[...] *Remembering the past is a form of mental time travel; it free us from the constraints of time and space and allows us to move freely along completely different dimension.* [...]

Eric R. Kandel, *In Search of Memory*

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A Dissertation in Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Toxicology

Modulation of memory processes: traveling through the endocannabinoid system

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CONTENTS

General introduction

The endocannabinoid system

The endocannabinoid system consists of G-protein coupled cannabinoid receptors that can be activated by cannabis-derived drugs and small lipids called endocannabinoids (ECs). Anandamide and 2-arachidonyl glycerol are considered as the principal ECs; however, the EC family also includes alsovirodhamine, noladin ether, *N*-arachidonoyldopamine (NADA), homo-linolenylethanolamide (HEA), docosatetraenylethanolamide (DEA), and other related compounds such as palmitoylethanolamide (PEA) and oleoylethanolamide (OEA). Moreover, the endocannabinoid system comprises the genes encoding CBRs, and the enzymes involved in their synthesis (NAPE-PLD, PLA_2, PLC, DAGL, PI-PLC and Lyso-PLC) and degradation (FAAH, MAGL) (Ahn *et al*, 2008)**.**

Until few years ago, CB1 has been believed to be the unique CB receptor subtype of the brain, limiting the expression of CB2 receptors to the immune system (Belvisi *et al*, 2008; Costa, 2007; Galiegue *et al*, 1995; Griffin *et al*, 1999; Howlett *et al*, 2002; Ibrahim *et al*, 2003; Lynn and Herkenham, 1994; Munro *et al*, 1993). More recently, CB2 expression in the brain and its potential involvement in addiction, eating disorders, neuropsychiatric disorders have

been provided (Ishiguro *et al*, 2010a; Ishiguro *et al*, 2010b; Ishiguro *et al*, 2007; Onaivi, 2009; Onaivi *et al*, 2008a; Onaivi *et al*, 2008b; Roche and Finn, 2010).

Given their lipidic nature, ECs are not stored in vesicles but are synthesized "on demand" from membrane phospholipid precursors in response to an increase in postsynaptic intracellular calcium ($[Ca^{2+}]_i$) alone, or combined with activation of postsynaptic GPCRs, such as group I metabotropic glutamate receptors (mGluRs) (Maejima *et al*, 2001; Varma *et al*, 2001) or M1/M3 muscarinic acetylcholine receptors (mAChRs) (Kim *et al*, 2002; Ohno-Shosaku *et al*, 2003) and immediately released from postsynaptic neurons. They travel retrogradely through the synaptic cleft and engage presynaptic cannabinoid receptors, generally suppressing neurotransmitter release from axon terminals (Wilson and Nicoll, 2001b). In some regions, e.g., hippocampus and amygdala, the highest densities of CB1 receptors are on axon terminals of interneurons co-expressing GABA and cholecystokinin (CCK) (Katona *et al*, 1999; Marsicano and Lutz, 1999). In other regions, such as in the cerebellum, CB1 receptors are more equally distributed on both excitatory and inhibitory terminals

Increases in postsynaptic $[Ca^{2+}]_i$ typically triggers short-term forms of CB1-mediated suppression of synaptic transmission: depolarization-induced suppression of inhibition (DSI) (Ohno-Shosaku *et al*, 2001; Wilson *et al*, 2001b) or excitation (DSE) (Kreitzer and Regehr, 2001) based on the type of terminals involved. Both DSI and DSE are mediated by brief (~secs) stimulation of CB1 receptors, which prevents transmitter release by increasing K^+ conductance (Kreitzer *et al*, 2002) or by inhibiting voltage-gated Ca^{2+} channels (Diana *et al*, 2002; Hoffman and Lupica, 2000; Kreitzer *et al*, 2001; Wilson *et al*, 2001a). On the other hand, pharmacological activation of mGlu receptors triggers CB1-mediated long-term synaptic depression (ECs-LTD), during which the synaptic transmission is reduced (Chevaleyre and Castillo, 2003; Gerdeman *et al*, 2002).

Endocannabinoid system, memory and cognition

Due to the localization of cannabinoid receptors in brain regions such as the hippocampus, the basolateral amygdala and the prefrontal cortex (Breivogel and Childers,

1998; Katona, 2009; Mackie, 2005), which are strictly associated with both cognitive and emotional processes (Laviolette and Grace, 2006a; McLaughlin and Gobbi, 2011; Tan *et al*, 2011; Viveros *et al*, 2007) and to the capability of the endocannabinoid system to modulate synaptic plasticity, it is not surprising that this system could play a pivotal role in the modulation of emotional memory processing.

It is well establish that CB1 receptors are importantly involved in neural plasticity mechanisms related to the processing, consolidation and extinction of emotionally salient cognitive events (Abush and Akirav, 2010; Akirav, 2011; Campolongo *et al*, 2009a; Campolongo *et al*, 2009b; Laviolette *et al*, 2006a; Laviolette and Grace, 2006b; Mackowiak *et al*, 2009; Marsicano *et al*, 2002). Animal studies have demonstrated that the endocannabinoid system may affects short-term memory by altering the mechanisms responsible for these processes within the hippocampus, by selectively affecting encoding processes (Barna *et al*, 2007). Moreover, the important involvement of other subcortical structures, for instance the amygdala, in the modulation of the memory consolidation mechanism in an endocannabinoid-dependent manner processes has been firmly established as well (Campolongo *et al*, 2009b).

Outline

In the first 2 years of the PhD program , at the Department. of Physiology and Pharmacology "V. Erspamer" – Sapienza University of Rome, I have investigated the role of the endocannabinoid system in the regulation of emotional memory processes in rats.

In a first line of research I have focused my attention on the effects induced by general anaesthetics on memory consolidation and their putative interaction with the endocannabinoid system. There is extensive evidence that the occurrence of traumatic experiences associated with perioperative awareness or intensive care unit ICU treatment could result in stress-related disorders such as posttraumatic stress disorder and impaired long-term health-related quality of life outcomes (Kapfhammer *et al*, 2004a; Schelling *et al*, 2003). In order to identify whether anaesthetic drugs, used in ICU, could be responsible of these effects, in **Chapter 1**, I have investigated the effects of propofol on memory consolidation of aversive events. Propofol is a a commonly used agent for

general anaesthesia and for sedation in patients undergoing intensive care treatment (Jones *et al*, 2007) and known to exert inhibitory action on fatty acid amide hydrolase, the enzyme that degradesthe endocannabinoid anandamide (Patel *et al*, 2003).

In **Chapter 2** I have investigated the effect induced by the prototypical endocannabinoid transport inhibitor, AM404, on memory recognition and short-term memory and whether such effects depend on different levels of emotional arousal. This study was expired by the evidence that cannabinoid compounds may influence emotional processes depending on the level of environmental aversiveness at the time of drug administration (Haller *et al*, 2009).

Growing evidence demonstrates that the endocannabinoid system in the basolateral complex of the amygdala (BLA) is one important actor generating and coordinating emotional cognitive responses (Campolongo *et al*, 2009b; Lee *et al*, 2006a, b; Milton *et al*, 2008a; Milton *et al*, 2013; Theberge *et al*, 2010).

In order to expand these findings, I spent the third year of my PhD program in the laboratory directed by Prof. Barry Everitt at Department of Psychology, Downing College, University of Cambridge, UK. My research in Everitt's lab evaluated the role of the endocannabinoid system in the BLA in the reconsolidation of conditioned fear memory using a classic Pavlovian conditioning approach. Memory reconsolidation is the process by which previously consolidated memories become destabilized at retrieval, and require restabilization in order to persist in the brain (Lewis, 1979; Nader, 2003). Previous findings suggest that pharmacological manipulation before or immediately after retrieval, could prevent (Debiec *et al*, 2002; Nader *et al*, 2000; Taubenfeld *et al*, 2009; Wang *et al*, 2009) or enhance the expression of conditioned fear responses (Lee *et al*, 2006b). In **Chapter 3** we firstly elucidated the neurochemical basis of reconsolidation processes - destabilization and restabilization - and the role of different subtypes of NMDAR, GluN2B-NMDARs andGluN2A-NMDARs in BLA. Subsequently, in **Chapter 4**, we studied how the manipulation of reconsolidation process by altering the endogenous cannabinoid tone in BLA after retrieval could influence expression of fear-related response.

Chapter 5, summarizes and discusses the findings of this thesis and provides conclusions and future perspectives.

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Chapter 1

Propofol enhances memory formation via an interaction with the endocannabinoid system

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ABSTRACT

Background: Propofol is associated with postoperative mood alterations and induces a higher incidence of dreaming compared with other general anesthetics. These effects might be mediated by propofol's inhibitory action on fatty acid amide hydrolase, the enzyme that degrades the endocannabinoid anandamide. Because propofol is also associated with a higher incidence of traumatic memories from perioperative awareness and intensive care unit treatment and the endocannabinoid system is involved in regulating memory consolidation of emotional experiences, the authors investigated whether propofol, at anesthetic doses, modulates memory consolidation via an activation of the endocannabinoid system.

Methods: Male Sprague-Dawley rats were trained on an inhibitory avoidance task in which they received an inescapable foot shock upon entering the dark compartment of the apparatus. Drugs were administered intraperitoneally immediately or 30, 90, or 180min after training. On the retention test 48 h later, the latency to reenter the dark compartment was recorded and taken as a measure of memory retention.

