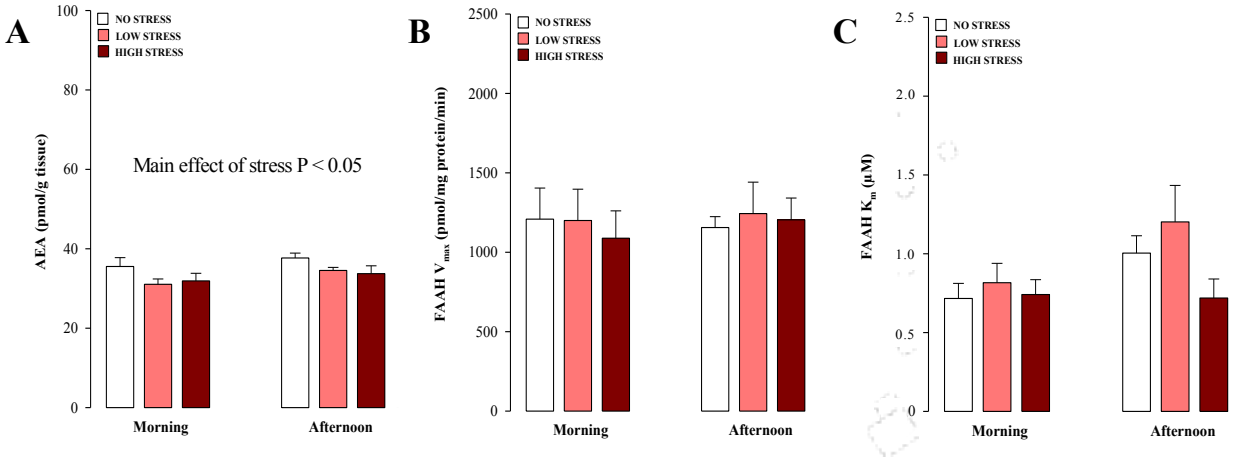

Fig. 2 – Time-of-day-dependent effects of stress on short-term recognition memory influence hippocampal AEA levels and its degradation. Hippocampal AEA levels (**A**), and FAAH V_{max} (**B**) and K_m (**C**) values, as assessed immediately after the testing trial in non-cannulated rats that were subjected to no, low or high stress conditions after the training trial performed in the morning or afternoon. P < 0.05 main effect of stress on hippocampal AEA levels. Data are expressed as mean ± SEM (n = 7-8 per group).

Stress intensity and time-of-day effects on short-term recognition memory

This experiment examined the effects of different stress intensities and times of the day (morning vs afternoon) on short-term memory retention performance. Each behavioral performance analyzed in the present section applies to animals treated with vehicle that were used in the subsequent KML29 experiments and is functional to discuss the effects of different stress intensities and time-of-day on short-term recognition memory. Consistently with our previous work involving non-cannulated rats [25], here we found impairing effects of stress on short-term recognition memory, which were stress intensity- and time-of-day-dependent. All the results concerning the behavioral performance on the training trial are shown in **Table S1**.

Two-way ANOVA for discrimination index revealed significant stress condition ($F_{(2.63)}$ = 5.517, P = 0.006) and time of the testing ($F_{(1.63)}$ = 7.463, P = 0.008) effects, but no significant interaction between these two factors. One-sample t-tests reported that intra-CA1 vehicle-treated rats displayed discrimination indexes significantly different from zero for both the no stress condition morning and afternoon groups ($t_{(11)}$ = 2.588, P = 0.025 and $t_{(10)}$ = 3.200, P = 0.010, respectively; **Fig. 3**) and only for the low stress condition group tested in the afternoon ($t_{(11)}$ = 3.976, P = 0.002; **Fig. 3**), suggesting that these experimental groups discriminated the novel object. Contrarily, rats belonging to the high stress condition groups tested either in the morning or afternoon and the low stress condition morning group did not express memory retention for the familiar object. *Post hoc* analysis indicated that exposure to the low stress condition in the morning decreased rat discrimination index as compared to the corresponding low stress condition group tested in the afternoon (P < 0.01; **Fig. 3**). Furthermore, animals belonging to both the low and high stress condition morning groups showed impaired discrimination indexes as compared to the no stress condition group that was tested at the same time of the day (P < 0.05 for both comparisons; **Fig. 3**). With respect to the total object exploration time on the testing trial, two-way ANOVA revealed a significant stress condition effect ($F_{(2.63)}$ = 4.892, P = 0.011), but no significant time of testing or stress condition × time of testing interaction effects (**Table S2**). Two-way

ANOVAs for number of crossings or rearings revealed a significant effect of the stress condition ($F_{(2.63)} = 7.579$, $P = 0.001$ and $F_{(2.63)} = 17.225$, $P < 0.0001$, respectively), but no time of testing or stress condition × time of testing interaction effects (**Table S2**).

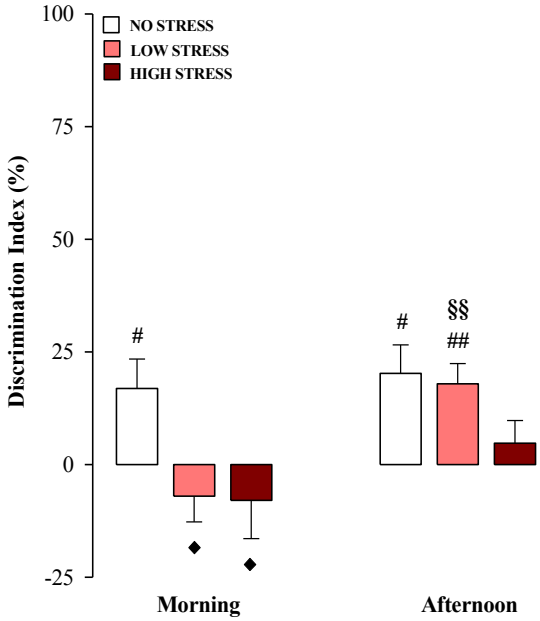

Fig. 3 – Different stress intensities and time-of-day effects on short-term memory. Discrimination index on the testing trial for intra-CA1 vehicle-treated rats belonging to the no, low or high stress condition groups that were tested in the morning or afternoon. *Post hoc* comparisons revealed significant differences between groups as follows: ♦ $P < 0.05$ vs the corresponding no stress group. §§ $P < 0.01$ vs the corresponding low stress morning group. # $P < 0.05$, ## $P < 0.01$, one-sample t-tests significantly different from zero. Data are expressed as mean ± SEM (n = 10-12 per group).

Effects of the 2-AG hydrolysis inhibitor KML29 on hippocampal modulation of short-term recognition memory performance in the no, low and high stress condition groups tested in the morning

This experiment investigated whether the 2-AG hydrolysis inhibitor KML29 (2 or 20 ng in 0.5 µl) bilaterally infused into the CA1 regions of the dorsal hippocampus, immediately after the training trial, modulates short-term memory performance in an

object recognition task and whether these effects are influenced by exposure to different stress conditions, in the morning. Results concerning the behavioral performance on the training trial are shown in **Table S1**.

Two-way ANOVA for the discrimination index revealed significant stress condition ($F_{(2.92)} = 3.186$, $P = 0.046$), treatment ($F_{(2.92)} = 8.520$, $P = 0.0004$) and stress condition × treatment interaction ($F_{(4.92)} = 3.134$, $P = 0.018$) effects. As shown in **Fig. 4A**, one-sample t-tests revealed that the discrimination indexes were significantly different from zero for all no stress treatment groups ($t_{(11)} = 2.588$, $P = 0.025$; $t_{(10)} = 8.064$, $P < 0.0001$ and $t_{(11)} = 2.993$, $P = 0.012$; vehicle, KML29 2 and 20 ng, respectively), while, for the low and high stress groups, only KML29 20 ng-treated rats discriminated the new object ($t_{(9)} = 2.811$, $P = 0.020$, $t_{(11)} = 3.208$, $P = 0.008$, for the low and high stress condition KML29 20 ng groups, respectively). In contrast, vehicle- and KML29 2 ng-treated groups in the low and high stress conditions did not express memory retention for the familiar object. *Post hoc* analysis showed that KML29 20 ng-treated rats subjected to low or high stress presented a better discrimination index relative to their corresponding vehicle groups ($P < 0.01$ and $P < 0.05$; low and high stress conditions, respectively; **Fig. 4A**). Moreover, rats that were treated with vehicle or KML29 2 ng and then exposed to the low or high stress condition showed impaired discrimination index as compared to their corresponding no stress groups ($P < 0.05$, for all comparisons; **Fig. 4A**). Concerning the total exploration time of the two objects on the testing trial, in accordance with our previous findings [25], two-way ANOVA revealed a significant stress condition effect ($F_{(2.92)} = 12.157$, $P < 0.0001$), but no significant treatment or stress condition × treatment effects (**Table S2**). Finally, rats' exploratory behavior of the arena during the test trial showed significant differences among experimental groups arisen from the different stress exposures. Two-way ANOVAs for number of crossings and rearings revealed a significant stress condition effect ($F_{(2.92)} = 9.387$, $P = 0.0002$ and $F_{(2.92)} = 42.565$, $P < 0.0001$, respectively), but no treatment or stress condition × treatment interaction effects (**Table S2**).

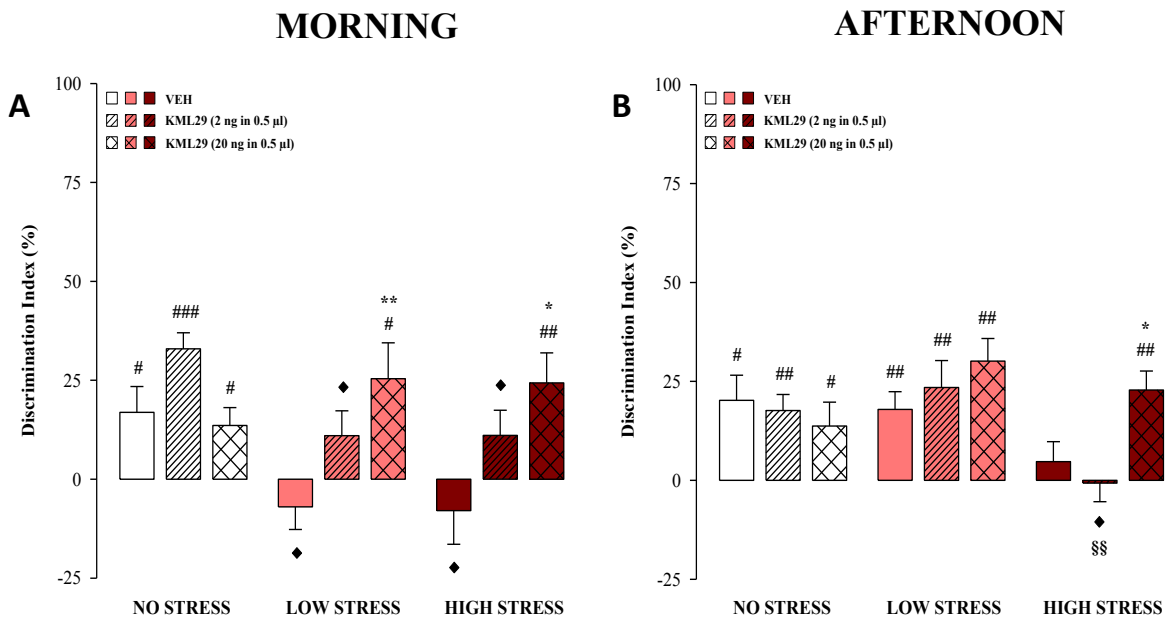

Fig. 4 – KML29 modulation of stress- and time-of-day-dependent effects on short-term memory. Discrimination index on the testing trial for rats that were administered in the CA1 region of the dorsal hippocampus with either vehicle or KML29 and then subjected to no, low or high stress conditions immediately after training, in the morning (**A**) or in the afternoon (**B**). *Post hoc* analysis reported significant group differences as follows: * $P < 0.05$, ** $P < 0.01$ vs the corresponding vehicle group. ♦ $P < 0.05$ vs the corresponding no stress group. §§ $P < 0.01$ vs the corresponding low stress group. # $P < 0.05$; ## $P < 0.01$, ### $P < 0.0001$, one-sample t-tests significantly different from zero. Data are expressed as mean ± SEM (n = 10-12 per group).

Effects of the 2-AG hydrolysis inhibitor KML29 on hippocampal modulation of short-term recognition memory performance in the no, low and high stress condition groups tested in the afternoon

This experiment investigated whether the 2-AG hydrolysis inhibitor KML29 (2 or 20 ng in 0.5 µl) bilaterally infused into the dorsal CA1, immediately after the training trial, modulates short-term memory performance in an object recognition task and whether these effects are influenced by exposure to different stress conditions, in the afternoon. Results concerning the behavioral performance on the training trial are shown in **Table**

S1. Two-way ANOVA for discrimination index revealed a significant stress condition ($F_{(2.95)}$ = 5.916, P = 0.004) effect, but no significant effect of treatment or interaction between these two factors. As shown in **Fig. 4B**, one-sample t-tests revealed that the discrimination indexes were significantly different from zero for the no stress and low stress vehicle, KML29 2 and KML29 20 ng groups ($t_{(10)}$ = 3.200, P = 0.010; $t_{(10)}$ = 4.336, P = 0.002 and $t_{(10)}$ = 2.274, P = 0.046, respectively for no stress groups; $t_{(11)}$ = 3.976, P = 0.002; $t_{(10)}$ = 3.446, P = 0.006 and $t_{(11)}$ = 5.258, P = 0.0003, respectively for low stress condition groups) and the high stress condition KML29 20 ng group ($t_{(11)}$ = 4.765, P = 0.0006), indicating that these animals were capable to discriminate the novel object. The remaining rats that were subjected to the high stress condition and administered with either vehicle or KML29 2 ng did not express memory retention for the familiar object (**Fig. 4B**). *Post hoc* comparisons showed that, among rats tested under the high stress condition, KML29 20 ng significantly increased the discrimination index as compared to animals that were treated with vehicle (P < 0.05; **Fig. 4B**), whereas KML29 2 ng impaired rat discrimination index in comparison to their corresponding no stress and low stress groups (P < 0.05 and P < 0.01, respectively; **Fig. 4B**). Consistently with our previous findings [25], two-way ANOVA for the total exploration time of the two objects on the testing trial revealed a significant effect of stress condition ($F_{(2.95)}$ = 4.116, P = 0.019), but no significant treatment or stress condition × treatment effects (**Table S2**). Two-way ANOVA for number of crossings and rearings revealed a significant effect of stress condition ($F_{(2.95)}$ = 9.065, P = 0.0002 and $F_{(2.95)}$ = 11.190, P < 0.0001, respectively), but no significant treatment or stress condition × treatment interaction effects (**Table S2**).

Discussion

The present findings show that different stress intensities and times of day differentially modulate hippocampal endocannabinoid tone. Exposure to high stress impairs short-term recognition memory selectively before the onset of the activity phase (afternoon), but not during the inactive phase (morning), and decreases hippocampal 2-AG content, presumably by increasing MAGL hydrolytic activity. Our results indicate that boosting hippocampal 2-AG signaling, with post-training bilateral intra-CA1 injections of the 2-AG hydrolysis inhibitor KML29, completely restores impaired memory performance, in accordance to the stress intensity and phase activity/inactivity.

Evidence has demonstrated the abundant expression of CB1 receptors within cortico-limbic regions, including the basolateral complex of the amygdala (BLA), hypothalamus, hippocampus and medial prefrontal cortex (mPFC) [26,27]. CB1 receptors activation regulates the HPA axis activity [20], but also stress and emotional arousal effects on memory [28]. It has been shown that exposure to stress activates corticotropin-releasing hormone (CRH) receptors 1 (CRHR1) in the amygdala, which increase the enzymatic activity of FAAH, resulting in a decrease of the inhibitory tone of AEA. Such mechanism contributes to the activation of the HPA axis and stress-related behavioral responses [20]. Conversely, elevations in corticosterone appear to be the primary mechanism by which stress increases 2-AG levels in the hypothalamus, which activating CB1 receptors contributes to negative-feedback inhibition of the HPA axis and termination of stress response [20].

The effects of stress on the endocannabinoid system are complex, regionally specific, and time-dependent [20]. Several studies demonstrated that exposure to acute stress generally causes a rapid reduction in AEA content in response to an array of stressors [29,30], whereas typically increases 2-AG signaling throughout different cortico-limbic regions [26,31], suggesting a bidirectional effect of stress on the endocannabinoid system. Specifically, within the hippocampus, acute restraint stress reduces AEA content and increases 2-AG levels [32,33]. In line with this evidence, our findings show

that independently of the time of the day and stress intensity, swim stress decreased hippocampal AEA levels. Concerning 2-AG's tone, however, we did not find any increase in hippocampal 2-AG immediately after acute stress exposure, as most of the studies in literature have normally documented. Indeed, we found a strong reduction of 2-AG content, along with a robust increase of the activity of its degrading enzyme, in rats exposed to the high stress condition and tested at the onset of the active phase.

According to the timing of stress exposure, stress-mediated secretion of glucocorticoids alters hippocampal functions and plasticity [34], thus affecting hippocampal-dependent memories in rodents and humans [35].

It is now well established that exposure to glucocorticoids, a stressor or emotional arousal, shortly before, during or immediately after training, impairs short-term memory performances in an object recognition task [25,36], likely by negatively interfering with memory retrieval [37]. Since corticosterone is still elevated at the time of the 1-h retention test, it is probable that it affected short-term retention performance via direct influences on the retrieval of memory processing [38].

By replicating our previously published findings [25], here we found that when animals were tested during the circadian low activity phase of the HPA axis (i.e. morning session), exposure to a stressor, regardless of its intensity, impaired memory performance. Conversely, when testing occurred at the beginning of their active phase (i.e. afternoon), when the HPA axis reaches its activity peak [39], under laboratory conditions of a regular light/dark cycle, only the high intensity stressor impaired memory performance. Similarly to corticosterone, activity of arousal system mediators is also influenced by circadian rhythm, with norepinephrine reaching its peak at the onset of the dark phase [40].

In the light of this evidence, as we had previously speculated [25], exposure to stress impairs memory retention only when it causes a more robust deviation from homeostasis, that is during the low activity phase of stress systems. Thus, it is likely that exposure to the low intensity stressor at the beginning of the active phase did not alter behavioral performance, because it did not cause a severe deviation from

homeostasis, being animals tested during their high HPA axis and arousal system activity phase.

Our surprising finding that only exposure to a high stress in the afternoon, but not in the morning, induces a strong reduction of hippocampal 2-AG levels might be the result of a compensatory and still unresolved mechanism that allows the system at the onset of the dark phase, which, thus, already presents high hippocampal corticosterone and norepinephrine levels, to perceive and initiate a proper stress response, by reducing both 2-AG inhibitory action at hippocampal noradrenergic fibers and its negative feedback regulation onto the HPA axis. Conversely, exposure to a lower intensity stressor, might have been not strong enough to activate this putative mechanism. This hypothesis is also supported by corticosterone plasma levels of the low stress exposure group tested in the afternoon, which did not differ from those of non-stressed controls [25].

It is important to note that studies examining the effects of stress on endocannabinoid content have often been performed ignoring that the timing of the experiments could influence stress modulation of the endocannabinoid system and memory processes. Thus, to our knowledge this is the first study documenting an interaction between stress exposure and time-of-day on hippocampal 2-AG levels, and this might explain the contrasting findings in literature concerning the endocannabinoid, and particularly 2-AG, modulation of memory. Further investigation is warranted to explore our novel findings.

We previously reported that systemic post-training injections of the AEA hydrolysis inhibitor URB597, which increases AEA levels, counteracted detrimental effects of stress on short-term memory, likely by restoring corticosterone to physiological levels, when altered by swim stress exposure. Our results highlight that URB597-mediated beneficial effects on memory are not hippocampus-dependent, as we found a consistent reduction of hippocampal AEA levels induced by stress in general. Evidence examining local manipulation of endocannabinoid signaling in the BLA has consistently found that increased AEA signaling is essential for enhancing the consolidation of emotional memories [41,42], making the BLA a possible candidate for AEA modulation of stress

effects on memory [24]. Simultaneously, since different contributions of the perirhinal, prefrontal and parahippocampal cortexes have been documented in memory processes [43], such brain areas could account for AEA beneficial effects on stress-induced alteration of recognition memory.

Extensive evidence demonstrated that glucocorticoids, through a rapid non-genomic mechanism, recruit 2-AG signaling within the hippocampus to impair memory retrieval of fear memories [22,38] through downstream activation of hippocampal noradrenergic system [20]. Our results show that intra-CA1 administration of the 2-AG hydrolysis inhibitor KML29 counteracts the detrimental effects of stress on memory. It should be noted, however, that the studies mentioned above evaluated the interaction between stress and hippocampal 2-AG in types of memory and behavioral tasks different from those employed in our current study. Future studies will evaluate whether stress intensities, time-of-day and endocannabinoid tone also affect cortical (e.g. parahippocampal or perirhinal) modulation of recognition memory. Literature data suggested that recognition memory reflects the contribution of recollection and familiarity as two separable memory retrieval processes, indicating the hippocampus and the parahippocampal cortex as brain regions crucial for recollection, whereas the perirhinal cortex is necessary for familiarity-based recognition [44,45]. The present paper focused on hippocampal modulation of memory because: i) compelling evidence demonstrated that the dorsal hippocampus is critical for object recognition memory [46]; ii) it has been repeatedly demonstrated that hippocampal vulnerability and sensitivity to stress affects memory and neuroplasticity [34], iii) endocannabinoids in the hippocampus crucially modulate stress effects on memory (i.e. short-term memory) [47]. Interestingly, it has been shown that activation of CB1 receptors on adrenergic and noradrenergic cells reduces the release of adrenaline and noradrenaline at both the peripheral and central level [48,49]. Besides the interaction with the arousal noradrenergic system, several studies report a mutual regulation between glucocorticoids and endocannabinoids, where glucocorticoids influence the endocannabinoid response, which in turn, modulates glucocorticoid secretion through

local and distal regulation of HPA axis activity [50].

Specifically, while the neuropeptide CRH, rapidly released in response to stress [51,52], reduces AEA signaling at glutamatergic neurons, which probably contributes to HPA axis activation [29], glucocorticoids enhance 2-AG's synthesis [19,53] to terminate the stress response throughout the HPA axis negative feedback regulation in limbic brain regions [26].

Collectively, these data seem to suggest that hippocampal 2-AG signaling might be responsible for the regulation of noradrenergic release, by exerting inhibitory control over noradrenergic fibers, and participate to the negative feedback regulation of the HPA axis. Supporting this hypothesis, the hippocampus represents an important site of negative feedback regulation of the HPA axis activity [54].

Therefore, it is tentative to speculate that our intervention might have reduced hippocampal norepinephrine release, which impairs memory retrieval [38], facilitating negative feedback on the HPA axis with a faster recovery from stress, and, ultimately, restored memory performance, highlighting that MAGL inhibition might be a potential therapeutic target for treating stress-induced memory performance deficits.

Materials and Methods

Animal Care and Use

Male adult Sprague-Dawley rats (10 weeks of age; 350–380 g at the time of behavioral experiments, Charles River Laboratories, Calco, Italy) were single housed in a temperature-controlled (21 ± 1°C) colony room and maintained under a 12h/12h light/dark cycle (07:00 AM to 7:00 PM lights on). Food and water were available *ad libitum*. All behavioral procedures were performed during the light phase of the cycle between 10:00 AM and 6:00 PM. All experimental procedures were performed in compliance with the ARRIVE guidelines, the European Union Directive on the protection of animals used for scientific purposes (2010/63/EU) and the D.L. 26/2014 of Italian Ministry of Health.

Surgery

Rats were anesthetized with ketamine hydrochloride (100 mg kg^{-1}) and xylazine (7 mg kg^{-1}), given intraperitoneally (i.p.). Successively, animals were subcutaneously injected with saline (3 ml) to facilitate clearance of drugs and prevent dehydration. Rats were then placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA), and two 23-gauge (11-mm-long) stainless-steel guide cannulae were implanted bilaterally 2 mm above the CA1 region of the dorsal hippocampus (AP, −3.3 mm; ML, ±1.7 mm; DV, −2.7 mm) [22,38,41]. The cannulae were affixed to the skull with two anchoring screws and dental cement. Stylets (11-mm-long 00 insect dissection pins) were inserted into each cannula to prevent clogging. After surgery, rats were retained on a heated pad to recover from anesthesia and were then returned to the home cage. Rats were allowed to recover from surgery for two weeks before testing.

Drug Administration

The 2-AG hydrolysis inhibitor KML29 (1,1,1,3,3,3-Hexafluoropropan-2-yl 4-[bis(1,3-benzodioxol-5-yl)-hydroxymethyl]piperidine-1-carboxylate) (2 ng or 20 ng in 0.5 µl per side; Tocris Bioscience, Bristol UK) or its vehicle were bilaterally infused into the CA1 region of the hippocampus immediately after the training trial and right before the swim stress procedure, in order to block any possible stress-induced increase in 2-AG hydrolyzation. Doses were selected on the basis of previous published papers and pilot experiments performed in our laboratory [23,55]. All drugs were dissolved in 5% polyethylene glycol, 5% Tween-80, and 90% saline (vol/vol). Post-training bilateral infusions of drugs or an equivalent volume of vehicle into the CA1 were made by using a 30-gauge injection needle connected by polyethylene tubing (PE-20) to a 10 ml Hamilton microsyringe driven by a minipump (KD Instruments, Canning Vale, Australia) over a period of 50 s [41]. The injection needles protruded 2 mm beyond each cannula tip and were retained within the cannulae for an additional 20 s after drug infusion to maximize diffusion and to prevent drug backflow into the cannulae. All drug solutions were freshly prepared before each experiment.

Behavioral Procedures

Object recognition task. A previously validated procedure described by [25] was used. All animals were randomly assigned to the no, low or high stress condition groups and tested either during rats' inactive phase (morning, 10:00 AM - 12:30 PM) or before the onset of the activity phase (afternoon, 3:30 PM - 6:00 PM). On the training trial, each rat was individually placed in the object recognition arena at the opposite end from the two identical objects. Memory retention was tested 1-h after training. On the testing trial, one copy of the familiar object (A3) and a new object (B) were placed in the same location as stimuli during the training trial (Fig. 5). To reduce potential biases due to preference for particular locations or objects, all combinations and locations of objects were used. Cognitive performance during the testing trial was assessed by calculating a discrimination index as the difference in time exploring the novel and the familiar object, expressed as the percentage ratio of the total time spent exploring both objects. See Supplementary Materials for additional details.

Swim stress procedure. Swim stress was used because its neurochemical and hormonal effects are well defined and meet the criteria of a stress-inducing agent [56]. Immediately after the training trial of the object recognition task, rats were forced to swim in a tank (50 cm in height × 20 cm in diameter), filled to a depth of 30 cm with water, in a separate room from the one where the object recognition task was performed. Subsequently, rats were removed from the tank and gently wiped to dryness with absorbent paper before returning to the home cage. Rats belonging to the low and high stress condition groups were subjected to a 1- or 5-min swim stress procedure at different water temperatures of 25 ± 1°C or 19 ± 1°C, respectively, known to elicit different plasma corticosterone levels [22], as we recently reported using the same behavioral procedure [25].

Endocannabinoid extraction and analysis

In a cohort of animals which did not receive cannulation surgery, hippocampal 2-AG and AEA content was measured in rats belonging to the no, low and high stress condition groups that were sacrificed immediately after the testing trial, in the morning or afternoon. After rapid decapitation, hippocampi were rapidly dissected, frozen on dry ice and stored at –80°C. The lipid extraction process and analysis of 2-AG and AEA were performed as previously described [22,55] and are detailed in the Supplementary Materials.

Membrane Preparation

To measure MAGL and FAAH activity, immediately after the testing trial, following rapid decapitation, the hippocampi were dissected from non-cannulated rats that were subjected to no stress, low stress or high stress conditions, in the morning or afternoon. Brains were stored at –80°C. Membrane samples were collected by homogenization of frozen tissue in TME buffer (50 mM Tris HCl, pH 7.4; 1 mM EDTA, and 3 mM $MgCl_2$; 10 volumes) [22,57]. Successively, homogenates were centrifuged at 18,000 × g for 20 min, and the resulting crude membrane fraction-containing pellet was resuspended in 10 volumes of TME buffer. To determine protein concentrations, the Bradford method (Bio-Rad) was used. Membranes were then used for MAGL and FAAH activity assays.

MAGL Activity Assay

MAGL activity was measured by conversion of 2-oleoylglycerol labeled with [^{3}H] ([^{3}H] 2-OG) in the glycerol portion of the molecule to [^{3}H] glycerol preparations [22]. A slightly modified procedure of that described by [58] was used. See Supplementary Materials for additional details.

FAAH Activity Assay

FAAH activity from hippocampal membranes was measured by conversion of AEA labeled with [^{3}H] in the ethanolamine portion of the molecule to [^{3}H] ethanolamine

preparations, as reported previously [22] (Supplementary Materials).

Histology

Cannulated rats were anesthetized with an overdose of ketamine hydrochloride (120 mg kg^{-1}, i.p.) and xylazine (20 mg kg^{-1}, i.p.) and perfused transcardially with 0.9% saline. Brains were removed and stored at room temperature in 4% paraformaldehyde solution for a minimum of 24 h, followed by storage in a 20% sucrose solution in saline for cryoprotection for additional 24-48 h before sectioning. Coronal sections of 40 μm were collected on a cryostat, mounted on gelatin-coated slides, and stained with cresyl violet. Brain sections were examined under a light microscope (Nikon 801 Microscope, Italy) and the location of infusion needle tips in the CA1 of the dorsal hippocampus were made according to the standardized atlas plates of [59] by an observer blind to drug treatment condition. For all experiments, only rats with infusion needle tips within the boundaries of the targeted brain region were included in the data analysis. Approximately 15% of the animals were excluded because of either cannula misplacement or damage to the targeted tissue.

Data and Statistical Analysis

Object recognition data, hippocampal endocannabinoid content and MAGL and FAAH activity parameters were analyzed by two-way ANOVAs. One-sample t-tests were used to determine whether the discrimination index was different from zero. Tukey-Kramer *post hoc* test was performed to control for significant differences between groups when appropriate. Significance was considered for $P < 0.05$. Prior findings indicate that only rats that reached a minimum criterion of total object exploration time > 10 s on either the training or testing trial adequately acquire the task and can be included in the statistical analysis [25,60]. Each measure is expressed as mean ± standard error of the mean (SEM).

Supplementary Materials

Results

Different stress intensities and time-of-day effects on short-term recognition memory retention performance

Training trial. Two-way ANOVA for total exploration time of the two identical objects on the training trial, before stress exposure, revealed no significant post-training stress condition effect, no significant effect of the time of training or an interaction between both factors (**Table S1**). Examination of rats' exploratory behavior of the experimental apparatus during the training trial indicated that there were no significant differences (**Table S1**) between groups. Specifically, two-way ANOVAs for number of crossings or rearings on the training trial revealed no significant post- training stress condition, time of the trial or post-training stress condition × time of the trial effects.

Effects of the 2-AG hydrolysis inhibitor KML29 on hippocampal modulation of short-term object recognition memory performance in the no, low and high stress condition groups tested in the morning

Training trial. Two-way ANOVA for total exploration time of the two identical objects on the training trial, before drug administration and stress exposure, revealed no significant post-training treatment effect, no significant post-training stress condition effect and no interaction between both factors (**Table S1**). Examination of rats' exploratory behavior of the experimental apparatus during the training trial revealed no significant differences for crossings and rearings among groups before drug treatment and stress exposure (**Table S1**). Two-way ANOVAs for number of crossings or rearings on the training trial revealed no significant post-training treatment effect, post-training stress effect and no significant post-training stress condition × treatment interaction effects.

Effects of the 2-AG hydrolysis inhibitor KML29 on hippocampal modulation of short-term object recognition memory performance in the no, low and high stress condition groups tested in the afternoon

Training trial. Two-way ANOVA for total object exploration time on the training trial, before drug administration and stress exposure, revealed no significant post-training treatment, post- training stress condition or post-training treatment × stress condition interaction effects (**Table S1**). Examination of rats' exploratory behavior of the experimental apparatus during the training trial indicated that there were no significant differences in terms of crossings and rearings (**Table S1**). In fact, two-way ANOVAs for number of crossings or rearings on the training trial revealed no post- training treatment, post-training stress condition or post-training treatment × stress condition interaction effects.

	Morning			Afternoon		
	Total object exploration time	**Number of crossings**	**Number of rearings**	**Total object exploration time**	**Number of crossings**	**Number of rearings**
NO STRESS						
VEHICLE	59.8 ± 3.5	45.5 ± 1.6	44.8 ± 4.2	50.4 ± 4.0	43.0 ± 3.3	32.0 ± 2.2
KML 2 ng	66.7 ± 3.9	52.4 ± 3.4	39.4 ± 2.9	54.2 ± 3.5	48.0 ± 3.4	41.4 ± 3.2
KML 20 ng	61.0 ± 3.5	48.8 ± 1.8	45.8 ± 2.8	58.8 ± 3.8	43.5 ± 3.9	37.0 ± 2.5
LOW STRESS						
VEHICLE	80.0 ± 11.7	51.1 ± 2.8	52.0 ± 4.8	53.1 ± 3.0	42.5 ± 2.8	35.2 ± 2.5
KML 2 ng	67.5 ± 8.1	59.2 ± 1.4	49.2 ± 3.9	56.9 ± 3.5	42.0 ± 2.1	37.6 ± 3.3
KML 20 ng	62.7 ± 6.6	49.1 ± 3.2	47.8 ± 3.7	54.2 ± 2.8	46.0 ± 2.2	37.1 ± 2.2
HIGH STRESS						
VEHICLE	62.8 ± 7.6	52.5 ± 4.7	46.3 ± 5.0	56.9 ± 3.3	46.1 ± 2.9	40.3 ± 2.8
KML 2 ng	62.3 ± 7.0	51.0 ± 3.6	43.1 ± 4.3	66.3 ± 5.5	48.8 ± 2.9	40.9 ± 2.3
KML 20 ng	63.6 ± 5.8	49.5 ± 2.6	39.8 ± 2.6	53.0 ± 3.1	48.2 ± 4.1	40.7 ± 3.3

Total time spent exploring the two objects (in seconds) and the number of crossings and rearings of all groups tested in the morning and in the afternoon. Data are expressed as mean ± SEM (n = 10-12 per group).

Table S1 – Exploratory behavior on the training trial for vehicle- and KML29-treated rats that were subjected to no, low or high stress conditions immediately after the training trial, in the morning or afternoon.

	Morning			Afternoon		
	Total object exploration time	**Number of crossings**	**Number of rearings**	**Total object exploration time**	**Number of crossings**	**Number of rearings**
NO STRESS						
VEHICLE	45.1 ± 3.4	20.7 ± 2.7	37.4 ± 3.5	40.6 ± 4.5	26.6 ± 3.9	33.6 ± 3.7
KML 2 ng	48.7 ± 4.6	19.2 ± 2.6	33.7 ± 3.1	29.7 ± 3.4	20.1 ± 3.7	29.1 ± 4.1
KML 20 ng	50.6 ± 4.1	22.3 ± 2.9	34.6 ± 3.4	42.3 ± 4.0	20.5 ± 3.3	32.6 ± 3.5
LOW STRESS						
VEHICLE	25.8 ± 3.1**	13.1 ± 2.8*	15.2 ± 2.1**	32.6 ± 3.8	12.6 ± 2.1*	24.6 ± 3.8
KML 2 ng	34.7 ± 3.7	14.1 ± 2.4	20.4 ± 2.8**	33.7 ± 4.2	11.6 ± 2.5	21.7 ± 3.1
KML 20 ng	29.3 ± 3.1*	12.4 ± 2.8*	20.1 ± 3.0**	33.3 ± 4.3	13.0 ± 2.5	18.3 ± 3.2*
HIGH STRESS						
VEHICLE	36.7 ± 4.4	19.0 ± 2.4	16.8 ± 3.4**	26.2 ± 4.3	15.4 ± 3.5	16.0 ± 3.3**
KML 2 ng	34.9 ± 4.5	20.5 ± 2.8	18.4 ± 2.5**	22.9 ± 3.0	13.7 ± 2.4	19.2 ± 3.3
KML 20 ng	28.1 ± 4.3**	17.0 ± 2.9	14.2 ± 2.6**	27.4 ± 5.2	16.2 ± 3.1	17.5 ± 3.8*

Total time spent exploring the two objects (in seconds) and the number of crossings and rearings of all groups tested in the morning and in the afternoon. * $P < 0.05$; ** $P < 0.01$ vs the corresponding no stress group. Data are expressed as mean ± SEM (n = 10-12 per group).

Table S2 – Exploratory behavior on the testing trial for vehicle- and KML29-treated rats that were subjected to no, low or high stress conditions immediately after the training trial, in the morning or afternoon.

Materials and Methods

Behavioral Procedures

Object recognition task. The experimental apparatus was a gray open-field box (in cm, 40 wide × 40 deep × 40 high) with the floor covered with sawdust, positioned in a dimly illuminated room. The objects to be discriminated were transparent glass vials (5.5 cm diameter and 5 cm height) and white glass light bulbs (6 cm diameter and 11 cm length). All rats were handled twice per day for 1 min each and extensively habituated to the experimental context twice per day for 3 min each for 7 days preceding the training day. During habituation, rats were allowed to freely explore the apparatus in the absence of objects. On the training trial, each rat was individually placed in the experimental apparatus at the opposite end from the objects. Rats were allowed to

explore two identical objects (A1 and A2) for 6 min, then were removed from the apparatus and, after drug treatment, if belonging to the low or high stress condition group, were subjected to a swim stress procedure; then, they were returned to the home cage. The no stress group was placed back to its home cage immediately after drug infusion. To avoid the presence of olfactory trails, sawdust was stirred, foecal boli were removed and the objects were cleaned with 70% ethanol after each trial. A rat's behavior was recorded by using a video camera positioned above the experimental apparatus and videos were analyzed with Observer XT 12 (Noldus Information Technology BV, Wageningen, The Netherlands) by a trained observer who was unaware of the treatment condition. Exploration of an object was defined as pointing the nose to the object at a distance of < 1 cm and/or touching it with the nose. Turning around or sitting on an object was not considered as exploration. During the training trial, the time spent exploring the two objects (total object exploration time, s) was taken as a measure of object exploration, and exploratory behavior of the experimental apparatus was analyzed by the measuring of total number of crossings and rearings. For crossings, the floor of the apparatus was divided into four imaginary squares and the total number of crossings between squares was determined. Memory retention was tested 1 h after the training trial. On the testing trial, one copy of the familiar object (A3) and a new object (B) was placed in the same location as stimuli during the training trial (Figure 1). All combinations and locations of objects were used to reduce potential biases due to preference for particular locations or objects. Each rat was placed in the apparatus for 6 min, and behavior was recorded. To analyze cognitive performance, during the test, a discrimination index was calculated as the difference in time exploring the novel and the familiar object, expressed as the percentage ratio of the total time spent exploring both objects.

Endocannabinoid extraction and analysis

The lipid extraction process was performed as previously detailed [22,55]. Brain tissue was weighed and placed into borosilicate glass culture tubes containing 2 ml of acetonitrile with 5 nmol of [$^{2}H_{8}$] 2-AG and 5 pmol of [$^{2}H_{8}$] AEA for extraction and homogenized with a glass rod. Tissue was sonicated for 30 min on ice water and incubated overnight at –20°C to precipitate proteins, then centrifuged at 1500 × g to remove particulates. The supernatants were transferred to a new glass tube and evaporated to dryness under N_2 gas. The samples were reconstituted in 300 µl of acetonitrile and dried again under N_2 gas. Lipid extracts were suspended in 20 µl of acetonitrile and stored at –80°C until analysis. Analysis of 2-AG and AEA was performed by liquid chromatography mass spectrometry analysis as previously detailed [22,55].

MAGL Activity Assay

Membranes were incubated in a final volume of 0.5 ml TME buffer (50 mM Tris-HCl, 3.0 mM $MgCl_2$, 1.0 mM EDTA, and 300 nM URB597, pH 7.4) that contained 1.0 mg ml^{-1} fatty acid-free BSA and 100,000 dpm [^{3}H] 2-OG. Isotherms were constructed using six concentrations of 2-OG at concentrations between 10 and 500 µM. Incubation was performed at 30°C, and the enzymatic reaction was stopped by the addition of 2 ml of chloroform/methanol (1:2). After remaining at room temperature for 30 min with frequent mixing, 0.67 ml of chloroform and 0.6 ml of water were added, and the aqueous and organic phases were separated by centrifugation at 1000 rpm for 10 min. The amount of [^{3}H] in 0.5 ml of each of the aqueous phases was determined by liquid scintillation counting and conversion of [^{3}H] 2-OG to [^{3}H] glycerol was calculated. The binding affinity (K_m) and maximal hydrolytic activity (V_{max}) values for this conversion were determined by fitting the data to a single-site Michaelis–Menten equation using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, USA).

FAAH Activity Assay

Membranes were incubated in a final volume of 0.7 ml TME buffer (50 mM Tris-HCl, 3.0 mM $MgCl_2$, 1.0 mM EDTA, pH 7.4) that contained 1.0 mg ml^{-1} fatty acid-free bovine serum albumin (BSA) and 0.2 nM [^{3}H] AEA. Isotherms were constructed using eight concentrations of AEA at concentrations between 0 and 1.5 μ M. Incubation was performed at 37°C, and the enzymatic reaction was stopped by the addition of 2 ml of chloroform/methanol (1:2). After remaining at room temperature for 30 min with frequent mixing, 0.67 ml of chloroform and 0.6 ml of distilled water were added, and the aqueous phases were separated by centrifugation at 1000 rpm for 10 min. The amount of [^{3}H] in 0.5 ml of each of the aqueous and organic phases was determined by liquid scintillation counting and conversion of [^{3}H] AEA to [^{3}H] ethanolamine was calculated. The K_m of AEA for FAAH and V_{max} of FAAH for this conversion were determined by fitting the data to a single-site Michaelis–Menten equation using GraphPad Prism.

