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MODULATION OF MEMORY FOR STRESSFUL EXPERIENCES: REVEALING HYPERMNESIA

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To Valentina

If in the twilight of memory we should meet once more, we shall speak again together and you shall sing to me a deeper song.
And if our hands should meet in another dream, we shall build another tower in the sky.

Kahlil Gibran, The Prophet

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GENERAL INTRODUCTION

The American Psychological Association defines Hypermnesia as a condition characterized by an extreme degree of memory retention and retrieval (American Psychiatric Association, 2013).

In the field of memory research, substantial attention has always been given to the completely opposite situation: memory loss. Memory loss has long been recognized as the principal characteristic of several mental disorders (e.g. Alzheimer Disease) and it is principally related to impairments in memory retrieval and more generally, to hypofunction of memory processes (Jahn, 2013). On the other hand, memory hyperfunction has to be considered an equally serious memory alteration which can lead, at the same way of memory loss, to many psychiatric disorders such as the Post-Traumatic Stress Disorder (PTSD) characterized by intrusive thoughts and flashbacks due to an excessive and involuntary memory retrieval of a negative past experience (McNally, 2006, Brewin, 2011, Parsons and Ressler, 2013).

Recently, some individuals with extraordinary autobiographical memory abilities have been identified. These subjects have a superior ability to recall specific details of autobiographical events and this memory phenomenon is called Highly Superior Autobiographical Memory (HSAM) (LePort et al., 2012). HSAM subjects have the natural ability to recall past autobiographical events independently from the emotional charge linked to the experience lived. Therefore, investigating the neurobiological mechanisms sustaining HSAM may expand our basic knowledge on maladaptive memory processes in psychiatric conditions such as PTSD, conducting to new scenario in the research for innovative therapeutic targets.

Currently, there is no effective drug treatment for PTSD cure (Hoskins et al., 2015) and the most practiced therapeutic approach is represented by the cognitive behavioral therapy (Milad and Quirk, 2012, Furini et al., 2014). The cognitive behavioral therapy is based on the repeated exposure of patients to trauma-related reminders, in a safe situation, thus lacking of negative consequences for the subject (Abramowitz, 2013). In the recent years, the research in the field of PTSD is more and more moving toward the identification of pharmacological approaches aimed at treating not only the symptoms but rather the causes of the pathology, which combined with the cognitive behavioral therapy, may improve efficacy (de Quervain et al., 2011, Trezza and Campolongo, 2013, Singewald et al., 2015). Considering the features of PTSD, a good drug should be able to interfere with the excessive retrieval and with the impaired extinction of the traumatic memory, and a great attention, in this regards, is nowadays given to drugs potentiating the endocannabinoid system (Campolongo et al., 2013, Morena and Campolongo, 2014, Rabinak and Phan, 2014). The endocannabinoid system is composed by endogenous lipidic retrograde neurotransmitters, called endocannabinoids, and by their receptors: the cannabinoid receptor type 1 (CB1) and type 2 (CB2). The two principal endocannabinoids are represented by N-arachidonoyl ethanolamine (anandamide, AEA) and 2-arachidonoyl glycerol (2-AG), which are metabolized by the enzymes fatty-acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL), respectively (Di Marzo, 2018). It has been shown in rodents that drugs acting as cannabinoid agonists enhance the extinction (Bowles et al., 2012, Gunduz-Cinar et al., 2013, Bitencourt et al., 2014) and attenuate the retrieval of aversive memory (Atsak et al., 2012, Morena and Campolongo, 2014, Morena et al., 2015), thus highlighting their positive potential role in the treatment of PTSD.

An important boundary to overcome, toward the identification of an optimal therapeutic approach for PTSD treatment, is represented by the susceptibility

issue. Most PTSD animal models do not take in consideration the individual differences in trauma response. The whole population is considered as belonging to a unique trauma response phenotype and only the anxiety outcomes of this pathology are studied, without considering the cognitive alterations related to PTSD. On the contrary, literature data demonstrate that among a human population exposed to a traumatic event, only a subset of people will ultimately develop PTSD, the others fully recover and mostly without any specific intervention (Javidi and Yadollahie, 2012). This evidence underlines the existence of different trauma response phenotypes among a population, and that genetic, epigenetic and environmental factors can play a crucial role in the susceptibility to PTSD development determining how the stress response to the traumatic experience can lead to aberrant and enduring alteration of memory processes (Skelton et al., 2012, Yehuda et al., 2015, Bolsinger et al., 2018).

It is widely known that normally, emotionally arousing experiences are well remembered over time with respect to no emotional events (McGaugh and Roozendaal, 2002, McGaugh, 2006, Roozendaal and McGaugh, 2011). Both positive and negative stressful situations can activate several physiological responses which end with stress mediators release (Roozendaal and McGaugh, 2011). Hundreds of preclinical studies have been conducted and indicate the key role of stress mediators, such as glucocorticoids and noradrenaline, in the modulation of memory processes (Barsegyan et al., 2014, Morena et al., 2015, Barsegyan et al., 2019). Particularly, it has been observed that enhanced levels of noradrenaline in specific brain areas are linked with improved memory performances (Barsegyan et al., 2014, Ferry et al., 2015).

Noradrenaline levels can be increased through psychostimulant drugs, such as amphetamine (Berridge, 2006, Faraone, 2018). Extensive research has proven the potent arousal-enhancing effect due to the noradrenergic modulation exerted by amphetamine (Berridge et al., 1999, Berridge and Morris, 2000), and, several

studies have shown the amphetamine enhancing effects on learning and memory processes (Martinez et al., 1980a, Martinez et al., 1980b).

Notwithstanding, the effect of amphetamine in the enhancement of memory is well-established, currently, its effect on memory generalization is poor investigated. An excessive memory retrieval in a situational reminder condition, may lead to memory generalization. From an ethological perspective, memory generalization may be both adaptive and maladaptive (Asok et al., 2018). For example, the excessive memory retrieval of a fear related situation in a context with high predatory risk, is a common adaptive response in animals, but this excessive memory retrieval in an environment lacking of an imminent threat is maladaptive and represents the core of fear memory generalization (Asok et al., 2018). The overgeneralization of a contextual fear memory is one of the most important features of trauma related disorders such as PTSD (Bian et al., 2019).

Outline

As mentioned above, recently individuals with a remarkable memory ability to recall accurately autobiographical information over time, were found. The study on HSAM provides a great opportunity to investigate the neurological underpinnings of hypermnesia. Under the supervision of Prof. Campolongo, during my PhD I have screened more than 200 subjects and identified the first European population of rare individuals with HSAM. This HSAM cohort is one of only two established HSAM populations in the world, the first study was conducted in USA by Prof. J. McGaugh (LePort et al., 2012), and up to today, 80 HSAM subjects in the USA have been identified. In **Chapter 1**, we carried out an fMRI investigation of a group of subjects identified in Italy as having HSAM. During fMRI scanning, subjects with HSAM and control subjects (subjects with normal autobiographical memory) were asked to retrieve autobiographical memories as well as non- autobiographical memories, in order

to elucidate the basic mechanisms and the brain networks which sustain hypermnesia in humans.

The excessive memory retention and retrieval (as seen in HSAM) are cardinal features of hypermnesia-related psychiatric disorders such as PTSD. As previously said, nowadays there is no specific treatment for PTSD, but the endocannabinoid system, with its peculiar role in the modulation of memory retention and retrieval, seems to be a promising therapeutic target. In **Chapter 2** we closely investigated the effects of a pharmacological manipulation of the endocannabinoid system in a preclinical model of PTSD. We recently proposed a footshock-based paradigm able to mimic both the enduring cognitive and emotional facets of PTSD pathology useful to model the extinction-based exposure therapy combined with pharmacological treatment (Berardi et al., 2014). Here, we evaluated whether activation of the endocannabinoid system, by facilitating extinction and dampening the retrieval of fear memory, could ameliorate the efficacy of extinction-based exposure therapy, leading to improvements that remain stable long after trauma exposure and pharmacological treatment termination.

Even if the endocannabinoid system looks as a promising target for the treatment of PTSD, it is also important to take in consideration the susceptibility aspect of this debilitating psychopathology. Animal models are a useful tool to investigate this issue (Musazzi et al., 2018). In **Chapter 3** we aimed at the development of an animal model of PTSD able to predict the susceptibility and the resilience phenotype, with a high translational value to human PTSD, considering both cognitive and emotional alteration long after trauma. For this purpose, we outstretched our previously validated animal model (Berardi et al., 2014) in order to identify before the extinction sessions and drug treatment, susceptible and

resilient rats in terms of over-consolidation, impaired extinction and social behavior alterations.

As previously said, not all people will respond at the same way to a traumatic experience and, moreover, among those who will develop the pathology, there are people that do not respond to the currently available treatments or relapse after the end of treatment. For this reason, it is tentative to hypothesized that there are also individual differences in PTSD's treatment success. Literature data indicate the close relationship between endocannabinoids and stress mediators, such as glucocorticoids (Morena et al., 2016, Harris et al., 2019, Siller-Perez et al., 2019). On the basis of these data the endocannabinoid system can be considered as an emotional buffer which modulates memory function differentially, depending on the level of stress and arousal lived by the subject and associated with the experimental context (Campolongo et al., 2013, Morena and Campolongo, 2014, Morena et al., 2015). Both endocannabinoids and glucocorticoids release are influenced by circadian rhythm (Vaughn et al., 2010, Spencer et al., 2018). In **Chapter 4** we investigated, in an object recognition task, how the exogenous augmentation of AEA levels at different times of the day, could influence the effect of different stress intensities on rat short-term memory.

Normally, only emotional arousing experiences can create memories that last a lifetime. Since it is well established the crucial role of stress in making some experiences unforgettable (McGaugh and Roozendaal, 2002) and that psychostimulants can enhance memory as well (Spencer et al., 2015), in **Chapter 5** we investigated in more details at preclinical level, how different stress intensities modulate the amphetamine effects on long term memory consolidation.

Memory generalization could be one of the consequences of excessive memory related to an emotional arousing experience. Psychostimulant outcomes on memory enhancement are known from many years and there are clinical data showing also their memory generalization effects (Easton and Bauer, 1997). However, the mechanisms through which psychostimulants affect memory quality is still poor investigated. In **Chapter 6** we explored the memory generalization effects induced by amphetamine and a new psychostimulant: the 3,4-methylenedioxypyrovalerone (MDPV), also called “Bath salt”. Both the psychostimulants share a similar, yet not identical mechanism of action augmenting noradrenaline and dopamine levels in the synaptic cleft. Thus, in a second experiment we aimed at evaluating the different involvement of the noradrenergic and dopaminergic system in the effects on memory enhancement, induced only by amphetamine, and on memory generalization, induced by both the two drugs.

Chapter 7 summarizes and discusses the findings of this thesis and provides conclusions and future perspectives.

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CHAPTER 1

FROM ORDINARY TO EXTRAORDINARY MEMORY: ENHANCED BRAIN ACTIVITY RELATED TO MEMORY ACCESS IN HIGHLY SUPERIOR AUTOBIOGRAPHICAL MEMORY

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Abstract

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 the first fMRI investigation of a group of subjects identified as having HSAM. During fMRI scanning, 8 HSAM and 21 control subjects were asked to retrieve autobiographical memories as well as non-autobiographic memories (e.g., examples of animals). Subjects were instructed to signal the "access" to the autobiographical memory (AM) by a key press and continue "reliving" it immediately after. Compared to controls, HSAM individuals 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 HSAM subjects as compared to controls. In contrast, HSAM subjects did not differ from controls in functional activity during the reliving phase. These findings, based on fMRI assessment, provide novel evidence of interaction of brain systems engaged in memory retrieval and suggests that enhanced activity of these systems is selectively involved in enabling more efficient access to past experiences in HSAM.

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 and semantic memories in HSAM and control subjects. While their brains were being scanned, subjects had to retrieve autobiographical as well as non-autobiographic memories (e.g. examples of animals). The HSAM subjects displayed superior ability to retrieve details of autobiographical memories, 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.

Introduction

The ability to remember personal experiences (i.e., autobiographical memory, AM) is essential for survival (Allen et al., 2013). Brain systems underlying human AM function have been classically investigated studying patients with selective memory impairments (Greenberg et al., 2003). The discovery of extremely rare individuals who spontaneously show highly superior autobiographical memory (HSAM) (Parker et al., 2006; LePort et al., 2012) provides, instead, an opportunity to investigate the brain processes underlying enhanced AM. HSAM individuals 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 (Parker et al., 2006; LePort et al., 2016; LePort et al., 2017). Prior MRI assessment of HSAM revealed that several brain regions differ in size and shape (e.g., parahippocampal gyrus, posterior insula, IPS; putamen and caudate) as well as in coherence of fiber tracts (e.g., uncinate fasciculus) when compared to those of control subjects (LePort et al., 2016). The present study investigated brain activity induced by AM using functional MRI (fMRI).

Prior evidence of detailed re-experiencing in HSAM subjects (Parker et al., 2006; LePort et al., 2016; LePort et al., 2017) suggests that HSAM subjects 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 continue to elaborate on the retrieved event (reliving phase) in as much detail as possible for the remaining part of the trial (Daselaar et a., 2008). Previous studies using this

approach have reported activity in prefrontal/medial temporal regions related to access and activity in sensory cortex related to reliving (Cabeza et al., 2007). Retrieval by HSAM subjects may therefore involve enhanced activity in sensory cortex associated with detailed reliving of re-activated 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 (Palombo et al., 2015). These findings may suggest that HSAM individuals show hyper-functioning of prefrontal/hippocampal regions. In the present study we addressed this question by performing the first fMRI examination of a group of HSAM subjects.

During fMRI, HSAM subjects and controls were asked to mentally retrieve "easy" AMs, thus to guarantee 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 went to a restaurant"). Participants confirmed the appearance of the AM through a response button (access phase) and then continue to relive the retrieved event in as much detail as possible (reliving phase; Fig. 1a) (Daselaar et al., 2008). 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" or "example of animals"; i.e., a semantic memory, SM, task). In comparison with controls, the HSAM subjects had faster access and more detailed retrieval of AMs. Memory access was associated with increased prefrontal/hippocampal functional connectivity and increased connectivity between the temporieparietal junction and sensory cortices. The prefrontal and hippocampal regions were found to be particularly involved with access to

remote AMs. In contrast, HSAM subjects did not differ from controls in brain activity during the reliving phase.

Methods

Participants

Eight HSAM individuals (5 males; mean age: 32.5 y.o.; range: 24-37 y.o.) recruited in accordance with the previous literature (LePort et al., 2012) (SI Appendix) and twenty-one control subjects (10 male; mean age: 32.5 y.o., range: 24-39 y.o.) participated in the study. All participants gave written consent to 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, which estimated a reliable statistical power of 84% for our sample size (8 HSAM subjects 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 (LePort et al., 2016)- and a significance level of 0.05.

Task and stimuli

During scanning, participants were asked to retrieve autobiographical and non-autobiographical – i.e., semantic – memories (AMs and SMs, respectively) (Fig. 1a and SI Appendix). The experiment included 3 functional runs, each including 18 memory cues: 12 AM (6 “first” and 6 “last” time events) and 6 SM trials, and a variable inter-trial interval (2-3 sec, uniformly distributed). Post-scanning, participants were asked to provide details about memories retrieved during the experiment (SI Appendix).

Magnetic Resonance Imaging and fMRI data analysis

A Siemens Allegra (Siemens Medical Systems, Erlangen, Germany) operating at

3T and equipped for echo-planar imaging (EPI) was used to acquire the functional magnetic resonance images (SI Appendix). We used SPM12 (Wellcome Department of Cognitive Neurology) implemented in MATLAB 7.4 (The MathWorks Inc., Natick, MA) for data pre-processing (SI Appendix) and statistical analyses. Each participant underwent three fMRI-runs, each comprising 477 volumes. Statistical inference was based on a two-steps random effects approach (see SI Appendix). Briefly, the first-level models included separated Access and Reliving regressors (Daselaar et al., 2008) for each of the three trial types: "first AM", "last AM", SM. For each subject we estimated contrast images that removed the activity associated with access to and reliving of SM (control condition) to the main AM conditions. For the second-level group-analysis, the single-subjects contrast images of parameter estimates were entered into a mixed design ANOVA with group (HSAM vs. control) as 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 AM in 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-unc. = 0.001), ensuring that we selected only regions activated by AM retrieval (see, e.g., Buchel et al., 1998). The statistical threshold was set to p-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 (Table 1 and Fig. 2a). The resulting activations were used to define regions of interest (ROIs) that were then used to test for condition-specific effects in interaction with the group variable, i.e., the two-way phase X group and AM type X group interactions, and the three-way phase X AM type X group interaction. For this, we considered spheres (radius, 8 mm; matching the FWHM of smoothing filter) centred on the regions activated by

AM retrieval in the two groups (Table 1) as the volume of interest (small volume correction) (Worsley et al., 1996).

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.

Functional connectivity analysis

The procedure described above allowed us to identify three regions selectively involved in AM access in HSAM subjects (i.e., the significant group X phase interaction), namely the left VMPFc, the left DMPFc, and the left TPJ (Fig. 2b). Given that these seed-regions are related 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 HSAM individuals (SI Appendix).

ROI correlations with memory access latencies and obssessiveness scores in HSAM subjects

We further exploratory analyses using multiple regression models to investigate whether the brain activity related to the access to remote or recent memories covariated as a function of the individual latency to access memories or as a function of obsessive-compulsive traits in HSAM subjects (SI Appendix).

Results

Behavioral data

Behavioral results are illustrated in Figure 1. First, we assessed whether HSAM subjects were faster than controls to access their AMs. We performed a Group (HSAM vs. control) by Trial type (first AM, last AM, SM) ANOVA on the response latencies, defining the timing of access to the specific autobiographical or semantic memories (Fig. 1b). This analysis revealed a main effect of trial type [$F(2, 54) = 21.2$, $p < .001$; $\eta^2 = .440$], indicating faster response latencies to access semantic (M = 3.368 secs) than first (4.545 secs) or last (5.059 secs) AMs. This effect was further qualified by the significant group x trial type interaction [$F(2, 54) = 5.8$, $p = .005$; $\eta^2 = .177$], indicating that HSAM subjects had faster access to autobiographical material than control subjects (first AM: 4.196 vs. 4.894 secs, $p = .029$; last AM: 4.504 vs. 5.614 secs, $p = .013$), but not to semantic information (3.681 vs. 3.056 secs, $p = .074$). The main effect of group was not significant [$F(1, 27) < 1$, n.s.]. A similar 2x3 ANOVA on the "no memory response" data (Fig. 1c) revealed a main effect of trial type [$F(2, 54) = 3.7$, $p = .032$; $\eta^2 = .120$], indicating that subjects failed more to retrieve semantic (9%) than first time (3.7%) and last (3.9%) autobiographical events. This effect was not further modulated by the group factor, as indicated by the absence of both main effect of group and group x type of trial interaction (both Fs < 1). No differences were observed between the groups in the self-evaluation ratings of the emotional level and reliving of AMs and easiness of generation and number of generated example for SMs during scanning, as assessed by 2-tailed independent sample t-tests (all Ts ranging between -1.4 and 0.9; all ps > .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. 1d) of the retrieved AMs using a Group by Remoteness of AM ("first" or "last" AM event) ANOVA. This analysis revealed a main effect of remoteness [$F(1, 27) = 188.4$, $p < .001$; $\eta^2 = .875$], indicating that first AMs were older than last AMs, 18.76 and 2.16 years, 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 x age of AM interaction (both Fs < 1.3; ps $> .257$). 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, emotional/though; (Levine et al., 2002). ANOVA. HSAM subjects retrieved a greater number of details than controls (2.0 vs. 1.4), as evidenced by the main effect of group [$F(1, 27) = 10.1$, $p = .004$; $\eta^2 = .272$]. This was particularly true for the remote events, as indicated by the three way interaction [$F(4, 108) = 2.5$, p 0 .047; $\eta^2 = .085$], showing more details reported in the event ($p = .005$), time ($p < .001$), and place ($p = .048$) categories relatively to remote AMs, but only more details in the time category ($p < .001$) relatively to recent AMs (Fig. 1e). HSAM subjects also provided higher vivid descriptions of their AMs when assessed qualitatively (all Ts ranging between 5.0 and 18.2; all ps $< .001$; SI Appendix, Fig. S2).

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

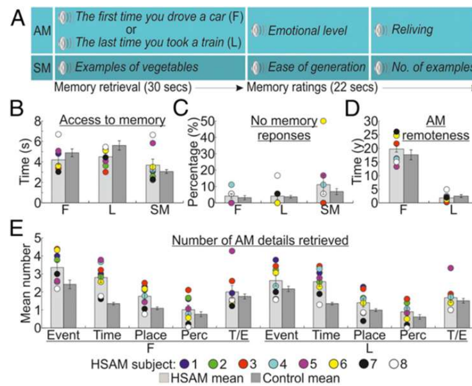

Figure 1. Task and behavioural results. a) Sequence of events in an example trial, involving 30 seconds to access and elaborate ("first", F, and "last", L) AMs or SMs, and 22 seconds to provided memory ratings; b) Time to access AMs and SMs in seconds; c) Percentages of no memory trials; d) Remoteness of reported AMs (in years); e) Mean number of details according to Levine et al.'s (2002) categories (event, time, place, perceptual, thought/emotion, T/E) and AM remoteness. In all graphs, for HSAM subjects are plotted individual scores. The error bars represent the standard error of the mean.

Assessment of Obsessive/Compulsive traits in HSAM individuals

The findings of previous studies suggest that HSAM subjects tend to have symptoms of obsessiveness/compulsiveness (LePort et al., 2017). To assess whether HSAM individual participating in the current study experienced an obsessive/compulsive symptomatology they were administered the Personality Assessment Inventory (PAI), which included – among others – the "Obsessive-Compulsive" (ARD-0) sub-scale. The average score at the obsessive-compulsive sub-scale for HSAM individuals was 67, corresponding to the 92nd percentile in terms of expressing obsessive/compulsive-related symptoms. We assessed whether faster access to AM (averaging across first and last AM type) and number of retrieved details correlated with obsessive-compulsive traits (averaging across the five categories). However, our analyses failed to reveale

any significant correlation ($r = .32$, $p = .444$ and $r = -.69$, $p = .58$, respectively).

fMRI data

The main aim of the present study was to investigate neural activation associated with AM retrieval in HSAM as compared to control subjects. In the HSAM group AM retrieval recruited a large network of areas extending along the fronto-parietal cortex (see Fig. 2a 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 (DLPFc), the left temporo-parietal junction (TPJ) and the left and right angular gyrus (ANG); plus the right insula. By contrast, we observed in the control group increased activity in few areas, involving the right superior frontal sulcus (SFS) 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 x group interaction. Three of the regions activated by AM retrieval in HSAM subjects were found to contribute selectively to memory access, namely, the left VMPFc (peaking at: x, y, z = -8, 58, 0; $Z = 3.44$; $p = .011$), the left DMPFc (x, y, z = -14, 42, 40; $Z = 3.14$; $p = .027$), and the left TPJ (x, y, z = -54, -44, 20; $Z = 3.23$; $p = .021$) (Fig. 2b). Activity in these regions selectively increased for the access vs. reliving phase (Fig. 2c-e: compare bar 5 & 7 vs. bar 6 & 8) in the HSAM group only (compare bar 5 to 8 vs. bar 1 to 4). None of the selected ROIs showed instead a selective involvement with the reliving phase in HSAM subjects. 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 x group interaction. However, the left VMPFc (peaking at: x, y, z = -12, 52, 2; $Z = 3.51$;

p = .009) showed a more selective contribution during access to remote AMs (i.e., the three way phase X AM type X group interaction). (The left DMPFc showed a similar trend, despite not statistically significant, peaking at: x, y, z = -10, 44, 44; Z = 2.75; p = .069). Activity in the left VMPFc selectively increased during access to first AMs in HSAM subjects (Fig. 2c).

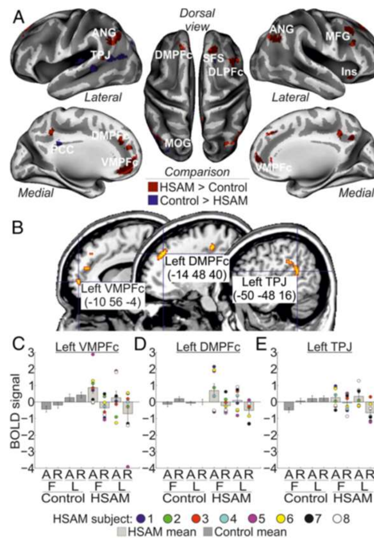

Figure 2. a) Regions activated by AM in the HSAM (red map) and in the control group (blue map) overlaid on an inflated template (see also Table 1); b) Sagittal sections on a standard MNI template showing the anatomical location of the regions selectively involved with the access phase to autobiographical memory in the HSAM group, i.e., the left VMPFc, the left DMPFc, and the left TPJ; c-e) bar plots summarizing the activity of the left VMPFc, DMPFc and TPJ, respectively, that showed increased activity during access to (vs. reliving of) AMs (compare bar 5 & 7 vs. bar 6 & 8), selectively in the HSAM group (compare bars 5 to 8 vs. bar 1 to 4). In all signal plots, the level of activation is expressed in arbitrary units (a.u., ± 90% confidence interval). For HSAM subjects are plotted the individual scores. For display purposes, all maps are displayed at a threshold of p-uncorrected = .001.

We then examined the functional coupling of the regions showing a selective involvement with AM access in HSAM subjects (i.e., the left VMPFc, the left DMPFc, and the left TPJ) with the rest of the brain. Analyses of inter-regional connectivity revealed large networks of areas functionally connected with the seed regions during AM access in HSAM subjects (Fig. 3a & Table 2). Specifically, the left VMPFc resulted connected with the medial temporal lobe, and, in particular with the left hippocampus (Hip) and with the rostral portion of the anterior cingulate cortex (rACC). The VMPFc was also found to be connected with another seed region, namely the left TPJ, and with the left and right sub-central gyrus (SCG). Activity in the left DMPFc was found instead to be synchronized during AM access in HSAM with the left and right intraparietal sulcus (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 (MCC), with the pre-central gyrus (PCG), and the middle occipital gyrus (MOG). Finally, the left TPJ showed increased coupling during AM access in HSAM subjects with adjacent regions of the left parietal cortex, such as the superior and inferior parietal lobe (SPL/IPL), plus other posterior regions in the occipital and temporal lobe, such as the left and right superior occipital gyrus (SOG) and in the left and right medial temporal cortex (MTc). The left TPJ also showed increased coupling with the ACC and the supplementary motor area (SMA), bilaterally. Despite a large variability across the individual values (see signal plots in Fig. 3b-d for some representative areas), the general pattern of activity of all of these regions showed increased coupling with the respective seed region during access to (vs. reliving of) autobiographical memories (compare bar 5 & 7 vs. bar 6 & 8), selectively in the HSAM group (compare bars 5 to 8 vs. bar 1 to 4).

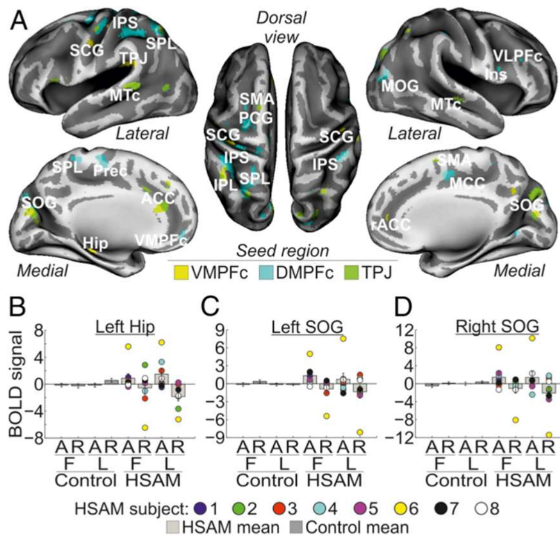

Figure 3. a) Regions showing functional connectivity with the left VMPFc (yellow map), the left DMPFc (cyan map), and the left TPJ (green map) during access to autobiographical memory in the HSAM group overlaid on an inflated template (see also Table 2). For display purposes, all maps are displayed at a threshold of p-uncorrected = .005, with a minimum cluster size of 20 voxels; b-l) signal plots showing the pattern of functional connectivity for some representative seed regions, namely, the left Hip (b), the left and the right SOG (c & d). All signal plots revealed increased coupling with the respective seed region during access to (vs. reliving of) autobiographical memories (compare bar 5 & 7 vs. bar 6 & 8), selectively in the HSAM group (compare bars 5 to 8 vs. bar 1 to 4). For HSAM subjects are plotted the individual scores. In all signal plots, the level of activation is expressed in arbitrary units (a.u., ± 90% confidence interval).

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.

Finally, we investigated whether the brain activity related to access remote or recent memories covariated with access latencies or as a function of obsessive-compulsive tendencies in HSAM subjects. We found that in HSAM (p = .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.

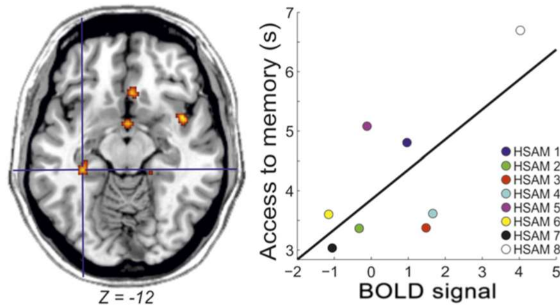

Figure 4. Increased activity in the left hippocampus as a function of increased latency to memory access in HSAM subjects.

Discussion

The HSAM subjects 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 HSAM subjects' superior access to details of past experiences. Additionally, the findings indicate that, in comparison with controls, the HSAM subjects remembered more autobiographical details of their past experiences, consistent with extensive prior investigations of HSAM (McGaugh et al., 2013; McGaugh et al., 2017), especially for the most remote AMs. The findings also confirmed those of previous research indicating that HSAM subjects 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, neither in response latency or underlying brain activity. While we estimated a reliable statistical power for the current experiment (see Methods), we cannot exclude that smaller effect sizes may be detected with larger sample sizes. Therefore, future experiments shall 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 HSAM subjects than in controls. During AM retrieval (irrespective to the access or reliving phase), when compared with controls, twice as many brain areas were activated in HSAM subjects. 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 HSAM subjects (Parker et al., 2006; LePort et al., 2016; LePort et al., 2017), we did not observe any neural difference between HSAM and control subjects during the reliving phase. In contrast, the findings suggest that the increase in neural activity was specifically involved in accessing AM, recruiting a left-lateralized fronto-parietal network (VMPFc, the DMPFc and the TPJ) in HSAM subjects only during memory access. Additionally, and importantly, during this phase, HSAM subjects exhibit an increased functional connectivity of these regions with brain areas crucial for memory retrieval. 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 HSAM than control subjects might confound the fMRI analysis, requiring a comparison between different amounts of BOLD signals (i.e. less signal for the access phase of HSAM than control subjects). Although the finding of greater

brain activity in HSAM subjects during memory access goes against this potential confound, future research will have to solve this possible limitation.

The enhanced AM access in HSAM individuals 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 Cabeza et al., 2007; Maguire 2001). AM retrieval is thought to be supported by an extensive network of brain regions – most pronounced on the left hemisphere (see, for a meta-analysis Svoboda et al., 2006) that has typically been interpreted as reflecting the variety of cognitive processes engaged during AM retrieval (Andrews-Hanna et al., 2014; Fossati 2013): executive control and retrieval monitoring (dorsolateral / dorsomedial prefrontal cortex); episodic remembering (hippocampus); emotion-related processes (ventromedial prefrontal cortex and amygdala); self-processing (dorsomedial / ventromedial prefrontal cortex, posterior cingulate); and visuospatial processing (retrosplenial cortex, precuneus, and parietal regions). Whereas the recruitment of the medial prefrontal cortex and TPJ reflects normal functioning of AM retrieval, the current findings provide the novel evidence of an increased activation of these regions associated with enhanced functioning of AM retrieval in HSAM individuals.

The increased activation of medial prefrontal regions might be related to enhanced self-reference processes in HSAM individuals. Recent literature reported consistent activations of both the ventral and the dorsal prefrontal cortex during the engagement of self-referential processes in AM retrieval (Martinelli et al., 2013). Moscovitch and Winocur (2002) 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 impaired VMPFc provides support for this suggestion (Turner et al., 2008). In contrast,

increased activity in the DMPFc may reflect recall of experienced events (Summerfield et al., 2009). The enhanced activation of the medial prefrontal cortex found in HSAM subjects during AM retrieval is in line with evidence of increased propensity of HSAM individuals to express self-referential processes as well as mental rumination of their prior experiences (LePort et al., 2016; McGaugh, 2013; McGaugh 2017).

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 HSAM subjects. Bonnici and Maguire (2011) reported evidence that, in normal subjects, the VMPFc is implicated in memory representation of events up to two years of remoteness, but not for more remote events. Here we found instead, an extended temporal window up to twenty years (Fig. 1d) in which the VMPFc contribute to access AMs (and, specifically, the most remote AM details, in line with the behavioral data, Fig. 1E) in HSAM subjects. The current findings also indicate that the VMPFc is functionally connected with the right hippocampus during AM access of HSAM individuals. 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 (Sheldon et al., 2018; for recent reviews see Jin et al., 2013; Sheldon et al., 2016). Consistent with that evidence, the present findings suggest that enhanced prefrontal/hippocampal coupling sustains enhanced memory performance in HSAM individuals. This evidence is consistent with findings of a single case HSAM study indicating greater than usual connectivity of the left hippocampus with prefrontal, but also premotor, and retrosplenial cingulate cortex (Brandt et al., 2018).

Importantly, an opposite pattern of results, i.e., decreased neural activity of the VMPFc and the hippocampus, was found in healthy subjects with severely deficient AM (SDAMs): Palombo and colleagues (2015) reported that three

SDAMs, who had otherwise preserved cognitive functions, expressed decreased neural activity in the left VMPFc cortex and reduced hippocampal volume. The evidence that the VMPFc and the hippocampus play a key role both in impaired AM subjects (Palombo et al., 2015) and normal subjects (Cabezza et al,.1007; Maguire, 2001; Svoboda et al., 2006; Fossati, 2013) as well as HSAM strongly suggests that the current level of prefrontal/hippocampal activity may play a critical role in determining the hypo- (i.e., SDAM) vs. hyper-functioning (i.e., HSAM) of AM retrieval. Although the increased hippocampal activity in HSAM subjects might potentially reflect task-related encoding activity (Wixted et al., 2017) 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 (Nadel et al., 1997) Together with the VMPFc, the hippocampus therefore appears to enable HSAM subjects to have faster and more detailed remote memory access (Bonnici et al 2018).

In addition to enhanced prefrontal/hippocampal functional connectivity, memory access in HSAM subjects was further supported by increased activity of the ventral parietal cortex (left TPJ) during AM retrieval. A growing body of evidence indicated that TPJ lesions entail dysfunctions related to self vs. other distinctions (Eddy, 2016). The increased activation of TPJ in HSAM might therefore be linked to an increased capability of HSAM subjects in selecting the correct AMs, better distinguishing between facts experienced by self or others. However, the findings also suggest a more parsimonious interpretation (Cabeza et al., 2015; Cabeza et al., 2008). TPJ activity during AM access in HSAM subjects might reflect internal attentional capture driven by information re-activated 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 (Beauchamp et al., 2012; Fiori et el., 2015), as well as in mental imagery (Grol et al., 2017). Accordingly, after internal focusing on re-activated memories, the TPJ might contribute to activate and maintain sensory representations in visual and auditory cortex, triggering visual/auditory imagery (Huijbers et al., 2011; Kraemer et al., 2005). These TPJ-centred mechanisms might contribute to the HSAM individuals' enhanced memory performance, 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 re-activation on the sensory representation developed at encoding (Folkerts et al., 2018, Grol et al., 2017). 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 (Cabeza et al., 2007; Rubin et al., 2007). In contrast, the present findings indicate that in HSAM individuals the recruitment of neural resources possibly devoted to visual or auditory imagery (i.e., the visual and auditory sensory cortices; (Huijbers et al., 2011; Kraemer et al., 2005) are anticipated in the access phase, thus contributing to their enhanced memory performance.

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

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CHAPTER 2

ENHANCING ENDOCANNABINOID NEUROTRANSMISSION AUGMENTS THE EFFICACY OF EXTINCTION TRAINING AND AMELIORATES TRAUMATIC STRESS-INDUCED BEHAVIORAL ALTERATIONS IN RATS

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Abstract

Exposure to a traumatic event may result in the development of Post-Traumatic Stress Disorder (PTSD). Endocannabinoids are crucial modulators of the stress response, interfere with excessive retrieval and facilitate the extinction of traumatic memories. Exposure therapy, combined with pharmacotherapy, represents a promising tool for PTSD treatment. We investigated whether pharmacological manipulations of the endocannabinoid system during extinction learning ameliorates the behavioral changes induced by trauma exposure. Rats were exposed to inescapable footshocks paired with social isolation, a risk factor for PTSD. One week after trauma, rats were subjected to three spaced extinction sessions, mimicking human exposure therapy. The anandamide hydrolysis inhibitor URB597, the 2-arachidonoylglycerol hydrolysis inhibitor JZL184 or the cannabinoid agonist WIN55,212-2 were administered before or after the extinction sessions. Rats were tested for extinction retention 16 or 36 days after trauma and 24-h later for social interaction. Extinction training alone reduced fear of the trauma-associated context but did not restore normal social interaction. Traumatized animals not exposed to extinction sessions exhibited reductions in hippocampal anandamide content with respect to home-cage controls. Noteworthy, all drugs exerted beneficial effects, but URB597 (0.1 mg/kg) induced the best improvements by enhancing extinction consolidation and restoring normal social behavior in traumatized rats through indirect activation of CB1 receptors. The ameliorating effects remained stable long after treatment and trauma exposure. Our findings suggest that drugs potentiating endocannabinoid neurotransmission may represent promising tools when combined to exposure-based psychotherapies in the treatment of PTSD.

Introduction

Post-Traumatic Stress Disorder (PTSD) is a chronic psychiatric disease of high prevalence and morbidity. It develops in vulnerable subjects after experiencing an intense and life-threatening traumatic event. The disorder is listed in the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) as a "trauma and stressor-related disorder" (American Psychiatric Association, 2013). Symptomatic features of PTSD include both cognitive and emotional alterations, primarily caused by an aberrant adaptation to the trauma (American Psychiatric Association, 2013). Cognitive symptoms include the inability to extinguish fear and anxiety responses, to suppress episodic memory retrieval and to acquire safety signals (Holmes and Singewald, 2013; Moore, 2009). Cognitive dysfunctions are linked to both PTSD development and chronicity and are strictly related to mnestic symptoms, which include spontaneous recollections, flashbacks, and dissociative amnesia (Moore, 2009; Parsons and Ressler, 2013). The dramatic changes in reactivity, arousal, mood and anxiety (Heim and Nemeroff, 2009) complete the symptomatological picture, causing severe impairments in patient's global functioning, which include reduced interest in social contact (American Psychiatric Association, 2013). The gold standard pharmacotherapy is represented by selective serotonin re-uptake inhibitors (SSRIs). However, the efficacy of these drugs is far from being satisfactory (Hoskins et al, 2015). Cognitive behavioral exposure-based psychotherapy, which is believed to engage extinction learning processes, in some cases represents an effective PTSD (Abramowitz, 2013; Furini et al, 2014; Milad and Quirk, 2012). This therapeutic approach is based on the repeated exposure of the patient to trauma-related reminders, in a safe controlled situation thus lacking the negative consequences for the subject (Abramowitz, 2013). Even if exposure therapy has proven some degree of efficacy in treating PTSD (Bisson et al, 2013),

drop outs are frequent among patients reaching rates of 39% of the population (Gutner et al, 2016). Moreover, PTSD symptoms can relapse when the exposure therapy ends leading to fear reinstatement. Therefore, it appears worth to investigate if drugs interfering with the excessive retrieval or enhancing the extinction of the traumatic memory could improve the exposure therapy efficacy by preventing spontaneous recovery (Choi et al, 2010; Hofmann, 2007; McGuire et al, 2014; de Quervain et al, 2011; Singewald et al, 2015; Trezza and Campolongo, 2013).