Results: The anesthetic doses of propofol administered after training significantly increased latencies of 48-h inhibitory avoidance performance (483.4 181.3, 432.89 214.06, 300 and 350 mg/kg, respectively; mean SD) compared with the corresponding vehicle group (325.33 221.22, mean SD), which is indicative of stronger memory consolidation in propofol treated rats. Administration of a non-impairing dose of the cannabinoid receptor antagonist rimonabant blocked the memory enhancement induced by propofol (123.39 133.10, mean SD). Delayed administration of propofol 90 and 180 min after training or immediate posttraining administration of the benzodiazepine midazolam or the barbiturate pentobarbital did not significantly alter retention.

Conclusions: These findings indicate that propofol, in contrast to other commonly used sedatives, enhances emotional memory consolidation when administered immediately after a stressful event by enhancing endocannabinoid signaling.

Introduction

Propofol is a commonly used agent for general anesthesia and for sedation in patients undergoing intensive care treatment (ICU). It is known to reduce postoperative nausea and vomiting[1] and is associated with postoperative mood alterations and a higher incidence of dreaming compared with other general anesthetics. However, the use of propofol for general anesthesia or for sedation of critically ill patients in the ICU is not universally successful with respect to preventing traumatic memories from perioperative awareness and ICU treatment[2]. There is extensive evidence that the occurrence of traumatic experiences associated with perioperative awareness or ICU treatment could result in stress-related disorders such as posttraumatic stress disorder and impaired long-term health-related quality of life out comes[3,4]. One clinical study, investigating propofol's effects on memory, reported that propofol inhibits conscious memory processing in human subjects soon after memory encoding and that it impairs the encoding of material into long-term memory.[5] In another study, propofol administration to rats induced amnesia of training on an inhibitory avoidance task.[6] However, in both studies propofol was administered before learning, thus revealing propofol's effect on the encoding of new information. No studies are available regarding propofol's effects on the consolidation of traumatic memories. However, because patients often have experienced stressful events, such as preoperative fear and anxiety, car accidents, myocardial infarctions, or acute respiratory distress shortly before induction of general anesthesia or sedation with propofol, it is crucial to investigate the effects of propofol administered shortly after the acquisition of new information, a time window when the memory trace is consolidated into stable long-term memory. Propofol inhibits the enzyme fatty acid amide hydrolase, which is known to degrade endocannabinoids, especially anandamide.[7] Like propofol, the endocannabinoid system recently has been shown to be crucially involved in mood control in animals [8,9] and the regulation of nausea and vomiting in humans during stress.[10] Thus, some of the mentioned propofol effects could be attributable to an activation of the endocannabinoid system. [11] Propofol administration to mice has been shown to increase endocannabinoid content within the brain, an effect that could not be detected with

other sedative agents, such as midazolam or thiopental.[7] In addition, endocannabinoid plasma concentrations increased moderately in patients undergoing propofol anesthesia but decreased in patients undergoing general anesthesia with a volatile agent such as sevoflurane [12] or isoflurane.[13] The endocannabinoid system consists of endocannabinoid ligands, the endogenous cannabinoid receptors 1 and 2 (CB1 and CB2), and enzymes involved in the synthesis and metabolism of endocannabinoids.[14] Endocannabinoids (i.e., anandamide and 2-arachidonoylglycerol) are synthesized on demand through cleavage of membrane precursors and serve as retrograde messengers at central synapses.[15] They bind to CB1 receptors on axon terminals to regulate ion channel activity and neurotransmitter release [16] and are degraded intracellularly by specific enzymes: anandamide is mainly degraded by fatty acid amide hydrolase and 2-arachidonoylglycerol by monoacylglycerol lipase.[17] CB1 receptors are highly expressed in several brain regions and in lower densities outside the brain.[18,19] In contrast, CB2 receptors have a more restricted distribution and are found mainly on immune cells and in low numbers in the brainstem[20] and some other brain regions.[21] Both CB1 and CB2 receptors primarily signal through inhibitory G proteins.[22] Recent evidence indicates an important role for endocannabinoids and CB1 receptor activation in enhancing the memory consolidation of emotionally arousing experiences.[23,24] Moreover, it recently has been shown that the fatty acid amide hydrolase inhibitor URB597 enhances memory acquisition and consolidation in rats.[2] These findings suggest that propofol might modulate memory consolidation of emotionally arousing experiences via an interaction with the endocannabinoid system. To investigate this issue, in a first experiment, anesthetic doses of propofol were administered to rats by intraperitoneal injection, immediately and 30, 90, and 180 min after aversively motivated inhibitory avoidance training, a widely used animal model to assess drug effects on emotional memory consolidation. In a second experiment, we evaluated whether the propofol effect on the consolidation of inhibitory avoidance memory is specific for this anesthetic by administering anesthetic doses of the benzodiazepine midazolam or the barbiturate pentobarbital immediately after inhibitory avoidance training. In the last experiment, we investigated whether the memory-enhancing effect of propofol depends on concurrent CB1 activity by administering a

nonimpairing dose of the CB1 receptor antagonist rimonabant 30 min before Propofol injection; we also studied whether propofol administration modulates endocannabinoid release in rats.

Materials and Methods

Animals

Male adult Sprague-Dawley rats (350–450 g at the time of training; Charles River Laboratories, Calco, Italy) were housed individually and maintained in a temperature-controlled environment (20° 1°C) under a 24-h light-dark cycle (7:00 AM to 7:00 PM lights on) with unlimited access to food and water. All procedures involving animal care or treatments were approved by the Italian Ministry of Health (Rome, Italy) and performed in compliance with the guidelines of the US National Institutes of Health and the Italian Ministry of Health (D.L. 116/92), the Declaration of Helsinki, and the Guide for the Care and Use of Mammals in Neuroscience and Behavioral Research (National Research Council 2004).

Drug Treatment

2,6-Diisopropyl phenol (propofol, 250, 300, or 350mg/kg), purchased from Sigma-Aldrich (Milan, Italy), was dissolved in a vehicle containing 100%sesame oil. Midazolam(30, 50, or 70 mg/kg; Ratiopharm, Ulm, Germany) was dissolved in saline, and pentobarbital (60, 70, or 80 mg/kg; Sigma-Aldrich, St. Louis, MO) was dissolved in a vehicle containing 40% propylene glycol (1,2-propanediol), 10% ethanol, and 50% distilled water. Drug solutions were freshly prepared before each experiment and administered by intraperitoneal injection in a volume of 1 ml/kg immediately after the training trial. To control for time specificity, propofol was administered to different groups of rats either 30, 90, or 180 min after the training trial. To assess whether CB1 receptors are involved in mediating the propofol effect on memory consolidation, the CB1 receptor antagonist rimonabant (1 mg/kg; donated by the National Institute of Mental Health, Chemical Synthesis and Drug Supply Program, Bethesda, MD) was dissolved in a vehicle containing 5% polyethylene

glycol, 5% TWEEN 80, and 90% saline[9] and administered immediately after training, whereas propofol was given 30 min later.

Behavioral Studies

Inhibitory Avoidance Apparatus and Procedures. Rats were trained and tested in an inhibitory avoidance apparatus consisting 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 long) was made of opaque white plastic and illuminated by a lamp; the shock compartment (60 cm long) was made of two dark, electrifiable metal plates and was not illuminated.[26] Training and testing were performed during the light phase, between 10:00 AM and 2:00 PM, and were conducted in dim light conditions in a sound-attenuated room. Animals were handled 1min each for 2 days before the training day. For training, the rats were placed into the starting compartment of the apparatus, facing away from the door, and were permitted to explore the apparatus. After the rats stepped completely into the dark compartment, the sliding door was closed and a single, inescapable foot shock (0.35 mA) was delivered for 1 s. The animals were removed from the shock compartment 15 s after termination of the foot shock. Retention was tested 48 h later. On the retention test trial, the rats were placed into the starting compartment, and the latency to reenter the shock compartment with all four paws (maximum latency of 600 s) was recorded and used as a measure of retention. Longer latencies were interpreted as indicating better retention.[27] Immediately after the training and testing of each animal, the apparatus was cleaned with a 70% ethanol solution. To be included in the test phase, rats they had to reach a minimum criterion on the training test (before treatment), which is 60 s maximum to step in the dark compartment of the maze. All the analyses were performed by the same observer, who was unaware of animal treatment.

Sleeping Time.

Sleeping parameters were determined in different groups of rats. To determine sleeping onset and recovery, immediately after anesthetic administration each rat was placed on

its back once every 30 s until it was unable to right itself within 30 s. Sleeping onset was defined as the interval between anesthetic injection and the time the rat 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. The time between loss and recovery of righting reflex for each rat was defined as sleeping time (cutoff180 min).[28] All of the analyses were performed by the same observer, who was unaware of animal treatment. Endocannabinoid Measurement In accordance with Patel's protocol in mice,[7] rats were treated with propofol (300 mg/kg, intraperitoneally) or with its vehicle and killed 8 or 40 min after administration. Brain and plasma samples were subjected to a lipid extraction process, and the endocannabinoid content of the lipid extracts was determined using isotope-dilution liquid chromatography-mass spectrometry as described previously.[12] The brain tissue was collected and stored at 80°C. Before the extraction process, tissues were weighted and homogenized in polypropylene tubes (Sarstedt, Numbrecht, Germany) and kept in ice water. Five hundred µl of the described homogenized tissue solution was transferred to a 2-ml Eppendorf tube, and 20 µl of internal standard and 1 ml methyl tertiary butyl ether (Sigma-Aldrich, Italy) were added to extract the endocannabinoids. The mixture was vortexed for 1min and centrifuged at 12,000g for 6min. The clear supernatant was transferred into a clean 5-ml polypropylene tube (Sarstedt) and evaporated under vacuum at 37°C. The residue of all evaporated samples was reconstituted in 100 µl acetonitrile, vortexed for 30 s, and sonicated in 4°C water for 15min. A 20 µl aliquot of the clear solution was used for liquid chromatography-tandem mass spectrometry analysis. All samples were injected in duplicates.