Data and Statistical Analysis

A between-subjects experimental design was used. Data were analyzed by two-way ANOVAs. Tukey–Kramer post hoc tests were used to determine the source of the detected significances. P values of < 0.05 were considered statistically significant. All data are expressed as mean ± SEM.

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CHAPTER 5

BIDIRECTIONAL INPUTS BETWEEN THE DORSAL HIPPOCAMPUS AND THE BLA ARE CRITICAL FOR ENABLING ENDOCANNABINOID MODULATION OF FEAR MEMORY RETRIEVAL

Alessia Santori[1,2], Patrizia Ratano[1], Krista L. Wahlstrom[3], Maida Ceman[1,2], James L. McGaugh[4,5], Ryan T. LaLumiere[3,6], Patrizia Campolongo[1,2]

[1]Department of Physiology and Pharmacology, Sapienza University of Rome, 00185 Rome, Italy; [2]Neurobiology of Behavior Laboratory, Santa Lucia Foundation, 00143 Rome, Italy; [3]Dept. of Psychological and Brain Sciences, University of Iowa, Iowa City, IA 52242-1407, USA; [4]Center for the Neurobiology of Learning and Memory, University of California, Irvine, CA 92697-3800; [5]Department of Neurobiology and Behavior, University of California, Irvine, CA 92697-3800; [6]Iowa Neuroscience Institute, University of Iowa, Iowa City, IA 52242, USA

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Abstract

The activation of CB1 receptors in the dorsal hippocampus and basolateral amygdala (BLA) modulates both excitatory and inhibitory signaling within specific neuronal circuits implicated in learning and memory processes for emotionally arousing experiences. Dysfunctional information processing is a common feature of stress-related disorders, which are frequently characterized by abnormal retrieval and insufficient extinction of traumatic memories. Of note, in psychiatric disorders hippocampal memory is compromised while amygdala-dependent memory is abnormally strengthened. Here, we aimed at evaluating whether the endocannabinoids anandamide (AEA) and 2-arachidonoylglycerol (2-AG), in the BLA or the CA1 region of the dorsal hippocampus, differentially regulate fear memory retrieval depending on the environment-associated emotional arousal, and if concurrent pharmacological / optogenetic inactivation of the CA1 or the BLA, respectively, influences any endocannabinoid modulation of fear memory retrieval.

To investigate whether the BLA-CA1 interplay plays any role in the modulation of either the basolateral amygdalar or hippocampal endocannabinoid effect on memory retrieval, rats were given bilateral lesions of the CA1 or the BLA through the administration of the $GABA_A$ receptor agonist muscimol or subjected to bilateral and temporal optogenetic inhibition during auditory and contextual fear memory retrieval.

In support of the view that such interplay is crucially involved in the endocannabinoid modulation of emotional memories, we found that AEA, in the BLA, and 2-AG, in the CA1, differentially impaired fear memory retrieval through a mechanism that involved both CB1 and CB2 receptors activation. This effect was reverted by inhibiting pharmacologically or optogenetically the dorsal CA1 for the basolateral amygdalar AEA effect on auditory fear memory retrieval and, conversely, by deactivating the BLA for the hippocampal 2-AG effect on the retrieval of contextual fear memory. Our results demonstrate that the dichotomic involvement of the dorsal hippocampus and BLA interplay sets endocannabinoids to retrieve auditory and contextual fear memories.

Introduction

Excessive fear and anxiety are hallmarks of a variety of disabling psychiatric disorders (Myers and Davis, 2007). The neural underpinnings of fear have been extensively studied by using the Pavlovian fear conditioning paradigm both in humans and rodents, thus allowing exploration of how response to a specific environmental stimulus is produced through associative learning processes (LeDoux, 2014). The neurocircuitry of fear memory involves the basolateral amygdala (BLA) as the key region modulating the acquisition, retrieval and extinction of fear response (Adolphs, 2013; Herry and Johansen, 2014; Johansen et al., 2011; Zelikowsky et al., 2014), by receiving inputs from somatosensory cortex, thalamus, and hippocampus that encodes contextual information and compares current contextual cues to previously encoded memories (Maren and Quirk, 2004). Within the hippocampus, many studies have demonstrated that the dorsal CA1 field is crucially required for contextual fear memory retrieval and extinction (Fanselow and Dong, 2010; Hunsaker and Kesner, 2008; Ji and Maren, 2008). Nevertheless, literature studies did not examine the influence of the BLA in any hippocampal-dependent modulation of fear processes.

The endocannabinoid system strongly regulates stress and emotional arousal effects on memory and cognition (Morena and Campolongo, 2014), as well as the neurophysiological states that mediate different behavioral outcomes to stress (i.e. fear reaction, anxiety, stress-coping, etc.; Lutz et al., 2015). Cannabinoid type 1 (CB1) receptors (Matsuda et al., 1990) were found to be highly expressed in brain regions that are critically associated with emotional memory processes (Marsicano and Lafenêtre, 2009), such as the hippocampus and the BLA (Katona and Freund, 2012), wherein their activation influences hippocampal and basolateral amygdalar endocannabinoid modulation of fear expression (Akirav, 2013; Morena et al., 2015, 2014; Ratano et al., 2014); additionally, cannabinoid type 2 (CB2) receptors activation was demonstrated to be recruited to process fear memory consolidation (Ratano et al., 2018, 2017). Hence, together with the two major endogenous ligands, 2-arachidonoyl glycerol (2-AG;

Sugiura et al., 1995) and N-arachidonyl ethanolamide (anandamide, AEA; Devane et al., 1992), and their corresponding hydrolytic enzymes, monoacylglycerol lipase (MAGL; Dinh et al., 2002) and fatty acid amide hydrolase (FAAH; Cravatt et al., 2001), respectively, the endocannabinoid system has been recognized as a promising tool to investigate stress-related disorders mechanisms, by influencing hippocampal (Atsak et al., 2012; Campolongo et al., 2013; Morena et al., 2015; Santori et al., 2020) and basolateral amygdalar (Atsak et al., 2015; Campolongo et al., 2009; Morena et al., 2014) modulation of fear memory retrieval and extinction (Morena et al., 2018; Segev et al., 2018). However, it still remains unexplored whether hippocampal or basolateral amygdalar endocannabinoids specifically modulate auditory and contextual fear memory retrieval, which mechanism regulates their action, and if the BLA and the dorsal CA1 interplay plays any role in such regulation.

Recent evidence demonstrated that optogenetic stimulation of the BLA projections to the ventral hippocampus regulates anxiety-mediated behavior (Felix-Ortiz et al., 2013) and emotional, but not contextual, memory consolidation (Huff et al., 2016). Simultaneously, manipulations of the GABAergic interneurons within the ventral hippocampus $\leftrightarrow$ BLA neurocircuitry were reported to be effective to modulate memory function in different protocols of fear conditioning (Herry et al., 2008; Müller et al., 2012). To date, only an important study reported that the connectivity between the dorsal hippocampus and the BLA is crucial to induce bidirectional switch of memory valence (Redondo et al., 2014), thus opening the avenue to further investigate the role of different components of the amygdalohippocampal circuits in fear memory processes.

Therefore, the first purpose of the present study was to investigate the effects of pharmacological manipulations of the endocannabinoid system within the BLA and the dorsal CA1 field in the retrieval of auditory or contextual fear conditioning (AFC or CFC, respectively) memories. To this aim, we evaluated i) the effects of both basolateral amygdalar and hippocampal enhancement of AEA and 2-AG signaling on auditory and contextual fear memory retrieval and whether ii) these effects were mediated by indirect

activation of CB1 and/or CB2 receptors. We then investigated whether the interplay between the BLA and the dorsal CA1 plays any role in such effect by testing if: iii) the muscimol-induced pharmacological inactivation of the dorsal CA1 could influence the basolateral amygdalar endocannabinoid modulation of the retrieval of auditory memory and, conversely, whether any muscimol-dependent BLA inactivation affected the dorsal CA1 endocannabinoid regulation of contextual fear memory retrieval; finally, we tested if iv) the activation of the endocannabinoid system in the BLA could ameliorate fear memory retrieval in the AFC when optogenetic inactivation of the dorsal CA1 field was performed during testing and, conversely, if optogenetic inhibition of the BLA influenced the hippocampal endocannabinoid modulation of contextual fear memory retrieval in the CFC.

Materials and Methods

Animal Care and Use

Male adult Sprague-Dawley rats (350-380 g at the time of behavioral experiments, Charles River Laboratories) were single housed in a temperature-controlled (21 ± 1°C) vivarium room and maintained under a 12h/12h light/dark cycle (07:00 A.M. to 7:00 P.M. lights on). Food and water were available *ad libitum*. All tests were performed during the light phase of the cycle between 10:00 A.M. and 4:00 P.M. All experimental procedures were performed in compliance with the ARRIVE guidelines, the European Union Directive on the protection of animals used for scientific purposes (2010/63/EU), the Italian law (D.L. 26/2014) and the National Institutes of Health guidelines for care of laboratory animals and were approved by the University of Iowa Institutional Animal Care and use Committee. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Surgery, viral vector microinjection, and drug administration

Rats were anesthetized with a mixture of Zoletil® (tiletamine hydrochloride + zolazepam, 80 mg/kg, i.p.) and Rompun® (xylazine, 10 mg/kg, i.p.). Subsequently, they were injected with 3 ml of saline subcutaneously to facilitate clearance of drugs and prevent dehydration. Animals were then placed in a stereotaxic frame (David Kopf Instruments), and two stainless-steel guide cannulae (23-gauge, 15-mm- or 11-mm-long into the BLA or the dorsal CA1 field, respectively) were implanted bilaterally, with the cannula tips 2 mm above either the BLA (coordinates: anteroposterior, AP, -2.8 mm; mediolateral, ML, ±5.0 mm; dorsoventral, DV, -6.5 mm) or the CA1 region of the dorsal hippocampus (AP, −3.3 mm; ML, ±1.7 mm; DV, −2.7 mm), according to the atlas of Paxinos and Watson (2014) and previous published studies (Atsak et al., 2012; Campolongo et al., 2009b, 2009a; Morena et al., 2016, 2015, 2014; Santori et al., 2020).

For the pharmacological inhibition experiments, animals underwent the implantation of four cannulae. Stainless-steel guide cannulae were implanted bilaterally with the cannula tips 2 mm above both the BLA (23 gauge, 15-mm-long; AP, -2.8 mm; ML, ±5.0 mm; DV, -6.5 mm) and the CA1 field (23 gauge, 11-mm-long; AP, −3.3 mm; ML, ±1.7 mm; DV, −2.7 mm). The cannulae were affixed to the skull with two anchoring screws and dental cement. Stylets (15-mm- or 11-mm-long 00 insect dissection pins) were inserted into each cannula to prevent clogging.

For the last two experiments involving optogenetic manipulations, rats received virus microinjections (rAAV5-CaMKIIα-eArchT3.0-eYFP or rAAV5-CaMKIIα-eYFP; University of North Carolina Vector Core). The rAAV5-CaMKIIα-eArchT3.0-eYFP virus or CaMKIIα-eYFP control vector were delivered bilaterally through a 10 µl Hamilton microsyringe driven by a stereotaxic injector set (Stoelting) into the BLA (0.35 µl with a rate of 0.1 µl/min; AP, -2.8 mm; ML, ±5.0 mm; DV, -8.5 mm) or the CA1 region of the dorsal hippocampus (0.50 µl with a rate of 0.37 µl/min; AP, −3.3 mm; ML, ±1.7 mm; DV, −4.7 mm). The CaMKIIα-eYFP control vector was used for control experiments to examine the effects of illumination (and, thus, possible heating)

alone. The injection volumes were chosen based on previous studies using these viral constructs (Hannapel et al., 2019; Wahlstrom et al., 2018). Two weeks later, allowing sufficient time for robust opsin expression, rats underwent a second surgery in which optical probes were aimed bilaterally at the BLA (AP, -2.8 mm; ML, ±5.0 mm; DV, -8.5 mm) or the CA1 region of the hippocampus (left AP, −4.1 mm; right AP, -2.5 mm; ML, ±1.7 mm; DV, −4.8 mm; 10° angled) and secured by surgical screws and dental acrylic. Furthermore, rats received cannula implantation in the CA1 field (AP, -4.1 mm; ML, ±1.7 mm; DV, −2.8 mm; 10° angled) or the BLA (AP, -2.8 mm; ML, ±5.0 mm; DV, -8.5 mm), according to the brain region that was not targeted by virus microinjection and optical probe implantation. After surgery, rats were retained on a heated pad until they recovered from anesthesia and were then returned to their home cages. Following surgical cannula or cannula and optic probe implantation, rats were allowed to recover from surgery for at least one week before testing.

All animals with surgical implant received bilateral infusions of drugs or an equivalent volume of vehicle into the BLA (0.20 µl/side) or the CA1 region of the dorsal hippocampus (0.50 µl/side) by using a 30-gauge injection needle connected by polyethylene tubing (PE-20) to a 10 µl Hamilton microsyringe driven by a minipump (KD Instruments) with a rate of 0.37 µl/min 1 h before testing; the injection needles protruded 2 mm beyond the tip of the cannula (Morena et al., 2014) and were retained within the cannulae for an additional 20 s after drug infusion to maximize diffusion and to prevent backflow of drug into the cannulae.

Drugs

The AEA hydrolysis inhibitor URB597 (3, 10 or 30 ng), the 2-AG hydrolysis inhibitor KML29 (0.2, 2 or 20 ng; Tocris Bioscience), the CB1 receptor antagonist AM251 (0.14 ng), the CB2 receptor antagonist SR144528 (0.48 ng), the $GABA_A$ agonist muscimol (500 ng; Tocris Bioscience) or their vehicles [5% polyethylene glycol, 5% Tween-80, 90% saline for cannabinoid drugs and phosphate buffered saline (PBS) for the muscimol

experiments] were bilaterally infused into the BLA (0.20 μl/side) or the CA1 region of the dorsal hippocampus (0.50 μl/side). Doses were chosen on the basis of pilot experiments performed in our laboratory and on literature data (Campolongo et al., 2009b; Chapman, 1999; Jhaveri et al., 2008; Morena et al., 2014; Qi et al., 2015; Santori et al., 2020; Yoon et al., 2009). All drug solutions were freshly prepared on the day of drug administration. URB597, AM251 and SR144528 were kindly donated by the National Institute of Mental Health (Chemical Synthesis and Drug Supply Program).

Optical Manipulations

Optical probes were constructed by gluing an optical fiber (200 μm core, multimode, 0.37 NA) into a metal ferrule (length: 7.95 + 8.00 mm, bore: 0.250 – 0.260 mm, concentricity: < 0.20 μm; Thorlabs). The fiber extended beyond the ferrule end for implantation into tissue. The other end of the optical probe was polished and, during light delivery, connected to an optical fiber via a ceramic split sleeve. The other end of the optical fiber (FC/PC connection) was threaded through a metal leash to protect the fiber from being damaged by the rat and attached to a 1:2 splitter to permit bilateral illumination. The splitter's single end was attached to an optical commutator (Doric Lenses) allowing free rotation of the optic leash connected to the rat. A fiber patch cable connected the commutator to the appropriate laser source (DPSS, 300 mW, 561 nm; CNI Optoelectronics Tech. Co.), with a multimode fiber coupler for an FC/PC connection (Wahlstrom et al., 2018). Based on previous work, light output was adjusted to allow for 10 mW at the fiber tip (Deisseroth, 2012; Huff et al., 2016; Mattis et al., 2012), as measured by an optical power meter. In all optogenetic experiments, a "sham-control" group of rats had received rAAV5-CaMKIIα-eYFP microinjections and was connected to optical leashes during the testing. Illumination was given bilaterally and provided continuously to rats for 1 minute during fear conditioning testing in a fear conditioning chamber that contained a weighted arm attached to the outside of the cubicle with the optical commutator at one end. In all cases, illumination was provided every 1 min to ensure intra-subject proper control.

Behavioral Procedures

Rats were trained and tested in fear conditioning chambers (30 × 24 × 21 cm^3, l × w × h) equipped with metal stainless-steel rod flooring connected to a shock generator. Each chamber was enclosed within ventilated and sound attenuating cubicles (Med Associates, Sandown Scientific). A video camera was located on the ceiling of the cubicle to record memory performance. Both auditory and contextual fear memory retrieval were evaluated by measuring percentage of freezing (i.e., absence of movement except for respiration) across auditory and contextual fear conditioning testing, respectively. To reduce stress, all animals were handled 1 min each for 3 days before conditioning (days 1-3).

Auditory fear conditioning (AFC). To assess auditory fear memory retrieval, a slightly modified procedure of that described by Asak et al. (2012) was used. Two different contexts were exploited (contexts A/B). Context A consisted of a chamber with a grid floor, back and side metal walls, clear Plexiglas front door and ceiling, and white light. To create a novel testing context (context B), both the original grid floor and the side metal walls was covered by a black plastic insert to modify the existing environment. Both context A and B were cleaned with 70% ethanol between rats. Auditory fear conditioning (day 4) was performed in context A. After a 2 min acclimation period, all rats were exposed to 2 conditioning trials. Each conditioning trial consisted of the conditioned stimulus (CS; 85 dB, 2.8 kHz tone) presentation for 30 s, which co-terminated with a 2 s unconditioned stimulus (US; 0.75 mA footshock). Intertrial interval (ITI) between two consecutive CS-US pairings was 1 min, and the conditioning session terminated 2 min after the last CS-US presentation. After conditioning, each rat was returned to its home cage. Twenty-four hours after conditioning, rats were administered with cannabinoid drugs or their corresponding vehicles 1 h before testing (day 5). For experiments involving pharmacological BLA or dorsal CA1 inactivation, animals were also administered with muscimol or its vehicle 2 h before the testing trial. On day 5, rats were tested in context B. After a 1 min acclimation period, all rats were exposed to a single CS presentation for 1 min. Freezing

time was assessed as a measure of fear to both the experimental context (first 60 s) and the CS presentation (last 60 s) and expressed as percentage.

For the last two experiments involving both pharmacological and optogenetic brain region inhibition, rats were tested in context B. After a 1 min acclimation period, they were exposed to a single CS for 2 min, for a total of three CS presentation over a 9 min trial. Optogenetic manipulations were given bilaterally and provided continuously for 1 min (light ON vs light OFF) during each CS presentation with a randomized fashion.

Contextual fear conditioning (CFC). To assess contextual fear memory retrieval, a slightly modified procedure of that described by Asak et al. (2012) was used. All rats were trained and tested in the same context (context A). During contextual fear conditioning (day 4), after a 2 min acclimation period, all animals were exposed to 3 conditioning trials of 2 s (0.90 mA footshock). ITI between footshocks was 2 min, and the conditioning session terminated 2 min after the last footshock presentation. After conditioning, each rat was returned to its home cage. Twenty-four hours after conditioning, rats were injected with cannabinoid drugs or their corresponding vehicles 1 h before testing (day 5). For experiments involving four cannulae, animals were also administered with muscimol or its vehicle 2 h before the test trial. On day 5, rats were tested in context A for 5 min. Freezing was determined as a measure of fear to the experimental context and expressed as percentage across the testing session.

For the last two experiments involving both pharmacological and optogenetic brain region inhibition, rats were tested in context A for 6 min. Optogenetic manipulations were given bilaterally and provided continuously for 1 min (light ON vs light OFF) with a randomized fashion.

Verification of Opsin Expression and Histology

Rats were anesthetized with an overdose of Zoletil® (tiletamine hydrochloride + zolazepam, 800 mg/kg, i.p.) and Rompun® (xylazine, 200 mg/kg, i.p.), and perfused transcardially with 0.9% saline. Brains were removed and stored at room temperature

in 4% paraformaldehyde PBS for a minimum of 24 h before sectioning. Coronal sections of 75 μm were collected on a vibratome and mounted onto either gelatin-coated slides for Cresyl violet staining or stored in anti-freeze solution at -20°C until immunohistochemical procedures began. Brain sections were examined with a standard stain preparation and light microscopy (Nikon 801 Microscope) and the location of infusion needle tips and optic probes in the hippocampus or the BLA were made according to the standardized atlas plates of Paxinos and Watson (2014) by an observer blind to drug treatment condition. For all experiments, only rats with infusion needle tips within the boundaries of the targeted brain region were included in the data analysis. Approximately 15% of the animals were excluded because of either cannula misplacement or damage to the targeted tissue. Expression in the CA1 field or the BLA cell bodies was confirmed by using immunohistochemistry procedures. Tissue sections were incubated in anti-GFP primary antibody solution for 24h [PBS, 2% goat serum, 0.4% Triton X, rabbit 1:20,000 primary antibody (Abcam)]. Sections were then incubated for 1 h in a biotinylated anti-rabbit secondary antibody solution (K-PBS; 0.3% Triton X; goat, 1:200; Vector Labs) and incubated in an ABC kit (Vector Labs) for 1h. Sections were developed in diaminobenzidine for ∼5–10 min before being mounted onto gelatin-subbed slides. Slides were allowed to dry before being dehydrated with reverse alcohol washes for 1 min each, soaked in Clearing solution for a minimum of 5 min, and coverslipped with DePeX (Electron Microscopy Sciences). GFP/eYFP expression was assessed by using a light microscope.

Statistical Analysis

Auditory and contextual fear conditioning data were analyzed in terms of freezing percentages with one-, two-way or repeated-measures ANOVAs, when appropriate. Tukey-Kramer *post hoc* tests were used to determine the source of the detected significances. P values of < 0.05 were considered statistically significant. All data are expressed as mean ± standard error of the mean (SEM) and each group's n is indicated in the corresponding figure legend.

Results

Enhancing endogenous levels of AEA, but not of 2-AG, into the BLA attenuates the retrieval of auditory fear memory

This set of experiments evaluated whether the enhancement of AEA or 2-AG levels into the BLA modulated auditory or contextual fear memory retrieval.

In a first batch of experiments, rats were bilaterally administered into the BLA with URB597 (3, 10 or 30 ng), KML29 (0.2, 2 or 20 ng), or their corresponding vehicles, 1 h before retention testing on the CFC task (Fig. 1A). As shown in Fig. 1C-D, one-way ANOVA for freezing time across the CFC testing sessions revealed no significant treatment effect in both the KML29 and URB597 experiments.

In a second set of experiments, different cohorts of rats were bilaterally administered into the BLA with URB597 (3, 10 or 30 ng), KML29 (0.2, 2 or 20 ng), or their corresponding vehicles, 1 h before retention testing on the AFC task (Fig. 2A). As shown in Fig. 2B concerning the KML29 experiments, repeated-measures ANOVA for freezing time across the AFC testing session revealed no significant treatment or treatment × CS presentation interaction effects. However, a significant difference for freezing time across the AFC testing session was revealed according to the CS presentation ($F_{(1.31)} = 24.955$; $P < 0.0001$). *Post hoc* analysis revealed that independently of the treatment groups, all animals showed higher percentages of freezing during the CS presentation with respect to the baseline condition (no CS; $P < 0.05$ for all comparisons; Fig. 2B).

Conversely, for URB597 experiments repeated-measures ANOVA indicated that URB597 induced a dose-dependent reduction in overall percent freezing during retention testing on the AFC task in response to the CS ($F_{(3.31)} = 3.239$; $P = 0.035$), but not to the baseline condition or according to the treatment × CS presentation interaction. *Post hoc* comparisons showed that rats belonging to the vehicle, URB597 3 ng and 30 ng groups reported higher levels of freezing during the CS presentation with respect to the baseline condition (no CS; $P < 0.05$ for all comparisons), and that only the 10 ng

dose of URB597 significantly decreased freezing rates during the CS presentation ($P < 0.05$ compared with vehicle; Fig. 2C).

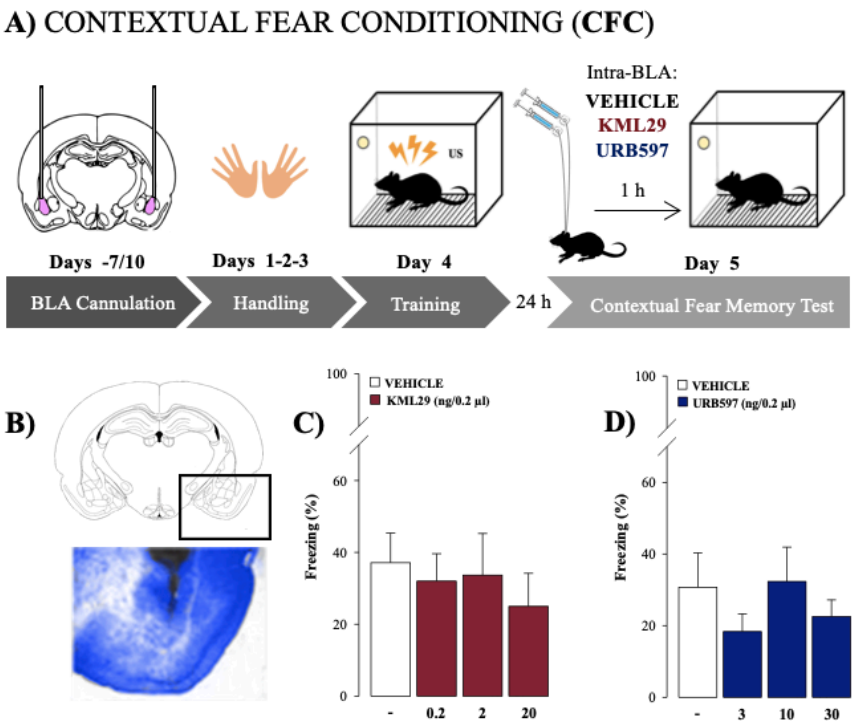

Fig. 1 – Basolateral amygdalar endocannabinoid modulation of contextual fear memory retrieval. Effects of different doses of the AEA hydrolysis inhibitor URB597 or the 2-AG hydrolysis inhibitor KML29, into the BLA, 1 h before testing, on the retrieval of contextual fear memory. Diagram of the experimental protocol for the CFC experiments (**A**). Representative photomicrograph (Nikon 801 microscope; original magnification 2×) illustrating the placement of the cannula and needle tip in the BLA (**B**). Freezing rates of rats treated into the BLA with KML29 (**C**), URB597 (**D**) or vehicle 1 h before the testing session (n = 10-11 per group).

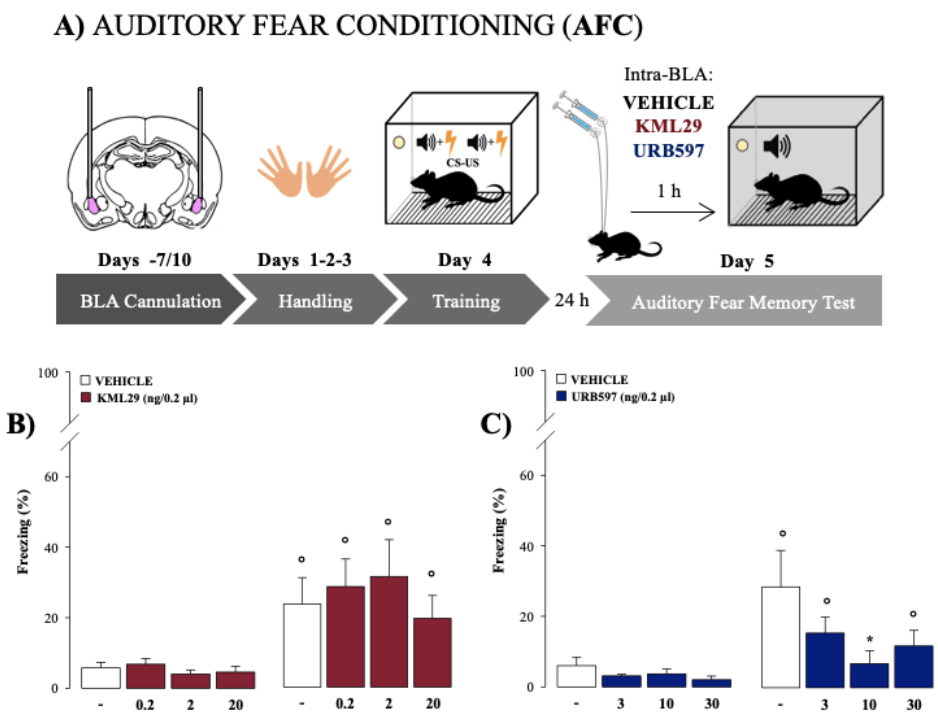

Fig. 2 – Basolateral amygdalar endocannabinoid modulation of auditory fear memory retrieval. Effects of different doses of the AEA hydrolysis inhibitor URB597 or the 2-AG hydrolysis inhibitor KML29, into the BLA, 1 h before testing, on the retrieval of auditory fear memory. Diagram of the experimental protocol for the AFC experiments (**A**). Freezing rates of rats intra-BLA administered with KML29 (**B**), URB597 (**C**) or vehicle 1 h before the testing session (º $P < 0.05$; vehicle, KML29 and URB597 groups vs the corresponding treatment groups during no CS presentation * $P < 0.05$, URB597 10 ng vs vehicle; n = 10-11 per group).

Basolateral amygdalar AEA impairs auditory fear memory retrieval through the activation of CB1 and CB2 receptors

In this set of experiments, we investigated whether the impairing effects of basolateral amygdalar URB597 (10 ng) on auditory fear memory retrieval were dependent on indirect activation of CB1 or CB2 receptors (Fig. 3A). To address this issue, we examined whether bilateral intra-BLA infusions of the selective CB1 receptor antagonist AM251 (0.14 ng) or the CB2 receptor antagonist SR144528 (0.48 ng), 1 h

before retention testing on the AFC task, would block the memory impairing induced by URB597 (10 ng).

Repeated-measures ANOVA for freezing time across the AFC testing sessions revealed no significant URB597 or AM251 effects, but significant CS presentation ($F_{(1.30)}$ = 27.665; $P < 0.0001$) and URB597 × AM251 interaction ($F_{(1.30)}$ = 4.608; $P = 0.040$) effects. No significant differences were reported for the CS presentation × URB597, CS presentation × AM251 or these three factors interaction effects. *Post hoc* analysis showed that, with the exception of those animals that were treated only with URB597, all experimental groups reported significantly higher percentages of freezing during the CS presentation with respect to the baseline condition (no CS; $P < 0.05$ for URB597-AM251 co-administered rats, $P < 0.01$ for vehicle groups; Fig. 3C). URB597 (10 ng) significantly decreased freezing levels in response to the CS as compared to rats given vehicle or to those co-administered with AM251 ($P < 0.01$ for both comparisons; Fig. 3C). As shown in Fig. 3D, repeated-measures ANOVA for freezing time across the AFC testing session revealed no significant effects of URB597 or SR144528 treatment, but significant CS presentation ($F_{(1.30)}$ = 49.385; $P < 0.0001$) and URB597 × SR144528 interaction ($F_{(1.30)}$ = 4.006; $P = 0.054$) effects. Moreover, significant differences were reported for the CS presentation × URB597 ($F_{(1.30)}$ = 4.992; $P = 0.033$), CS presentation × AM251 ($F_{(1.30)}$ = 4.510; $P = 0.042$) and these three factors interaction ($F_{(1.30)}$ = 6.321; $P = 0.018$) effects. *Post hoc* comparisons indicated that, with the exception of the animals that were treated only with URB597, all experimental groups reported significantly higher percentages of freezing during the CS presentation with respect to the baseline condition (no CS; $P < 0.01$ for all comparisons; Fig. 3D). Furthermore, rats that were administered only with URB597 displayed significantly lower freezing during the CS than their corresponding vehicle and SR144528 co-infused groups ($P < 0.01$ for both comparisons; Fig. 3D).

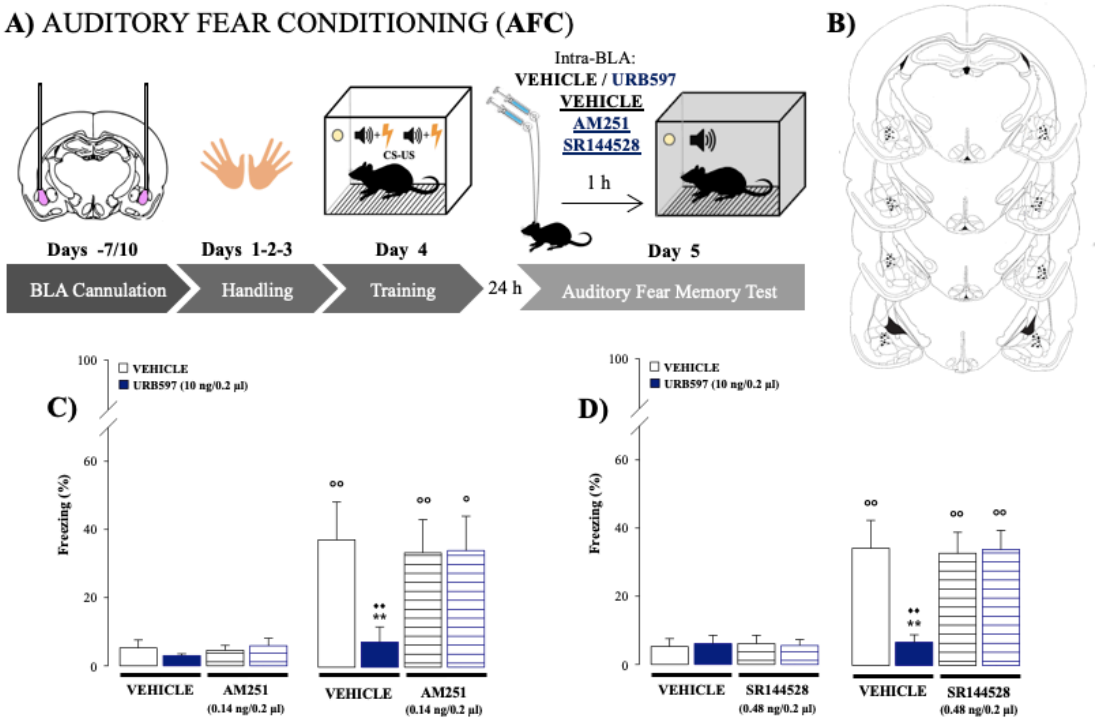

Fig. 3 – AEA-mediated impairment of auditory fear memory retrieval was dependent on intra-BLA activation of CB1 and CB2 receptors. Effects of the effective dose of the AEA hydrolysis inhibitor URB597 on auditory fear memory retrieval, when co-administered into the BLA with either the CB1 receptor antagonist AM251 or the CB2 receptor antagonist SR144528. Diagram of the experimental protocol for the AFC experiments involving co-administration of drugs (**A**). Diagram of rat brain coronal sections demonstrating injection sites randomly selected among rats included in the final analysis (• Vehicle/URB597; ▲ Vehicle/AM251/SR144528) (**B**). Freezing rates of rats co-infused into the BLA with URB597 10 ng and AM251 0.14 ng (**C**) or their corresponding vehicles 1 h before the testing session (** $P < 0.01$, URB597 10 ng vs vehicle; ♦♦ $P < 0.01$, URB597 10 ng vs URB597 10 ng + AM251 0.14 ng; n = 12 per group). Freezing rates of rats co-infused into the BLA with URB597 10 ng and SR144528 0.48 ng (**D**) or vehicles 1 h before the testing session (º $P < 0.05$; ºº $P < 0.01$, vehicle, URB597, AM251 and SR144528 groups vs the corresponding treatment groups during no CS presentation; ** $P < 0.01$, URB597 10 ng vs vehicle; ♦♦ $P < 0.01$, URB597 10 ng vs URB597 10 ng + SR144528 0.48 ng; n = 11 per group).

Enhancing endogenous levels of 2-AG, but not of AEA, into the dorsal hippocampus, attenuates the retrieval of contextual fear memory

This set of experiments evaluated whether the enhancement of AEA or 2-AG levels into the CA1 region of the dorsal hippocampus influenced auditory or contextual fear memory retrieval.

To address this question, rats were bilaterally treated into the dorsal CA1 with URB597 (3, 10 or 30 ng), KML29 (0.2, 2 or 20 ng), or their relative vehicles, 1 h before retention testing on the AFC task (Fig. 4A). Repeated-measures ANOVA for freezing time across the AFC testing sessions reported no significant effects of treatment or treatment × CS presentation interaction, concerning both the KML29 and URB597 experiments (Fig. 4B-C). However, significant differences for freezing time across the AFC testing session were revealed during the CS presentation in both the KML29 and URB597 experiments ($F_{(1.31)} = 58.990$; $P < 0.0001$ and $F_{(1.31)} = 61.509$; $P < 0.0001$, respectively). *Post hoc* analysis indicated that independently of the treatment group, all animals showed higher percentages of freezing during the CS presentation with respect to the baseline condition (no CS; $P < 0.01$ for all comparisons in the KML29 experiments, Fig. 4B; $P < 0.05$ for URB597 3 and 30 ng groups; $P < 0.01$ for vehicle- and URB597 10 ng-treated rats, Fig. 4C).

In a second batch of experiments, different cohorts of rats were bilaterally administered into the dorsal CA1 with URB597 (3, 10 or 30 ng), KML29 (0.2, 2 or 20 ng), or their vehicles, 1 h before retention testing on the CFC task (Fig. 5A). As shown in Fig. 5C, one-way ANOVA indicated that KML29 induced a dose-dependent reduction in freezing levels during retention testing on the CFC task ($F_{(3.46)} = 6.077$; $P = 0.001$). *Post hoc* comparisons showed that intra-CA1 KML29 (2 ng) administration significantly reduced freezing levels as compared to control rats ($P < 0.01$). In contrast to KML29, intra-CA1 URB597 treatment did not alter freezing levels during retention on the CFC task (Fig. 5D).

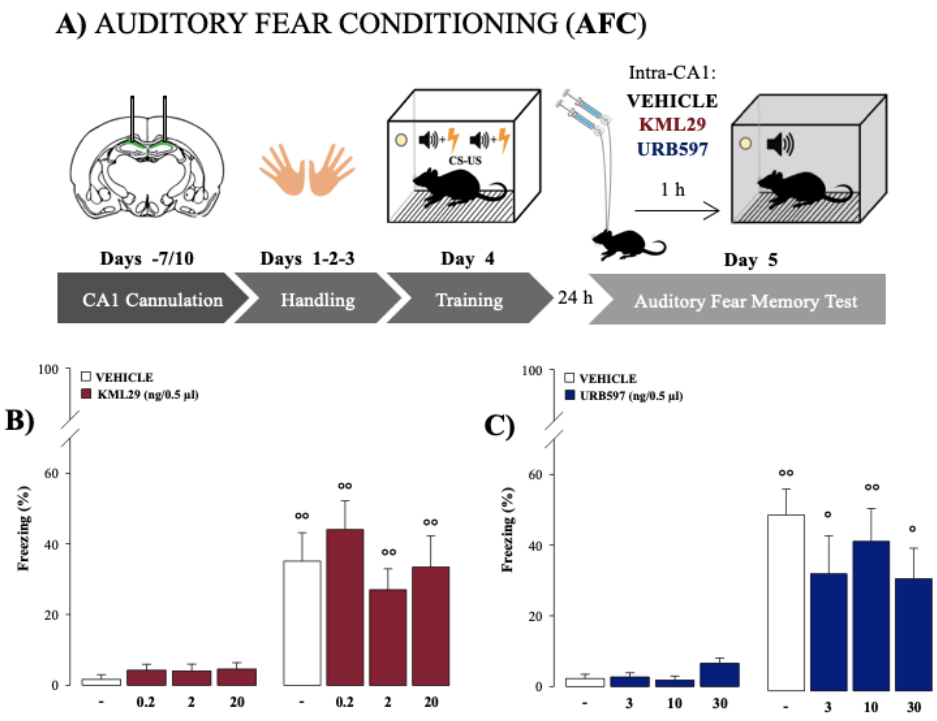

Fig. 4 – Hippocampal endocannabinoid modulation of auditory fear memory retrieval. Effects of different doses of the AEA hydrolysis inhibitor URB597 or the 2-AG hydrolysis inhibitor KML29, into the dorsal CA1, 1 h before testing, on the retrieval of auditory fear memory. Diagram of the experimental protocol for the AFC experiments (**A**). Freezing rates of rats infused into the dorsal CA1 field with KML29 (**B**), URB597 (**C**) or vehicle 1 h before the testing session (º $P < 0.05$; ºº $P < 0.01$, vehicle, KML29 and URB597 groups vs the corresponding treatment groups during no CS presentation; n = 11 per group).