High attention in this regard has been lately given to drugs potentiating the endocannabinoid signaling (Campolongo et al, 2013; Morena and Campolongo, 2014; Rabinak and Phan, 2014; Trezza and Campolongo, 2013). The endocannabinoid system is composed by the cannabinoid receptors type 1 (CB1) and type 2 (CB2), two principal endogenous ligands, namely N-arachidonoyl ethanolamine (anandamide, AEA) and 2-arachidonoyl glycerol (2-AG) (Kano et al, 2009; Di Marzo, 1999), and their primarily degrading enzymes the fatty-acid amide hydrolase (FAAH) (Cravatt et al, 1996) and the monoacylglycerol lipase (MAGL) (Dinh et al, 2002), for AEA and 2-AG, respectively. The endocannabinoid system can be exogenously targeted by means of the FAAH inhibitor URB597 that elevates endogenous levels of AEA (Kathuria et al, 2003), of the MAGL inhibitor JZL184 that elevates endogenous levels of 2-AG (Long et al, 2009; Morena et al, 2015) or by direct cannabinoid receptor agonists such as WIN55,212-2. Genetic deletion or pharmacological blockade of CB1 receptors in rodents result in impaired fear memory extinction (Chhatwal et al, 2005; Marsicano et al, 2002; Morena and Campolongo, 2014; Pamplona et al, 2008). Consistently, cannabinoid agonists enhance extinction (Bitencourt et al, 2014; Bowers and Ressler, 2015; Gunduz-Cinar et al, 2013; Pamplona et al, 2008) and attenuate retrieval of aversive memory (Atsak et al, 2012; Morena et al, 2015; Morena and Campolongo, 2014).

We recently proposed a footshock-based paradigm able to mimic both the enduring cognitive and emotional facets of PTSD pathology useful to model the extinction-based exposure therapy combined with pharmacological treatment (Berardi et al, 2014). The main purpose of the present study was to evaluate the effects of pharmacological manipulation of the endocannabinoid system in a preclinical model of PTSD, by targeting extinction learning processes. To this aim, we first adapted our model (Berardi et al, 2014) by including multiple extinction sessions and we verified whether the extinction procedure alone was able to improve the behavioral alterations displayed by traumatized rats. We also evaluated the effects induced by trauma exposure on AEA and 2-AG levels in brain regions critically involved in extinction and emotional behavior, i.e. hippocampus, prefrontal cortex and amygdala (Furini et al, 2014). We then evaluated whether activation of the endocannabinoid system, by facilitating extinction and dampening the retrieval of fear memory, could ameliorate the efficacy of "extinction-based exposure therapy" leading to improvements which remain stable long after trauma exposure and pharmacological treatment termination.

Materials and Methods

Subjects

Male Sprague-Dawley rats (350-450 g at the time of testing; Charles River Laboratories, Calco, Italy) were kept in an air-conditioned room (temperature: 21° ± 1° C; lights on 7:00 a.m. - 7:00 p.m.) with food and water available *ab libitum*. All the experiments were performed during the light phase of the cycle. Rats were handled for 1 minute for 7 consecutive days before behavioral testing. All procedures were performed in compliance with the European Union Directive on the protection of animals used for from the scientific purposes

(2010/63/EU) and the Italian law (D.L. 26/2014).

Behavioral procedures

The PTSD model we first described (Berardi *et al*, 2014) was adapted for the assessment of memory extinction.

The experimental apparatus consisted in a metal trough-shaped alley (60 cm long, 15 cm deep, 20 cm wide at the top, and 6.4 cm wide at the bottom) connected to an animal shocker. All the experimental sessions were video-recorded and subsequently scored by two well trained researchers blind to the experimental conditions. After each session, fecal boli were removed and the apparatus was cleaned with a 70% ethanol solution.

Housing. All rats were individually housed for 3 days prior to the habituation session and remained singly-housed until the end of the behavioral testing. We have previously shown that social isolation is necessarily required to develop enduring signs of emotional distress upon exposure to a traumatic event (Berardi et al, 2014).

Habituation. On the first day of testing (day -1), rats were individually taken from the home-cage and habituated for 5 minutes to the test apparatus. Rats were then returned to their home-cages.

Exposure session. On day 0, rats were individually placed in the apparatus and were left undisturbed for 2 minutes. Then, 5 footshocks (2 sec, 0.8 mA) were randomly delivered. After the last shock, rats were kept in the apparatus for 60 seconds to facilitate context association to the aversive stimuli. A control group was only exposed to the apparatus for 10 minutes without any footshock presentation.

Extinction sessions. Each of the 3 extinction sessions consisted in a 10-min re-exposure to the experimental apparatus, with the first carried out 7 days after the exposure session (day 7). Each subsequent session was separated from the

preceding one by a 72-hour interval (days 10, 13). A control group was not exposed to the extinction procedure.

Extinction retention test. To evaluate memory retention, rats were exposed to the experimental apparatus 16 or 36 days after trauma exposure (3 or 20 days after the last extinction session), for a 10-min period, during which contextual freezing behavior was evaluated (Chen et al, 2012; Wheeler et al, 2013).

Social interaction test. To evaluate the level of emotional distress, rats were tested in the social interaction test (SI), which was carried out 24 hours after the extinction retention test (day 17 or day 37).

Couples for the SI were established according to the following criteria: 1) belonging to the same drug treatment/experimental condition; 2) unfamiliarity; 3) least weight difference. Each couple was put for 10 minutes in a quadrangular arena (40 x 40 x 60 cm) made of Plexiglas with clean sawdust covering the floor, under red light conditions (~10 lux). After each session, rats were returned to their home cage, fecal boli were removed, sawdust was blended, and the arena's walls were cleaned with a 70% ethanol solution. In this test, social behavior was scored as previously described (Berardi *et al*, 2014).

Drugs

The FAAH inhibitor URB597 (0.1, 0.3 mg/kg), the MAGL inhibitor JZL184 (0.5, 1 mg/kg), the CB agonist WIN55,212-2 (1, 3 mg/kg) and the CB1 antagonist SR141716 (1 mg/kg) were dissolved in a vehicle containing 5% polyethylene glycol, 5% Tween80 and 90% saline solution. Doses were chosen on the basis of pilot experiments performed in our lab and on literature data showing behavioral efficacy and lack of locomotor alterations in rats (Campolongo et al, 2013; Haller et al, 2009; Järbe et al, 2007; Manduca et al, 2016; Morena and Campolongo, 2014; Robinson et al, 2003; Sciolino et al, 2011). All drugs were freshly prepared on the day of the experiment and administered i.p. at a volume of 1 ml/kg. On

the basis of the experimental procedure, WIN55,212-2, URB597 and JZL184 (or vehicle) were administered either 1 hour before or immediately after each extinction session on days 7, 10 and 13. SR141716 (or vehicle) was administered 30 min before the extinction sessions on days 7, 10 and 13. In all the experiments neither drugs nor vehicle were administered on the extinction retention session on day 16 or 36. Drugs were kindly donated by the National Institute of Mental Health (Chemical Synthesis and Drug Supply Program, Bethesda, MD, USA).

Endocannabinoid extraction and analysis

AEA and 2-AG extraction and analysis were performed as previously described (Morena et al, 2015). Briefly, animals were sacrificed immediately after the SI test and the hippocampus, prefrontal cortex and amygdala were dissected by a well-trained experimenter. The brain areas were put into 2-ml Eppendorf tubes and immediately stored at –80 °C in order to avoid endocannabinoid degradation (Hauer et al, 2011). Brain tissue was weighed and placed into borosilicate glass culture tubes containing 2 ml of acetonitrile with 5 pmol of [$^{2}H_{8}$] AEA and 5 nmol of [$^{2}H_{8}$] 2-AG 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 X 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 AEA and 2-AG was performed by liquid chromatography mass spectrometry analysis as previously detailed (Qi et al, 2015).

Statistical analysis

Statistical analysis was performed using SPSS statistical software. Each measure

is expressed as mean ± SEM. For each measure on experiment 1, independent samples Student's t-test was performed. In the evaluation of freezing percentage across the extinction sessions, a repeated measures ANOVA was performed with sessions as the repeated factor and treatment and shock condition as between-subjects factors. In all the other cases, data were analyzed with one- or two-way ANOVA, when appropriate. Tukey-Kramer post-hoc test was performed to control for significant differences between groups when appropriate. Significance was considered for $P < 0.05$.

Results

Extinction training attenuates the behavioral alterations of traumatized rats

In this experiment, we examined the effects of the extinction training procedure on behavioral alterations displayed by footshock-exposed rats (Extinction group), compared with a control group never exposed to the extinction sessions (No-Extinction group). In our previous work, we showed that the exposure to a single session of multiple footshocks caused in rats a long-lasting form of traumatic memory along with a sustained reduction of social behavior when compared to unexposed control rats (Berardi et al, 2014).

Here, we found that the extinction procedure alone reduced conditioned freezing 16 days after the exposure to footshocks, but failed to promote an enhancement of social behavior. Indeed, when analyzing freezing percentage at day 16, Student's t-test revealed that rats receiving the full extinction protocol displayed significantly lower levels of freezing than rats never exposed to the extinction sessions ($t_{18} = -3.57$, $P = 0.002$) (Fig. 1a). Conversely, for the social interaction time, t-test revealed only a trend toward significance ($t_{18} = 1.89$, $P = 0.075$) (Fig. 1b).

We next measured endocannabinoid AEA and 2-AG levels immediately after the

SI test in rats subjected to the extinction sessions, in rats never exposed to the extinction sessions and in a home-cage control group, in the hippocampus, prefrontal cortex and amygdala. In the hippocampus, ANOVA for AEA levels revealed a significant effect of condition ($F_{2,27} = 3.53$, $P = 0.043$) and post-hoc tests showed that rats not exposed to the extinction procedure had significantly lower AEA levels when compared to home-cage controls (Table 1). No differences were found in the hippocampus for 2-AG levels ($F_{2,27} = 0.008$, $P = 0.99$, Table 1). In the prefrontal cortex, ANOVA for AEA (Table 1) and 2-AG (Table 1) levels did not reveal any significant effect of the condition ($F_{2,24} = 0.34$, $P = 0.72$; $F_{2,24} = 0.80$, $P = 0.46$, respectively). Similarly, in the amygdala, ANOVA for AEA (Table 1) and 2-AG (Table 1) levels did not reveal any significant condition effect ($F_{2,26} = 0.057$, $P = 0.95$; $F_{2,26} = 0.41$, $P = 0.67$, respectively).

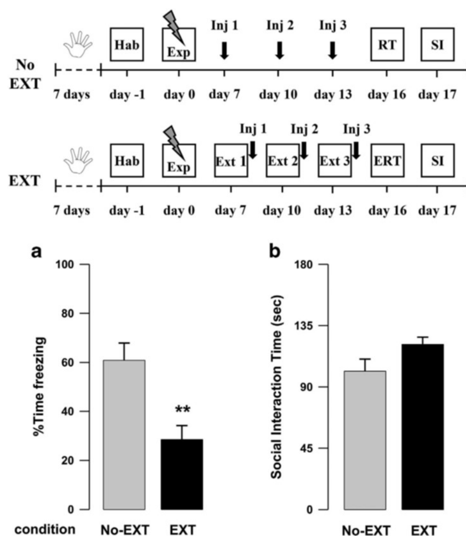


Figure 1. (a) Freezing rates of vehicle-treated rats exposed to the footshock experience (Exp) but not subjected to the extinction sessions (No-EXT) and of rats exposed to the extinction sessions (EXT 1, 2 and 3), as assessed during the extinction retention test (ERT)

or retention test (RT) on day 16. **(b)** Social interaction (SI) time of No-EXT and EXT rats during the SI test on day 17. (** $P < 0.01$ vs the No-EXT group). Data are expressed as mean + SEM (n = 10 per group).

Table 1 AEA and 2-AG levels in rats subjected to the extinction sessions (Ext), in rats never exposed to the extinction sessions (No-Ext) and in a home-cage control group (CTRL), in the hippocampus (HIPP), prefrontal cortex (PFC) and amygdala (AMY).

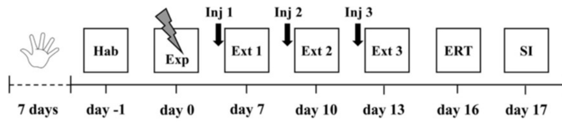
	AEA (pmol/g tissue)			2-AG (nmol/g tissue)		
	CTRL	No-Ext	Ext	CTRL	No Ext	Ext
HIPP	36.43 ± 2.30	29.42 ± 1.24 *	34.84 ± 2.15	15.93 ± 1.44	15.79 ± 0.93	15.72 ± 1.13
PFC	37.04 ± 4.59	40.45 ± 5.74	35.52 ± 2.76	6.28 ± 0.70	7.78 ± 1.14	6.75 ± 0.61
AMY	26.44 ± 2.33	26.13 ± 2.95	27.31 ± 2.50	12.00 ± 0.97	10.72 ± 0.72	11.43 ± 1.17

* $P < 0.05$ vs the corresponding CTRL group. Data are expressed as mean ± SEM (n = 8-10 per group).

Effects of pre-extinction cannabinoid drug administration on trauma-induced behavioral alterations

In these experiments, we investigated the effects of the tested drugs given 1 hour before extinction training sessions, on both cognitive and emotional modification following trauma exposure. Results showed only a limited efficacy of the tested substances in modifying rats' behaviors. Indeed, the lower dose of URB597 (0.1 mg/kg) increased social behavior, and both doses of JZL184 (0.5 and 1 mg/kg) reduced global freezing percentages. Concerning URB597 effects, ANOVA for freezing time across sessions showed a significant effect of sessions ($F_{3,81}$ = 19.45, $P < 0.001$) but no significant effects of drug treatment and sessions X drug

treatment interaction ($F_{2,27} = 0.80$, $P = 0.46$; $F_{6,81} = 0.16$, $P = 0.99$ respectively) (Fig. 2a). Conversely, in the SI, ANOVA showed a significant effect of drug treatment ($F_{2,27} = 6.029$, $P = 0.007$) and post-hoc analysis revealed that animals treated with the 0.1 mg/kg dose spent significantly more time interacting with the social partner when compared to controls ($P < 0.05$) (Fig. 2b). ANOVA for freezing time across sessions showed a significant effect of both sessions and drug treatment for JZL184 ($F_{3,81} = 42.82$, $P < 0.001$; $F_{2,27} = 4.25$, $P = 0.025$, respectively), but no effect of sessions X drug treatment interaction ($F_{6,81} = 0.552$, $P = 0.77$). Post-hoc comparisons for the total freezing showed that animals treated with both doses of JZL184 displayed significantly less freezing than control rats ($Ps < 0.05$) (Fig. 2c). In the SI, ANOVA for social interaction time showed a significant effect of drug treatment ($F_{2,27} = 4.27$, $P = 0.024$) (Fig. 3d). No differences between JZL184 and vehicle treated rats were observed in this test. Finally, for WIN55,212-2, ANOVA for freezing showed a significant effect of sessions ($F_{3,81} = 28.40$, $P < 0.001$) but no significant effects of drug treatment and sessions X drug treatment interaction ($F_{2,27} = 0.76$, $P = 0.48$; $F_{6,81} = 1.44$, $P = 0.21$, respectively) (Fig. 2e). In the SI, ANOVA did not show any significant effect ($F_{2,27} = 0.32$, $P = 0.73$) (Fig. 2f).



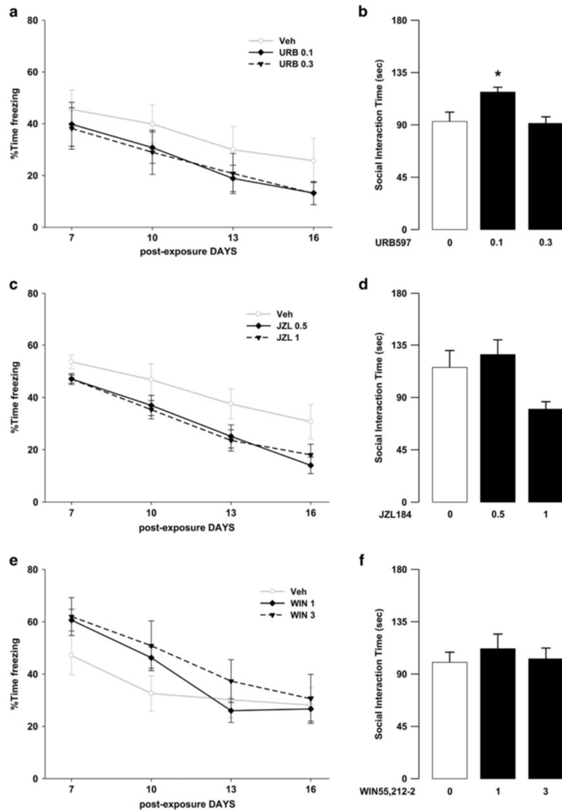


Figure 2. Freezing rates across **(a)** the 3 extinction sessions (Ext 1, 2 and 3) and the extinction retention test (ERT) and **(b)** social interaction (SI) time of rats treated with URB597 or vehicle (Veh) 1 hour before the extinction sessions on day 7, 10 and 13. Freezing rates across **(c)** the 3 extinction sessions and the extinction retention test and **(d)** social interaction time of rats treated with JZL184 or vehicle 1 hour before the extinction sessions on day 7, 10 and 13. Freezing rates across **(e)** the 3 extinction sessions and the extinction retention test and **(f)** social interaction time of rats treated with WIN55,212-2

or vehicle 1 hour before the extinction sessions on day 7, 10 and 13. * $P < 0.05$ URB597 0.1 mg/kg vs Veh. Data are expressed as mean ± SEM (n = 8-11 per group).

Effects of post-extinction cannabinoid drug administration on trauma-induced behavioral alterations

In these experiments, we investigated the effects of the tested drugs, given immediately after the extinction sessions, on both cognitive and emotional modifications induced by trauma exposure.

Concerning URB597, ANOVA for freezing time showed a significant effect of sessions, drug treatment and sessions X drug treatment interaction ($F_{3,81} = 38.14$, $P < 0.001$; $F_{2,27} = 4.32$, $P = 0.024$; $F_{6,81} = 2.22$, $P = 0.050$, respectively). Post-hoc comparisons showed that animals treated with the higher dose of URB597 (0.3 mg/kg) displayed significantly less freezing on the second ($P < 0.05$) and third ($P < 0.05$) extinction sessions when compared to vehicle-treated rats. In addition, animals treated with the 0.1 mg/kg dose displayed significantly less freezing on the third extinction session and the extinction retention session (Ps < 0.05) when compared to vehicle-treated rats. Post-hoc tests for freezing time showed reduced total freezing in URB597-treated animals with respect to controls ($P < 0.05$) (Fig. 3a). In the SI, ANOVA showed a significant effect of drug treatment ($F_{2,27} = 5.80$, $P = 0.008$). Post-hoc analysis revealed that URB597 (0.1 mg/kg) treated rats interacted significantly more with the social partner than controls ($P < 0.01$) (Fig. 3b). Concerning JZL184, ANOVA for freezing showed a significant effect of sessions ($F_{3,81} = 44.70$, $P < 0.001$) and no significant effects of drug treatment and sessions X drug treatment interaction ($F_{2,27} = 0.79$, $P = 0.47$; $F_{6,81} = 1.75$, $P = 0.12$ respectively) (Fig. 3c). In the SI, one-way ANOVA showed no significant drug treatment effect ($F_{2,27} = 0.092$, $P = 0.91$) (Fig. 3d). Finally, ANOVA for WIN55,212-2 effects showed a significant effect of sessions and of sessions X drug treatment interaction ($F_{3,99} = 28.48$, $P < 0.001$; $F_{6,99} = 2.91$, $P = 0.012$, respectively) but no drug treatment effect ($F_{2,33} = 1.29$, $P = 0.29$). Post-hoc tests

revealed that animals treated with the higher dose of WIN55,212-2 (3 mg/kg) displayed significantly less freezing during the fourth session (extinction retention session) when compared to vehicle-treated animals ($P < 0.05$) (Fig. 3e). In the SI, ANOVA did not show any significant drug treatment effect ($F_{2,33} = 0.86$, $P = 0.43$) (Fig. 3f).

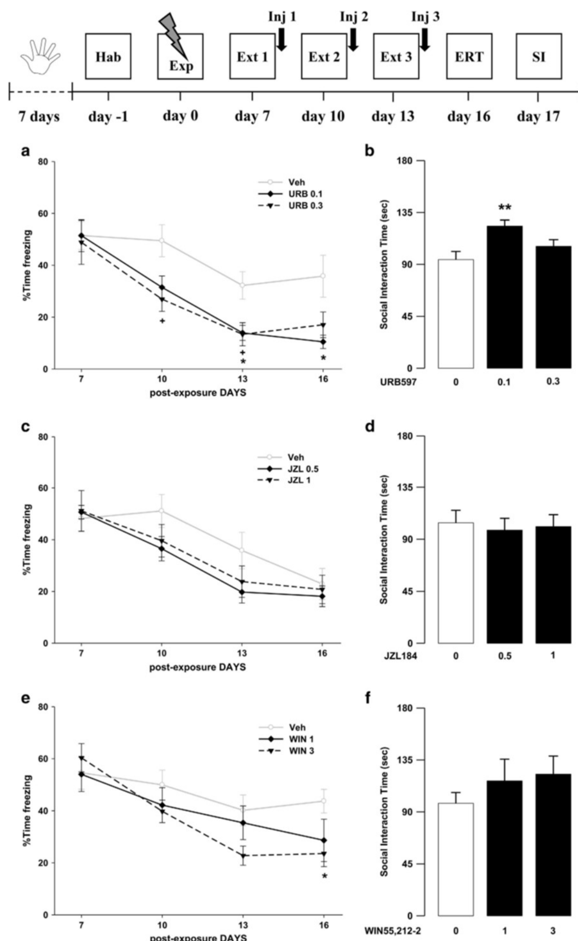


Figure 3. Freezing rates across **(a)** the 3 extinction sessions (Ext 1, 2 and 3) and the

extinction retention test (ERT) and **(b)** social interaction (SI) time of rats treated with URB597 or vehicle (Veh) immediately after the extinction sessions on day 7, 10 and 13 (* $P < 0.05$, ** $P < 0.01$, URB597 0.1 mg/kg vs Veh; + $P < 0.05$, URB597 0.3 mg/kg vs Veh). Freezing rates across **(c)** the 3 extinction sessions and the extinction retention test and **(d)** social interaction time of rats treated with JZL184 or vehicle immediately after the extinction sessions on day 7, 10 and 13. Freezing rates across **(e)** the 3 extinction sessions and the extinction retention test and **(f)** social interaction time of rats treated with WIN55,212-2 or vehicle immediately after the extinction sessions on day 7, 10 and 13 (* $P < 0.05$, WIN55,212-2 3 mg/kg vs Veh). Data are expressed as mean + SEM (n = 10-13 per group).

The ameliorating effects of post-extinction URB597 treatment are specific for trauma-exposed animals and depend on facilitation of memory extinction

In this experiment, the effective dose of URB597 (0.1 mg/kg) was administered in rats never subjected to footshock session, but only exposed 3 times to the experimental apparatus. Memory retention and SI were tested on days 16 and 17, respectively. We found that vehicle-treated rats exposed to the shock experience presented higher levels of freezing and reduction in social interaction as compared to their non-shocked controls. Moreover, URB597 did not affect freezing or social behavior in rats not exposed to the shock experience, yet it ameliorated both cognitive and emotional trauma-induced alterations present in the shock-exposed animals. ANOVA for freezing did not reveal a significant effect of drug treatment ($F_{1,28} = 3.08$, $P = 0.090$), but did show a significant effect of the shock exposure, the sessions and drug treatment X shock exposure X sessions interaction ($F_{1,28} = 154.20$, $P < 0.0001$; $F_{3,28} = 17.30$, $P < 0.0001$; $F_{3,84} = 5.09$, $P < 0.028$, respectively; Fig. 4a). Post-hoc tests showed that vehicle-treated rats exposed to the shock experience presented higher levels of freezing across the extinction sessions as compared to the non-shock vehicle group ($P < 0.05$, for all comparisons). Furthermore, shock-exposed rats given URB597 presented

less freezing on the third extinction session and on the extinction retention session when compared to shock-exposed rats treated with vehicle ($Ps < 0.05$). No significant differences were detected among shock-exposed rats treated with URB597 and rats not exposed to the trauma given vehicle or URB597, during the extinction retention session. Thus, URB597 treatment in shock-exposed animals completely restores animals' behavior, rendering it comparable to that observed in rats never exposed to the traumatic experience. Similarly, there were no significant differences across sessions between the two non-shocked animal groups (Fig. 4a). When analyzing the total freezing independently from the sessions, post-hoc tests showed that, within vehicle-treated groups, shock-exposed animals showed significantly more freezing than no-shocked rats ($P < 0.01$). Similarly, within URB597-trated groups, shock-exposed animals showed significantly more freezing than URB597 no-shocked rats ($P < 0.01$). Moreover, among the shock-exposed groups, URB597-treated rats displayed significantly less freezing than the vehicle-treated group ($P < 0.01$). ANOVA for SI did not reveal a significant drug treatment ($F_{1,28} = 0.491$, $P = 0.4893$) or shock exposure ($F_{1,28} = 3.143$, $P = 0.0872$) effect, but showed a significant drug treatment X shock exposure interaction effect ($F_{1,28} = 5.427$, $P = 0.0273$). Post-hoc comparisons showed that vehicle treated rats exposed to the shock experience presented reduced social interaction time ($P < 0.05$). URB597-treated rats exposed to the shock displayed higher levels of social interaction than vehicle-treated rats exposed to the same shock condition ($P < 0.05$), and comparable to those of non-shocked rats given either vehicle or URB597 (Fig. 4b).

We next examined the effects of URB597 (0.1, 0.3 mg/kg) administered in animals exposed to the trauma but not subjected to the repeated extinction procedure and exposed to the conditioned context only on day 16 (retention test). Rats were injected with URB597 on post-shock exposure day 7, 10 and 13. Results showed that neither freezing nor social behavior were affected by

URB597 treatment when extinction sessions were omitted ($F_{2,26}$ = 0.228, P = 0.797) (Fig. 4c). ANOVA did not reveal any significant effect of the drug treatment on social interaction time in the SI ($F_{2,26}$ = 0.593, P = 0.560) (Fig. 4d).

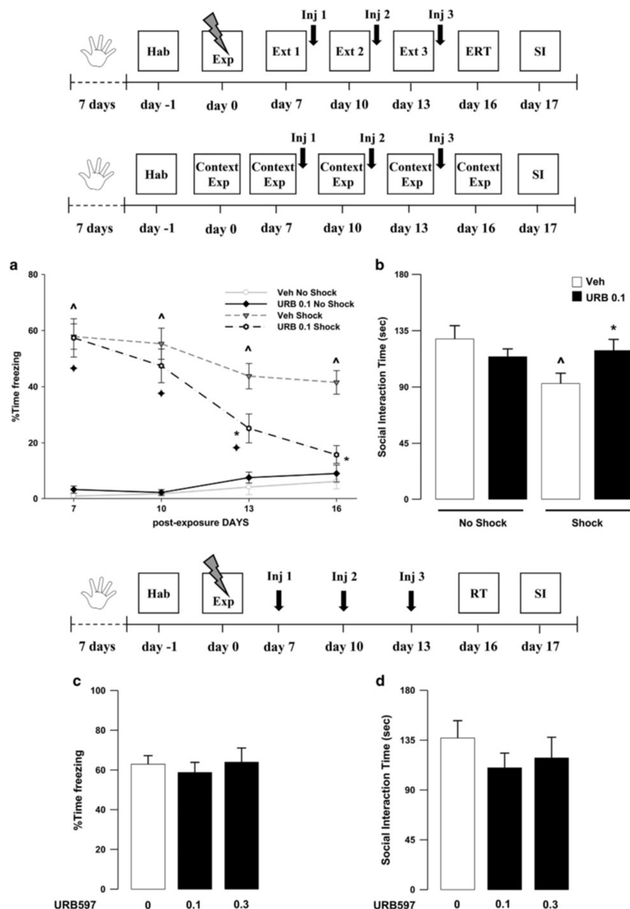


Figure 4. Freezing rates across **(a)** the 3 extinction sessions (Ext 1, 2 and 3) and extinction retention test (ERT) and **(b)** social interaction (SI) time of rats treated with URB597 (0.1 mg/kg; URB 0.1 Shock) or vehicle (Veh Shock) immediately after the extinction sessions on day 7, 10 and 13, exposed to the traumatic experience (Exp) or never presented the

shock but only exposed to the experimental context (Context Exp; URB 0.1 No Shock, Veh No Shock). **(c)** Freezing rates of rats exposed to the shock experience (Exp), but not to the extinction sessions and treated with URB597 or vehicle on day 7, 10 and 13, as assessed during the retention session (RT) on day 16. **(d)** Social interaction time of the same rats during the SI test on day 17. ^ $P < 0.05$, Veh Shock vs Veh No Shock; * $P < 0.05$, URB 0.1 Shock vs Veh Shock; ◆$P < 0.05$, URB 0.1 Shock vs URB 0.1 No Shock. Data are expressed as mean ± SEM (n = 7-11 per group).

The ameliorating effects of post-extinction URB597 administration are long-lasting and blocked by pre-treatment with the CB1 receptor antagonist SR141716A

In this experiment, we investigated whether post-extinction URB597 effects on both freezing and social behavior were dependent on indirect activation of CB1 receptors by increased AEA. We found that SR141716, at a dose (1 mg/kg) not altering the behavior *per se*, blocked the ameliorating effects of URB597 (0.1 mg/kg) on both freezing and social behavior, at day 16 and 17 post-trauma exposure. ANOVA for freezing revealed a significant effect of the sessions, of the drug treatment and of sessions X drug treatment interaction ($F_{3,108} = 57.16$, $P < 0.001$; $F_{3,36} = 3.82$, $P = 0.018$; $F_{3,108} = 2.23$, $P = 0.026$ respectively). Post-hoc tests showed that URB597-treated rats displayed less freezing on the second and third extinction sessions and on the extinction retention session when compared to rats receiving SR141716 alone (Ps < 0.05). Moreover, URB597-treated rats displayed less freezing than SR141716/URB597 treated rats on the third extinction session and on the extinction retention session (Ps < 0.05). Finally, URB597-treated rats displayed less freezing than vehicle-treated rats on the extinction retention session ($P < 0.01$). When analyzing total freezing independently from the sessions, post-hoc tests showed that URB597-treated animals displayed significantly less freezing than the vehicle-treated group ($P < 0.05$). No differences were observed among the groups of rats treated with SR141716/URB597, SR141716 or vehicle alone (Fig. 5a). ANOVA for social

interaction time also revealed a significant effect of the drug treatment ($F_{3,36}$ = 4.549, P = 0.008). Post-hoc analysis showed that rats treated with URB597 interacted longer with the social partner than rats from the other groups did (Ps < 0.05). No significant differences were observed among SR141716/URB597, SR141716 or vehicle-treated groups (Fig. 5b).

We next examined whether post-extinction URB597 effects on both freezing and social behavior were long-lasting. Animals underwent the same drug administration regimen and three-day extinction session exposure, as described for the previous experiment, except that the extinction retention test and social behavior were examined more than a month after trauma exposure (day 36 and 37, for freezing and social interaction, respectively). We found that control rats still presented robust trauma-related behavioral alterations at day 36 and 37 post-trauma exposure. However, URB597 treatment (0.1 mg/kg) combined with the extinction exposure sessions restored normal freezing and social interaction which remain stable long after treatment termination. Additionally, these effects were blocked by pre-administration of SR141617 (1 mg/kg), thus showing that URB597 effects are dependent on indirect activation of CB1 receptors through increased levels of AEA.

ANOVA for freezing revealed a significant effect of the sessions, of the drug treatment and of sessions X drug treatment interaction ($F_{3,28}$ = 30.02, P < 0.0001; $F_{3,28}$ = 3.39, P = 0.032; $F_{3,84}$ = 2.050, P = 0.044, respectively). Post-hoc tests showed that URB597-treated rats displayed less freezing on the third extinction session and on the extinction retention session when compared to rats receiving vehicle alone or SR141716/URB597 (Ps < 0.05). Moreover, URB597-treated rats displayed less freezing than rats treated with SR141716 alone on the third extinction session (P < 0.05) and on the extinction retention session (P < 0.01). When analyzing the total freezing independently from the sessions, post-hoc tests showed that URB597-treated animals displayed significantly less freezing than

the controls (P < 0.01). No differences were observed among the other groups of rats (Fig. 5c). ANOVA for social interaction time also revealed a significant effect of the drug treatment ($F_{3,28} = 8.370$, P = 0.0004). Post-hoc analysis showed that rats treated with URB597 interacted longer with the social partner than rats from the other groups did (Ps < 0.01). No significant differences were observed among the other groups (Fig. 5d).

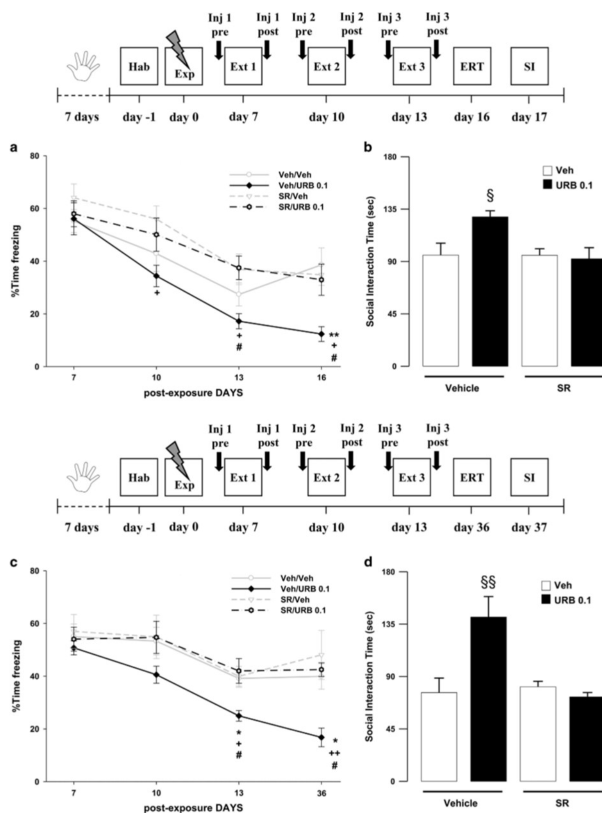


Figure 5. Freezing rates across **(a)** the 3 extinction sessions (Ext 1, 2 and 3) and extinction retention test (ERT, day 16) and **(b)** social interaction (SI) time (day 17) of rats treated with SR141716 (SR, 1 mg/kg) or vehicle (Veh) 30 min before, and with URB597 (0.1

mg/kg, URB 0.1) or vehicle immediately after the extinction sessions on day 7, 10 and 13. Freezing rates across **(c)** the 3 extinction sessions and extinction retention test (day 36) and **(d)** social interaction time (day 37) of rats treated with SR141716 (1 mg/kg) or vehicle 30 min before, and with URB597 (0.1 mg/kg) or vehicle immediately after the extinction sessions on day 7, 10 and 13. * $P < 0.05$, ** $P < 0.01$, Veh/URB 0.1 vs Veh/Veh; + $P < 0.05$, ++ $P < 0.01$, Veh/URB 0.1 vs SR/Veh; # $P < 0.05$, Veh/URB 0.1 vs SR/URB 0.1; § $P < 0.05$, §§ $P < 0.01$, Veh/URB 0.1 vs each other group). Data are expressed as mean + SEM (n = 8-10 per group).

Discussion

The present findings show that facilitation of endocannabinoid signaling augments the efficacy of extinction training and ameliorates the behavioral alterations caused by traumatic stress in rats. We previously showed that exposure to footshocks in rats, together with social isolation, induced a long-lasting reduction in social behavior paired with excessive retrieval for the traumatic experience, leading to chronic dysfunction (Berardi et al, 2014). In particular, we showed that exposure to footshocks induces a long-lasting form of contextual fear memory characterized by extremely high freezing rates, still present even three months after stress exposure. This highlights the chronic nature of the fear memory, a main feature of the human PTSD pathology. It is of interest, also from a translational point of view, that the stressful experience caused a chronic alteration of social behavior as well (Berardi et al, 2014).
Here, we aimed at mimicking a classical setting of exposure therapy in humans by establishing, in rats, a form of spaced extinction learning induced by repeated exposures to the context in the absence of footshocks and paralleled by drug administrations (Abramowitz et al, 2013; de Quervain et al, 2011). We observed a reduction in freezing behavior 2 weeks after exposure to trauma in animals that were repeatedly exposed to a no longer dangerous context when compared to rats

that were not subjected to the extinction sessions, thus highlighting that the exposure sessions can dampen *per se* fear memory recall. Furthermore, compared with home cage control animals, rats never exposed to the repeated extinction procedure presented decreased hippocampal AEA levels when measured immediately after the SI session, at the end of our behavioral paradigm. Interestingly, in the same brain region, AEA levels in animals subjected to the spaced extinction sessions were comparable to those observed in home cage control animals. Since we did not observe any change in the Extinction group, subjected to the same SI testing before measuring endocannabinoid levels, it is unlikely that the reduction of AEA levels in the No-extinction group might be ascribed to acute phasic changes due to the prior SI testing. Our finding rather suggests that the absence of the repeated extinction procedure might have caused a tonic reduction of AEA levels in the hippocampus. Consistently with human data on extinction therapy in PTSD patients (Bisson et al, 2013; Quirk et al, 2010), the present finding suggests that repeated exposure to trauma-related reminders, in the absence of negative consequences, represents a useful approach to dampen the excessive recall of the traumatic memory. However, the repeated exposure to trauma-related reminders alone is not sufficient to normalize stress-induced aberrations in social behavior (Berardi et al, 2014). Therefore, we tested whether administrations of drugs enhancing endocannabinoid signaling before or after the three extinction sessions, could be effective in restoring a normal behavioral phenotype. Drugs were administered either one hour before, or immediately after, each extinction session, in order to disentangle the impact of drug treatment on the retrieval or the consolidation of extinction, respectively. To reduce the development of drug tolerance rats were exposed to the extinction sessions on spaced days. Of note, this procedure was reported to be more effective than extinction sessions carried out on consecutive days (Matsuda et al, 2014). When drug injections were given 1 hour before the extinction sessions,

none of the tested compounds were able to improve extinction learning and social behavior at the same time. Noteworthy, boosting AEA signaling with URB597 administration, promoted social behavior while leaving the recall of the traumatic event unaltered. Conversely, JZL184 administration, slightly reduced the traumatic memory recall but failed to improve social behavior.
Different results were observed with post-extinction treatment. Although JZL184 did not produce any significant effect, the CB receptor agonist WIN55,212-2 facilitated memory extinction while leaving the social behavior unaltered. Interestingly, URB597, through indirect activation of CB1 receptors, enhanced the consolidation of extinction, as well as ameliorated the trauma-induced alterations in social behavior by increasing the levels of social interaction. We also found that these ameliorating effects of URB597 were dependent on the traumatic experience. By replicating our previous findings (Berardi et al, 2014), our results showed that vehicle-treated rats presented impairments in social behavior and, clearly, higher levels of freezing, 2 weeks after trauma exposure as compared to rats that never experienced the traumatic event. Treatment with URB597 did not affect freezing or social behavior in non-shocked rats, thus showing that the ameliorating effects of URB597 were specific for rats exposed to the footshock experience. Most importantly, URB597 treatment in traumatized animals completely restored cognitive and emotional behavior, rendering it comparable to that observed in rats never exposed to the traumatic experience.
In addition to this, in a separate experiment we found that the efficacy of URB597 treatment was also strictly dependent on the repeated exposure-based extinction approach. Indeed, when animals were treated at the same time intervals of those of the extinction procedure, but without re-exposing them to the context, URB597 did not produce any effect on freezing rates or social behavior. This clearly demonstrates that the beneficial effects of URB597 cannot be attributed solely to the drug, but instead appear to augment the efficacy of the

extinction training.