Statistical Analysis

The training and retention latencies of rats were analyzed with one-way ANOVA. Time-dependent effects of propofol, the interactions between propofol and rimonabant, and propofol effects on endocannabinoid concentrations were analyzed with two-way ANOVAs. The source of the detected significances was determined by Tukey–Kramer post hoc tests. To determine whether learning had occurred, paired t-tests were used to

compare the training and retention latencies of the vehicle groups. Sleeping parameters were analyzed with Kruskal-Wallis one-way ANOVA on ranks or Mann–Whitney U test because of their non normal distribution. StatView software (SAS Institute, Cary, NC) was used to conduct statistical analyses. Normal data are expressed as mean ± SD; nonparametric data are expressed as median and percentiles. Two-tailed testing was used for all the analyses. P values of 0.05 were considered statistically significant. The number of rats per group is indicated in the figures and tables.

Results

Posttraining Administration of Propofol Enhances 48-h Inhibitory Avoidance Retention Performance

This experiment examined whether immediate posttraining administration of propofol would enhance 48-h retention performance of inhibitory avoidance training. Average stepthrough latencies for all groups during training (i.e., before footshock and drug treatment) were 17.6±13.7 s (mean±SD). One-way ANOVA for training latencies revealed no significant differences between groups ($F_{3,46}$=0.93, P=0.43). The 48-h retention latencies of rats given vehicle immediately after training were significantly longer than their entrance latencies during the training trial (t=5.59, P 0.0002), indicating that the rats retained memory of the shock experience. As shown in figure 1, propofol induced dose-dependent retention enhancement. One-way ANOVA for 48-h retention latencies revealed a significant treatment effect ($F_{3,43}$=7.82, P=0.0003). Post hoc analysis indicated that rats treated with the higher doses of propofol (300 or 350 mg/kg) had significantly longer retention than did those treated with vehicle or with 250 mg/kg propofol (P<0.01 and P 0.05 for 300 and 350mg/kg, respectively). The lower dose of propofol (250 mg/kg), which did not induce anesthesia, did not induce retention enhancement. Three of 12 rats given 350 mg/kg propofol died of respiratory depression.

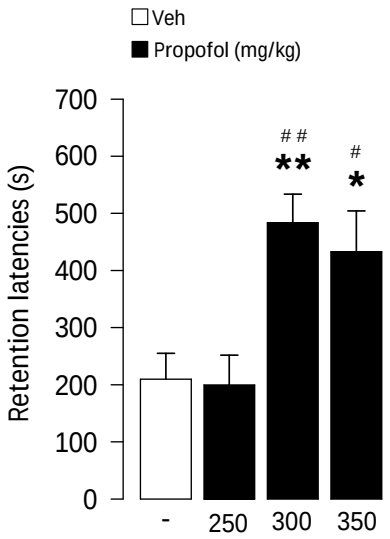

Fig. 1. Effects of posttraining administration of propofol on retention of an inhibitory avoidance response. Step-through latencies (mean±SD) on a 48-h retention test. Immediate posttraining administration of propofol (300 mg/kg) enhanced memory retention. Data are expressed as mean ± SEM * P<0.05; ** P<0.01 versus vehicle; # P<0.05; ## P<0.01 versus 250 mg/kg propofol (n=12, vehicle; n=13, 250 and 300 mg/kg propofol; n=9, 350 mg/kg propofol).

Propofol Administered Immediately or 30 min (but Not 90 or 180 min) after the Training Enhanced 48-h Inhibitory Avoidance Retention Performance

To examine whether propofol influences the consolidation phase of memory processing, rats were treated with Propofol (300 mg/kg) immediately or 30, 90, or 180 min after training. Average step-through latencies for all groups during training, before foot shock and drug treatment, were 16.6 ± 13.0 s (mean ± SD). Two-way ANOVA for training latencies revealed no significant differences between groups (main effect of treatment $F_{1,78}$=0.77, P=0.38; main effect of time of administration $F_{3,78}$=2.0, P=0.12; interaction $F_{3,78}$=1.54, P=0.21). Two-way ANOVA for 48-h retention latencies revealed a significant main effect of Propofol ($F_{1,78}$=17.64, P=0.0001) as well as a significant main effect of time of administration ($F_{3,78}$=3.76, P=0.014). Moreover, there was a statistically significant

interaction effect between treatment and time of administration ($F_{3,78}$=4.76, P=0.0042). As shown in figure 2, post hoc analysis indicated that rats treated with propofol either immediately or 30 min after training had significantly longer retention latencies than did those given vehicle (P<0.01). Retention latencies of rats injected with propofol immediately or 30 min posttraining were significantly longer than were those of rats given propofol 180 min after the training (P<0.01).

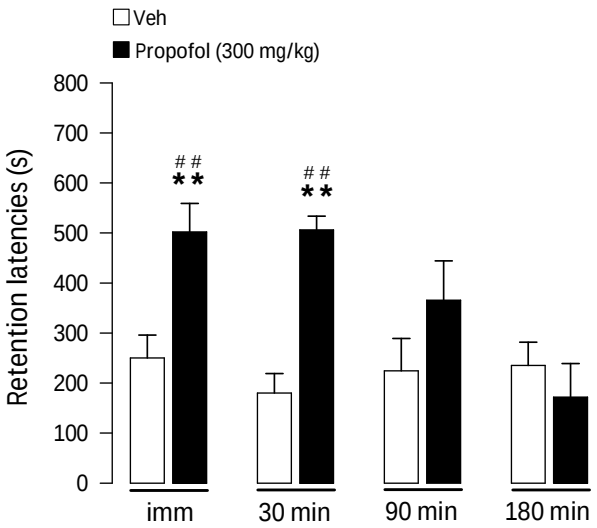

Fig. 2.
Effects of immediate and delayed posttraining administration of propofol on retention of an inhibitory avoidance response. Step-through latencies (mean±SD) on a 48-h retention test. Rats injected with propofol immediately or 30 min posttraining showed retention latencies longer than those of rats injected with vehicle at the corresponding time point and with propofol 180 min after training. Data are expressed as mean ± SEM ** P<0.01 versus the corresponding vehicle group; ## P<0.01 versus rats injected with propofol 180 min after training (n=10, vehicle 30 min and 300 mg/kg propofol 90 min; n=11, all other groups).

Posttraining Administration of Midazolam or Pentobarbital Does Not Enhance 48-h Inhibitory Avoidance Retention Performance

To determine whether the propofol effect on inhibitory avoidance memory enhancement is specific for this anesthetic, rats were treated with anesthetic doses of midazolam (30, 50, or 70mg/kg, intraperitoneally) or pentobarbital (60, 70, or 80 mg/kg, intraperitonelly) immediately after inhibitory avoidance training. For midazolam, average stepthrough latencies for all groups during training, before footshock and drug treatment, were 17.7±13.9 s (mean±SD). One-way ANOVA for training latencies revealed no significant differences between groups ($F_{3,34}$=0.17, P=0.92). As shown in figure 3A, one-way ANOVA for 48-h retention latencies indicated that midazolam did not significantly enhance retention latencies ($F_{3,34}$=0.09, P=0.97). For pentobarbital, average step-through latencies for both groups during training, before foot shock and drug treatment, were 17.2±14.2 s (mean±SD). One-way ANOVA for training latencies revealed no significant differences between groups ($F_{3,34}$=0.34, P=0.79). As shown in figure 3B, one-way ANOVA for 48-h retention latencies indicated that pentobarbital did not significantly enhance retention latencies ($F_{3,34}$=0.21, P=0.89).

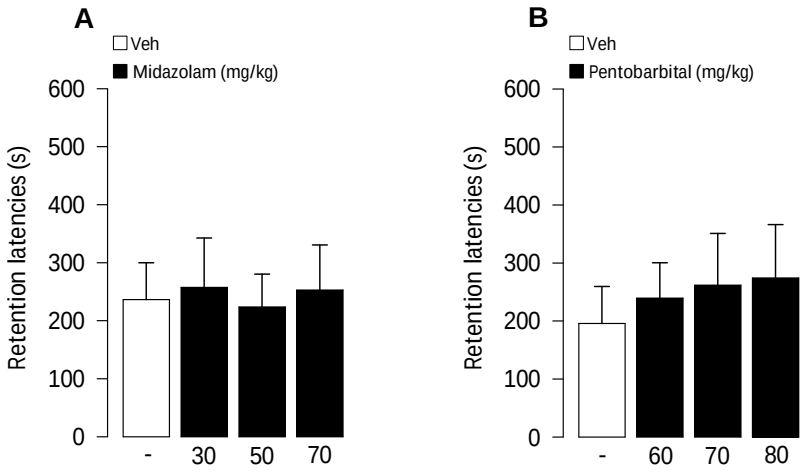

Fig. 3. Effects of posttraining administration of midazolam or pentobarbital on retention of an inhibitory avoidance response. Step-through latencies (mean±SD) on a 48-h retention test.