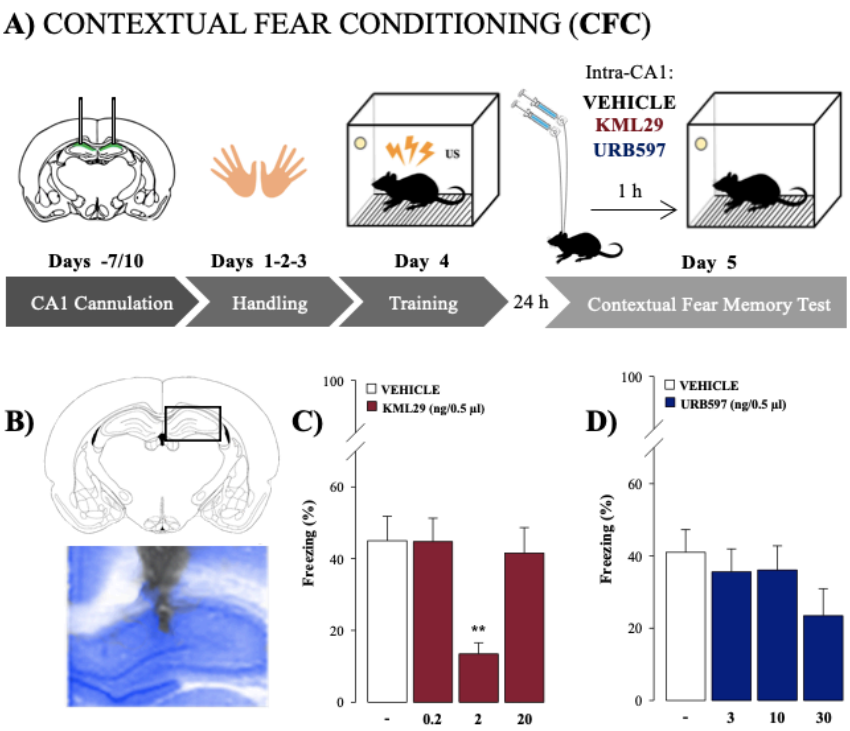

Fig. 5 – Hippocampal endocannabinoid modulation of contextual fear memory retrieval. Effects of different doses of the AEA hydrolysis inhibitor URB597 or the 2-AG hydrolysis inhibitor KML29, into the dorsal CA1, 1 h before testing, on the retrieval of contextual fear memory. Diagram of the experimental protocol for the CFC experiments (**A**). Representative photomicrograph (Nikon 801 microscope; original magnification 2×) illustrating the placement of the cannula and needle tip in the CA1 region of the dorsal hippocampus (**B**). Freezing rates of rats intra-CA1 administered with KML29 (**C**), URB597 (**D**) or vehicle 1 h before the testing session (** $P < 0.01$, KML29 2 ng vs vehicle; n = 10-11 per group).

Hippocampal 2-AG impairs contextual fear memory retrieval through the activation of CB1 and CB2 receptors

To investigate whether the CB1 or CB2 receptors mediated the impairing effects of hippocampal KML29 (2 ng) on contextual fear memory retrieval, the CB1 receptor antagonist AM251 (0.14 ng) or the CB2 receptor antagonist SR144528 (0.48 ng), were

administered together with the effective dose of KML29 (2 ng) into the dorsal CA1 1 h before retention testing on the CFC task (Fig. 6A).

As shown in Fig. 6C, two-way ANOVA for freezing time across the CFC testing sessions indicated no significant effects of KML29 or AM251 treatment, but revealed a significant KML29 × AM251 interaction effect ($F_{(1.35)} = 6.206$; $P = 0.018$). *Post hoc* comparisons reported that KML29 (2 ng) significantly decreased levels of freezing as compared to rats given vehicle or co-administered with AM251 ($P < 0.05$ for both comparisons). Two-way ANOVA for freezing time across the CFC testing sessions reported no significant SR144528 treatment effect, but significant KML29 treatment and KML29 × SR144528 interaction effects ($F_{(1.41)} = 6.306$; $P = 0.016$ and $F_{(1.41)} = 6.756$; $P = 0.013$, respectively). *Post hoc* analysis indicated that rats that were administered with KML29 (2 ng) showed significantly lower freezing levels as compared to their corresponding vehicle and SR144528 co-infused groups ($P < 0.01$ for both comparisons; Fig. 6D).

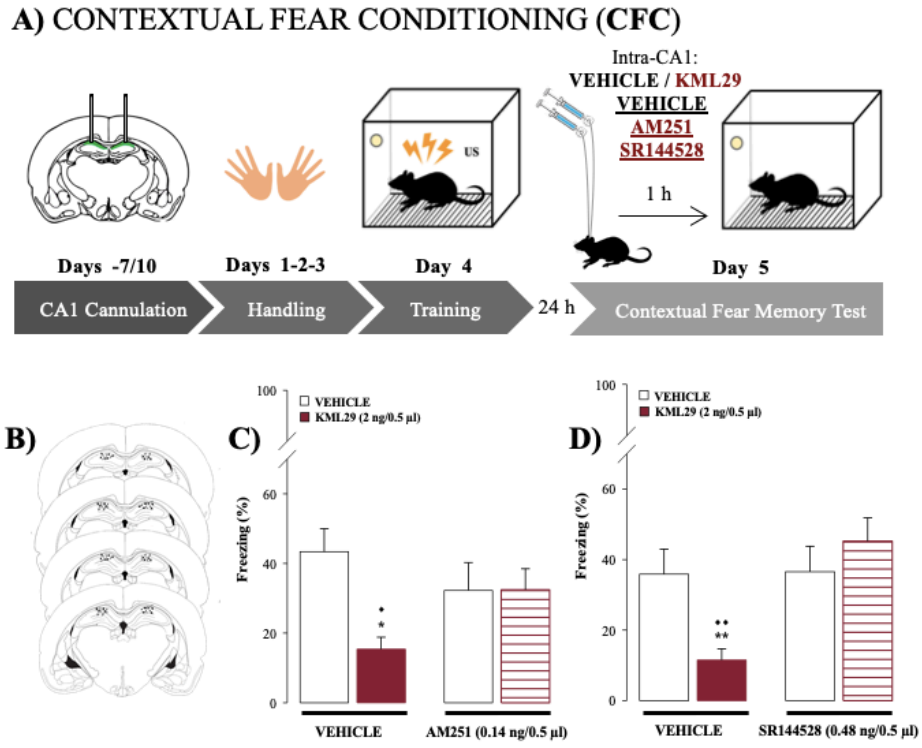

Fig. 6 – 2-AG-mediated impairment of contextual fear memory retrieval was dependent on intra-CA1 activation of CB1 and CB2 receptors. Effects of the effective dose of 2-AG hydrolysis inhibitor KML29 on contextual fear memory retrieval, when co-administered into the dorsal CA1 with either the CB1 receptor antagonist AM251 or the CB2 receptor antagonist SR144528. Diagram of the experimental protocol for the CFC experiments involving co-administration of drugs (**A**). Diagram of rat brain coronal sections demonstrating injection sites randomly selected among rats included in the final analysis (• Vehicle/KML29; ▲ Vehicle/AM251/SR144528) (**B**). Freezing rates of rats co-infused into the CA1 region of the dorsal hippocampus with KML29 2 ng, or vehicle, and AM251 0.14 ng (**C**) or vehicle 1 h before the testing session (* $P < 0.05$, KML29 2 ng vs vehicle; ♦ $P < 0.05$, KML29 2 ng vs KML29 2 ng + AM251 0.14 ng; n = 10-11 per group). Freezing rates of rats co-infused into the dorsal CA1 field with KML29 2 ng, or vehicle, and SR144528 0.48 ng (**D**) or vehicle 1 h before the testing session (** $P < 0.01$, KML29 2 ng vs vehicle; ♦♦ $P < 0.01$, KML29 2 ng vs KML29 2 ng + SR144528 0.48 ng; n = 11-12 per group).

Pharmacological inactivation of the dorsal hippocampus reverts the basolateral amygdalar AEA-mediated impairment of auditory fear memory retrieval

This experiment evaluated whether a muscimol-dependent inactivation of the dorsal CA1 modulated the basolateral amygdalar AEA-mediated impairment of auditory fear memory retrieval in the AFC task. Consequently, to ensure bilateral hippocampal lesions and basolateral amygdalar AEA enhancement, rats were bilaterally administered 2 h before retention testing into the dorsal CA1 with muscimol (500 ng) and treated into the BLA 1 h before retention testing with the effective dose of URB597 (10 ng) (Fig. 7A). Repeated-measures ANOVA for freezing time across the AFC testing session revealed no significant effect of the dorsal CA1 inactivation, but significant CS presentation, URB597 treatment and interaction between dorsal CA1 inactivation × URB597 treatment ($F_{(1.30)} = 61.565$; $P < 0.0001$; $F_{(1.30)} = 4.298$; $P = 0.047$ and $F_{(1.30)} =$

8.623; P = 0.006, respectively) effects. No significant differences were reported for the CS presentation × URB597 or CS presentation × dorsal CA1 inactivation interaction effects, but repeated-measures ANOVA revealed a significant effect of the interaction between the CS presentation × dorsal CA1 inactivation × URB597 treatment ($F_{(1.30)}$ = 8.853; P = 0.006). *Post hoc* comparisons indicated that, with the exception of the animals that were treated only with URB597, all experimental groups reported significantly higher percentages of freezing during the CS presentation with respect to the baseline condition (no CS; P < 0.01 for all comparisons; Fig. 7C). Moreover, when rats were treated with URB597 (10 ng) into the BLA, but displayed active dorsal CA1, their freezing levels were significantly lower than those of the corresponding vehicle-treated group and animals that were administered with URB597 (10 ng) but reported an inactivated dorsal CA1 because of muscimol infusion (P < 0.01 for both comparisons; Fig. 7C).

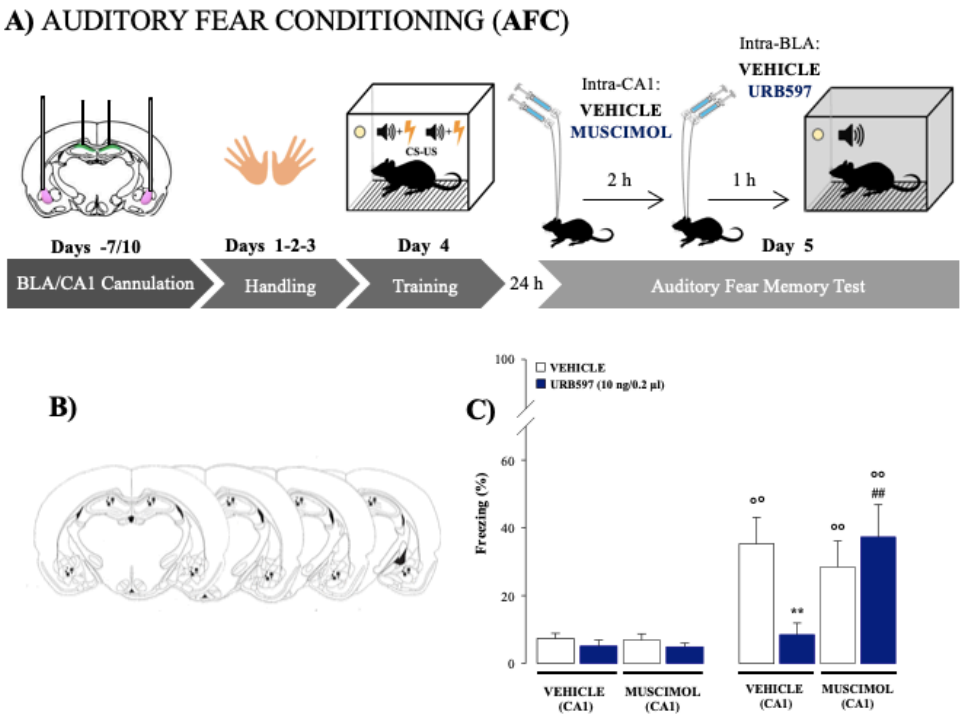

Fig 7 – Muscimol-mediated inhibition of the dorsal CA1 field reverts the basolateral amygdalar AEA modulation of auditory fear memory retrieval. Effects

of muscimol-induced pharmacological lesions of the CA1 region of the dorsal hippocampus on the basolateral amygdalar URB597 effects on auditory fear memory retrieval. Diagram of the experimental protocol for the AFC experiments involving hippocampal administration of muscimol and intra-BLA infusion of URB597 (**A**). Diagram of rat brain coronal sections demonstrating injection sites randomly selected among rats included in the final analysis (● Vehicle; ■ URB597/Muscimol) (**B**). Freezing rates of rats that were treated into the dorsal CA1 with muscimol 500 ng, or vehicle, 2 h before the testing session, and subjected to intra-BLA administration of URB597 10 ng (**C**) or vehicle 1 h before testing (º $P < 0.05$; ºº $P < 0.01$, vehicle and URB597 10 ng groups vs the corresponding treatment groups during no CS presentation; ** $P < 0.01$, URB597 10 ng vs vehicle; ## $P < 0.01$, URB597 10 ng displaying inhibited CA1 vs URB597 10 ng with functional dorsal CA1; n = 11 per group).

Pharmacological inactivation of the BLA reverts the hippocampal 2-AG-mediated impairment of contextual fear memory retrieval

This experiment evaluated whether a muscimol-dependent inactivation of the BLA modulated the hippocampal 2-AG-mediated impairment of contextual fear memory retrieval in the CFC task. Consequently, to ensure bilateral basolateral amygdalar lesions and intra-CA1 2-AG signaling enhancement, rats were bilaterally administered 2 h before retention testing into the BLA with muscimol (500 ng) and treated 1 h before retention testing into the dorsal CA1 field with the effective dose of KML29 (2 ng) (Fig. 8A). Fig. 8C shows freezing time across the CFC testing session. Two-way ANOVA for freezing time across the CFC testing session revealed no significant effect of the BLA inactivation, but significant KML29 effects and KML29 × BLA inactivation interaction effect ($F_{(1.42)} = 4.285$; $P = 0.042$ and $F_{(1.42)} = 4.795$; $P = 0.034$, respectively). *Post hoc* comparisons reported that KML29 (2 ng)-treated rats that presented functional BLA showed significantly lower freezing levels than their corresponding vehicle-treated group and those of animals that were administered with KML29 (2 ng) but had

an inactivated BLA because of muscimol administration ($P < 0.01$ for both comparisons).

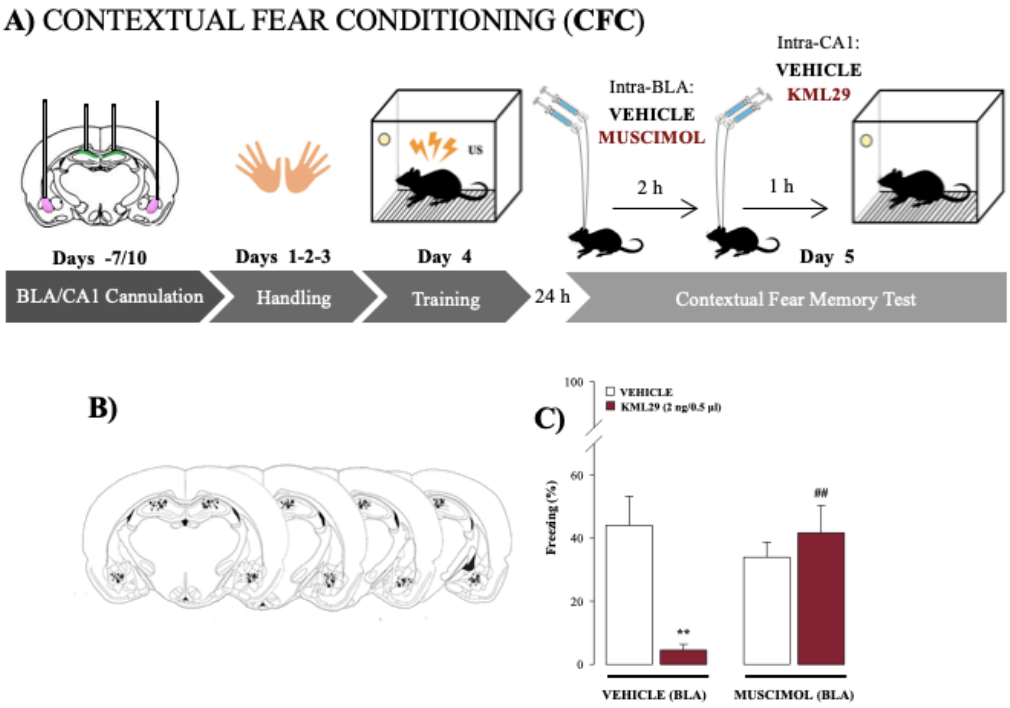

Fig 8 – Muscimol-mediated inhibition of the BLA reverts the hippocampal 2-AG modulation of contextual fear memory retrieval. Effects of muscimol-induced pharmacological lesions of the BLA on the hippocampal KML29 effects on contextual fear memory retrieval. Diagram of the experimental protocol for the CFC experiments involving basolateral amygdalar administration of muscimol and intra-CA1 infusion of KML29 (**A**). Diagram of rat brain coronal sections demonstrating injection sites randomly selected among rats included in the final analysis (● Vehicle; ■ KML29/Muscimol) (**B**). Freezing rates of rats that were infused into the BLA with muscimol 500 ng, or vehicle, 2 h before the testing session, and subjected to intra-CA1 administration of KML29 2 ng (**C**) or vehicle 1 h before testing (** $P < 0.01$, KML29 2 ng vs vehicle; ## $P < 0.01$, KML29 2 ng displaying inhibited BLA vs KML29 2 ng with functional BLA; n = 11-12 per group).

Optogenetic inhibition of the dorsal hippocampus restores the impairing effects of basolateral amygdalar AEA on auditory fear memory retrieval

To further characterize whether the dorsal hippocampus mediates the basolateral amygdalar endocannabinoid modulation of the retrieval of auditory fear memory, we optogenetically inhibited the dorsal CA1.

In these experiments, rats were bilaterally administered into the BLA with URB597 (10 ng), or vehicle, 1 h before retention testing on the AFC task and underwent bilateral optogenetic inhibition of the dorsal CA1, during the AFC testing session, with optical illumination of the CA1 field just during the CS presentation (light ON vs light OFF) (Fig. 9A).

Repeated-measures ANOVA for freezing time across the AFC testing session revealed significant treatment ($F_{(1.45)}$ = 13.291; P = 0.001) and temporal inhibition ($F_{(2.90)}$ = 28,185; P < 0.0001) effects, but no significant interaction between both factors. Furthermore, repeated-measures ANOVA reported significant effects of the temporal inhibition × virus ($F_{(2.90)}$ = 3.732; P = 0.028) and temporal inhibition × treatment ($F_{(2.90)}$ = 4.127; P = 0.019) interactions, but no significant interaction between these three factors. *Post hoc* analysis revealed higher freezing rates during the CS and light ON for both intra-BLA vehicle- and URB597-treated rats that expressed eArchT3.0 in the dorsal CA1 with respect to no CS presentation (P < 0.05 for vehicle; P < 0.01 for URB597 group; Fig. 9C). Animals expressing eArchT3.0 in the dorsal CA1, which were administered with URB597 into the BLA, also showed higher levels of freezing than those that were reported during the CS presentation but light OFF (P < 0.05; Fig. 9C). *Post hoc* analysis additionally indicated that when eYFP-sham control and eArchT3.0 expressing rats were treated with URB597 10 ng into the BLA, but displayed active dorsal CA1 (light OFF), their freezing levels were significantly lower than those of the corresponding vehicle-treated groups (P < 0.05 for eYFP-sham control; P < 0.01 for eArchT3.0 expressing rats; Fig. 9C). However, among the eYFP-sham control groups, intra-BLA URB597-treated animals showed significantly lower freezing levels with respect to the corresponding vehicle group also when displaying inhibited dorsal

hippocampus for optical illumination (light ON; $P < 0.05$; Fig. 9C). Finally, *post hoc* comparisons reported significant higher levels of freezing for intra-BLA URB597-treated rats that expressed eArchT3.0 in their dorsal hippocampus and underwent optogenetic inhibition of the dorsal CA1 (light ON) with respect to their corresponding eYFP-sham control group ($P < 0.05$; Fig. 9C), indicating that the optogenetic inhibition of the dorsal CA1 field through the selective expression of eArchT3.0 influences basolateral amygdalar URB597 modulation of auditory fear memory retrieval.

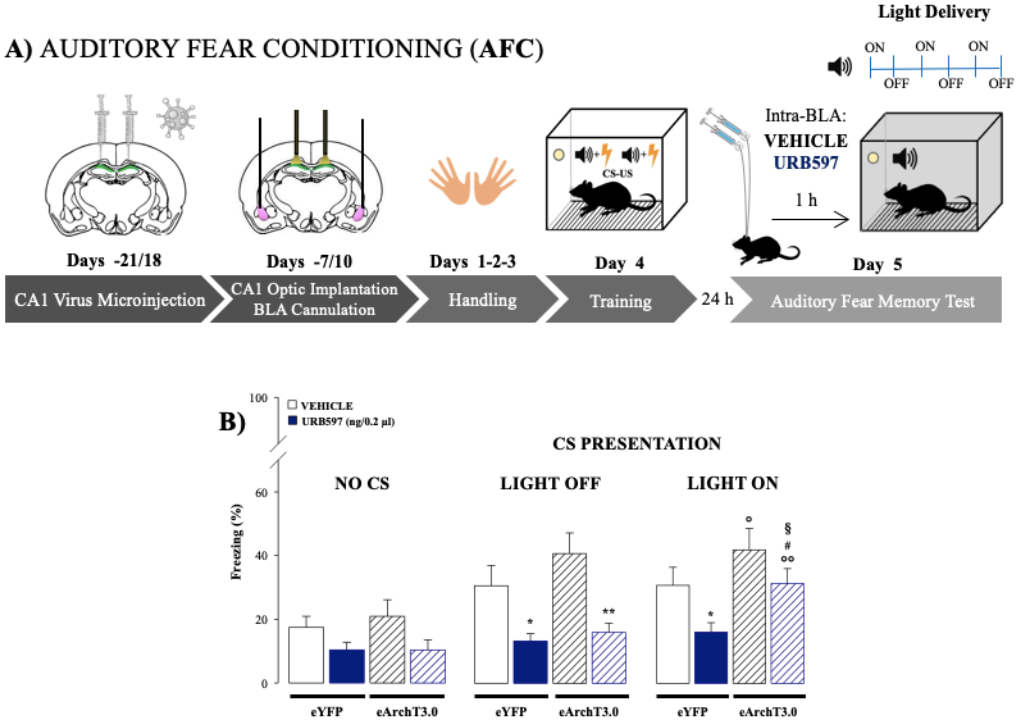

Fig. 9 – Optogenetic inhibition of the dorsal hippocampus restores the impairing effects of intra-BLA URB597 on auditory fear memory retrieval

Effects of optogenetic reversible inhibition of the dorsal CA1 on the basolateral amygdalar URB597 effects in auditory fear memory retrieval. Diagram of the experimental protocol for the AFC experiments involving intra-BLA administration of URB597 and optogenetic inhibition of the dorsal CA1 field (**A**). Freezing rates of rats treated into the BLA with URB597 10 ng, or vehicle, 1 h before testing and subjected to optogenetic inhibition of the dorsal hippocampus (**B**) during the testing session (º P

< 0.05; ºº P < 0.01, vehicle and URB597 10 ng groups during light ON vs the corresponding treatment groups during no CS presentation; # P < 0.05, URB597 10 ng reporting inhibited CA1 for laser light ON vs URB597 10 ng with functional dorsal CA1 – laser light OFF; * P < 0.05; ** P < 0.01, URB597 10 ng vs vehicle; § P < 0.05, intra-CA1 eArchT3.0 expressing animals vs the corresponding eYFP-sham control group; n = 12-13 per group).

Optogenetic inhibition of the BLA restores the impairing effects of hippocampal 2-AG on contextual fear memory retrieval

To further characterize whether the BLA mediates the hippocampal endocannabinoid modulation of the retrieval of auditory fear memory, we optogenetically inhibited the BLA.

In these experiments, rats were bilaterally administered into the dorsal CA1 with KML29 (2 ng), or vehicle, 1 h before retention testing on the CFC task and underwent bilateral optogenetic inhibition of the BLA, during the CFC testing session, with optical illumination of the BLA every 1 min (light ON vs light OFF) (Fig. 10A).

Repeated-measures ANOVA for freezing time across the CFC testing session revealed significant treatment ($F_{(1.49)}$ = 18.596; P < 0.0001) and virus × treatment interaction ($F_{(1.49)}$ = 4.978; P = 0.030) effects, but no significant effects of the virus. Furthermore, repeated-measures ANOVA reported significant effects of the temporal inhibition ($F_{(1.49)}$ = 30.636; P < 0.0001), the temporal inhibition × virus ($F_{(1.49)}$ = 4.295; P = 0.044) and the temporal inhibition × treatment ($F_{(1.49)}$ = 4.578; P = 0.037) interactions, but no significant interaction between these three factors.

Post hoc analysis revealed higher freezing rates during optogenetic inhibition (light ON) for intra-CA1 KML29-treated rats that expressed eArchT3.0 in the BLA with respect to no optogenetic manipulations (light OFF; P < 0.05; Fig. 10C). *Post hoc* comparisons additionally indicated that when eYFP-sham control and eArchT3.0 expressing rats were treated with KML29 2 ng into the dorsal CA1, but displayed active BLA (light OFF), their freezing levels were significantly lower than those of the

corresponding vehicle-treated groups ($P < 0.05$ for both comparisons; Fig. 10C). However, among the eYFP-sham control groups, intra-CA1 KML29-treated animals showed significantly lower freezing levels with respect to the corresponding vehicle group also when displaying inhibited BLA for optical illumination (light ON; $P < 0.01$; Fig. 10C). Finally, *post hoc* comparisons reported significant higher levels of freezing for intra-CA1 KML29-treated rats that expressed eArchT3.0 in their BLA and underwent optogenetic inhibition of the BLA (light ON) with respect to their corresponding eYFP-sham control group ($P < 0.05$; Fig. 10C), indicating that the optogenetic inhibition of the BLA field through the selective expression of eArchT3.0 influences hippocampal KML29 modulation of contextual fear memory retrieval.

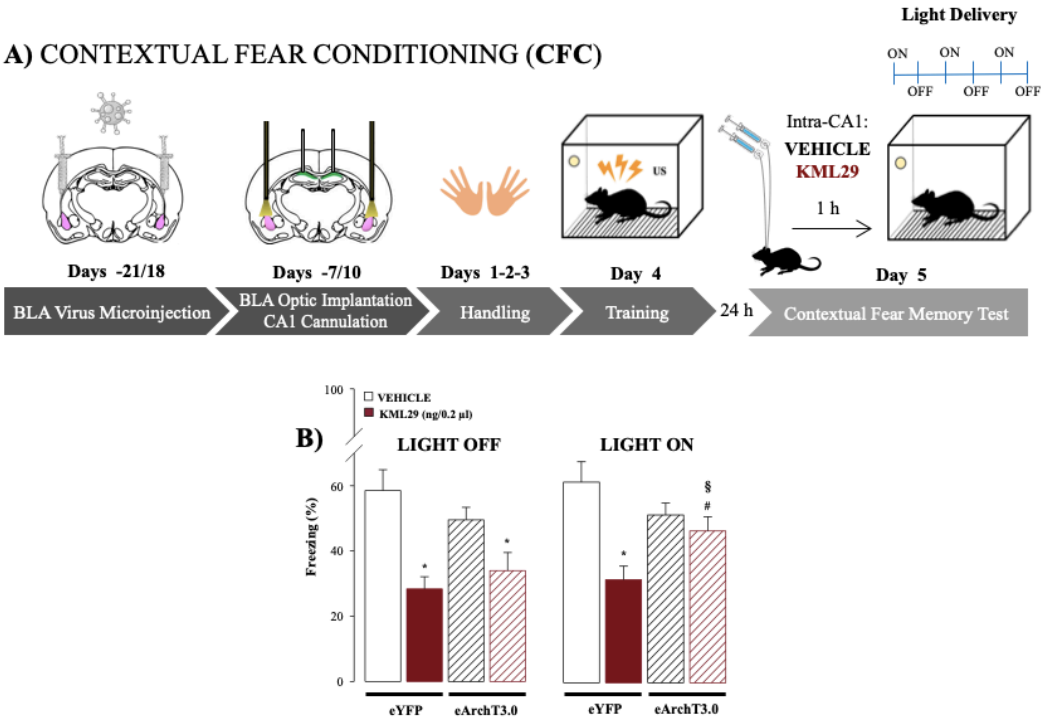

Fig. 10 – Optogenetic inhibition of the BLA restores the impairing effects of intra-CA1 KML29 on contextual fear memory retrieval

Effects of optogenetic reversible inhibition of the BLA on the basolateral amygdalar KML29 effects in contextual fear memory retrieval. Diagram of the experimental protocol for the CFC experiments involving intra-CA1 administration of KML29 and

optogenetic inhibition of the BLA (**A**). Freezing rates of rats that were infused into the dorsal CA1 field with KML29 2 ng, or vehicle, 1 h before testing and subjected to optogenetic inhibition of the BLA (**B**) during the testing session (# $P < 0.05$, KML29 2 ng reporting inhibited BLA for laser light ON vs KML29 2 ng with functional BLA – laser light OFF; * $P < 0.05$, KML29 2 ng vs vehicle; § $P < 0.05$, intra-BLA eArchT3.0 expressing animals vs the corresponding eYFP-sham control group; n = 12-13 per group).

Discussion

The present findings show that endocannabinoids in the basolateral amygdala and in the hippocampus differentially modulate fear memory retrieval processes depending on the level of environment-associated emotional arousal. The AEA hydrolysis inhibitor URB597, when bilaterally infused into the BLA 1 h before testing, impairs auditory, but not contextual, fear memory retrieval, by indirect activation of basolateral amygdalar CB1 and CB2 receptors, no effects were observed when infused in the hippocampus. Conversely, the 2-AG hydrolysis inhibitor KML29 impairs the retrieval of contextual, but not auditory, fear memory, when infused into the CA1 field 1 h before testing, by indirect activation of hippocampal CB1 and CB2 receptors. Our results also indicate that the basolateral amygdalar AEA-mediated impairment of auditory fear memory retrieval, requires an intact dorsal hippocampus, since pharmacological or optogenetic inactivation of the dorsal CA1 field abolishes URB597 effects on memory. At the same time, the hippocampal 2-AG-mediated impairment of contextual fear memory retrieval, requires an intact BLA, as its pharmacological or optogenetic inactivation reverts KML29 effects on memory.

Substantial amount of research indicates that AEA and 2-AG have differential roles in the endocannabinoid neurotransmission, with AEA providing a tonic signal that offsets excess excitability and 2-AG contributing more to the phasic endocannabinoid

response (Ahn et al., 2008). Endocannabinoids are neuromodulator lipids that exhibit extremely rapid on-demand biosynthesis in response to neuronal activation and are subsequently degraded by specialized catabolic enzymes (Gunduz-Cinar et al., 2013a). Such ligands primarily signal through CB1 receptors, which are largely distributed on GABAergic and glutamatergic terminals in corticolimbic regions that mediate fear and anxiety such as the hippocampus and the BLA (Rubino et al., 2008 Hill et al., 2011). Additionally, endocannabinoids activate CB2 receptors, mainly expressed in the periphery and in some microglia and neuronal populations in the central nervous system (CNS) (i.e. cerebral cortex, hippocampus, striatum, amygdala) (García-Gutiérrez et al., 2012; Xi et al., 2011; Zhang et al., 2014). Although evidence of CB2 receptors involvement in stress response is currently lacking, it is well known that CB1 receptors activation regulates the hypothalamic–pituitary–adrenal (HPA) axis activity (Hill et al., 2011) and stress-mediated modulation of memory (Akirav, 2013). It has been extensively demonstrated that both CB1 and CB2 receptors are required to influence different sorts of memory (Kruk-Slomka et al., 2017).

BLA has long been known to be the hub of fear memory (Fanselow and LeDoux, 1999; Roozendaal et al., 2009), it is still debated, however, whether it plays a role in contextual fear conditioning to represent emotional significance, context-shock associations, or simply to reflect arousal that modulates memory formation in other brain regions (Cahill et al., 1999; Fanselow and LeDoux, 1999). Recently, Zelikowsky et al. (2014) demonstrated that spatial and emotional properties of an environment are differentially encoded by the dorsal hippocampus and BLA, respectively, with BLA cells that were activated during Pavlovian fear conditioning being preferentially reactivated during memory retrieval (Reijmers et al., 2007), suggesting that further investigation about these brain regions interplay is worthy.

Despite sparse studies demonstrated that optimal levels of 2-AG in the BLA are required for appropriate processing of fear responses (Hartley et al., 2016; Lutz et al., 2015), basolateral amygdalar AEA signaling has been extensively shown to attenuate stress response, anxiety, fear expression (Gunduz-Cinar et al., 2013a), and contextual

memory processes (Morena et al., 2014). Moreover, although there is only one report showing that reduced basolateral amygdalar AEA levels diminish fear behavior (Morena et al., 2019), literature studies mostly indicated that stress rapidly mobilizes FAAH, depletes the signaling pool of AEA, and increases BLA pyramidal neurons excitability to drive fear and anxiety responses (Gunduz-Cinar et al., 2013b, 2013a; Hill et al., 2009).

We and others have previously demonstrated that intra-BLA administration of the FAAH inhibitor URB597 enhanced consolidation (Morena et al., 2014) and impaired the retrieval of aversive memories through CB1 receptors activation (Morena et al., 2015; Munguba et al., 2011; Segev et al., 2018). Our findings that URB597, when selectively administered in the BLA, impairs auditory, but not contextual, fear memory retrieval, and that such effect is counteracted by intra-BLA administration of a low dose of the CB1 receptors antagonist AM251, is in line with this evidence. Furthermore, our result that intra-BLA administration of KML29 does not influence the retrieval of auditory or contextual fear memory confirms literature evidence reporting the predominant role of the AEA in basolateral amygdalar memory processing.

A recent study found that overexpression of FAAH within postsynaptic compartments of hippocampal CA1-CA3 glutamatergic neurons produced increased long-term potentiation (LTP) along with reduced cognitive performance in a hippocampus-dependent spatial memory task (Zimmermann et al., 2019). Moreover, Segev et al. (2018) demonstrated that URB597, when given into the CA1 of the dorsal hippocampus, attenuates fear memory retrieval in a CB1 receptors activation-dependent manner. However, such limited evidence is restricted to particular types of behavioral paradigms that differ from the ones employed in our study.

Surprisingly, we did not find any influence of intra-CA1 administration of URB597 on both auditory and contextual fear memory retrieval, rather we found that the MAGL inhibitor KML29 impairs the retrieval of contextual fear memory by activating hippocampal CB1 receptors. This is in line with evidence demonstrating that stress recruits 2-AG signaling through CB1 receptors activation within the hippocampus to

impair the retrieval of fear memories (Atsak et al., 2012; Morena et al., 2015), and the corroboration that intra-CA1 administration of URB597 is incapable to counteract such memory impairments (Morena et al., 2015), differentially from KML29 that when is administered into the dorsal CA1 field, is able to specifically counteract the detrimental effects of stress on memory (Santori et al., 2020). Our and other studies thus highlight a major involvement of 2-AG in hippocampus-dependent memory processing. It should be noted, however, that the present studies evaluated the role of the interaction between endocannabinoids and the CA1 activity on the retrieval of spatial and recognition memory leaving 2-AG effects on fear memory retrieval barely investigated.

By employing two different Pavlovian fear conditioning paradigms, the results of the present study indicate that both AEA, in the BLA, and 2-AG, in the dorsal CA1 field of the hippocampus, modulate fear memory retrieval by triggering both CB1 and CB2 receptors. To date, only few studies demonstrated CB2 receptors involvement in memory function, mainly reporting that cannabinoid modulation of different fear memories consolidation involves CB2 receptors (Ratano et al., 2018, 2017) and that their activation modulates excitatory and inhibitory synaptic transmissions within the hippocampus (García-Gutiérrez et al., 2013, 2012), influencing primarily spatial and contextual fear memory processed by this brain region (Li and Kim, 2016; Stern et al., 2017). Thus, to our knowledge, this is the first study documenting basolateral amygdalar and hippocampal CB2 receptors involvement in fear memory retrieval and has a high impact potential.

Over the past two decades, much progress has been made in understanding the core mechanisms of the BLA and the hippocampus in mediating memory function, by determining how they independently exert distinct functions in cognition and also act synergistically in emotion and memory processes (Yang and Wang, 2017).

Exposure to fear increases amygdalar and hippocampal theta rhythm synchronization during memory retrieval (Seidenbecher et al., 2003). Moreover, neurons in the BLA (Wang et al., 2011) and the hippocampus (Adhikari et al., 2011) have been found to fire actively during anxiety-related behaviors, indicating their neural correlation. Literature

studies, investigating these brain regions interplay, revealed that inactivation of the hippocampus with muscimol attenuates amygdala-mediated enhancement of recognition memory (Bass et al., 2014), whereas muscimol-dependent inactivation of the BLA in a contextual fear conditioning task diminishes the consolidation of contextual memory, which is mainly modulated by the hippocampus (Huff and Rudy, 2004).

Our results that basolateral amygdalar AEA- and hippocampal 2-AG-mediated impairment of auditory and contextual fear memory retrieval requires functional BLA-dorsal hippocampus interplay are in line with this evidence and additionally indicate that muscimol-dependent inactivation of the BLA-dorsal hippocampus cross-talk crucially influences other neurotransmissions modulation of a broad range of memory types, such as fear memory processing that was barely considered so far.

To date, numerous pharmacological and optogenetic studies have demonstrated that the dorsal hippocampus transmits contextual information to the BLA to process contextual fear conditioning memory (de Oliveira Coelho et al., 2013; Goshen et al., 2011; Ramirez et al., 2013). Specifically, lesions of the dorsal hippocampus block fear conditioning to contextual, but not to unconditioned (i.e. tones), stimuli (Phillips and LeDoux, 1992), while basolateral amygdalar lesions prevent fear conditioning to both types of stimuli (Maren and Fanselow, 1997; Phillips and LeDoux, 1992). Similarly, optogenetic inhibition of the dorsal CA1 field reversibly blocks the retrieval of contextual fear conditioning (Goshen et al., 2011), whereas optogenetic inhibition of the BLA pyramidal neurons prevents acquisition of both auditory and contextual fear memory (Goshen et al., 2011). It has been shown that optogenetic stimulation of the glutamatergic projections from the BLA to the dorsal CA1 is necessary and sufficient for stress-associated molecular changes and memory impairments (Rei et al., 2015). However, neuroanatomical studies of the amygdalohippocampal circuits indicated that the output portions of the dorsal hippocampus (dorsal subiculum/CA1) have no direct projections to the BLA, rather probably activate the BLA via a relay in the entorhinal or perirhinal cortexes (McDonald and Mott, 2017). Our finding that optogenetic

inhibition of the BLA-CA1 interplay interferes with basolateral amygdalar AEA and hippocampal 2-AG modulation of auditory and contextual fear memory retrieval opens the avenue to further investigate which neurons and neural connections modulate the endocannabinoid modulation of fear memory processing.

In humans, when emotional information is retrieved, the amygdala–hippocampal connectivity increases bidirectionally (Fastenrath et al., 2014). The fibers arising from the CA1 and projecting to the BLA are NMDA receptors-dependent (Mello et al., 1992) and, interestingly, play a specific role in Pavlovian fear conditioning, as they convey information about contextual stimuli (Maren and Fanselow, 1995).

Exposure to stressful stimuli, such as a context or a tone previously paired with an aversive event, alters glutamate neurotransmission both in the BLA and hippocampus, thereby modulating cognitive and emotional processing (Lowy et al., 1995; Popoli et al., 2011).

In the hippocampus GABAergic interneurons regulate local glutamatergic fibers (Lovett-Barron et al., 2014). It is thus tentative to speculate that muscimol- or inhibitory optogenetic-induced activation of these interneurons prevented glutamate release from NMDA-dependent fibers projecting to the BLA. Consequently, the suppression of inhibitory transmission through the endocannabinoid signaling was overcome and the impairing effects on fear memory retrieval prevented.

Although its mechanism is still unknown, the BLA also modulates hippocampal retrieval processes. Inhibition of glutamatergic projections from the BLA to the dorsal hippocampus impaired acquisition of contextual fear memory (Sparta et al., 2014). Neurons projecting from the amygdala to the hippocampus are active during retrieval of extinction memory (Herry and Johansen, 2014; Knapska et al., 2012), and the hippocampus regulates the context-specific firing of BLA neurons after fear memory extinction (Maren and Hobin, 2007). Local GABAergic interneurons regulate the activity of BLA projections (Bissieére et al., 2003; Shaban et al., 2006; Wolff et al., 2014). Hence, we hypothesize that muscimol- or inhibitory optogenetics-dependent activation of this GABAergic network switched off glutamatergic connections to the

hippocampus recovering freezing response after context exposure.

Collectively, our findings indicate that drugs potentiating endocannabinoid neurotransmission might represent promising tools when combined with optogenetics in the study of the mechanisms underlying fear- and stress-related disorders, with potential important consequences in the development of a proper treatment for such diseases.

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CHAPTER 6

SEX-DEPENDENT EFFECTS OF ENDOCANNABINOID MODULATION OF CONDITIONED FEAR EXTINCTION IN RATS

Maria Morena[1,2,3], Andrei S. Nastase[1,2,4], Alessia Santori[5], Benjamin F. Cravatt[6], Rebecca M. Shansky[7], Matthew N. Hill[1,2,3]

[1]Hotchkiss Brain Institute, [2]Mathison Centre for Mental Health Research, [3]Departments of Cell Biology and Anatomy & Psychiatry, [4]Neuroscience Program, Cumming School of Medicine, University of Calgary, 3330 Hospital Dr. NW, T2N 4N1 Calgary, AB, Canada; [5]Department of Physiology and Pharmacology, Sapienza University of Rome, P.le Aldo Moro, 5, 00185 Rome, Italy; [6]The Skaggs Institute for Chemical Biology and Department of Chemistry, The Scripps Research Institute, 10550 North Torrey Pines Road La Jolla, CA 92037, USA; [7]Department of Psychology, Northeastern University, 360 Huntington Ave, 125 NI, Boston, MA 02115, USA

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Abstract

Background and Purpose. Women are twice as likely as men to develop post-traumatic stress disorder (PTSD) making the search for biological mechanisms underlying these gender disparities especially crucial. One of the hallmark symptoms of PTSD is an alteration in the ability to extinguish fear responses to trauma-associated cues. In male rodents, the endocannabinoid system can modulate fear extinction and has been suggested as a therapeutic target for PTSD. However, whether and how the endocannabinoid system may modulate fear expression and extinction in females remains unknown.