Given the changes in representation of memory traces over time (Wheeler et al, 2013), we further evaluated the long-term efficacy of URB597 treatment, combined with the repeated extinction session procedure. When vehicle-treated rats were tested for extinction retention and social behavior more than one month after shock-exposure, they still presented significant behavioral dysfunctions. Of note, treatment with URB597 considerably decreased freezing behavior and increased the time spent in social interaction, more than a month after trauma exposure and 3 weeks after the last drug injection. Moreover, we also found that these effects were blocked by pre-treatment with the CB1 receptor antagonist SR141716, thus showing that URB597 effects are dependent on indirect activation of CB1 receptors through increased levels of AEA.

These behavioral results are consistent with our biochemical data indicating that reductions in AEA content in the hippocampus produced by traumatic stress, were absent in the animals exposed to extinction training. Consistent with previous reports (Gunduz-Cinar et al, 2013; Marsicano et al, 2002), this suggests that extinction training increases endogenous AEA signaling and that augmentation of this effect, through inhibition of AEA hydrolysis, enhances the efficacy of extinction training. Taken together, these results demonstrate that URB597, by increasing AEA levels, can promote extinction consolidation, reducing the emotional consequences of traumatic stress as well, through increasing AEA signaling capacity at CB1 receptors.

The fact that traumatic stress produced a sustained reduction in hippocampal AEA content is consistent with previous reports that stress or glucocorticoids can cause delayed and lasting reductions in hippocampal AEA content (Bowles et al, 2012; Hill et al, 2008a, 2008b; Wang et al, 2012). It is interesting to speculate that the reduction of AEA hippocampal levels might be responsible of the long-lasting effects on memory and social behavior observed in rats exposed to the

trauma. However, given that extinction procedure alone restored normal levels of hippocampal AEA and was sufficient to reduce the cognitive effects of trauma, but at the same time insufficient to significantly increase social behavior, this suggests that the restoration of AEA signaling may be more specific to the cognitive effects as opposed to social behavior. That being said, amplification of AEA signaling during the extinction training session did normalize alterations in social behavior indicating that these changes in AEA signaling may indeed be relevant for alterations in social behavior. These data are particularly interesting in light of recent findings indicating that AEA signaling in the hippocampus is critical for safety learning (Micale et al, 2017), suggesting that augmentation of hippocampal AEA signaling may have broad spanning therapeutic mechanisms in the suppression of emotionally aversive memories.

Our results may be of help in understanding why the URB597-mediated AEA enhancement is more efficacious with respect to the JZL184-mediated 2-AG enhancement in the promotion of the consolidation of extinction of the traumatic memories. Even though AEA and 2-AG act on the same receptors, distinct behavioral effects are frequently reported in literature (Manduca et al, 2015; Morena et al, 2015). For example, exposure to stress generally results in a decrease in AEA and a parallel increase in 2-AG levels (Morena et al, 2016b). Here we did not observe any changes in the tissue content of 2-AG, at least at the discrete time points where we analyzed endocannabinoid levels, but the reduction of freezing induced by JZL184 administered 1 hour before the extinction session, is in line with the retrieval-dampening effects of JZL184 (Morena et al, 2015). The present results provide evidence as to why AEA elevation could represent a better therapeutic approach than synthetic direct cannabinoid agonists in PTSD treatment. Indeed, the synthetic cannabinoid agonist WIN55,212-2, by indistinctly activating cannabinoid receptors, did not produce any significant improvement in the behaviors of traumatized rats except

for a reduction of freezing rates when given post-extinction. Conversely URB597, by acting only on those brain areas where there is an active endocannabinoid transmission, can selectively promote AEA modulatory influences on memory and behavior. It is also important to note that URB597, differently from direct cannabinoid agonists, lacks the classical cannabinoids side-effects such as catalepsy, hypothermia, hyperphagia as well as abuse potential (Piomelli et al, 2006).

In support to our findings, a large body of preclinical and clinical research reports the potential benefit of cannabinoids in the treatment of PTSD symptomatology, including hyperarousal, sleep disturbances and heightened anxiety (Hill et al, 2017; Jetly et al, 2015; Morena et al, 2016a).

Studies on preclinical traumatic stress/PTSD models have suggested that cannabinoid treatment could prevent the development of PTSD-like symptomatology (increased startle response, impairments in hippocampus- and amygdala-dependent memory) when administered in proximity to trauma exposure (Korem and Akirav, 2014; Shoshan et al, 2017). Although these studies give important insights into the mechanisms involved in stress-related processes, from a translational point of view, however, it would be difficult to predict whether a person would develop PTSD after a traumatic event. However, a successful therapeutic approach would be represented by enhancement of extinction learning through exposure psychotherapy. Growing evidence reveals the importance of cognitive enhancers augmenting the efficacy of exposure therapy to treat trauma and anxiety-related disorders (Singewald et al, 2015). In this scenario, our results suggest that drugs elevating AEA signaling at CB1 receptors could be successfully coupled with the exposure therapy to increase its efficacy and reduce both the cognitive and emotional dysfunctions induced by exposure to traumatic stress, thus potentially restoring normal behavior, which we found to remain stable long after trauma-exposure and pharmacological

treatment termination. The fact that URB597 drug intervention could be potentially coupled and limited only to the behavioral exposure psychotherapy, preserving its efficacy and, at the same time, avoiding a chronic administration regimen, would represent a considerable advantage both in terms of promoting drug compliance and minimizing potential side effects on other physiological and/or psychological functions. Taken together our findings have a remarkable translational relevance to the potential importance of FAAH inhibition in the treatment of PTSD. Further studies are needed to confirm the present findings in additional animal models of traumatic stress, but also in clinical settings where the utility of FAAH inhibitors for the treatment of PTSD has become a potential novel therapeutic target.

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CHAPTER 3

PREDICTING SUSCEPTIBILITY AND RESILIENCE IN AN ANIMAL MODEL OF POST-TRAUMATIC STRESS DISORDER (PTSD)

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In preparation

Abstract

Post-traumatic stress disorder (PTSD) is a psychiatric disorder whose pathogenesis relies on a maladaptive expression of the memory for a life-threatening experience, characterized by over-consolidation, generalization and impaired extinction, which in turn are responsible of dramatic changes in arousal, mood, anxiety and social behavior. Even if human subjects experiencing a traumatic event during lifetime all show an acute response to the trauma, only a subset (susceptible) of them ultimately develops PTSD, meanwhile the others (resilient) fully recover after the first acute response. Animal models of PTSD lacks of a dynamic dissection to better understand how this acute response can turn into PTSD-related maladaptive changes. Here we have implemented the PTSD model we previously developed, to make it suitable to differentiate between susceptible (high responders, HR) and resilient (low responders, LR) rats in terms of over-consolidation, impaired extinction, enhanced anxiety and social impairment after trauma experience. Rats were exposed to inescapable footshocks paired with social isolation. One week after trauma and before starting the extinction sessions, animals were tested in the Open Field and Social Interaction tests to search for a predictive variable for HR and LR classification. Our findings show that the locomotor activity in the Open Field test is a reliable predictive variable in term of resilience or susceptibility to develop a PTSD-like phenotype after trauma exposure in rats.

Introduction

The fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DMS-5) defines post-traumatic stress disorder (PTSD) as belonging to the category of "trauma and stressor-related disorders" (American Psychiatric Association, 2013). PTSD has a significant prevalence and morbidity and the abnormal adaptation to a traumatic experience lived represents its specific pathogenic starting point (Berardi et al., 2016, Trezza and Campolongo, 2013, Yehuda et al., 2015). Normally, the formation, consolidation, retrieval and extinction of fear memories associated to a traumatic or stressful situation, is orchestrated by multiple brain regions that act in a coordinate manner (Asok et al., 2018, Herry and Johansen, 2014, Johansen et al., 2011). However, after experiencing a life threatening event, in certain conditions the brain may switch to a state in which there is a maladaptive expression of memory specificities, characterized by over-consolidation, memory generalization and impaired extinction (Desmedt et al., 2015, Finsterwald et al., 2015). These cognitive alterations lead to a persistent reminding of the traumatic memories which in turn is responsible of PTSD development and consecutive symptoms, (e.g., dramatic changes in arousal, mood and social behavior) (Berardi et al., 2016, Yehuda et al., 2015). It has been estimated that more than 50% of the world population will encounter a trauma-causing experience once in their lifetime (Javidi and Yadollahie, 2012) and even if all the traumatize persons will show an acute response to the trauma, not everyone will develop PTSD and the majority of them will recover without intervention (Lewis et al., 2019). Therefore, the important question arises on why do some people develop PTSD after a life-threatening experience, whereas others do not? It is well known, that stress response cannot be sustained for a long time and the organism needs to develop effective physiological and psychological changes (McEwen, 2007). Thus, stressful

experiences lead to adaptation, which in some individuals may be either healthy or pathogenic (Del Giudice et al., 2011, Ullmann et al., 2019).

Animal models of psychiatric diseases are generally useful tools to understand the pathophysiological mechanisms characterizing a specific disorder, thus ultimately leading to a better therapeutic intervention in humans (Nestler and Hyman, 2010). Notwithstanding there are several pre-clinical PTSD models nowadays available and aimed at a better understanding of the etiological mechanisms that may contribute to PTSD development, it still remains difficult to reach a good translational value to humans. Commonly, all the stress-exposed animals in PTSD models, are considered as a homogeneous group for PTSD-like phenotype, the lack of focus on individual variability toward PTSD-like phenotype development causes poor construct, face and predictive validity (Holly and Miczek 2015). Furthermore, another important limit of the majority experimental models of PTSD, is that only the symptoms (e.g. anxiety, anhedonic behavior) are studied (Musazzi et al., 2018), while the causes (e.g. cognitive alterations such as the excessive memory retention and retrieval and the impaired extinction) are underinvestigated. The development of an animal model which is able to predict individual differences in developing a PTSD-like phenotype, remarking both the cognitive and emotional features of PTSD observed in humans, would greatly help the understanding of the neurobiological substrates underlying the individual variability to PTSD, leading to better therapeutic approaches in humans.

We recently developed an animal model that is able to capture both some of the cognitive etiological alterations of the PTSD together with a peculiar set of emotional dysfunctions related to the social domain, alterations also characterizing the human pathology (Berardi et al., 2014, Morena et al., 2018). Here, we outstretched this animal model to disentangle etiological factors that make some individuals vulnerable and others resilient to PTSD development and

we aimed at identifying a predictive variable to rigorously and systematically differentiate, before extinction learning, susceptible (high responders, HR) and resilient (low responders, LR) rats in term of over-consolidation, impaired extinction and social impairment.

Materials and Methods

Animal Care and Use

Male adult Sprague-Dawley rats (350–450 g at the time of testing), Charles River Laboratories, Calco, Italy) were kept 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*. All the experiments were performed during the light phase of the cycle. Rats were handled for 1 min for 3 consecutive days before behavioral testing. 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.

Behavioral Procedures - Experiment 1

The PTSD model we first described (Berardi et al., 2014, Morena et al., 2018) was adapted to make it suitable to distinguish between HR and LR animals in term of over-consolidation, impaired extinction and social impairment (for the experimental design see Fig. 1a). The experimental apparatus consisted in a metal trough shaped alley (60 cm long, 15 cm deep, 20 cm wide at the top, and 6.4 cm wide at the bottom) connected to an animal shocker. All the experimental sessions were video-recorded and subsequently scored by two well-trained researchers blind to the experimental conditions. After each session, fecal boli were removed and the apparatus was cleaned with a 70 % ethanol solution.

Housing. All rats were individually housed for 2 days prior to the habituation session and remained singly housed until the end of the behavioral testing. We have previously shown that social isolation is necessarily required to develop enduring signs of emotional distress upon exposure to a traumatic event (Berardi et al., 2014).

Habituation. On the first day of testing (day −1), rats were individually taken from the home-cage and habituated for 5 min to the test apparatus. Rats were then returned to their homecages.

Exposure session. On day 0, rats were individually placed in the apparatus and were left undisturbed for 2 min. Then, five footshocks (2 s, 0.8 mA) were randomly delivered. After the last shock, rats were kept in the apparatus for 60 s to facilitate context association to the aversive stimuli.

Screening of HR and LR populations. 5 and 6 days after trauma exposure rats were subjected to the OF and Social Interaction (SI) tests respectively, with the purpose of identify a predictive variable for the screening of HR and LR phenotypes in accordance to their behaviour.

Open Field Test. Each rat was placed into the center of the Open Field arena (80×80×60 cm) for 15 min. The floor of the apparatus was divided into sixteen imaginary equal-sized squares and the total number of crossings between squares, as well as the time spent in the centre of the arena, were determined as parameters indicating the rat locomotor activity and emotionality, respectively. After each session, rats were returned to their home cage, fecal boli were

removed and the arena's walls and floor were cleaned with a 70% ethanol solution.

Social Interaction Test. In the Social Interaction test couples of rats were put for 10 min in a quadrangular arena (40×40×60 cm) made of Plexiglas with clean sawdust covering the floor, under red light conditions. After each session, rats were returned to their home cage, fecal boli were removed, sawdust was blended, and the arena's walls were cleaned with a 70% ethanol solution. Each couple was established according to the unfamiliarity criteria (the 2 rats of each pair have never been housed in the same cage). In this test, social behavior was scored as previously described (Berardi et al., 2014).

Extinction sessions and Extinction retention test. Each of the four extinction sessions consisted in a 10-min re-exposure to the experimental apparatus, with the first carried out 7 days after the exposure session (day 7). Each subsequent session was separated from the preceding one by a 72-h interval (days 10, 13 and 16). During each extinction session the contextual freezing behavior was evaluated (Chen et al., 2012, Morena et al., 2018, Wheeler et al., 2013). Particularly, the freezing behavior analyzed during the first extinction session (7 days after trauma exposure) was considered as an index of the over-consolidation of memory related to the traumatic experience, whereas, the last extinction session (16 days after trauma exposure) was considered as the extinction retention test, and the contextual freezing behavior here evaluated was considered as an index of the impaired-extinction in our PTSD animal model.

Social Interaction Test. To evaluate the social impairment, rats were tested in the Social Interaction test, which was carried out 72 h after the extinction retention test (day 19). Couples of rats for this Social Interaction were different from the

previous couples in the Social Interaction at 6 days after trauma exposure. The test was conducted as above described.

Behavioral Procedures - Experiment 2

In a second experiment, a second group of rats was submitted to the rotarod test 5 days after trauma exposure (day 5), instead to the Open Field test, which was run 2 days before the trauma (day -2), accordingly with the experimental design shown in Fig. 1b. The Open Field test and the other behavioral procedures (Habituation, Exposure, Extinction sessions, Extinction retention test and Social Interaction test) as well as the Housing condition, were run and maintained at the same way as described for the experiment 1.

Rotarod Test. The Rotarod test was performed as previously described (Hadadianpour et al., 2017). The rotarod speed was regularly accelerated each 30 s from 10 to 60 rpm. The cut-off time was fixed at 300 s. Rats were given three trials with 30 min inter-trial rest intervals. The mean time, across the three trials, taken from each animal to fall from the rotarod apparatus was measured and it represented a measure of the motor activity, since it is the time taken by each rat to maintain its balance walking on the revolving rod.

Statistical Analysis

Statistical analysis was performed using SPSS statistical software. Each measure is expressed as mean ± SEM. For each correlation analysis performed, R coefficient and the relative P value were evaluated and a good correlation between variables was considered for $R \geq 0.35$ and $P < 0.05$. Behavioral data were analyzed through a repeated measures ANOVA (RM ANOVA) or one-way ANOVA when appropriate. Tukey–Kramer post hoc test was performed to

control for significant differences between groups and significance was considered for P < 0.05.

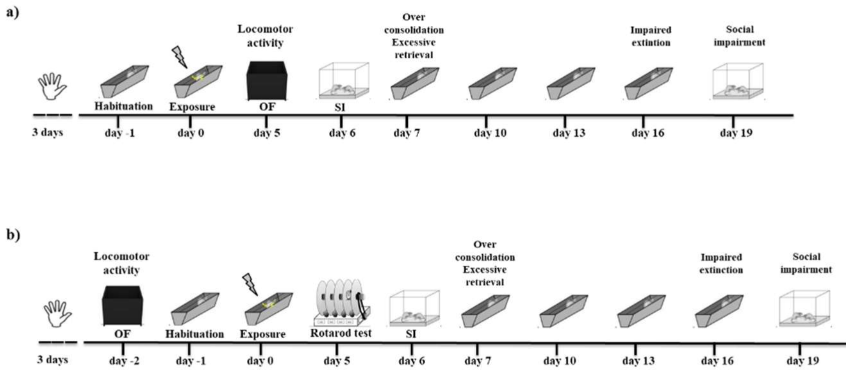

Figure 1. Experimental designs for the Experiment 1 (a) and the Experiment 2 (b)

Results

The number of crossings in the Open Field test is a reliable predictive variable in term of resilience and susceptibility to develop a PTSD-like phenotype after trauma exposure in rats

In the first experiment, we aimed at identifying a predictive variable for HR and LR phenotypes screening. To this aim we performed a correlation analysis between potentially predictive variables (number of crossings in the Open Field test, time spent in the centre in the Open Field test and time spent in social interaction in the Social Interaction test performed before the extinction learning) and other behavioral outcomes associated with PTSD (such as: over-consolidation, excessive retrieval, impaired extinction and social alteration). Particularly, we found a significant correlation between the number of crossings in the Open Field test performed 5 days after trauma exposure and the freezing behavior shown by rats during the first extinction session (day 7) (R = 0.491, P

< 0.0001) (Fig. 2a), index of the over-consolidation of the trauma experience in the PTSD model. The same result was obtained for the correlation analysis between this parameter and the freezing behaviour shown by rats during the extinction retention test (day 16) ($R = 0.532$, $P < 0.0001$) (Fig. 2b), index of the impaired extinction of the trauma experience in the PTSD model. Moreover, a significant correlation was found between this parameter and the time spent in social interaction in the Social Interaction test performed 19 days after trauma exposure ($R = 0.384$, $P < 0.001$) (Fig. 2c), index of social alterations in the PTSD model. Subsequently, we performed the same correlation analysis, taking in consideration, as a potentially predictive variable, the time spent in the centre of the Open Field arena during the Open Field test performed 5 days after trauma exposure (Tab. 1), and no significant correlations were found between this parameter and the freezing behavior shown by rats during the first extinction session (day 7) ($R = 0.011$, $P = 0.921$), the freezing behavior shown by rats during the extinction retention test (day 16) ($R = 0.040$, $P = 0.727$) and the time spent in social interaction in the Social Interaction test performed 19 days after trauma exposure ($R = 0.027$, $P = 0.812$). Finally, we performed the correlation analysis taking in consideration the time spent by each rat in social interaction, during the Social Interaction test performed 6 days after trauma exposure, as a potentially predictive variable (Tab 1). The statistical analysis revealed no significant correlation between this factor and the freezing behavior shown by rats during the first extinction session (day 7) ($R = 0.107$, $P = 0.345$), and the freezing behavior shown by rats during the extinction retention test (day 16) ($R = 0.057$, $P = 0.617$). No significant correlations were also found, between this parameter and the time spent in social interaction, in the Social Interaction test performed 19 days after trauma exposure ($R = 0.032$, $P = 0.776$).

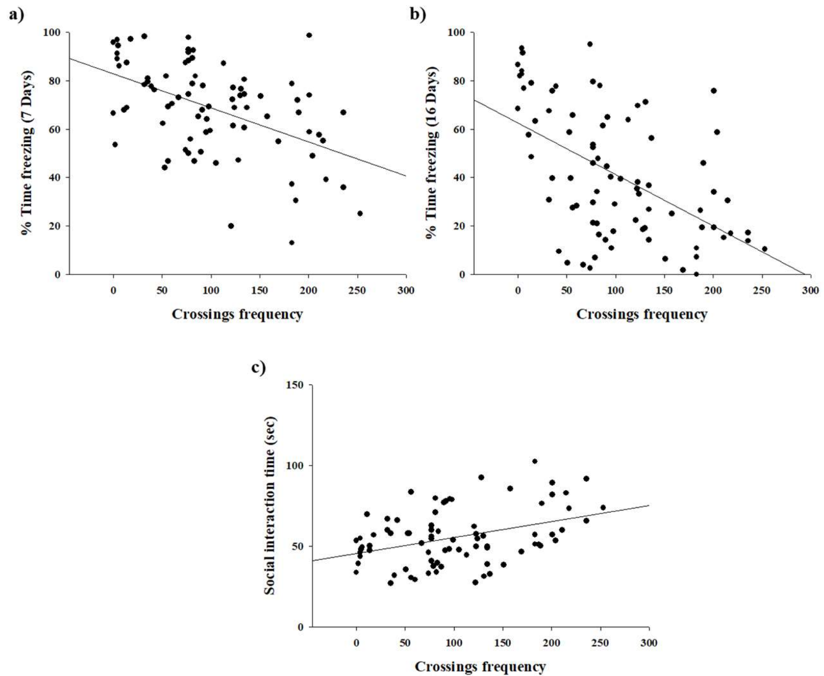

Figure 2. The number of crossings in the Open Field test is a reliable predictive variable in term of resilience and susceptibility to develop a PTSD-like phenotype after trauma exposure in rats. The number of crossings evaluated in the Open Field test performed 5 days after trauma exposure, significantly correlated with freezing behaviors at 7 and 16 days after trauma, index of the over consolidation and impaired extinction of memory for the traumatic experience, respectively **(a, b)** and with the social interaction time, evaluated in the Social Interaction test performed 19 days after trauma exposure as an index of social behavior alterations in the PTSD-like phenotype. **(c).** N = 80.

Predictive variables	Dependent variables		
	Freezing (day 7)	**Freezing (day 16)**	**Social Interaction Time (day 19)**
Time spent in the centre of the OF arena (day 5)	R = 0.011 P = 0.912	R = 0.040 P = 0.727	R = 0.027 P = 0.812
Social Interaction Time (day 6)	R = 0.107 P = 0.345	R = 0.057 P = 0.617	R = 0.032 P = 0.776

Tab. 1. Correlation analysis considering the time spend in the centre of the OF arena and the Social Interaction Time as predictive variables in term of resilience and susceptibility to develop a PTSD-like phenotype after trauma exposure in rats. N = 44.

Rats classification according to the number of crossings in the Open Field test, revealed behavioral alterations associated with the PTSD-like phenotype after trauma exposure in rats

Thereafter the identification of the number of crossings in the Open Field test as a promising predictive variable for the HR and LR phenotypes screening, we defined the rats' classification according to the extremes (25th or 75th percentile) of the experimental group's distribution for this parameter. Every rat scored above 25th or over 75th percentiles has been considered as HR or LR respectively, whereas every rat scored between 25th and 75th percentiles has been considered Normal Responder (NR). The subsequent RM ANOVA for the freezing behavior analysis across the extinction sessions in the three experimental groups (Fig. 3a), revealed a significant effect of the phenotype HR, LR or NR ($F_{(2,77)} = 18.189$, $P < 0.0001$), a significant effect of the extinction sessions ($F_{(3,77)} = 48.510$, $P < 0.0001$) and tendency toward significance for the interaction between this two factors ($F_{(6,231)} = 2.071$, $P = 0.058$). Post hoc tests shown that HR rats presented higher levels of freezing across the extinction sessions and at extinction retention

test as compared with the LR group (Ps < 0.01, for all days of extinction), and they presented higher levels of freezing with respect to the NR group only during the third extinction session (day 13) and the extinction retention test (day 16) (Ps < 0.01). Moreover, the post hoc tests revealed that the LR group presented lower levels of freezing across all the extinction sessions as compared with the NR group (P < 0.05, days 7 and 13; P < 0.01, day 10), excepted the extinction retention test (day 16) in which no significant differences between these two groups were found. Then, through a one-way ANOVA analysis, we analyzed the time spent in social interaction during the Social Interaction test performed 19 days after the trauma exposure (Fig. 3b) and a significant difference between the three experimental groups has been revealed ($F_{(2,77)} = 5.801$, $P = 0.005$). Particularly, post hoc analysis revealed that HR and NR groups spent less time in social interaction with respect to the LR group (P < 0.01 and P < 0.05, respectively).

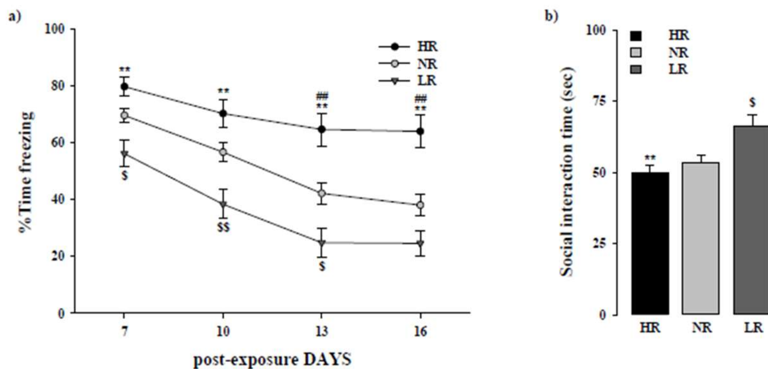

Figure 3. Rats classification according to the number of crossings in the Open Field test, revealed behavioral alterations associated with the PTSD-like phenotype after trauma exposure in rats. Freezing rates across the three extinction sessions (days: 7, 10 and 13) and the extinction retention test (day 16) in rats segregated in HR, NR and LR according to the 75th and 25th percentile of the experimental group's distribution for

number of crossings in the OF test performed 5 days after trauma exposure. HR rats displayed increased freezing response compared to LR rats, across the extinction sessions and at extinction retention test, where they showed higher level of freezing also with respect to the NR group. LR rats shown an exactly opposite freezing profile, with lower levels of freezing across the extinction session and no significant differences with respect to the NR group at the extinction retention test. **(a).** Social interaction time of HR, NR and LR rats. HR and NR rats spent less time interacting with a conspecific with respect to LR rats **(b).** *, $P < 0.05$ HR vs LR group; §, $P < 0.05$ HR vs NR group; #, $P < 0.05$ LR vs NR group; **, $P < 0.01$ HR vs LR group; ##, $P < 0.01$ HR vs NR group §, $P < 0.05$; §§, $P < 0.01$ LR vs NR group. N = 20-40 per group.

Trauma induced changes in the natural tendency to explore a new environment made the number of crossings a reliable predictive variable for the development of PTSD-like phenotype

In the second experiment, in order to better understand the role of the pure motor activity in making the number of crossings in the Open Field performed 5 days after trauma a reliable predictive variable of PTSD-like phenotype, we performed a correlation analysis between the mean time taken by each animal to fall from the rotarod apparatus and the behavioral outcomes associated with PTSD. The same correlation analysis was also performed between the number of crossings in the Open Field test pre-trauma and the behavioral outcomes associated with PTSD-like phenotype, in order to establish the role of the natural tendency to explore a new environment as a reliable predictive variable of PTSD-like phenotype. The correlation analysis between the mean time, across the three trials, taken from each animal to fall from the rotarod apparatus and the freezing behavior shown by rats during the first extinction session (day 7) and during the extinction retention test (day 16) did not reveal any significant correlation ($R = 0.133$, $P = 0.389$; $R = 0.106$, $P = 0.494$; respectively). No significant correlation was also found between this parameter of pure motor activity and the time spent in social interaction during the Social Interaction test performed 19 days after

trauma exposure (R = 0.073, P = 0.637) (Tab 2). The correlation analysis between the number of crossings in the Open Field pre-trauma and the behavioral outcomes associated with the PTSD-like phenotype did not revealed any significant correlation between this parameter and the freezing behavior shown during the first extinction session (day 7) (R = 0.047, P = 0.760), the freezing behavior shown during the extinction retention test (day 16) (R = 0.036, P = 0.817) and the time spent in social interaction during the Social Interaction test performed 19 days after trauma (R = 0.081, P = 0.600) (Tab 2).

Predictive variables	**Dependent variables**		
	Freezing (day 7)	**Freezing (day 16)**	**Social Interaction Time (day 19)**
Mean time to fall from rotarod apparatus (day 5)	R = 0.133 P = 0.389	R = 0.106 P = 0.494	R = 0.073 P = 0.637
Crossing frequency (day -2)	R = 0.047 P = 0.760	R = 0.036 P = 0.817	R = 0.081 P = 0.600

Tab. 2. Correlation analysis considering the time to fall from the rotarod apparatus and the number of crossing in the OF test performed before trauma exposure as predictive variables. N = 44.

Discussion

In this study we aimed at identifying a predictive variable to rigorously and systematically differentiate, between susceptible and resilient subjects in an animal models of PTSD. Here, by introducing specific behavioral tests suitable to disentangle individual differences with regards to the emotionality, such as the Open Field test and Social Interaction test, performed after the traumatic

experience and before the extinction sessions, we outstretched our previously validated protocol and we found a predictive variable for the screening of PTSD susceptibility and resilience in term of over-consolidation, impaired extinction, and social impairment. Particularly, we found as a reliable predictive variable the number of crossings (locomotor activity) in the Open Field test. The analysis of the number of crossings in the Open Field test is an easy and valuable tool to study at the same time the motor activity and the natural tendency of rodents to explore a new environment (Denenberg, 1969). Through a correlation analysis, we found a negative dependence between this parameter and the percentage of time spent in freezing behavior, during the first extinction session (7 days after trauma) and during the extinction retention test (16 days after trauma). Maladaptive memory processes are specific trait of PTSD pathology (Trezza and Campolongo, 2013) and they can be studied in rodents (Berardi et al., 2014). Contextual freezing behavior represents a fear and anxiety related response shown by rodents when exposed to the context in which they lived a traumatic experience (Phillips and LeDoux, 1992). This behavior is linked to fear memory retention and indeed, we previously found that it is reduced across consecutive extinction sessions, in which each rat is repeatedly exposed, at specific time points, to the context of trauma in the absence of trauma (Berardi et al., 2014, Morena et al., 2018). The fear memory elicited at the first extinction session is considered as a measure of memory retrieval. In fact, at this time point, the extinction learning has not started yet, and an excessive memory retrieval can be due to a maladaptive memory consolidation processes induced by the traumatic experience and known as over-consolidation (Kida, 2019). Conversely, freezing behavior at the extinction retention test can be considered as a measure of extinction learning after each extinction session and high levels of freezing at this time later points represent an index of deficit in fear extinction of the traumatic experience (Singewald and Holmes, 2019). Our results highlight that

the lower is the number of crossings the higher is the freezing behavior of rats both at the retrieval or extinction sessions. This result shows that this behavioral parameter is strictly related to the PTSD-like cognitive maladaptive changes of over-consolidation and impaired extinction.

One of the overarching features of PTSD, in addiction to the cognitive alterations, is represented by the reduction in social functioning (American Psychiatric Association, 2013). Several clinical reports, indicate social withdrawal and social isolation as common hallmarks of PTSD patients (Hofmann et al., 2003, Kashdan et al., 2009, LaMotte et al., 2017). In the animal model we previously validated we found that rats exposed to footshock trauma paired with social isolation displayed reduced social interaction time in the Social Interaction test (Berardi et al., 2014). Here we found that the number of crossings in the OF test positively correlates with the time spent in social interaction during the Social Interaction test performed long after trauma, suggesting that this parameter is also predictive of the trauma induced maladaptive changes in the social domains. Taken together these results indicate the number of crossings as a very good predictive variable for both the cognitive and emotional alterations in a PTSD-like phenotype after trauma exposure in rats. Therefore, rats were segregated in HR, NR and LR according to the 75th and 25th percentile of the experimental group's distribution for number of crossings in the Open Field test. Our results indicate that HR rats displayed increased freezing response compared to LR rats, across retrieval and extinction sessions, where they showed higher level of freezing also with respect to the NR group. Thus, resembling what we know about PTSD clinical settings, where an over-consolidation of fear memory related to a traumatic event and an impaired extinction learning after exposure therapy sessions are robust endophenotypes of susceptibility to PTSD pathology, whereas, the absence of these maladaptive changes is typical of a resilient phenotype (Hill et al., 2018). The same classification revealed significant

differences among experimental groups also in the Social Interaction test. Particularly, we found that HR rats spent less time interacting with a conspecific with respect to LR rats after trauma. Thus, indicating that the rat's classification according to the number of crossings distribution is able to reproduce the trauma induced social alteration in susceptible subjects, giving a high translational value to our PTSD animal model with respect to human PTSD.

Motor activity has a good translational value since it may resemble the strategies adopted by human subjects to deal with stressful situations, known as coping strategies (Feder et al., 2009, Koolhaas et al., 2007, Puglisi-Allegra and Andolina, 2015). The number of crossings, as previously said, is a measure of motor activity but also of the innate tendency of the animal to explore a new environment moving across it (Denenberg, 1969). Prompted by the results we obtained in the first set of experiments, we aimed to dissociate these two aspects, in order to better understand if it is the motor activity *per se,* or the natural tendency to explore a new environment that made the number of crossings a reliable predictive variable for the development of PTSD-like phenotype. Literature data indicate a reduction in the motor activity in animals exposed to contextual fear conditioning regardless of their individual susceptibility (Daviu et al., 2010, Radulovic et al., 1998). The results of a second set of experiments, did not reveal any significant correlation between the pure motor activity assessed through the rotarod test performed 5 days after trauma exposure and behavioral outcomes associated with the PTSD-like phenotype, thus allowing us to exclude the role of the pure motor activity in making the number of crossings a predictive variable for the development of a PTSD-like phenotype.

Interestingly, we did not find any significant correlation between the number of crossings in the Open Field test performed before the traumatic experience and behavioral outcomes associated with the PTSD-like phenotype. Specifically, the number of crossings here evaluated represent an index of the rat's innate

tendency to explore a new environment. The results we obtained, clearly indicate that only the changes induced by the trauma exposure on the number of crossings, make it a predictive variable for susceptibility and resilience towards developing PTSD-like phenotype. It is tentative to hypothesize that the footshock trauma may enhance the stress responsiveness, specifically in HR rats, to other potentially stressful situation, such as a novel environment and, as a consequence of this, a reduction of their innate tendency to explore a novel environment is showed. Consequently, it may be possible to assume that the trauma induced changes in the natural tendency of the rats to explore a new environment, evaluated by the number of crossings in the Open Field test performed 5 days after trauma, make this parameter a predictive variable for screening of susceptible and resilient phenotype to PTSD development.

In conclusion, here we developed an animal model able to predict individual differences in developing PTSD-like phenotype with a high translational value remarking the same differences observed in humans. Our findings pave the road to further studies in which HR, NR and LR rats in such way screened, can be differently manipulated to a better understanding of the neurobiological underpinnings of susceptibility and resilience to PTSD, leading to greater therapeutic interventions in humans.

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CHAPTER 4

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

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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.

Material 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 ± 1C; 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).

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 x 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 (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).

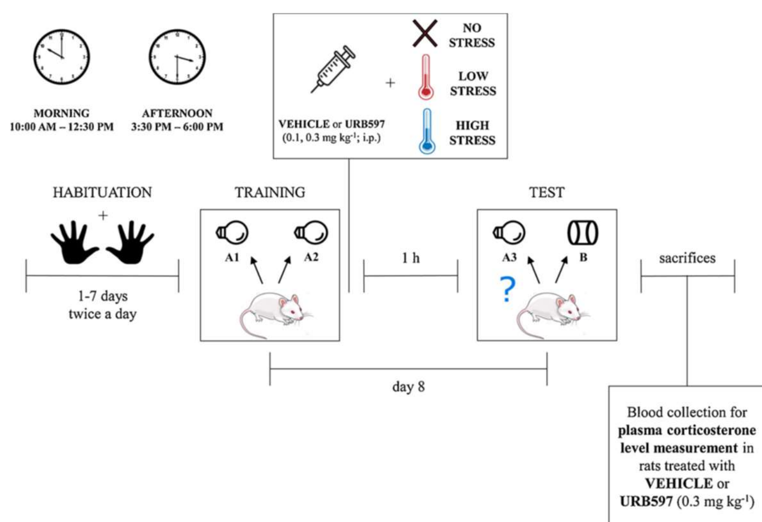

Figure 1 – Diagram of the experimental procedure.

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).

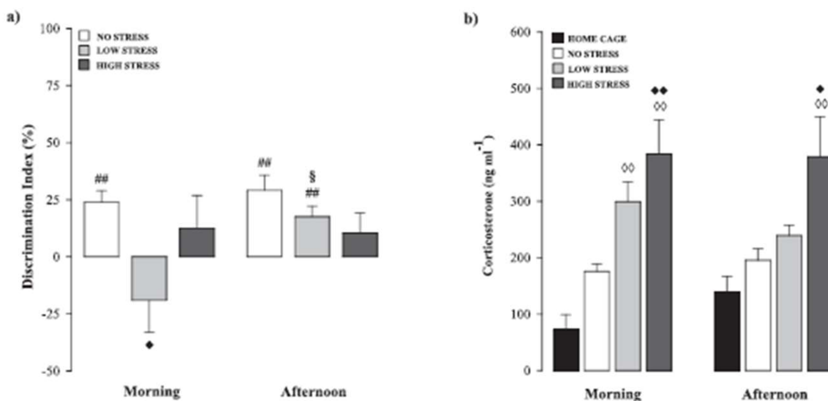

Figure 2 – **Circadian-dependent effects of different stress conditions on short-term memory**. **a)** Discrimination index on the testing trial for vehicle-treated rats that were subjected to no, low or high stress conditions immediately after the training trial performed in the morning or afternoon. *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).

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.

	Morning			Afternoon		
	Total object exploration time (s)	Number of crossings	Number of rearings	Total object exploration time (s)	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).

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.

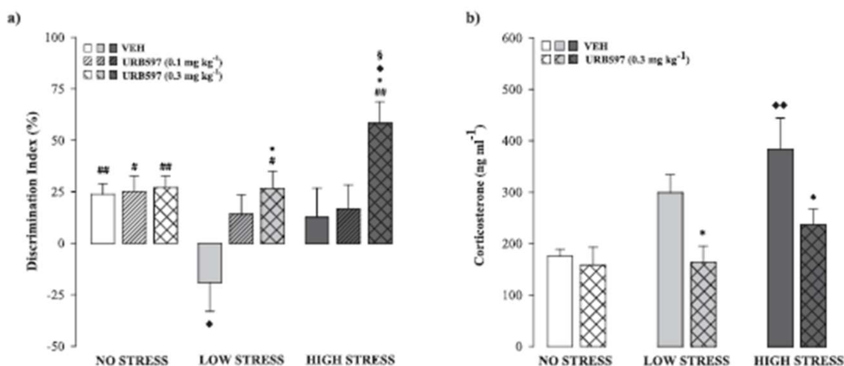

Figure 3 – URB597 modulation of stress-dependent effects on short-term memory in the morning. **a)** Discrimination index on the testing trial for vehicle- and URB597-treated rats that were subjected to no, low or high stress conditions immediately after the training trial performed in the morning. *Post 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.