Immediate posttraining administration of midazolam (A) or pentobarbital (B) did not enhance memory consolidation. Data are expressed as mean ± SEM. (n=9, 30 mg/kg midazolam and 70 or 80 mg/kg pentobarbital; n=10 vehicle, 50 or 70 mg/kg midazolam and 60 mg/kg pentobarbital)

The CB1 Antagonist Rimonabant Blocks the Memory-enhancing Effect Induced by Propofol

This experiment examined whether the memory-enhancing effect of propofol depends on a concurrent activation of CB1 receptors. To address this issue, we investigated whether the CB1 receptor antagonist rimonabant (1 mg/kg) administered intraperitoneally immediately after inhibitory avoidance training would block the retention enhancement induced by propofol given 30 min later. Average step-through latencies for all groups during training, before foot shock and drug treatment, were 15.2±11.8 s. The 48-h retention latencies of rats given vehicle after training were significantly longer than their entrance latencies during the training trial (P=0.0001). As shown in figure 4, posttraining administration of rimonabant blocked the retention enhancement induced by propofol (300 mg/kg). Two-way ANOVA for 48-h retention latencies revealed a significant rimonabant plus propofol interaction effect ($F_{1,27}$=11.70, P=0.002). Post hoc comparison revealed that retention latencies of rats given propofol alone were significantly longer than were those of vehicle-treated rats (P<0.01). Most importantly, retention latencies of rats given an otherwise non impairing dose of rimonabant together with propofol were significantly shorter than those of rats treated with propofol alone (P<0.01).

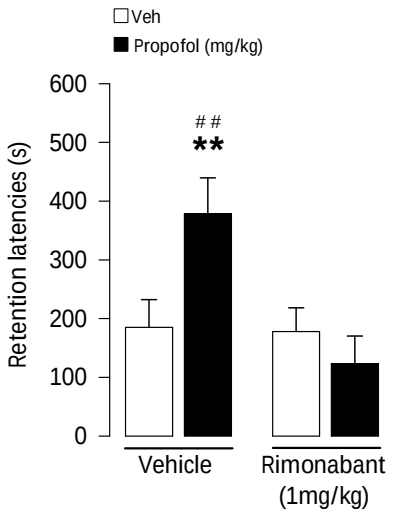

Fig. 4. Effects of the CB1 antagonist rimonabant on the memory-enhancing effects induced by propofol. Stepthrough latencies (mean±SD) on a 48-h retention test. Immediate posttraining administration of the cannabinoid receptor antagonist rimonabant (1 mg/kg) blocked the memory enhancing effects of propofol (300 mg/kg). Data are expressed as mean ± SEM. ** P<0.01 versus the corresponding vehicle group; ## P<0.01 versus the corresponding propofol group (n=7, 1 mg/kg rimonabant+vehicle propofol; n=8, all other groups)

Sleeping Time

Table 1 shows the effects of propofol, midazolam, and pentobarbital on sleeping parameters. Kruskal-Wallis ANOVA revealed no statistically significant effect on sleeping onset (H_6=10.27, P=0.11). However, Kruskal-Wallis ANOVA revealed a statistically significant effect for sleeping time (H_6=19.64, P=0.002). Post hoc comparisons (Mann–Whitney U test with Bonferroni correction) revealed that rats given 50 mg/kg midazolam slept for a shorter amount of time than did rats given 70 or 80 mg/kg pentobarbital or those given 350 mg/kg propofol. None of the rats treated with the lower doses of midazolam (30 mg/kg) or propofol (250 mg/kg) lost righting reflex. Table 2 shows the effects of rimonabant on propofol in inducing anesthesia. Mann–Whitney U test showed no difference between rats pretreated with rimonabant compared with rats pretreated

with vehicle on sleeping onset or time induced by propofol (U=5.0, P=0.11; U=11.000, P=0.75, respectively), indicating that the anesthetic effect of propofol is independent from the indirect activation of the endocannabinoid system.

Table 1. Sleeping Parameters of Propofol-, Midazolam-, and Pentobarbital-treated Rats

	Treatment, mg/kg						
—	Propofol 300 (n = 6)	Propofol 350 (n = 5)	Midazolam 50 (n = 6)	Midazolam 70 (n = 6)	Pentobarbital 60 (n = 6)	Pentobarbital 70 (n = 5)	Pentobarbital 80 (n = 6)
Onset time (min)							
Median	12.0	20.0	11.0	10.5	4.0	4.0	14.0
25th percentile	6.3	9.25	6.0	9.0	3.0	3.0	9.0
75th percentile	13.8	20.3	24.0	15.0	13.0	7.5	24.0
Sleep time (min)							
Median	180	180	86.5*	113	113	144	165
25th percentile	31.8	180	50.0	97.0	92.0	135	150
75th percentile	180	180	102	158	163	180	180

*P<0.05 vs. 350 mg/kg propofol and 70 or 80 mg/kg pentobarbital.

Table 2. Sleeping Parameters of Rats Treated with Propofol Alone or Together with Rimonabant

	Onset Sleeping (min)			Sleep Time (min)		
Treatment	Median	25th Percentile	75th Percentile	Median	25th Percentile	75th Percentile
Vehicle rimonabant + propofol (n = 5)	16.0	11.0	17.0	180	142	180
Rimonabant + propofol (n = 5)	11.0	9.0	13.5	180	134	180

Endocannabinoid Measurement

Two-way ANOVA for propofol effects on Endocannabinoid content revealed a statistically significant interaction between treatment and time of administration ($F_{1,19}$=7.1, P=0.015). Post hoc comparisons revealed that Propofol increases anandamide concentrations in rat brains 8 min after administration (P< 0.05, table 3).

Table 3. Endocannabinoid Concentrations

Treatment	Anandamide		2-Arachidonoylglycerol (2-AG)	
	Brain (ng/g Tissue)	Plasma (ng/ml)	Brain (μg/g Tissue)	Plasma (ng/ml)
Vehicle (8 min, n = 6)	13.8 ± 7.1	0.9 ± 0.3	28.4 ± 27.1	0.3 ± 0.1
Propofol (8 min, n = 6)	50.7 ± 38.3*	0.9 ± 0.2	29.1 ± 19.9	0.3 ± 0.1
Vehicle (40 min, n = 5)	33.7 ± 29.2	1.1 ± 0.5	45.9 ± 41.7	0.4 ± 0.2
Propofol (40 min, n = 6)	16.3 ± 6.5	1.1 ± 0.5	46.3 ± 36.3	0.4 ± 0.2

* P<0.05 versus vehicle-treated rats (8 min).

Discussion

The current findings indicate that propofol, at anesthetic doses, enhances memory consolidation of inhibitory avoidance training in rats when administered immediately after the training experience. This memory enhancement is blocked by coadministration of the CB1 cannabinoid receptor antagonist rimonabant, suggesting that the enhancing effect of propofol on memory consolidation depends on an indirect activation of CB1 receptors. In contrast, midazolam and pentobarbital, two anesthetics that do not increase endocannabinoid signaling,[7]did not enhance the consolidation of memory of inhibitory avoidance training. The current findings may appear at odds with preclinical and clinical findings indicating that propofol induces amnesia. For example, Veselis et al. [5] reported that propofol inhibits conscious memory processes in human subjects soon after memory encoding and that it impairs the acquisition or encoding of material into long-term memory. In addition, propofol has been reported to induce amnesia of training in rats on the same inhibitory avoidance task used in the current study.[6] However, a critical difference between these investigations and the current study is that in the human studies, memory function was assessed shortly after drug administration, whereas in the preclinical study, rats were given the drug before training. Therefore, acute pharmacologic effects could have influenced directly both the acquisition and retention of the training. In contrast, in our study the drug was administered after the training and was not present during the acquisition phase. Thus, the enhancing effects of propofol on retention performance in our study are likely mediated by specific influences on the

consolidation of memory of the training experience.[29] The use of posttraining drug manipulation is a widely accepted method for effectively dissociating memory processes from secondary behavioral effects of non-associative nature, such as those related to sensory sensitivity.[30] Because retention testing took place 48 h after training and drug treatment, these findings further exclude residual pharmacologic effects as having a direct influence on behavior during retention testing. Moreover, the effect of post-training propofol administration on retention enhancement was time dependent: propofol administration immediately or 30 min after inhibitory avoidance training resulted in memory enhancement, whereas administration of Propofol 90 or 180 min after training was ineffective. Together these findings provide evidence that propofol enhances time-de pendent processes underlying the consolidation of memory for emotionally arousing experiences. The posttraining drug administration protocol used in the current article has a translational value to humans. Acute sedation or even the induction of anesthesia immediately after a traumatic experience (e.g., in the consolidation phase of a traumatic memory) is a common clinical scenario in emergency medicine and in the ICU. Our findings demonstrate that propofol is able to enhance memory consolidation when administered immediately after the exposure to a traumatic event and that this effect on memory depends on an indirect activation of the endocannabinoid system. In accordance with the behavioral data, we also found that propofol administration increases anandamide concentrations in the rat brain 8 min after injection, whereas anandamide plasma concentrations remain unaffected. Our data are in accordance with preclinical and clinical evidence. Patel et al.[7] demonstrated increased concentrations of anandamide in the mouse brain after systemic administration of propofol in contrast to the administration of benzodiazepines, barbiturates, or volatile anesthetics; the effect of propofol on anandamide concentrations is mediated by an inhibition of fatty acid amide hydrolase, the major degradation enzyme of anandamide.[7] In humans undergoing general anesthesia, plasma concentrations of the endocannabinoid anandamide remained unchanged during Propofol anesthesia but were significantly reduced during anesthesia with volatile agents.[12,13] The basolateral complex of the amygdala (BLA) appears to be a critical site for mediating drug effects on memory performance, including those of