Experimental Approach. To answer this question, we pharmacologically manipulated endocannabinoid signalling in male and female rats prior to extinction of auditory conditioned fear and measured both passive (freezing) and active (darting) conditioned responses.

Key Results. Surprisingly, we found that acute systemic inhibition of the endocannabinoid anandamide (AEA) or 2-arachidonoyl glycerol (2-AG) hydrolysis did not significantly alter fear expression or extinction in males. However, the same manipulations in females produced diverging effects. Increased AEA signalling at vanilloid TRPV1 receptors impaired fear memory extinction. In contrast, inhibition of 2-AG hydrolysis promoted active over passive fear responses acutely via activation of cannabinoid type-1 receptors. Measurement of AEA and 2- AG levels after extinction training revealed sex- and brain region-specific changes.

Conclusion and Implications. We provide the first evidence that AEA and 2-AG signalling affect fear expression and extinction in females in opposite directions. These findings are relevant to future research on sex differences in mechanisms of fear extinction and may help develop sex-specific therapeutics to treat trauma-related disorders.

Introduction

Impaired fear extinction contributes to the development and persistence of post-traumatic stress disorder (PTSD) (Milad et al., 2009; Jovanovic and Norrholm, 2011). While only a small proportion of trauma-exposed individuals develop PTSD, women have a two-fold greater risk, prevalence, and duration of PTSD than men (Breslau, 2009). The biological mechanisms underlying these gender disparities remain unclear and controversial. Yet, most preclinical studies on fear memory processes are exclusively performed in males and studies comparing the sexes are few and inconsistent (Shansky, 2015). In rodents, learned fear responses are traditionally assessed by quantifying freezing behaviour, a passive fear response defined as the absence of movements except for respiration (Fanselow, 1980), predominately expressed by males. In contrast, females generally exhibit lower freezing and express darting behaviour, a rapid, forward movement that resembles an active and escape-like fear response (Gruene et al., 2015a; Colom-Lapetina et al., 2019). A better understanding of the mechanisms that mediate these sex differences in fear responding may inform sex-specific pharmacological approaches to the management of PTSD (Velasco et al., 2019).

Compelling evidence from studies in males demonstrates the importance of the endocannabinoid system in modulating fear responses and memory for aversive experiences (Lutz et al., 2015; Morena et al., 2016b). The endocannabinoid system consists of the cannabinoid type-1/2 receptors (CB1R and CB2R), two main endogenous ligands anandamide (AEA) and 2-arachidonoylglycerol (2-AG), and their respective degrading enzymes fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) (Blankman and Cravatt, 2013). In addition to binding to cannabinoid receptors, AEA is also an endogenous ligand for the non- selective cation channel, transient potential receptor of vanilloid type-1 channel (TRPV1R) (Zygmunt et al., 1999). Both CB1R and TRPV1R are widely expressed in brain areas involved in anxiety and fear (Tsou et al., 1999; Mezey et al., 2000; Cristino et al., 2008)

and like CB1R, AEA activation of TRPV1R can regulate synaptic plasticity (Chávez et al., 2010; Grueter et al., 2010). While CB1R activation has overall inhibitory effects through reduction of neurotransmitter release (Katona and Freund, 2012; Yasmin et al., 2020), activation of TRPV1R promotes membrane depolarization, increases neuronal firing rate and facilitates neurotransmitter release (Marinelli et al., 2003; Xing and Li, 2007; Musella et al., 2009; Bialecki et al., 2020). Behaviourally, activation of CB1R or TRPV1R in male rodents has also been shown to induce opposing responses. Specifically, CB1R stimulation reduces anxiety and facilitates fear extinction, while TRPV1R activation promotes anxiety-like behaviour and increases fear expression (Moreira et al., 2012). Interestingly, compelling preclinical evidence has shown sex differences in endocannabinoid content and CB1R binding and affinity in different stress- and fear-related brain areas (de Fonseca et al., 1994; Bradshaw et al., 2006; Riebe et al., 2010; Castelli et al., 2014; Cooper and Craft, 2018). In humans, sex differences have been reported as well, showing higher CB1R binding in the limbic system and cortico-striato-thalamic-cortical circuit in males compared to females (Van Laere et al., 2008), but higher AEA levels in females compared to males (Neumeister et al., 2013). In parallel, cannabinoid compounds have been reported to have sex-divergent effects both in animal and human studies, due to direct gonadal hormone influence on the endocannabinoid system, and pharmacokinetic and pharmacodynamic differences in drug metabolism (Wiley and Burston, 2014) and potency (Craft et al., 2012), reviewed in (Cooper and Craft, 2018).

We recently reported that repeatedly enhancing AEA signalling accelerated extinction learning in male rats (Morena et al., 2018). Specifically, the amygdala represents an important brain region for AEA regulation of fear extinction (Gunduz-Cinar et al., 2013). Recent evidence has shown that the endocannabinoid signaling regulates plasticity within the amygdala-prefrontal cortex (PFC) circuit under stressful experiences (Marcus et al., 2020), and fundamental sex differences have been identified within this amygdala-PFC fear circuit, underlying differences in fear expression (Gruene et al., 2015b). Together with the amygdala and PFC, the periaqueductal grey

(PAG) represents an important fear-related brain area (Maren, 2001), wherein the dorsal subregion (dPAG) primarily regulates innate and active fear responses (Bandler et al., 2000; Watson et al., 2016), more prominent in females (Gruene et al., 2015a), while the ventral PAG (vPAG) seems to be more involved in the regulation of freezing behavior (Watson et al., 2016), more prominent in males (Gruene et al., 2015a). To date, little is known about whether endocannabinoids regulate conditioned fear extinction in females. To answer this question, we employed systemic pharmacological inhibition of either AEA or 2-AG in male and female rats and determined the role of CB1R or TRPV1R in mediating any potential behavioural changes observed. Finally, we measured post-extinction AEA and 2-AG levels in the amygdala, PFC, dPAG and vPAG, to identify potential sex differences in these extinction-related brain regions (Maren, 2001). Results from this study may inform future research aiming at investigating sex- differences in endocannabinoid regulation of fear memory dynamics within specific brain regions and neuronal circuits.

Materials and Methods

Animals

Male and female Sprague Dawley rats (10-11 weeks old at the time of testing; Charles River, Montreal, QB, Canada) were pair housed in clear plastic cages (47 × 25 × 20 cm) in separate temperature-controlled (20 ± 1°C) rooms and maintained under a 12 h/12 h light/dark cycle (8:00 A.M. to 8:00 P.M. lights on) with *ad libitum* access to food and water. This animal model was chosen because processing of emotional information, including memory of aversive experiences and expression of fear, rely on the activation of an evolutionary primitive subcortical and cortical circuit, highly conserved across species, including humans, and pattern of fear response expressed by rats parallels that observed in humans (Lang et al., 2000). All tests were performed during the light phase of the cycle between 10:00 A.M. and 5:00 P.M. Animals were randomly assigned to the experimental groups. Male and female rats were tested separately, in different cohorts

and different days. All experimental procedures were in compliance with protocols approved by the University of Calgary Animal Care Committee, guidelines from the Canadian Council on Animal Care and ARRIVE guidelines (Kilkenny et al., 2010). All efforts were made to minimize animal suffering and to reduce the number of animals used. Recommendations set out in the BJP editorials, where relevant, were followed by the authors.

Experimental procedures

Auditory fear conditioning and extinction paradigm. Rats underwent auditory fear conditioning, extinction, and extinction retrieval with a slightly different procedure as the ones previously described (Gruene et al., 2015a; Morena et al., 2019) (Fig. 1A). Behavioural testing occurred in two different contexts (A and B). Context A consisted of a chamber with a grid floor, back and side metal walls, clear Plexiglas front door and ceiling, and white light. Context A was cleaned with 70% ethanol between rats. Context B consisted of a white opaque plastic floor and curved walls and was cleaned with Virkon solution between rats. To habituate the animals to the behavioural testing room, rats were transferred to the behavioural room and their home cages were placed in sound attenuating, ventilated, and lighted cabinets for at least 30 minutes before and after the handling on day 1, 2, and 3, and for at least 90 min before and after testing, the following days. Fear conditioning chambers and cabinets were cleaned thoroughly with soapy water and ethanol at the end of each experimental run, in between male and female experimental cohorts. Rats were handled for 1 min each. On day 2 and 3, immediately after the handling procedure animals were habituated to context A and B for 10 minutes. Auditory fear conditioning (day 4), was performed in context A. After a 5-minute acclimation period, all rats were exposed to seven conditioning trials. Each conditioning trial involved presentation of the conditioned stimulus (CS; 80dB, 4Hz tone) for 30 s, co-terminating with a 1 s unconditioned stimulus (US; 0.65 mA shock). Inter-trial interval (ITI) between two consecutive CS-US pairings was 3 min. After conditioning, each rat was returned to its home cage. On day 5, rats underwent the extinction training

consisting of 20 CS presentations with an ITI between CSs of 2 min, in context B. On day 6, rats received an extinction retrieval session in context B. After a 2-min acclimation period, rats were presented with five CSs (2-min ITI). Behaviour was video- recorded, scored, and analyzed for freezing (i.e. absence of any movement except for those necessary for respiration) using Video Freeze software (Med Associates Inc., St. Albans, VT, USA; RRID:SCR_014574). Darting behaviour (i.e. rapid, forward movement across the chamber that resembles an escape-like response) was scored manually as number of discrete darting events and expressed as darting rate (dart/min), by two trained observers blinded to the experimental conditions.

To test the effects of the AEA or 2-AG hydrolysis inhibitors on fear extinction, rats were injected intraperitoneally (i.p.) with URB597 or MJN110 (respectively), or their vehicle, 60 min prior to the extinction training. The CB1R antagonist AM251, or its vehicle, was injected i.p. 30 min before URB597 or MJN110 administration (i.e. 90 min before extinction training). A separate group of rats was injected with AM251 in combination with the TRPV1R antagonist Capsazepine (CPZ), 30 min before URB597 injection.

Endocannabinoid extraction and analysis

To assess whether learned fear expression and extinction learning elicits sex-specific patterns of endocannabinoid release, male and female rats were randomly assigned to either extinction [Ext] or no-extinction [No-Ext] groups. Ext groups underwent fear conditioning and extinction training as described above. No-Ext groups underwent fear conditioning but were exposed to the extinction context for an equivalent amount of time without CS presentations. Immediately after the extinction training, rats underwent rapid decapitation, and the brain regions of interest (amygdala, PFC, dPAG and vPAG) were dissected, frozen on dry ice and stored at - 80°C until endocannabinoid level determination. Lipid extraction to determine AEA and 2-AG levels was performed as described previously (Morena et al., 2015; Qi et al., 2015).

Data and Statistical analysis

The manuscript complies with BJP's recommendations and requirements on experimental design and analysis (Curtis et al., 2018). All data were analyzed using GraphPad Prism 6 (RRID:SCR_002798) and are expressed in all Figures as mean ± standard error of the mean (SEM). Statistical analysis was run using independent values and outliers were included in data analysis and presentation. To better evaluate any difference in drug effects in the early or late phases of the behavioral sessions, percentage of freezing or darting rate during extinction training sessions were averaged in four blocks of five consecutive CSs each (CS1-5, CS6-10, CS11-15, CS16-20); behavioural measures for extinction retrieval were averaged in two blocks (CS1-2 and CS3-5). CS-US- or CS-evoked freezing and darting were analyzed with repeated measures (RM) ANOVA. Freezing and darting during the pre-CS period were analyzed with Student's t test or one-way ANOVA, when appropriate. Student's t test was used to analyze brain endocannabinoid levels. Adjusted Bonferroni's multiple comparison post-hoc tests were run when F achieved $P < 0.05$ and there was no significant variance in homogeneity. The correlation analyses were performed with the Pearson correlation test. A probability level of < 0.05 was accepted as statistically significant. Group size, shown in the figure legends, is the number of independent values (i.e. number of rats). To achieve a power of 0.80-0.95, a sample size of at least 10 (for behavioural experiments), or 8 (for biochemical experiments) animals per group was calculated. Studies were designed to generate groups of equal size, using randomisation and blinded analysis. However, sizes for the Vehicle, URB597 and MJN110 groups are higher than those of the remaining groups, as they were combined from separate sets of experiments which were originally run separately to generate pilot data and then replicated when the remaining groups were added to the study. Statistical analysis was undertaken only for studies where each group size was at least $n = 5$. All experiments and data analyses were carried out by operators blinded to the experimental conditions.

Materials

The AEA hydrolysis inhibitor URB597 (0.3 mg/kg; Cayman Chemical, Cedarlane□, Burlington, ON, Canada), the 2-AG hydrolysis inhibitor MJN110 (10 mg/kg; provided by B.F. Cravatt), the CB1R antagonist/inverse agonist AM251 (1 mg/kg; Tocris, Cedarlane□, Burlington, ON, Canada), the TRPV1R antagonist Capsazepine (CPZ; 5 mg/kg; Cayman Chemical, Cedarlane□, Burlington, ON, Canada) or their vehicle (5% polyethylene glycol, 5% Tween-80, 90% saline) were injected i.p. at a volume of 1 ml/kg. URB597, MJN110 or their vehicle were injected 60 min before the extinction training session, AM251, CPZ or their vehicle were injected 90 min before the extinction training session. Doses and timing were chosen based on previously published papers (Kathuria et al., 2003; Colangeli et al., 2017; Ratano et al., 2017; Morena et al., 2018; Sticht et al., 2019) and pilot experiments performed in our laboratory. All drug solutions were freshly prepared before each experiment.

Results

Sexually divergent expression of fear responses during auditory fear conditioning and extinction

We first examined whether sex-specific conditioned fear strategies emerged across the different sessions of the auditory fear conditioning paradigm by assessing CS-US- and CS- evoked freezing and darting behaviour of all rats that received an i.p. injection of vehicle used in the subsequent experiments, pooled together (Fig. 1). As shown in Fig. 1B-D and Table S1, we found significant main effects of CS trial across all three test days, and significant main effect of sex at conditioning and extinction retrieval indicating higher freezing in males. We also observed significant sex x trial interaction for fear conditioning training. Post-hoc comparisons indicated that male rats showed significant higher freezing levels as compared to females during presentations of CS-US 2, 3, 5 and 7. Student's t tests for freezing before CS presentations (pre- CS period)

at conditioning, extinction training and retrieval indicated that male rats showed significant higher freezing levels as compared to females, potentially suggesting higher innate fear and context generalization in males. Figure 1E-G shows CS-US-evoked darting during conditioning, CS-evoked darting during extinction training, and extinction retrieval. Analysis of darting behaviour during fear conditioning revealed significant main effects of trial and sex, and a significant trial x sex interaction. Post-hoc comparisons revealed that females darted more than males at CS-US 3, 4, 5, 6 and 7. During both extinction training and retrieval we found a main effect of sex. Student's t tests for darting during the pre-CS period indicated higher darting in females as compared to males at conditioning and extinction retrieval. These results indicate that, as we have shown previously (Gruene et al., 2015a), males and females engage different fear responses; males consistently show greater freezing than females, while females consistently show higher darting than males.

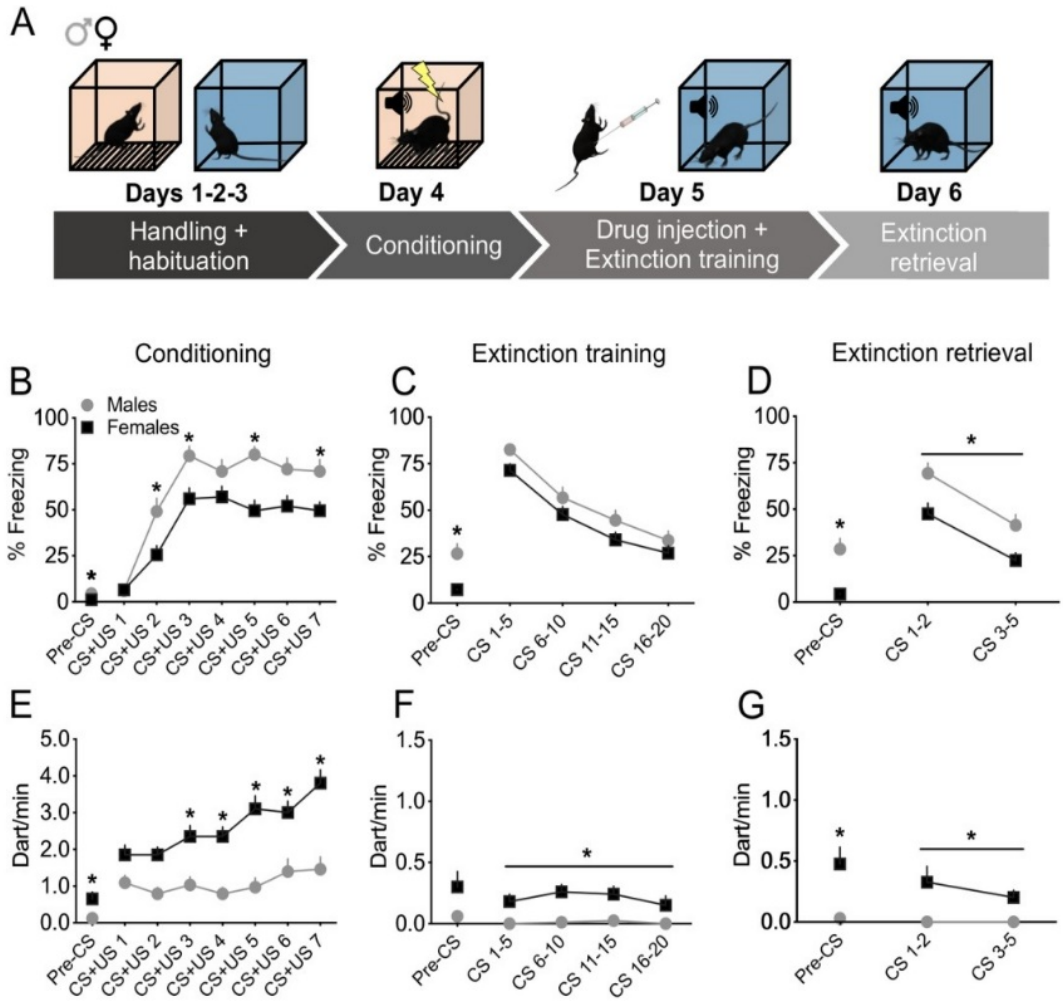

Figure 1. Sexually divergent expression of fear responses during the auditory fear conditioning paradigm. (**A**) Schematic representation of the experimental design. (**B-D**) Except during CS presentations at extinction training (**C**), male rats consistently showed higher freezing behaviour than females during the auditory fear conditioning paradigm. Percentage of freezing during auditory fear conditioning (**B**), extinction training (**C**) and extinction retrieval (**D**). (**E-G**) Female rats consistently showed higher darting behaviour than males throughout the auditory fear conditioning paradigm. Number of darting events/min (dart/min) during auditory fear conditioning (**E**), extinction training (**F**) and extinction retrieval (**G**). Data are expressed as mean ± SEM. * $P < 0.05$, males vs females, the horizontal line below the star indicates main effect of sex; (males, n = 33; females, n = 40).

AEA hydrolysis inhibition does not significantly affect auditory fear memory expression and extinction in males

Figure 2 shows behavioural data for pre-extinction administration of AEA hydrolysis inhibitor URB597 alone, CB1R antagonist AM251 alone and URB597+AM251 (A-F) in males; statistics are in Table S2. In freezing measures (Fig. 2A-C), we observed significant main effects of trial for all three days of testing, suggesting successful fear conditioning and extinction learning. We found a main effect of drug at extinction training and a significant trial x drug interaction for extinction retrieval (Fig. 2B-C). However, although URB597 treatment trended to decrease freezing, post-hoc analyses did not reveal a significant difference compared to the vehicle group, but did show a significant difference compared to the URB597+AM251 group at CS3-5 at extinction retrieval (Fig. 2C). In darting measures (Fig. 2D-F), we found a significant main effect of trial during fear conditioning only, and observed very little or no darting at all during extinction or extinction retrieval. Importantly, there was no main effect of drug during fear conditioning, suggesting that there were no pre-existing differences in these cohorts (Fig. 2A,D). One-way ANOVAs for freezing or darting during the pre-CS period did not show significant effects in any of the testing sessions.

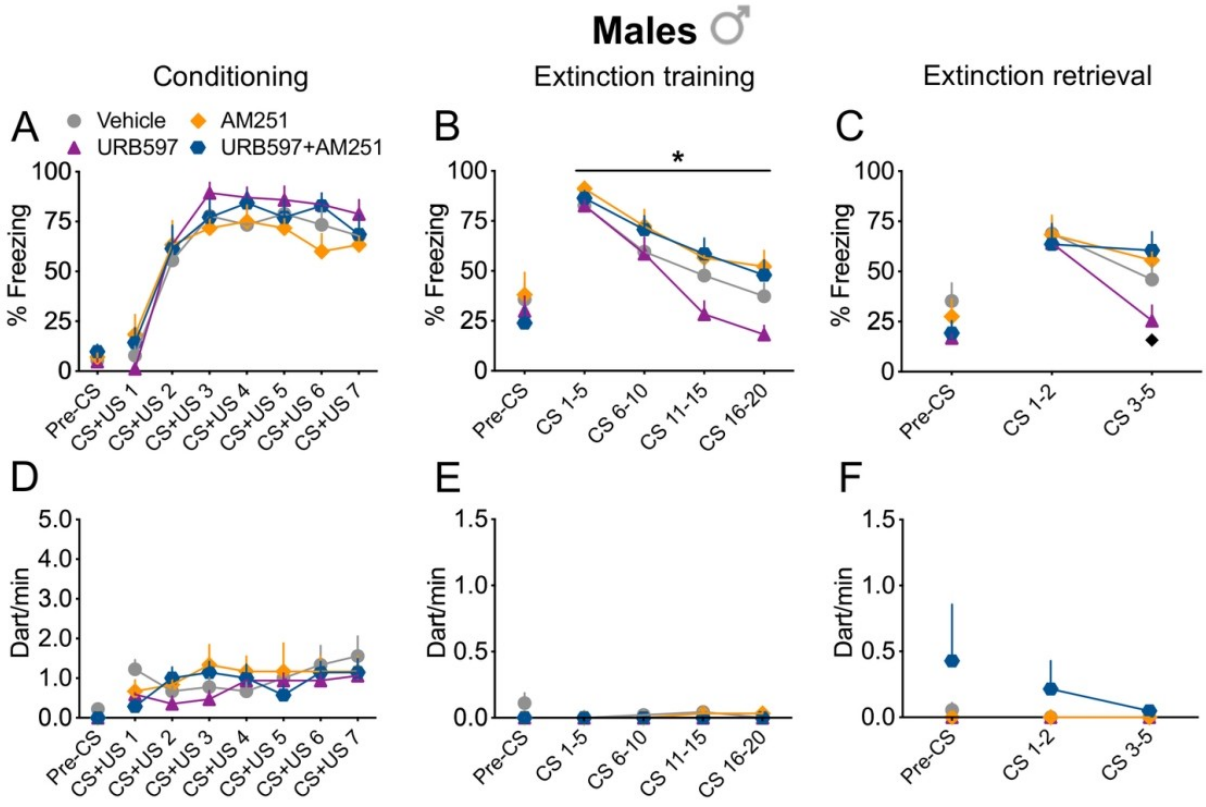

Figure 2. Increased AEA signalling did not significantly alter auditory fear memory expression and extinction in males. (**A-F**) Treatment with the AEA hydrolysis inhibitor URB597, the CB1R antagonist AM251 or their combination (URB597+AM251) did not significantly alter freezing or darting behaviour in male rats, although URB597 showed a trend toward reducing conditioned freezing as compared to the vehicle group during the late phases of extinction training and retrieval and significantly reduced freezing compared to URB597+AM251 (at extinction retrieval). Percentage of freezing during auditory fear conditioning (**A**), extinction training (**B**) and extinction retrieval (**C**). Number of darting events/min (dart/min) during auditory fear conditioning (**D**), extinction training (**E**) and extinction retrieval (**F**). Vehicle, n = 18; URB597, n = 17; AM251, n = 12; URB597+AM251, n = 14. Data are expressed as mean ± SEM. * $P < 0.05$ main effect of drug; ◆ $P < 0.05$ vs URB597+AM251.

2-AG hydrolysis inhibition does not affect auditory fear memory expression and extinction in males

Figure 3 shows behavioural data for pre-extinction administration of 2-AG hydrolysis inhibitor MJN110 alone, AM251 alone and MJN110+AM251 (A-F) in males; statistics are in Table S3. We only found significant main effects of trial for freezing at conditioning, extinction training and retrieval, indicating successful fear conditioning and extinction learning. We did not observe any main effects of drug or drug x trial interactions for freezing at conditioning, extinction training or retrieval (Fig. 3A-C). No statistically significant effects were observed in darting measures (Fig. 3D-F) or during the pre-CS period for freezing or darting in all the three testing sessions.

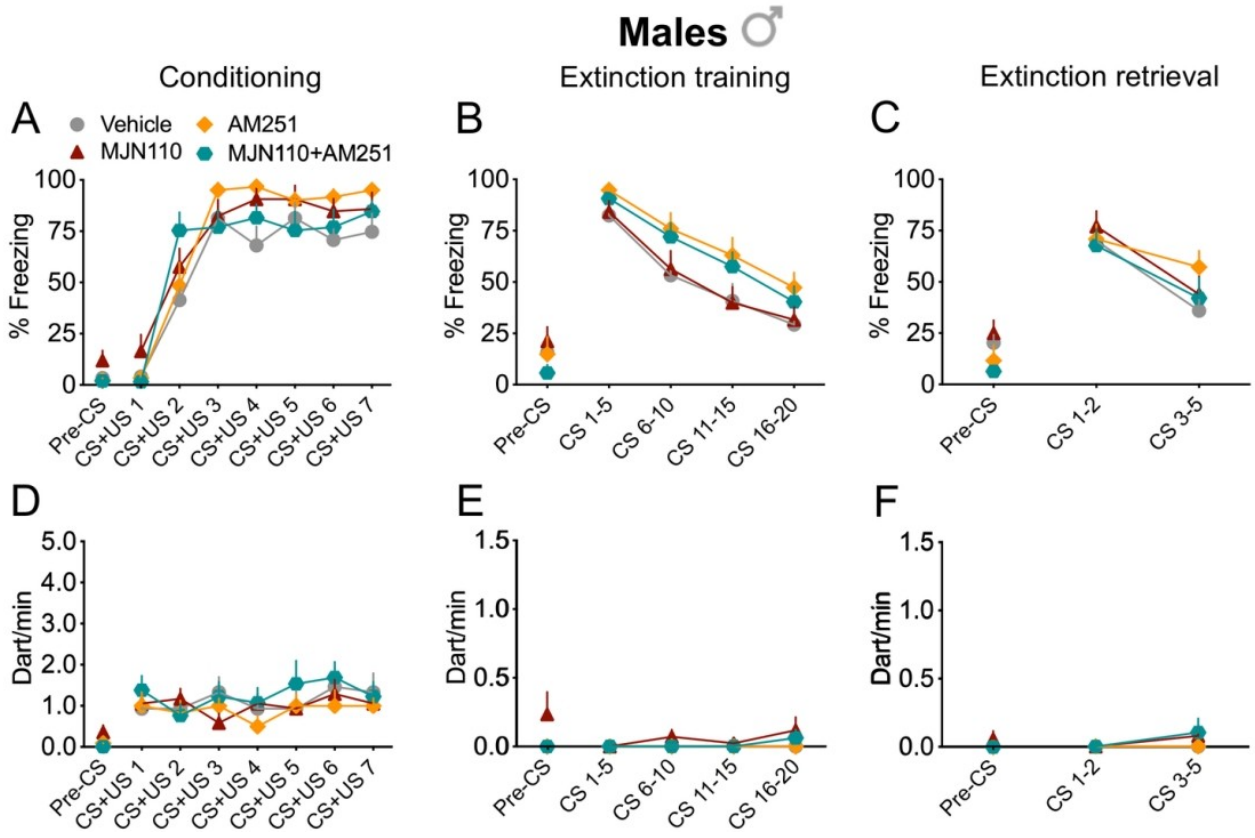

Figure 3. Increased 2-AG signalling did not alter auditory fear memory expression and extinction in males. (**A-F**) Treatment with the 2-AG hydrolysis inhibitor MJN110, the CB1R antagonist AM251 or their combination (MJN110+AM251) did not alter freezing or darting behaviour in male rats. Percentage of freezing during auditory fear

conditioning (**A**), extinction training (**B**) and extinction retrieval (**C**). Number of darting events/min (dart/min) during auditory fear conditioning (**D**), extinction training (**E**) and extinction retrieval (**F**). Vehicle, n = 15; MJN110, n = 17; AM251, n = 12; MJN110+AM251, n = 13. Data are expressed as mean ± SEM.

Increased AEA signalling at TRPV1R augments freezing behaviour at extinction training and retrieval in females

Figure 4 shows the effects of systemic pre-extinction administration of URB597 alone, AM251 alone, URB597+AM251 and URB597+AM251 together with the TRPV1R antagonist CPZ on freezing (Fig. 4A-C) and darting (Fig. 4D-F) behaviour during the auditory fear conditioning paradigm. Detailed statistics is reported in Table S4. Analysis of freezing during fear conditioning revealed a significant main effect of trial, but no significant drug treatment or drug x trial interaction (Fig. 4A), or differences during the pre-CS period. This confirms no pre-existing differences between groups before drug treatment and shows that all groups exhibited fear learning. Analysis of freezing behaviour during extinction (Fig. 4B) revealed significant main effects of trial, drug, and a trial x drug interaction. Post-hoc comparisons showed that rats treated with URB597+AM251 exhibited higher freezing at later time blocks compared to vehicle (CS6-10, CS11-15, CS16-20), URB597 alone (CS11-15 and CS16-20), AM251 alone (CS16-20) and URB597+AM251+CPZ groups (CS11-15 and CS16-20; Fig. 4B). The same group also showed higher freezing levels during the pre-CS period than vehicle-, URB597- and AM251- treated rats, suggesting higher context generalization. During extinction retrieval (Fig. 4C), we found significant main effects of trial and drug, but no interaction. Post-hoc comparisons showed that URB597+AM251 rats exhibited overall higher freezing levels than vehicle-treated rats (Fig. 4C). No differences were observed for freezing during the pre-CS period at extinction retrieval. Analysis of darting behaviour during conditioning, extinction training, and retrieval (Fig. 4D-F) revealed a significant main effect of trial for conditioning, and a significant main effect of drug at extinction training, but no other significant effects. Post-hoc comparisons revealed that

URB597-treated rats exhibited overall lower darting than the vehicle group did, across all the CS trials presented during the extinction training (Fig. 4E). One-way ANOVAs for darting during the pre-CS period did not reveal any significant effects for conditioning, extinction training and retrieval.

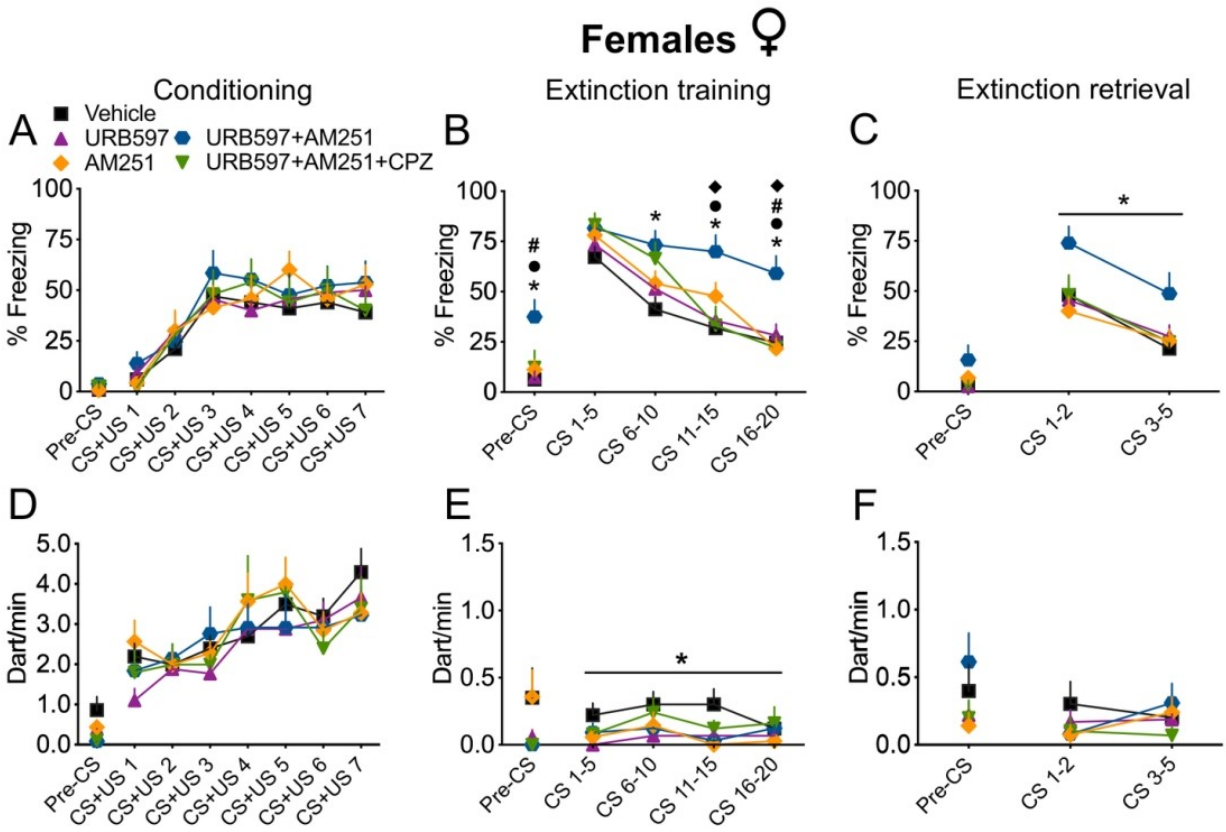

Figure 4. Increased AEA signalling at TRPV1Rs augmented freezing behaviour at extinction training and retrieval in females. (**A-F**) Treatment with the AEA hydrolysis inhibitor URB597 with concurrent blockade of CB1R with the antagonist AM251 (URB597+AM251) induced fear generalization, impaired within-session extinction and extinction retrieval. These effects were mediated by AEA signalling at TRPV1Rs, as they were completely blocked by concomitant injection with the TRPV1R antagonist CPZ (URB597+AM251+CPZ). Furthermore, treatment with URB597 alone induced an overall reduction of darting behaviour during CS presentation at extinction training compared to the vehicle group. Percentage of freezing during auditory fear conditioning (**A**), extinction training (**B**) and extinction retrieval (**C**). Number of darting

events/min (dart/min) during auditory fear conditioning (**D**), extinction training (**E**) and extinction retrieval (**F**). Vehicle, n = 20; URB597, n = 18; AM251, n = 14; URB597+AM251, n = 13; URB597+AM251+CPZ, n = 10. Data are expressed as mean ± SEM. * $P < 0.05$ vs Vehicle; • $P < 0.05$ vs URB597; # $P < 0.05$ vs AM251; ◆ $P < 0.05$ vs URB597+AM251+CPZ. Horizontal line below the star indicates a main effect of drug: $P < 0.05$, URB597+AM251 group vs Vehicle group (**C**) and URB597 group vs Vehicle group (**E**).

Increased 2-AG signalling at CB1R reduces freezing and enhances darting behaviour at extinction training in females

Figure 5 shows the effects of systemic pre-extinction administration of MJN110 alone, AM251 alone and MJN110+AM251 on freezing (Fig. 5A-C) and darting (Fig. 5D-F) behaviour during the auditory fear conditioning paradigm. Detailed statistics is reported in Table S5. Analysis of freezing during conditioning (Fig. 5A) revealed a significant main effect of trial, but no significant drug treatment effect or significant trial x drug interaction, or differences during the pre-CS period, thus confirming no pre-existing differences between groups before drug treatment. Analysis of freezing during extinction training (Fig. 5B) revealed significant main effects of both trial and drug, but no interaction. Post-hoc comparisons showed that the MJN110 alone group exhibited overall less freezing than vehicle-treated rats did across all the CS presentations, and there were no significant differences between the vehicle group and the MJN110+AM251 group, suggesting that MJN110 reduces freezing behaviour through a CB1R- mediated mechanism. Analysis of freezing during extinction retrieval (Fig. 5C) showed only a significant main effect of trial. One-way ANOVAs for freezing during the pre-CS period at extinction training and retrieval did not reveal any significant effects. Analysis of darting (Fig. 5D-F) revealed a significant main effect of trial during conditioning (Fig. 5D), significant effects of trial, and trial x drug interaction at extinction training (Fig. 5E), but no significant effects at extinction retrieval (Fig. 5F). Post-hoc analyses for extinction training showed that treatment with MJN110 alone

significantly increased darting during CS11-15 as compared with both the vehicle and the AM251 groups (Fig. 5E). One-way ANOVAs for darting during the pre-CS periods for all the three testing days revealed a significant effect only for extinction training. Post-hoc analysis showed that the MJN110 group presented higher darting than the vehicle group (Fig. 5E).

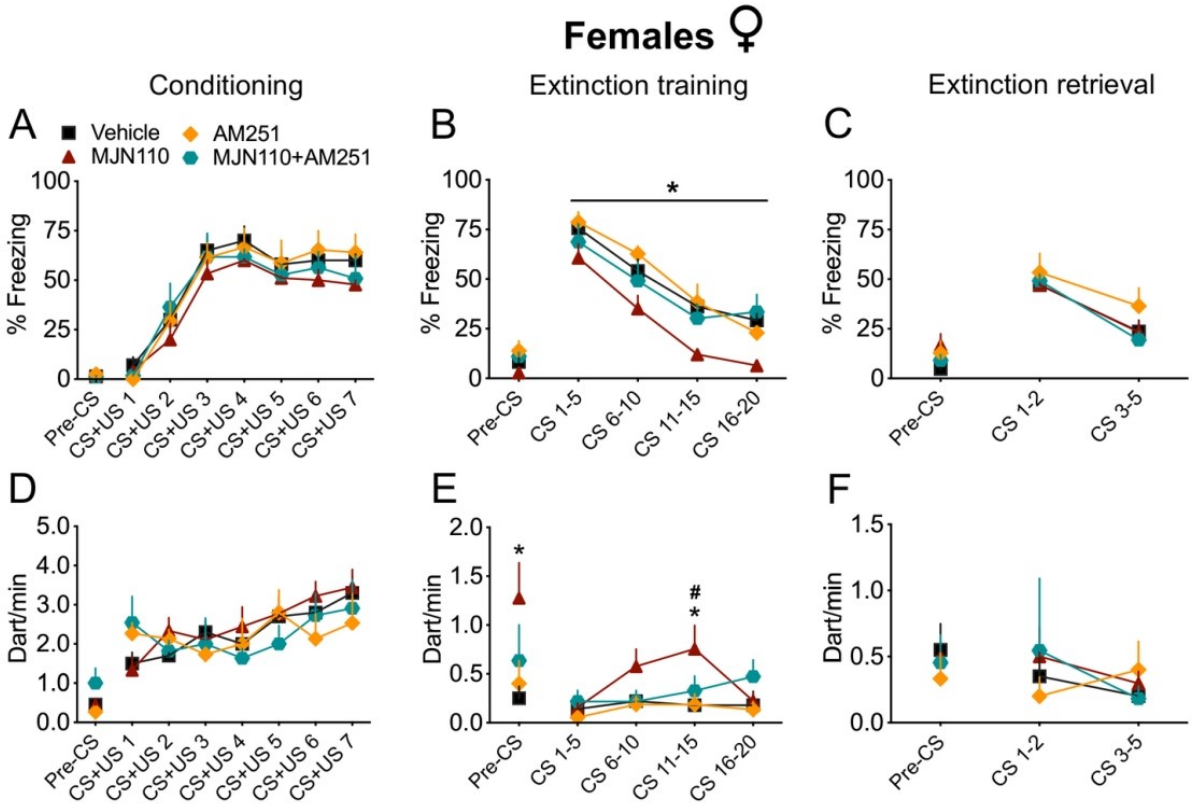

Figure 5. Increased 2-AG signalling at CB1Rs reduced freezing and augmented darting behaviour at extinction training in females. (**A-F**) The 2-AG hydrolysis inhibitor MJN110 decreased freezing and increased darting behaviour at extinction training. These effects were mediated by activation of CB1Rs, as they were blocked by concurrent injection with the CB1R antagonist AM251 (MJN110+AM251). Percentage of freezing during auditory fear conditioning (**A**), extinction training (**B**) and extinction retrieval (**C**). Number of darting events/min (dart/min) during auditory fear conditioning (D), extinction training (**E**) and extinction retrieval (**F**). Vehicle, n = 20; MJN110, n = 18; AM251, n = 15; MJN110+AM251, n = 11. Data are expressed as

mean ± SEM. * $P < 0.05$ vs Vehicle; # $P < 0.05$ vs AM251. Horizontal line below the star indicates a main effect of drug: $P < 0.05$, MJN110 group vs Vehicle group (**B**).