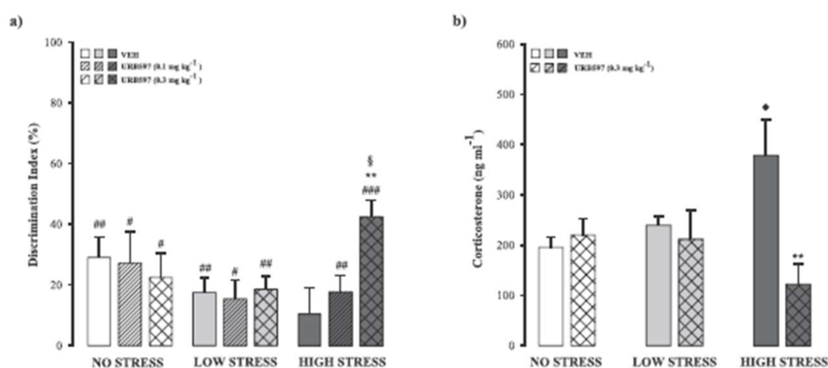

Figure 4 – URB597 modulation of stress-dependent effects on short-term memory in the afternoon. a) Discrimination index on the testing trial for vehicle- and URB597-treated rats that were subjected to no, low or high stress conditions immediately after the training trial, in the afternoon session. *Post 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 5

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

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In Preparation

Abstract

Amphetamine is a potent psychostimulant that increases brain levels and activity of monoamine neurotransmitters, such as norepinephrine, dopamine and serotonin. Among these, norepinephrine is a neurotransmitter crucially involved in the regulation of memory consolidation for stressful experiences. Here, we investigated amphetamine effects on the consolidation of rat long-term recognition memory, when animals were exposed to different intensities of forced swim stress. Furthermore, we evaluated whether such effects are dependent on the activation of the peripheral adrenergic system. To this aim, immediately after an object recognition training, adult male Sprague Dawley rats were injected with amphetamine (0.5 or 1 mg/kg), or its corresponding vehicle, and 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 is capable to enhance memory consolidation, only when rats were subjected to the mild stress condition, while impairs memory performance after exposure to strong stress. These dichotomic effects appear to depend on the stress-induced activation of the peripheral adrenergic system response. Indeed, we found opposite amphetamine-mediated effects on the consolidation of long-term recognition memory in adrenal medullectomized rats. So far, our results underlie that the peripheral adrenergic response is a key player in the modulation of amphetamine effects on long-term memory.

Introduction

The psychostimulant amphetamine was discovered more than 100 years ago (Heal et al., 2013). The analogy in the chemical structure between amphetamine and the monoamine neurotransmitters, such as norepinephrine (NE), dopamine (DA) and serotonine (5-HT), is crucial not only for its mechanism of action, but also for the pharmacological properties of this compound (Ferris and Tang, 1979). Amphetamine acts as a competitive substrate of monoamine neural membrane transporters: NET, DAT and SERT, for NE, DA and 5-HT re-uptake, respectively (Sulzer et al., 2005). Once entered in the presynaptic neuron, amphetamine distrupts the monoamine storage vesicles and, as a consequence, increases the levels of monoamines in the neural cytosolic pool (Teng et al., 1998). Such enhanced cytosolic concentration of monoamines reverts the transport direction of NET, DAT and SERT, increasing the amount of NE, DA and 5-HT in the synaptic cleft (Robertson et al., 2009). Enhanced levels of the monoamines, in particular NE and DA, at the synaptic terminal, is responsible of 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 (Martinez et al., 1980a, Ballard et al., 2014, Bardgett et al., 2019). Interestingly, it has been shown that these effects are dependent on the amphetamine-induced activation of the noradrenergic system (Lee and Ma, 1995).

The noradrenergic system is broadly involved in the modulation of long-term memory consolidation (Ferry et al., 1999, Roozendaal and McGaugh, 2011, LaLumiere et al., 2017). It is widely recognized that emotional 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 glucorticoids, released 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 mediate effects on cognitive function, by promoting an inverted U-shaped dose-effect curve (Schilling et al., 2013), in which optimal levels of stress hormones are responsible of memory performance potentiation, whereas an over-expression of them leads to memory impairment (Baldi and Bucherelli, 2005).

Considering the amphetamine modulation of noradrenergic system, and in turn of memory processes, and taking in account also that different stress intensities prompt to different stress hormones levels with distinct effects on cognitive functions, here we first aimed at investigating amphetamine effects on consolidation of long-term object recognition (OR) memory in rats exposed to different intensities of stress. To this aim, immediately after the training trial in an OR task, rats were treated with amphetamine, or its vehicle, and then subjected to a forced swim stress (FSS) procedure, for 1 or 5 minutes of swimming at water temperature of 25 (mild) or 19 ± 1°C (strong stress condition), respectively, in order to elicit different plasma stress hormone levels (Morena et al., 2015; Santori et al., 2019). In a second set of experiment, we evaluated whether amphetamine effects on the consolidation of long-term OR memory, in rats exposed to different stressful conditions, were dependent on the activation of the peripheral adrenergic system. To this aim, before starting the experimental procedures, rats were subjected to surgical removal of the adrenal medulla and thereafter subjected to the same experimental protocol of the first set of experiments.

Material 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 trial of the cycle between 11:00 AM and 2:00 PM. All procedures involving animal care or treatments were performed in compliance withthe ARRIVE guidelines, the Directive 2010/63/EU of the European Parliament, and the D. L. 26/2014 of Italian Ministry of Health.

Drug Treatment

Amphetamine ((RS)-1-phenylpropan-2-amine) (0.5 and 1 mg/kg) were dissolved in saline 0.9% (vehicle) and administered intraperitoneally (i.p.), at the volume of 1 ml/kg, immediately after the training session. Doses were chosen on the basis of pilot experiments performed in our laboratory and on literature data (PMID: 8643648). The solutions were freshly prepared on the day of the experiment.

Behavioral Procedures

Object recognition task. The object recognition task was performed accordingly to a slightly modified procedure of that described by Santori et al.(2019). 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 (Campolongo et al., 2003). During habituation, rats were allowed to freely explore the apparatus in the absence of objects. The animals were randomly assigned to two different groups: mild stress and strong stress conditions. 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, it was subjected to a mild or strong FSS session; then, it was returned to the home cage. 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 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. Long-term memory retention was tested 24-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. 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. FSS procedure was carried out accordingly to Santori et al., (2019). Immediately after the training trial of the OR 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 mild and strong stress condition groups were subjected to a mild or strong 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; Santori et al., 2019).

Surgical procedures

Adrenal medullectomy. In a second set of experiments, rats were subjected to adrenal medullectomy, which was performed as previously described (Martinez et al., 1980b, Wilkinson et al., 1981, Shin et al., 2017; Khasar et al., 2009). Briefly, rats were anesthetized with a mixture of Zoletil and Domitor (40 mg/kg and 35 µg/kg respectively). Each animal was 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 the

removal of adrenal medullae. Accordingly to leterature data, rats were provided with 0.45% saline to drink for 7 days after surgery (Khasar et al., 2009). Experimental procedures were started 1 week after surgery.

Data and Statistical Analysis

One-sample t-tests were used to determine whether the discrimination index was different from zero. Object recognition data were analyzed by one or two-ANOVA, 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). All data are expressed as mean ± standard error of the mean (SEM).

Results

Amphetamine enhances long-term memory consolidation in rats subjected to a mild FSS condition

This experiment investigated amphetamine effects on the consolidation of rat long-term recognition memory, when animals were exposed to a mild FSS condition immediately after the training trial.

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 post-training treatment effect ($F_{(2,23)} = 1.074$, $P = 0.358$; Table 1). Examination of rats' exploratory behavior of the experimental apparatus during the training trial indicated that there were no significant differences for crossings and rearings among groups before drug treatment and stress exposure (Table 1). In fact, one-way ANOVA for the number of crossings

and rearings on the training trial revealed no significant post-training treatment effect ($F_{(2,23)} = 0.675$, $P = 0.519$ 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 revelead that the DI of rats administered with vehicle was not significantly different form zero ($t_{(8)} = 0.028$, $P = 0.978$). Similarly, the same results were obtained for rats treated with amphetamine at the dose of 0.5 mg/kg ($t_{(7)} = 1.378$, $P = 0.211$) . On the contrary, rats treated with amphetamine at the dose of 1 mg/kg, significantly discriminated the novel object from the familiar one ($t_{(8)} = 5.078$, $P = 0.010$). Furthermore, one-way ANOVA for the DI reported significant differences among the drug treatment groups ($F_{(2,23)} = 4.341$, $P = 0.025$) and the following *post hoc* analysis indicated that the DI of rats treated with 1 mg/kg of amphetamine was significantly higher from the one of vehicle-treated rats ($P < 0.05$) (Fig 1). One-way ANOVA for total exploration time of the two identical objects 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).

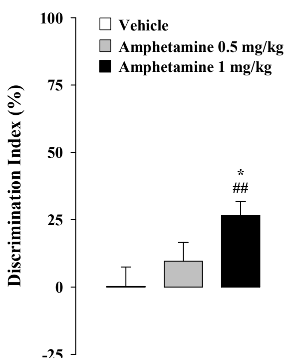

Figure 1. Amphetamine effects on the consolidation of long-term OR memory in rats exposed to the mild FSS condition immediately after the training trial. Discrimination

index on the retention test trial for vehicle- and amphetamine-treated rats that were subjected to the mild stress condition immediately after the training trial. *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).

Amphetamine impairs long-term memory consolidation in rats subjected to a strong FSS condition

This experiment investigated amphetamine effects on the consolidation of rat long-term recognition memory, when animals were exposed to a strong FSS condition immediately after the training trial.

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

Testing trial. One sample t-test revelead that the DI of vehicle-treated rats was significantly different from zero ($t_{(9)} = 3.007$, $P = 0.015$), indicating that these animals were able to express long-term retention for the familiar object. 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), pointing out that such rats were not able to discriminate the two objects. Moreover, one-way ANOVA for the DI revealed significant differences between experimental groups ($F_{(2,28)} = 3.889$, $P =0.032$) and the following *post hoc* analysis indicated that the DI of rats treated with 1 mg/kg of amphetamine, was significantly lower from that of vehicle treated rats ($P < 0.05$) (Fig 2). No

significant differences were found, in the retention test trial, for the total exploration time ($F_{(2,28)} = 0.206$, $P = 0.815$), the number of crossings ($F_{(2,28)} = 2.079$, $P = 0.144$) and the number of rearings ($F_{(2,28)} = 1.478$, $P = 0.245$) (Table 2).

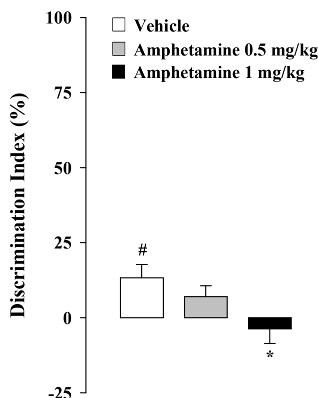

Figure 2. Amphetamine effects on the consolidation of long term OR memory in rats exposed to the strong FSS condition immediately after the training trial. Discrimination index on the retention test trial for vehicle- and amphetamine-treated rats that were subjected to the strong stress condition immediately after the training trial. *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 a mild FSS condition

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

Training trial. Two-way ANOVA for total object exploration time 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 adrenal medullectomy x treatment ($F_{(1,33)}$ = 1.252, P = 0.271) effects. *Post hoc* analysis showed that medullectomized and post-training amphetamine-treated animals showed higher total exploration time with respect to the relative sham group (P < 0.05) (Table 1). Two-way ANOVA for the number of crossings revealed no significant effects of post-training drug treatment ($F_{(1,33)}$ = 0.052, P = 0.821), adrenal medullectomy ($F_{(1,33)}$ = 0.261, P = 0.613) or the interaction between these two factors ($F_{(1,33)}$ = 0.384, P =0.540) (Table 1). The same results were obtained for two-way ANOVA concerning the number of rearings (treatment: $F_{(1,33)}$ = 0.137, P = 0.714; adrenal medullectomy: $F_{(1,33)}$ = 0.284, P = 0.598; treatment x adrenal medullectomy $F_{(1,33)}$ = 0.058, P = 0.812) (Table 1).

Testing trial. One sample t-test revelead that the DI of both sham and medullectomized rats treated with vehicle were no significant different from zero ($t_{(7)}$ = 0.774, P = 0.464; $t_{(7)}$ = 2.007, P = 0.085), indicating that they were not able to express long-term retention of the familiar object. On the contrary, the DI of animals treated with amphetamine and belonging to the sham group was significantly different from zero ($t_{(7)}$ = 8.423, P < 0.0001). The same result was obtained for medullectomized rats treated with amphetamine ($t_{(12)}$ = 4.519, P = 0.0007), thus suggesting that both experimental groups were able to discriminate the two objects Two-way ANOVA analysis 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 he interaction between the two factors ($F_{(1,33)}$ = 6.081, P = 0.019). As expected, the *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, the *post hoc* analysis indicated that medullectomized rats treated with amphetamine showed lower DI than the respective sham group (P < 0.01) (Fig. 3). Two-way ANOVA analysis for the total exploration time did not reveal any significant differences between experimental groups due to

amphetamine treatment ($F_{(1,33)} = 0.967$, $P = 0.333$), but a significant effect of the adrenal medullectomy was found ($F_{(1,33)} = 10.757$, $P = 0.003$). No significant differences between experimental groups were detected for the interaction between these two factors ($F_{(1,33)} = 0.702$, $P = 0.408$). *Post hoc* analysis revealed that adrenal medullectomized rats treated with amphetamine or with vehicle, showed higher total exploration time than the respective sham groups ($P < 0.05$) (Table 2). No significant differences, between the experimental groups, were found for the two-way ANOVA analysis of 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 of the number of 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).

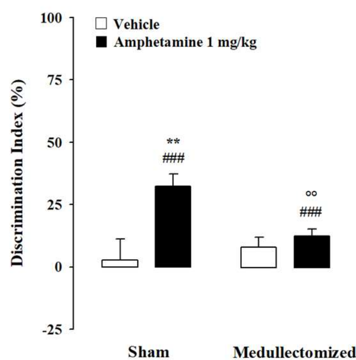

Figure 3. Peripheral adrenergic system influences on amphetamine effects on the consolidation of long-term OR memory in rats exposed to the mild FSS condition immediately after the training trial. Discrimination index on the retention test trial for sham and medullectomized rats treated with vehicle or amphetamine and subjected to the mild stress condition immediately after the training trial. *Post hoc* comparisons reported significant differences between groups as follows: ** $P < 0.01$ vs the corresponding vehicle group. °°, $P < 0.01$ vs the corrisponding 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 ameliorated long term memory consolidation in adrenal medullectomized rats subject to a strong FSS condition

In this experiment we sought to determine whether amphetamine impairing effects on long-term memory consolidation in rats exposed to the strong condition of FSS 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 medullectomized and post-training vehicle-treated animals showed lower number of crossings with respect to the relative sham group ($P < 0.05$) (Table 1). Concerning the number of rearings, no significant effects of treatment ($F_{(1,40)} = 0.532$, $P = 0.470$), adrenal medullectomy ($F_{(1,40)} = 0.507$, $P = 0.481$) and of the interaction between these two factors ($F_{(1,40)} =1.683$, $P = 0.202$) were found according to two-way ANOVA (Table 1).

Testing trial. One sample t-test revelead that in the sham group, only vehicle-treated animals were able to express long-term retention of the familiar object ($t_{(9)} = 2.275$, $P = 0.049$) the sham animals treated with amphetamine not ($t_{(11)} = -0.127$, $P = 0.901$). In the medullectomized groups, rats treated with vehicle or with amphetamine significantly discriminated the two objects ($t_{(10)} = 7.003$, $P < 0.0001$; $t_{(10)} = 2.775$, $P = 0.020$, respectively). Two way ANOVA analysis for the DI, revealed a significant effect of treatment and of 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 the two factors ($F_{(1,40)}$ = 0.264, P = 0.610). *Post hoc* analysis indicated that medullectomized animals treated with vehicle showed higher DI with respect to their sham group (P < 0.01), and the same results were found for amphetamine treated groups (P < 0.05) (Fig 4). Two-way ANOVA for the analysis of the total exploration time during the retention test trial, did not reveal any significant differences between experimental groups for the treatment and for the adrenal medullectomy effects ($F_{(1,40)}$ = 0.579, P = 0.451; $F_{(1,40)}$ = $6.81x10^{-5}$, P = 0.993, respectively) but a significant effect for the interaction between these two factors was found ($F_{(1,40)}$ = 4.722, P = 0.036) (Table 2). No significant differences, between the experimental groups, were found for the two-way ANOVA analysis of the 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).

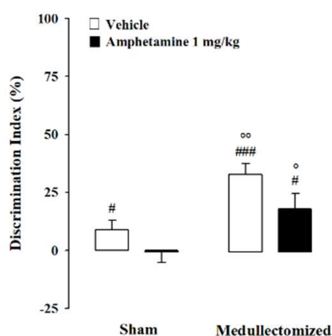

Figure 4. Peripheral adrenergic system influences on amphetamine effects on the consolidation of long-term OR memory in rats exposed to the strong FSS condition immediately after the training trial. Discrimination index on the retention test trial for

sham and medullectomized rats treated with vehicle or amphetamine and subjected to the strong stress condition immediately after the training trial. *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).

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

	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

*, $P < 0.05$ vs the relative sham group

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

	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	24.0 ± 3.8	19.7 ± 2.7	46.0 ± 5.9
Amphetamine 1	23.1 ± 1.4	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	32.8 ± 5.3	15.0 ± 1.9	24.0 ± 3.3
Amphetamine 1	48.5 ± 6.0	19.2 ± 2.3	27.5 ± 3.4

Discussion

The present findings show that the psychostimulant amphatamine exerts dichotomic effects on long-term OR memory, which are strictly related to the level of stress presented during the consolidation phase of memory. Particularly, we found that amphetamine, at the dose of 1 mg/kg, improves long-term memory consolidation, when rats are exposed to the mild stress condition, whereas, impairs memory performance in case of strong stress exposure. In the course of time, amphetamine has become greatly famous for its powerful central nervous system stimulation properties (Heal et al., 2013). For this reason it is nowadays, one of the most commonly abused drugs (Berman et al., 2008). Psychostimulant properties of amphetamine are due to its mechanism of action aimed at the modulation of the noradrenergic and dopaminergic systems (Fleckenstein et al., 2007). Among these, the noradrenergic system involvement in cognitive functions has been widely demonstrated (McIntyre et al., 2002, Ferry and McGaugh, 2008, Wichmann et al., 2012) and recognized as crucial lead of memory consolidation for emotional experiences (McIntyre et al., 2012; Roozendaal and McGaugh 2011; McGaugh 2013). Indeed, amphetamine modulation of memory processes has been studied from many decades (Martinez et al., 1983, Oscos et al., 1988, Bardgett et al., 2019). It has been shown that amphetamine-dependent enhancement of memory consolidation depends on the noradrenergic system stimulation properties (Lee and Ma, 1995).

During the stress response, the HPA axis is activated and stress mediators, such as epinephrine and glucocorticoids (specially cortisol in humans and corticosterone in rats) are released; these mediators act as endogenous modulators of memory consolidation (McIntyre and Roozendaal, 2007; Schwabe et al., 2012, Wolf et al., 2016, de Quervain et al., 2017). Furthermore, it is extensively known, that the relationship existing between stress intensity and

memory function follows an inverted U-shaped curve, with improved memory performance with optimal levels of stress and decreased memory performance with extremely high levels of stress (Salehi et al., 2010). An inverted U-shaped dose-response curve has been also documented for amphetamine (White, 1998). Our results provide new insights to this issue, highlighting the existence of a modulatory interaction between amphetamine and different stress intensities in the modulation of long-term memory consolidation. This hypothesis could be explained in view of the noradrenergic tone modulation induced by both amphetamine and stress, and considering the inverted U-shape dose-response curve existing between NE and memory performances (Baldi and Bucherelli, 2005). As our results suggested, exposure to the mild stress condition immediately after the training trial in an OR task does not allow vehicle-treated rats to discriminate the novel object from the familiar one during the retention test trial performed 24-h later, but treatment with amphetamine at the dose of 1 mg/kg turns the table, making rats able to well remember which object is familiar and which one is not. It can be hypothesized that the NE levels elicited by the mild stress condition are not sufficient enough to enhance the memory consolidation process, and that the association of the treatment with amphetamine, specifically at the higher dose of 1 mg/kg, rises the NE concentration to an optimal level for the long term-memory consolidation improvement. Conversely, the strong stress condition, in itself, is able to determine an optimal level of NE, making vehicle-treated rats able to discriminate the two objects, but the concomitant treatment with amphetamine at the dose of 1 mg/kg, extremely increases NE levels, leading to a long-term memory consolidation impairment.

Early studies suggested the key role of epinephrine in the modulation of NE 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 extensively involved in memory consolidation (Roozendaal and McGaugh, 2011). Previous findings have demonstrated that the surgical removal of adrenal medulla prevents the amphetamine enhancing effects on memory consolidation in rats not exposed to any stressful condition (Martinez et al., 1980b), thus suggesting that amphetamine effects on memory consolidation are influenced by the peripheral adrenergic tone. Basing on these evidences, in a second set of experiments, here we aimed at examining the potential role of the peripheral adrenergic tone in the modulation of long-term memory consolidation exerted by amphetamine and different stress intensities. Our results plainly indicate that the peripheral adrenergic system has a key role in such modulatory effects. Particularly, here we found that in medullectomized rats, which were unable to synthetize epinephrine, exposure to the mild stress condition, immediately after the treatment with amphetamine (1 mg/kg), not only was sufficient to revert the amphetamine capability to enhance memory consolidation, but impaired memory performance; on the contrary, exposure to strong stress immediately after training ameliorated memory discrimination at the testing time. Basing on these results, it might be assumed that the release of epinephrine, strongly modulated by the different stress conditions, influences through the vagal nerve-NTS-PGi-LC pathway the brain levels of NE. Such influence, together with the amphetamine mediated modulation of the noradrenergic system, finely tunes the NE levels in specific brain areas extensively involved in memory consolidation (e.g. hippocampus, amygdala) determining, according to the NE dose response U-shaped curve, the impairing or enhancing effects on long-term memory consolidation. Thus, taken together

these results highlight the importance of the peripheral adrenergic modulation in the amphetamine modulations of long-term memory consolidation.

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CHAPTER 6

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

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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 underinvestigated. 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 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 occurs, 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 (31 cm) was made of opaque white plastic and was well lit; the shock 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. The 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 the training or testing of each animal, each apparatus was wiped clean with a 70% ethanol solution. The experimental design is illustrated in Fig. 1.

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.

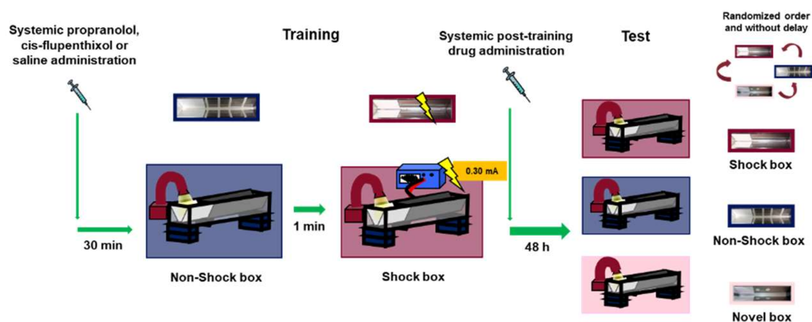

Figure 1: Schematic representation of the experimental design.

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 1.

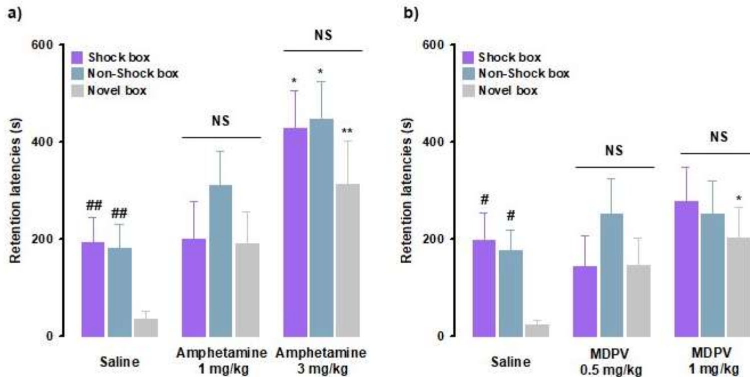

Figure 2: Amphetamine and MDPV induce memory generalization of inhibitory avoidance discrimination task. On the 48-h retention test, rats were sequentially tested in all three contextually modified inhibitory avoidance apparatuses in a random order and their retention latencies were analyzed. **a)** 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 2.

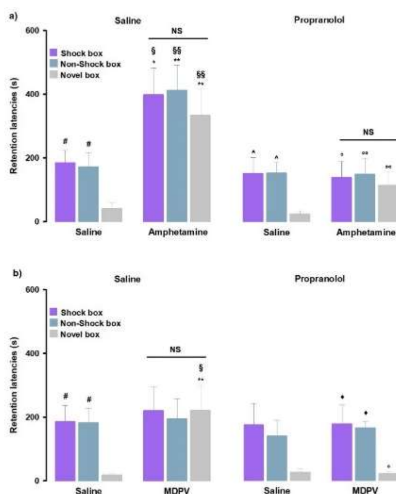

Figure 3: Noradrenergic activation mediates amphetamine and MDPV effects on memory generalization. On the 48-h retention test, rats were sequentially tested in all

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 3.

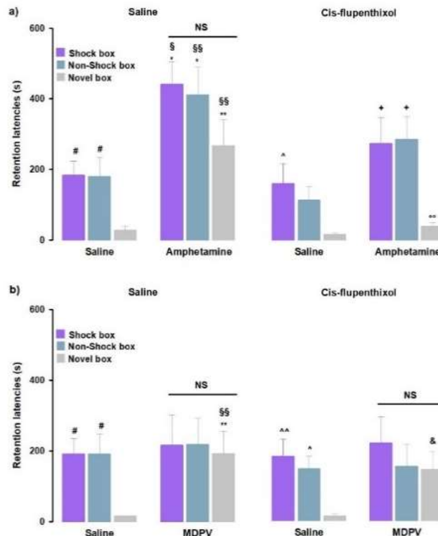

Figure 4: Dopaminergic activation mediates the effects induced by amphetamine, but not MDPV, on memory generalization. On the 48-h retention test, rats were sequentially tested in all three contextually modified inhibitory avoidance apparatuses in a random order and their retention latencies were analyzed. **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 two other 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.

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CHAPTER 7

GENERAL DISCUSSION AND CONCLUSION

This thesis aimed at investigating a still unexplored dark side of memory: hypermnesia.

Here we first investigated, in Chapter 1, the basic mechanisms and the brain networks sustaining hypermnesia. To this aim we conducted an fMRI study in the first and unique Highly Superior Autobiographical Memory (HSAM) European population. Our results indicate that the superior ability to retrieve details of autobiographical memory in HSAM subjects is supported by enhanced activation of several brain regions, including the medial prefrontal cortex and temporoparietal junction. Moreover, we found increased connectivity of the prefrontal cortex with the hippocampus, a region well known to be involved in memory representation (Bonnici et al., 2018). Taken together these findings suggest that activation of these systems may play a critical role in enabling HSAM, letting these people able to recall past autobiographical events independently from the emotional charge linked to the experience they lived.

Memory hyperfunction should be considered a serious memory alteration which can lead to many psychiatric disorders (e.g. Post-Traumatic Stress Disorder (PTSD)). The excessive and involuntary memory retrieval, of traumatic experience, seriously compromise PTSD patient's life with deleterious cognitive and behavioral alterations (McNally, 2006, Brewin, 2011, Parsons and Ressler, 2013). Nowadays there is no specific treatment for PTSD, but the

endocannabinoid system, with its peculiar role in the modulation of memory retention and retrieval, seems to be a promising therapeutic target. Here, in Chapter 2, we evaluated in rats exposed to inescapable footshocks paired with social isolation, a PTSD animal model we previously validated, whether activation of the endocannabinoid system, by facilitating extinction and dampening the retrieval of fear memory, could ameliorate the efficacy of extinction-based exposure therapy. We found that traumatized animals not exposed to extinction sessions exhibited reductions in hippocampal anandamide content with respect to home-cage controls. Noteworthy, all drugs here tested and acting on the endocannabinoid system exerted beneficial effects, the FAAH inhibitor URB597 induced the best improvements by enhancing extinction consolidation and restoring normal social behavior in traumatized rats through indirect activation of CB1 receptors. Moreover, we found that the ameliorating effects remained stable long after treatment and trauma exposure. Our findings suggest that drugs potentiating endocannabinoid neurotransmission may represent promising tools, leading to an improved memory extinction processes when combined to exposure-based psychotherapies in the treatment of PTSD.

Even if the endocannabinoid system looks as a promising target for the treatment of PTSD, in which stress-induced memory alterations lead to intrusive thoughts and flashbacks due to an excessive and involuntary memory retrieval of the negative past experience, it is also important to take in consideration the susceptibility aspect of this debilitating psychopathology. Literature data demonstrate the existence of different trauma response phenotypes among a population (Javidi and Yadollahie, 2012). Then, an animal model able to predict the susceptibility and the resilience to the development of a PTSD-like phenotype taking in consideration the cognitive alteration of the pathology, will improve, with a high translational value to human, our knowledge on the neurobiological underpinnings of the excessive memory retention and retrieval. Here, in Chapter

3, by introducing specific behavioral tests suitable to disentangle individual differences with regards to PTSD development, we outstretched our previously validated PTSD animal model. We identified a predictive variable for the screening of PTSD susceptibility and resilience in term of over-consolidation, impaired extinction, and social impairment. Particularly, we found as a reliable predictive variable the number of crossings (locomotor activity) in the Open Field test. The analysis of the number of crossings in the Open Field test is an easy and valuable tool to study at the same time the motor activity and the natural tendency of rodents to explore a new environment (Denenberg, 1969). Here we further dissociated these two aspects, in order to better understand if it is the motor activity *per se,* or the natural tendency to explore a new environment that made the number of crossings a reliable predictive variable for the development of PTSD-like phenotype. Our results clearly indicate that the changes induced by the trauma exposure on the number of crossings make it a predictive variable for susceptibility and resilience towards developing a PTSD-like phenotype.

As previously said in Chapter 3 not all the people will respond at the same way to a traumatic experience and, moreover, among those who will develop the pathology, there are people that do not respond to the currently available treatments or relapse after the end of the treatment. For this reason, it is tentative to hypothesized that there are also individual differences in PTSD's treatment success. We showed in Chapter 2 the potential role of the endocannabinoid system as a therapeutic target in PTSD. The endocannabinoid system has a close relationship with glucocorticoids (Morena et al., 2016, Harris et al., 2019, Siller-Perez et al., 2019), for this reason it can be considered as an emotional buffer which modulates memory function differentially, depending on the level of stress and arousal lived by the subject and associated with the experimental context (Campolongo et al., 2013, Morena and Campolongo, 2014, Morena et al., 2015). Endocannabinoids and glucocorticoids release is also influenced by the circadian

rhythm (Rueda-Orozco et al., 2008; Dickmeis, 2009). Here, in Chapter 4, 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, through URB597 treatment, could restore any observed impairment. The results we obtained show that stress impairing effects on short-term recognition memory are dependent on the intensity of stress and circadian rhythm. The FAAH inhibitor URB597 treatment rescued the altered memory performance and decreased corticosterone levels in all the impaired groups yet leaving memory unaltered in the non-impaired groups. These results highlight the role of the endocannabinoid system as an emotional buffer, suggesting that FAAH inhibition may be a potential therapeutic target for stress-induced memory alterations.

Normally, both positive and negative stressful situations can activate several physiological responses ending with stress mediators release which in turn may conduct to improved memory performances (Roozendaal and McGaugh, 2011). Psychostimulant drugs, like amphetamine, act as potent arousal-enhancers by increasing brain noradrenaline levels with important consequences on memory performances (Berridge et al., 1999, Berridge and Morris, 2000; Martinez et al., 1980a, Martinez et al., 1980b). Thus, we here investigated, in Chapter 5, in an object recognition task, how different stress intensities modulate the amphetamine effects on long term memory consolidation in rats. Our results suggest the existence of dichotomic effects of amphetamine on memory performances. These dichotomic effects are strictly dependent on the level of stress presented during the consolidation phase of memory. Particularly, we found that amphetamine enhanced memory consolidation in rats submitted to a post-training mild stress condition, whereas impaired it in rats submitted to a post-training strong stress condition. It is extensively known, that the relationship

existing between stress intensity and memory function follows an inverted U-shaped curve, with improved memory performance with optimal levels of stress and decreased memory performance with extremely high levels of stress (Salehi et al., 2010). An inverted U-shaped dose-response curve has been also documented for amphetamine (White, 1998). Our results provide new insights to this issue, highlighting the existence of a modulatory interaction between amphetamine and different stress intensities in the modulation of long-term memory consolidation. This hypothesis could be explained in view of the noradrenergic tone modulation induced by both amphetamine and stress, and considering the inverted U-shape dose-response curve existing between NE and memory performances (Baldi and Bucherelli, 2005). Indeed, it can be hypothesized that the noradrenaline levels elicited by the mild stress condition are not sufficient enough to enhance the memory consolidation process, and that the association of the treatment with amphetamine rises noradrenaline concentration to an optimal level for boosting long term-memory consolidation. Conversely, the strong stress condition, by itself, is able to determine an optimal level of noradrenaline, making vehicle-treated rats able to discriminate between the two objects, but the concomitant treatment with amphetamine, increases noradrenaline levels to such higher levels that cause memory dysfunction, leading to a long-term memory consolidation impairment. Taken together these results highlight how amphetamine and different stress-mediated adrenaline levels, finely tune the brain noradrenaline concentration.

The enhanced memory consolidation may lead to the excessive memory retrieval in a situational reminder condition leading to memory generalization. Notwithstanding, the memory enhancing effects of amphetamine are well-established, currently, its effect on memory generalization is poor investigated. Thus, in Chapter 6 we aimed at investigating the memory generalization effects induced by a known consolidation enhancer, such as amphetamine, and a new

psychostimulant: the 3,4-methylenedioxypyrovalerone (MDPV), also called "Bath salt". Our results indicate that both amphetamine and MDPV induce generalization of fear memory, but only amphetamine enhances memory consolidation. Since both amphetamine and MDPV modulate noradrenaline and dopamine tone, we also aimed at evaluating the involvement of the noradrenergic and dopaminergic systems in mediating their effects on fear memory generalization. Our results indicate that the noradrenergic system is responsible for the memory consolidation enhancement exerted by amphetamine. Of 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 memory generalization are partially blocked by preventive administration of the β-adrenoceptor antagonist propranolol, while MDPV effects are totally blocked by propranolol administration. Conversely, we found that the generalization effect induced by amphetamine is strongly regulated by the dopaminergic system, whereas the MDPV effects on memory generalization are due to a selective activation of the noradrenergic system. Although these results require further investigation, it can be hypothesized that these memory generalization effects are due to a differential recruitment induced by amphetamine and MDPV on the monoamine systems in different brain areas particularly involved in memory generalization, such as medial prefrontal cortex, nucleus reunions, and hippocampus (Xu and Sudhof, 2013).

All together our results provide new insights in the field of memory research. Particularly, investigating the mechanisms supporting hypermnesia, also under the pathological point of view of PTSD's memory alterations, and studying how memory enhancement may be modulated, open the road to a better understanding of the mechanism sustaining not only good memory function but memory loss as well.

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2. A. Santori*, **P. Colucci***, G. F. Mancini, M. Morena, M. Palmery, V. Trezza, MN. Hill, P. Campolongo, Anandamide modulation of circadian- and stress-dependent effects on rat short-term memory. Psychoneuroendocrinology. 2019; 108:1055-162. * equal contribution.

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3. **P. Colucci**, E. Marchetta, G. F. Mancini, F. Marangio, S. Puglisi Allegra, P. Campolongo, Predicting susceptibility and resilience in an animal model of Post-Traumatic Stress Disorder (PTSD). 7th Mediterranean Neuroscience Society Meeting 2019, June 23-27, Marrakech, Marocco. Invited speaker.

4. **P. Colucci**, F. Fortini, M. Splendori, S. Scaccianoce, P. Campolongo, Amphetamine modulation of long term object recognition memory in rats: Influence of

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5. **P. Colucci,** G. F. Mancini, M. Splendori, B. Roozendaal, P. Campolongo, Amphetamine and the “bath salt” 3,4-methylenedioxypyrovalerone (MDPV) differentially affect the accuracy of memory for emotional experiences in rats. International Conference on Learning and Memory 2018, April 18-22, Huntington Beach, California USA.

6. **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.

7. **P. Colucci**, G. F. Mancini, A. Santori, P. Campolongo, Amphetamine and the “bath salt” 3,4-methylenedioxypyrovalerone (MDPV) alter accuracy of memory for emotional arousing experiences in rats. Italian Society of Pharmacology 38th National Congress, 25-28 Ott 2017, Rimini, Italy.

8. **P. Colucci**, G. F. Mancini, A. Santori, B. Roozendaal, P. Campolongo, Amphetamine and the 'bath salt' MDPV enhance generalization of memory for emotional experiences in rats. 6th Mediterranean Neuroscience Society Meeting 2017, June 12-15, St Julian’s, Malta.

APPENDIX

KETAMINE ANESTHESIA ENHANCES FEAR MEMORY CONSOLIDATION VIA NORADRENERGIC ACTIVATION IN THE BASOLATERAL AMYGDALA

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In preparation

Abstract

Trauma patients treated with ketamine during emergency care have been shown to present aggravated early post-traumatic stress reaction which is highly predictive of post-traumatic stress disorder (PTSD) development and severity. The use of ketamine in the acute trauma phase may directly or indirectly interfere with neural processes of memory consolidation of the traumatic event, thus leading to the formation of maladaptive memories, a hallmark symptom of PTSD. We have recently shown that ketamine anesthesia, immediately after a traumatic event, enhances long-term memory consolidation and leads to long-lasting alterations of social behavior in rats. Based on the evidence that ketamine induces a robust central and peripheral adrenergic/noradrenergic potentiation and that activation of this system is essential for the formation of memory for stressful events, we explored the possibility that the strong sympathomimetic action of ketamine, at both the peripheral and central levels, might underlie the memory enhancing effects of this anesthetic agent, in rats. Confirming our hypothesis, we found that rats given immediate, but not delayed, post-training ketamine anesthesia (125 mg/kg) presented enhanced 48-h memory retention in an inhibitory avoidance task and that these effects were blocked by adrenal medullectomy, lesions of the locus coeruleus, systemic or intra-basolateral amygdala ß-adrenergic receptor antagonism. Our results indicate that the memory enhancing effects of ketamine anesthesia are time-dependent and mediated by a combined peripheral and central sympathomimetic action. Taken together, here we elucidated a mechanism by which ketamine exacerbates acute post-traumatic reaction and leads to development of PTSD symptomatology later in life. These findings will help guide for a better management of sedation/anesthesia in emergency care to promote the prophylaxis and reduce the risk of developing trauma-related disorders in trauma victims.