propofol. One study reported that permanent neurotoxic lesions of the BLA produced with N-methyl-D-aspartate blocked the amnestic effect of pretraining propofol administration of rats trained on an inhibitory avoidance task.[6] We recently have shown that the endocannabinoid system in the BLA is involved in the enhanced consolidation of inhibitory avoidance memory and that CB1 activity within the BLA is essential for mediating glucocorticoid effects on long-term memory.[23–31] Based on these findings, a newmodel has emerged.[32–33]In this model, stress-induced glucocorticoids bind to membrane-bound receptors in the BLA that activate a G-protein signaling cascade that induces endocannabinoid synthesis. The ensuing release of endocannabinoid ligands could diffuse to local -aminobutyric acid–mediated (GABAergic) terminals and inhibit -aminobutyric acid release onto noradrenergic terminals in the BLA. The end result of this process is an increased norepinephrine release within the BLA and subsequently an enhancement of emotional memory consolidation. Many sedative and anamnestic effects of general anesthetics, including those of propofol, crucially depend on -aminobutyric acid release. The current findings demonstrate that the enhancing effects of propofol onmemory consolidation depend on concomitant CB1 receptor activity, so we hypothesize that the anamnestic effects of propofol are mediated by an endocannabinoid-induced inhibition of γ-aminobutyric acid release, resulting in a more pronounced memory consolidation during stressful conditions when glucocorticoid signaling is high.[34] The pharmacokinetic properties of midazolam, pentobarbital, and propofol differ to a large extent, but all three drugs share the pharmacodynamic capability to potentiate -aminobutyric acid neurotransmission.[35] Our results showing that rats treated with midazolam (50 mg/kg) slept less than did rats treated with propofol (350 mg/kg) or pentobarbital (70 or 80 mg/kg) are in accordance with clinical evidence showing that midazolam has a shorter half-life than Propofol and barbiturates.[35] However, neither rats treated with the higher dose of midazolam nor the ones treated with pentobarbital showed differences in the sleeping parameters compared with those treated with propofol. Although Propofol enhances memory consolidation through an activation of the endocannabinoid system, the anesthetic effect of Propofol does not depend on this activation. The CB1 receptor antagonist rimonabant blocks the propofol-enhancing effect

on memory consolidation but does not influence propofol's effects on sleeping. On the whole, these data suggest that, unlike midazolam and pentobarbital, propofol induces selective effects on memory consolidation, which are linked to the activation of the endocannabinoid system and not related to the potentiation of GABAergic neurotransmission. These findings, together with the results showing that midazolam and pentobarbital, at anesthetic doses, did not influence memory consolidation strongly corroborate the hypothesis that propofol's effects on memory consolidation are not attributable to a general nonspecific anesthetic effect. In summary, our study demonstrates that propofol enhances memory consolidation via an endocannabinoid-mediated mechanism. These effects are markedly different from those of other direct GABAergic agents such as midazolam or pentobarbital. These findings from animal experiments suggest that propofol should be used with caution in individuals during the aftermath of an acute traumatic event and may help to explain the increased incidence of aversive memories from intraoperative awareness seen in patients undergoing total intravenous anesthesia with propofol.[36] Likewise, the findings suggest that pharmacologic manipulation of endocannabinoid signaling could be a useful intervention aimed at blocking memory consolidation immediately after a traumatic event.

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Chapter 2

The endocannabinoid transport inhibitor AM404 differentially modulates recognition memory in rats depending on environmental aversiveness

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Abstract

Cannabinoid compounds may influence both emotional and cognitive processes depending on the level of environmental aversiveness at the time of drug administration. However, the mechanisms responsible for these responses remain to be elucidated. The present experiments investigated the effects induced by the endocannabinoid transport inhibitor AM404 (0.5-5 mg/kg, i.p.) on both emotional and cognitive performances of rats tested in a Spatial Open Field task and subjected to different experimental settings, named *High Arousal* and *Low Arousal* conditions.

The two different experimental conditions influenced emotional reactivity independently of drug administration. Indeed, vehicle-treated rats exposed to the *Low Arousal* condition spent more time in the centre of the arena than vehicle-treated rats exposed to the *High Arousal* context. Conversely, the different arousal conditions did not affect the cognitive performances of vehicle-treated animals such as the capability to discriminate a spatial displacement of the objects or an object substitution.

AM404 administration did not alter locomotor activity of the animals exposed to both environmental conditions. Interestingly, AM404 administration increased the emotional reactivity of rats exposed to the *High Arousal* condition but did not influence emotionality of rats exposed to the *Low Arousal* condition. Moreover, AM404 administration influenced the cognitive parameters depending on the level of emotional arousal: it impaired the capability of rats exposed to the *High Arousal* condition to recognize a novel object while it did not induce any impairing effect in rats exposed to the *Low Arousal* condition.

These findings suggest that drugs that enhance endocannabinoid signalling induce different effects on recognition memory performance depending on the level of emotional arousal induced by the environmental conditions.

Keyword: Cannabinoid system, endocannabinoids, AM404, emotionality, short-term memory, cognition.

Introduction

The endocannabinoid system is a crucial regulator of central nervous system (CNS) function (Cravatt et al., 1996; Di Marzo and Matias, 2005; Pacher et al., 2006; Trezza et al., 2008b; Campolongo et al., 2009b,c, 2011; Bisogno and Di Marzo, 2010; Hill and McEwen, 2010). Endocannabinoids are released from post-synaptic neurons in an activity-dependent manner, travel retrogradely through the synaptic cleft and activate presynaptic cannabinoid type 1 receptors (CB1), thus suppressing neurotransmitter release from axon terminals (Wilson and Nicoll, 2002). Among the endogenous cannabimimetic signaling molecules, anandamide (N-arachidonoylethanolamine, AEA) and 2-arachidonoylglycerol (2-AG) stand out as the first identified and most intensively studied (Ueda et al., 1995, 2011; Di Marzo, 1998; Piomelli, 2003; Waku, 2006). Receptor activation by endocannabinoids ends by the removal from the synaptic cleft operated by a transport system present in neural and non-neural cells (Di Marzo et al., 1994; Beltramo et al., 1997; Hillard et al., 1997) followed by hydrolysis operated by fatty-acid amide hydrolase (FAAH, that hydrolyzes anandamide) or monoacylglycerol lipase (MAGL, that cleaves 2-AG) (Desarnaud et al., 1995; Hillard et al., 1995; Ueda et al., 1995; Cravatt et al., 1996). Interestingly, while the endocannabinoid hydrolyzing enzymes have been fully identified and cloned, the functional properties of the putative transporter have been only partially characterized (Hillard and Jarrahian, 2003; Yates and Barker, 2009; Fu et al., 2011) and its molecular identity remains still unknown. CB1 receptor is crucially involved in neural plasticity mechanisms related to the processing, consolidation, and extinction of emotionally salient cognitive events (Marsicano et al., 2002; Laviolette and Grace, 2006a,b; Campolongo et al., 2009a,b; Mackowiak et al., 2009; Abush and Akirav, 2010; Akirav, 2011; Hauer et al., 2011). This fits well with the notion that CB1 receptors are highly expressed in brain structures including the basolateral amygdala (BLA), the medial prefrontal cortex (mPFC) and the hippocampus (Breivogel and Childers, 1998; Mackie, 2005; Katona, 2009), strictly associated with both cognitive and emotional processes (Laviolette and Grace, 2006a; Viveros et al., 2007; McLaughlin and Gobbi, 2011; Tan et al., 2011).

Animal studies have demonstrated that the endocannabinoid system modulates recognition memory by altering the mechanisms responsible for this process within the hippocampus and selectively affecting the encoding stage (Barna et al., 2007). Moreover, the important involvement of other structures, for instance the amygdala, in the modulation of memory consolidation and extinction for emotional events has been firmly established (McGaugh, 2000; Vianna et al., 2004; Clarke et al., 2008; de Oliveira Alvares et al., 2008, 2010; Campolongo et al., 2009b; Ganon-Elazar and Akirav, 2009;Manwell et al., 2009; Roozendaal and McGaugh, 2011). In line with the widespread distribution of CB1 receptors throughout the limbic system, it has been extensively demonstrated that cannabinoid compounds also induce diverse effects on anxiety- and fear-related behaviors (Trezza et al., 2008a, 2012; Micale et al., 2009; Moreira and Wotjak, 2010; Terzian et al., 2011). Interestingly, cannabinoid effects on emotionality are biphasic, as it is also reported by cannabis abusers (Fant et al., 1998;Hall and Solowij, 1998; Bolla et al., 2002; Curran et al., 2002). The classical explanation to this phenomenon is often provided by the use of different doses of cannabinoid drugs, with low doses generally inducing anxiolytic-like effects and high doses often causing the opposite. A new and appealing explanation to this phenomenon is now emerging, underlying that these opposite effects may also depend on previous experiences, the context of use and the level of emotional arousal at the time of drug administration/consumption (Akirav, 2011; Sciolino et al., 2011). Drugs that interfere with endocannabinoid degradation increase ongoing endocannabinoid signaling in a temporarily and spatially restricted manner (Janero et al., 2009). However, preclinical evidence has shown that indirect cannabinoid agonists can also induce biphasic effects on behavior, depending on the emotional state of the subject. For instance, it has been recently demonstrated that the FAAH inhibitor URB597 does not affect anxiety under mildly stressful circumstances but has robust anxiolytic-like effects in highly aversive testing conditions (Haller et al., 2009). These finding leaves open the possibility thatinhibitors of endocannabinoid transport, which prolong endocannabinoid actions by preventing endocannabinoid access to intracellular hydrolyzing enzymes (Beltramo et al., 1997; Kathuria et al., 2003), may influence both emotional and cognitive

processes depending on the level of environmental aversiveness at the time of drug administration.