Sex-dependent effects of auditory fear extinction training on endocannabinoid brain levels

To assess how extinction training may differentially alter endocannabinoid levels in males and females, we fear conditioned new cohorts of animals and measured AEA and 2-AG in the amygdala, PFC, dPAG, and vPAG immediately after extinction training (Ext) or a no-CS control session (No-Ext). All data are shown in Fig. 6 and statistics are in Table S6. Surprisingly, we observed effects of extinction on endocannabinoid levels in males only. In the amygdala, we found that male Ext rats had significantly higher AEA levels than male No-Ext rats, while no

differences in AEA levels between the No-Ext and Ext groups in females were detected (Fig. 6A). No significant differences were found for amygdala 2-AG levels in either sex (Fig. 6B). In the PFC, we did found no significant differences in AEA or 2-AG levels between No-Ext and Ext groups in males or females (Fig. 6C,D). In the dPAG, we found a reduction in AEA levels in Ext males compared to No-Ext males, but no significant differences for AEA levels in females (Fig. 6E). No significant effects were observed for 2-AG levels in the dPAG (Fig. 6F) or for AEA or 2-AG in the vPAG (Fig. 6G,H). We also found significant positive correlations between amygdala 2-AG levels and darting rate ($r = 0.72$, $P < 0.05$) and between vPAG AEA levels and freezing behaviour ($r = 0.74$, $P < 0.05$) and a negative correlation between PFC AEA levels and darting rate ($r = -0.67$, $P < 0.05$) shown during CS presentations at the extinction session in females (Table S7). Freezing and darting behaviour for rats in the No-Ext and Ext groups is shown in Fig. S1.

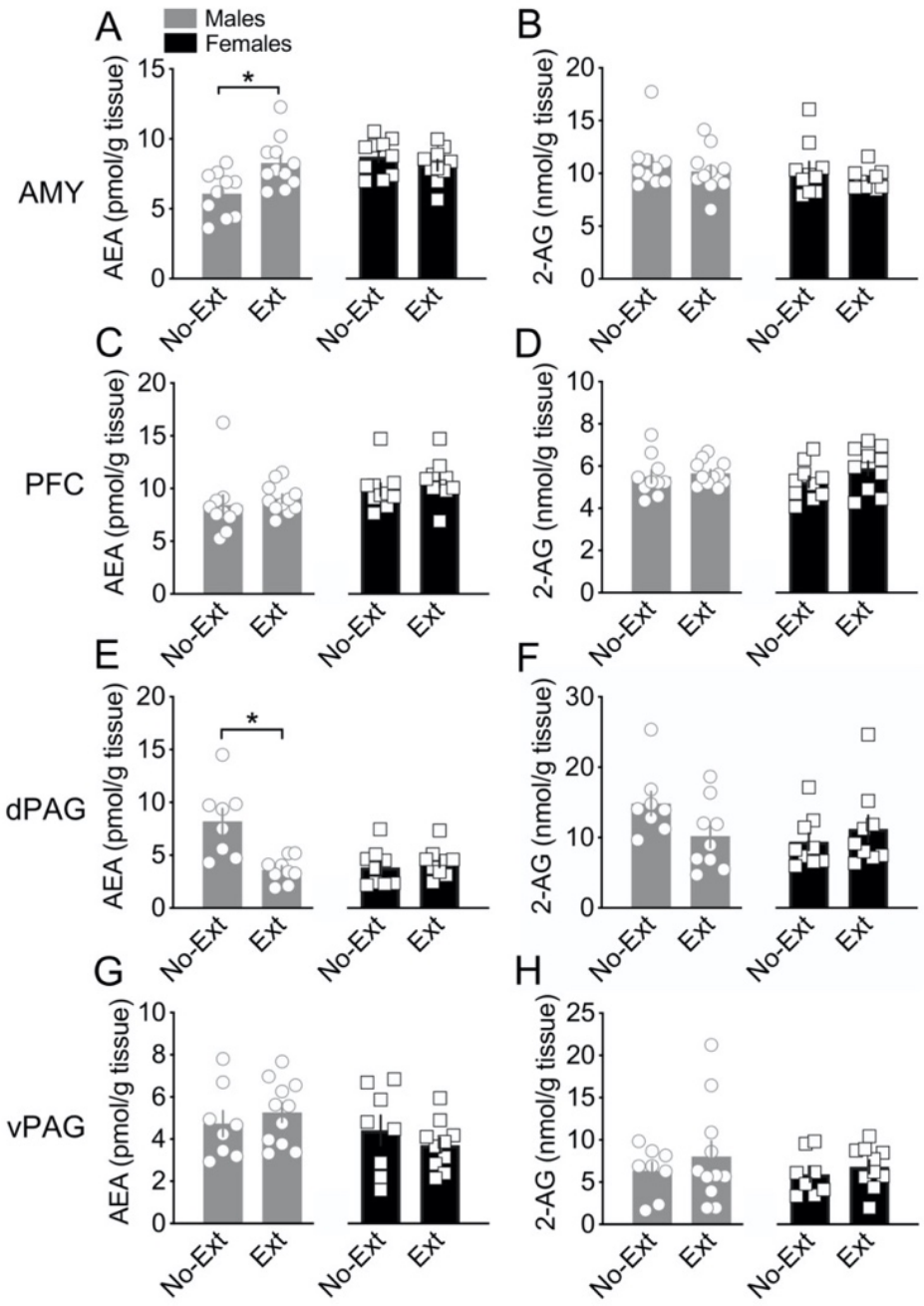

Figure 6. Sex-dependent effects of auditory fear extinction training on endocannabinoid brain levels. AEA and 2-AG brain levels (pmol/g tissue and nmol/g tissue, respectively) in the amygdala (AMY; **A, B**; for AEA and 2-AG: males No-Ext, n = 10, males Ext, n = 11, females No-Ext, n = 10, females Ext, n = 10), prefrontal cortex (PFC; **C, D**; for AEA and 2-AG: males No-Ext, n = 10, males Ext, n = 11, females No-Ext, n = 10, females Ext, n = 10), dorsal periaqueductal grey (dPAG; **E, F**; for AEA and 2-AG: males No-Ext, n = 8, males Ext, n = 9, females No-Ext, n = 9, females Ext, n = 9) and ventral periaqueductal grey (vPAG; **G, H**; for AEA and 2-AG: males No-

Ext, n = 8, males Ext, n = 11, females No-Ext, n = 8, females Ext, n =10 in male and female rats immediately after the extinction training session (Ext group) or in control groups only exposed to the extinction context without the CS presentations (No-Ext group). Data are expressed as mean ± SEM. * $P < 0.05$.

Discussion

Although the effects of endocannabinoid system manipulation on fear memory in males have been well investigated, our study provides the first systematic pharmacologic examination of endocannabinoid regulation of fear extinction in both sexes and reveals for the first time a strong sex-dependent effect of endocannabinoids in the acute modulation of fear extinction. Quite surprisingly, we found that acutely elevating AEA or 2-AG signalling at extinction training did not significantly alter fear expression or extinction in males, although increased AEA tended to facilitate fear extinction. Experiments in females revealed an opposite picture to what has been previously reported for males. We observed divergent effects of AEA versus 2-AG signalling manipulations, each mediated by distinct mechanisms.

Consistent with previous findings (Gruene et al., 2015a), we show a robust sexual dimorphism in behavioural expression of fear. While males predominately expressed freezing behaviour, females exhibited both freezing and darting. Although darting increased over time with CS-US presentations at conditioning, it remained mostly unvaried with progression of CS presentations during both extinction training and retrieval, thus, under our experimental conditions, darting did not seem to strictly reflect a learned fear response. Moreover, accordingly with previous studies comparing conditioned freezing behaviour between sexes (Maren et al., 1994; Pryce et al., 1999; Gupta et al., 2001), we found that males showed significantly higher freezing than females at conditioning and extinction retrieval.

The endocannabinoid system has been consistently reported to modulate fear memory

extinction and stress/fear coping strategies, in male rodents (Marsicano et al., 2002; Metna- Laurent et al., 2012; Gunduz-Cinar et al., 2013; Llorente-Berzal et al., 2015; Morena et al., 2016a, 2018, 2019; Heinz et al., 2017; Colangeli et al., 2020). Increased AEA has been shown to promote fear extinction by reducing expression of freezing (Marsicano et al., 2002; Chhatwal et al., 2005; Bitencourt et al., 2008; Pamplona et al., 2008; Gunduz-Cinar et al., 2013), via activation of CB1Rs on forebrain glutamatergic neurons (Llorente-Berzal et al., 2015); elevated 2-AG signalling, however, has been reported to impair within-session extinction (Hartley et al., 2016) and increase freezing, via activation of CB1Rs on forebrain GABAergic neurons (Llorente-Berzal et al., 2015), in male rodents. Surprisingly, our pharmacological manipulations did not significantly alter fear responses in males. However, consistent with previously published findings using somewhat different approaches (Chhatwal et al., 2005; Bitencourt et al., 2008; Pamplona et al., 2008; Gunduz-Cinar et al., 2013), elevating AEA signalling did tend to facilitate fear extinction. Differences in species, experimental protocol, type, doses and administration regimen of drugs used, likely contributed to these discrepancies. Indeed, in the work by Gunduz- Cinar et al. (2013) were used mice and, specifically, an inbred strain with impaired fear extinction learning and retrieval. Furthermore, in all the above mentioned studies in rats by Chhatwal et al. (2005), Bitencourt et al., (2008), and Pamplona et al. (2008), to explore the effects of increased AEA levels was used AM404, which, in addition to inhibit AEA uptake, has been shown to increase 2-AG signaling and act on many other different sites including TRPV1R and sodium channels (Zygmunt et al., 2000; Nicholson et al., 2003; Hájos et al., 2004; Wiskerke et al., 2012). Consistent with our results, however, pre-extinction injection of URB597, at the same dose we used in the present study, has been reported to not affect fear extinction in male rats under basal conditions, but to only prevent the impairment in fear extinction induced by stress (Zer-Aviv and Akirav, 2016).

It is also possible that doses different from the ones used in the present study or repeated dosing are necessary to produce consistent effects as repeated FAAH inhibition enhanced fear extinction in both male rats (Morena et al., 2018) and a mixed sample of

males and females in humans (Mayo et al., 2020).

Interestingly, in females, elevated AEA signalling at TRPV1Rs increased freezing behaviour both acutely during the extinction training and the following day during extinction retrieval, unveiling an impairment of within-session extinction and recall of extinction memory. Furthermore, the same manipulation induced a strong fear generalization as indicated by elevated freezing shown before CS presentations in the extinction context, which was never associated to the aversive experience. Specifically, although it reduced darting across all CS trials at extinction training, we did not find that inhibition of AEA hydrolysis per se increased freezing behaviour. Surprisingly, concurrent blockade at CB1R while elevating AEA signalling robustly increased freezing response. Additional treatment with CPZ revealed that this effect was mediated by activation of TRPV1R. Since inhibition of AEA hydrolysis together with CB1R blockade did not influence fear responses and memory in males, the TRPV1R antagonism experiment was carried out exclusively in females. These data indicate, for the first time, that AEA signalling at TRPV1Rs might be biased toward facilitating freezing in female rats, thus, unveiling sex differences in the affinity, expression and/or functionality of TRPV1Rs and endocannabinoid system components. An alternate possibility is that females could exhibit an upregulation of TRPV1Rs in response to the noxious footshocks delivered during fear conditioning itself, which then favors AEA signalling at these receptors. Future work is required to understand this relationship in more depth, exploring the effects of direct TRPV1R agonism in discrete brain regions, as it would be challenging to examine this mechanism with a systemic manipulation and avoid confounding pain-related effects due to peripheral TRPV1R activation.

Elevated 2-AG signalling at CB1Rs in females modulated learned fear expression in the opposite direction. Pre-extinction treatment with MJN110 acutely reduced freezing at the last CS presentations, thus accelerating within-session extinction. This effect was CB1R-mediated as it was blocked by CB1R antagonism. Interestingly, MJN110 affected darting behaviour in females in the opposite direction. Therefore, increased 2-AG signalling promoted active over passive fear responses acutely, without affecting

the consolidation of extinction, as treatment with MJN110 did not affect rats' behaviour at extinction retrieval. This shift from passive to active forms of acute fear coping is consistent with an established role of CB1Rs on glutamatergic neurons (Metna-Laurent et al., 2012), suggesting that in females elevated 2-AG signaling may preferentially engage this receptor population to promote this behavioral transition. These collective findings in females are very reminiscent of a study in a line of male mice, bred to exhibit a high degree of anxiety, where elevated AEA signalling increased passive fear responses whereas inhibition of 2-AG hydrolysis increased active responses (Heinz et al., 2017). While it is not immediately apparent as to why pharmacological manipulations of endocannabinoids in these anxious male mice parallel our results with female rats, it does indicate that bidirectional effects of manipulating endocannabinoid signalling on fear behaviours can occur across species and sexes.

Sex differences were also observed in AEA levels in several brain regions involved in the regulation of fear memory and fear responses. Corroborating previous findings in mice (Marsicano et al., 2002; Gunduz-Cinar et al., 2013), male rats undergone fear extinction exhibited higher amygdala AEA levels than males never exposed to CS extinction. Furthermore, among males, we found decreased dPAG AEA levels in rats undergone fear extinction. Interestingly, a previous study showed increased dPAG AEA levels following a 3 min re- exposure to a context previously associated with a footshock (Olango et al., 2012), thus potentially indicating an opposing role in the regulation of early fear expression/extinction vs late extinction phases of fear memory. Fear extinction did not affect AEA levels in females nor 2-AG levels in either sex in the brain regions examined. However, correlational analyses in females revealed that rats presenting higher amygdala 2-AG levels showed increased darting during extinction training, paralleling our behavioural findings with MJN110 treatment. Interestingly, within the PFC, AEA levels negatively correlated with darting, which paralleled our finding that treatment with URB597 decreased darting across all CS presentations at extinction training in females. Furthermore, accordingly to our behavioural results in females showing increased freezing following AEA-mediated activation of TRPV1Rs,

a positive correlation was also detected between freezing during extinction training and AEA levels in the vPAG, a brain region strongly involved in freezing and learned fear responses (Watson et al., 2016). Future work will employ site-specific pharmacological manipulations to establish the sites of action of AEA and 2-AG and to further identify subregion-specific endocannabinoid changes in the amygdala and PFC, also known to play important roles in different phases of fear memory. Previous studies have shown that, although darting is not affected by estrous cycle (Gruene et al., 2015a), freezing behaviour at extinction varies with estrous phases (Zeidan et al., 2011; Gruene et al., 2015b). Moreover, the estrous cycle has been reported to modulate CB1R density and affinity (de Fonseca et al., 1994), and AEA and 2-AG levels across different brain regions (González et al., 2000; Bradshaw et al., 2006). However, in the present study estrous cycle was not monitored, thus future investigations are warranted to examine the influence of estrous phases on endocannabinoid modulation of fear memory expression and extinction.

The opposing effects of enhanced AEA versus 2-AG signalling in the modulation of fear responses and the biphasic effects of cannabinoid drugs have been largely documented (Moreira et al., 2012; Morena and Campolongo, 2014). Beside the involvement of CB1Rs at different neuronal subpopulations (Rey et al., 2012; Metna-Laurent et al., 2012; Llorente-Berzal et al., 2015; Lutz et al., 2015; Heinz et al., 2017), these opposing effects have also been ascribed to the recruitment of receptors other than CB1R (Casarotto et al., 2012; Moreira et al., 2012; Patel et al., 2017; Colangeli et al., 2019; Di Maio et al., 2019), such as TRPV1Rs, which can be activated by high AEA levels (Zygmunt et al., 1999; Di Marzo, 2008; Bialecki et al., 2020). Both CB1Rs and TRPV1Rs are widely expressed in brain areas involved in anxiety and fear, including the PFC, hippocampus, amygdala and PAG (Tsou et al., 1999; Mezey et al., 2000; Cristino et al., 2008; Bialecki et al., 2020). Consistent with our results, compelling evidence has reported opposing roles for CB1Rs and TRPV1Rs in the modulation of fear and anxiety-related responses, where activation of TRPV1R has been shown to increase fear and anxiety-like behaviour, whereas CB1R activation attenuates these

behavioural responses, in male rodents (Rubino et al., 2008; Campos and Guimarães, 2009; Moreira et al., 2012). In agreement with our results, Laricchiuta et al. (2013) found that a systemic-induced augmentation of AEA signalling at TRPV1R increased freezing and impaired extinction in a contextual fear conditioning paradigm in mice (Laricchiuta et al., 2013). Moreover, TRPV1 knock-out (male) mice show low anxiety-like behaviour and conditioned fear responses compared to their wild-type controls (Marsch et al., 2007). Further corroborating our findings, it has been recently reported in males that both antagonism of CB1Rs or activation of TRPV1Rs in the dorsolateral PAG increased fear response, through a mechanism that seemed to involve increased glutamatergic transmission induced by either manipulation (Uliana et al., 2016).

Further supporting our sex-divergent results, a number of preclinical studies have reported sex-differences in the expression and functionality of endocannabinoid system components in fear-related brain regions in both baseline conditions and in models for stress/trauma-related disorders (Reich et al., 2009; Fattore and Fratta, 2010; Xing et al., 2014; Zer-Aviv and Akirav, 2016; Cooper and Craft, 2018).

In conclusion, our data provide the first evidence supporting fundamental sex differences of the endocannabinoid system in the modulation of fear expression and extinction. Augmenting AEA or 2-AG signalling did not significantly alter fear expression in male rats, whereas it did affect fear expression and extinction in females in opposite directions. While increased 2-AG signalling acutely reduced conditioned freezing, facilitated within-session extinction and enhanced darting via activation of CB1Rs, elevated AEA signalling at TRPV1Rs increased conditioned freezing, fear generalization and impaired fear extinction. Processes of fear extinction are profoundly altered in PTSD, and clinical literature provides evidence that the prevalence of PTSD is twice as high in women compared to men (Breslau, 2009), with documented sex differences found in both disease severity and treatment efficacy. Moreover, human studies have reported sex-related changes of endocannabinoid system components in patients suffering from PTSD, showing a more pronounced upregulation of CB1R in the amygdala-hippocampal-cortico-striatal neural circuit in women than men and a

decrease in peripheral AEA levels in both sexes (Neumeister et al., 2013). Therefore, understanding how endocannabinoids modulate fear responses and processes of extinction in both sexes will provide new insights into the sex dimorphism documented in the pathophysiology of PTSD and possibly help facilitate the development of sex-specific therapeutic interventions.

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CHAPTER 7

CIRCADIAN REGULATION OF MEMORY UNDER STRESS: ENDOCANNABINOIDS MATTER

Alessia Santori[1,2], Maria Morena[3,4,5], Patrizia Campolongo[1,2]

[1]Department of Physiology and Pharmacology, Sapienza University of Rome, P.le Aldo Moro 5, 00185 Rome, Italy; [2]Neurobiology of Behavior Laboratory, Santa Lucia Foundation, Via del Fosso di Fiorano 64, 00143 Rome, Italy; [3]Hotchkiss Brain Institute, [4]Mathison Centre for Mental Health Research, [5]Departments of Cell Biology and Anatomy & Psychiatry, University of Calgary, 3330 Hospital Dr. NW, T2N 4N1 Calgary, AB, Canada

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Abstract

Organisms ranging from plants to higher mammals have developed 24-hour oscillation rhythms to optimize physiology to environmental changes and regulate a plethora of behavioral processes, including learning and memory function. In this review, we explore how time-of-day differentially influences memory performance.

Extensive evidence indicated that a wide array of memories can be influenced by stress- and emotional arousal-activated neurobiological systems, and that boosting endocannabinoid levels counteracts impaired memory performance. We evaluate the stress memory literature for evidence of circadian influence on stress-dependent modulation of memory, also highlighting the circadian functioning pattern of the endocannabinoid system. Our discussion illustrates how the endocannabinoid system contribution may pervasively prevent stress detrimental effects on memory in a circadian-dependent fashion. We suggest that endocannabinoids might regulate molecular mechanisms that control memory function under circadian and stress influence, with potentially important clinical implications for both neurodevelopmental disorders and psychiatric conditions involving memory impairments.

Introduction

Circadian rhythms are near-24-hour oscillations found to be essential in many physiological, biological and behavioral processes occurring in organisms ranging from invertebrates to higher mammals (Logan and McClung, 2019). In humans, time-of-day does influence the cognitive processing, including memory retrieval, which usually declines in the late afternoon-early evening (Ebbinghaus, 1985). While this effect has been recognized for decades, the underlying neurobiological mechanisms are currently not completely understood.

The suprachiasmatic nucleus (SCN) in the anterior hypothalamus serves as the master pacemaker that sets the timing of rhythms by regulating neuronal activity, body

temperature and hormonal signals (Colwell, 2011). Disruption of circadian rhythms is associated with altered stress response (Koch et al., 2017) and higher risk of several neurodevelopmental disorders such as attention-deficit hyperactivity disorder (ADHD) and autism spectrum disorders (ASDs) (Logan and McClung, 2019). Moreover, disrupted circadian timekeeping and altered daily hormone release profiles were reported in conditions involving memory impairment, including old age (Krishnan and Lyons, 2015; Reinhart and Nguyen, 2019), Alzheimer's disease (Videnovic et al., 2014), major depression, bipolar disorder and psychosis (Jones and Benca, 2015). Much of the currently available evidence linking brain disorders to circadian dysfunction is correlational. Less clear is the exact nature of time-of-day effects by which memory is modulated under stress: both enhancing and impairing effects have been reported. Corroborations from human and rodent studies indicated that an inverted U-shaped relationship between stress intensity and memory exists, with optimal memory strength at an intermediate level of stress (de Quervain et al., 2017). Recent evidence further reported that stress modulation of memory depends on time-of-day and that interventions on the endocannabinoid system might counteract the detrimental effects of stress in a time-of-day-dependent fashion (Santori et al., 2020, 2019). Noteworthy, extensive research has identified the glucocorticoid-endocannabinoid crosstalk as crucial mediator of stress-dependent modulation of memory (Hill et al., 2018), but literature data currently lacks information about whether and how circadian rhythms influence such mediation.

Accordingly, in this review we first aim at briefly describing the effects of circadian rhythms on the stress system and those of time-of-day on memory function. To shed light on the underlying mechanisms of contrasting results, we portray how stress-dependent modulation of memory is influenced by circadian rhythms. We argue that a manipulation of the endocannabinoid system is capable to effectively modulate the circadian-dependent effects of stress on memory and to prevent its detrimental effects on memory function. Lastly, we comment how these lines of evidence collectively indicate that endocannabinoids might regulate molecular mechanisms that control

memory processes under circadian and stress influence, with potentially important clinical implications.

Circadian Rhythms of the Stress System

Circadian Rhythmicity of Life

Rhythmicity of physiological mediators is found in virtually all living systems. Regulatory systems such as the hypothalamus-pituitary-adrenal (HPA) axis and the autonomic nervous system, both being important for the regulation of stress responses, receive strong circadian input (Koch et al., 2017).

In order to synchronize single-cell oscillators with each other, the mammalian circadian system is organized hierarchically. Light is received by specialized melanopsin producing photoreceptive retinal ganglion cells (ipRGCs) in the eye. These ipRGCs project through the retinohypothalamic tract to the SCN, which relays timing information to other brain regions via direct [i.e. paraventricular nucleus of the hypothalamus (PVN), paraventricular nucleus of the thalamus (PVT), lateral habenula (LHb)] and indirect [locus coeruleus (LC), nucleus accumbens (NAc), ventral tegmental area (VTA)] projections.

The molecular mechanisms mediating circadian rhythm generation by a transcriptional–translational feedback loop in the SCN are well studied: the transcriptional activators BMAL1 (brain and muscle ARNT-like protein 1) and CLOCK (circadian locomotor output cycles kaput) form a heterodimer and regulate the expression of many genes, including those encoding period (PER) and cryptochrome (CRY), which, once translated, inhibit their own transcription (Hartsock and Spencer, 2020; Hasegawa et al., 2019). Many other proteins, including various kinases, phosphatases and other transcriptional factors, such as CREB (cAMP response element-binding protein), interact among themselves and regulate this core molecular clock (Eckel-Mahan et al., 2008; O'Neill et al., 2008; Rawashdeh et al., 2014). Humoral signals and the autonomic

nervous system convey information from the SCN to orchestrate peripheral clocks (Logan and McClung, 2019).

Neurocircuitry of Stress

Stress – in its many forms – is generally perceived as an excessive demand on human psychological and/or physiological adaptive capabilities, and can have a direct influence on different physiological, biological and behavioral processes due to the integration of neuronal and hormonal pathways of the stress reaction and the circadian regulation (Steinach and Gunga, 2020).

The brain triggers stress responses that are commensurate with the nature of the stimulus (Ulrich-Lai and Herman, 2009). Decades of basic and clinical research have delineated several well-defined brain circuits that are important for the manifestation of the physiological response to psychological stressors. Sensory information regarding the external environment is processed by the thalamus and primary sensory cortical centers and funneled to the amygdala through a network of corticothalamic afferents. Of particular importance to stress is the transmission of information to the nuclei of the amygdala and extended amygdala where preconscious threat detection occurs, emotional valence is ascribed, and reference to previous experiences occurs through the crosstalk with the medial prefrontal cortex (mPFC) and the hippocampus (Roozendaal et al., 2009). This triadic circuit of the amygdala–mPFC–hippocampus has been found to be relevant for almost every neurobehavioral response to psychological stress (McEwen, 2012). In general, activation of output pyramidal neurons of the basolateral amygdala (BLA) contributes to many aspects of stress, including HPA axis activation, anxiety, pain sensitivity and alterations in cognitive processes through the trans-synaptic recruitment of downstream circuits, such as the central amygdala (CeA), medial amygdala, bed nucleus of the stria terminalis (BNST), NAc and distinct hypothalamic nuclei such as the lateral, anterior and dorsomedial hypothalamus (Hermans et al., 2014). The hippocampus and mPFC, on the other hand, have been identified as inhibiting the HPA axis, and are also involved in the extra-hypothalamic

glucocorticoid-mediated negative feedback of the HPA axis-mediated stress response (Hill et al., 2010).

Circadian Rhythms and Stress Interactions

Among all peripheral oscillators, the adrenal gland displays a striking circadian feature that can influence 24-hour rhythms in other peripheral tissues via rhythmic release of hormones (i.e. glucocorticoids) with clock-modulating properties (Koch et al., 2017). Specifically, glucocorticoids are secreted upon adrenocorticotropic hormone (ACTH) binding to melanocortin-2 receptors (MC2R) in the adrenal gland. ACTH itself is secreted from the anterior pituitary upon the signaling of the corticotropin-releasing hormone (CRH), which is produced by the PVN, together with the arginine vasopressin (AVP), under the SCN projecting neurons control. Collectively, these elements constitutively represent the HPA axis, and circadian oscillations are detectable for all its components (Nicolaides et al., 2017). Activation of the HPA axis elevates circulating glucocorticoids (i.e. cortisol in humans and corticosterone in rats), which reach their peak plasma levels tens of minutes after the initiation of stress (Ulrich-Lai and Herman, 2009). Normally, the circadian pattern of glucocorticoid secretion involves higher hormone concentrations during the day for diurnal species and at night for nocturnal species, and can be abolished by specifically disrupting the circadian clock in the adrenal gland (Nicolaides et al., 2017; Son et al., 2008).

Experiments investigating the circadian aspect of acute or chronic stress have frequently suggested that sex differences in the circadian timing system are critical to unveil core mechanisms regulating the response to both endogenous and exogenous stress factors (Bailey and Silver, 2014), with potential important implications for understanding behavior and physiology (McCarthy et al., 2012). Morphological sex differences in the SCN are well established in both animals and humans (Bailey and Silver, 2014). Thus, circadian rhythms have been indicated to differentially influence both acute stress response (Bangasser and Wiersielis, 2018; Morena et al., 2020; Verma et al., 2010) and anxiety (Meseguer Henarejos et al., 2020; Verma et al., 2010) in rodent males and

females that were tested in different behavioral paradigms. Similarly, literature evidence suggested that chronic stress might lead to circadian rhythm dysfunctions, which in turn alter HPA axis activity and glucocorticoid concentrations, possibly causing severe brain disorders like major depression and post-traumatic stress disorder (PTSD) (Koch et al., 2017; Steinach and Gunga, 2020). Particularly, it has been shown that exposure to repeated stress has a more detrimental impact if applied during the inactive phase compared with the active phase (Koch et al., 2017), with sex differences playing an additional role in the different types of response to chronic stress exposure (Morley-Fletcher et al., 2019).

Circadian and Time-of-Day Effects on Memory

Cognitive performance fluctuates during the course of the day. In humans, circadian rhythms have been demonstrated in mental activities, such as mood (McClung, 2013), vigilance (Maire et al., 2018) and cognitive throughput (Chellappa et al., 2018), so it may not be surprising that memory also falls under circadian control, with different cognitive functions showing optimal performance at different times of the day.

Evidence suggested that time-of-day-dependent relay exists between clock pacemaker neurons and memory-forming cells, since diurnal cyclical changes have been observed in baseline physiological properties of SCN pacemaker cells in both mammals and flies (Gerstner and Yin, 2010). Particularly, SCN neurons in nocturnal rodents display circadian changes in terms of spontaneous firing rate (SFR) and resting membrane potential (RMP), with an augmented SFR and more depolarized RMP in the light phase than the dark (Green and Gillette, 1982; Kuhlman and McMahon, 2004; Pennartz et al., 2002). Such conserved mechanism might influence time-of-day-dependent expression of physiological events downstream of SCN pacemaker cells, such as stimulation of brain regions that are involved in learning and memory (Gerstner and Yin, 2010). Importantly, there is increasing evidence that molecular clocks in these extra-SCN

regions exert autonomous circadian regulation of many structure-specific functions, like progressively memory retrieval has been designated to be controlled by the hippocampal clock (Cermakian and Sassone-Corsi, 2000; Eckel-Mahan et al., 2008; Hasegawa et al., 2019; McDearmon et al., 2006; Wang et al., 2009).

To date, time-of-day and circadian effects on different cognitive performance and memory sorts have been observed in various behavioural paradigms (Flyer-Adams et al., 2020; Gritton et al., 2012; Hasegawa et al., 2019; Hauber and Bareiß, 2001; Hoffmann and Balschun, 1992; Meseguer Henarejos et al., 2020; Morales-Delgado et al., 2018; Poveda et al., 2020; Santori et al., 2020, 2019; Snider et al., 2016; Valentinuzzi et al., 2001; Table 1).

Circadian modulation of different hippocampal-dependent forms of learning and memory has been previously reviewed (Snider et al., 2018). Different studies explicitly examined time-of-day differences in behaviors corresponding to encoding (Chaudhury and Colwell, 2002; Flyer-Adams et al., 2020; Gritton et al., 2012; Harrison et al., 2017; Meseguer Henarejos et al., 2020; Poveda et al., 2020; Snider et al., 2016; Takahashi et al., 2013; Winocur and Hasher, 2004), with not all the experiments showing time-of-day influence on such memory phase, thus encouraging future work in this field. Recent studies investigated whether the temporal dynamics of protein synthesis-dependent memory consolidation vary depending on time-of-day, highlightening a special role for the hippocampus in mediating *de novo* protein synthesis that is crucial for the formation and consolidation of long-term memories (Raven et al., 2020; Shahmoradi et al., 2015; Shimizu et al., 2016). The molecular mechanisms of memory encoding, consolidation and retrieval are distinct, and might partially clarify which memory process is primarily impacted by circadian rhythms. Many studies showing time-of-day differences in memory retrieval have been reported (Chaudhury and Colwell, 2002; Hasegawa et al., 2019; Hauber and Bareiß, 2001; Le Glou et al., 2012; Martin-Fairey and Nunez, 2014; Santori et al., 2020, 2019; Snider et al., 2016; Snider and Obrietan, 2018; Tam et al., 2017). Snider and Obrietan (2018) indicated that circadian disruption can impair memory retrieval. Additionally, recent evidence suggested that time-of-day memory

retrieval profile is controlled by the circadian-dependent transcription factor BMAL1, under the regulation of the hippocampal clock (Hasegawa et al., 2019). To date, only a report indicated that circadian rhyhtms also influence memory extinction (Woodruff et al., 2015), which is selectively facilitated during the dark phase than the light, being in line with previous data showing that increased mice extinction rates occur when extinction training was performed during the dark phase (Chaudhury and Colwell, 2002).

Process	Memory paradigm	Organism	Phase	Reference
Encoding	Sustained attention task	Sprague-Dawley rats	Inactive/Active	Gritton et al., 2012
Encoding	Morris water maze	Sprague-Dawley rats	Inactive/Active	Gritton et al., 2012
Encoding	Passive avoidance test	Swiss mice	Inactive/Active	Meseguer Henarejos et al., 2020
Encoding	Open-field test	Wistar rats	Inactive/Active	Poveda et al., 2020
Encoding/retrieval	Radial arm radial maze	Sprague-Dawley rats	Inactive/Active	Hauber and Bareiß, 2001
Encoding/retrieval	Barnes maze/Tail suspension test	Transgenic mice	Inactive/Active	Snider et al., 2016
Encoding/Short- and long-term memory	Appetitive/Aversive associative olfactory assay	Drosophila Melanogaster	Inactive	Flyer-Adams et al., 2020
Short-term memory	Object recognition task	Sprague-Dawley rats	Inactive/Active	Santori et al., 2020, 2019
Long-term memory	Alley maze	C57BL/6 Ola mice	Inactive/Active	Hoffmann and Balschun, 1992
Retrieval	Elevated plus-maze	Wistar rats	Inactive/Active	Morales-Delgado et al., 2018
Retrieval	Auditory/Contextual fear conditioning	C57BL/6J mice	Inactive/Active	Valentinuzzi et al., 2001
Retrieval	Social recognition task	Transgenic mice	Inactive/Active	Hasegawa et al., 2019

Table 1 – Different paradigms of studies examining time-of-day differences in memory processes.

Stress Modulation of Memory

The ability to learn and remember is essential for our survival (McGaugh, 2013). The strength of memories varies with the emotional significance of the events; indeed, emotionally arousing experiences tend to be very well remembered (Brown and Kulik, 1977). The neurocircuitry underlying emotional memory involves brain regions that belong to the corticolimbic system, including the amygdala, hippocampus, mPFC (Barsegyan et al., 2010; Campolongo et al., 2009; Fastenrath et al., 2014; Roozendaal et al., 2009).

The finding that emotion influences memory at multiple levels has been so far consistent in both human and animal studies, with emotional arousal enhancing long-term memory consolidation, when experienced during or after learning, while impairing memory retieval, when stress exposure occurs before memory retention testing, through a modulation that involves both epinephrine and glucocorticoids (de Quervain et al., 2017, 1998; Roozendaal, 2002). Although most studies have focused on the effects of stress before or after learning, or eventually before memory testing, there is extensive evidence that stress can influence subsequent memory also if it is presented after retrieval, thus suggesting that stress affects also extinction and/or reconsolidation processes (Morena et al., 2018; Morena and Campolongo, 2014; Segev et al., 2018).

How stress influences memory depends on when and by what an individual is stressed (Schwabe et al., 2012). The contribution of glucocorticoids to the regulation of memory was first found in adrenalectomized rats, which showed reduced corticosterone levels, as well as spatial memory deficits (Roozendaal et al., 1996). Indeed, glucocorticoids are critical regulators in the activation and cooperation of the brain regions that are involved in encoding memory acquisition, consolidation and expression (Schwabe et al., 2012). For example, stress-mediated secretion of glucocorticoids and/or activation of glucocorticoid receptors directly affects hippocampal functions, thus modulating the consolidation of several types of hippocampal-dependent memories, including spatial and contextual memories in rodents and declarative memory in humans (Donley et al.,

2005). Stress-induced secretion of glucocorticoids also targets the amygdala (Roozendaal et al., 2009). Particularly, activation of amygdalar glucocorticoid receptors is important for fear memory consolidation, whereas their inhibition impairs contextual fear memories (Donley et al., 2005). Within the mPFC, glucocorticoid receptors activation has been found to both enhance memory consolidation and impair working memory, through a mechanism that depend on norepinephrine-mediated increase of the cAMP-dependent proteinkinase in the mPFC (Barsegyan et al., 2010).

Of note, in both humans and rodents, stress intensity and memory performance are known to follow an inverted U-shaped relationship, with maximal memory strength at an intermediate level of stress (Yerkes and Dodson, 1908). In line with this evidence, exposure to an mild level of stress that leads to optimal cognitive performance triggers the glucocorticoid secretion in a fashion that completely activates the high affinity mineralcorticoid receptors and just partially triggers the low affinity glucocorticoid receptors, which, conversely, are activated under higher arousal or stress intensity conditions (Finsterwald and Alberini, 2014; Morena and Campolongo, 2014). Furthermore, whereas memory performance associated with complex cognitive tasks is sensitive to stress in an inverted-U fashion, simple forms of fear memory induced by traumatic experiences can be strong and persistent (Finsterwald and Alberini, 2014). In this regard, literature evidence revealed that the noradrenergic system might be responsible for the persistence of traumatic memories in stress-related disorders, such as post-traumatic stress disorder (PTSD) (Liu et al., 2019).

Although epinephrine and glucocorticoids affect brain function through different specific mechanisms and pathways, they converge in regulating memory processes by influencing central noradrenergic mechanisms (de Quervain et al., 1998; McGaugh and Roozendaal, 2002). The amygdala is a critical site of the converging modulatory influences of adrenal stress hormones on memory consolidation (Ferry et al., 1999; Roozendaal, 2000). Extensive evidence indicated that epinephrine affects memory consolidation by modulating the amygdalar noradrenergic system, through the activation of both α- and β-adrenoceptors in the BLA (Ferry et al., 1999; Ferry and

McGaugh, 1999). Noradrenergic activation may activate glutamatergic mechanisms in the BLA (Lennartz et al., 1996) and facilitate NMDA-dependent neuroplasticity in BLA pyramidal neurons (Wang et al., 1996). Noradrenergic activation involves β-adrenoceptor influences on cAMP and cAMP-dependent PKA formation, which in turnen phosphorylates the transcription factor CREB (Carew, 1996); manipulations of the amygdalar CREB levels have been reported to influence long-term memory formation for aversive conditioning (Lamprecht et al., 1997).

Additionally, the amygdala is richly interconnected with other brain regions, including the cortex, which is known to be involved in processing different aspects of memory (LeDoux, 1992). There is now considerable evidence that the amygdala influences memory consolidation through its projections to other brain regions (Cahill et al., 1995; McGaugh, 2000). Findings from many studies indicate that the hippocampus is involved in spatial learning in rodents and declarative memory in humans (Eichenbaum, 2000), whereas the caudate nucleus is involved in the learning of specific cues associated with responses (Packard and Goodman, 2012). Packard and colleagues found that post-training activation of the amygdala (using microinfusions of D- amphetamine) enhanced memory for both place learning and cued response learning in a water maze (Packard et al., 1994). In contrast, hippocampal infusions selectively enhanced spatial memory, and caudate infusions selectively enhanced cued response memory.

Circadian Rhythms of the Endocannabinoid System

The endocannabinoid signaling is broadly utilized throughout the body as a mechanism to regulate intercellular communication and considerable evidence supports its modulatory role in many fundamental physiological processes (Hillard, 2018). It has been demonstrated that pharmacological manipulation of the cannabinoid (CB) receptor signaling affects sleep/wake cycles (Murillo-Rodríguez et al., 2017), learning and memory (Marsicano and Lafenêtre, 2009), temperature regulation (Maccarrone and

Wenger, 2005), food consumption and fat storage (de Kloet and Woods, 2009), central nervous system regulation of autonomic (Pacher et al., 2005) and endocrine functions (Maccarrone and Wenger, 2005), reward-driven behavior (Solinas et al., 2008), gastrointestinal function (Camilleri, 2018), mood (Hill and Gorzalka, 2009) and sensory perception (Bíró et al., 2009). All of these processes are altered in a cyclical manner (Vaughn et al., 2010).

Circadian Gating of the Endocannabinoid Signaling

Literature evidence indicated that the endocannabinoid signaling exhibits circadian rhythms with variations reported in endocannabinoid tissue contents (Valenti et al., 2004), CB1 receptor number (Rueda-Orozco et al., 2008) and in the enzymes controlling the synthesis and degradation of the endocannabinoids anandamide (AEA) and 2-arachidonoylglycerol (2-AG) (Valenti et al., 2004). Examination of 24-hour rhythms of the endocannabinoid serum concentrations has revealed that mean diurnal concentrations of AEA were significantly lower than those previously reported for 2-AG, but that their values were also highly correlated (Hanlon, 2020; Hanlon et al., 2016). Of note, 24-hour profile of AEA is quantitatively distinct from that of 2-AG and of lower amplitude (Hanlon, 2020).

Because AEA and 2-AG are mobilized “on-demand”, their concentrations in lipid extracts of isolated brain regions are hypothesized to be proportional to their concentrations in the synapse. In rats, significant diurnal variations in AEA and 2-AG contents have been demonstrated in cerebrospinal fluid (CSF), hypothalamus, hippocampus, pons, NAc, prefrontal cortex and striatum (Valenti et al., 2004). With respect to AEA concentrations, two patterns have been reported. In the pons, NAc, prefrontal cortex, hippocampus and striatum (Valenti et al., 2004), AEA content was higher in tissues harvested during the active phase of the rats (i.e. dark), than during the inactive phase. An opposite pattern was seen in CSF and hypothalamus, where AEA concentrations were higher in the inactive than in the active phase (Murillo-Rodriguez et al., 2006). Interestingly, the tissue contents of 2-AG were opposite to those of AEA

in tissues where both endocannabinoids were measured. 2-AG contents were higher during the inactive phase in NAc, mPFC, striatum and hippocampus (Valenti et al., 2004). Morning changes in 2-AG paralleled the rise in glucocorticoids, and data evidence has indicated that corticosterone can rapidly increase 2-AG's synthesis in the hypothalamus (Hill et al., 2010).

There is evidence that cannabinoid type 1 (CB1) receptor density in rat brain is regulated in a circadian manner. In both pons (Martinez-Vargas et al., 2003) and hippocampus (Rueda-Orozco et al., 2008), the density of CB1 receptor protein is approximately 5% higher during the inactive than the active phase. The changes in both endocannabinoid content and CB1 receptor density with time-of-day in the pons and hippocampus display interesting relationships. In both brain regions, AEA content and CB1 receptor protein concentration are nearly perfectly out of phase with each other. For the majority of the inactive phase, CB1 receptor density is high and AEA content is low in the hippocampus. The finding that hippocampal 2-AG content is higher in the inactive phase suggests that its synthesis is greater or clearance is reduced during this phase, which could also contribute to a situation in which CB1 receptor activation by 2-AG is potentiated. When the animals are awake and active, AEA tone is high, while CB1 receptor density is slightly lower.