Introduction

Trauma victims are at increased risk of developing post-traumatic stress disorder (PTSD), a highly debilitating disease characterized, among other symptoms, by altered memory processing of the traumatic experience, thus leading to excessive retrieval and flashbacks of the trauma [1]. Drugs commonly used in emergency care can interfere with memory formation for the traumatic event, and thus influence the risk to develop PTSD later in life. Asides from its relatively recent discovered antidepressant effects [2], ketamine is an intravenous anesthetic drug commonly used in clinics for premedication, sedation, and induction and maintenance of general anesthesia, and it is widely administered in emergency care [3,4]. Moreover, ketamine is a suitable anesthetic agent for trauma victims, patients with pulmonary diseases, septic shock, and hypovolemia [5]. Ketamine principally acts as an antagonist of N-methyl-ᴅ-aspartate (NMDA) glutamate receptors, which determines its anesthetic action [6]. However, it has several off-site targets [7,8], which have been shown to mediate different effects. Among these effects, it is well known the ability of ketamine to increase heart rate and blood pressure and to alter mechanisms of attentional processes, which have been attributed to the indirect sympathomimetic action of ketamine [9]. Blockade of both peripheral and central adrenaline/noradrenaline re-uptake has been reported as the primary mechanism by which ketamine increases noradrenergic transmission [10–12]. At the central level it has also recently been shown that acute doses of ketamine increase the firing rate of noradrenergic neurons in the locus coeruleus (LC), the primary source of noradrenaline in the central nervous system (CNS), through a mechanism that seems to involve activation of AMPA receptors [13].
Compelling evidence has reported the essential role of the noradrenergic system in the modulation of memory consolidation for emotionally arousing experiences, particularly in the basolateral amygdala (BLA), a brain region crucially involved in fear memory processing [14–16]. At the peripheral level, during

emotionally arousing experiences, epinephrine is released from the adrenal medulla into the blood stream and, through the vagus nerve, activates the central noradrenergic system in the nucleus of the solitary tract and LC, the main source of norepinephrine in the brain [17], which in turn activate noradrenergic mechanisms in the BLA, resulting in enhanced memory for these emotionally arousing events [14,18–21].

As trauma victims and patients in emergency care have often experienced stressful events (i.e. car accidents, myocardial infarctions, acute respiratory distress) shortly before treatment with ketamine, it is of crucial importance to investigate the effects of this anaesthetic agent when administered shortly after experiencing a traumatic event, a time window characterized by a rapid surge of peripheral and central adrenaline/noradrenaline levels and when the memory trace for the trauma is consolidated into stable long-term memory. Excessive consolidation of highly stressful events could lead to the development of trauma-related disorders, including PTSD, which is difficult to treat and able to evoke distress for a lifetime and impair long-term health-related quality of life outcomes [22].

Growing evidence exists that ketamine modulates or aggravates early post-traumatic stress reactions when given in the acute trauma phase, and its use during emergency care correlates with onset of sustained PTSD symptomatology [23–25]. Whereas, recent findings indicate that treatment with ketamine, at sub-anesthetic doses, improves psychiatric symptoms when given to individuals already suffering from PTSD and comorbid treatment-resistant depression [26,27]. Therefore, this suggests that ketamine anesthesia might be detrimental and increase the risk of PTSD development when given in close proximity of the trauma, plausibly by potentiating the noradrenergic-mediated enhancement of memory consolidation. Interestingly, treatment with the β-adrenergic receptor antagonist propranolol, although not always effective, has been shown to reduce

fear and PTSD development when paired with behavioral therapy soon after trauma and to ameliorate PTSD symptomatology when the psychiatric disorder is already developed [28]. Furthermore, reduction of noradrenergic release with dexmedetomidine sedation, an α_2-adrenergic receptor agonist, has been shown to decrease the duration of delirium in intensive or post-anesthesia care unit, or after cardiac surgery [29–31]. Accordingly with these human findings, we have recently shown that dexmedetomidine anesthesia reduces aversive memory retention while ketamine anesthesia, enhances memory retention in an inhibitory avoidance task and exacerbates PTSD symptomatology in a rat model, when injected soon after a traumatic event [32]. Building on these previous findings, here, we first examined whether ketamine enhancing effects on aversive memory retention were specific for the early phases of memory consolidation processing. To this aim, we tested the effects of post-training immediate, or delayed, ketamine anesthesia on inhibitory avoidance memory retention in rats. Then, we explored the neural mechanisms sustaining ketamine effects on fear memory consolidation. Therefore, we performed a series of parallel experiments aimed at examining the effects of ketamine anesthesia on memory retention and its interaction with the adrenergic system at both the peripheral and central levels. Specifically, ketamine was injected immediately after an inhibitory avoidance training (1) in rats undergone adrenal medullectomy surgery, (2) in the presence of a systemic injection of propranolol, (3) in rats with post-training temporary lesions of the LC, (4) in the presence of intra-BLA ß-adrenergic blockade.

Materials and methods

Animals

Male Sprague-Dawley rats (350–450 g at the time of behavioral experiments; Charles River Laboratories, Calco, Italy) were kept in an air-conditioned

controlled room (temperature: 21°±1°C; lights on 7:00 A.M.–7:00 P.M.) with food and water available ad libitum. Training and testing were performed during the light phase of the cycle between 10:00 A.M. and 3:00 P.M. All procedures were 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

Propranolol hydrochloride (Tocris, Milan, Italy), when injected systemically, and ketamine (Ratiopharm, Ulm, Germany) were dissolved, or diluted, respectively, in saline solution (0.9%). Lidocaine (Tocris, Milan, Italy) and propranolol hydrochloride (Tocris, Milan, Italy), when injected centrally, were dissolved in phosphate buffered saline 1X (PBS, pH = 7.4). Drug solutions were freshly prepared the day of the experiment. Ketamine (125 mg/kg) was administered by intraperitoneal injection (i.p.) immediately or 3h after the inhibitory avoidance training trial. Propranolol (2 mg/kg) was given i.p. 30 min before training. In a separate experiment, propranolol (0.5 µg/0.2 µl/side) was administered bilaterally into the BLA immediately after the inhibitory avoidance training. Lidocaine (4% wt/vol; 4 g/100 ml of PBS) was administered bilaterally into the LC immediately after the inhibitory avoidance training. Drug doses were chosen on the basis of previous studies performed in our laboratory and by others [20,32–36]. Post-training central infusions of drugs or their respective vehicles were made 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, Canning Vale, Australia). The injection needles protruded 2.0 mm beyond the tip of the cannula, and a 0.2-µL, or 0.5-µl, injection volume per hemisphere was infused over a period of 25–85 s in the BLA, or LC, respectively. The injection needles were retained within the cannulae for an additional 20 s after infusion to maximize diffusion and to prevent backflow of drug into the

cannulae. The infusion volumes were chosen on the basis of previous experiments [37,38].

Cannulation

Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.), given atropine sulfate (0.4 mg/kg, i.p.) to maintain respiration, and subsequently injected with 3 mL of saline subcutaneously to facilitate clearance of drugs and prevent dehydration. The rats were then placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA), and two stainless-steel guide cannulae (23-gauge) were implanted bilaterally, with the cannula tips 2 mm above the BLA [15 mm-long; coordinates: anteroposterior (AP), −2.8 mm from Bregma; mediolateral (ML), ±5.0 mm from midline; dorsoventral (DV), −6.5 mm from skull surface] or 2 mm above the LC [13 mm-long; coordinates: AP, −9.8 mm; ML, ±1.2 mm; DV, −5.1 mm] [38,39]. Stylets (15 mm- or 13 mm-long 00 insect dissection pins) were inserted into each cannula to maintain patency. Rats were allowed to recover from surgery for at least 1 week before training and handled three times for 1 min before training.

Adrenal medullectomy

Adrenal medullectomy (ADMX) was performed as previously described [40–42]. Briefly, rats were anesthetized with isoflurane (2.5 % isoflurane in O_2). Each animal was placed on a flat surface with their limbs in the extended position and their dorsal area was trichotomized. Incisions of 2 cm were made on the right and left dorsal lateral surface of the animals just over each kidney. Small incisions were made on the adrenal capsule and the medulla was removed. Sham ADMX rats only received small skin incisions. Wounds were closed with suture clips. Rats were allowed to recover from surgery for 5 weeks before starting behavioral experiments. ADMX rats were given 0.50% saline to drink in place

of water for 1 week after surgery to compensate for salt and water losses during the period of corticosteroid deficiency [41,42].

Inhibitory avoidance test

Rats were trained and tested in an inhibitory avoidance apparatus as previously described [32,43]. Briefly, the apparatus consisted of a trough-shaped alley divided into two compartments, separated by a sliding door. The starting compartment was made of opaque white plastic and illuminated; the shock compartment was made of two dark, electrifiable metal plates and was not illuminated. Animals were handled 1 min each for 3 days before training. For training, the rats were placed into the starting compartment of the apparatus, after rats stepped completely into the dark compartment, the sliding door was closed and a single, inescapable footshock (0.35 mA) was delivered for 1 s. Cannulated rats received higher FS intensity (0.60-0.65 mA) to ensure a good memory performance in all experimental groups [44–46]. The animals were removed from the shock compartment 15 s after the footshock termination. Retention was tested 48-h later. On the retention test, rats were placed into the starting compartment, and the latency to reenter the shock compartment with all four paws (retention latency; 600 s cut-off) was recorded. Longer latencies were interpreted as indicating better memory retention [47]. After each session, the apparatus was cleaned with a 70% ethanol solution.

Sleep parameters

Sleep onset time and duration for rats given ketamine were measured as previously described [32,48]. Immediately after the anesthetic administration each rat was placed on its back once every 30 s until it was unable to right itself within 30 s. Sleep onset time was defined as the interval between anesthetic injection and the time the rat was unable to turn itself upright. The time between loss and recovery of righting reflex for each rat was defined as sleep duration (180 min

cut-off). The loss of righting reflex was defined when the rat, after ketamine injection, was unable to turn itself upright at least twice within 1 min. Then, each rat was left undisturbed on its back until it spontaneously regained its righting reflexes, defined as having at least three paws under its body. Complete recovery of the righting reflex was defined as the rat being able to turn itself upright.

Histology

To check for cannula placement, cannulated rats were anesthetized with an overdose of sodium pentobarbital (100 mg/kg, i.p.) and perfused transcardially with 0.9% saline. The brains were then removed and immersed in a 4% (wt/vol) formaldehyde solution. At least 48 h before sectioning, the brains were transferred to a 20% (wt/vol) sucrose solution in saline for cryoprotection. Coronal sections of 35 μm were cut on a cryostat, mounted on gelatin-coated slides, and stained with cresyl violet. The sections were examined under a light microscope (Nikon 801 microscope, Nikon instruments SPA, Milan, Italy) and the location of infusion needle tips in the BLA or LC were made by an observer blind to treatment conditions.

Statistical analysis

The person who performed and analyzed the test was blinded to the condition of the animals. Statistical analysis was performed using GraphPad Prism Software (La Jolla, CA, USA). To determine whether learning had occurred in the inhibitory avoidance training, paired t-tests were used to compare the training and retention latencies of vehicle control groups. Sleep duration was analyzed with unpaired t-tests and correlation analyses were performed with the Pearson correlation test. For the other behavioral measures, unpaired t tests or two-way ANOVAs with the treatments as between-subjects factors, followed by Tukey-Kramer post-hoc tests, were used when appropriate. Data are expressed as mean ± standard error of the mean (SEM). P values of < 0.05 were considered

statistically significant. The number of rats per group is indicated in the figure legends.

Results

Immediate post-training, but not delayed, ketamine injection enhances aversive memory retention

We have previously shown that an anesthetic dose of ketamine (125 mg/kg, i.p.) given immediately after an inhibitory avoidance training enhances 48-h memory retention in rats [32]. To examine whether ketamine specifically enhances the early consolidation phase of aversive memory processing, rats were treated with ketamine, or its vehicle, immediately, or 3h after the inhibitory avoidance training (experiment 1; Fig. 1A). By replicating our previous results [32], we found that immediate post-training ketamine injection enhanced 48-h memory retention (Fig. 1B). Average step-through latencies for all groups during training, before footshock and immediate post-training drug treatment, were 10.11 ± 1.36 s (mean ± SEM). Unpaired t test for training latencies did not reveal any significant difference between vehicle and ketamine groups injected immediately post-training (t_{14} = 0.52, P = 0.61), indicating that there were not pre-existing behavioral differences between the two experimental groups. Retention latencies of vehicle-treated rats were significantly longer than their training latencies (t_{7} = 2.68, P = 0.03), indicating that rats correctly retained memory of the shock experience. As, shown in Fig. 1B, unpaired t test revealed that rats given immediate post-training ketamine injection presented significantly higher retention latencies than controls (t_{14} = 2.51, P = 0.03). Average step-through latencies during training, before footshock, for vehicle and ketamine groups injected 3-h post-training, were 9.54 ± 1.12 s (mean ± SEM). Unpaired t test for training latencies did not reveal any significant difference between vehicle and ketamine groups injected 3-h post-training (t_{14} = 1.97, P = 0.07), showing no pre-

existing differences between groups before drug treatment. Retention latencies of vehicle-treated rats were significantly longer than their training latencies (t_7 = 3.16, P = 0.02), indicating that rats correctly retained memory of the shock experience. Unpaired t test for retention latencies did not reveal any significant difference between vehicle- and ketamine-treated rats injected 3-h after training (t_{14} = 1.47, P = 0.16; Fig. 1C). These results show that ketamine anesthesia enhances 48-h retention latencies when given immediately, but not 3-h after training.

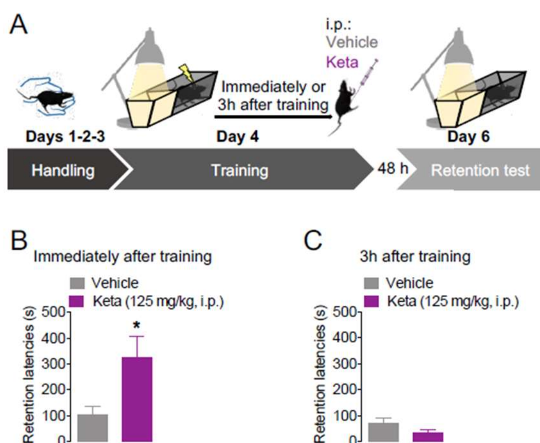

Figure 1. Immediate, but not delayed, post-training ketamine anesthesia enhances 48-h inhibitory avoidance memory retention.

Schematic representation of experiment 1 (A). Ketamine injection (Keta; 125 mg/kg, i.p.) enhanced 48-h memory retention when injected immediately (B), but not 3-h after training (C). *, $P < 0.05$ vs vehicle group. Results represent mean ± SEM (n = 8 per group).

Memory enhancing effects of immediate post-training ketamine injection are dependent on adrenal catecholamines

To examine whether the memory enhancing effects of immediate post-training ketamine injection were dependent on adrenal catecholamines, the anesthetic

agent, or its vehicle, was given to sham ADMX or ADMX rats immediately after the inhibitory avoidance training (experiment 2; Fig. 2A). Average step-through latencies for all groups during training, before footshock and drug treatment, were 13.34 ± 1.78 s (mean ± SEM). Two-way ANOVA for training latencies did not reveal any significant ketamine treatment ($F_{1,35} = 0.06$, $P = 0.82$), ADMX ($F_{1,35} = 2.38$, $P = 0.13$), or ketamine x ADMX interaction ($F_{1,35} = 0.01$, $P = 0.92$) effects, indicating that there were not pre-existing behavioral differences among all experimental groups. Retention latencies of sham ADMX rats treated with vehicle were significantly longer than their training latencies ($t_{12} = 3.09$, $P = 0.009$), indicating that rats correctly retained memory of the shock experience. As, shown in Fig. 2C, two-way ANOVA for retention latencies revealed significant ketamine ($F_{1,35} = 9.54$, $P = 0.004$) and ADMX ($F_{1,35} = 12.86$, $P = 0.001$) effects and a significant interaction between both factors ($F_{1,35} = 7.49$, $P = 0.009$). Post-hoc tests indicated that retention latencies of sham ADMX rats treated with ketamine (125 mg/kg) were significantly longer than those of both sham ADMX rats given vehicle and ADMX rats treated with ketamine ($P < 0.001$ for both comparisons; Fig. 2C). There were no significant differences between sham ADMX and ADMX rats treated with vehicle. These results indicate that a functional adrenal medulla capable to release adrenaline in the blood stream after a fearful experience is required for enabling the enhancing effects of ketamine on fear memory consolidation.

Memory enhancing effects of immediate post-training ketamine injection are dependent on β-adrenergic receptor activation

This experiment examined whether memory enhancing effects of immediate post-training ketamine anesthesia were dependent on β-adrenergic receptor activation (experiment 3; Fig. 2B). Immediate post-training injections of ketamine (125 mg/kg, i.p.), or its vehicle, were given to rats pre-treated with systemic injections of the β-adrenergic receptor antagonist propranolol (2 mg/kg,

i.p.), or its vehicle, 30 min before the inhibitory avoidance training. Average step-through latencies for all groups during training, before footshock and drug treatment, were 11.78 ± 1.37 s (mean ± SEM). Two-way ANOVA for training latencies did not reveal any significant ketamine ($F_{1,42} = 0.97$, $P = 0.33$) or propranolol ($F_{1,42} = 0.49$, $P = 0.49$) treatment effects or an interaction between both factors ($F_{1,42} = 1.69$, $P = 0.20$). Retention latencies of vehicle-treated rats were significantly longer than their training latencies ($t_{11} = 2.45$, $P = 0.03$), indicating that rats retained memory of the shock experience. As shown in Fig. 2D, two-way ANOVA for retention latencies revealed significant propranolol ($F_{1,42} = 9.05$, $P = 0.004$) and ketamine x propranolol interaction ($F_{1,42} = 11.42$, $P = 0.002$) effects, but no significant effect of ketamine ($F_{1,42} = 2.87$, $P = 0.10$). Post-hoc tests indicated that retention latencies of rats treated with ketamine alone were significantly longer than those of both vehicle-treated rats ($P < 0.01$; Fig. 2D) and rats given propranolol together with ketamine ($P < 0.001$; Fig. 2D). There were no significant differences between rats treated with vehicle alone and rats given propranolol alone. These results indicate that memory enhancing effects of ketamine require indirect β-adrenergic receptor activation.

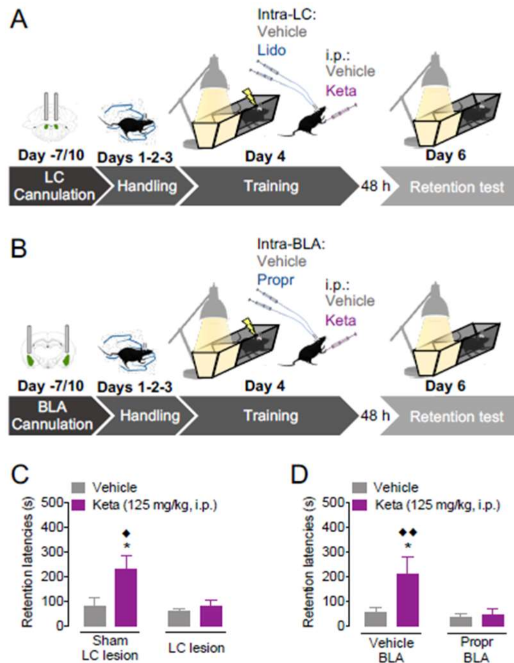

Figure 2. Systemic adrenergic blockade prevents ketamine enhancing effects on 48-h inhibitory avoidance retention.

Schematic representations of experiment 2 (A) and experiment 3 (B). Immediate post-training administration of ketamine (Keta; 125 mg/kg, i.p.) enhanced memory retention and either adrenal medullectomy (ADMX) (C) or systemic β-adrenergic receptor antagonism with propranolol (Propr; 2 mg/kg, i.p.) (D) blocked the memory enhancing effects of post-training ketamine. **, $P < 0.01$; ***, $P < 0.001$ vs the corresponding vehicle group; ♦♦♦, $P < 0.001$ vs the corresponding ADMX or propranolol group. Results represent mean ± SEM (n = 8 - 13 per group).

An intact Locus coeruleus is required to enable memory enhancing effects of immediate post-training ketamine injection

To examine whether memory enhancing effects of immediate post-training ketamine anesthesia were dependent on central adrenergic activity, two separate

groups of rats received either post-training bilateral sham LC lesions with vehicle or temporary LC lesions with lidocaine (4%, 0.5 μl/side) infusion. Immediately after LC infusions, each group was also administered with either ketamine (125 mg/kg, i.p.), or its vehicle (experiment 4; Fig. 3A). Average step-through latencies for all groups during training, before footshock and drug treatment, were 7.51 ± 0.47 s (mean ± SEM). Two-way ANOVA for training latencies did not reveal any significant ketamine ($F_{1,43} = 2.60$, $P = 0.11$), LC lesion ($F_{1,43} = 3.15$, $P = 0.08$) or ketamine x LC lesion interaction ($F_{1,43} = 2.51$, $P = 0.12$) effects. Retention latencies of sham lesioned vehicle-treated rats were significantly longer than their training latencies ($t_{14} = 2.15$, $P = 0.04$), indicating that rats retained memory of the shock experience. As shown in Fig. 3C, two-way ANOVA for retention latencies revealed significant ketamine ($F_{1,43} = 4.84$, $P = 0.03$) and LC lesion ($F_{1,43} = 5.25$, $P = 0.03$), but no significant interaction between both factors ($F_{1,43} = 2.98$, $P = 0.09$). Post-hoc analyses indicated that retention latencies of sham lesioned rats treated with ketamine were significantly longer than those of both sham lesioned vehicle-treated rats and LC lesioned rats given ketamine ($P < 0.05$, for both comparisons; Fig. 3C). There were not significant differences between sham lesioned and LC lesioned rats treated with vehicle. These results indicate that memory enhancing effects of ketamine require central norepinephrine release.

Memory enhancing effects of immediate post-training ketamine injection are dependent on β-adrenergic receptor activation in the BLA

This experiment examined whether memory enhancing effects of immediate post-training ketamine injection were dependent on β-adrenergic receptor activation in the BLA (experiment 5; Fig. 3B). Post-training injections of ketamine (125 mg/kg, i.p.), or its vehicle, were administered to rats given bilateral intra-BLA infusions of either propranolol (0.5 μg/0.2 μl/side), or its

vehicle, immediately post-training and before ketamine injection. Average step-through latencies for all groups during training, before footshock and drug treatment, were 11.00 ± 1.12 s (mean ± SEM). Two-way ANOVA for training latencies did not reveal any significant ketamine ($F_{1,38}$ = 1.14, P =0.29) or propranolol ($F_{1,38}$ = 0.69, P =0.41) treatment effects or an interaction between both factors ($F_{1,38}$ = 3.01, P =0.09). Retention latencies of vehicle-treated rats were significantly longer than their training latencies (t_9 = 2.62, P = 0.03), indicating that rats retained memory of the shock experience. As shown in Fig. 3D, two-way ANOVA for retention latencies revealed significant ketamine ($F_{1,38}$ = 6.11, P = 0.02), propranolol ($F_{1,38}$ = 7.63, P = 0.009) and ketamine x propranolol interaction ($F_{1,38}$ = 4.58, P = 0.04) effects. Post-hoc tests indicated that retention latencies of rats treated with ketamine alone were significantly longer than those of both vehicle alone-treated rats ($P < 0.05$) and rats given intra-BLA propranolol together with ketamine ($P < 0.01$). There were no significant differences between rats treated with vehicle alone and rats given propranolol alone. These results indicate that memory enhancing effects of ketamine require indirect β-adrenergic receptor activation in the BLA.

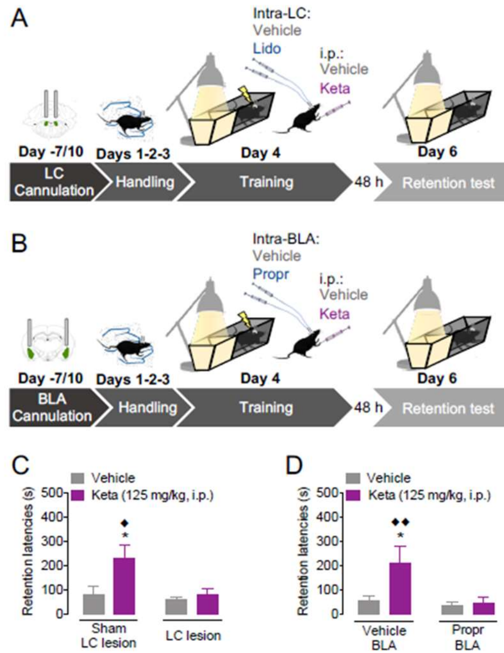

Figure 3. Central adrenergic blockade within the LC or BLA prevents ketamine enhancing effects on 48-h inhibitory avoidance retention.

Schematic representations of experiment 4 (A) and experiment 5 (B). Immediate post-training LC temporary lesions (C) or intra-BLA β-adrenergic receptor antagonism with propranolol (Propr; 0.5 µg/0.2 µl/side) (D) blocked ketamine (Keta; 125 mg/kg, i.p.) enhancing effects on memory retention. *, $P < 0.05$ vs the corresponding vehicle group; ♦, $P < 0.05$; ♦♦, $P < 0.01$ vs the corresponding LC lesioned group or intra-BLA propranolol group. Results represent mean ± SEM (n = 8 - 15 per group).

Memory enhancing effects of post-training ketamine injection are not influenced by sleep parameters

Many studies have documented a close relationship between sleep and memory, showing, in particular, that sleep optimizes processes of memory consolidation

[49]. We examined whether the different experimental conditions used in our study altered the onset and/or the duration of ketamine-induced anesthesia and whether this influenced 48-h memory retention.

Table 1 shows sleep onset time and duration for rats given ketamine in all the experimental groups. Unpaired t-tests for sleep onset time of rats given ketamine (alone or in combination with other treatments) showed no significant differences between groups within each experiment (t_{14} = 1.13, P = 0.28; t_{15} = 1.00, P = 0.33; t_{20} = 1.88, P = 0.08; t_{20} = 1.00, P = 0.33; t_{19} = 0.48, P = 0.64; for experiment 1, 2, 3, 4 and 5, respectively; Table 1). Similarly, unpaired t-tests for sleep duration of rats given ketamine (alone or in combination with other treatments) showed no significant differences between groups within each experiment (t_{14} = 1.32, P = 0.21; t_{15} = 0.12, P = 0.90; t_{20} = 1.69, P = 0.11; t_{20} = 0.45, P = 0.66; t_{19} = 0.89, P = 0.38; for experiment 1, 2, 3, 4 and 5, respectively; Table 1), thus, showing that neither the different experimental conditions nor the treatments combined with ketamine altered sleep onset or duration induced by the anesthetic injection. Moreover, Pearson's analyses did not reveal any significant correlation between sleep onset time, or duration, with 48-h retention latencies shown by all rats treated with ketamine (Table 1), thus indicating that the memory enhancing effects of ketamine are not related to the anesthetic properties of the drug.

Table 1. Sleep parameters of ketamine-treated rats

	Experiment 1		Experiment 2		Experiment 3		Experiment 4		Experiment 5	
Experimental group	Keta i.p. imm. post-training	Keta i.p. 3h post-training	Sham ADMX + Keta i.p.	ADMX + Keta i.p.	Veh i.p. + Keta i.p.	Propr i.p. + Keta i.p.	Sham LC lesion + Keta i.p.	LC lesion + Keta i.p.	Veh BLA + Keta i.p.	Propr BLA + Keta i.p.
Sleep onset time (min)	5.75 ± 1.28	3.88 ± 1.06	5.88 ± 1.30	10.00 ± 3.70	4.44 ± 0.65	6.23 ± 0.65	6.73 ± 1.53	5.09 ± 0.56	5.75 ± 1.00	6.54 ± 1.14
Correlation: Sleep onset time vs Retention latencies	r = -0.17, P = 0.69	r = -0.35, P = 0.40	r = 0.15, P = 0.73	r = -0.33, P = 0.38	r = -0.06, P = 0.88	r = 0.38, P = 0.21	r = 0.03, P = 0.92	r = 0.13, P = 0.70	r = 0.64, P = 0.09	r = -0.29, P = 0.34
Sleep duration (min)	42.25 ± 5.59	51.38 ± 4.08	39.13 ± 5.40	39.89 ± 3.29	45.11 ± 6.21	35.77 ± 1.77	48.64 ± 5.14	52.45 ± 6.75	40.38 ± 3.05	47.23 ± 5.69
Correlation: Sleep duration vs Retention latencies	r = -0.03, P = 0.95	r = 0.12, P = 0.77	r = -0.20, P = 0.63	r = 0.22, P = 0.56	r = 0.41, P = 0.27	r = 0.29, P = 0.33	r = -0.10, P = 0.76	r = -0.01, P = 0.98	r = -0.43, P = 0.29	r = -0.28, P = 0.36

Effects of intraperitoneal ketamine injections on rats' sleep onset time and duration tested under the different experimental conditions and Pearson's correlations analyses between sleep onset time, or duration, of ketamine-treated rats and their respective 48-h retention latencies. Experiment 1: Rats injected with ketamine, i.p., immediately after training (Keta i.p. imm. post-training) and rats injected with ketamine, i.p., 3h after training (Keta i.p. 3h post-training). Experiment 2: Sham adrenal medullectomized rats + ketamine i.p. (Sham ADMX + Keta i.p.) and adrenal medullectomized rats + ketamine i.p. (ADMX + Keta i.p.). Experiment 3: Rats injected with vehicle, i.p. 30 min before training and ketamine immediately after training (Veh i.p. + Keta i.p.) and rats injected with propranolol, i.p. 30 min before training and ketamine immediately after training (Propr i.p. + Keta i.p.). Experiment 4: Sham Locus Coeruleus lesioned rats + ketamine i.p. (Sham LC lesion + Keta i.p.) and Locus Coeruleus lesioned rats + ketamine i.p. (LC lesion + Keta i.p.). Experiment 5: Rats given intra-basolateral amygdala vehicle injection + ketamine i.p. (Veh BLA + Keta i.p.) and rats given intra-basolateral amygdala propranolol injection +

ketamine i.p. (Propr BLA + Keta i.p.). Data are expressed as mean ± SEM (n =8 - 13 per group).

Discussion

The present findings indicate that ketamine anesthesia enhances memory consolidation of fear experiences when administered shortly after encoding of an inhibitory avoidance training via a combined peripheral and central sympathomimetic action which terminates with the activation of the BLA noradrenergic system.

We have recently shown that ketamine anesthesia strengthens the traumatic memory trace when given immediately after a stressful event and induces pronounced and persistent emotional dysfunction. Rats treated with ketamine after a traumatic experience presented a long-lasting social anxiety-like behavior, shown by reduced social interaction with a conspecific, sixteen days after trauma and ketamine injection [32]. By adding to our previous findings, here we unveiled the mechanism underlying the traumatic memory enhancing properties of ketamine anesthesia by showing that these effects are time-dependent and arise from the strong sympathomimetic action of the anesthetic agent.

Specifically, our results reveal that ketamine anesthesia modulates time-dependent processes of aversive memory consolidation together with a combined peripheral and central mechanism of ketamine in potentiating adrenergic/noradrenergic signaling to promote aversive memory retention. Adrenal medullectomy or blockade of ß-adrenergic receptors, with systemic injections of propranolol, although non altering 48-h memory retention *per se*, prevented the memory potentiating effects of immediate post-training ketamine injection. Furthermore, our findings show that temporary post-training lesions of the LC were able to completely block the memory enhancing effects of ketamine. Our last experiment identified the BLA as the brain area responsible for ketamine

enhancing effects on traumatic memory retention. Selective blockade of ß-adrenergic receptors within the BLA completely prevented the effects of post-training ketamine anesthesia.

The sympathomimetic action of ketamine, via inhibition of the noradrenaline transporter or by increasing LC noradrenergic neuronal firing, has been widely demonstrated at both the peripheral and central levels [11–13,50]. The LC represents a main source of noradrenaline in the CNS [17] and sends dense projections to nearly the whole brain, including the BLA [51], which is engaged when fear stimuli elicit an arousal effect to modulate anxiety and processes of fear memory formation [14,15,21,39]. Increased noradrenergic release in the rat BLA has been observed during an inhibitory avoidance training, which was predictive of the strength of the aversive memory for the training experience [21]. Furthermore both systemic and central activation of the adrenergic/noradrenergic system have been shown to enhance fear memory retention through activation of ß-adrenergic receptors in the BLA [15]. Since peripheral epinephrine enters the brain poorly, and peripherally restricted ß-adrenergic antagonism has been shown to block the memory enhancing effects of adrenaline [52], it has been proposed that both peripheral and central ß-adrenergic receptor activation influence memory consolidation. Accordingly, our results show that either adrenal medullectomy or central blockade of noradrenergic transmission prevent the effects of ketamine. It is known that systemic adrenaline induces norepinephrine release within the brain [53], likely by activation of ß-adrenergic receptors on the ascending vagus nerve that projects to brain stem noradrenergic nuclei, thus inducing norepinephrine release throughout the forebrain, including the BLA [15]. In the light of this evidence, our results demonstrate that ketamine, administered immediately after a trauma, by increasing noradrenergic transmission peripherally and within the CNS, strongly potentiates memory consolidation for the traumatic event via activation of ß-adrenergic receptors in the BLA. Our

finding that the memory enhancing effects were observed only when ketamine anesthesia was given immediately, but not 3-h, after training further confirms that the ketamine-induced noradrenergic potentiation underlyes this mechanism. Indeed, it has been shown that soon after a stressful experience the BLA receives a strong noradrenergic input from the LC, which is relatively short lasting (less than 1-h) [54]. During this short time-window the BLA is concomitantly exposed to high levels of other stress hormones, neurotransmitters and neuromodulators which act in concert to enhance memory consolidation of the stressful event [44,46,54]. Intriguingly, our present result resembles what we have previously shown with propofol anesthesia being able to enhance aversive memory consolidation only when given shortly after training, by potentiating the memory enhancing effects induced by endocannabinoids rapidly released after stress [48].

Sleep has a profound effect on memory consolidation [49], therefore it might be argued that the effects of ketamine anesthesia on sleep might have indirectly influenced memory performance in our experiments. However, the fact that there were no correlations between sleep onset or duration with retention latencies measured during the 48-h memory tests, allows us to exclude any potential contribution of sleeping property alterations induced by ketamine on its memory enhancing effects. Moreover, we did not find any significant difference when comparing sleep properties of anesthetized rats within all the experiments performed, thus showing that the different conditions (i.e. immediate vs delayed ketamine injection, adrenal medullectomy, LC lesion, systemic or intra-BLA injection of propranolol) had no influence on sleep onset and duration induced by ketamine.

Previous studies examining the effects of ketamine on aversive memory processes have principally focused on the use of sub-anesthetic doses and have led to discrepant findings. In rats, pre-training injection of ketamine impaired acquisition of a cued fear conditioning paradigm [55]. Conversely, other studies in

the same species have found no effects of ketamine on acquisition or consolidation of cued fear conditioning [56,57]. Moreover, Bolton et al. (2012) reported that ketamine impaired trace cued fear conditioning but had no effect of contextual fear conditioning. Clifton et al. (2018) also reported no effects of sub-anesthetic ketamine administration on contextual fear memory consolidation [58]. Another study in mice has shown no effects of anesthetic doses on inhibitory avoidance memory retention [59]. However, corroborating our previous [32] and present findings, one study showed that ketamine was able to increase fear memory retention at sub-anesthetic doses when administered to rats in an active avoidance paradigm [60]. Additionally, another study reported that repeated ketamine administration at sub-anesthetic doses given after trauma exposure did not prevent PTSD symptomatology, and increased fear behavior to the trauma cue assessed a month later [61]. Differences in doses, route and time of administration, animal species and strains used and behavioral paradigms might have accounted for these discrepancies.

Conversely, there seems to be a general agreement in human studies showing that treatment with ketamine soon after accidental trauma is associated with increased symptoms of PTSD, such as dissociation, reexperiencing, hyperarousal and avoidance in the aftermath of the event [24], which contribute to the development of a long-lasting PTSD symptomatology [25] and are highly predictive of the severity and duration of subsequent PTSD [62]. These reports corroborate our previous [32] and present findings that the use of ketamine anesthesia shortly after a traumatic event may represent a risk factor for the development of trauma-related disorders. Conversely, several evidence has reported that when ketamine is given in animal models for PTSD or in PTSD patients, not in proximity of the stressful event or at sub-anesthetic doses, reduces PTSD symptomatology, including fear memory, anxiety- and depressive-like behavior in animals [63,64], while in humans seems to be particularly effective in

ameliorating comorbid depressive symptoms [26,27,63,65,66]. Therefore, there seems to be a dual, time-dependent effect of ketamine. When ketamine is given to patients suffering from PTSD, it has beneficial effects, particularly in the presence of comorbid depression disorder, however, when given, at anesthetic doses, shortly after a traumatic event, in patients at risk of developing PTSD, it seems to facilitate and contribute to the development of a long-lasting PTSD symptomatology. Our present results strongly support this clinical evidence and indicate that the increased risk of PTSD development, associated with ketamine anesthesia, may arise from its memory consolidation enhancing properties. Furthermore, we also identify the neural underpinnings of the effects of ketamine anesthesia on memory when administered immediately after stressful experiences by providing a neurological mechanism by which ketamine, through a strong, indirect peripheral and central noradrenergic activation, might potentiate traumatic memory consolidation, and contribute to the development of PTSD later in life. Accordingly, in the clinical setting the combination of ketamine anesthesia with dexmedetomidine, which reduces noradrenergic activation, has been shown to reduce both the sympathomimetic effects, including increased cardiovascular response, and postoperative psychiatric adverse reaction induced by ketamine [67].

Given that PTSD is a highly debilitating psychiatric disorder and very difficult to treat, our findings present a strong translational value, as they have the potential to inform clinicians for a better management of ketamine anesthesia in emergency care and trauma victims. For instance, coupling ketamine injection with ß-blockers or α_2-adrenoceptor agonists, such as dexmedetomidine, which have already been shown to reduce traumatic memory consolidation and PTSD symptomatology [28,29,32], would potentially have a prophylactic effect and help to prevent the occurrence and development of trauma-related disorders later in life.

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PERINATAL EXPOSURE TO OMEGA-3 FATTY ACID IMBALANCE LEADS TO EARLY BEHAVIORAL ALTERATIONS IN RAT PUPS

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In preparation

Abstract

Background: Polyunsaturated long-chain omega-3 fatty acids (n3-PUFAs) are crucially involved in brain development and function. Inadequate n3-PUFA intake in rats during the perinatal period leads to behavioral deficits in adulthood, but early behavioral changes have not yet been investigated.

Objective: The present study aimed to investigate potential behavioral alterations in neonatal rats exposed to a perinatal n3-PUFA imbalance.

Methods: Female Sprague Dawley rats were fed an n3-PUFA–enriched or an n3-PUFA–deficient diet throughout mating, pregnancy, and lactation. Controls were fed an n6/n3-PUFA–balanced diet. We observed maternal behavior from postnatal day (PND) 2 to PND 13 and tested pups in the isolation-induced ultrasonic vocalization (USV) emission task at PNDs 3, 5, 9 and 13 to evaluate the impact of perinatal n3-PUFA on early emotional traits.

Results: Both the n3-PUFA–enriched and n3-PUFA–deficient diets profoundly decreased maternal behavior. At PNDs 3 and PND 5, pups of dams fed either the n3-PUFA–deficient or –enriched diet emitted significantly fewer USVs compared with control pups whose dams were fed a standard diet. Further, the sonographic pattern of the USVs was altered in the test pups compared with that of the control pups at PND 9 and PND 13.

Conclusions: The present findings indicate that both n3-PUFA deficiency and n3-PUFA supplementation during development induce early behavioral alterations.