To address this issue, in the present study we investigated the effect of the prototypical endocannabinoid transport inhibitor, AM404 in a non-aversive task, the Spatial Open Field test under two experimental conditions differing by the level of emotional arousal at the time of testing. The Spatial Open Field task has been extensively used (Poucet et al., 1986; Thinus-Blanc et al., 1987; Poucet, 1989, 1993; Ricceri et al., 1999, 2002; Scattoni et al., 2004; de Bartolo et al., 2010) and permits to assess both emotional and cognitive parameters, in terms of reactivity to a spatial or an object novelty, by exploiting the natural propensity of rodents to explore the environment. The High Arousal condition (HA) was obtained by testing rats in an empty arena under white light illumination without previous handling, while the Low Arousal emotional processes (Laviolette and Grace, 2006a; Viveros et al., 2007; McLaughlin and Gobbi, 2011; Tan et al., 2011).

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Materials and Methods

Animals

Male adult Wistar rats (300 g at the time of testing, Charles River Laboratories, Italy) were housed in groups and maintained in a temperature-controlled environment (20 ± 1°C) under a 12-h light/12-h dark cycle (7:00 am to 7:00 pm lights on) with unlimited access to food and water. All procedures involving animal care or treatments were approved by the Italian Ministry of Health and performed in compliance with the guidelines of the US National Institutes of Health (NIH) and the Italian Ministry of Health (D.L. 116/92), the Declaration of Helsinki, the Guide for the Care and Use of Mammals in Neuroscience and Behavioral Research (National Research Council 2004) and the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes.

Drug Treatments

N-(4-Hydroxyphenyl)-5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenamide (AM404, 0.5-1-5 mg/kg), purchased from Tocris Bioscience (UK), was dissolved in a vehicle containing 10% polyethylene glycol, 10% Tween-80 and 80% saline. Drug solutions were freshly prepared before each experiment and administered by intraperitoneal injection in a volume of 1 ml/kg 15 minutes before the beginning of the task.

Spatial Open Field procedures

The apparatus consisted in an open-field arena made of black Plexiglas (80 x 80 x 60 cm) surrounded with a visually uniform environment. A video camera above the field was connected to a video recorder. Experiments were performed between 10.00 am and 2.00 pm. The test schedule consisted of six 5-min sessions, separated by 3-min delays during which the subjects were returned to their home cage (fig.1). During session 1, each rat was placed into the centre of the empty arena to allow it to become familiar with the apparatus and to record baseline levels of locomotor and exploratory activity. Starting from session 2, three different objects were simultaneously present in the open field:

Object A, a dark metal parallelepiped (4 cm high x 13 cm wide x 9 cm long); Object B, a transparent Plexiglas cube with holes regularly distributed on the sides (height = 10 cm); Object C, a grey plastic square (10 x 10x 10 cm) with a central triangle forming a 90° angle. During sessions 2-3, the A, B and C objects were placed in the arena. In session 4, the spatial test session, the configuration was changed by moving two objects: object B replaced object A which was itself displaced at the periphery of the apparatus. In session 5, the configuration of the objects was unchanged to let the rats habituate to the new arrangement of the objects. In the last session (session 6) one of the familiar, non-displaced objects (object C) was replaced by a new object (object D, which consisted of a black-and-white plastic cylinder, height = 13 cm; diameter = 6 cm (fig. 1).

We exposed the rats to two experimental conditions, named *High Arousal* (HA) and *Low Arousal* (LA) conditions. In the HA condition (experiment 1), the test was performed under normal light (30-40 lux), rats were not handled and tested in an empty arena (no bedding). In the LA condition (experiment 2), the test was performed under dim red light (2 lux) condition, rats were extensively habituated to the experimenter and to the injection procedure for 1 week before the experiment (every day, 1 min per each rat) and tested in an arena with the ground loaded with familiar bedding.

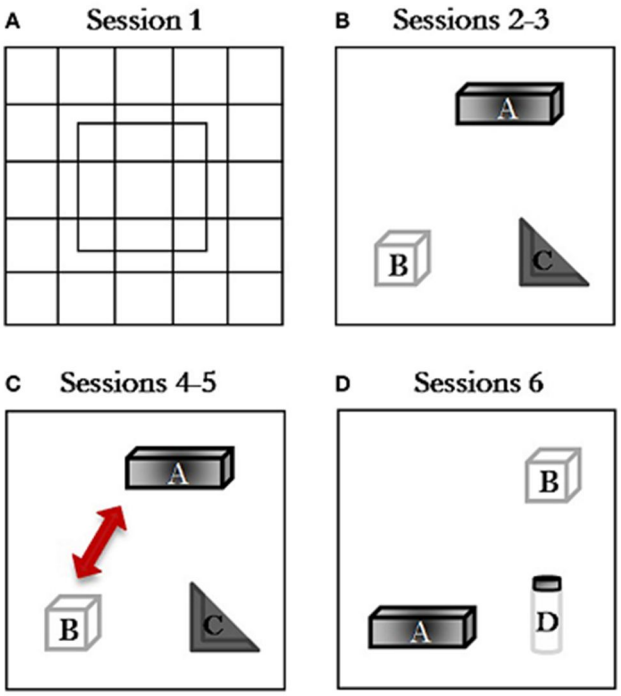

FIGURE 1 Spatial Open-Field procedure. Schematic diagram representing the object configuration in the Spatial Open-Field test: (A) session 1, open field without objects; (B) session 2, habituation session with three stable objects; (C) sessions 3–4, spatial change discrimination sessions where object B displaced object A (session 4); (D) session 5–6, object novelty sessions where object D replaced object C (session 6).

Statistical Analysis

Data collection was performed from the same observer who was unaware of animal treatment using the Observer XT software (Noldus, the Netherland). During the first session, frequency and/or duration of the following responses were measured: crossings, rearings and time spent in the centre of the apparatus. From sessions 2 to 6, object exploration was measured as total time spent by the animal in contact with an object (1 sec as minimal contact was considered) throughout all sessions 2-6.

The total time spent by rats investigating all objects throughout all sessions has been considered as an indicator of general investigative activity. A contact was defined as the subject's snout actually touching an object. In session 4, the spatial arrangement of the objects was modified and response to spatial change was assessed by comparing the mean time spent in contact with both Displaced (DO) and Non-Displaced (NDO) Objects in session 4 minus the mean time spent in contact with the same object in session 3. A discrimination index of the response to the spatial change was obtained by subtracting the NDO value to DO value. Finally, the response to the non-spatial novelty was assessed by comparing mean time in contact with the Substituted Object (SO, unfamiliar) and Non-Substituted Objects (NSO, familiar) in session 6 minus the mean time spent with objects located in the corresponding position in session 5. A discrimination index of the response to the non-spatial novelty was obtained by subtracting the NSO value to SO value. Unpaired t-test was used to compare the behavioural performance of vehicle groups. One-sample t tests were used to determine whether the discrimination index was different from zero. A probability level of <0.05 was accepted as statistically significant.

Results

Different arousal conditions influenced emotional behaviour and object exploration but did not alter cognitive performances of vehicle-treated animals

Unpaired t-test showed that the different arousal context did not affect the locomotor activity of the vehicle groups. Both crossing (fig. 2A) and rearing (fig. 2B) frequencies did not statistically differ between the two groups ($t=-0.66$; $p=0.52$; $t=1.09$; $p=0.29$). However, unpaired t-test showed that the different arousal conditions influenced the emotional behaviour of vehicle-treated animals exposed to the different experimental contexts. Rats treated with vehicle and exposed to a *High Arousal* condition (HA group) spent less time in the centre of the arena than vehicle-treated rats exposed to a *Low Arousal* context (LA group) ($t=-4.11$; $p=0.0005$, fig. 2C).

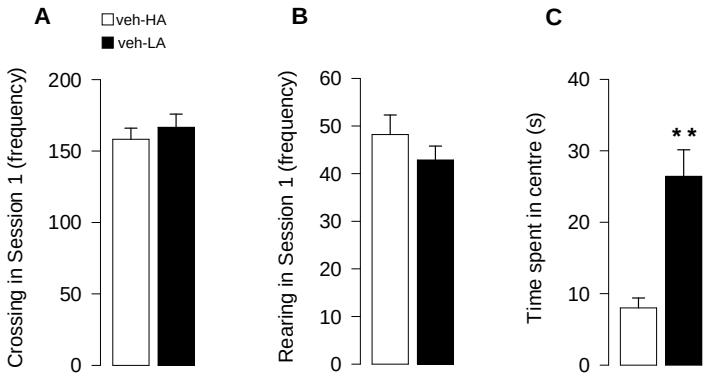

FIGURE 2 Effects induced by different arousal conditions on locomotor activity and emotional behavior of vehicle-treated rats. Locomotor activity: number of crossing (A) and rearing (B) in session 1. Emotional behavior: time spent in the center of the arena in session 1 (C). **P < 0.01. Data are expressed as mean ± SEM. (High Arousal: HA n = 10; Low Arousal: LA n = 15)

Unpaired t-test showed that rats treated with vehicle and exposed to a HA context spent less time investigating objects than vehicle-treated rats exposed to a LA context (t=-4.41; p<0.0001, fig. 3A). Additionally, unpaired t-test showed that both vehicle groups did not differ in the discrimination index for a spatial object displacement in session 4 (t=0.60; p=0.55, fig. 3B) and for the substitution of the objects in session 6 (t=0.47; p=0.64, fig. 3C). However, One-sample t tests revealed that while both vehicle groups were able to discriminate the object novelty (veh-HA, t=4.49, P=0.0015; veh-LA, t14=2.61, P=0.02, fig. 3C) they did not respond to a spatial rearrangement (veh-HA, t=1.10, P=0.30; veh-LA, t=0.16, P=0.88, fig. 3B).