Endocannabinoid System in the Stress Response

There is also considerable evidence that the endocannabinoid signaling regulates the activation of the HPA axis by stress (Patel et al., 2004), and is required for normal glucocorticoid-mediated feedback on the HPA axis (Di et al., 2003). Specifically, while the neuropeptide CRH, rapidly released in response to stress (Merlo Pich et al., 1995; Roozendaal et al., 2002), reduces AEA signaling at glutamatergic neurons, which probably contributes to HPA axis activation (Gray et al., 2015), glucocorticoids enhance 2-AG's synthesis (Di et al., 2003; Hill et al., 2010) to terminate the stress response throughout the HPA axis negative feedback regulation in limbic brain regions (Hill et al., 2011). Studies using CB1 receptor null mice indicated that the fundamental rhythms

of circulating glucocorticoids are intact in the global absence of the endocannabinoid signaling. However, relative to wild-type mice, CB1 receptor null mice exhibited significantly higher circulating glucocorticoid concentrations at the onset of the active phase (Cota et al., 2007). These data are consistent with the role for the endocannabinoid signaling to negatively regulate HPA axis activation, likely as a downstream mediator of glucocorticoid receptor activation (Di et al., 2003).

Endocannabinoid Regulation of Emotional Memories

The endocannabinoid system strongly regulates stress and emotional arousal effects on memory and cognition (Morena and Campolongo, 2014). CB1 receptors (Matsuda et al., 1990) were found to be highly expressed in corticolimbic brain regions (Marsicano and Lafenêtre, 2009), wherein they modulate both excitatory and inhibitory signaling within specific neuronal circuits that are implicated in learning and memory processes for emotionally arousing experiences (Akirav, 2013; Morena et al., 2014; Tasker et al., 2015); additionally, cannabinoid type 2 (CB2) receptors activation was demonstrated to be recruited to process fear memory consolidation (Ratano et al., 2018, 2017). Hence, together with the two major endogenous ligands, 2-AG (Sugiura et al., 1995) and N-arachidonyl ethanolamide (AEA; Devane et al., 1992), and their corresponding hydrolytic enzymes, monoacylglycerol lipase (MAGL; Dinh et al., 2002) and fatty acid amide hydrolase (FAAH; Cravatt et al., 2001), respectively, the endocannabinoid system has been recognized to crucially interact with glucocorticoids to mediate the emotional arousal-dependent modulation of memory under stress (Campolongo et al., 2009).

Compelling evidence indicates that drugs that target the endocannabinoid system induce biphasic effects on cognitive and emotional behavior depending on the level of stress and emotional arousal at the time of encoding and drug consumption (Campolongo et al., 2013; Manduca et al., 2014; Morena et al., 2015, 2014; Santori et al., 2019).

Specifically, glucocorticoid and endocannabinoid signaling interacts to enhance consolidation and extinction, while impairing the retrieval of emotional memories (Morena et al., 2016).

With respect to memory acquisition, both enhancing and impairing effects have been documented. Campolongo et al. (2012) showed that systemic injection of the AEA transport inhibitor AM404 impaired the acquisition of the novel object recognition task, only when rats were tested under high emotional arousal conditions, without altering memory performance of low aroused rats (Campolongo et al., 2012). Moreover, it has been reported that while lower doses of the CB1 receptor agonist WIN55,212-2 potentiated the emotional salience of normally subthreshold fear memory, a relatively higher dose completely blocked fear memory acquisition (Draycott et al., 2014).

Extensive research has demonstrated that glucocorticoids facilitate consolidation of aversive memories through slow acting (genomic) and fast acting (non-genomic) mechanisms (Atsak et al., 2012b). Although there has been considerable study focusing on the genomic actions of glucocorticoids, a role for endocannabinoid signaling in the non-genomic effects of glucocorticoids on aversive memories has been identified. Importantly, endocannabinoid signaling in the BLA has generally been shown to facilitate the consolidation of aversive memories in male rats (Morena et al., 2014), paralleling the effects of glucocorticoid signaling (de Quervain et al., 2017). The dependence of glucocorticoids on endocannabinoid signaling in the context of aversive memory consolidation was first discovered via CB1 blockade, through intra-BLA AM251 administration, which abrogated the facilitating effects of systemic corticosterone on aversive memory consolidation in male rats (Campolongo et al., 2009). In a different study, these findings were replicated by injecting directly into the BLA the membrane impermeable glucocorticoid, corticosterone:BSA (Atsak et al., 2015). The authors also showed that activation of the CB1 receptors, with intra-BLA CB1 receptor agonist WIN55,212-2 infusions, mimicked the memory enhancing effects of glucocorticoids when glucocorticoid receptor activity was simultaneously blocked using the glucocorticoid receptor antagonist RU38466 (Atsak et al., 2015). These data

suggested that, within the BLA, glucocorticoids facilitate aversive memory consolidation through non-genomic actions via downstream activation of endocannabinoid signaling. A similar interplay has also been identified within the hippocampus, whereby intra-hippocampal administration of the CB1 receptor antagonist AM251 blocked glucocorticoid-induced memory facilitation (De Oliveira Alvares et al., 2010).

In a similar vein of research, post-training delivery of WIN55,212-2 increased plasma corticosterone levels and enhanced long-term object recognition memory performance (tested 24-hour later) in high aroused animals, but decreased levels in low aroused rats, yet leaving their memory retention unaltered (Campolongo et al., 2013). Similarly, we recently showed that exposure to a low stress immediately after the training trial selectively impairs short-term memory retention when animals were tested in the morning, while exposure to high stress impairs short-term performance independently of the testing time; systemic administration of the AEA hydrolysis inhibitor URB597 or intra-hippocampal infusions of the 2-AG hydrolysis inhibitor KML29 counteracted the stress impairing effects on short-term object recognition memory, in a stress intensity- and circadian-dependent fashion (Santori et al., 2020, 2019).

While glucocorticoid and endocannabinoid signaling interact to enhance consolidation and extinction, they impair the retrieval of emotional memories. Indeed, there is evidence illustrating that intra-hippocampal infusions of the cannabinoid agonist WIN55,212-2 impair the retrieval of memory (Morena et al., 2015); however, antagonism of the β-adrenoceptor activity blocks the memory retrieval impairment induced by WIN55,212-2 (Atsak et al., 2012a). Comparable to mechanisms described for memory consolidation and extinction, interaction between these two systems appear to impair memory retrieval through their influence on downstream noradrenergic activity (Balsevich et al., 2017).

Lastly, extensive research has revealed a role for endocannabinoids and glucocorticoids in the extinction of aversive memories. Over a decade ago, Marsicano et al. (2002) showed that disruption of the CB1 receptor via genetic deletion or pharmacological

blockade greatly hinders extinction of auditory fear memory (Marsicano et al., 2002). Subsequent studies have pointed out that AEA appears to selectively promote this memory process (Gunduz-Cinar et al., 2013; Morena et al., 2018; Segev et al., 2018). Moreover, it has been demonstrated that the fear extinction facilitating effect of elevated AEA tone requires the synthesis of corticosterone and glucocorticoid receptor activation (Bitencourt et al., 2014).

Taken together, these data clearly indicate that a conserved, bidirectional loop of endocannabinoid and glucocorticoid crosstalk has a significant influence on the acquisition, consolidation, retrieval and extinction of emotionally salient memories (Balsevich et al., 2017).

Time-of-Day's Role in the Endocannabinoid Modulation of Memory under Stress

Stress influences the endocannabinoid system with effects that are quite complex, regionally specific, and time-dependent relative to stress exposure and its chronicity (Morena et al., 2016).

Exposure to acute stress generally causes a rapid reduction in amygdalar and hippocampal AEA content in response to an array of psychological stressors (Gray et al., 2015; Wang et al., 2012). This reduction in AEA levels is also consistent in the hypothalamus (Dubreucq et al., 2012) and is, at least in part, corroborated by an increase in AEA hydrolysis by FAAH (Hill et al., 2009). Unlike the consistency seen in the amygdala and hippocampus, the mPFC seems to be somewhat of a more complex structure, as exposure to acute stress has been found to produce a robust reduction of AEA content only relatively to some paradigms of stress (Hill et al., 2011; McLaughlin et al., 2012).

Regarding 2-AG, unlike the effects of stress on AEA, the majority of studies suggested that stress acts to increase its signaling within the mPFC (Hill et al., 2011), hippocampus (Wang et al., 2012) and hypothalamus (Evanson et al., 2010). Conversely, several

studies have shown that acute stress does not increase 2-AG content within the amygdala (Patel et al., 2009), and eventually impairs 2-AG levels in the hippocampus in response to high stress exposure (Santori et al., 2020), thus indicating significant differences in the temporal dynamics of 2-AG, but also AEA, changes following stress exposure.

The LC, the main source of norepinephrine in the mammalian forebrain, provides norepinephrine to different brain regions, including the BLA (McCall et al., 2017) and mPFC (Sara, 2009), wherein activation of CB1 receptors results in decreased cortical norepinephrine release , when it is normally potentiated by acute stress exposure (Morilak, 2012). Evidence suggested that under high levels of stress the LC promotes fear learning by enhancing BLA function, while simultaneously blunting prefrontal function. Conversely, low levels of arousal are sufficient for the LC to facilitate mPFC function and promote downstream inhibition of the amygdala (Giustino and Maren, 2018).

Circadian modulation of the activity of noradrenergic neurons within the LC, with neuron SFR depending on time-of-day, has been documented (González and Aston-Jones, 2006). Particularly, circadian fluctuations in the LC requires an intact dorsomedial hypothalamus, which has been shown to serve as a relay of the functional circuit SCN-LC, higly implicated in sleep/wake cycles (Aston-Jones et al., 2007).

Evidence supporting the endocannabinoid modulation of the stress-integrative LC-NE system revealed the biphasic nature of CB1 receptor activation within the LC depending on their neuronal subpopulations: CB1 receptors on glutamatergic terminals have higher sensitivity to CB1 receptors agonists compared to CB1 receptors on GABAergic terminals (Ohno-Shosaku et al., 2002; Rey et al., 2012; Scavone et al., 2010). However, different studies reported that basal tonic CB1 receptor activation strongly involves GABAergic terminals rather than glutamatergic neurons (Katona and Freund, 2008; Marsicano et al., 2003).

It has been therefore hypothesized that within the LC, tonic endocannabinoid release primarily regulates GABAergic synapses. Following postsynaptic depolarization,

phasic release of endocannabinoids would help gating further glutamatergic excitation of LC neurons (Wyrofsky et al., 2019).

We previously demonstrated that stress exposure differentially decreased hippocampal endocannabinoid levels according to the stress intensity and time-of-day (Santori et al., 2020). We speculated that stress of different intensities at two times of the day differentially regulated LC-NC action on the mPFC, which enhances or hinders memory performance depending on different arousal states (Giustino and Maren, 2018; Santori et al., 2019), through the involvement of the amygdalo-hippocampal activity. Specifically, when animals were tested during the circadian low activity phase of the HPA axis (i.e., morning session), exposure to a stressor, regardless of its intensity, impaired memory performance, probably by causing a more robust deviation from homeostasis, that is during the low activity phase of stress systems. Conversely, when testing occurred at the beginning of their active phase (i.e., afternoon), when the HPA axis reaches its activity peak, only the high intensity stressor impaired memory performance. Thus, it is likely that exposure to the low intensity stressor at the beginning of the active phase did not alter behavioral performance, because it did not cause a severe deviation from homeostasis, with animals tested during their high HPA axis and arousal system activity phase. Our experiments highlighted that boosting AEA signaling and hippocampal 2-AG tone counteracted the detrimental effects of stress on memory in a stress intensity- and time-of-day-dependent manner (Santori et al., 2020, 2019).

Therefore, here we argue that low stress exposure minimally affects the endocannabinoid system, and that high levels of endocannabinoids allow CB1 receptor activation within the LC on its GABAergic terminals with the consequent decrease of norepinephrine concentrations. Conversely, exposure to high stress impairs endocannabinoid contents, which at a basal tone preferentially bind CB1 receptors on glutamatergic neurons, with a resulting enhancement of norepinephrine levels in the LC. Literature evidence indicated that after LC activation, norepinephrine is released in the dorsal hippocampus and BLA, but diminished in the mPFC (Hansen, 2017). We

demonstrated that augmenting endocannabinoid levels might reduce hippocampal norepinephrine release, which impairs memory retrieval (Atsak et al., 2012a), facilitating negative feedback on the HPA axis with a faster recovery from stress, and, ultimately, restored memory performance (Santori et al., 2020). Our findings opened the avenue to further investigate how a minipulation of the endocannabinoid system might be a potential target for treating stress-induces memory performance deficits.

However, evidence supported that when large levels of CB1 receptors agonists are present, GABAergic CB1 receptors also become activated. This hypothesis might account for the biphasic nature of CB1 receptor activation within the LC (Wyrofsky et al., 2019) and encourages future studies in this field to unveil the exact mechanisms underlying endocannabinoid modulation of circadian- and stress intensity-dependent effects on memory.

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The present PhD thesis sought to determine how different drugs of abuse, particularly cannabinoids, modulate memory processes under different stress intensities, circadian and sex influence.

In **Chapter 1**, we first investigated whether the psychostimulants amphetamine and MDPV influence fear memory generalization in rats. Our results indicated that both amphetamine and MDPV induce generalization of fear memory, whereas only amphetamine enhances memory strength. To further explore the neurobiological underpinnings, we demonstrated that the co-administration of the β-adrenoceptor antagonist propranolol prevents the effects of both drugs on memory strength and generalization, whereas the dopaminergic receptor blocker cis-flupenthixol selectively reverses the amphetamine effect on the generalization of fear memory, suggesting that different modulations of the noradrenergic and dopaminergic system are required for amphetamine and MDPV to induce fear memory generalization. Although these results require further investigation, it can be hypothesized that the differential recruitment of the monoamine systems induced by amphetamine and MDPV strictly engages those brain areas that are particularly involved in memory generalization, such as the mPFC, nucleus reuniens, and hippocampus (Xu and Südhof, 2013).

Enduring forms of memories are generally caused by either traumatic events or drug experiences (Gisquet-Verrier and Le Dorze, 2019). Administration of amphetamine, in different brain regions, has been shown to ameliorate numerous memory processes, including consolidation (McGaugh and Roozendaal, 2009). However, the effects of stress on such mediation are underinvestigated. Consequently, we aimed at evaluating how different stress intensities, soon after encoding in an object recognition task,

modulate amphetamine effects on long-term memory consolidation (**Chapter 2**). Our findings revealed that the psychostimulant amphetamine exerts dichotomic effects on long-term recognition memory, which are strictly related to the level of stress experienced in the early phase of memory consolidation. Specifically, amphetamine enhanced long-term memory consolidation, when rats were subjected to a mild stress condition immediately after training, whereas impaired long-term recognition memory performance in rats subjected to strong stress exposure. Furthermore, we evaluated whether such effects are dependent on the activation of the peripheral adrenergic system and demonstrated that surgical removal of the adrenal medulla not only abolishes amphetamine effects on memory consolidation but also reverts its dichotomic effects according to the different stress intensities influence

Glucocorticoids are stress mediators that modulate memory consolidation and retrieval of emotionally arousing experiences (de Quervain et al., 2017) through non-genomically mediated interactions with the noradrenergic transmission within the BLA and the hippocampus, wherein the endocannabinoid system has been shown to play an important role in mediating such effects (Atsak et al., 2015, 2012; Campolongo et al., 2009; Jiang et al., 2014). Glucocorticoid and endocannabinoid release is influenced by circadian rhythm (Dickmeis, 2009; Vaughn et al., 2010). In **Chapter 3**, we investigated how different stress intensities, immediately after encoding, influence rat short-term memory in an object recognition task, whether the effects depend on circadian rhythm and if administration of URB597, which increases levels of the endocannabinoid anandamide at active synapses, could restore any observed impairment. Mild stress exposure elevated plasma corticosterone levels and impaired 1-h recognition memory performance when animals were tested in the morning. Exposure to the higher stress condition elevated plasma corticosterone levels and impaired memory performance, independently of the testing time. These findings show that stress impairing effects on short-term recognition memory are dependent on the intensity of stress and circadian rhythm. URB597 rescued the stress-induced altered memory performance and

decreased corticosterone levels in all the impaired groups yet leaving memory unaltered in the non-impaired groups.

To further characterize the endocannabinoid signaling role in the modulation of circadian- and stress intensity-dependent regulation of recognition memory, we tested if two different stress intensities, and the time-of-day, alter hippocampal endocannabinoid tone, and whether these changes modulate short-term recognition memory (**Chapter 4**). Consistent with our previous findings (Chapter 3), mild stress impaired 1-h memory performance only in the morning, whereas exposure to strong stress impaired memory independently of testing time. Stress exposure decreased hippocampal AEA levels independently of memory alterations. Interestingly, exposure to high stress decreased hippocampal 2-AG content and, accordingly, increased MAGL activity, selectively in the afternoon. Thus, to further evaluate 2-AG's role in the modulation of short-term recognition memory, rats were given bilateral intra-hippocampal injections of the 2-AG hydrolysis inhibitor KML29 immediately after training, then subjected to mild or strong stress conditions and tested 1-h later. Our results suggested that KML29 abolished the time-of-day-dependent impairing effects of stress on short-term memory, ameliorating short-term recognition memory performance.

In **Chapter 5**, we examined endocannabinoid modulation of fear memory retrieval in accordance to different environment-associated emotional arousal. A hallmark symptom of stress-related disorders is that hippocampal memory is compromised while amygdala-dependent memory is abnormally strengthened (Segev et al., 2018). Endocannabinoids primarily signal through CB1 receptors, which are largely distributed on GABAergic and glutamatergic terminals in cortico-limbic regions that mediate fear and anxiety, such as the hippocampus and the BLA (Rubino et al., 2008 Hill et al., 2011). Additionally, AEA and 2-AG activate CB2 receptors, mainly expressed in the periphery and in some microglia and neuronal populations in the

central nervous system (CNS) (i.e. cerebral cortex, hippocampus, striatum, amygdala) (García-Gutiérrez et al., 2012; Xi et al., 2011; Zhang et al., 2014). In this Chapter, we evaluated i) the effects of both basolateral amygdalar and hippocampal enhancement of AEA and 2-AG signaling on auditory and contextual fear memory retrieval and ii) whether these effects were mediated by indirect activation of CB1 and/or CB2 receptors. We then investigated whether the interplay between the BLA and the dorsal CA1 plays any role in such effect by testing if: iii) the muscimol-induced pharmacological inactivation of the dorsal CA1 could influence the basolateral amygdalar endocannabinoid modulation of the retrieval of auditory memory and, conversely, whether the muscimol-dependent BLA inactivation affected the dorsal CA1 endocannabinoid regulation of contextual fear memory retrieval; finally, we tested whether iv) the activation of the endocannabinoid system in the BLA could ameliorate auditory fear memory retrieval when optogenetic inactivation of the dorsal CA1 field was performed during testing and, conversely, if optogenetic inhibition of the BLA influenced the hippocampal endocannabinoid modulation of contextual fear memory retrieval. Our results revealed that AEA, in the BLA, and 2-AG, in the CA1, differentially impaired fear memory retrieval through a mechanism that involved both CB1 and CB2 receptors activation. This effect was reverted by inhibiting pharmacologically or optogenetically the dorsal CA1 for the basolateral amygdalar AEA effect on auditory fear memory retrieval and, conversely, by deactivating the BLA for the hippocampal 2-AG effect on the retrieval of contextual fear memory. We clearly demonstrate that bidirectional inputs between the dorsal CA1 and the BLA are critical for enabling endocannabinoid modulation of fear memory retrieval.

Impaired fear extinction contributes to the development and persistence of PTSD (Yehuda et al., 2015). While only a small proportion of trauma-exposed individuals develop PTSD, women have a two-fold greater risk, prevalence, and duration of PTSD than men (Breslau, 2009). Despite the endocannabinoid system has been suggested as a therapeutic target for PTSD (Morena et al., 2018), whether and how the

endocannabinoid signaling may modulate fear expression and extinction in both males females remains unknown. To answer this question, we pharmacologically manipulated endocannabinoid signalling in male and female rats prior to extinction of auditory conditioned fear and measured both passive (freezing) and active (darting) conditioned responses (**Chapter 6**). Our results highlighted that acute systemic inhibition of the endocannabinoid AEA or 2-AG hydrolysis did not significantly alter fear expression or extinction in males. However, in females increased AEA signalling at vanilloid TRPV1 receptors impaired fear memory extinction. Conversely, inhibition of 2-AG hydrolysis promoted active over passive fear responses acutely via activation of CB1 receptors, collectively suggesting that AEA and 2-AG signaling affect fear expression and extinction in females in opposite directions. Furthermore, measurement of AEA and 2-AG levels after extinction training revealed sex- and brain region-specific changes. These findings are relevant to future research on sex differences in mechanisms of fear extinction and may help develop sex-specific therapeutics to treat trauma-related disorders.

In **Chapter 7**, we evaluated the stress memory literature for evidence of circadian influence on stress-dependent modulation of memory, also highlighting the circadian functioning pattern of the endocannabinoid system. We illustrated how the endocannabinoid system contribution may pervasively prevent stress detrimental effects on memory in a circadian-dependent fashion.

All together our results provide new insights in the field of memory research. We suggest that endocannabinoids might regulate molecular mechanisms that control memory function under circadian, stress and sex influence, with potentially important clinical implications for both neurodevelopmental disorders and psychiatric conditions involving memory dysfunction.

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CURRICULUM VITAE

PERSONAL INFORMATION

Name: Alessia Santori
Date and Place of Birth: December 05th 1992, Rome (Italy)
Nationality: Italian
Address: Via Camillo Porzio 11, 00179, Rome (Italy)
Phone Number: +39 346 2252759
E-mail: alessia.santori@uniroma1.it

EDUCATION

Dec 2017 — **Board-Certified Pharmacist**. Sapienza University of Rome (Italy).

Oct 2017 — **Master of Science in Medicinal Chemistry** (final mark: 110/110 *cum laude*). Sapienza University of Rome (Italy). Research Thesis title: *"Endocannabinoid modulation of short-term recognition memory in rats: influence of stress and circadian rhythm"*.

July 2011 — **High School Diploma** (PNI – National Plan of Computer Studies). Liceo Scientifico "Bertrand Russell", Rome (Italy).

PROFESSIONAL EXPERIENCE

Nov 2017 – Dec 2020 — **PhD student** in Pharmacology and Toxicology at the Dept. of Physiology and Pharmacology "V. Erspamer". Course Director: Prof. Maura Palmery; PhD Director: Prof. Patrizia Campolongo. Sapienza University of Rome (Italy).

Oct 2019 — ***on merit*** **External Teacher** at Middle and High Schools of Lazio joining the project "Smart Drugs? No thanks!" promoted by ASL Roma 6 (Lazio, Italy), October 21-31 (2019). Project Manager: Dr. Marina Bruno.

Sep 2018 – Dec 2018 — **Visiting PhD student** at the Dept. of Psychological &

	Brain Sciences (LaLumiere's lab). Director: Prof. Ryan LaLumiere. University of Iowa (IA), USA.
Jan 2016 – Oct 2017	**Master student**. Research Thesis in Pharmacology and Toxicology at the Dept. of Physiology and Pharmacology "V. Erspamer". Director: Prof. Patrizia Campolongo. Sapienza University of Rome (Italy).
April 2017 – Aug 2017	**Visiting student** at The Mathison Centre for Mental Health Research & Education and Hotchkiss Brain Institute (Hill's lab). Director: Dr. Matthew N. Hill. University of Calgary (AB), Canada.
Oct 2015 – Jan 2016	**Part time placement** at the Dept. of Pharmacy. Sapienza University of Rome (Italy).

TRAINING COURSES

- Advanced Course: "Learning and Memory – Cellular and System Mechanisms". Neuroscience School of Advanced Studies (NSAS) 2019, September 7-14, Venice, Italy.
- "Use of statistics in Biomedical Research: basic principles". Centro Europeo di Ricerca sul Cervello (CERC) & Santa Lucia Foundation 2019, June 5-7, Rome, Italy.
- Course on Laboratory Animal Science: "Working with Rats in Research Settings". University of Iowa 2018, Iowa City (IA), USA.
- Course on Laboratory Animal Science: "Rat Husbandry, Handling and Procedures; Institutional Animal User Training Program (IAUTP)". University of Calgary 2017, Calgary (AB), Canada.

FELLOWSHIPS/AWARDS

2020	"Trainee Travel Award". 30th Annual ICRS Symposium on the Cannabinoids, July 4th – 9th 2020, Galway, Ireland.
2017	Individual three years PhD fellowship. Ministry of Education (Italy).
2017	"Visiting Trainees Award". The Mathison Centre for Mental Health Research & Education of Calgary (Canada).
2016	Thesis abroad fellowship. Sapienza University of Rome (Italy).

2016 Part time placement fellowship. Dept. of Pharmacy, Sapienza University of Rome (Italy).

PUBLICATIONS (PEER REVIEWED JOURNALS)

1. P. Colucci, A. Santori, L. Romanelli, C. Zwergel, A. Mai, S. Scaccianoce, P. Campolongo (2021). Amphetamine modulation of long-term object recognition memory in rats: Influence of stress. *Front in Pharmacol, online ahead of print.*
2. HA. Vecchiarelli, M. Morena, CM. Keenan, V. Chiang, K. Tan, M. Qiao, K. Leitl, **A. Santori**, QJ. Pittman, KA. Sharkey, MN. Hill (2021). Comorbid Anxiety-Like Behavior in a Rat Model of Colitis is Mediated by an Upregulation of Central Fatty Acid Amide Hydrolase. *Neuropsychopharmacology,* 1-12.
3. M. Morena, AS. Nastase, **A. Santori**, BF. Cravatt, RM. Shansky, MN. Hill (2020). Sex-dependent effects of endocannabinoid modulation of conditioned fear extinction in rats. *Br J Pharmacol,* 178(4):983-996.
4. **A. Santori**, M. Morena, MN. Hill, P. Campolongo (2020). Hippocampal 2-arachidonoyl glycerol signaling regulates time-of-day- and stress-dependent effects on rat short-term memory. *Int J Mol Sci*, 21(19):1-16, 7316.
5. P. Colucci, GF. Mancini, **A. Santori**, C. Zwergel, A. Mai, V. Trezza, B. Roozendaal, P. Campolongo (2019). Amphetamine and the Smart Drug 3,4-Methylenedioxypyrovalerone (MDPV) Induce Generalization of Fear Memory in Rats. *Front Mol Neurosci*, 12:292.
6. **A. Santori**, P. Colucci, GF. Mancini, M. Morena, M. Palmery, V. Trezza, S. Puglisi-Allegra, MN. Hill, P. Campolongo (2019). Anandamide modulation of circadian- and stress-dependent effects on rat short-term memory. *Psychoneuroendocrinology*, 108:155-162.
7. V. Santangelo, C. Cavallina, P. Colucci, A. Santori, S. Macrì, JL. McGaugh, P. Campolongo (2018). Enhanced brain activity associated with memory access in highly superior autobiographical memory. Proc Natl Acad Sci USA, 115(30):7795-7800.

ABSTRACTS AT NATIONAL AND INTERNATIONAL MEETINGS

1. **A. Santori**, M. Morena, C. Visentin, MN. Hill, P. Campolongo, Hippocampal endocannabinoid signaling regulates short-term recognition

memory in rats: Influence of stress and circadian rhythm. PhD Neuroscience Webinar 2020, September 29-30.

2. **A. Santori**, P. Ratano, M. Ceman, KL. Wahlstrom, RT. LaLumiere, P. Campolongo, Optogenetic inhibition of the basolateral amygdala influences hippocampal endocannabinoid modulation of fear memory retrieval in rats. International Cannabinoid Research Society (30th Annual Symposium) 2020, July 4-9, Galway, Ireland.
3. **A. Santori**, P. Ratano, KL. Wahlstrom, RT. LaLumiere, P. Campolongo, Role of the basolateral amygdala in the hippocampal endocannabinoid modulation of fear memory retrieval in rats. Società Italiana di Farmacologia 2019 (39° Congresso Nazionale), November 20-23, Florence, Italy.
4. AS. Nastase, M. Morena, **A. Santori**, R. Shansky, MN. Hill, Sex differences in the endocannabinoid modulation of fear memory dynamics. Society for Neuroscience 2019, October 19-23, Chicago, Illinois, USA.
5. **A. Santori**, P. Ratano, KL. Wahlstrom, RT. LaLumiere, P. Campolongo, Role of the basolateral amygdala in the hippocampal endocannabinoid modulation of fear memory retrieval in rats. Italian Society for Neuroscience (18th National Congress) 2019, September 26-29, Perugia, Italy.
6. **A. Santori**, M. Splendori, M. Morena, MN. Hill, P. Campolongo, Endocannabinoid modulation of short-term recognition memory in rats: Influence of stress and circadian rhythm. International Society of Psychoneuroendocrinology 2019, August 29-31, Milan, Italy.
7. **A. Santori**, P. Colucci, GF. Mancini, P. Ferrante, M. Morena, MN. Hill, P. Campolongo, Endocannabinoid modulation of circadian- and stress-dependent effects on short-term memory in rats. Mediterranean Neuroscience Society Meeting 2019, June 23-27, Marrakech, Morocco.
8. **A. Santori**, P. Colucci, N. Marinelli, M. Morena, MN. Hill, P. Campolongo, Endocannabinoid modulation of circadian- and stress-dependent effects on rat short-term memory. Pavlovian Society Meeting 2018, October 4-6, Iowa City, Iowa, USA.
9. H. Vecchiarelli, T. Kaitlyn, C. Vincent, M. Morena, **A. Santori**, C. Keenan, K. Leitl, M. Sticht, W. Ho, M. Qiao, K. Sharkey, MN. Hill, Colitis promotes anxiety through a corticotropin-releasing factor receptor 1 mediated suppression of central anandamide signaling. Federation of European Neuroscience Societies 2018, July 7-11, Berlin, Germany.
10. **A. Santori**, P. Colucci, N. Marinelli, M. Morena, MN. Hill, P. Campolongo, Endocannabinoid modulation of short-term recognition memory in rats:

Influence of stress and circadian rhythm. Federation of European Neuroscience Societies 2018, July 7-11, Berlin, Germany.

11. K. Leitl, M. Morena, **A. Santori**, M. Sohn, MN. Hill, Modulation of anxiety by the endocannabinoid anandamide signaling in the prefrontal cortex is dependent on the emotional arousal state. Federation of European Neuroscience Societies 2018, July 7-11, Berlin, Germany.
12. P. Colucci**, A. Santori**, M. Morena, M. Palmery, V. Trezza, MN. Hill, P. Campolongo, Effects of different post-encoding stress intensities on short-term recognition memory in rats: The endocannabinoid buffering. International Conference on Learning and Memory 2018, April 18-22, Huntington Beach, California, USA.
13. AS. Nastase, **A. Santori**, M. Morena, MN. Hill, Sex-specific effects of endocannabinoid hydrolysis inhibition on fear memory dynamics. Canadian Spring Conference on Behaviour and Brain 2018, February 22-24, Fernie, British Columbia, Canada.
14. P. Colucci, GF. Mancini, **A. Santori**, P. Campolongo, Amphetamine and the "bath salt" 3,4-methylenedioxypyrovalerone (MDPV) alter accuracy of memory for emotional arousing experiences in rats. Società Italiana di Farmacologia 2017 (38° Congresso Nazionale), October 25-28, Rimini, Italy.
15. AS. Nastase, **A. Santori**, M. Morena, MN. Hill, Sex-specific effects of the endocannabinoid anandamide hydrolysis inhibition on fear memory dynamics. HBI Summer Student Symposium 2017, August 15, Calgary, Alberta, Canada.
16. **A. Santori**, P. Colucci, M. Morena, MN. Hill, P. Campolongo, Endocannabinoid modulation of short-term recognition memory in rats: Influence of stress and circadian rhythm. HBI Summer Student Symposium 2017, August 15, Calgary, Alberta, Canada.
17. M. Sohn, **A. Santori**, M. Morena, MN. Hill, Modulation of anxiety by the endocannabinoid anandamide signaling in the prefrontal cortex is dependent on the emotional arousal state. HBI Summer Student Symposium 2017, August 15, Calgary, Alberta, Canada.
18. P. Colucci, GF. Mancini, **A. Santori**, B. Roozendaal, P. Campolongo, Amphetamine and the 'bath salt' MDPV enhance generalization of memory for emotional experiences in rats. Mediterranean Neuroscience Society Meeting 2017, June 12-15, Malta.

APPENDIX

Enhanced brain activity associated with memory access in highly superior autobiographical memory

Valerio Santangelo[a,b,1], Clarissa Cavallina[a,b], Paola Colucci[c], Alessia Santori[c], Simone Macrì[d], James L. McGaugh[e,f,1], and Patrizia Campolongo[c,g]

[a]Department of Philosophy, Social Sciences & Education, University of Perugia, 06123 Perugia, Italy; [b]Neuroimaging Laboratory, Santa Lucia Foundation, 00179 Rome, Italy; [c]Department of Physiology and Pharmacology, Sapienza University of Rome, 00185 Rome, Italy; [d]Centre for Behavioural Sciences and Mental Health, Istituto Superiore di Sanità, 00161 Rome, Italy; [e]Center for the Neurobiology of Learning and Memory, University of California, Irvine, CA 92697-3800; [f]Department of Neurobiology and Behavior, University of California, Irvine, CA 92697-3800; and [g]Neurobiology of Behavior Laboratory, Santa Lucia Foundation, 00143 Rome, Italy

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Brain systems underlying human memory function have been classically investigated studying patients with selective memory impairments. The discovery of rare individuals who have highly superior autobiographical memory (HSAM) provides, instead, an opportunity to investigate the brain systems underlying enhanced memory. Here, we carried out an fMRI investigation of a group of subjects identified as having HSAM. During fMRI scanning, eight subjects with HSAM and 21 control subjects were asked to retrieve autobiographical memories (AMs) as well as non-AMs (e.g., examples of animals). Subjects were instructed to signal the "access" to an AM by a key press and to continue "reliving" it immediately after. Compared with controls, individuals with HSAM provided a richer AM recollection and were faster in accessing AMs. The access to AMs was associated with enhanced prefrontal/hippocampal functional connectivity. AM access also induced increased activity in the left temporoparietal junction and enhanced functional coupling with sensory cortices in subjects with HSAM compared with controls. In contrast, subjects with HSAM did not differ from controls in functional activity during the reliving phase. These findings, based on fMRI assessment, provide evidence of interaction of brain systems engaged in memory retrieval and suggest that enhanced activity of these systems is selectively involved in enabling more efficient access to past experiences in HSAM.

long-term memory | prefrontal cortex | hippocampus | fMRI | functional connectivity

The ability to remember personal experiences [i.e., autobiographical memories (AMs)] is essential for survival (1). Brain systems underlying human AM function have been classically investigated studying patients with selective memory impairments (2). The discovery of extremely rare individuals who spontaneously show highly superior autobiographical memory (HSAM) (3, 4) provides, instead, an opportunity to investigate the brain processes underlying enhanced AMs. Individuals with HSAM demonstrate an extraordinary ability to recall vividly and accurately many remote autobiographical events, irrespective of their emotional saliency, and without the explicit use of mnemonic strategies. In contrast, their performance is generally comparable to that of control subjects in performance assessed by laboratory memory tests (3–5). Prior MRI assessment of HSAM revealed that several brain regions differ in size and shape [e.g., parahippocampal gyrus, posterior insula, intraparietal sulcus (IPS), putamen, caudate] as well as in coherence of fiber tracts (e.g., uncinate fasciculus) compared with those of control subjects (4). The present study investigated brain activity induced by AMs using fMRI.

Prior evidence of detailed reexperiencing in subjects with HSAM (3–5) suggests that subjects with HSAM may express increased neural activity underlying memory reliving. Previous fMRI investigations of normal (i.e., not superior) AM retrieval assessed memory access and memory reliving by asking participants to confirm elicitation of an AM through a response button (access phase) and then to continue to elaborate on the retrieved event (reliving phase) in as much detail as possible for the remaining part of the trial (6). Previous studies using this approach have reported activity in prefrontal/medial temporal regions related to access and activity in sensory cortex related to reliving (7). Retrieval by subjects with HSAM may therefore involve enhanced activity in sensory cortex associated with detailed reliving of reactivated experiences. Alternatively, HSAM might entail enhanced prefrontal/medial temporal resources devoted to AM access. This interpretation is consistent with recent findings showing a selective decrease of neural activity in the medial prefrontal cortex as well as a reduced hippocampal volume in individuals who have impaired AM retrieval (8). These findings may suggest that individuals with HSAM show hyperfunctioning of prefrontal/hippocampal regions. In the present study, we addressed this question by performing an fMRI examination of a group of subjects with HSAM.

During fMRI, subjects with HSAM and controls were asked to mentally retrieve "easy" AMs, thus guaranteeing good performance in controls. Subjects were presented with memory cues pointing to specific spatiotemporal coordinates that emphasize the difference between very old and more recent AMs (e.g., "the first time you drove a car" or "the last time you took a train"). Participants

Significance

Recent research has identified human subjects who have highly superior autobiographical memory (HSAM). Here, we investigated, using fMRI, the neural activation induced by retrieval of autobiographical memories (AMs) and semantic memories (SMs) in subjects with HSAM and control subjects. While their brains were being scanned, subjects had to retrieve AMs as well as SMs (e.g., examples of animals). The subjects with HSAM displayed a superior ability to retrieve details of AMs, supported by enhanced activation of several brain regions, including the medial prefrontal cortex and temporoparietal junction, as well as increased connectivity of the prefrontal cortex with the hippocampus, a region well known to be involved in memory representation. These findings suggest that activation of these systems may play a critical role in enabling HSAM.

Author contributions: V.S., S.M., J.L.M., and P. Campolongo designed research; V.S., C.C., P. Colucci, and A.S. performed research; V.S. analyzed data; and V.S., S.M., J.L.M., and P. Campolongo wrote the paper.

Reviewers: B.L., Rotman Research Institute of Baycrest and University of Toronto; and J.T.W., University of California, San Diego.

The authors declare no conflict of interest.

[1]To whom correspondence may be addressed. Email: valerio.santangelo@unipg.it or james.mcgaugh@uci.edu.

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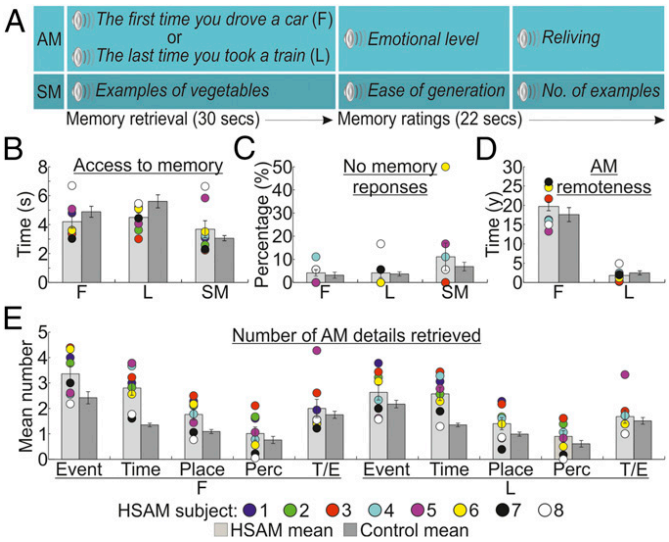

Fig. 1. Task and behavioral results. (*A*) Sequence of events in an example trial, involving 30 s to access and elaborate [first (F) and last (L)] AMs or SMs and 22 s to provide memory ratings. (*B*) Time to access AMs and SMs in seconds. (*C*) Percentages of no memory trials. (*D*) Remoteness of reported AMs (in years). (*E*) Mean number of details according to the categories of Levine et al. (9) [event, time, place, perceptual (Perc), thought/emotion (T/E)] and AM remoteness. In all graphs, individual scores for subjects with HSAM are plotted. The error bars represent the SEM.

confirmed the appearance of the AM through a response button (access phase) and then continued to relive the retrieved event in as much detail as possible (6) (reliving phase; Fig. 1*A*). The specificity of AM activations was controlled by subtracting neural activity induced by accessing and generating examples of specific semantic categories [e.g., "example of vegetables"; i.e., a semantic memory (SM) task]. In comparison to controls, the subjects with HSAM had faster access and more detailed retrieval of AMs. Memory access was associated with increased prefrontal/hippocampal functional connectivity and increased connectivity between the temporoparietal junction (TPJ) and sensory cortices. The prefrontal and hippocampal regions were found to be particularly involved with access to remote AMs. In contrast, subjects with HSAM did not differ from controls in brain activity during the reliving phase.

Results

Behavioral Data. Behavioral results are illustrated in Fig. 1. First, we assessed whether subjects with HSAM were faster than controls to access their AMs. We performed a group (HSAM vs. control) by trial type (first AM, last AM, and SM) ANOVA on the response latencies, defining the time needed to access a specific AM or SM (Fig. 1*B*). This analysis revealed a main effect of trial type [$F(2, 54) = 21.2$, $P < 0.001$; $\eta^2 = 0.440$], indicating faster response latencies to access SMs (mean = 3.368 s) than first (4.545 s) and last (5.059 s) AMs. This effect was further qualified by the significant group × trial type interaction [$F(2, 54) = 5.8$, $P = 0.005$; $\eta^2 = 0.177$], indicating that subjects with HSAM had faster access to AMs than control subjects (first AM: 4.196 vs. 4.894 s, $P = 0.029$; last AM: 4.504 vs. 5.614 s, $P = 0.013$), but not to SMs (3.681 vs. 3.056 s, $P = 0.074$). The main effect of group was not significant [$F(1, 27) < 1$]. A similar 2 × 3 ANOVA on the "no memory response" data (Fig. 1*C*) revealed a main effect of trial type [$F(2, 54) = 3.7$, $P = 0.032$; $\eta^2 = 0.120$], indicating that subjects failed more often to retrieve SMs (9%) than first (3.7%) and last (3.9%) AMs. This effect was not further modulated by the group factor, as indicated by the absence of both main effect of group and group × type of trial interaction (both $F < 1$). No differences were found between the groups in the self-evaluation ratings of the emotional level and reliving of AMs and the ease of generation and number of generated example for SMs during scanning, as assessed by two-tailed independent sample t tests (t values ranging between −1.4 and 0.9, $P > 0.181$; *SI Appendix*, Fig. S1).