Introduction

The central nervous system is abundant in lipid molecules (1) and is particularly rich in polyunsaturated fatty acids (PUFAs), specifically the n3-PUFA docosahexaenoic acid (DHA) and the n6-PUFA arachidonic acid (AA) (2-4). Linoleic acid, the n6-PUFA precursor, and α-linolenic acid, the n3-PUFA precursor, are essential dietary nutrients for maintaining adequate levels of long-chain PUFAs (5). The Western diet, however, often leads to n3-PUFA deficiencies, and more generally, to an imbalance of fatty acids (6). For this reason, n3-PUFAs are added in large amounts, as supplements, to several products, particularly newborn and infant foods. Evidence suggests that n3-PUFAs are critical for neuronal (7, 8) and cognitive development in children (9-12). In rats, n3-PUFA deficiency affects emotional behaviors through alterations in synaptic plasticity (13), and induces cognitive and memory impairments (14, 15) in adulthood. Normal fetal development is highly dependent on the perinatal fatty acid intake, especially the dietary n6/n3 ratio (16-18). Further, normal emotional and cognitive development of the offspring depends on the quantity and quality of maternal care received during early-life stages (19). The ultrasonic vocalizations (USVs) emitted by rodent pups are an essential communicative tool for mother-pup interaction (20) and are considered a measure of emotional reactivity in rodent pups (21-23). This dyadic interaction between mother and infant has a profound influence on emotional and cognitive development (20, 24), and may be influenced by maternal nutrition (25, 26). The effects of a maternal n6/n3-PUFA intake imbalance during gestation and lactation on perinatal development, however, have not yet been investigated.

In the present study, we analyzed maternal behavior in rats during the entire lactation period and offspring USV emissions at postnatal days (PNDs) 3, 5, 9 and 13, to compare the effects of an n3-PUFA–enriched or n3-PUFA–deficient

diet with those of a diet having an n6/n3 balanced ratio on early emotional functions in young rat offspring.

Material and methods

Animal care and use

Multiparous Sprague-Dawley rats were purchased from a commercial breeder (Charles River, Calco, Italy). Upon arrival, animals were pair-housed (cage: 42 × 27 × 14-cm) in an air-conditioned room (temperature 21±1 °C, lights on 7:00 a.m. - 7:00 p.m.) with access to food and water *ad libitum.* After 1 week of habituation, females were mated with males (2 females and 1 male per cage). 5 days after, pregnant females were individually housed. Newborn litters found up to 5.00 p.m. were considered to be born on that day (PND 0). On PND 1, litter weight, total number of pups and gender ratio per litter were evaluated. Every litter was culled to 8 pups (4 males and 4 females). All procedures involving animal care or treatments were performed in compliance with the ARRIVE guidelines, the Directive 2010/63/EU of the European Parliament, the D. L. 26/2014 of 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).

Diets

Rats were fed a diet containing 6% fat in the form of peanut oil rich in linoleic acid (n3-PUFA-deficient diet), a diet containing 6% fat in the form of rapeseed oil rich in α-linolenic acid (n3-PUFA-enriched diet) or a standard diet composed of 3% peanut oil and 3% rapeseed oil (n6/n3-PUFA-balanced diet) starting 1 week before mating and throughout gestation and lactation periods (14). All diets

were provided by Dr Piccioni Laboratories (Milan, Italy), were isocaloric and did not differ with respect to the n6 content. Diet composition is reported in Table 1.

Table 1: Diet composition.

	n6/n3-PUFA–Balanced Diet	**n3-PUFA–Enriched Diet**	**n3-PUFA–Deficient Diet**
n6-PUFA (linoleic acid)	32	27	33
n3-PUFA (alpha-linolenic acid)	4	6	1
Monounsaturated Fats	53.8	61.8	45.8
Polyunsaturated Fats	33.1	31.5	34.7
n6/n3 Ratio	8.6 : 1	4.5 : 1	23.9 : 1
Energy Content (Kcal/g)	4.09	4.05	4.12

All diets were isocaloric, had the same amount of n6-PUFA and differed only in the n3-PUFA content. Data are expressed as relative percent of total fatty acids.

Maternal behavior observation

Maternal behavior was observed daily in the colony room from PND 2 to PND 13. Observation was carried out by single well-trained experimenter and occurred at regular times in 3 sessions of 72 min each during the light phase (09:00 a.m., 01:00 p.m., 05:00 p.m.). During each session, each dam and its litter

were observed every 3 min (25 observations per 3 sessions per day for a total of 75 observations per day).

We measured 7 maternal parameters: 1) arched nursing (dam adopting nursing posture with its back and ventral surface arched over its pups), 2) blanket nursing (dam over the pups in nursing posture but not arched), 3) passive nursing (dam adopting nursing posture lying either on its back or side), 4) licking pups (dam licking pups), 5) pup retrieval (dam moving the pups in another cage position), 6) building nest (dam manipulating nest shavings), 7) maternal self-grooming (dam grooming its breasts); and 4 non-maternal parameters: 1) feeding, 2) exploring (exploring the cage), 3) not-exploring without pups (dam away from the pups), 4) self-grooming (grooming its body but not the breast). Data are reported as the percentage of observations (33).

USV measurement

USV emission was measured at PNDs 3, 5, 9 and 13. 1 male and 1 female per litter for each experimental group were randomly removed from the nest and placed in the center of a Plexiglas arena (30 ×30 ×30 cm). The recording session lasted 3 min. During the test, an ultrasonic microphone sensitive to frequencies between 10 and 180 kHz was suspended 10 cm above the arena. The USVs were quantitatively and qualitatively analyzed using Avisoft Recorder software (Avisoft Bioacoustics, Berlin, Germany) (27). For quantitative analysis, we measured the total number of calls (USV frequency), whereas for qualitative analysis, we identified each call according to its waveform, as belonging to one of the following categories accordingly to (28, 29): a) Flat calls displaying a constant beginning and a constant ending at the same frequency (<=3 kHz of each other); b) Frequency steps with instantaneous frequency changes appearing as a vertically discontinuous ‘‘step’’ on a spectrogram, but with no interruption in time; c) 2-Syllable (2-Syl) calls consisting of two components: a main call

(flat) with an additional punctuated component towards the end; d) 3-Syl calls consisting of 3 components: a main call (flat) with two additional punctuated components. We calculated the percentage of each waveform with respect to the total number of USVs emitted. The axillary temperature of each pup was measured before and after the test by a digital thermometer for rodents.

Statistical analysis

Data are expressed as mean ± standard error medium (SEM). Reproduction parameters, maternal behavior and USV frequency were analyzed by Repeated Measures (RM) Analysis of Variance (ANOVA). One-way ANOVA was used for the analysis of litter weight, total number of pups, gender ratio and USV waveform among the 3 experimental groups. Post-hoc comparisons were performed using Tukey-Kramer Test. For all comparisons, P values of < 0.05 were considered statistically significant.

Results

Reproduction parameters

RM ANOVA revealed a significant effect of PND on food consumption ($F_{18,27} = 14.77$, $P < 0.0001$), while no significant effect of diet or of the interaction between these 2 factors were observed (diet: $F_{2,27} = 0.35$, $P = 0.71$; diet x PND interaction $F_{36,486} = 0.53$, $P = 0.99$). With regard to litter weight ($F_{2,74} = 0.54$, $P = 0.59$), total number of pups ($F_{2,74} = 0.03$, $P = 0.94$) and gender ratio ($F_{2,74} = 0.49$, $P = 0.61$ for female and $F_{2,74} = 0.51$, $P = 0.60$ for male) of each litter, one-way ANOVA did not show any significant difference among the 3 experimental groups. Reproduction parameters are summarized in table 2 as mean ± SEM.

Table 2: Reproduction parameters

Diet	Dams' food intake (g)	Litter weight (g)	Number of pups		
			Total	Males	Females
n6/n3-PUFA–balanced	23.9 ± 0.4	101.6 ± 4.6	14.9 ± 0.8	7.4 ± 0.6	7.4 ± 0.5
n3-PUFA–enriched	23.5 ± 0.3	100.1 ± 3.2	14.8 ± 0.5	7.0 ± 0.5	7.3 ± 0.4
n3-PUFA–deficient	22.6 ± 0.4	105.6 ± 3.5	14.6 ± 0.7	7.8 ± 0.5	6.8 ± 0.4

No significant effects of diet were detected in maternal food intake during pregnancy, litter weight, and number of pups. Results are expressed as mean ± SEM. (Dams n = 10/group, Pups n = 80/group).

Maternal behavior

RM ANOVA showed a significant effect of PND ($F_{11,31} = 24.50$, $P < 0.0001$) and of diet ($F_{2,31} = 8.97$, $P = 0.0008$) and a tendency towards significance for the interaction between these 2 factors ($F_{22,341} = 1.93$, $P = 0.08$) in the sum of maternal behaviors. Post-hoc analysis showed that both n3-PUFA-enriched and n3-PUFA-deficient diets reduce the total amount of maternal behavior compared to the n6/n3-PUFA-balanced diet ($P < 0.01$) (Fig 1 a). One-way ANOVA for each PND of observation, showed significant differences at PND 3 ($F_{2,32} = 7.76$, $P = 0.0018$); PND 6 ($F_{2,32} = 3.79$, $P = 0.0333$); PND 7 ($F_{2,32} = 7.48$, $P = 0.0022$); PND 8 ($F_{2,32} = 8.26$, $P = 0.0013$); PND 9 ($F_{2,32} = 16.91$, $P < 0.0001$) and PND 13 ($F_{2,32} = 7.48$, $P = 0.0022$). Post-hoc analysis showed that, at PND 3 and PND 6, n3-PUFA-enriched nourished dams spent less time in performing maternal behavior with respect to controls ($P < 0.01$ and $P < 0.05$ respectively); at PNDs

7, 8 and 9 both n3-PUFA-enriched ($P < 0.01$) and deficient diets reduce the total amount of maternal behavior compared to the control diet; at PND 13 n3-PUFA deficient nourished dams spent less time in performing maternal behavior compared to n3-PUFA-enriched nourished dams ($P < 0.05$) and to n6/n3-PUFA balanced nourished dams ($P < 0.01$) (Fig 1 a). RM ANOVA for passive nursing behavior showed a significant effect of PND ($F_{2,28} = 9.44$, $P < 0.0001$), of diet ($F_{2,28} = 4.94$, $P = 0.0145$) and of the interaction between these 2 factors ($F_{22,308} = 2.43$, $P = 0.0004$). Post-hoc analysis revealed that the n3-PUFA-deficient group showed decreased levels of passive nursing behavior than the n6/n3-PUFA-balanced group ($P < 0.05$) (Fig 1 b). A significant statistical difference was found at PND 8 ($F_{2,32} = 7.46$, $P = 0.002$); at PND 9 ($F_{2,32} = 15.32$, $P < 0.0001$); at PND 10 ($F_{2,31} = 4.13$, $P = 0.026$) and at PND 13 ($F_{2,31} = 4.65$, $P = 0.017$). Post-hoc analysis showed that at PND 8 both n3-PUFA-enriched and n3-PUFA-deficient groups performe less passive nursing behavior with respect to the n6/n3-PUFA-balanced group ($P < 0.01$ and $P < 0.05$ respectively); the same happened at PND 9 (Ps < 0.01). At PND 10 n3-PUFA-deficient group did less passive nursing behavior than the n6/n3-PUFA-balanced group ($P < 0.05$); and finally, at PND 13 n3-PUFA-deficient group showed a reduction in passive nursing behavior with respect to n3-PUFA-enriched group ($P < 0.05$) (Fig 1 b).

Statistical analysis performed for each type of maternal behavior revealed no significant differences for arched nursing behavior, blanket nursing behavior, maternal self-grooming behavior and licking pup behavior. For the non-maternal behaviors see Supplementary data.

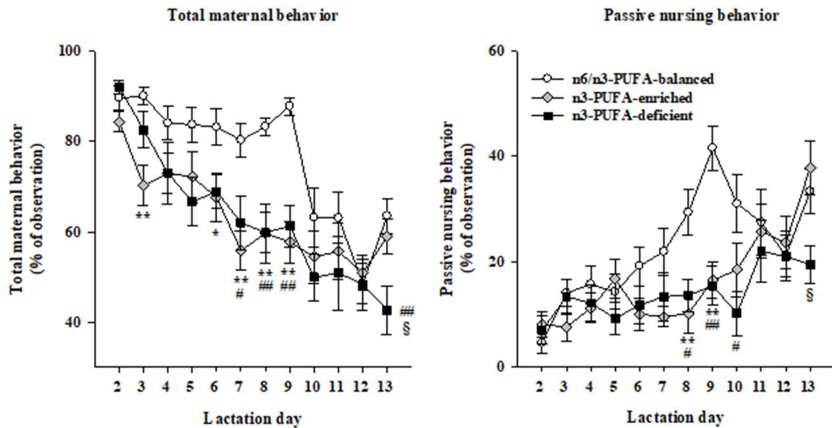

Figure 1: Maternal behavior. Total maternal behavior during the lactation period a) and Passive nursing behavior b). Both the n-3 PUFA–enriched and n3-PUFA–deficient groups exhibited less total maternal behavior compared with the n6/n3-PUFA–balanced group. Particularly, statistically significant differences were detected in passive nursing behavior. *, P <0.05, **, P < 0.01, n3-PUFA–enriched vs n6/n3-PUFA–balanced; #, P < 0.05, ##, P < 0.01 n3-PUFA–deficient vs n6/n3-PUFA–balanced; §, P< 0.05, n3-PUFA–enriched vs n3-PUFA–deficient. (n = 11-12/group).

USV frequency

RM ANOVA showed a significant difference among experimental groups in USV frequency for the effect of PND ($F_{2,75} = 13.71$, P < 0.0001), diet ($F_{2,25} = 2.15$, P = 0.01) and a tendency toward significance for the interaction between these 2 factors ($F_{6,75} = 5.54$, P = 0.06). Post-hoc analysis showed that both n3-PUFA-enriched and n3-PUFA-deficient diets reduce USV frequency compared to the n6/n3-PUFA-balanced diet (P < 0.05) (Fig 2 a). One-way ANOVA for each PND of observation showed statistical significant differences for USV frequency at PND 3 and PND 5 ($F_{2,25} = 7.24$, P = 0.003; $F_{2,25} = 7.90$, P = 0.002, respectively) (Fig 2 b, c) but not at PND 9 and PND 13 ($F_{2,25} = 0.48$, P = 0.62,$F_{2,25}$

= 0.10, P = 0.90, respectively) (Fig 2 d, e). At PND 3 and 5, n3-PUFA-enriched and n3-PUFA-deficient groups emitted fewer USVs than the n6/n3-PUFA-balanced group (Post-hoc, $P < 0.01$ e $P < 0.05$ respectively) (Fig 2 b, c).

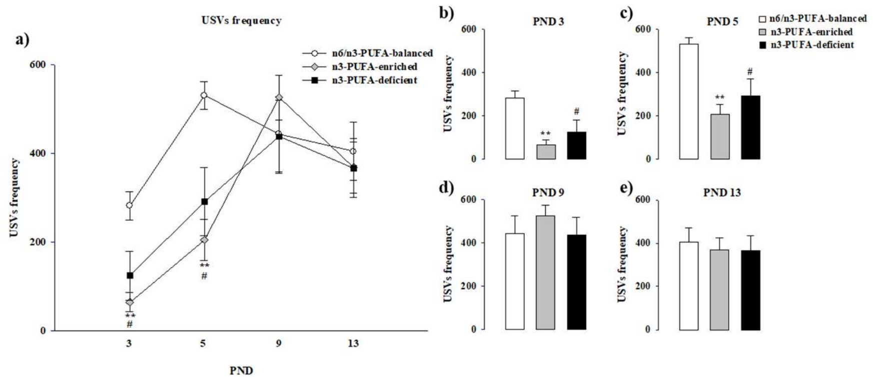

Figure 2: USV frequency. USV emission analysis at PND 3, 5, 9, and 13 a). At PND 3 b) and PND 5 c) both n3-PUFA–enriched and n3-PUFA–deficient groups had decreased USV frequency compared with the n6/n3-PUFA–balanced group. No significant differences between experimental groups were detected at PND 9 d) and PND 13 e). **, $P < 0.01$, n3-PUFA–enriched vs n6/n3-PUFA–balanced; #, $P < 0.05$ n3-PUFA–deficient vs n6/n3-PUFA–balanced. (n = 8-10/group).

USV waveform

One-way ANOVA did not show significant differences in USV waveform at PND 3 and PND 5: Flat ($F_{2,25} = 2.37$; P = 0.11; $F_{2,25} = 0.17$; P = 0.84, respectively); Frequency step ($F_{2,25} = 2.30$; P = 0.12; $F_{2,25} = 0.26$; P = 0.77, respectively); 2-Syl ($F_{2,25} = 0.89$; P = 0.42; $F_{2,25} = 0.67$; P = 0.52, respectively); no 3-Syl calls were recorded at this PND. At PND 9 statistical significant differences were found for the Flat calls ($F_{2,25} = 12.72$; P = 0.0002) and for the 2-Syl calls ($F_{2,25} = 4.60$; P = 0.02) but not for the Frequency step calls ($F_{2,25} = 0.64$;

$P = 0.54$) and for the 3-Syl calls ($F_{2,25} = 0.52$; $P = 0.60$). Post-hoc analysis showed that the n3-PUFA-enriched group emitted fewer Flat calls than the n3-PUFA-deficient and the n6/n3-PUFA-nourished balanced groups ($P < 0.05$; $P < 0.01$ respectively). The n3-PUFA-deficient group emitted reduced numbers of Flat calls with respect to the n6/n3-PUFA-balanced group ($P < 0.05$) but higher numbers of the 2-Syl calls with respect to the n6/n3-PUFA-balanced group ($P < 0.05$). Statistically significant differences were also found at PND 13 for Flat calls ($F_{2,25} = 6.26$; $P = 0.006$) and for 2-Syl calls ($F_{2,25} = 3.55$; $P = 0.044$) but not for Frequency step calls ($F_{2,25} = 1.82$; $P = 0.18$) and 3-Syl calls ($F_{2,25} = 0.34$; $P = 0.71$). Post-hoc analysis showed that the n3-PUFA-deficient group emitted fewer numbers of Flat calls than the n6/n3-PUFA-balanced group ($P < 0.01$) and greater numbers of 2-Syl calls than the n6/n3-PUFA-balanced group ($P < 0.05$) (Fig 4 d). USV waveform data are summarized in table 3 as mean value ± SEM. USV waveform distribution is showed in Fig 3.

Table 3: USV waveform analysis performed at PND 3, 5, 9, and 13. Pups in both the n-3 PUFA–enriched and n3-PUFA–deficient groups exhibited an altered sonographic pattern compared with the n6/n3-PUFA–balanced group.

PND 3	**Flat**	**Frequency steps**	**2-Syl**	**3-Syl**
n6/n3-PUFA–balanced	100.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
n3-PUFA–enriched	99.93 ± 0.08	0.08 ± 0.08	0.00 ± 0.00	0.00 ± 0.00
n3-PUFA–deficient	97.74 ± 1.06	2.23 ± 1.06	0.03 ± 0.03	0.00 ± 0.00
PND 5				
n6/n3-PUFA–balanced	75.69 ± 7.07	24.13 ± 7.00	0.19 ± 0.09	0.00 ± 0.00
n3-PUFA–enriched	78.31 ± 6.42	20.83 ± 6.19	0.20 ± 0.12	0.00 ± 0.00
n3-PUFA–deficient	80.49 ± 3.29	18.26 ± 3.56	1.25 ± 1.21	0.00 ± 0.00
PND 9				
n6/n3-PUFA–balanced	85.19 ± 2.17	8.96 ± 2.50	5.69 ± 1.19	0.16 ± 0.09
n3-PUFA–enriched	52.48 ± 4.70$^{** §}$	14.42 ± 4.06	31.17 ± 6.55*	0.57 ± 0.40
n3-PUFA–deficient	68.17 ± 5.19$^{\#}$	12.62 ± 4.49	18.90 ± 6.60	0.31 ± 0.25
PND 13				
n6/n3-PUFA–balanced	76.68 ± 5.25	0.51 ± 0.27	18.32 ± 4.66	4.49 ± 1.28
n3-PUFA–enriched	64.21 ± 1.70	4.84 ± 1.82	25.56 ± 2.56	33.61 ± 4.58
n3-PUFA–deficient	56.52 ± 4.50$^{\#\#}$	3.84 ± 1.86	33.61 ± 4.58$^{\#}$	6.03 ± 1.39

No significant differences among experimental groups were detected at PND 3 and PND 5. At PND 9, the n3-PUFA–enriched group emitted fewer Flat calls than the n3-PUFA–deficient and n6/n3-PUFA–balanced groups. The n3-PUFA–deficient group emitted fewer Flat calls than the n6/n3-PUFA–balanced group, but more 2-Syl calls than the

n6/n3-PUFA balanced group. At PND 13, the n3-PUFA–deficient group emitted fewer Flat calls than the n6/n3-PUFA–balanced group and more 2-Syl calls than the n6/n3-PUFA balanced group. *, P <0.05, **, P < 0.01, n3-PUFA–enriched vs n6/n3-PUFA–balanced; #, P < 0.05, ##, P < 0.01 n3-PUFA–deficient vs n6/n3-PUFA–balanced; §, P< 0.05, n3-PUFA–enriched vs n3-PUFA–deficient. Results are expressed as mean ± SEM. (n = 8-10/group).

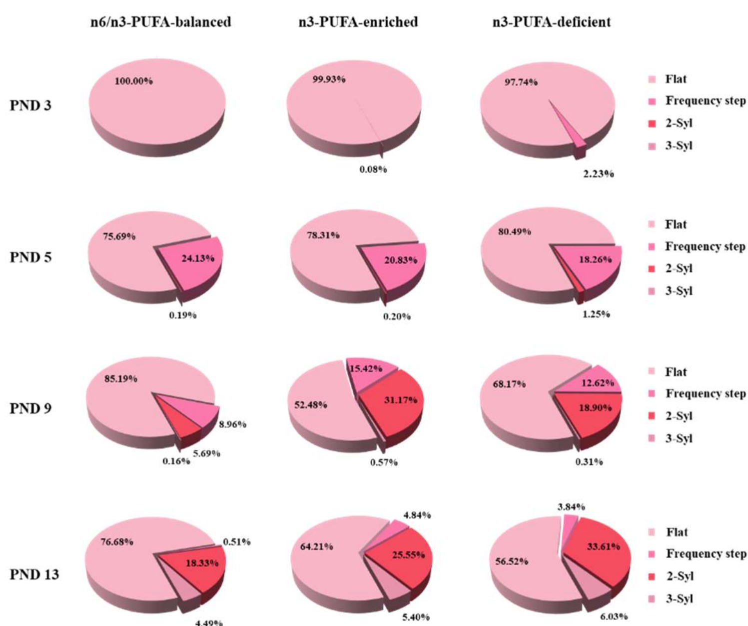

Figure 3: USV waveform distribution. Pie graphs of the different call categories show the percentages the different call categories emitted by the three experimental groups. Percentages in each experimental group were calculated as the number of calls in each category for each subject/total number of calls analyzed for each subject (n = 8-10/group).

Discussion

The present findings indicate that perinatal exposure to an unbalanced n3-PUFA diet –either enriched or deficient – induces early behavioral disturbances in neonatal rats. Dams fed either the n3-PUFA–deficient diet or n3-PUFA–enriched diet exhibited decreased maternal care compared with those fed an n6/n3 PUFA-balanced diet. Further, pups nourished with the unbalanced diets produced USVs with altered frequency and altered sonographic patterns compared with pups nourished with the n6/n3 PUFA-balanced diet.

Maternal behavior is a complex behavioral response that naturally occurs in mammals upon the first filial generation (30-32). Several studies have documented the importance of maternal behavior for brain development in the offspring (33-35), as well as a link between maternal care and stress response maturation in the offspring (36-39). Rodent pups emit USVs under environmental stimuli such as cold temperature (40-42) to attract maternal attention. These USVs are an essential form of communication between pups and their mothers and serve as a useful tool for studying emotionality and communicative/social skills in infant rodents (21). The link between maternal care and USVs in pups is mutual (43, 44). When separated from the mother and the nest, newborn rodents emit USVs with frequencies between 30 and 90 KHz (29). USVs stimulate maternal behavior, which strongly influences the development of the emotional, cognitive, and social behavior in the offspring (19, 45). Therefore, the emotional and cognitive development of the offspring depends upon functional interactions between mothers and infants (35, 46). In the present study, we evaluated the functional interactions between dams and their offspring by analyzing the maternal behavior and pup USVs during early life under 3 different n3-PUFA dietary regimens. Daily analysis of maternal behavior was performed from PND 2 through PND 13. On the basis of previous studies (47, 48), we identified 7 maternal parameters and 4 non-maternal

parameters, and the sums of the parameters are reported as total maternal behavior and total non-maternal behavior, respectively. Our findings revealed that both n3-PUFA supplementation and n3-PUFA deficiency reduce total maternal behavior compared with dams fed an n6/n3-PUFA-balanced diet. Dams fed either an n3-PUFA–deficient or an n3-PUFA–enriched diet exhibited decreased passive nursing behavior, indicating that both n-3 PUFA supplementation and deficiency lead to alterations in specific maternal behaviors. On the other hand, compared with dams fed the n6/n3-PUFA-balanced diet, the total amount of non-maternal behaviors was increased in dams fed either unbalanced diet. Overall, these results demonstrate that an n6/n3 PUFA unbalanced diet affects maternal care.

Dietary n3-PUFA fatty acids contribute to the prevention of affective disorders in the mother (49, 50). Such disorders compromise maternal attachment, leading to deleterious consequences for the development of their offspring (51). Our findings are consistent with those of a previous study in mice showing that dietary n3-PUFA supplementation leads to abnormal maternal behavior (53) and support the main concept that a balanced n6/n3 PUFA intake is required for normal, healthy mother-infant interactions. In the present study, 3- and 5-day old pups nourished with either an n3-PUFA–enriched or an n3-PUFA–deficient diet emitted fewer USVs compared with the pups nourished with an n6/n3-PUFA-balanced diet, indicating altered communication between mothers and pups depending on the n6/n3-PUFA ratio in the diet. This altered communication between the mother and pups during early ontogenic phases also affects the development of normal adolescent and adult behavior. In a previous study, we evaluated the cognitive and emotional skills of adult offspring nourished as pups with the same altered diets used in the present study. Consistent with the present findings, cognitive alterations were observed in both the n3-PUFA–enriched and n3-PUFA–deficient groups (14).

Not only do pups nourished with an n3-unbalanced diet emit fewer USVs, but more sophisticated analysis revealed that the USV sonographic pattern also differed from that of normal rat pups. Rat pups emit different types of USVs, some of which have additional components that make the syllable more complex than a simple whistle (53). Consistent with a previous report (54), we found that the waveform complexity increased with the pup's age independent of the diet, likely a result of the progressive development of the anatomical structures in the respiratory tract and, represents an index of cognitive development that allows rats to vocalize in a more complex manner (54). Moreover, the production of differently shaped USVs may reflect the stress state of the pups (55, 29) and may depend on the amount of maternal care experienced during early life (21). Pup USV waveforms are finely tuned by the maternal diet (56, 57). Our findings demonstrated that the n3-PUFA unbalanced diets affected the normal USV repertoire; that is, pups nourished with an n3-PUFA–enriched diet emitted more complex USV waveforms than pups nourished with a balanced diet, while at PND 13 pups nourished with an n3-PUFA–deficient diet emitted more complex USVs than pups nourished with the balanced diet. Although precocious development toward complexity with respect to controls may seem to be a positive attribute, it must be considered a negative effect represented by a deviation from normal development that may disrupt emotional, cognitive, and social outcomes. In support of this concept, short-term memory deficits in the novel object recognition task was recently reported in adult rats nourished throughout life with the unbalanced diets used in the present study (14).

Although n3-PUFA food supplementation has become an attractive alternative source for balancing the n6/n3 ratio in the diet to promote health, emerging studies highlight some controversy with regard to its potential beneficial effects (58-62). Recent evidence indicates the lack of efficacy of n3-PUFA supplementation on heart disease, leading to re-assessment of the marketing

authorizations concerning the use of n3-PUFA medicines for the prevention of health and blood vessel problems (63). Moreover, in humans, prenatal supplements with DHA do not result in improved cognitive development in the offspring or prevent mood disorders in the mothers (64-66). Here we show for the first time that n3-PUFA dietary supplementation, rather than being beneficial, negatively affects the maternal-offspring interaction, which leads to behavioral alterations in early ontogenetic phases, similar to the effects of an n3-PUFA–deficient diet. These findings suggest that a diet with a balanced n6/n3 PUFA ratio is necessary for healthy development and indicate that caution needs to be paid to the extensive use of n3-PUFA–enriched foods for newborns, infants, and toddlers.

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RESEARCH ARTICLE

Effects of sevoflurane and clonidine on acid base status and long-term emotional and cognitive outcomes in spontaneously breathing rat pups

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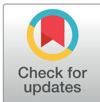

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Abstract

Background

Numerous experiments in rodents suggest a causative link between exposure to general anaesthetics during brain growth spurt and poor long-lasting neurological outcomes. Many of these studies have been questioned with regard of their translational value, mainly because of extremely long anaesthesia exposure. Therefore, the aim of the present study was to assess the impact of a short sevoflurane anaesthesia, alone or combined with clonidine treatment, on respiratory function in spontaneously breathing rat pups and overall effects on long-lasting emotional and cognitive functions.

Methods

At postnatal day (PND) 7, male Sprague Dawley rat pups were randomized into four groups and exposed to sevoflurane for one hour, to a single dose of intraperitoneal clonidine or to a combination of both and compared to a control group. Blood gas analysis was performed at the end of sevoflurane anaesthesia and after 60 minutes from clonidine or saline injection. Emotional and cognitive outcomes were evaluated in different group of animals at infancy (PND12), adolescence (PND 30–40) and adulthood (PND 70–90).

Results

Rat pups exposed to either sevoflurane or to a combination of sevoflurane and clonidine developed severe hypercapnic acidosis, but maintained normal arterial oxygenation. Emotional and cognitive outcomes were not found altered in any of the behavioural task used either at infancy, adolescence or adulthood.

Conclusions

Sixty minutes of sevoflurane anaesthesia in newborn rats, either alone or combined with clonidine, caused severe hypercapnic acidosis in spontaneously breathing rat pups, but was devoid of long-term behavioural dysfunctions in the present setting.

Introduction

Multiple studies have raised concerns regarding direct neurotoxic effects of general anaesthetics on the developing brain with possible long lasting impact on neurocognitive function [1]. Alterations, such as attention deficit/hyperactivity disorders (ADHD) [2], learning deficits [3], behavioural disturbances [4] and language deficits [5] have been reported in retrospective cohort follow up studies in children. Time of exposure as well as the cumulative anaesthetic dose have both been identified as determinant factors of neurotoxicity. There is clear evidence from animal studies that the time of synaptogenesis is the most vulnerable period for anaesthetic neurotoxicity. Different brain regions are vulnerable at different time windows. In rats, extensive synaptogenesis takes place in the thalamus, hippocampus and neocortex at postnatal day (PND) 7 [6]. Therefore, most studies in this field have been performed by exposing rodents to general anaesthetics at this specific time frame. A recent study in young rodents suggested that physiological disturbances during anaesthesia might contribute to volatile anaesthetic induced neurotoxicity [7]. A higher overall mortality as well as a significant increase in hippocampal cell death and worse performance in the Morris water maze test were reported in spontaneously breathing, anaesthetized rats compared to those who were mechanically ventilated or control group animals.

Stratmann and coworkers observed a similar pattern of neuronal cell death in rat pups exposed to either 4h of isoflurane or 4h of hypercarbia suggesting that part of isoflurane induced cell death might be due to isoflurane induced hypercarbia [8]. However these findings did not translate consistently into neurocognitive outcome as only those animals treated with 4 h of isoflurane anaesthesia showed long-term neurocognitive deficits.

The impact of hypercapnic acidosis on neurocognitive outcome has also been questioned in neonates undergoing thoracoscopic surgery for repair of congenital diaphragmatic hernia and oesophageal fistula [9,10].

Interestingly, the European paediatric anaesthesia community is currently leading an active debate on how to define 'safe conduct of anaesthesia' in neonates and young children, pointing out the primary importance of controlling and maintaining stability of physiological parameters [11]. So the current question is, if it is the anaesthetic agent itself or rather the anaesthesia induced derangement of body homeostasis having detrimental effects on the developing brain [12,13].

In the search of preventive and protective strategies, alpha-2-agonists gained an important role being attributed with neuroprotective properties attenuating the apoptotic and neurodegenerative effects of NMDA-antagonists and $GABA_A$-agonists [14–16]. The use of clonidine in paediatric anaesthesia is expanding as a result of its' sedative and analgesic properties [17–19]. On this background we tested the hypothesis that clonidine and 1h of 2.5% sevoflurane, either alone or in combination, would differently affect the respiratory system in spontaneously breathing animals. In order to evaluate if early exposure to anaesthesia—induced hypercarbia could lead to long-lasting behavioural alterations, emotional and cognitive functions were assessed as secondary outcomes not only at infancy (PND12), but also at adolescence (PND 30–40) and adulthood (PND 70–90).

Materials and methods

Animals

All animal experiments were approved by the Italian Ministry of Health (Rome, Italy) and performed according to the guidelines of the Italian Ministry of Health (D.L. 96/1992) and the European Community Directive 2010/63/EU of September 22nd 2010.

Multiparous pregnant female Sprague-Dawley rats (Charles River®, Calco, Italy) were housed in an air-conditioned room (temperature 21±1˚C) in a standard 12-h light-dark cycle (lights on at 06:00 h), with access to food and water ad libitum.

Newborn litters, found up to 5 pm, were considered to be born on that day (PND0). On PND1 all litters were reduced to a size of 6 males and 2 females per litter as previously described [20].

On PND21, pups were weaned and housed in groups of three in 42 × 27 × 14 cm Plexiglas cages in air-conditioned rooms. One male pup per litter from different litters per treatment group was used in each experiment. Each male rat was tested only once. Females were not tested.

Randomization

At PND7 rats were randomized by a computer generated list (Graph Pad Software, Inc., California, USA) into four groups receiving: sevoflurane (group S), clonidine (group C), sevoflurane/clonidine (group SC), saline (control group).

Anaesthesia protocol

Animals anaesthetized with sevoflurane (Sevorane, Abbott, Italy) were placed in a heated anaesthesia chamber connected to a vaporizer system and to a gas anaesthesia machines (Fluovac System, Harvard Apparatus, Holliston, US) breathing sevoflurane 2.5% in oxygen and air (1:1) with a fresh gas flow of 2 l/min. Animals treated with clonidine (Catapresan 150 mcg/ml, Böhringer Ingelheim, Italy) received an intraperitoneal injection of 400 μg/kg diluted in sterile saline to a standard volume of 200 l immediately before being placed in the anaesthetic chamber. Control animals and those exposed to sevoflurane received an intraperitoneal administration of 200 l of sterile saline. The duration of stay in the gas chamber was set to a total time of 60 minutes for comparison with sevoflurane exposure. The chamber was heated and the temperature maintained constant by an infrared light and a heating pad. The concentration and duration of sevoflurane administration were established after preliminary results and previous published studies on rat pups. We decided to omit anaesthesia induction with a high concentration of sevoflurane in order to minimize cardiorespiratory depressant effects. Anaesthesia induction was defined as time from the beginning of sevoflurane until loss of righting reflex. The clonidine dose was chosen according to Kesavan and coworkers [21]. Arterial pulse oximetry and heart rate (MouseOx Plus®, Starr Life Sciences Corp., Oakmont, US) were recorded continuously in one animal every set of four animals undergoing anaesthesia. All other animals were clinically observed for any sign of distress during the entire procedure.

Blood gas analysis

Blood samples for blood gas analysis were taken from the left ventricle after 60 minutes of drug or saline administration or after 60 minutes of placement in the gas chamber. Arterial blood was analysed immediately after blood collection with a blood gas analyser (GEM Premier 4000, Instrumentation Laboratory SpA, Milan, Italy). After blood sampling animals were sacrificed by cervical dislocation.

Behavioural testing

The series of behavioural tests was chosen on the basis of the relatively well established anatomical areas involved in these tasks. All behavioural tests were performed by the same operator, who was blinded to group assignment. Adolescent and adult rats were handled three times for one minute each day, starting 72 hours before testing. Emotional and cognitive assessments were performed at infancy (PND12), adolescence (PND30-40) and at adulthood (PND70-90).

Behavioural tests at infancy

Ultrasonic vocalization (USV). The ultrasonic vocalization (USV) analysis is a useful tool to assess pup social communication skills in the first few days of life [22]. The USV test has previously been studied to assess autism-like behaviour in rodents after neonatal exposure to sevoflurane [23]. When separated from the mother and the nest, newborn rats emit USVs with frequencies between 30 and 90 kHz. These USVs are essential for the mother/pup interaction and are critical for pups survival [22]. At PND 12, pups were removed from the nest and placed in a soundproof arena (30 × 30 × 30 cm). The recording session lasted 3 min [20]. USV calls were recorded by the Avisoft recorder (Avisoft Bioacoustics, Berlin, Germany).

Homing test. One of the earliest expressions of spatial behaviour is the pups' ability to return towards their nest if separated from mother and littermates. Hippocampal formation plays a pivotal role in spatial cognition and navigation [24]. The homing test is a useful task for the evaluation of these rudimentary cognitive abilities in infant rats [25]. At PND 12, each pup was placed in a Plexiglas arena (36×22.5×10 cm) with the bottom covered by 2/3 clean litter and by 1/3 of litter from the mother's cage, with the nest odour, representing the nest area. The pup was placed close to the wall on the clean litter side and video-recorded for 4 min. The latency to reach the nest area and the time spent in the nest area were recorded to evaluate homing performance.

Behavioural tests at adolescence and adulthood

Inhibitory avoidance. The inhibitory avoidance test is a commonly used task to investigate learning and long-term memory processes in rodents [26]. This test has been used previously to measure sevoflurane-induced impairment of memory consolidation in rodents [27]. The procedure was carried out as previously described by Morena [28]. For training, adolescent and adult rats were placed into the starting compartment and were allowed to explore the apparatus. After the rats stepped into the dark compartment, the sliding door was closed, and a single footshock (0.4 mA) was delivered for 1 s. Animals were removed from the shock compartment 15 s after termination of the footshock. Retention was tested 48 h later. On the retention test, rats were placed into the starting compartment, and the latency to re-enter the shock compartment (maximum latency of 600 s) was recorded. Longer latencies were interpreted as indicators of better retention.

Elevated plus maze. The elevated plus maze (EPM) is a validated assay to study hippocampus dependent behaviour performance in rodents [29] and has been applied previously to assess anxiety related behaviour in rodents after inhalational [30] and intravenous anaesthetics [31]. The EPM apparatus comprised two open arms (50 × 10 × 40 cm) and two closed arms (50 × 10 × 40 cm) that extended from a common central platform (10 × 10 cm). Confinement to the closed arms was associated with the observation of significantly more anxiety-related behaviours than confinement to the open arms [32]. The EPM test was performed following the procedure described by Manduca and colleagues [33]. Adolescent and adult rats were individually placed on the central platform facing a closed arm and a 5-min test period was recorded. Behavioural analysis was carried out using the Observer® XT 13.0 software (Noldus, Wageningen, The Netherlands). The following parameters were analysed:

Table 1. Demographic data.

	CONTROL	CLONIDINE	SEVOFLURANE	SEVOFLURANE/ CLONIDINE
BW (g)	17.90 ± 0.78	16.89 ± 0.72	16.30 ± 0.79	17.85 ± 0.80
T˚C pre- treatment	36.70 ± 0.08	36.63 ± 0.18	36.68 ± 0.11	36.68 ± 0.08
T˚C post- treatment	36.71 ± 0.08	36.64 ± 0.17	36.93 ± 0.05	36.89 ± 0.05

Data are presented as mean ± standard error of mean (SEM). There was no difference of body weight and temperature among groups (n = 10 per group; p > 0.05). BW = body weight. T˚C = axillary body temperature.