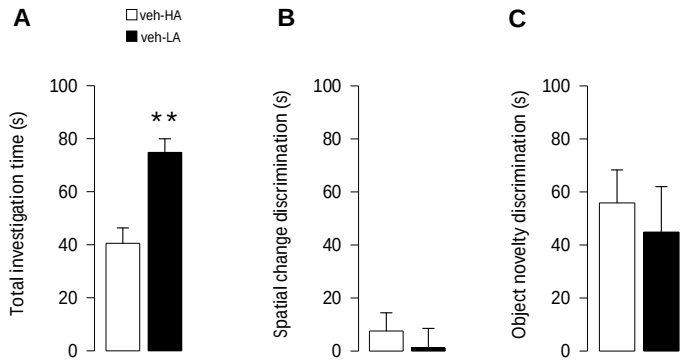

FIGURE 3 Effects induced by different arousal conditions on object investigation and cognitive performances of vehicle-treated rats. Total investigation time of all objects through sessions (A) spatial change discrimination (B) and object novelty discrimination (C).**P < 0.01. Data are expressed as mean ± SEM. (High Arousal: HA n = 10; Low Arousal: LA n = 15)

AM404 administration did not alter locomotor activity and emotional behaviour in rats exposed to different arousal conditions

AM404 administration did not alter the locomotor activity of rats exposed to either a HA or LA condition. One–way ANOVA for crossing (fig. 4A) and rearing (fig. 4B) frequencies in session 1 for AM404-treated rats exposed to a HA condition did not show a statistically significant difference ($F_{3,36}$=0.60; p=0.62; $F_{3,36}$=1.44; p=0.25, respectively). Moreover, one–way ANOVA for the number of crossings (fig. 4C) or rearings (fig. 4D) in session 1 did not show a statistically significant difference between vehicle- and AM404-treated rats exposed to a LA condition ($F_{3,50}$=0.97; p=0.42; $F_{3,50}$=2.21; p=0.10, respectively). AM404 administration did not affect the emotional reactivity in rats exposed to either a HA or LA condition. Indeed, one-way ANOVA showed that vehicle- and AM404-treated rats did not differ for the time spent in the centre of the arena in session 1 (HA condition: $F_{3,36}$=1.25; p=0.31; fig. 5A; LA condition: $F_{3,50}$=1.18; p=0.33; fig. 5B).

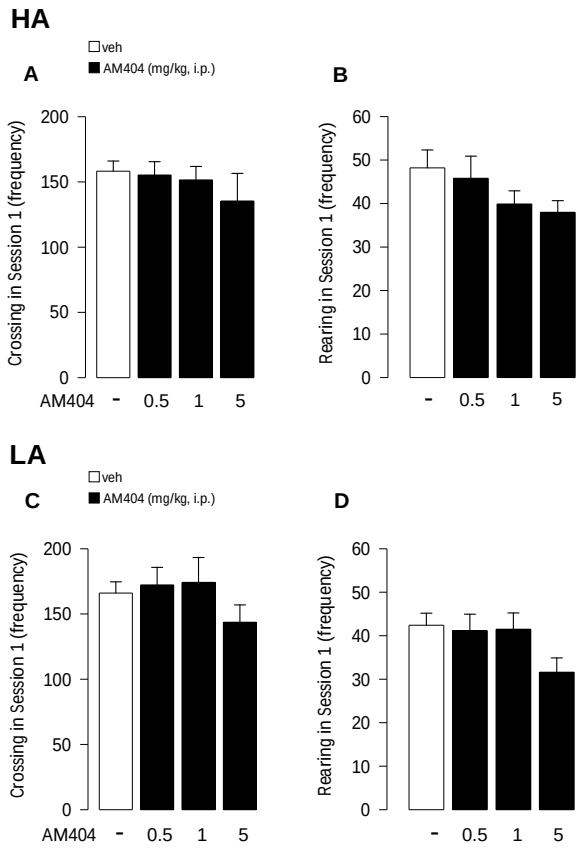

FIGURE 4 Effects of AM404 administration on locomotor activity in rats exposed to high arousal (HA) or low arousal (LA) conditions. Number of crossing (A) and rearing (B) of rats exposed to HA or LA conditions (C, D, respectively) in session 1. Data are expressed as mean ± SEM. (HA: veh n = 10, 0.5mg/kg n = 11, 1 mg/kg n = 10, 5 mg/kg n = 9;LA: veh n = 15, 0.5mg/kg n = 12, 1 mg/kg n = 14, 5 mg/kg n = 13).

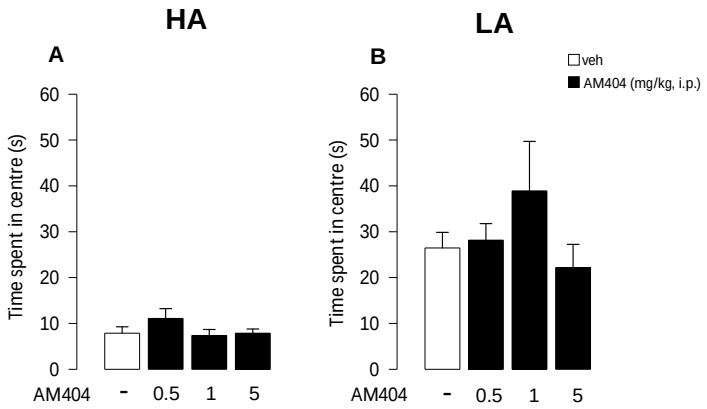

FIGURE 5 Effects of AM404 administration on emotional behavior in rats exposed to high arousal (HA) or low arousal (LA) conditions. Time spent in the center of the arena by rats exposed to HA (A) or LA conditions (B) in session 1. Data are expressed as mean ± SEM.(HA:veh n = 10, 0.5mg/kg n = 11, 1 mg/kg n = 10, 5 mg/kg n = 9; LA: veh n = 15, 0.5mg/kg n = 12, 1 mg/kg n = 14, 5 mg/kg n = 13).

AM404 administration influenced object exploration depending on the different arousal condition

One–way ANOVA showed that administration of AM404 influenced the object investigation measured over all sessions in rats exposed to a HA context ($F_{3,193}$=2.62; p=0.05). Post-hoc analysis revealed that rats treated with a lower dose of the indirect agonist spent less time investigating the objects compared to their respective vehicle group (p<0.05, fig. 6A). On the other hand, one –way ANOVA revealed that AM404-treated rats exposed to a LA context spent the same amount of time investigating the objects as the vehicle-treated animals ($F_{3,265}$=0.54; p=0.66; fig. 6B).

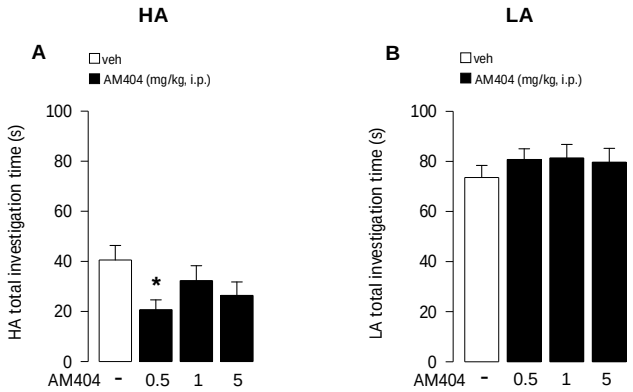

FIGURE 6 Effects of AM404 administration on object investigation in rats exposed to high arousal (HA) or low arousal (LA) conditions. Total investigation time of all objects by rats exposed to HA (A) or LA (B) conditions through sessions 2–6. *P < 0.05. Data are expressed as mean ± SEM. (HA: veh n = 10, 0.5mg/kg n = 11, 1 mg/kg n = 10, 5 mg/kg n = 9; LA: veh n = 15, 0.5mg/kg n = 12, 1 mg/kg n = 14, 5 mg/kg n = 13).

AM404 administration did not influence *spatial change* discrimination while it altered *object novelty* recognition in rats exposed to different arousal conditions.

One–way ANOVA showed that administration of AM404 did not influence the rat capability to discriminate the object displacement under both the HA (fig. 7A) or LA (fig.7B) experimental conditions ($F_{3,36}$=1.176; p=0.34; $F_{3,50}$=2.24; p=0.095, respectively). However, one–way ANOVA showed a statistical significant effect on the capability of the rats to discriminate a novel object under a HA condition ($F_{3,36}$=4.32; p=0.01; fig. 8A). Post-hoc comparisons revealed that rats administered with AM404 0.5 and 1 mg/kg were not able to discriminate the new object as vehicle-treated rats did (p<0.05). One–way ANOVA revealed that AM404 administration to LA exposed rats did not influence the capability of

the rats to discriminate the new object compared to the vehicle group ($F_{3,50}$=0.26; p=0.85; fig. 8B).

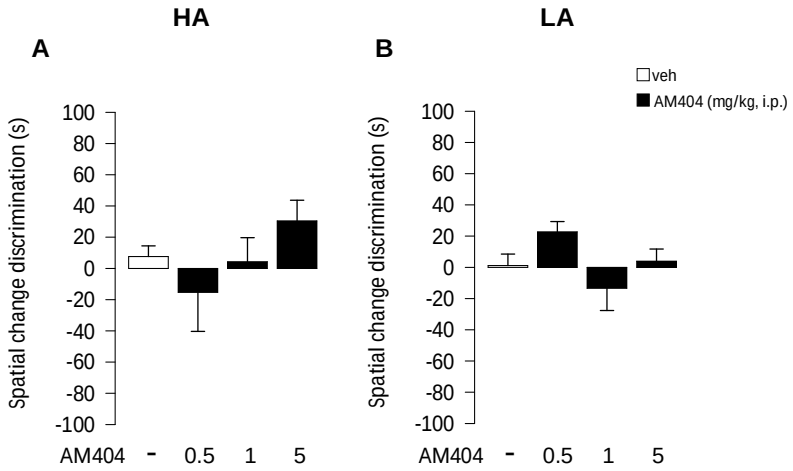

FIGURE 7 Effects of AM404 administration on spatial change discrimination in rats exposed to high arousal (HA) or low arousal (LA conditions. Spatial change discrimination index of rats exposed to HA (A) or LA (B) conditions. Tim spent in contact with Displaced (DO) and Non-Displaced (NDO) Objects in session 4 minus the mean time spent in contact with the same object in session 3. A discrimination index was obtained by subtracting the NDO value to DO value. Data are expressed as mean ± SEM.(HA:veh n = 10, 0.5mg/kg n = 11, 1 mg/kg n = 10, 5 mg/kg n = 9; LA: veh n = 15, 0.5mg/kg n = 12, 1 mg/kg n = 14, 5 mg/kg n = 13).