After the fMRI scanning, participants were presented again with the memory cues and asked to provide a verbal account of their memories. We analyzed the temporal distribution (Fig. 1*D*) of the retrieved AMs using a group by remoteness of AM (first or last AM) ANOVA. This analysis revealed a main effect of remoteness [$F(1, 27) = 188.4$, $P < 0.001$; $\eta^2 = 0.875$], indicating that first AMs were older than last AMs: 18.76 y and 2.16 y, respectively. The two groups did not differ in the remoteness of AMs reported, as indicated by the absence of both main effect of group and group × age of AM interaction (both $F < 1.3$, $P > 0.257$). Finally, we compared how detailed were the AMs reported by the two groups, using a group by remoteness of AM by type of detail [event, time, place, perceptual, thought/emotion (9)] ANOVA. Subjects with HSAM retrieved a greater number of details than controls (2.0 vs.

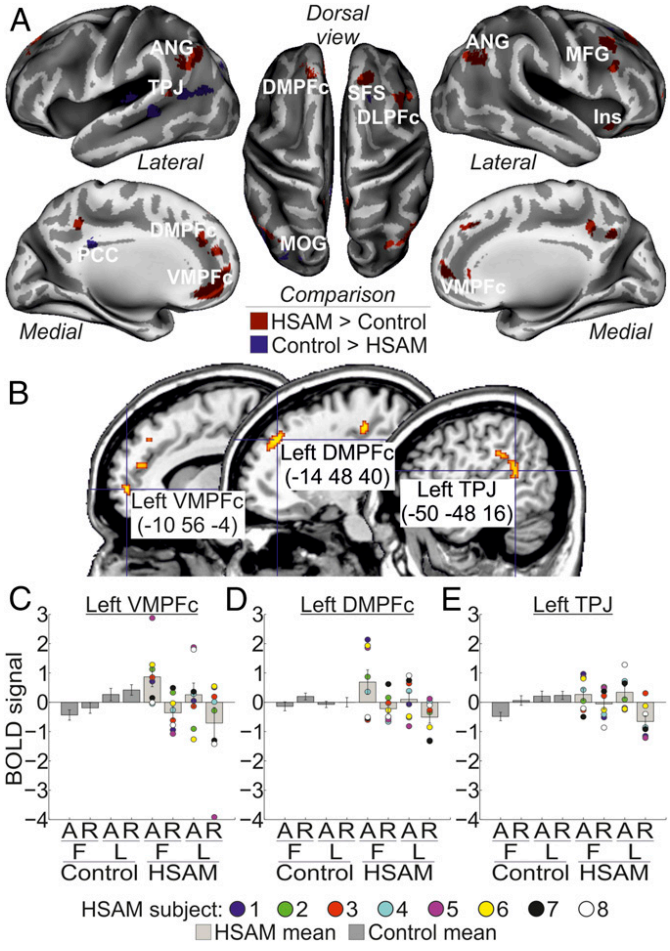

Fig. 2. (*A*) Regions activated by AM in the HSAM group (red map) and in the control group (blue map) overlaid on an inflated template. ANG, angular gyrus; DLPFc, dorsolateral prefrontal cortex; Ins, insula; MFG, middle frontal gyrus; SFS, superior frontal sulcus. (*B*) Sagittal sections on a standard Montreal Neurological Institute template showing the anatomical location of the regions selectively involved with the access phase to AM in the HSAM group (i.e., left VMPFc, left DMPFc, left TPJ). For display purposes, all maps are displayed at a threshold of *P*-uncorrected = 0.001. (*C*–*E*) Bar plots summarizing the activity (expressed in arbitrary units ± 90% confidence interval) of the left VMPFc, DMPFc, and TPJ, respectively, which showed increased activity during access to (A) [vs. reliving of (R)] AMs (compare bars 5 and 7 vs. bars 6 and 8), selectively in the HSAM group (compare bars 5–8 vs. bars 1–4). Individual scores are plotted for subjects with HSAM. BOLD, blood oxygen level-dependent.

1.4), as evidenced by the main effect of group [$F(1, 27) = 10.1$, $P = 0.004$; $\eta^2 = 0.272$]. This was particularly true for the remote events, as indicated by the three-way interaction [$F(4, 108) = 2.5$, $P = 0.047$; $\eta^2 = 0.085$], showing more details reported in the event ($P = 0.005$), time ($P < 0.001$), and place ($P = 0.048$) categories relative to remote AMs, but only more details in the time category ($P < 0.001$) relative to recent AMs (Fig. 1*E*). Subjects with HSAM also provided higher vivid descriptions of their AMs when assessed qualitatively (t values ranging between 5.0 and 18.2, $P < 0.001$; *SI Appendix*, Fig. S2).

Overall, these findings indicate that, compared with controls, subjects with HSAM had faster and more vivid access to AMs, especially for the most remote AMs, but had normal SMs.

Assessment of Obsessive/Compulsive Traits in Individuals with HSAM. The findings of previous studies suggest that subjects with HSAM tend to have symptoms of obsessiveness/compulsiveness (10). To assess whether individuals with HSAM participating in the current study experienced obsessive/compulsive symptomatology, they were administered the Personality Assessment Inventory, which included, among others, the "obsessive-compulsive" subscale. The average score at the obsessive-compulsive subscale for individuals with HSAM was 67, corresponding to the 92nd percentile in terms of expressing obsessive/compulsive-related symptoms. We assessed whether faster access to AMs (averaging across first and last AM types) and number of retrieved details correlated with obsessive/compulsive traits (averaging across the five categories). However, our analyses failed to reveal any significant correlation ($r = 0.32$, $P = 0.444$ and $r = -0.69$, $P = 0.58$, respectively).

fMRI Data. The main aim of the present study was to investigate neural activation associated with AM retrieval in subjects with HSAM compared with control subjects. In the HSAM group, AM retrieval recruited a large network of areas extending along the frontoparietal cortex (Fig. 2*A* and Table 1). This activation included dorsomedial regions, such as the left dorsomedial prefrontal cortex (DMPFc; the right DMPFc was marginally significant) and the left and right ventromedial prefrontal cortex (VMPFc), and lateral regions, such as the right dorsolateral prefrontal cortex, the left TPJ, and the left and right angular gyrus, plus the right insula. By contrast, we observed in the control group increased activity in a few areas involving the right superior frontal sulcus and the posterior cingulate cortex (PCC), plus activation of visual areas [i.e., the left middle occipital gyrus (MOG)].

Next, we assessed the contribution of these group-specific activations in the access or reliving phase (i.e., phase × group interaction). Three of the regions activated by AM retrieval in subjects with HSAM were found to contribute selectively to memory access, namely, the left VMPFc (peaking at: $x, y, z = -8, 58, 0$; $Z = 3.44$; $P = 0.011$), the left DMPFc ($x, y, z = -14, 42, 40$; $Z = 3.14$; $P = 0.027$), and the left TPJ ($x, y, z = -54, -44, 20$; $Z = 3.23$; $P = 0.021$) (Fig. 2*B*). Activity in these regions selectively increased for the access vs. reliving phase (Fig. 2 *C–E*; compare bars 5 and 7 vs. bars 6 and 8) in the HSAM group only (compare bars 5–8 vs. bars 1–4). None of the selected regions of interest (ROIs) showed a selective involvement with the reliving phase in subjects with HSAM instead. Similarly, none of the regions activated by AM retrieval in the control group was found to contribute selectively to the access or reliving phase. Analogously, no ROIs were found to reveal any AM type × group interaction. However, the left VMPFc (peaking at: $x, y, z = -12, 52, 2$; $Z = 3.51$; $P = 0.009$) showed a more selective contribution during access to remote AMs (i.e., the three-way phase × AM type × group interaction). The left DMPFc showed a similar trend, despite not being statistically significant, peaking at: $x, y, z = -10, 44, 44$; $Z = 2.75$; $P = 0.069$. Activity in the left VMPFc selectively increased during access to first AMs in subjects with HSAM (Fig. 2*C*).

We then examined the functional coupling of the regions showing a selective involvement with AM access in subjects with HSAM (left VMPFc, left DMPFc, and left TPJ) with the rest of the brain. Analyses of interregional connectivity revealed large networks of areas functionally connected with the seed regions during AM access in subjects with HSAM (Fig. 3*A* and Table 2). Specifically, the left VMPFc connected with the medial temporal lobe and, in particular, with the left hippocampus and rostral portion of the anterior cingulate cortex (ACC). The VMPFc was also found to be connected with another seed region, namely, the left TPJ, and with the left and right subcentral gyrus. Activity in the left DMPFc was found instead to be synchronized during AM access in subjects with HSAM with the left and right IPS along the dorsal frontoparietal network and with prefrontal regions, such as the right ventrolateral prefrontal cortex (VLPFc) extending anteriorly and medially to the left and right VMPFc. The left DMPFc was also functionally connected with the medial portion of the cingulate cortex, the precentral gyrus, and the MOG. Finally, the left TPJ showed increased coupling during AM access in subjects with HSAM with adjacent regions of the left parietal cortex, such as the superior and inferior parietal lobe, plus other posterior regions in the occipital and temporal lobes, such as the left and right superior occipital gyrus and the left and right medial temporal cortex. The left TPJ also showed increased coupling with the ACC and the supplementary motor area bilaterally. Despite a large variability across the individual values (some representative areas are shown in signal plots in Fig. 3 *B–D*), the general pattern of activity of all of these regions showed increased coupling with the respective seed region during access to (vs. reliving of) AMs (compare bars 5 and 7 vs. bars 6 and 8), selectively in the HSAM group (compare bars 5–8 vs. bars 1–4).

Finally, we investigated whether the brain activity related to access remote or recent memories covaried with access latencies or as a function of obsessive/compulsive tendencies in subjects with HSAM. We found that in subjects with HSAM ($P = 0.026$), but not in control subjects, the activity of the left hippocampus increased as a function of individual latencies to access remote memories (Fig. 4). No other effects were observed in the other ROIs or as a function of the individual scores of obsessiveness.

Table 1. MNI coordinates (x, y, z), Z-values, and P-FWE–corrected values for areas showing a main effect of group, HSAM vs. control group or vice versa

Area	x, y, z	Z-value	P-FWE–corrected
HSAM > control			
Left TPJ	−50, −48, 16	7.00	<0.001
Left ANG	−50, −64, 40	6.42	<0.001
Right ANG	46, −68, 38	5.36	0.002
Left VMPFc	−10, 56, −4	5.75	<0.001
Right VMPFc	18, 62, 2	5.31	0.002
Right DLPFc	44, 22, 42	5.66	<0.001
Left DMPFc	−14, 48, 40	5.64	<0.001
Right Ins	30, 24, −14	4.93	0.048
Control > HSAM			
Left MOG	−36, −66, 30	6.25	<0.001
Right SFS	18, 20, 44	4.85	0.016
Left PCC	−12, −38, 28	4.68	0.033

ANG, angular gyrus; DLPFc, dorsolateral prefrontal cortex; Ins, insula; SFS, superior frontal sulcus.

Discussion

The subjects with HSAM were faster and more efficient in retrieving AMs. In contrast, they did not differ from control subjects in retrieving semantic information. The findings strongly suggest that the shorter latencies in providing AMs reflect superior access to details of past experiences on the part of subjects with HSAM. Additionally, the findings indicate that, in comparison to controls, the subjects with HSAM remembered more autobiographical details of their past experiences, consistent with extensive prior investigations of HSAM (11, 12), especially for

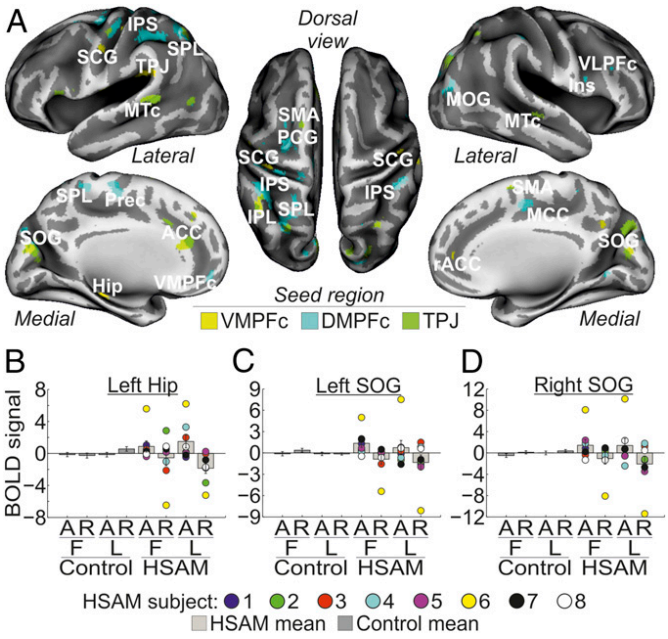

Fig. 3. (*A*) Regions showing functional connectivity with the left VMPFc (yellow map), left DMPFc (cyan map), and left TPJ (green map) during access to AM in the HSAM group overlaid on an inflated template. For display purposes, all maps are displayed at a threshold of *P*-uncorrected = 0.005. Hip, hippocampus; Ins, insula; MCC, medial cingulate cortex; MTc, medial temporal cortex; PCG, precentral gyrus; Prec, precuneus; rACC, rostral anterior cingulate cortex; SCG, subcentral gyrus; SMA, supplementary motor area; SOG, superior occipital gyrus; SPL, superior parietal lobe. Signal plots show the pattern of functional connectivity for some representative seed regions, specifically, the left Hip (*B*) and the left and right SOG (*C* and *D*). All signal plots revealed increased coupling (expressed in arbitrary units ± 90% confidence interval) with the respective seed region during access to (A) [vs. reliving of (R)] AMs (compare bars 5 and 7 vs. bars 6 and 8), selectively in the HSAM group (compare bars 5–8 vs. bars 1–4). Individual scores are plotted for subjects with HSAM. BOLD, blood oxygen level-dependent.

the most remote AMs. The findings also confirmed those of previous research indicating that subjects with HSAM tend to express obsessive/compulsive symptoms. However, we did not find evidence in our HSAM sample that the individual level of obsessiveness is related to the memory access, in either response latency or underlying brain activity. While we estimated a reliable statistical power for the current experiment (*Methods*), we cannot exclude that smaller effect sizes may be detected with larger sample sizes. Therefore, future experiments will determine whether the null effects reported in this study reflect a lack of difference or limited statistical power.

The major aim of the present investigation was to determine whether HSAM is associated with enhanced activation of brain systems as assessed by fMRI. The findings provide supporting evidence. Cortical activity increased in several areas, selectively in association with autobiographical remembering, and the increase was greater in subjects with HSAM than in controls. During AM retrieval (irrespective to the access or reliving phase), compared with controls, twice as many brain areas were activated in subjects with HSAM. However, while it might be expected that the increased brain activity in HSAM is specifically devoted to memory reliving, given the richness of details provided by subjects with HSAM (3–5), we did not observe any neural difference between subjects with HSAM and control subjects during the reliving phase. In contrast, the findings suggest that the increase in neural activity was specifically involved in accessing AMs, recruiting a left-lateralized frontoparietal network (VMPFc, DMPFc, and TPJ) in subjects with HSAM only during memory access. Additionally, these HSAM-related regions showed enhanced functional coupling with brain areas crucial for memory retrieval selectively during memory access vs. reliving. These results suggest that HSAM may involve enhanced activation of specific brain areas involved in accessing representations of autobiographical experiences. One may argue that the quicker memory access of subjects with HSAM than control subjects might confound the fMRI analysis, requiring a comparison between different amounts of blood oxygen level-dependent signals (i.e., less signal for the access phase of HSAM than control subjects). Although the finding of greater brain activity in subjects with HSAM during memory access goes against this potential confound, future research will have to solve this possible limitation.

The enhanced AM access in individuals with HSAM involved increased brain activity within core regions of the frontoparietal cortex, namely, the medial prefrontal cortex and TPJ. These areas have been associated with the retrieval of autobiographical material (7, 13). AM retrieval is thought to be supported by an extensive network of brain regions, most pronounced in the left hemisphere (a meta-analysis is provided in ref. 14), that has typically been interpreted as reflecting the variety of cognitive processes engaged during AM retrieval (15, 16): executive control and retrieval monitoring (dorsolateral prefrontal cortex/DMFCc), episodic remembering (hippocampus), emotion-related processes (VLPFc and amygdala), self-processing (DMPFc/VMPFc, PCC), and visuospatial processing (retrosplenial cortex,

Table 2. MNI coordinates (*x*, *y*, *z*), *Z*-values, and *P*-FWE values for areas showing increased functional connectivity with the seed regions (left VMPFc, left DMPFc, left TPJ)

Area	*x*, *y*, *z*	*Z*-value	P-FWE–corrected
Left VMPFc functional connectivity			
Left Hip	−32, −30, −12	6.82	<0.001
Left TPJ	−50, −32, 24	6.35	<0.001
Left SCG	−44, −6, 14	6.93	<0.001
Right SCG	46, −6, 22	6.02	<0.001
Right PostCG	50, −16, 34	6.44	<0.001
Left SMG	−52, −46, 30	6.04	<0.001
rACC	16, 46, 4	5.96	<0.001
Left STc	−56, −12, 14	5.84	<0.001
Left DMPFc functional connectivity			
MCC	12, −8, 50	Inf.	<0.001
Left PCG	−12, −30, 70	7.83	<0.001
Right IPS	44, −38, 56	7.56	<0.001
Left IPS	−40, −38, 52	7.44	<0.001
Right Ins	48, 2, 16	7.32	<0.001
Left SFG	−24, −8, 60	7.27	<0.001
Left Prec	−14, −42, 70	7.16	<0.001
Left SPL	−26, −62, 56	6.74	<0.001
Right VLPFc	38, 34, 22	6.98	<0.001
Right VMPFc	14, 56, −14	6.51	<0.001
Left VMPFc	−8, 54, −12	5.91	<0.001
Right MOG	42, −78, 14	7.32	<0.001
Left TPJ functional connectivity			
Left SPL	−16, −66, 42	6.95	<0.001
Left IPL	−40, −52, 50	5.90	<0.001
ACC	−6, 38, 16	6.08	<0.001
Left SOG	−22, −74, 26	6.68	<0.001
Right SOG	22, −80, 36	6.50	<0.001
Left MTc	60, −16, −12	6.53	<0.001
Right MTc	−60, −40, 6	6.05	<0.001
Right SMA	14, −8, 52	6.86	<0.001
Left SMA	−12, −8, 68	6.02	<0.001

Hip, hippocampus; Inf., infinite; Ins, insula; IPL, inferior parietal lobe; MCC, middle cingulate cortex; MTc, medial temporal cortex; PCG, precentral gyrus; PostCG, postcentral gyrus; Prec, precuneus; rACC, rostral anterior cingulate cortex; SCG, subcentral gyrus; SFG, superior frontal gyrus; SMA, supplementary motor area; SMG, supramarginal gyrus; SOG, superior occipital gyrus; SPL, superior parietal lobe; STc, superior temporal cortex.

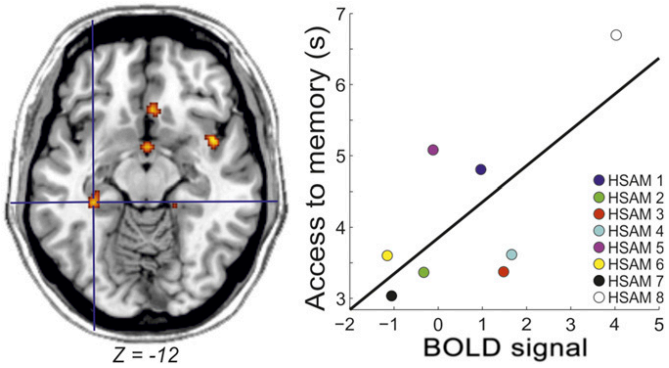

Fig. 4. Increased activity in the left hippocampus as a function of increased latency to memory access in subjects with HSAM. BOLD, blood oxygen level-dependent.

precuneus, and parietal regions). Whereas the recruitment of the medial prefrontal cortex and TPJ reflects normal functioning of AM retrieval, the current findings provide evidence of increased activation of these regions associated with enhanced functioning of AM retrieval in individuals with HSAM.

The increased activation of medial prefrontal regions might be related to enhanced self-reference processes in individuals with HSAM. Recent literature reported consistent activations of both the ventral and dorsal prefrontal cortex during the engagement of self-referential processes in AM retrieval (17). Moscovitch and Winocur (18) suggested that the VMPFc is selectively involved with monitoring the "truthfulness" of AMs during retrieval, providing the feeling of having recollected the correct AM. The enhanced production of confabulation and false memories found in patients with an impaired VMPFc provides support for this suggestion (19). In contrast, increased activity in the DMPFc may reflect recall of experienced events (20). The enhanced activation of the medial prefrontal cortex found in subjects with HSAM during AM retrieval is in line with evidence of an increased propensity of individuals with HSAM to express self-referential processes as well as mental rumination of their prior experiences (10–12).

The present design allowed differentiating brain activity related to retrieval of remote vs. more recent AMs. We found a selective increased activity in the VMPFc during access to remote AMs in subjects with HSAM. Bonnici and Maguire (21) reported evidence that the VMPFc in normal subjects is implicated in memory representation of events up to 2 y of remoteness, but not for more remote events. Here, we found instead an extended temporal window up to 20 y (Fig. 1*D*) in which the VMPFc contributes to access AMs (and, specifically, to the most remote AM details, in line with the behavioral data; Fig. 1*E*) in subjects with HSAM. The current findings also indicate that the VMPFc is functionally connected with the right hippocampus during AM access of individuals with HSAM. Extensive findings of both human and animal subjects have suggested that functional coupling between these two regions is essential for episodic/long-term memory retrieval (22, recently reviewed in refs. 23, 24). Consistently, the present findings suggest that enhanced prefrontal/hippocampal coupling sustains enhanced memory performance in individuals with HSAM. This evidence is consistent with the findings of a single HSAM case study, indicating greater than usual connectivity of the left hippocampus with prefrontal, but also premotor, and retrosplenial cingulate cortex (25).

Importantly, an opposite pattern of results (i.e., decreased neural activity of the VMPFc and hippocampus) was found in subjects with severely deficient AMs (SDAMs). Palombo et al. (8) reported that three subjects with SDAMs, who had otherwise preserved cognitive functions, expressed decreased neural activity in the left VMPFc and reduced hippocampal volume. The evidence that the VMPFc and hippocampus play a key role in both subjects with impaired AM (8) and normal subjects (7, 13–15), as well as in subjects with HSAM, suggests that the current level of prefrontal/hippocampal activity may play a critical role in determining the hypofunctioning (i.e., SDAM) vs. hyperfunctioning (i.e., HSAM) of AM retrieval. Although the increased hippocampal activity in subjects with HSAM might potentially reflect task-related encoding activity (26), the fact that the hippocampal activity increased as a function of longer latencies (indicating increased difficulty) to access the most remote (first-time) events appears to indicate a selective role of the hippocampus in AM retrieval. This latter finding is in agreement with the hypothesis that AMs might permanently depend on the hippocampal activity (27). Together with the VMPFc, the hippocampus therefore appears to enable subjects with HSAM to have faster and more detailed remote memory access (21).

In addition to enhanced prefrontal/hippocampal functional connectivity, memory access in subjects with HSAM was further supported by increased activity of the ventral parietal cortex (left TPJ) during AM retrieval. A growing body of evidence indicates that TPJ lesions entail dysfunctions related to self vs. other distinctions (28). The increased activation of TPJ in HSAM might therefore be linked to an increased capability of subjects with HSAM to select the correct AMs, better distinguishing between facts experienced by self or others. However, the findings also suggest a more parsimonious interpretation (29, 30). TPJ activity during AM access in subjects with HSAM might reflect internal attentional capture driven by information reactivated from long-term memory by the search mechanisms (i.e., the prefrontal/hippocampal cortex). The functional coupling between the left TPJ and the visual and auditory sensory cortex is consistent with this "attentional" account. Recent findings revealed the causal role played by the TPJ in the modulation of sensory representations (31, 32), as well as in mental imagery (33). Accordingly, after internal focusing on reactivated memories, the TPJ might contribute to activate and maintain sensory representations in visual and auditory cortex, triggering visual/auditory imagery (34, 35). These TPJ-centered mechanisms might contribute to the enhanced memory performance of individuals with HSAM, allowing these subjects to check, as early as during AM access, the validity of recollected AMs through visual/auditory imagery. This possibility is consistent with the prevailing view that episodic memories are based (at least in part) on the reactivation of the sensory representation developed at encoding (36, 37). However, previous findings suggest that imagery-related activity in sensory cortex occurs after full access to the memory trace in normal subjects, progressively increasing during explicit reliving of memory details (7, 38). In contrast, the present findings indicate that in individuals with HSAM, the recruitment of neural resources possibly devoted to visual or auditory imagery [i.e., the visual and auditory sensory cortices (34, 35)] is anticipated in the access phase, thus contributing to their enhanced memory performance.

These findings have identified brain activation that differs in subjects with HSAM and control subjects, and they suggest that the differential activation may play a role in enabling more efficient access, with subsequent enhanced retrieval, to autobiographical information. These findings provide targets for brain stimulation and/or therapeutic interventions to enhance memory retrieval in conditions related to altered memory functioning.

Methods

Participants. Eight individuals with HSAM (five male, mean age = 32.5 y, age range: 24–37 y) recruited in accordance with the previous literature (4) (*SI Appendix*) and 21 control subjects (10 male, mean age = 32.5 y, age range: 24–39 y) participated in the study. All participants gave written consent to participate in the study, which was approved by the independent Ethics Committee of the Santa Lucia Foundation (CE/PROG.540). Before conducting the experiment, we performed a power analysis that estimated a reliable statistical power of 84% for our sample size (eight subjects with HSAM plus a minimum of 20 controls) based on an effect size of 0.5, in line with those reported by the previous literature on HSAM (10), and a significance level of 0.05.

Task and Stimuli. During scanning, participants were asked to retrieve AMs and non-AMs (SMs) (Fig. 1*A* and *SI Appendix*). The experiment included three functional runs, each including 18 memory cues: 12 AM trials (six first-time and six last-time events) and six SM trials, and a variable intertrial interval (2–3 s, uniformly distributed). After scanning, participants were asked to provide details about memories retrieved during the experiment (*SI Appendix*).

MRI and fMRI Data Analysis. A Siemens Allegra (Siemens Medical Systems) operating at 3 T and equipped for echoplanar imaging was used to acquire the fMRI scans (*SI Appendix*). We used SPM12 (Wellcome Department of Cognitive Neurology) implemented in MATLAB 7.4 (The MathWorks, Inc.) for data preprocessing (*SI Appendix*) and statistical analyses. Each participant underwent three fMRI runs, each comprising 477 volumes. Statistical inference was based on a two-step random effects approach (*SI Appendix*). Briefly, the first-level models included separated access and reliving regressors (6) for each of the three trial types: first AM, last AM, and SM. For each subject, we estimated contrast images that removed the activity associated with access to and reliving of SMs (control condition) to the main AM conditions. For the second-level group analysis, the single-subject contrast images of parameter estimates were entered into a mixed-design ANOVA with group (HSAM vs. control) as a between-subjects variable and phase (access vs. reliving) and AM type (first vs. last) as within-subjects variables. First of all, we highlighted the regions involved with AMs in the HSAM vs. control group (and vice versa), irrespective of phase and AM type. As an additional constraint, we considered only voxels showing an overall activation across all conditions and groups (T-contrast, *P*-uncorrected = 0.001), ensuring that we selected only regions activated by AM retrieval (e.g., ref. 39). The statistical threshold was set to *P*-family-wise error (FWE)–corrected < 0.05 at the voxel level, considering the whole brain as the volume of interest. This comparison allowed us to highlight different circuits recruited by AM retrieval in the HSAM and control groups (Fig. 2*A* and Table 1). The resulting activations were used to define ROIs that were then used to test for condition-specific effects in interaction with the group variable (i.e., the two-way phase × group and AM type × group interactions and the three-way phase × AM type × group interaction). For this, we considered spheres (8-mm radius, matching the FWHM of the smoothing filter) centered on the regions activated by AM retrieval in the two groups (Table 1) as the volume of interest (small volume correction) (40).

Functional connectivity analysis. The procedure described above allowed us to identify three regions selectively involved in AM access in subjects with HSAM (i.e., the significant group × phase interaction), namely, the left VMPFc, left DMPFc, and left TPJ (Fig. 2*B*). Given that these seed regions are related to HSAM, we did not expect any increased functional connectivity in the control group. The main goal of this analysis was to understand whether additional neural resources supported access to memory in individuals with HSAM (*SI Appendix*).

ROI correlations with memory access latencies and obsessiveness scores in subjects with HSAM. Finally, we conducted exploratory analyses using multiple regression models to investigate whether the brain activity related to the access to remote or recent memories covaried as a function of the individual latency to access memories or as a function of obsessive/compulsive traits in subjects with HSAM (*SI Appendix*).

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ARTICLE OPEN

Comorbid anxiety-like behavior in a rat model of colitis is mediated by an upregulation of corticolimbic fatty acid amide hydrolase

Haley A. Vecchiarelli[1,2,3], Maria Morena[2,3,4,5], Catherine M. Keenan[2,6,7], Vincent Chiang[2,3,4,5], Kaitlyn Tan[2,3,4,5], Min Qiao[2,3,4,5], Kira Leitl[2,3,4,5], Alessia Santori[2,3,4,5], Quentin J. Pittman[2,3,6,7], Keith A. Sharkey[2,6,7] and Matthew N. Hill[2,3,4,5]

Peripheral inflammatory conditions, including those localized to the gastrointestinal tract, are highly comorbid with psychiatric disorders such as anxiety and depression. These behavioral symptoms are poorly managed by conventional treatments for inflammatory diseases and contribute to quality of life impairments. Peripheral inflammation is associated with sustained elevations in circulating glucocorticoid hormones, which can modulate central processes, including those involved in the regulation of emotional behavior. The endocannabinoid (eCB) system is exquisitely sensitive to these hormonal changes and is a significant regulator of emotional behavior. The impact of peripheral inflammation on central eCB function, and whether this is related to the development of these behavioral comorbidities remains to be determined. To examine this, we employed the trinitrobenzene sulfonic acid-induced model of colonic inflammation (colitis) in adult, male, Sprague Dawley rats to produce sustained peripheral inflammation. Colitis produced increases in behavioral measures of anxiety and elevations in circulating corticosterone. These alterations were accompanied by elevated hydrolytic activity of the enzyme fatty acid amide hydrolase (FAAH), which hydrolyzes the eCB anandamide (AEA), throughout multiple corticolimbic brain regions. This elevation of FAAH activity was associated with broad reductions in the content of AEA, whose decline was driven by central corticotropin releasing factor type 1 receptor signaling. Colitis-induced anxiety was reversed following acute central inhibition of FAAH, suggesting that the reductions in AEA produced by colitis contributed to the generation of anxiety. These data provide a novel perspective for the pharmacological management of psychiatric comorbidities of chronic inflammatory conditions through modulation of eCB signaling.

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INTRODUCTION

In peripheral inflammatory conditions, such as inflammatory bowel diseases (IBD), comorbid anxiety and depression are associated with increased disease activity, greater rate of relapse and reduced responsiveness to therapies [1–5], significantly reducing patient quality of life [6, 7]. It is established in cohorts from around the world that patients with IBD (ulcerative colitis and Crohn's disease; combined, and each, separately,) show a 2–3 times greater incidence of anxiety and depression [2–4, 8–29]. It is likely that the driving force behind these psychiatric comorbidities is disease activity [8, 13, 30–35], implying, at least partially, that inflammation and a dysregulation of the gut-brain axis may be involved in the pathogenesis of psychiatric comorbidities in IBD. Both basally and during disease states, the gut-brain axis allows for bidirectional communication between the brain and the gut, including at the levels of the autonomic nervous system and circumventricular organs [36]. As such, understanding the neural mechanisms that underlie the generation of anxiety and depression in peripheral inflammatory disorders may allow for the development of novel treatment approaches to manage these comorbid symptoms that severely impact individuals with these disorders.

Peripheral inflammation, particularly within the gut, is known to be a potent activator of the hypothalamic-pituitary-adrenal (HPA) axis [37, 38]. Sustained elevations in circulating glucocorticoid hormones can modulate central processes, including those involved in the regulation of emotional behavior [39, 40]. One system known to be sensitive to hormonal components of the HPA axis, and that is a significant regulator of emotional behavior, is the endocannabinoid (eCB) system [41–43].

Constitutive eCB signaling constrains anxiety, as acute pharmacological disruption of eCB function rapidly produces a state of anxiety [44–46]. Similarly, exposure to stress is known to increase activity of the enzyme fatty acid amide hydrolase (FAAH), which metabolizes the eCB ligand anandamide (AEA) [47–49], through the release of the neuropeptide corticotropin-releasing factor (CRF; alternatively corticotropin-releasing hormone (CRH)) and subsequent activation of the CRF type 1 receptor (CRF-R1) [50]. This suppression of AEA signaling by CRF-R1 activity promotes the development of anxiety, largely through coordinated actions in

[1]Neuroscience Graduate Program, University of Calgary, Calgary, AB T2N4N1, Canada; [2]Hotchkiss Brain Institute, University of Calgary, Calgary, AB T2N4N1, Canada; [3]Mathison Centre for Mental Health Research and Education, University of Calgary, Calgary, AB T2N4N1, Canada; [4]Department of Cell Biology and Anatomy, University of Calgary, Calgary, AB T2N4N1, Canada; [5]Department of Psychiatry, University of Calgary, Calgary, AB T2N4N1, Canada; [6]Snyder Institute for Chronic Diseases, University of Calgary, Calgary, AB T2N4N1, Canada and [7]Department of Physiology and Pharmacology, Cumming School of Medicine, University of Calgary, Calgary, AB T2N4N1, Canada
Correspondence: Haley A. Vecchiarelli (haleyvecchiarelli@uvic.ca) or Matthew N. Hill (mnhill@ucalgary.ca)

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corticolimbic circuits encompassing the amygdala [50], medial prefrontal cortex [51], and hippocampus [52]. Interestingly, CRF signaling is also known to be important for the development of anxiety in response to inflammation, as blockade of CRF signaling can dampen anxiety and other adverse behavioral responses to a variety of experimental inflammatory conditions such as cerebral ischemia [53], arthritis [54, 55], and inflammatory pain [54, 56, 57]. As sustained inflammation is known to produce an upregulation of central CRF [57–59], it seems plausible that this could result in a suppression of AEA signaling that in turn could contribute to the development of comorbid anxiety in colitis.

To further examine the relationship between eCBs and peripheral inflammation, we utilized a rat model of colitis to investigate the potential role that the eCB system plays in the mechanisms underlying psychiatric comorbidity in chronic inflammatory diseases. Colitis represents an ideal condition for this investigation, as humans afflicted with colitis exhibit considerable psychiatric comorbidities, particularly anxiety [1–5], and antagonism of CRF-R1 in humans with IBD has been found to normalize both alterations in neural connectivity and changes in emotional behavior [60, 61]. Rodent models of colitis produce a sustained state of systemic inflammation [62–65], exhibit upregulation of central CRF [66–69] and recapitulate the anxiety phenotype [70–73] seen in the human condition, making them an ideal model to explore the role of eCBs in these processes.

METHODS AND MATERIALS

Animals

All experiments utilized adult (~300–350 g at time of colitis induction), male or female, Sprague Dawley rats from Charles River (Saint Constant, QC, Canada, RGD Cat# 734476; RRID: RGD_734476). Animals were allowed to acclimate for at least one week prior to experiment onset. Rats were paired-housed under specified pathogen free conditions on a 12:12 h light/dark cycle with *ad libitum* access to food and water. All experiments were conducted during the light phase of the cycle. All animal protocols were approved by the University of Calgary Animal Care Committee and followed guidelines from the Canadian Council for Animal Care. For each set of experiments described below, animals from a minimum of 2, and up to 4, cohorts were used, aside from locomotor activity which was assessed in a single cohort.

Colitis induction and assessment

Under brief isoflurane anesthesia, rats received an intracolonic bolus of 2,4,6-trinitrobenzenesulfonic acid (TNBS) (Millipore Sigma, Darmstadt, Germany, #92822; 0.45 mL, 50 mg/mL, 50% [vol/vol] in ethanol/water), via a cannula, inserted 7 cm proximal to the anus [74–77]. TNBS haptenizes self and microbial proteins, which makes them available to initiate an immune response in the host's own immune system [78–80]. Control animals received the same volume of saline delivered similarly, as is standard in the field. Body weight was monitored. Behavioral testing took place 1-week after the induction of colitis after which rats were euthanized by decapitation. Colons were quickly removed, rinsed with ice-cold physiological saline (0.9%) and cut open longitudinally to enable macroscopic scoring for damage and inflammation, including adhesions, diarrhea and degree of ulceration. This score was adapted from those previously reported [74, 77] and is described in the Supplementary Materials. An ~100 mg sample of colon was excised, snap frozen and stored at −80 °C until assayed for myeloperoxidase (MPO) activity, as previously described [74–77] and in the Supplementary Materials.

Behavioral measures

Locomotor activity. Ambulatory activity was assessed using the Opto-Varimex-5 Auto Track (Columbus Instruments, Columbus, OH, USA) infrared beam activity monitor with a 17.5"x17.5" arena as previously described [81]. Day 0 testing occurred prior to TNBS or saline administration. Data were normalized within an animal to a percentage of its Day 0 activity.

Elevated plus maze (EPM). Animals were subjected to handling and body weight measurement in the behavior testing room at least 5 days prior to anxiety testing. EPM (Med Associates, Fairfax, VT, USA) testing occurred on Day 7 following colitis induction under dim light and with a white noise background. EPM was performed for 5 min as previously described [82] and is detailed in the Supplementary Materials.

Biochemical and molecular measures

Corticosterone enzyme-linked immunosorbent assay (ELISA). After behavioral experiments were completed on Day 7 after the induction of colitis, trunk blood was collected as previously described [83, 84] and plasma corticosterone levels were assayed using a commercially available ELISA kit (Cayman Chemical Company, Ann Arbor, Michigan, USA, #500655), according to the manufacturer's protocol.

Endocannabinoid measurements. Excisions of brain structures were performed on ice as described previously [85] and samples were immediately snap frozen and stored at −80 °C. Analysis of AEA, and the other primary eCB 2-arachidonoylglycerol (2-AG), was conducted through liquid chromatography/tandem mass spectrometry on an Eksigent ekspert micro liquid chromatographer 200 coupled to an AB Sciex Qtrap 5500 mass spectrometer (SCIEX, Framingham, MA, USA) as previously described [82, 86] and in the Supplementary Materials.

Enzyme activity assays. Brain structures were excised on ice [85] and samples were then immediately snap frozen and stored at −80 °C. Brain tissues were homogenized and membrane fractions were isolated as described previously [50].

The activity of the enzyme FAAH, which is responsible for the degradation of AEA, was measured as the conversion of [^{3}H]-AEA to [^{3}H]-ethanolamine [87]. Similarly, monoacylglycerol lipase (MAGL) activity was measured as the conversion of [^{3}H]-2-oleoylglycerol (2-OG) to [^{3}H]-glycerol [88]. The maximal hydrolytic activity (V_{max}) of FAAH and MAGL and the binding affinities (K_m) of AEA for FAAH and 2-AG for MAGL were determined by fitting the data to the Michaelis-Menten equation using Prism v8 (GraphPad, San Diego, CA, USA, RRID:SCR_002798).

Gene expression analysis. mRNA isolation and cDNA synthesis was performed as previously described [50, 83, 84] and detailed in the Supplemental Materials, using magnetic bead homogenization with a TissueLyser LT (Qiagen, Hilden, Germany) and the RNeasy Plus Universal Mini Kit (Qiagen, #73404) on a Qiacube (Qiagen, RRID:SCR_018618) followed by the QuantiTect Reverse Transcription Kit (Qiagen, #205314) according to the manufacturer's protocols. Primers for genes of interest were designed using IDTDNA PrimerQuest (Coralville, Iowa, USA) and acquired from IDTDNA (Table S1). qPCR was performed as previously described (1 min at 90 °C, 40 cycles of 95 °C for 5 s and 60 °C for 30 s, before a final melt step) using PerfeCTa SYBR Green Fast Mix (QuantaBio, Beverly, MA, USA, #95072) on a RotoGene Q light cycler (Qiagen). Data were analyzed using the $2^{-\Delta\Delta CT}$ method. Data were normalized so the average of the saline group was 1.