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a. % Time spent on the open arms (% TO);

b. % Open entries (% OE);

c. Number of exploratory head dippings (HDIPS);

d. Number of stretched-attend postures (SAP).

Statistical analysis. Data are expressed as mean ± standard error of mean (SEM). To assess the effects of different treatment, data were analysed using one-way ANOVA (Graph Pad Prism version 6.0) followed by Tukey's multiple comparison post-hoc test where appropriate. For all comparisons, p values of less than 0.05 where considered statistically significant.

Results

There were no differences in demographic data between groups (Table 1). Mean time and SEM of anaesthesia induction was 4.8 ± 0.5 minutes in the sevoflurane group and 4.1 ± 0.3 minutes in the sevoflurane/clonidine group (p = 0.3). There was no anaesthesia related mortality.

Blood gas analysis

Mean and SEM values for all parameters are reported in Table 2.

pH and pCO_2. Rat pups treated with either sevoflurane or a combination of sevoflurane and clonidine developed severe respiratory acidosis, while there was no difference in pH and pCO2 between control animals and those receiving clonidine ($F_{(3,34)} = 46.82$, $P < 0.0001$; $F_{(3,34)} = 41.16$, $P < 0.0001$ for pH and pCO_2, respectively).

Table 2. Blood gas analysis.

	CONTROL	CLONIDINE	SEVOFLURANE	SEVOFLURANE/CLONDINE
pH	7.39 ± 0.01	7.39 ± 0.01	7.25 ± 0.04*	6.99 ± 0.04*
pCO_2 mmHg	47.50 ± 1.53	50.70 ± 2.10	81.40 ± 7.81*	133.50 ± 9.70*
lactate mmol/L	3.55 ± 0.25	2.68 ± 0.14*	1.10 ± 0.13*	0.66 ± 0.11*
HCO_3 mmol/L	26.39 ± 0.57	27.84 ± 0.58	27.79 ± 0.65	26.45 ± 1.66
BE mmol/L	3.89 ± 0.61	5.57 ± 0.83	7.25 ± 1.2	6.13 ± 1.37
glucose mg/dL	113 ± 4.27	141 ± 7.77	127 ± 7.82	112 ± 16.36
pO_2 mmHg	93.40 ± 2.71	92.70 ± 2.68	88.30 ± 1.48	90.13 ± 1.7

Values are reported as mean ± standard error of mean (SEM).

* p<0.05. BE = base excess, pH, pCO_2 and lactate levels were significantly lower in rat pups treated with sevoflurane or sevoflurane and clonidine. There were no differences among groups for bicarbonate, pO_2 and glucose levels (p > 0.05).

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Table 3. Ultrasound vocalization (USV) and homing test at infancy.

		CONTROL	CLONIDINE	SEVOFLURANE	SEVOFLURANE/ CLONIDINE
USV	Frequency (n)	691.08 ± 100.37	619.20 ± 104.39	448.80 ± 123.71	624.10 ± 107.17
HOMING TEST	Latency (s)	83.34 ± 23.25	91.34 ± 28.53	82.50 ± 27.27	136.95 ± 32.51
	Nesting time (s)	145.42 ± 21.39	152.25 ± 21.37	190.49 ± 23.78	115.90 ± 27.48

Data are expressed as mean ± standard error of mean. There were no differences of behavioural tests among groups (n = 10 per group; p > 0.05).
s = seconds; n = numbers of vocalizations

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Lactate. Lactate levels were significantly lower in animals treated with sevoflurane alone or in combination with clonidine compared to control and clonidine treated rats. Lactate levels of rat pups treated with clonidine alone were significantly lower compared to the control group ($F_{(3,34)} = 61.35$; $P < 0.0001$).

HCO_3. One-way ANOVA did not reveal any significant difference in bicarbonate levels among groups ($F_{(3,34)} = 0.8338$; $P = 0.48$)

pO_2. No cases of hypoxia were observed. There was no statistically significant difference in pO_2 between groups ($F_{(3,34)} = 1.115$; $P = 0.36$)

Base excess. One-way ANOVA did not show any significant difference among groups ($F_{(3,34)} = 2.004$; $P = 0.13$)

Glucose levels. No significant differences were observed among groups ($F_{(3,34)} = 2.205$; $P = 0.10$)

Behavioural tests

USV and homing test. No significant effects of treatment on USVs were observed ($F_{(3, 38)} = 0.91$, $P = 0.45$, Table 3). As concerning the homing test, one-way ANOVA did not reveal any significant effect of treatment on the latency time ($F_{(3, 38)} = 0.85$, $P = 0.48$) and on the nesting time ($F_{(3, 38)} = 0.44$, $P = 0.73$) in the homing test (Table 3).

Inhibitory avoidance. No significant differences were found in the inhibitory avoidance test among groups either at adolescence or adulthood respectively (training: $F_{(3, 28)} = 0.55$, $P = 0.65$; test: $F_{(3, 28)} = 1.01$, $P = 0.40$; training: $F_{(3, 38)} = 0.44$, $P = 0.72$; test: $F_{(3, 38)} = 0.61$, $P = 0.61$, Table 4).

Elevated plus maze. One-way ANOVA showed that there was no significant effect of treatment at adolescence on %TO ($F_{(3, 38)} = 0.55$, $P = 0.65$; Fig 1A), on %OE ($F_{(3, 38)} = 0.62$, $P = 0.60$; Fig 1B), on HDIPS frequency ($F_{(3, 38)} = 0.74$, $P = 0.54$) and on SAP Frequency ($F_{(3, 38)} = 0.55$, $P = 0.65$) and at adulthood on: %TO ($F_{(3,34)} = 0.89$, $P = 0.46$; Fig 1A), %OE ($F_{(3,34)} = 0.27$, $P = 0.85$; Fig 1B), in HDIPS frequency ($F_{(3,34)} = 1.51$, $P = 0.23$), and in SAP frequency ($F_{(3,34)} = 1.25$, $P = 0.31$).

Table 4. Inhibitory avoidance test (IA) at adolescence and adulthood.

		CONTROL	CLONIDINE	SEVOFLURANE	SEVOFLURANE/ CLONIDINE
ADOLESCENCE	Training Latency Time (s)	9.07 ± 1.75	8.13 ± 1.31	8.29 ± 2.36	6.06 ± 1.25
	Test Latency Time (s)	140.42 ± 71.30	352.95 ± 97.78	285.52 ± 89.43	294.36 ± 98.42
ADULTHOOD	Training Latency Time (s)	10.03 ± 1.41	10.83 ± 2.10	12.66 ± 2.62	9.83 ± 1.41
	Test Latency Time (s)	352.44 ± 74.76	295.91 ± 59.13	356.48 ± 69.81	239.12 ± 73.12

Latency times are measured in seconds (s). Results are expressed as mean ± standard error of mean (SEM). There was no difference among groups (n = 10 per group; p > 0.05).

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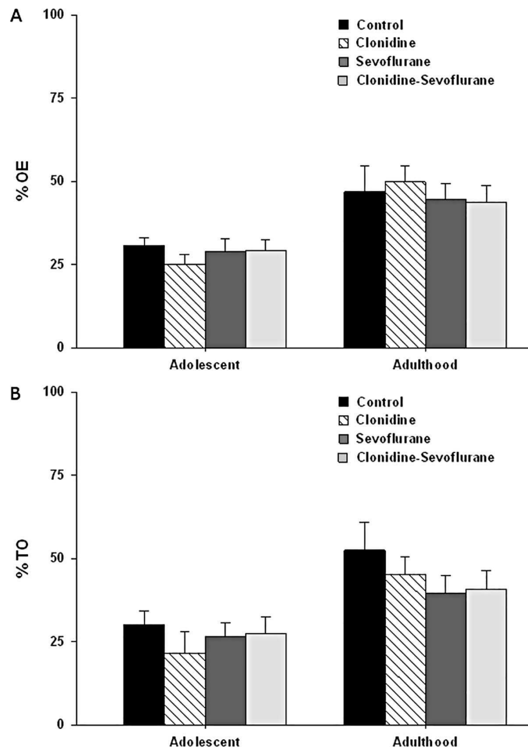

Fig 1. (A): Elevated Plus Maze (EPM) at adolescence and adulthood. Percentage of entries in the open arms (OE). Results are expressed as mean ± standard error of mean (SEM). There was no difference among groups (n = 10 per group; p > 0.05). (**B): Elevated Plus Maze (EPM) at adolescence and adulthood** Percentage of time spent in the open arms (TO). Results are expressed as mean ± standard error of mean (SEM). There was no difference among groups (n = 10 per group; p > 0.05).

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Discussion

To date there is an open debate on whether impaired long-term neurocognitive outcome after anaesthesia in newborn rodents and humans might be a result of a direct drug related neurotoxic effect or rather a consequence of physiological disturbances occurring during anaesthesia [7,11,34].

The present findings show that even a relatively brief anaesthesia with sevoflurane causes profound hypercapnic acidosis (HCA) in spontaneously breathing rat pups. This effect is aggravated by the contemporary administration of clonidine. However, no correlation could be found between anaesthesia–induced hypercapnic acidosis at PND 7 and emotional or cognitive alterations at infancy, adolescence and adulthood in any of the behavioural tasks used in

the current study. HCA has been suggested as a determinant factor in anaesthesia related neurotoxicity and long-term cognitive outcome [7,8].

Stratmann and coworkers showed that neonatal exposure to either two and four hours of isoflurane or four hours of carbon dioxide caused a similar pattern and distribution of cell death in the cortex, hippocampus and thalamus [8]. However long term neurocognitive deficits were only found in rodents after four hours of isoflurane exposure suggesting a dose-dependent threshold.

In contrast Wu and co-workers [7] compared spontaneously breathing to mechanically ventilated 14-day old rats that were exposed to four hours of either sevoflurane or isoflurane. Self—ventilating animals showed a higher percentage of overall mortality (30%), neuronal cell death and alterations of spatial learning compared to mechanically ventilated and control animals. HCA has important biochemical and physiological effects [35].

The effect of HCA on brain function in neonates is of uttermost interest among neonatologist when applying protective ventilation strategies with permissive hypercapnia. Extremes of hypercapnia and especially rapid changes of $PaCO_2$ have been linked to adverse neurological outcome in premature neonates [36]. Furthermore, HCA was shown to inhibit neuronal metabolism and to increase expression of pro-apoptotic mediators in the cerebral cortex of newborn piglets [37]. Notwithstanding the above concerns, several animal studies have suggested neuroprotective effects of hypercapnia on impaired neuronal function in the injured neonatal rat brain [38–40].

Vannucci and coworkers showed that hypercapnia protects the immature rat brain from hypoxic—ischaemic damage. Improved cerebrovascular perfusion and oxygen delivery as well as enhanced cerebral glucose utilization were observed in animals exposed to hypoxia and hypercapnia. Besides a marked attenuation in cerebral tissue lactacidosis, a reduction of the excitatory amino acid neurotransmitter glutamate in the CSF was found [38,39].

There has been recently a debate on safety of thoracoscopic surgery for congenital diaphragmatic hernia (CDH) and oesophageal atresia in the newborn [9,10]. A prospective pilot randomized controlled trial at Great Ormond Street Hospital provided evidence that thoracoscopy in neonates is associated with significant hypercapnia and acidosis. The levels of hypercapnia and acidosis were of such concern that thoracoscopic repair of CDH in this age group was suspended. Six out of 10 neonates experienced $paCO_2$ levels above 105 mmHg for a duration ranging from 30 minutes up to three and a half hours. There were no changes of arterial oxygenation. Results from neurodevelopmental follow-up of these kids are still awaited and might elucidate long-term effects of hypercapnia and acidosis on the developing brain [10]. There are no reports in the literature as yet indicating a deleterious cerebral effect of acidosis and hypercapnia in the presence of normoxia.

A case report of an 8—year old, asthmatic boy suffering more than 14 hours of severe hypercapnic acidosis due to respiratory failure was followed by complete recovery [41]. A second case of severe hypercapnia under sevoflurane anaesthesia was reported in a three—week old baby undergoing incarcerated hernia repair. No neurological sequelae were observed [42]. A reduction in cerebral metabolic requirements due to deep sedation with sevoflurane as well as adequate tissue perfusion and oxygenation were proposed as protective mechanisms for tolerating severe hypercapnia.

Volatile anaesthetic preconditioning (APC) of the brain has been demonstrated both, in vitro and in vivo, yet the underlying mechanism is not fully understood. Suppression of energy requirements seems not sufficient to explain the neuroprotective effects of sevoflurane. Further mechanisms such as inhibition of glutamate release, direct scavenging of free radicals, anti-oxidative and anti-inflammatory effects as well as prevention of apoptotic cell death seem to play an important role for increasing cerebral ischaemic tolerance [43].

In the present study neonatal rodents were exposed to sevoflurane and as a consequence of anaesthesia induced respiratory depression also to hypercapnic acidosis. However, oxygenation and tissue perfusion were guaranteed as shown by normal serum paO_2 and lactate levels. The battery of neurocognitive tests utilized focused on the detection of more subtle changes related to social communication, autism-like behaviour and anxiety.

More recent studies in neonatal rodents assessing autism-like behaviour, social behaviour and fear conditioning after sevoflurane exposure [23,30] reported conflicting results. Noteworthy, most of these experiments used extremely long anaesthesia protocols with up to six hours of exposure to inhalational anaesthetics. When translating time from newborn rat pups to human beings, six hours in a rat pups' life would have an equivalent of more than two days in human life [44]. Not surprisingly very high mortality rates up to 30%[7] are reported in these experiments, especially in the final hours of the anaesthesia. A direct correlation between the cumulative dose of general anaesthetics and the risk of neurocognitive impairment has been postulated [1,2,45].

The role of alpha-2-agonists in the context of neuroprotection needs still to be defined. Few studies in rodents have been performed suggesting protective properties [14–16].

Pre- and postconditioning with dexmedetomidine and clonidine has been reported to attenuate cerebral ischaemia–reperfusion injury in rats [46–48].

Conversely, in relation to the possible neuroprotective effects of clonidine [15,16], our findings suggest that clonidine alone has no impact either on respiratory function nor on neurocognitive outcome in rat pups. In combination with sevoflurane a rather synergistic depressant effect on the ventilatory response to carbon dioxide was observed. This is in accordance with findings of Kesavan and coworkers [21] showing a 35% reduction in respiratory rate after 90 minutes of intraperitoneal injection of clonidine 400 mcg/kg in neonatal rats. No conclusions regarding possible neuroprotective effects can be drawn from the present study as the battery of behavioural tests did not reveal any deficit in any group treated. More detailed studies in this field are warranted.

An unexpected finding of our study was the inverse relationship between serum lactate and carbon dioxide. The regulation of serum lactate in rat pups is not fully understood and it has been suggested that lactate may play a pivotal role as substrate of cerebral metabolism during the neonatal period [49]. It has been shown that repeated tactile stimulation of neonatal rats increases serum lactate levels by 207%. However, on PD7 this effect was reduced to 11%. No increase in serum lactate levels was observed in anaesthetized rat pups [50]. This observation is in accordance with our findings showing lower serum lactate levels in anaesthetized rat pups compared to controls.

Limits of the study

The present study design does not allow a clear separation between anaesthesia and hypercarbia related effects as a hypercarbic (CO_2) control group is lacking. However, results give insight in an 'overall' combined effect of general anaesthesia and hypercapnic acidosis on the developing brain opening the hypothesis that general anaesthesia might be protective under these conditions.

Conclusions

Results of the present study suggest that a brief exposure to sevoflurane and severe hypercarbia might not affect long-term cognitive and emotional functions during the period of brain growth spurt. However, the absence of behavioural and emotional deficits does not preclude other forms of behavioural impairments, which might have been detected by more specific tasks others than those used in the present study.

Author Contributions

Conceptualization: NA PCo PCa.

Data curation: NA PCo VDC DV.

Formal analysis: NA PCo PCa.

Funding acquisition: PCa.

Investigation: NA PCo VDC DV.

Methodology: NA PCo VT MP PCa.

Project administration: PCa.

Resources: PCa.

Supervision: PCa.

Validation: PCa.

Visualization: NA PCo VT PCa.

Writing – original draft: NA PCo PCa.

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Lifelong imbalanced linoleic/alpha-linolenic acid intake impairs emotional and cognitive behavior in adult rats via changes in brain endocannabinoid system

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Running title: dietary alpha-linolenic-induced alterations in behavior and brain endocannabinoid system

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Abstract

Imbalanced dietary n-3 and n-6 PUFA content has been associated with a number of neurological conditions. Endocannabinoids are n-6 PUFA derivatives, whose brain concentrations are sensitive to modifications of fatty acid composition of the diet, and play a central role in the regulation of mood and cognition. As such, the endocannabinoid system appears to be an ideal candidate for mediating the effects of dietary fatty acids on mood and cognition.

Lifelong administration of isocaloric alpha linolenic acid (ALA) deficient and enriched diets induced short-term memory deficits, whereas only dietary ALA enrichment altered emotional reactivity in adult male rats compared to animals fed a standard diet, balanced in ALA/linoleic (LA) ratio. In the prefrontal cortex, both diets reduced 2-AG levels and increased MAG lipase expression, whereas only the enriched diet reduced AEA levels, simultaneously increasing FAAH expression. In the hippocampus, ALA enriched diet decreased AEA content and NAPE-PLD expression, and reduced 2-AG content while increasing MAG lipase expression.

These findings highlight the importance of a diet balanced in fatty acid contents for normal brain functions and support a link between dietary ALA, brain endocannabinoid system and behavior, indicating that dietary ALA intake is a sufficient condition for altering the endocannabinoid system in brain regions modulating mood and cognition.

Keywords: diet, alpha linolenic acid, endocannabinoid system, mood, cognition, rats

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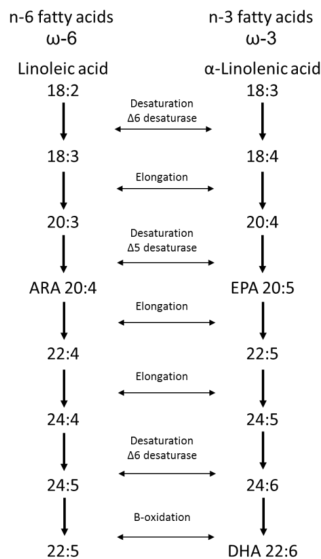
Introduction

Among nutrients that can play a role in improving human health, two distinct classes of essential fatty acids, n-6 and n-3, have received wide attention during the last decades. The n-6 and n-3 fatty acids are fundamental nutrients that must be ingested as foods or dietary supplements.

Linoleic acid (LA) and alpha-linolenic acid (ALA) are the parent fatty acids of the n-6 and n-3 groups, respectively. These 18-carbon fatty acids are required for the synthesis of longer polyunsaturated fatty acids (PUFAs), such as arachidonic acid (AA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Conversion of n-6 and n-3 fatty acids to PUFAs occurs as a result of enzymatic desaturation and elongation steps. In addition, since the enzymes that are involved in these mechanisms have the same functions in the two fatty acid groups, and therefore the n-6 and n-3 fatty acids compete for them for their biosynthesis, in experimental animals it has been shown that formation of long chain n-3 PUFAs (n-3 LCPUFAs) is dependent on LA and total dietary PUFA concentration. However, the conversion rate in humans of ALA to EPA, and particularly to DHA, is very low in humans (1). Thus, while both n-6 and n-3 fatty acids are essential for health, the balance of the two is critical as well the dietary intake of n-3 LCPUFAs, EPA and DHA (2).



Schematic representation of n-6 and n-3 metabolism

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Over the last decades, the ratio of n-6 to n-3 fatty acid intake in the overall population, including children and adolescents, has dramatically shifted in the Western diet. In the eating habits of Western industrial countries, food tends to be rich in n-6 fatty acids and low in long-chain n-3 fatty acids (3, 4). This dysregulation can affect the equilibrium of several organs and particularly of the brain, that is highly enriched in LCPUFAs. Brain's LCPUFAs are involved in the maturation of neuronal structures, and are essential throughout the entire lifespan for maintaining normal brain activities (5, 6). Consequently, it is unsurprising that a lack of n-3 LCPUFAs or an imbalance between n-3 and n-6 ratio have been associated with a number of neurological and psychiatric disorders, including depression, anxiety, schizophrenia and attention deficit hyperactivity disorder (ADHD), in both children and adults (7-12).

Despite the growing body of evidence suggesting a link between n-3 LCPUFAs dysregulation and neuropsychiatric diseases, how dietary fatty acids can actually affect the brain and behavior is still poorly understood.

The endocannabinoid system appears to be an ideal candidate for mediating the effects of dietary fatty acids on mood and cognition. Indeed, endocannabinoids are local neuromodulators that are metabolic derivatives of the n-6 LCPUFA, AA, and their concentrations in the brain are sensitive to modifications of fatty acid composition of the diet (13, 14). Furthermore, the endocannabinoid system plays a role in the regulation of mood and cognition under physiological conditions, and its dysregulation is believed to contribute to the development of several neuropsychiatric pathologies (15).

In this context, endocannabinoids shape neuronal architecture (16), and recent preclinical studies comparing the effects of lifelong n-3 deficient to regular diets have pointed out that long-term ALA dietary insufficiency impairs endocannabinoid mediated long-term synaptic depression in brain areas associated with the development of anxiety- and depressive-like behaviors (17, 18), suggesting that lifelong deficiency in n-3 PUFA may influence cerebral areas controlling mood, through alteration of the endocannabinoid system functionality. However, the above-cited studies investigated the consequences of diets deficient in ALA and without comparing the effects of such dietary imbalances to those present after administration of a healthy diet balanced in n-3 and n-6 fatty acid contents.

The present study aimed at comparing the effects of lifelong administration of isocaloric diets enriched or deficient in ALA, but containing similar amounts of LA, to that of a standard diet, with a balanced and

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recommended n-3/n-6 ratio. On this basis, we investigated whether (1) imbalanced amounts of ALA in the diet could be a sufficient condition for the development of altered emotional and cognitive behaviors at adulthood, and (2) these potential behavioral alterations could occur via dysregulations of the endocannabinoid system in selected brain regions regulating mood and cognition.

Diets were administered to healthy rats from pregnancy to adulthood and their consequences on measures of recognition and emotional memory, depressive-, anxiety- and psychotic-like behaviors were investigated in the adult male offspring. Subsequent biochemical analyses were carried out in the prefrontal cortex, hippocampus and amygdala, to check for possible alterations in 1) CB_1 receptor density and functionality, 2) protein expression of endocannabinoid synthetic and degradative enzymes and 3) tissue levels of n-3- and n-6-derived endocannabinoids.

Materials and Methods

Animals

Time-mated Female Sprague Dawley rats, starting from gestation day (GD) 1, were housed singularly in clear plastic cages on a 12 h light-dark cycle (lights on 08:00h) and in a temperature (22 ± 2°C) and humidity controlled environment (50±10%) with a plastic tube for environmental enrichment. All animals had free access to food and water. Newborn litters found up to 5.00 p.m. were considered to be born on that day (PND 0). On PND 1, all litters were reduced to a standard size of eight pups per litter (four males and four females). At birth, litter weights, the total number of pups and the number of males and females were measured. On PND 21, pups were weaned. All experiments took place on the male adult offspirng during the light phase and were performed in accordance with the guidelines released by the Italian Ministry of Health (D.L. 2014/26), and the European Community directives regulating animal research (2010/63/EU). All efforts were made to minimize the number of animals used and their suffering.

Diet composition

Diets were provided by Dr Piccioni Laboratories (Milan, Italy) and their composition was further assessed as follows.

Lipids extraction

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Food pellets were separately mixed with a mixture of $CHCl_3$/MeOH (2:1 v/v) at a ratio of 1 to 10 (w/v). After homogenization, samples were filtered and transferred to a new test tube. The extraction was done twice, and the two filtrates were combined together. A 0.9% NaCl solution was added to the recovered solution and centrifuged. The lower organic phase was removed, dried *in vacuo* and stored at −80°C until analysis. Prior to analysis, dried lipid extracts were reconstituted in methanol.

Gas chromatography-flame ionization detector (GC-FID) analysis of fatty acid methyl esters (FAMEs)

Lipids were derivatized to generate methyl esters of fatty acids (FAMEs). The FAMEs were prepared as follows: 100 μL of extracted lipids were saponified with 1 ml of sodium methylate in methanol (0.5% w/v) at 100°C for 15 min. After cooling, 1 mL of boron trifluoride-methanol reagent was added and the solution was heated at 100°C for 15 min. The FAMEs were extracted by adding 1 ml of n-hexane, 4 mL of a saturated NaCl solution, and agitating manually for 2 minutes before a 5-min centrifugation (3000 rpm). Finally, the n-hexane layer was transferred to a GC injector vial.

GC–FID analyses were carried out on a GC2010 (Shimadzu, Milan, Italy) equipped with aa split–splitless injector (260°C) and a FID detector. FAMEs separation was performed on a Supelcowax column (Sigma-Aldrich/Supelco, Bellefonte, PA, USA; 30 m × 0.25 mm ID × 0.25 μm df). GC parameters: initial pressure of carrier gas (helium) (constant linear velocity: 30 cm/s), 99.5 kPa; temperature program: 120–260°C at 3 °C/min; injection volume, 1 μL; split ratio, 1:100. FID parameters: temperature, 265°C; H_2 flow rate, 40 mL/min; air flow rate, 400 mL/min; make-up gas (He) flow rate, 50 mL/min. Data acquisition was performed using the GCsolution software.

An aliquot of the PFC and hippocampus lipid extract for each sample was mildly saponified and fatty acids were analysed by HPLC (Agilent 1100 HPLC system with diode array detector, Palo Alto, Calif., USA) as previously described (19). Spectra (195–315 nm) of the eluate were obtained every 1.28 s and were electronically stored. These spectra were acquired to confirm identification of the HPLC peaks (20).

Since saturated fatty acids are transparent to UV, after derivatization, they were measured as fatty acid methyl esters, by a gas chromatograph (Agilent, Model 6890, Palo Alto, CA) as described in (21).

Diet administration

Diets were administered according to the timeline reported in figure 1, panel A. Time-mated Sprague-Dawley rats were fed a diet containing 6% fat in the form of peanut oil rich in linoleic acid (n-3 deficient diet), a diet

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containing 6% fat in the form of rapeseed oil rich in α-linolenic acid (n-3 enriched diet) or a standard diet composed of 3% peanut oil and 3% rapeseed oil throughout gestation and lactation. Diets were isocaloric and did not differ with respect to the n-6 content. After weaning, the offspring received the same diet throughout the rest of their life to model a lifetime of n-3 deficiency or supplementation.

Behavioral Tests

Classic and spatial versions of the novel object recognition (NOR) test

The experimental apparatus used for the object recognition test was an open-field box (43 x 43 x 32 cm) made of Plexiglas, placed in a dimly illuminated room. The experiment was performed and analyzed as previously described (22). Animals performed each test individually. Briefly, each animal was placed in the arena and allowed to explore two identical previously unseen objects for 5 minutes (familiarization phase). After an inter-trial interval of 3 minutes one of the two familiar objects was replaced by a novel, previously unseen object and rats were returned to the arena for the 5-minute test phase. In the spatial variant of the test, the familiar object was placed in a different position compared to the familiarization phase, that is a spatial cue was added in the test. During the test phase the time spent exploring the familiar object (Ef) and the new object (En) was videotaped and recorded separately by two observers blind to the treatment groups and the discrimination index was calculated as follows: [(En-Ef)/(En+Ef)] x 100.

Social Interaction Test

The test was carried out as previously reported (22). During the test, each animal was allowed to freely explore an unfamiliar congener in an open-field box made of Plexiglas (60 x 60 x 60 cm) for 10 minutes and we recorded the total time spent in active social behaviors (as the sum of time spent in sniffing, following, grooming, mounting and nosing during the test session) and the total number of aggressive episodes (attacking, biting, tail rattling and aggressive grooming).

Forced Swim Test (FST)

Animals were tested in a modified version of the FST with only the first session of swimming as previously reported (22, 23) to measure a preexisting behavioral deficit induced by diet administration.

Rats were forced to swim for 15 minutes inside a clear 50 cm tall, 20 cm diameter glass cylinder filled to 30 cm with 25°C water. During the test, the following parameters were monitored: immobility (time spent by the animal floating in the water making only those movements necessary to keep its head above the water),

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swimming (active swimming movements to the center of the cylinder), climbing (forceful thrashing movements with forelimbs against the walls of the cylinder).

Elevated plus maze test

The test was performed as previously described (24). The elevated plus-maze apparatus comprised two open arms (50×10×0 cm) and two closed arms (50×10×40 cm) that extended from a common central platform (10×10 cm). The apparatus, made of Plexiglas (gray floor, clear walls), was elevated to a height of 60 cm above the floor level. A video camera above the maze was connected to a television monitor connected to a videorecorder. Briefly, rats were individually placed on the central platform facing a closed arm for 5 minutes. The following parameters were analyzed: % Time spent on the open arms, number of open arm entries, total arm entries.

Inhibitory Avoidance

The inhibitory avoidance apparatus 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 2 compartments separated by a sliding door. The starting compartment (31 cm long) was made of opaque white plastic and illuminated by a lamp; the shock compartment (60 cm long) was made of 2 dark, electrifiable metal plates and was not illuminated. The procedure was carried out as previously described by Morena and coworkers (25). For training, rats were placed into the starting compartment facing away from the door and were allowed to explore the apparatus. After the rats stepped completely in to the dark compartment, the sliding door was closed, and a single footshock (0.4mA) was delivered for 1 s. Animals were removed from the shock compartment 15 s after termination of the footshock. Retention was tested 24h later. On the retention test trial, rats were placed into the starting compartment, and the latency to reenter the shock compartment with all 4 paws (maximum latency of 600s) was recorded. Longer latencies were interpreted as indicating better retention.

Prepulse inhibition of startle reflex

The test was carried out as previously descripted (26). Briefly, each rat was placed in a PPI apparatus (Med Associates, St Albans, USA) for a 5 min acclimatization period with a 70dB background noise, which continued for the remainder of the session. Each session consisted of 3 consecutive sequences of trials. During the first and the third sequence, the rats were presented with five pulse-alone trials of 115dB. The second sequence consisted of 50 trials in pseudo-random order, including 12 pulse-alone trials, 30 trials of pulse

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preceded by 73, 76, or 82dB prepulses (10 for each level of prepulse loudness), and eight no stimulus trials, where only the background noise was delivered. Intertrial intervals were selected randomly between 10s and 15s. Acoustic devices and startle cages were connected to a computer, which detected and analyzed all chamber variables using customized software. Percent PPI was calculated with the following formula: 100 - [(mean startle amplitude for prepulse–pulse trials/mean startle amplitude for pulse-alone trials) x 100].

Biochemical studies

The long-term effects of diet administration on components of the endocannabinoid system were evaluated in the adult male offspring (PND 75). Biochemical analyses were performed on animals that underwent novel object recognition, social interaction and forced swim tests, 24 hours after the last test.

Autoradiographic-binding assays

Rats were decapitated and brains were rapidly removed, frozen in liquid nitrogen and stored at -80°C until processing. Coronal sections (20µm thick) were cut on a cryostat and mounted on gelatin-coated slides. The sections were stored at –80°C until processing.

[^{3}H]CP-55,940 receptor autoradiographic binding

The [^{3}H]CP-55,940 receptor autoradiographic binding was performed as previously described (27, 28).

CP-55,940-stimulated [^{35}S]GTPγS binding in autoradiography

This was determined as previously described (27, 28).

Image analysis

The intensity of the autoradiographic films was assessed by measuring the grey levels with an image analysis system consisting of a scanner connected to a PC running Microsoft Windows. The images were analyzed using Image-Pro Plus 7.0 (MediaCybernetics, Silver Spring, USA) as previously described in Viganò et al. (29).

Western blot analyses

For western blot analyses, rats were decapitated and brains quickly removed. The cerebral areas of interest (PFC, hippocampus and amygdala) were obtained by regional dissection on ice, immediately frozen in liquid nitrogen and stored at -80°C until processing.

The experiments were carried out as previously reported (30). Briefly, equal amount of protein lysates (30 µg) were run on a 10% SDS-polyacrylamide gel. The proteins were then transferred to polyvinylidene difluoride

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(PVDF) membranes, blocked for 2 h at room temperature in 5% dry skimmed milk in TBS1x 0.1% tween-20 before incubation overnight at 4°C with the primary antibody. The following primary antibodies were used: rabbit polyclonal anti-CB_1 (1:1000; Cayman Chemical, Ann Arbor, MI), rabbit polyclonal anti-NAPE-PLD (1:3000; Cayman Chemical, Ann Arbor, MI), rabbit polyclonal anti-FAAH (1:2000; Cayman Chemical, Ann Arbor, MI), goat polyclonal anti-DAGLα (1:1000; Abcam, Cambridge, UK), rabbit polyclonal anti-MAGL (1:1000; Cayman Chemical, Ann Arbor, MI),. Bound antibodies were detected with horseradish peroxidase (HRP) conjugated secondary anti-rabbit or anti-goat antibody (1:2000-1:5000; Chemicon International, Temecula, CA). For normalization, the blots were stripped with Restore Western Blot Stripping Buffer (Thermo Scientific, Rockford, IL) and re-blotted with mouse anti-β-actin monoclonal antibody (1:10000; Sigma Aldrich, Italy) overnight at 4°C. Bound antibodies were visualized using Clarity Western ECL Substrate (Bio-Rad Laboratories, Hercules, CA, USA) and bands were detected with a GBOX XT camera (Syngene, Cambridge, UK). Optical density of the bands was quantified using Image Pro Plus 7.0 software (MediaCybernetics, Bethesda, MD, USA). The density of the bands was normalized to β-actin and expressed as arbitrary units.

Lipid Extraction and Endocannabinoid Measurement

PFC, hippocampus and amygdala were dounce-homogenized and extracted with chloroform/methanol/Tris-HCl 50 mM pH 7.4 (2:1:1, v/v) containing internal deuterated standards for AEA, PEA, OEA, DHA-EA and 2-AG, quantification by isotope dilutiond [8-AEA, d4-PEA, d4-OEA, d4-DHA-EA and d5-2-AG, (Cayman Chemicals, MI, USA)], as well as 17:0-AE (Cayman Chemicals, MI, USA) and 2-17:0-G (Larodan AB, Malmo, Sweden) for 18:2-EA, EPA-EA and 2-22:6-G measurement, respectivelly.

The lipid-containing organic phases were then purified by open bed chromatography on silica and fractions were obtained by eluting the column with 99:1, 90:10 and 50:50 (v/v) chloroform/methanol.Fractions eluted with chloroform/methanol 90:10 were collected, the excess solvent evaporated with a rotating evaporator, and aliquots analysed by isotope dilution-liquid chromatography/atmospheric pressure chemical ionization/mass spectrometry (LC–APCI–MS) carried out in the selected ion monitoring mode by using a Shimadzu HPLC apparatus (LC-10ADVP) coupled to a Shimadzu (LCMS-2020) quadrupole MS via a Shimadzu APCI interface.

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MS detection were performed by using m/z values of 356 and 348 (molecular ions + 1 for d8-AEA and AEA), 304 and 300 (molecular ions + 1 for d4-PEA and PEA), 330 and 326 (molecular ions + 1 for d4-OEA and OEA), 376 and 372 (molecular ions + 1 for d4-DHA-EA and DHA-EA), 384 and 379 (molecular ions +1 for d5-2-AG and 2-AG), 346 (molecular ions + 1 for EPA-EA), 324 (molecular ions + 1 for 18:2-EA), 314 (molecular ions + 1 for 17:0-EA), 403 (molecular ions + 1 for 2-22:6-G), and 345 (molecular ions + 1 for 2-17:0-G).

AEA, PEA, OEA, DHA-EA and 2-AG levels were therefore calculated on the basis of their area ratios with the internal deuterated standard signal areas. EPA-EA,18:2-EA and 2-22:6-G were quantified against 17:0-EA and 2-22:6-G respectively, the internal standards for which calibration curves had been constructed in pilot experiments. Lipid amounts espressed as pmol were then normalized per gram or milligram of wet tissue.

Statistical analysis

Results were expressed as mean ± SEM and analyzed by two- or one-way ANOVA, followed up by Bonferroni's post hoc test. The level of statistical significance was set at $p<0.05$.

Results

Diet composition

Table 1 reports the fatty acid composition of the diets used in this study. The n-3 deficient diet contained 6% fat in the form of peanut oil rich in linoleic acid (n-3:n-6 ratio=1:23,9), the n-3 enriched diet contained 6% fat in the form of rapeseed oil rich in α-linolenic acid (n-3:n-6 ratio=1:4,5) and the standard diet was composed of 3% peanut oil and 3% rapeseed oil (n-3:n-6 ratio=1:8,6).

Effect of lifelong dietary n-3 enrichment or deficiency on fatty acid contents in the PFC and hippocampus

To assess whether altered dietary ALA/LA ratio could actually affect brain fatty acid profile in the offspring, we quantified the fatty acid contents in the PFC and hippocampus of adult male rats after lifelong diet administration. The results are shown in table 2 and 3. In the PFC (table 2), consumption of n-3 enriched diets significantly decreased 18:2 n-6 and 20:3 n-6 contents by about 20 and 21% with respect to animals fed a standard diet. Administration of n-3 deficient diets significantly reduced 22:5 n-3 and 18:2 n-6 levels by about 32 and 35% compared to rats fed a standard diet. Lifelong dietary n-3 deficiency also significantly increased 20:3 n-6, 22:4 n-6 and 22:5 n-6 contents by about 60, 10 and 142% respectively. Both dietary regimens did

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not affect DHA 22:6 n-3 and ARA 20:4 n-6 levels in this brain region. In the hippocampus (table 3), n-3 enriched diets significantly increased 22:5 n-3 and 22:4 n-6 levels by about 29 and 15% compared to the standard diet. Significant decreases in DHA 22:6 n-3 (8%) and 22:5 n-6 (33%) levels were also present. Administration of n-3 deficient diets resulted in significant reductions by about 27 and 12% of 22:5 n-3 and DHA 22:6 n-3 contents, and in significant increases by about 9 and 108% of 22:4 n-6 and 22:5 n-6 levels. Neither enriched nor deficient diets altered hippocampal 18:2 n-6, 20:3 n-6 and ARA 20:4 n-6 levels.

Effect of diet administration on body weight, food intake and pregnancy outcome in time-mated Sprague-Dawley rats

As shown in Figure 1, panel B, administration of n-3 deficient and enriched diets during gestation did not affect body weight gain and food intake in time-mated rats when compared to controls fed a standard chow diet. Furthermore, the number of fetuses delivered per dam was similar for all the diets considered and no changes in body weight gain were observed in the offspring (data not shown).

Behavioral consequences of lifelong dietary n-3 imbalances in the male offspring

Male adult (PND 75) offspring were submitted to a series of behavioral tests to evaluate the long-term consequences of dietary n-3 imbalances from pregnancy on cognitive performances, emotional reactivity and psychotic-like symptoms.

Figure 2 shows the consequences of diet administration on recognition and emotional memories, as measured through the novel object recognition test (panel A) and the inhibitory avoidance task (panel B), respectively. One-way ANOVA showed significant effects of diet administration both in the classic ($F_{2,17}$=9.392; p=0.0035) and in the spatial ($F_{2,17}$=5.892; p=0.0121) versions of the novel object recognition test. Lifelong administration of both n-3 deficient and enriched diets resulted in impaired cognitive performance in the classic version of the NOR test in the adult male offspring, as stated by the significant reductions of the discrimination index by about 76% and 60%, respectively, compared to controls fed a standard diet. A similar effect was also observed in the spatial version of the test, the discrimination index being reduced by about 64% and 50% in both experimental groups.