Pharmacological intervention - behavioral studies

As global FAAH inhibition is associated with suppression of colonic inflammation [89–97], we administered the FAAH inhibitor intracerebroventricularly (icv) to be able to establish the importance of central FAAH inhibition on colitis-induced anxiety. Rats underwent intracranial cannulations as previously described

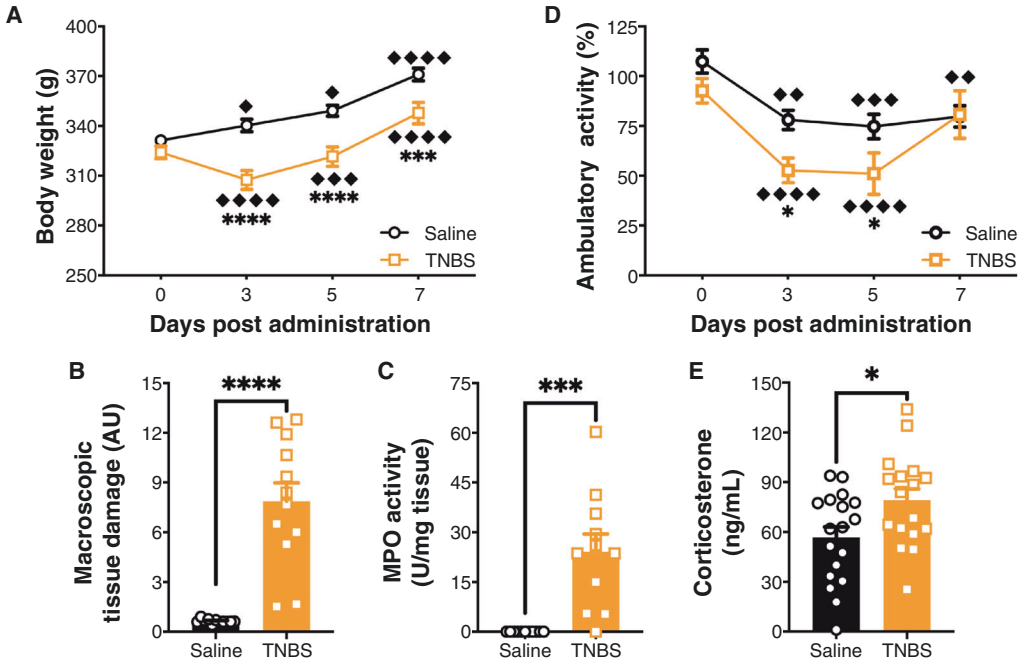

Fig. 1 TNBS-induced colitis phenotype. A There was a significant interaction on body weight between time post-administration and trinitrobenzene sulfonic acid (TNBS) administration, and a main effect of both time and colitis. Saline animals gained weight each day. TNBS animals initially lost weight, but gained after Day 3. There were no differences at baseline between conditions, but there were at Days 3, 5, and 7. $n = 12$/group. ♦ $p < 0.05$, ♦♦♦ $p < 0.001$, ♦♦♦♦♦ $p < 0.0001$ compared to previously recorded weight in same condition. ***$p < 0.001$, ****$p < 0.0001$ saline vs. TNBS on the same day. **B** TNBS administration at Day 7 post-administration led to a significant increase in macroscopic tissue damage. $n = 12$/group. ****$p < 0.0001$ *t* test saline vs. TNBS. **C** TNBS administration at Day 7 post-administration led to a significant increase in myeloperoxidase activity (MPO). Each 1 Unit (U) of MPO activity was the amount of enzyme required to split 1μmol H_2O_2 per min at 25 °C. $n = 12$/group. ***$p < 0.001$ *t* test saline vs. TNBS. **D** There were no differences in locomotor activity between saline and TNBS groups at baseline, but there was a reduction in ambulatory activity at Day 3 and Day 5, but not at Day 7. Saline and TNBS administered animals both showed reductions in ambulatory activities compared to their baselines. $n = 4$–6/group. ♦♦ $p < 0.01$, ♦♦♦ $p < 0.001$, ♦♦♦♦ $p < 0.0001$ compared to Day 0 in same condition. *$p < 0.05$ saline vs. TNBS on the same day. **E** TNBS led to a significant increase in plasma corticosterone levels. $n = 17$–18/group at Day 7 post-administration. *$p < 0.05$ *t* test saline vs. TNBS. Saline = left, black bars with circles. TNBS = right, orange bars with squares.

[50]. Briefly, under isoflurane anesthesia and analgesic treatment (meloxicam (1 mg·kg^{-1}, subcutaneously)), rats were implanted with a 12 mm unilateral cannula into the lateral ventricle (coordinates: −0.90 mm anteroposterior, 1.4 mm mediolateral, and −2.8 mm dorsoventral from Bregma). Rats were given one week of recovery before colitis induction, and as in the previous experiments, anxiety-like behavior was tested 7 days later. On the three consecutive days before drug infusion and testing, rats were exposed to daily mock infusions. Two hours prior to EPM testing, animals received icv infusions (2 μL; 1 μL/min) of solutions containing vehicle (0.9% saline:dimethylsulfoxide (DMSO):Tween-80 [80:10:10; vol:vol:vol]) or a FAAH inhibitor (PF-04457845 (PF); Pfizer, New York, NY, USA; 100 ng and 1 μg) [98–100]. Infusers extended 2 mm past guide cannula and were left in place 1 min following infusion. Two hours following drug administration all animals were tested for 5 min in the EPM as described above. Following testing, animals were euthanized and ventricular cannula placement was confirmed with dye infusion post-mortem.

Pharmacological intervention - biochemical studies

To understand the role of CRF signaling on the colitis-induced reductions of AEA signaling, we examined the impact of sustained disruption of CRF-R1 signaling during the entire duration of colitis (i.e., 7 days) utilizing continuous drug infusion with an osmotic mini-pump (Alzet, Cupertino, CA, USA; Model 2002; 0.5 μL/h) connected to a 5 mm cannula (Alzet, Brain Infuser Kit 2) [101]. The osmotic mini-pumps were pre-loaded with vehicle (artificial cerebral spinal fluid [102]: DMSO [90:10; vol:vol]) or a CRF-R1 antagonist (antalarmin (Cayman Chemical Company, 15147); 10 μg/day) [103] and were incubated at 37 °C submerged in sterile physiological saline for 1–3 days prior to implantation. Under isoflurane anesthesia and analgesic (meloxicam (1 mg·kg^{-1}, subcutaneously)) treatment, the unilateral cannula was placed into the lateral ventricle, −0.90 mm anteroposterior and 1.4 mm mediolateral from Bregma, and the pump was placed subcutaneously. Surgeries were performed on the same day, but prior to, TNBS or saline administration. One week following surgery and colitis onset, brain regions were isolated as described above for eCB analysis. Ventricular cannula placement was confirmed post-mortem with dye infusion.

Statistical analyses

All statistics were carried out using Prism v8. For comparison of two groups, one-tailed (phenotypic confirmation of damage, corticosterone and anxiety-like behavior) or two-tailed Student's *t* tests were used (remaining data, including correlations). For comparison of repeated measures, a repeated measure analysis of variance (ANOVA) or mixed-effect analysis was performed. For comparisons between two independent variables, two-way ANOVAs were performed. For all ANOVA analyses, significant interactions and main effects were reported, and specific group comparisons were made using Fisher's Least Significant Difference tests. Planned comparisons based on a priori hypothesis were performed using independent *t* tests. t- or F-values, *p* values and eta squared (R^2) are reported, as well as Pearson correlation coefficients (r) (weak = 0.1 < 0.3; moderate = 0.3 < 0.05; strong = 0.5 < 0.7; very strong = >0.7). Data are presented as mean ± standard error of the mean (SEM). Outliers were removed using the ROUT

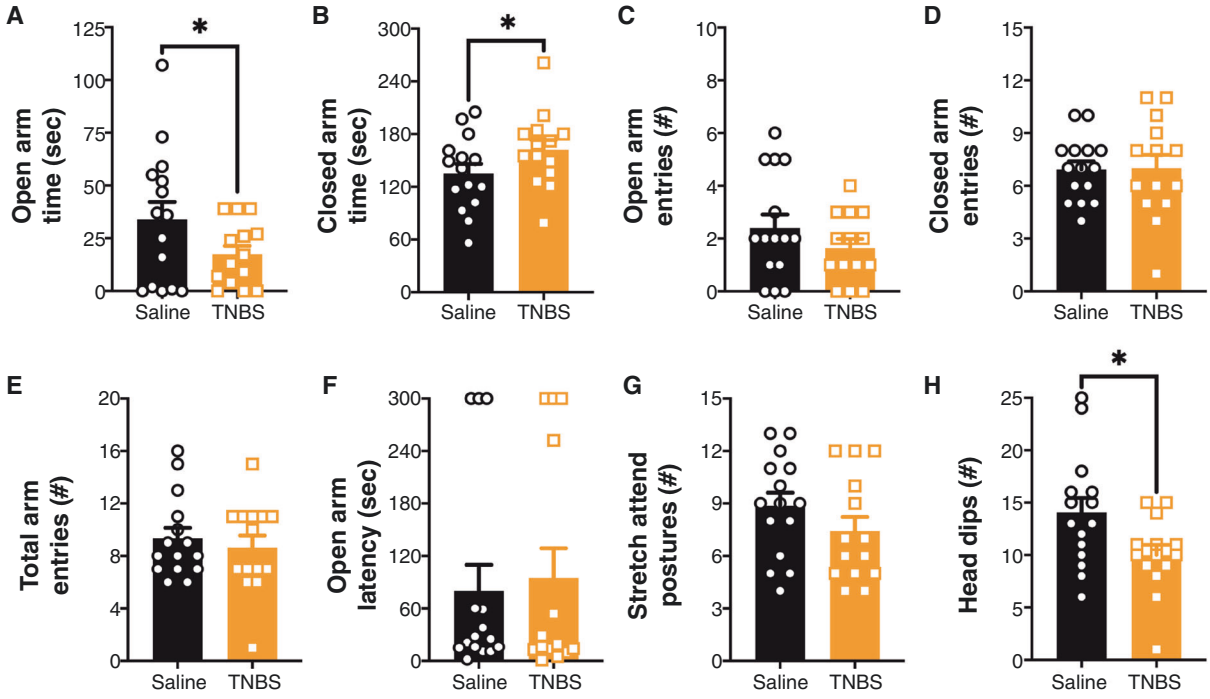

Fig. 2 TNBS-induced colitis leads to anxiety-like behavior in the EPM at Day 7 post-administration. At Day 7 post-trinitrobenzene sulfonic acid (TNBS) administration there was an increase in anxiety-like behavior as indicated by a (**A**) reduction in time spent in the open arms, (**B**) increase in time spent in the closed arms and (H) a reduction in head dips; however, there were no effects on (**C**) open arm entries, (**D**) closed arm entries, (**E**) total arm entries, (**F**) latency to open arm or (**G**) stretch attend postures. $n = 14–15$/group. *$p < 0.05$ t test saline vs. TNBS. Saline = left, black bars with circles. TNBS = right, orange bars with squares.

method [104], set to a 1% threshold, as previously described [83]. $p < 0.05$ was considered statistically significant. Detailed statistics for data represented in figures, as well as correlation values, are reported in Tables S2–4.

RESULTS

Colitis induction produced behavioral indices of increased anxiety-like behavior

Data presented on colitis phenotype (i.e., weight loss, macroscopic tissue damage and MPO activity; Fig. 1A–C) are a representative set of data from the rats used for AEA and 2-AG analysis, but these effects were consistent across all experimental cohorts. Animals administered TNBS lost weight between Day 0 and Day 3 but started gaining it again thereafter (Fig. 1A); whereas controls gained weight daily. There were no differences at baseline between saline and TNBS-treated animals, but TNBS-treated animals weighed less than saline-treated animals all other days (Fig. 1A).

Rats administered TNBS also show an increase in colonic inflammation as measured by macroscopic tissue damage (Fig. 1B) and MPO activity (Fig. 1C) 7 days after treatment. Together these results indicate that peak weight loss occurred at Day 3, and gut inflammation was sustained at Day 7 after treatment.

Before initiating behavioral tasks, we wanted to verify that there would be no locomotor deficits (Fig. 1D), as reductions in locomotor activity can be a significant confound in behavioral tests of anxiety [105]. Using a mixed-effect model to analyze, we found that animals in both saline and TNBS groups had reduced locomotor activity after the first test day, likely due to habituation to the task. Animals administered TNBS showed reduced activity at Days 3 and 5 compared to saline-treated rats, but this difference was not present at baseline or Day 7. TNBS-treated animals also exhibited an elevation in circulating levels of corticosterone (Fig. 1E), the hormonal endpoint of the HPA axis, at Day 7. Corticosterone levels were strongly positively correlated with damage. Based on these results, we proceeded with anxiety-like behavior testing on Day 7, as at this time point animals showed no locomotor deficits, but TNBS-treated animals had sustained gut inflammation.

In the EPM, TNBS-treated animals had increased anxiety-like behavior as indicated by a reduction in the time spent in the open arms, increased time spent in the closed arms and reduction in head dips (Fig. 2A, B, H). There were no changes in open arm entries, closed arm entries, total arm entries, latency to enter open arms, or stretch attend postures (Fig. 2C–G). There was no correlation between damage score and any of these measures.

TNBS-induced colitis altered central endocannabinoid levels

In order to investigate the role of the eCB system on colitis-induced anxiety-like behavior, we analyzed whether the eCB system was altered in Day 7 TNBS-treated rats. We found that AEA levels were reduced in the amygdala, medial prefrontal cortex and hippocampus but not in the hypothalamus in animals treated with TNBS (Fig. 3A–D). AEA levels, overall, were moderately or strongly, negatively correlated with macroscopic tissue damage, except in the hypothalamus. Concomitantly, TNBS treatment led to an increase in the hydrolytic activity of AEA's metabolic enzyme, FAAH (V_{max}), in the amygdala and medial prefrontal cortex, but not in the hypothalamus or hippocampus (Fig. 3E–H). TNBS treatment resulted in no differences in the binding affinity of AEA for FAAH (K_m) (Table S3) in the amygdala, hypothalamus or hippocampus; however, animals with colitis had an increase in K_m in the medial prefrontal cortex. Similar to AEA levels, FAAH hydrolytic activity, but not binding affinity, overall, was strongly, but positively, correlated with damage score, particularly in the amygdala and medial prefrontal cortex. These data indicate that colitis is associated with an increase in corticolimbic FAAH-activity and a decline in the pool of AEA.

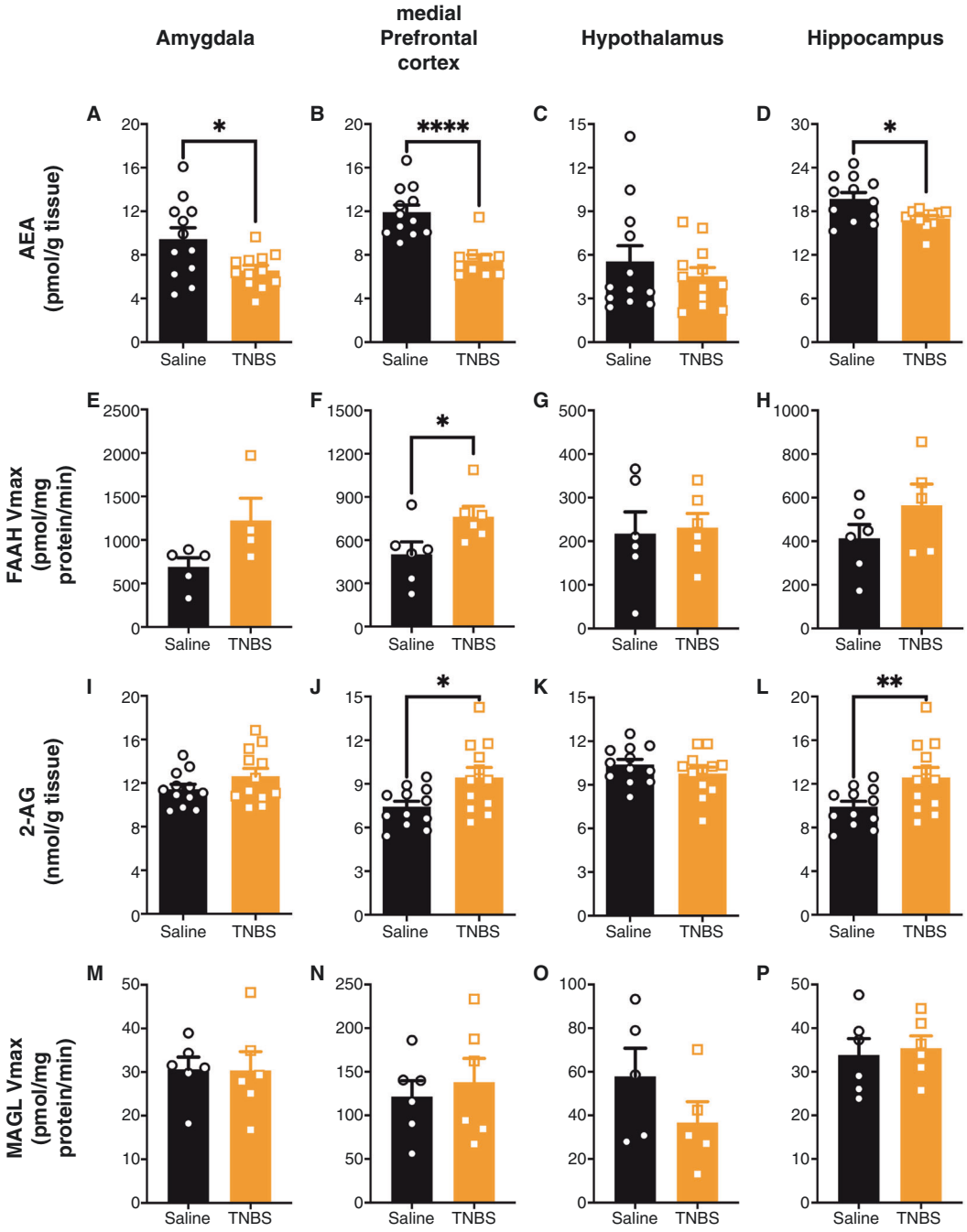

Fig. 3 Colitis altered central endocannabinoid levels. Following trinitrobenzene sulfonic acid (TNBS) administration, at Day 7, anandamide (AEA) levels were reduced in the (**A**) amygdala, (**B**) medial prefrontal cortex and (**D**) hippocampus, but not the (**C**) hypothalamus. Concomitantly, there was an increase in AEA's metabolic enzyme's (fatty acid amide hydrolase (FAAH)) activity (V_{max}), in the (**E**) amygdala, (**F**) medial prefrontal cortex, with no differences in the (**H**) hippocampus or (**G**) hypothalamus. In contrast to AEA levels, Day 7 2-arachindonylyl glycerol (2-AG) levels were increased in the (**J**) medial prefrontal cortex, (**L**) hippocampus, and no significant changes occurred in the (**I**) amygdala or (**K**) hypothalamus with TNBS administration. There were no differences at Day 7 post-administration in the activity of 2-AG's metabolic enzyme (monoacylglycerol lipase (MAGL); V_{max}) in the (**M**) amygdala, (**N**) medial prefrontal cortex, (**O**) hypothalamus or (**P**) hippocampus from colitis. $n = 9–12$/group for levels and $n = 4 = 6$/group for enzyme activity. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$, *t* test saline vs. TNBS. Saline = left, black bars with circles. TNBS = right, orange bars with squares.

In contrast to AEA levels, 2-AG levels were increased in the medial prefrontal cortex and hippocampus, but not significantly changed in the amygdala or hypothalamus (Fig. 3I–L), following TNBS administration. There was no impact of TNBS-colitis on the activity of 2-AG's metabolic enzyme (V_{max}; Fig. 3M–P), MAGL, or its K_m (Table S3) in the amygdala, medial prefrontal cortex, hypothalamus or hippocampus. Overall, only hippocampal 2-AG was strongly, positively, correlated with damage; neither MAGL activity (excepting the hypothalamus) nor binding affinity correlated with damage score.

In addition, we examined the gene expression levels of a number of the molecular components of the eCB system (Table S4). There were no significant changes in any genes examined.

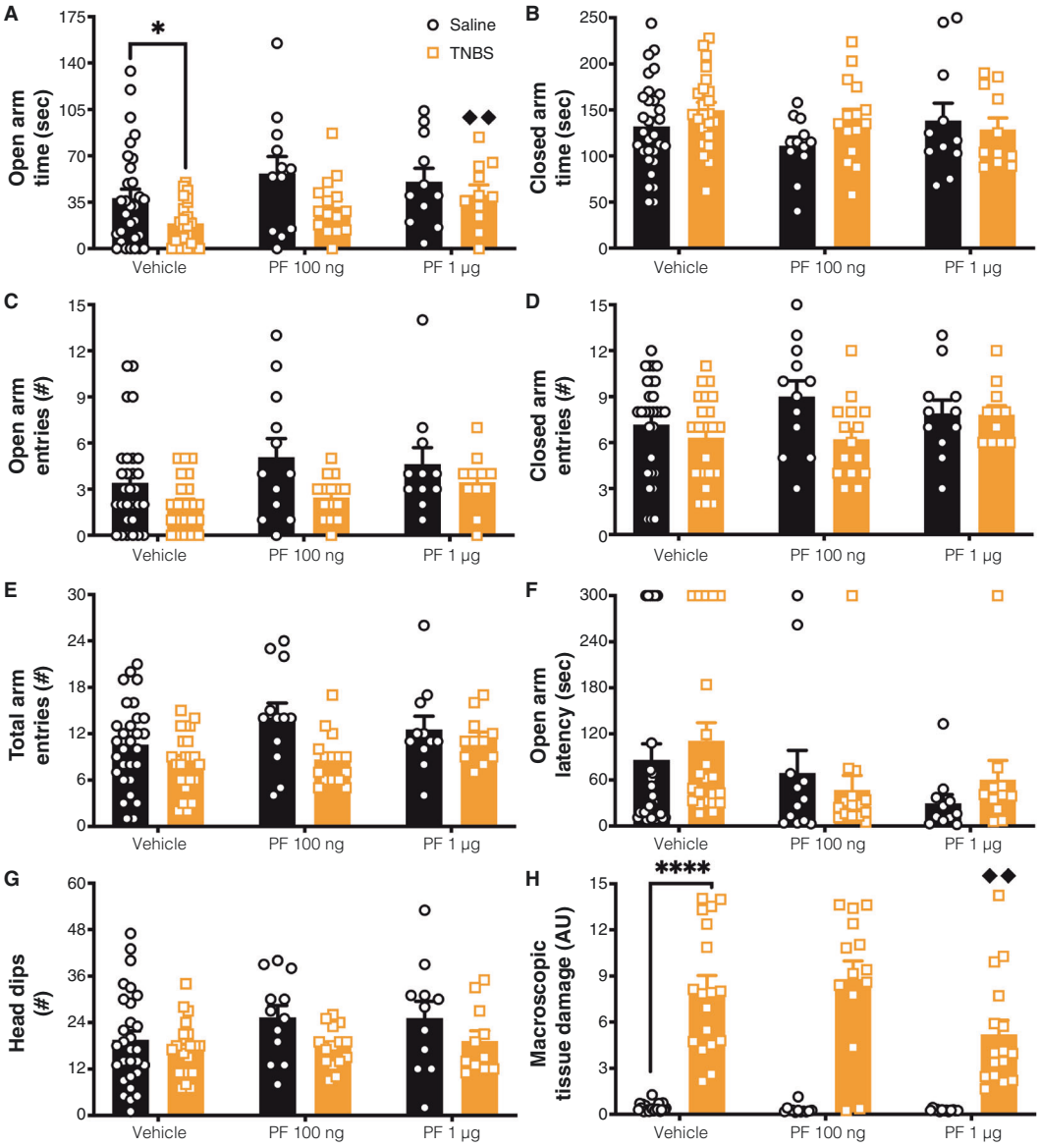

Fig. 4 Central FAAH inhibition reversed colitis-induced anxiety-like behavior. We examined (**A**) time in the open arms and found main effects of colitis reducing open arm time, and PF administration increasing it. Planned comparisons revealed that there was a reduction in open arm time between the vehicle saline vs. TNBS groups. TNBS animals treated with 1 µg PF had significantly increased open arm time relative to vehicle treated animals. Colitis reduced (**C**) open arm entries and (**G**) head dips, but there was no effect of FAAH inhibition or interaction between colitis and PF on these measures. For (**B**) closed arm time, (**D**) closed arm entries, (**E**) total arm entries and (**F**) open arm latency there was not a significant effect of colitis, FAAH inhibition or interaction between PF and TNBS. **H** Macroscopic tissue damage was increased with colitis, but this was modulated by PF administration, specifically, with the 1 µg dose, which had reduced damage scores compared to the TNBS vehicle group. $n = 12–24$/group. *$p < 0.05$, ****$p < 0.0001$. saline vs. TNBS within same treatment, ◆◆ $p < 0.01$ vs. vehicle of same condition. Saline = left, black bars with circles. TNBS = right, orange bars with squares.

Central FAAH inhibition reversed colitis-induced anxiety-like behavior

To determine if the elevated FAAH activity and reduced AEA levels contributed to the increase in anxiety-like behavior, we examined if acute inhibition of FAAH, to elevate AEA signaling, would counter the colitis-induced anxiety. Given that FAAH activity was broadly increased following colitis, we opted to perform a central inhibition of FAAH to determine the impact of widespread central elevations in AEA signaling. We administered a FAAH inhibitor (PF) acutely at two doses and examined anxiety-like behavior in the EPM in order to investigate the relevance of changes in eCB levels to the increase in anxiety-like behavior.

Open arm time was reduced with TNBS-induced colitis and increased with administration of the FAAH inhibitor (Fig. 4A). Based on the outcomes of the initial experiments in this study, we made the a priori hypothesis that colitis would increase anxiety-like behavior and that treatment with a FAAH inhibitor would reverse that. Analysis of these planned comparisons demonstrated that, even following cranial surgery, there was a reduction in open arm time in TNBS-treated animals treated with saline vs. control animals treated with saline, supporting the robustness of this behavioral effect (as was seen in Fig. 2A). Administration of PF dose-dependently reversed the reduction of open arm time in the EPM; whereas 100 ng PF treatment in TNBS-treated animals partially reversed the anxiety phenotype (as it was no longer significantly different relative to vehicle-control animals, but not different from TNBS-vehicle animals), TNBS-treated animals

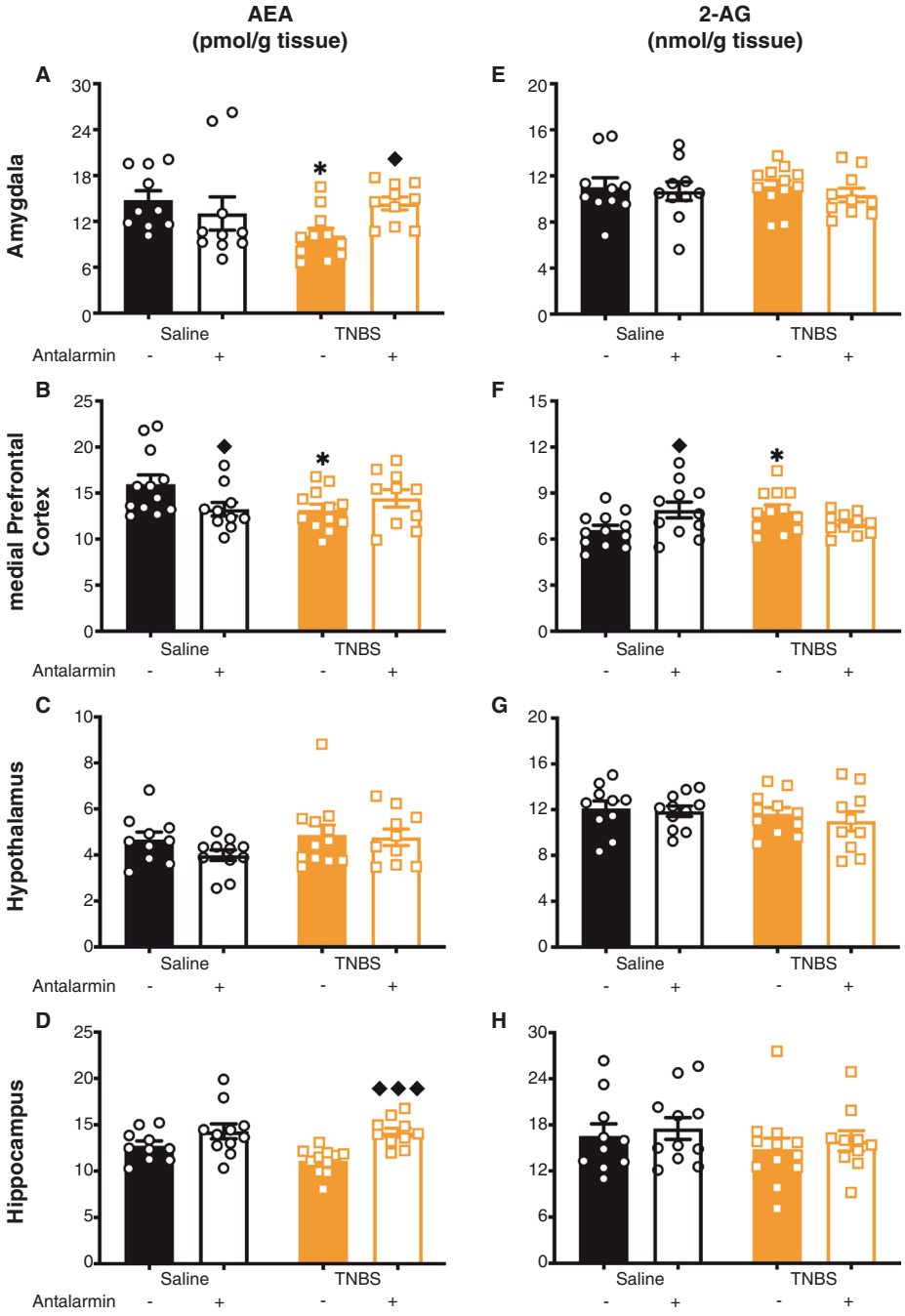

Fig. 5 Colitis-induced reductions in AEA levels were mediated by CRF-R1. Antalarmin (an antagonist for the corticotropin releasing factor receptor type 1 (CRF-R1)) reversed the colitis-induced reduction in anandamide (AEA) levels in the (**A**) amygdala and (**D**) hippocampus. In the (**C**) hypothalamus, there was no TNBS effect (as in Fig. 3C), and no effect of antalarmin. In the (**B**) medial prefrontal cortex, there was an interesting effect where antalarmin in the saline animals reduced AEA levels, but did not alter the TNBS-induced reduction of AEA levels. However, antalarmin had no effect on 2-arachidonylglycerol (2-AG) levels in the (**E**) amygdala, (**G**) hypothalamus or (**H**) hippocampus; however, in the (**F**) medial prefrontal cortex, antalarmin increased 2-AG levels in saline animals, but did not alter 2-AG levels in TNBS animals. $n = 10–12$/group. *$p < 0.05$ saline vs. TNBS of same treatment, ◆ $p < 0.05$, ◆◆◆ $p < 0.001$ vs. vehicle of same condition. Saline = left, black bars with circles. TNBS = right, orange bars with squares. Vehicle = left of each pair, filled bars. Antalarmin = right of each pair, open bars.

treated with 1 µg PF exhibited significantly elevated time in the open arms relative to the TNBS-vehicle treated animals (Fig. 4A). We found that TNBS-treated rats had reduced open arm entries and head dips, but neither of these were influenced by administration of PF (Fig. 4C, G). There were no significant effects on closed arm time, closed arm entries, total arm entries or open arm latency as a result of TNBS-treatment or PF administration (Fig. 4B, D–F).

Macroscopic damage of the colon was increased in TNBS-treated rats, but this was lower in the 1 µg PF dose compared to its vehicle (Fig. 4H). Specifically, there was a significant reduction in the 1 µg PF group in most scorable items, including ulceration score, diarrhea and bowel thickness (data not shown). MPO activity was also increased with TNBS-treated animals, but was not influenced by central administration of 1 µg PF (Table S2). In addition, in most indices measured, there were weak, negative

correlations with damage score. Together these results indicate that central FAAH inhibition reverses colitis-induced suppression of open arm time and reduces macroscopic colonic tissue damage score.

Central CRF-R1 signaling regulates colitis-induced alterations in AEA

As increased FAAH activity was found to contribute to the generation of colitis-induced anxiety, and given previous work that showed that activation of CRF-R1 can induce FAAH hydrolysis of AEA during psychological stress [50, 106], we examined CRF signaling as a potential upstream mechanism in our model [68].

We investigated if blocking central CRF-R1 with an antagonist, antalarmin, for the 7-days post-TNBS administration altered colitis-induced changes in AEA levels. While this caused no changes to TNBS-induced increases in macroscopic tissue damage (Table S2), antalarmin reversed colitis-induced reductions in AEA levels (Fig. 5A, D) in the amygdala and hippocampus. As in Fig. 3C, there was no effect of TNBS on hypothalamic AEA levels, nor was there an effect of antalarmin (Fig. 5C). In the medial prefrontal cortex, antalarmin alone reduced AEA levels, but did not alter TNBS-induced changes in AEA levels (Fig. 5B). CRF-R1 antagonism had no effect on 2-AG levels in the amygdala, hypothalamus and hippocampus (Fig. 5E, G, H). However, in the medial prefrontal cortex (Fig. 5F), antalarmin administration in saline animals increased 2-AG but did not alter 2-AG levels in the TNBS-treated animals. No overall pattern emerged with regards to correlation with macroscopic damage. Together these data demonstrated that the colitis-induced reductions in AEA, at least within the amygdala and hippocampus, were driven through CRF-R1 signaling.

TNBS-colitis also reduced central AEA levels in female rats

Comorbid anxiety with IBD is not restricted to males and is also observed in females [16, 27]. To this end, we examined if there was a similar alteration in the eCB system as a result of TNBS administration in female rats in order to understand potential generalizability across sexes of the phenomenon we have demonstrated. Female rats also showed an increase in macroscopic tissue damage and MPO activity. Similar to male rats, there was a reduction (although smaller) of AEA levels in the medial prefrontal cortex (Fig. S1B) and no change in the hypothalamus (Fig. S1C), but reductions seen in males in the amygdala and hippocampus ($p > 0.05$) (Fig. S1AD) were not seen. Also, in contrast to male levels, there were no alterations ($p > 0.05$) in 2-AG levels in any of these areas (Fig. S1E–H). Unlike what was seen in males, neither AEA nor 2-AG levels were correlated with macroscopic damage.

DISCUSSION

We demonstrate that the TNBS-model of colitis in rats, consistent with other rodent models of colitis [70–73, 107–109], produces an increase of anxiety-like behavior (Fig. 2), similar to the well-established comorbidity of colitis and anxiety in humans [1–5, 22, 27]. Colitis also resulted in an increase in FAAH-mediated hydrolysis of AEA across corticolimbic structures (Fig. 3) important for the regulation of affective behavior [105]. The magnitude of reductions in AEA and increases in FAAH activity is overall correlated with macroscopic damage, suggesting that the greater the disease severity the larger the impact on FAAH and AEA. This reduction in AEA signaling was mediated by central CRF-R1 (Fig. 5), and it contributed to the development of colitis-induced anxiety, as this was reversed by central inhibition of FAAH (Fig. 4). Together these data indicate that sustained peripheral inflammation can modulate affective behavior through an attenuation of central AEA signaling, which is driven by a recruitment of stress-responsive signaling systems. As such, this would suggest that inhibition of FAAH could represent a novel therapeutic approach to managing comorbid anxiety in peripheral inflammatory diseases.

Endocannabinoid signaling is well established to regulate affective behavioral processes such as anxiety through actions localized within the amygdala, medial prefrontal cortex and hippocampus [43, 110]. The current data extend these findings to demonstrate that a sustained inflammatory state results in a loss of central AEA signaling that contributes to the development of anxiety. Previous work has suggested that AEA and FAAH may be involved in behavioral changes produced by inflammation. For example, administration of the viral mimetic poly I:C produces changes in thermoregulation, pain sensitivity and anxiety, which are reversed by administration of a FAAH inhibitor [111]. In addition, acute early life inflammatory events have been shown to reduce social behavior during adolescence, a process that is also reversible through pharmacological inhibition of FAAH [112]. The current data, however, are the first demonstration that a sustained peripheral inflammatory insult reduces AEA levels and increases FAAH activity via central CRF-R1 activity, to increase anxiety, and thereby provides a putative model by which peripheral inflammation can modulate the central regulation of affective behavior.

Recent work from our group shows that both male and female mice exhibit anxiety-like behavior in a dextran sulfate sodium model of colitis [73]. Here we show in males that anxiety-like behavior induced by TNBS colitis is mediated through an CRF-R1 suppression of AEA levels. We also demonstrate in female rats that TNBS administration leads to a reduction of AEA levels, albeit to a lesser magnitude than in the males. It is possible that in females this reduction of AEA levels also contributes to the anxiety like-behavior observed across models, as previous work has demonstrated a correlation between AEA levels and anxiety-like behavior [113]; and, the difference in magnitude of AEA changes between sexes may contribute to the sex differences in anxiety-like behavior previously observed [73].

The finding that CRF-R1 activity mediates the colitis-induced reduction in AEA content broadens previous work indicating that CRF and FAAH exhibit an intricate relationship in the regulation of affective behavior [50, 106]. Chronic exposure to glucocorticoids results in sustained elevations in central FAAH hydrolysis and this is mediated by the elevated CRF/CRF-R1 activity as this effect of glucocorticoids is blocked by continuous administration of a CRF-R1 antagonist and is replicated by genetic overexpression of forebrain *Crh* [50, 106]. Inflammation is well-established to increase drive on the HPA axis, likely in an auto-regulatory manner where the elevations in potent anti-inflammatory glucocorticoids act to dampen inflammation itself [35, 114, 115]. Consistent with this, our data replicate previous studies [116, 117] showing that TNBS-colitis results in chronic elevations in corticosterone secretion, which is in line with the established increase in central *Crh* expression in rodent models of gut inflammation [54, 57, 59, 66, 67, 69, 89]. These data would suggest colitis-induced inflammation produces sustained adrenocortical responses, which result in the upregulation of CRF levels in the brain, producing an increase in FAAH activity and a reduction in AEA signaling. Consistent with previous work [50], this effect of glucocorticoids and CRF-R1 signaling on FAAH does not appear to be mediated by transcriptional changes in gene expression.

An unexpected finding of this study was that acute central inhibition of FAAH reduced the severity of colitis. Endocannabinoids are well-established anti-inflammatory molecules [118, 119], and FAAH inhibitors have been repeatedly found to be capable of reducing multiple aspects of gut inflammation across several animal models [89–91, 96, 120, 121]. While these anti-inflammatory effects of AEA signaling in colitis are largely due to peripheral actions on colonic tissue directly or local immune cells, there is evidence that central cannabinoid type 1 receptors (CB1) contribute to reducing inflammation in colitis

[122]. It is also possible that PF entered the circulation, elevating AEA levels outside of the brain to influence the damage score. That said, the magnitude of reduction of colitis damage in the current study from central FAAH inhibition was relatively minor. Regardless, these data support previous findings that central FAAH inhibition is capable of modulating colonic inflammation.

In addition to reduced AEA, we also found that colitis was associated with elevations in 2-AG throughout several corticolimbic structures (Fig. 2). Prolonged elevations in CRF signaling have been found to produce elevations in tissue 2-AG levels [106]. This effect was not as robust as the reduction in AEA, as it was largely lost following cannulation surgery, and not seen in females. Unlike the reductions in AEA, the relevance of these increases in 2-AG during colitis has yet to be elucidated. As stress-induced elevations in 2-AG signaling have been proposed to produce both anxiolytic and anxiogenic effects [43], future work is required to examine this question in more depth.

We did not investigate which receptors mediate the anxiolytic effect observed herein. Previous work with psychological stress points to a role for CB1 in this regard [43]. AEA's anxiolytic effect seems to be due to its signaling at the CB1 receptor [43]. Furthermore, differently from AEA, the ability of 2-AG to buffer anxiety is linked to signaling at the CB1 receptor, but also the CB2 receptor [123–126]. Given our anxiolytic effect was observed with FAAH inhibition, which elevates AEA and not 2-AG, it was likely through a CB1 mechanism; however, future work will have to elucidate these specifics, especially as AEA can also act on the transient receptor potential cation channel subfamily V member 1 (TRPV1) and peroxisome proliferator-activated receptors (PPARs). More so, FAAH also metabolizes oleoylethanolamide (OEA) and palmitoylethanolamide (PEA) which have also been implicated in anxiety and inflammation [127–133].

Together, these data demonstrate that the induction of colitis results in a suppression of central AEA signaling via a CRF-R1 mediated increase in FAAH activity, which then promotes the development of anxiety. Given the similarities seen to chronic stress and glucocorticoid exposure [50, 106], this suggests that compromised central AEA signaling may be a broad mechanism favoring the development of anxiety in response to a host of psychological or physiological insults, particularly those that produce increased demand on the HPA axis. As such, these data would support the investigation of FAAH inhibitors as a treatment approach in chronic inflammatory disease states, both for the inflammatory pathology itself but also the psychiatric comorbidities. FAAH inhibitors have already been established in humans to reduce anxiety that develops during cannabis withdrawal [134], dampen the subjective and physiological responses to stress [135] and produce clinically relevant anxiolysis in social anxiety disorder [136], indicating their feasibility and potential efficacy for the management of affective disturbances in humans. In line with this, many individuals with chronic inflammatory diseases use cannabis which is associated with broad improvements in affective state and quality of life [137, 138], suggesting that cannabinoids may also have some therapeutic value in this domain. Therefore, there is potential for FAAH inhibition on both the primary outcomes of inflammatory diseases, as well as comorbid psychiatric issues and quality of life measures, serving as a dual-pronged therapeutic.

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AUTHOR CONTRIBUTIONS

HAV, CMK, QJP, KAS, and MNH were involved in the design of the manuscript. HAV, MM, CMK, VC, KT, MQ, KL, AS, and MNH performed experiments. HAV, CMK, QJP, KAS, and MNH analyzed data. HAV prepared figures. HAV, QJP, KAS, and MNH wrote the manuscript with input from all authors.

ADDITIONAL INFORMATION

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