In contrast, diet administration did not significantly affect emotional memory in the inhibitory avoidance task, as demonstrated by the presence of similar retention performances in all the experimental groups.

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Figure 3 reports the effects of diet administration on measures of depressive- and anxiety-like behaviors, such as the social interaction test (panel A), the forced swim test (panel B) and the elevated plus maze (panel C), as well as on psychotic-like symptoms, through the prepulse inhibition test (panel D).

Statistical analysis revealed a significant effect of diets in the social interaction test ($F_{2,17}$=6.628; p=0.0062) and forced swim test (Immobility: $F_{2,17}$=4.468; p=0.0257. Climbing: $F_{2,17}$=7.512; p=0.0039). Administration of n-3 enriched diets induced a significant reduction in the time spent in active social behaviors in the 10-minute test session by about 52% when compared to controls. Similarly, in the forced swim test, rats fed n-3 enriched diet displayed a significant increase by about 60% in the time spent in immobility during the 15-minute test session, paralleled by a significant 36% reduction of the time spent in climbing activity. In contrast, n-3 deficient diet administration did not alter animals' behavior in the social interaction and forced swim tests. Conversely, no significant effects were instead observed in the elevated plus maze test, since administration of n-3 deficient and enriched diets did not change the time spent in the open arms and the percentage of open arm entries with respect to controls. This effect did not appear to be due to motoric effects as there was no change in total arm entries (data not shown).

Finally, dietary fatty acid composition had no effect on PPI responses in the adult offspring.

Behavioral consequences of n-3 enrichment or deficiency restricted to gestation and lactation

In order to dissect between the behavioral effect of lifelong or perinatal administration of n-3 deficient or enriched diets, we performed a separate experiment in which diets were given either lifelong or during gestation and lactation only. The offspring belonging to all experimental groups was then tested for behavior at adulthood, i.e. PND 75.

Figure 4 represents the effects of lifelong or perinatal diet administration on recognition memory (panel B) and depressive-like behaviors, as measured through the social interaction test (panel C) and forced swim test (panel D).

Statistical analysis revealed main effects of diet, time and diet x time interaction in the novel object recognition test (diet: $F_{2,30}$=48.61; p<0.0001; time: $F_{1,30}$=12.92; p=0.0011; diet x time interaction: $F_{2,30}$=11.54; p=0.0002). As expected, lifelong administration of both n-3 deficient or enriched diets significantly reduced the discrimination index in the adult offspring by about 70% with respect to animals fed a standard diet. Interestingly, n-3 deprivation during gestation and lactation was sufficient to induce a significant impairment

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of recognition memory in adult animals. In contrast, administration of n-3 enriched diet restricted to gestation and lactation did not alter recognition memory at adulthood.

In the social interaction test, two-way ANOVA showed significant main effects of diet, time and diet x time interaction (diet: $F_{2,30}$=3.738; p=0.0355; time: $F_{1,30}$=12.01; p=0.0016. diet x time interaction: $F_{2,30}$=20.96; p<0.0001). Lifelong administration of n-3 enriched diets induced a significant reduction in the time spent in active social behaviors by about 50% when compared to controls whereas lifelong administration of n-3 deficient diet did not alter sociability in the long-term. Interestingly, n-3 enriched diets given during gestation and lactation only did not alter social behaviors in the adult offspring. A similar effect was also observed in the forced swim test. Indeed, statistical analysis revealed main effects of diet, time and diet x time interaction in the time spent in immobility (diet: $F_{2,30}$=8.016; p=0.0016; time: $F_{1,30}$=11.46; p=0.0020; diet x time interaction: $F_{2,30}$=17.54; p<0.0001) and climbing (diet: $F_{2,30}$=11.33; p=0.0002; time: $F_{1,30}$=9.502; p=0.0044; diet x time interaction: $F_{2,30}$=12.73; p<0.0001) activities. Lifelong administration of n-3 enriched diets led to a significant increase by about 50% in the time spent in immobility, paralleled by a significant 35% reduction of the time spent in climbing activity, in the adult offspring. In contrast, no alterations were observed in adult animals that were fed n-3 enriched diets only during perinatal life. Finally, neither lifelong nor perinatal administration of n-3 deficient diets did not alter animals' behavior in the forced swim tests.

Diet-induced changes in the endocannabinoid system in the PFC, hippocampus and amygdala of adult male offspring

Biochemical analyses were carried out in the brains of the adult offspring in order to determine whether lifelong dietary n-3 deficiency or supplementation could impact the endocannabinoid system in the long-term. Specifically, CB_1 receptor density and functionality, protein expression of endocannabinoid synthetic and degradative enzymes (NAPE-PLD, DAGLα, FAAH and MAGL) as well as the levels of n-3- and n-6-derived endocannabinoids were monitored in the PFC, hippocampus and amygdala, brain regions closely involved in the modulation of cognition and emotionality.

Figure 5 reports the effect of diet administration on the endocannabinoid system in the PFC of adult male rats. The composition of dietary fatty acids did not alter CB_1 receptor expression and G protein coupling within this brain area (Figure 5A). In contrast, statistical analysis revealed that both n-3 deficiency and supplementation significantly affected the expression of the degradative enzymes for endocannabinoids, FAAH ($F_{2,14}$=7.450;

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p=0.0241) and MAGL ($F_{2,14}$=11.10; p=0.0037). n-3 supplementation significantly increased the expression of both FAAH and MAGL by about 50% and 180% respectively when compared to control rats. Similarly, administration of n-3 deficient diet resulted in a significant 200% increase in MAGL expression without altering FAAH protein levels. No alterations on NAPE-PLD and DAGLα expression were observed in any of the experimental group considered (figure 5B). Changes in degradative enzymes were paralleled by significant alterations of endocannabinoid levels within the PFC (Figure 6). One-way ANOVA showed that modifications of dietary n-3 intake triggered significant changes in the levels of AEA ($F_{2,9}$=5.824; p=0.0238), 2-AG ($F_{2,9}$=5.377; p=0.0291) as well as the n-3 derivative, MAG 2-22:6 ($F_{2,9}$=8.389; p=0.0151). Levels of AEA and 2-AG significantly decreased by about 70% and 40% in the PFC after administration of n-3 enriched diets. A significant 50% reduction of MAG 2-22:6 was also observed in this experimental group. In contrast, administration of n-3 deficient diets led to reductions of 2-AG (-30%) and MAG 2-22:6 (-40%), without affecting AEA levels. PEA, OEA, DHA-EA, EPA-EA and 18:2-EA levels were not altered by the different dietary conditions.

Alterations of the endocannabinoid system were also present in the hippocampus of adult male rats after administration of both n-3 deficient and enriched diets (Figures 7 and 8). Administration of both deficient and enriched diets significantly reduced CB_1 receptor functionality by about 30% within the hippocampus of the adult male offspring ($F_{2,9}$=4.452; p=0.0194), whereas no effect was observed on CB_1 receptor density (Figure 7A). Diet administration also affected the expression of the enzymes responsible for endocannabinoid synthesis and degradation (Figure 7B). Specifically, statistical analysis showed an effect of dietary n-3 intake on NAPE-PLD ($F_{2,14}$=5.746; p=0.0247) and MAGL ($F_{2,14}$=7.911; p=0.0127) expression. Lifelong administration of n-3 enriched diets significantly reduced NAPE-PLD protein levels by about 40% and increased MAGL expression by about 60% when compared to controls fed a standard diet. Instead, no alterations were observed after administration of n-3 deficient diets.

As shown in Figure 8, diet administration also affected endocannabinoid levels within the hippocampus (AEA: $F_{2,9}$=5.245; p=0.0277; 2-AG: $F_{2,9}$=4.467; p=0.0449; MAG 2-22:6: $F_{2,9}$=8.114; p=0.0097; DHA-EA: $F_{2,9}$=11.55; p=0.0033). Levels of AEA and 2-AG decreased in this brain region after administration of n-3 enriched diet. A similar decrease was also observed for MAG 2-22:6, its levels being reduced by about 60% after administration of the enriched diet. In contrast, n-3 enrichment resulted in a significant increase by about

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60% of the levels of the n-3 derivative, DHA-EA. No significant changes were observed in animals that underwent administration of n-3 deficient diets and dietary n-3 content did not affect PEA, OEA, EPA-EA and 18:2-EA levels.

Finally, in the amygdala dietary fatty acid composition did not affect CB_1 receptor density and functionality nor AEA, 2-AG, PEA, OEA levels within this brain region (data not shown).

Discussion

The present results indicate that sustained imbalances in dietary ALA from pregnancy have long-term consequences on adult offspring behavior in terms of cognitive and emotional functions. Specifically, we found that lifelong administration of both ALA deficient and enriched diets induced lasting short-term memory deficits in the novel object recognition test, whereas dietary ALA enrichment also resulted in abnormal emotional reactivity in the social interaction and forced swim tests in adult male rats with respect to animals fed a standard diet, balanced in n-3 content. Accordingly, we found a decrease of DHA in PFC of rats fed both deficient and enriched ALA diets with respect to control diet. The decrease of DHA in brain of rats fed an ALA deficient diet has already been described in mice (17, 18). However, also the decrease of DHA in brain of rats fed the ALA enriched diet is not surprising, because it has been shown that high dietary ALA inhibits delta 6 desaturase impairing the formation of the direct precursor of DHA, c24:6n-3 fatty acid (31). In fact, we found an increase of the other precursor the 22:5n-3. Interestingly, in our experimental conditions, we didn't detect any change in AA content in contrast with previous findings in mice (17, 18). Thus, we may suggest that long term feeding imbalance n-3/n-6 ratio diets from the gestational period may result in a physiological response to maintain a steady AA concentration in the brain by modulating its metabolism including endocannabinoid biosynthetic and degrading enzymes.

n-3 and n-6 LCPUFAs are essential elements in the assembly, maturation and physiological function of neuronal structures, and optimal neuronal development is highly dependent on the supply of LC PUFAs (2). Thus, dysregulations in brain n-3 LCPUFAs content could adversely affect brain maturation and neuronal activity, and a diet balanced in n-6 and n-3 contents should be recommended to ensure normal brain functions. Accordingly, our data demonstrate that when optimal n-6/n-3 ratio is lost, both following dietary ALA deficiency and enrichment, long-term behavioral abnormalities in cognition and emotionality arise.

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However, literature data on the effects of dietary n-3 deficiency and supplementation are often conflicting. Impairments in several aspects of learning and memory have been reported as a consequence of pre- and post-natal dietary n-3 deficiency (32-35), while transient or maternal n-3 PUFA-deficient diet has been reported to induce depressive- and anxiety-like symptoms as well as abnormal social behavior in adult offspring (17, 18, 36-40). Notably, other studies found opposite or no effects of different n-3 deficient and supplemented diets on behavior (41-46). Interestingly, the discriminant is DHA concentration. In fact, is well establish that dietary ALA deficiency results in DHA brain deficiency, whereas the most efficient strategy to increase DHA levels in brain is its dietary intake, more efficiently in the phospholipid form as in Krill oil (21). In our hands, lifelong administration of n-3 deficient diets impairs short-term memory performance in adult offspring when compared to controls fed a standard diet without inducing any depressive- or anxiety-like symptom.

Discrepancies in the literature data could be attributed to differences in diet compositions, as the type of n-3 fatty acid, and protocols of administration used. Indeed, to reach an imbalanced n-6/n-3 ratio in the n-3 deficient diets, in some studies overall n-6 content was markedly increased (17, 18, 39, 45), while in others n-3 fatty acids were completed depleted (37, 40). Moreover, great variability in the time window and duration of administration was also present. Diets were administered either starting before mating (42, 46), during gestation and lactation (37, 43, 45), after weaning (38), from pregnancy throughout adulthood (17, 18, 44) or across consecutive generations (41). Our findings further highlight the importance of the time window of administration in determining the detrimental behavioral effects of dietary ALA enrichment or deficiency. In fact, when isocaloric diets enriched or deficient in ALA but with similar overall LA were administered from pregnancy throughout adulthood, both dietary regimens led to significant behavioral abnormalities in the adult male offspring. In contrast, when ALA enrichment or deficiency was restricted to gestation and lactation, a different picture was observed. In fact, while administration of n-3 deficient diets restricted to this period was sufficient to impair recognition memory in adult animals, no behavioral alterations in terms of neither cognitive deficits nor depressive-like behaviors were observed in the adult offspring after administration of n-3 enriched diets. Thus, it appears that the perinatal phases are the more sensitive period for the negative consequences of n-3 deficiency on cognition, whereas the adverse effects of n-3 enrichment arise only when enriched diets are administered for a longer period (i.e. lifelong).

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Among others, one of the added value of our study is that it also investigated the effects of lifelong ALA enrichment *per se* in comparison to a standard diet. This finding further underlines the importance of the right PUFA composition for proper brain development and behavior. Thus, clinical studies investigating the efficacy of n-3 PUFAs for the treatment of neurological conditions should take into account the initial n-3 LCPUFA status of the subjects in order to determine the optimal dose required to achieve any potential benefit. Indeed, n-3 LCPUFA supplementation could exert some benefits by restoring a balanced n-6/n-3 ratio in those subjects in which n-3 intake is deficient.

In the context of the possible molecular mechanisms involved in the observed behavioral alterations, the endocannabinoid system could represents an ideal candidate to mediate the effects of dietary fatty acids on brain and behavior (47).

Endocannabinoids include the fatty acid ethanolamides AEA, OEA and PEA, as well as the ester 2-AG, biosynthesized from PUFAs of the n-6 family, and those biosynthesized from n-3 PUFAs, such as eicosapentaenoyl ethanolamide (EPA-EA) and docosahexaenoyl ethanolamide (DHA-EA) (48-50). Endocannabinoids are important regulators of synaptic function (16). They suppress neurotransmitter release by acting as retrograde messengers at presynaptic CB_1 receptors. Retrograde endocannabinoid signaling mediates short-term forms of synaptic plasticity as well as presynaptic forms of long-term depression at both excitatory and inhibitory synapses (51). Moreover, the endocannabinoid system has been shown to play a central role in the modulation of mood and cognition both at adulthood and during critical developmental stages (52), and it is engaged in food addiction (53).

In the present paper, we demonstrated that dietary ALA profoundly alter the brain endocannabinoid system, in terms of both endocannabinoid levels as well as their synthetic and degrading enzymes. Both ALA deficiency and enrichment increase MAGL levels in the PFC and hippocampus with respect to rats fed the standard diet and this is associated with reductions of 2-AG and MAG 2-22:6 levels. In the same brain regions, lifelong administration of ALA enriched diets also reduces AEA levels, possibly via increased AEA degradation by FAAH in the PFC and reduced AEA synthesis by NAPE-PLD in the hippocampus. Intriguingly, ALA deficient and enriched diets alter AEA and 2-AG contents without affecting the concentrations of "entourage compounds", such as PEA and OEA. The observation that whenever MAGL expression is increased, reductions of both 2-AG and MAG 2-22:6 are found, strongly suggests that MAGL could be also

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responsible for the degradation of this n-3-derived endocannabinoid. As mentioned above, the modulation of biosynthetic and degrading enzymes of AEA and 2-AG as well of MAG 2-22:6 might be envisaged as a physiological mechanism to maintain brain AA and DHA steady concentrations.

As recent evidence indicates that MAGL deletion and subsequent elevation of 2-AG levels enhance memory in the NOR test (54), it is tempting to speculate that the cognitive impairments induced by both ALA deficient and enriched diets, other than DHA decrease, could possibly be a consequence of deficient 2-AG signaling within the PFC and hippocampus. Conversely, reduced AEA levels as well as FAAH dysfunction have been linked to depression both in animals and humans (55-58), consistent with the phenotype observed in rats administered with ALA enriched diets. Of course, these hypotheses are merely speculative at this time, and further studies are needed to clearly establish any specific correlation. Of note, the expression of endocannabinoid system components can be regulated by epigenetic mechanisms (59), and food intake has been shown to modulate indeed CB_1 expression via DNA methylation (47). Therefore, it is tempting to speculate that modulation of endocannabinoid system protein expression by ALA shown here occurs through epigenetic mechanisms.

Finally, both diets did not alter animals' performance in the inhibitory avoidance task and, accordingly, no changes in CB1 receptor and endocannabinoid content was observed in the amygdala, a key area involved in the modulation of this behavioral response.

Endocannabinoids, both n-6 and n-3 derived, can bind and activate CB_1 receptors, although with different affinity (60, 61). Both ALA deficient and enriched diets reduce CB_1 receptor/G protein coupling in the hippocampus whereas no changes were present in the PFC and amygdala. Accordingly, it has been recently shown that lifelong administration of diets rich in ALA and LA reduced ERK1/2 phosphorylation in the hippocampus after treatment with WIN55212-2 (18), suggesting an impairment in the CB_1 signaling pathway after administration of both ALA enriched and deficient diets. In our hands, both ALA enriched and deficient diets resulted in CB_1 receptor desensitization in the presence of decreased or unchanged 2-AG and AEA contents in the hippocampus. Given that membrane lipid composition has been demonstrated to affect CB_1 cannabinoid receptor binding and signaling (62), it is possible that the effect of diets on CB_1 receptor functionality could be the consequence of alterations of brain fatty acid composition triggered by dietary PUFAs.

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Taken together, these findings support the critical role of n-3 PUFAs in brain maturation and functioning by comparing the effects of diets enriched or deficient in ALA to that of a standard diet, thus highlighting the importance of a balanced diet that ensures adequate intake of key nutrients for mental health. More interestingly, our data support a link between dietary intake of ALA, brain endocannabinoid system and behavior, indicating that dietary ALA is a sufficient condition for altering the endocannabinoid system in brain regions modulating mood and cognition. Dietary n-3 PUFAs act as homeostatic regulators of the endocannabinoid system and adequate levels of dietary n-3 fatty acids are required for proper endocannabinoid signaling. Overall, modulation of the endocannabinoid system by dietary n-3 PUFAs could represent an interesting approach for future clinical investigations. However, attention should be paid when prescribing n-3 PUFA supplementation without specifying the type of n-3 PUFA as adjuvant treatment for psychiatric conditions, as its effect in terms of CB_1 receptor and/or endocannabinoids can be difficult to predict, especially after chronic intakes.

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Table 1

Fatty acid composition of the dietary lipids (relative % of total fatty acids). All diets were isocaloric, had the same amount of n-6 and differ only for n-3 content.

	Standard Diet	*n-3 enriched Diet*	*n-3 deficient Diet*
linoleic acid	**32.0**	**27.5**	**33.3**
alpha-linolenic acid	**3.7**	**6.0**	**1.4**
Monounsaturated fats	**35.7**	**48.3**	**38.7**
Polyunsaturated fats	**35.7**	**33.6**	**34.7**
***n*-3 : *n*-6 ratio**	**1 : 8.6**	**1 : 4.5**	**1 : 23.9**
Energy Content (Kcal/g)	**4.09**	**4.05**	**4.12**

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Table 2

Effect of lifelong administration of n-3 enriched or deficient diets on the fatty acid profile in the PFC of adult rats. Data represent mean ± SEM for four rats per group, and are expressed as nanomoles per milligram of lipid. Statistical significance: ***p<0.001, **p<0.01, *p<0.05 vs standard diet.

	Standard Diet	*n-3 enriched Diet*	*n-3 deficient Diet*
22:5 n-3	0,752 ± 0,024	0,508 ± 0,040 **	0,883 ± 0,053
DHA 22:6 n-3	12,745 ± 0,167	11,595 ± 0,480	13,571 ± 0,288
18:2 n-6	2,319 ± 0,136	1,496 ± 0,039 ***	1,845 ± 0,042 *
20:3 n-6	0,418 ± 0,017	0,667 ± 0,008 ***	0,328 ± 0,016 **
ARA 20:4 n-6	12,041 ± 0,259	12,080 ± 0,344	12,343 ± 0,081
22:4 n-6	2,197 ± 0,048	2,427 ± 0,068 **	2,303 ± 0,022
22:5 n-6	0,617 ± 0,016	1,492 ± 0,141 ***	0,422 ± 0,010

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Table 3

Effect of lifelong administration of n-3 enriched or deficient diets on the fatty acid profile in the hippocampus of adult rats. Data represent mean ± SEM for four rats per group, and are expressed as nanomoles per milligram of lipid. Statistical significance: ***p<0.001, **p<0.01, *p<0.05 vs standard diet.

	Standard Diet	*n-3 enriched Diet*	*n-3 deficient Diet*
22:5 n3	0,248 ± 0,014	0,180 ± 0,009 *	0,319 ± 0,044 *
DHA 22:6 n3	11,394 ± 0,043	10,048 ± 0,199 ***	10,423 ± 0,164 **
18:2 n6	1,444 ± 0,136	1,302 ± 0,095	1,651 ± 0,082
20:3 n6	0,522 ± 0,033	0,631 ± 0,031	0,442 ± 0,019
ARA 20:4 n6	12,665 ± 0,232	12,733 ± 0,316	12,218 ± 0,353
22:4 n6	2,284 ± 0,045	2,491 ± 0,044 *	2,641 ± 0,089 **
22:5 n6	0,579 ± 0,028	1,202 ± 0,041 ***	0,385 ± 0,023 **

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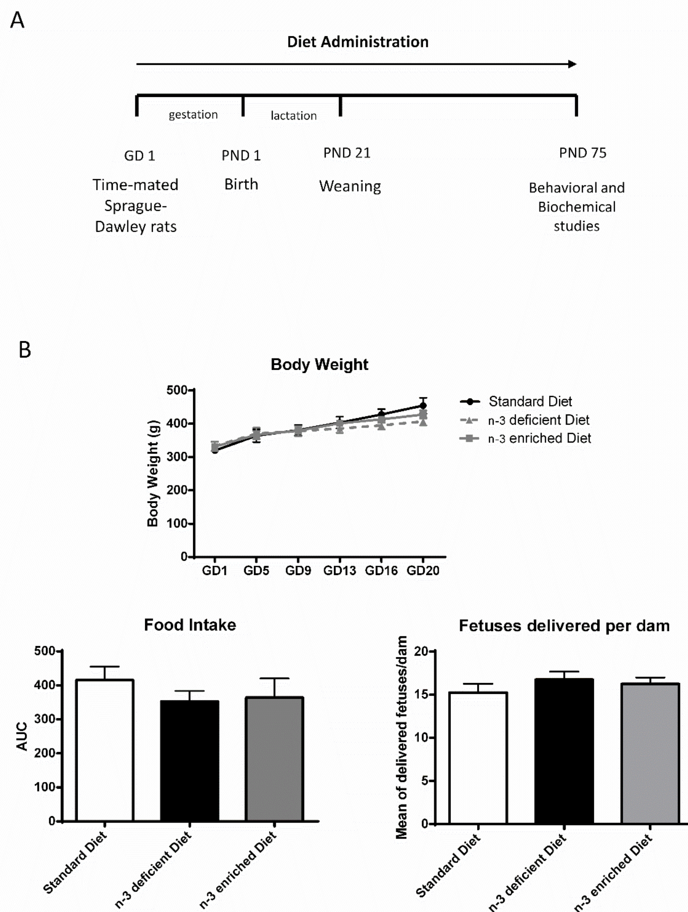

Figure 1: (**A**) Scheme of diet administration. Diets were administered to time-mated Sprague-Dawley rats throughout gestation and lactation. After weaning, the offspring received the same diet throughout the rest of their life to model a lifetime of n-3 deficiency or supplementation. (**B**) Body weight gain, food intake in dams during gestation and the number of fetuses delivered per dam. Data represent mean ± S.E.M. of 8 animals per group.

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Figure 2

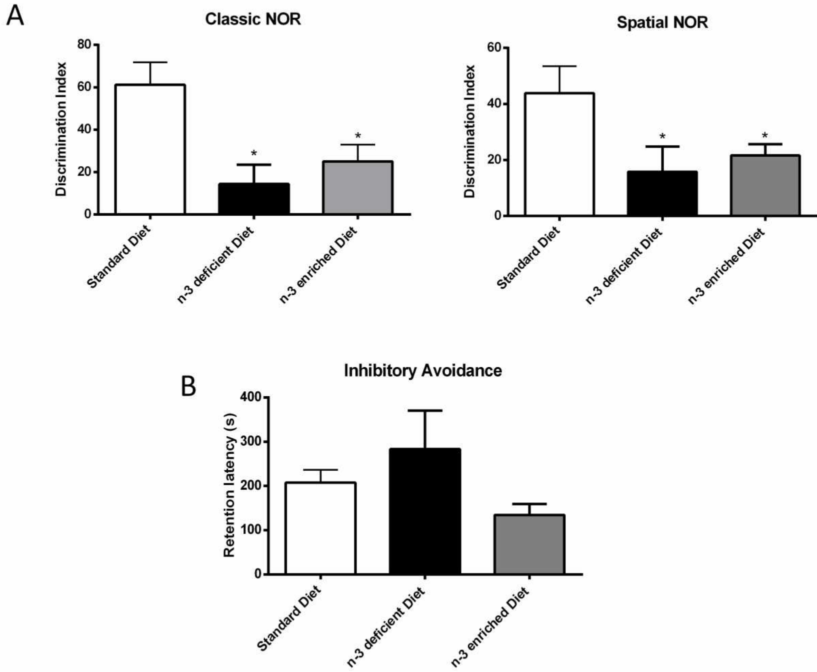

Figure 2: Effect of diet administration on non-emotional and emotional memories in the adult male offspring. (**A**) Cognitive performance in the Novel Object Recognition test (NOR), either in the classic (left panel) and in the spatial (right panel) versions of the task. The discrimination index was calculated as follows: [(En-Ef)/(En+Ef)] x 100, where Ef and En represent the Exploration time of the familiar and the new object respectively. (**B**) Inhibitory avoidance retention latencies in seconds. Rats were given footshock in Box A and 48 hours later they were tested for retention latencies in the same apparatus. Data represent mean ± S.E.M. of 8 animals per group for the NOR test and mean ± S.E.M. of 10-11 animals per group for the inhibitory avoidance task. *p<0.05 vs standard diet (Bonferroni's post hoc test).

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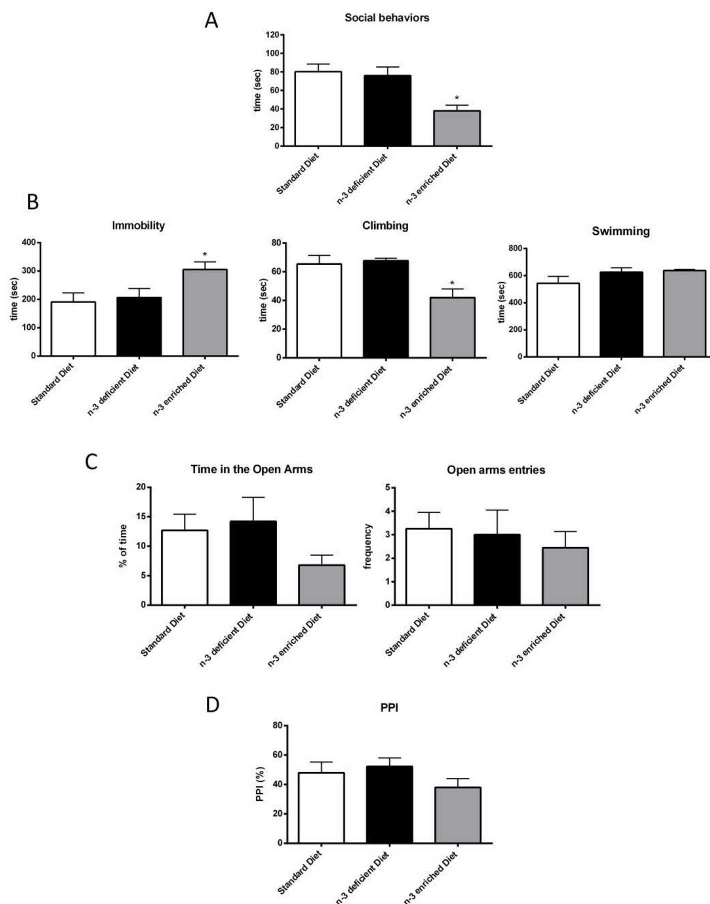

Figure 3: Effect of diet administration on depressive- and anxiety-like behaviors and psychotic-like signs in the adult male offspring. (**A**) Active social behaviors in the social interaction test. (**B**) Time spent in immobility, climbing and swimming in the forced swim test. (**C**) Time spent in open arms and open arms entries in the elevated plus maze test. (**D**) Prepulse inhibition response. Data represent mean ± S.E.M. of 8 animals per group for the social interaction and forced swim tests and mean ± S.E.M. of 10-11 animals per group for the elevated plus maze and prepulse inhibition tasks. *$p<0.05$ vs standard diet (Bonferroni's post hoc test).

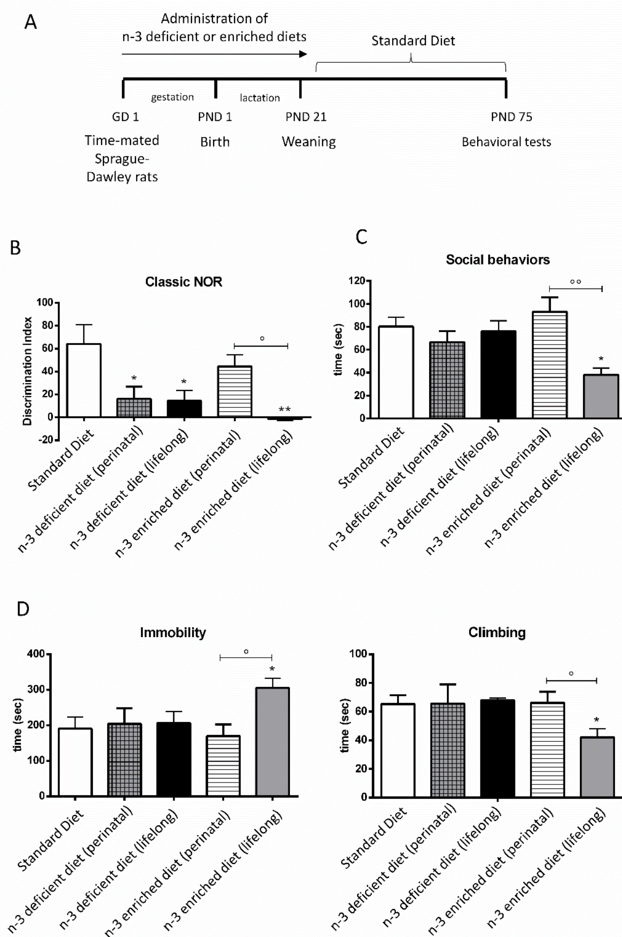

Figure 4: Long-term consequences of lifelong or perinatal administration of n-3 deficient or enriched diets (experimental procedure as for panel A) on recognition memory (panel B) and depressive-like behaviors, as measured through the social interaction test (panel C) and forced swim test (panel D). Data represent mean ± S.E.M. of 6 animals per group. **p<0.01; *p<0.05 vs standard diet; °°p<0.01, °p<0.05 vs lifelong administration of n-3 enriched diet (Bonferroni's post hoc test).

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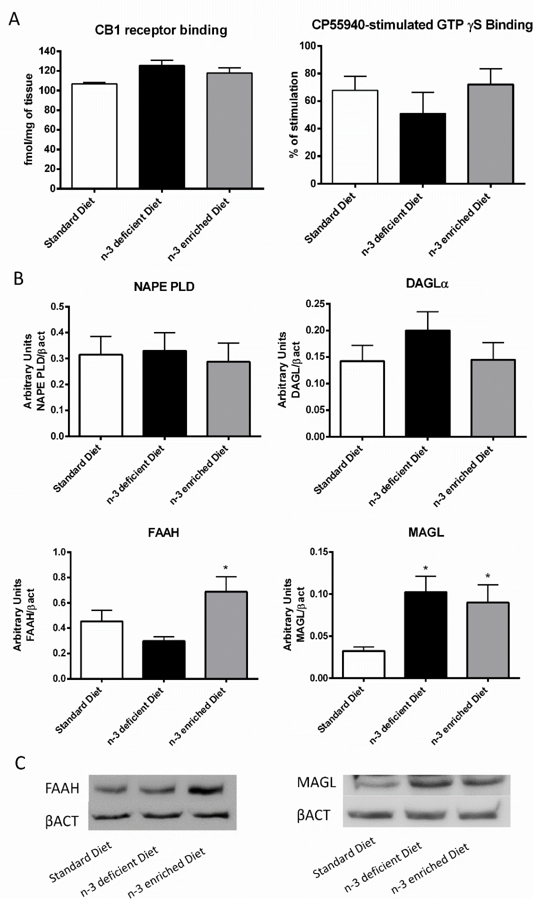

Figure 5: Effect of dietary n-3 intake on the endocannabinoid system within the PFC of adult male offspring. (**A**) [^{3}H]CP-55,940 receptor binding in autoradiography (left panel) and CP-55,940-stimulated [^{35}S]GTPγS binding in autoradiography (right panel). Results are expressed as mean ± S.E.M. of 4 animals per group. (**B**) Protein levels of synthetic and degradative enzymes for endocannabinoids. Data are expressed as arbitrary units and represent mean ± S.E.M. of 5 animals per group. *p<0.05 vs standard diet (Bonferroni's post hoc test). (**C**) Representative western blot images of FAAH and MAGL. β-actin was probed as loading control.

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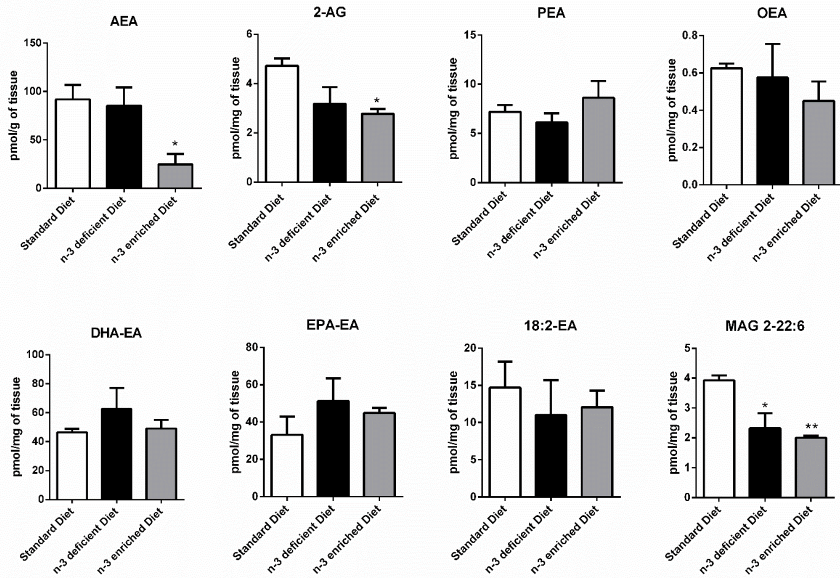

Figure 6: Tissue concentrations of n-6-derived endocannabinoids (AEA, 2-AG, PEA, OEA) and n-3-derivatives (DHA-EA, EPA-EA, 18:2-EA, MAG 2-22:6) in the prefrontal cortex of adult male rats after lifelong administration of deficient and enriched diets. Data are expressed as the mean ± S.E.M. of 4 animals per group. *p<0.05; **p<0.01 vs standard diet (Bonferroni's post-hoc test).

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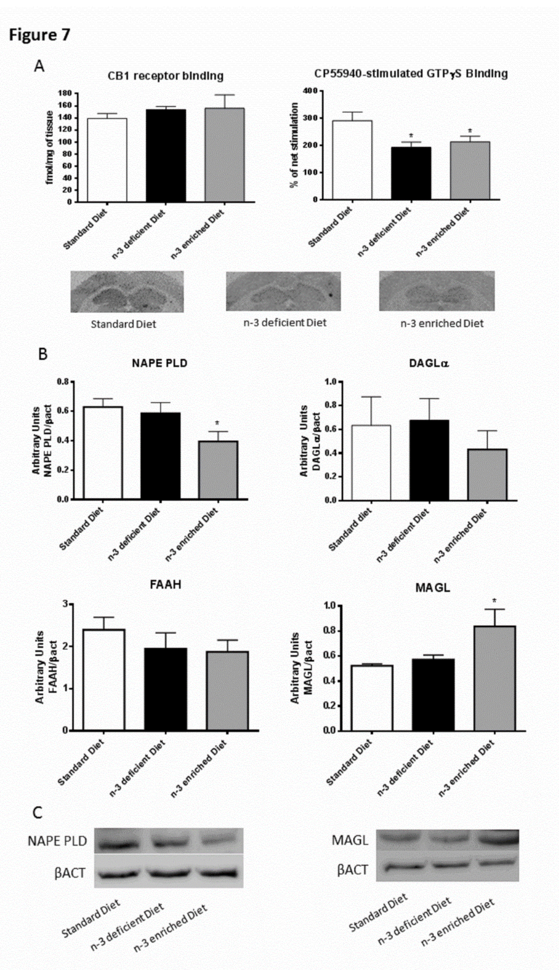

Figure 7: Effect of dietary n-3 intake on the endocannabinoid system within the hippocampus of adult male offspring. (**A**) [^{3}H]CP-55,940 receptor binding in autoradiography (left panel) and CP-55,940-stimulated [^{35}S]GTPγS binding in autoradiography (right panel). Results are expressed as mean ± S.E.M. of 4 animals per group. (**B**) Protein levels of synthetic and degradative enzymes for endocannabinoids. Data are expressed as arbitrary units and represent mean ± S.E.M. of 5 animals per group. *p<0.05 vs standard diet (Bonferroni's post hoc test). (**C**) Representative western blot images of NAPE-PLD and MAGL. β-actin was probed as loading control.

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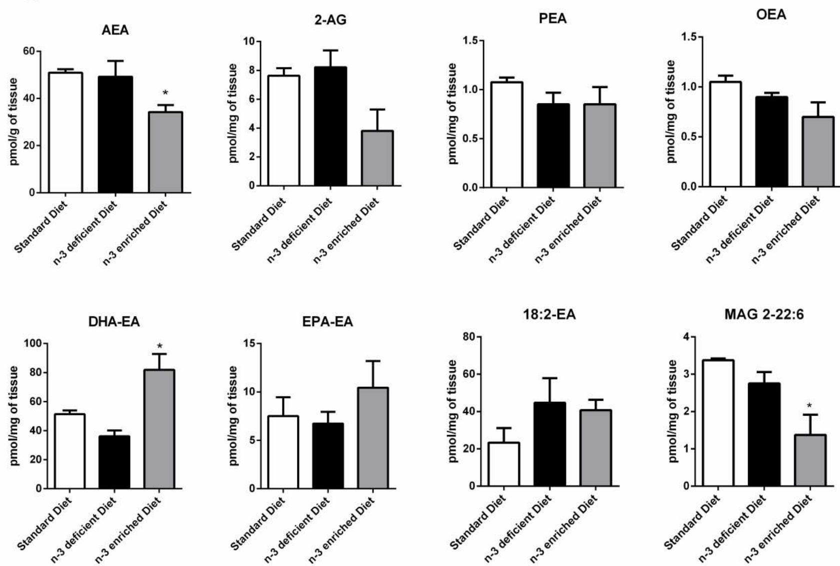

Figure 8: Tissue concentrations of n-6-derived endocannabinoids (AEA, 2-AG, PEA, OEA) and n-3-derivatives (DHA-EA, EPA-EA, 18:2-EA, MAG 2-22:6) in the hippocampus of adult male rats after lifelong administration of deficient and enriched diets. Data are expressed as the mean ± S.E.M. of 4 animals per group. *p<0.05 vs standard diet (Bonferroni's post-hoc test).

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