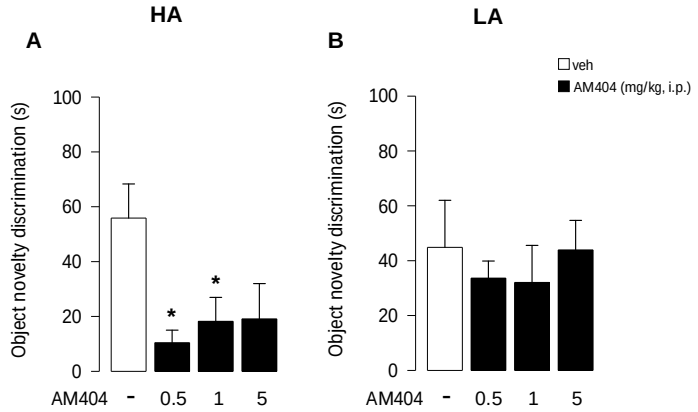

FIGURE 8 Effects of AM404 administration on object novelty discrimination in rats exposed to high arousal (HA) or low arousal (LA) conditions. Object novelty discrimination index of rats exposed to HA (A) or LA (B) conditions. Time spent in contact with Substituted Object (SO, unfamiliar) and Non-Substituted Objects (NSO, familiar) in session 6 minus the mean time spent with objects located in the corresponding position in session 5. A discrimination index was obtained by subtracting the NSO valuetoSOvalue.*P < 0.05. Data are expressed as mean ± SEM. (HA: veh n = 10, 0.5mg/kg n = 11, 1 mg/kg n = 10, 5 mg/kg n = 9; LA: veh n = 15, 0.5mg/kg n = 12, 1 mg/kg n = 14, 5 mg/kg n = 13

Discussion

The present findings demonstrate that: (1) different levels of environmental aversiveness strongly influence the emotional reactivity of untreated rats without affecting the cognitive performance in the Spatial Open-Field test; (2) endocannabinoids affect recognition memory of rats in the Spatial Open Field test depending on the level of emotional arousal induced by the environmental conditions. The Spatial Open-Field is a non-aversive test that permits to assess several behaviors which are indicative of the emotional state of the animal as well as the reactivity to both spatial rearrangement (spatial novelty) or the replacement of one familiar object with a new one (object novelty, as in the classical object recognition task) (Poucet et al., 1986; Thinus-Blanc et al., 1996). This test exploits the natural propensity of rodents to explore the environment without using rewards or punishments.

Previous studies have shown that naive rodents respond to a new spatial displacement or substitution by renewed exploration of the entire environment and/or by selective reinvestigation of the displaced/substituted objects (Poucet et al., 1986; Thinus-Blanc et al., 1987; Poucet, 1989, 1993; Ricceri et al., 1999, 2000, 2002; Scattoni et al., 2004; de Bartolo et al., 2010). The one-day six-session assessment of the task used in our study permits to determine pharmacological effects on short-term memory as well as on emotional reactivity of the subject. Activation of emotional responses, triggered by stressful stimuli, is crucial in the modulation of contextual learning and memory

performances (McGaugh and Roozendaal, 2002; McGaugh, 2004; Morris, 2006; Campolongo et al., 2009b; Hill et al., 2010). There is evidence that behavioral responses to the environmental stimuli are strictly dependent on the emotional reactivity induced by the environment itself (Blanchard et al., 2001; Haller et al., 2009). The environmental–induced arousal is critically involved in assessing the novelty and salience of the external stimuli in terms of relevance for the adaptation and survival (Poucet, 1993; Biegler and Morris, 1996; Breivogel and Childers, 1998). Thus, when compared with a previous experience, a novel information recognized as highly relevant is committed to and stored by the memory (Lemaire et al., 1999). However, the mechanisms underlying themodulation of responsiveness to the environment and its evaluation in evolutionary terms both under LA or HA contexts remain to be elucidated. Based on previous findings (Szeligo and Leblond, 1977; Sahakian et al., 1982; Morato and Castrechini, 1989; Griebel et al., 1993; Escorihuela et al., 1994; Hall et al., 1998; Varty et al., 2000; Haller et al., 2009), in order to characterize the behavioral responses to different environmental situations, we manipulated the experimental context to create two opposite arousal conditions by using two different protocols: (1) rats either extensively handled or not handled by the experimenter before testing, (2) isolated- or grouped-housed rats; (3) bright or dim red light conditions; (4) without or with familiar bedding during the testing phase for HA or LA conditions, respectively (for a comprehensive description see Materials and Methods). By using these different experimental conditions, we were able to induce a high or a low state in the animal, independently of any drug administration.

To first characterize the behavioral responses of rats to different environmental situations in the Spatial Open Field task, regardless of any drug administration, we analyzed the performance of vehicle-treated rats exposed to a HA or a LA context. The analysis of the first session of the Spatial Open Field task (when no objects were present) showed that locomotor activity was not influenced by the two different arousal conditions, while the different environmental situations influenced the level of emotional reactivity of the animals. Vehicle-treated rats, exposed to the LA context, spent indeed more time in the center of the open field than vehicle-treated rats exposed to the HA context. This result indicates that the LA environment may induce a lower level of emotional activation (Prut

and Belzung, 2003). The view that LA condition induces a lower level of emotional activation is also supported by behavioral analysis derived from sessions 2 to 6 of the task, in which the rats encountered different objects, also located in different positions in the open-field arena. Rats exposed to the LA context spent more time investigating the objects than rats exposed to the HA context, suggesting that a lower state of anxiety urges animals to better explore the objects (Crawley, 1985). Concerning the cognitive performance, the different level of emotional activation derived by exposure to the two environmental conditions did not influence the cognitive parameters measured in the task. Indeed, vehicle-treated rats exposed to either HA or LA conditions were equally able to recognize the object substitution but failed to respond to the object displacement. Interestingly, Ricceri and co-workers (Ricceri et al., 2000) showed that only 90-day-old mice were able to discriminate a spatial object rearrangement, while 46-day-old mice were not. In our study, we used young adult rats; this leaves open the possibility that the ability to discriminate a spatial change has to be still developed by rats at this age. Moreover, our findings are in accordance with the general assumption that the capability to recognize a new setting of the environment is important for the species survival, but the impact of the object novelty is more salient than a spatial rearrangement with the same objects (Mumby et al., 2002).

Extensive evidence demonstrates that the Endocannabinoid system is a crucial regulator of emotionality and cognition (Marsicano et al., 2002; Laviolette and Grace, 2006a,b; Campolongo et al., 2009a,b; Mackowiak et al., 2009; Abush and Akirav, 2010; Akirav, 2011; Trezza et al., 2012). Although the neurobiological mechanisms underlying cannabinoid manipulation of emotional and cognitive functions have not yet been completely elucidated, previous evidence demonstrates that the anxiolytic effects induced by pharmacological enhancement of endocannabinoid tone strongly depend on the emotional state at the time of testing (Patel and Hillard, 2006)and that these effects are modulated by the level of emotional reactivity induced by high or low aversive experimental conditions (Haller et al., 2009). To further shed light on the role of environmental aversiveness in cannabinoid modulation of emotionality and cognitive performance, we investigated whether exogenous manipulation of the endocannabinoid

system influences rat behavior in the Spatial Open Field task in experimental conditions characterized by either a HA or LA state. Our findings clearly show that the effects of the endocannabinoid transport inhibitor AM404 on cognitive responses in the Spatial Open Field test strongly depend on the level of emotionality at the time of testing. Indeed, AM404 administration impaired the rat capability to discriminate between a familiar and a new object only in rats exposed to the HA condition. Several studies have shown that CB1 receptor agonists produce anxiolytic- (Patel and Hillard, 2006; Scherma et al., 2008) or anxiogenic-like (Viveros et al., 2005; Patel and Hillard, 2006) effects, depending on the dose tested. Conversely, indirect cannabinoid agonists, that increase ongoing Endocannabinoid signaling by interfering with their deactivation, induce anxiolytic-like effects without anxiogenic responses also when administered at high doses. For instance, the FAAH inhibitor URB597 produces anxiolytic-like effects in the elevated zero-maze and in the ultrasonic vocalization test in rats (Kathuria et al., 2003). In accordance with these findings, FAAH knockout mice exhibit an anxiolytic-like phenotype in the elevated plus-maze and in the light-dark box tests (Naidu et al., 2007; Moreira et al., 2008, 2009). Anxiolytic-like effects can also be induced by the inhibition of the endocannabinoid transport operated by endocannabinoid uptake inhibitors like AM404 (Beltramo et al., 1997; Beltramo and Piomelli, 2000). Thus, it has been demonstrated that the systemic administration of AM404 produces anxiolytic-like effects in three rat models of anxiety: elevated plus maze, defensive withdrawal, and separation-induced ultrasonic vocalization tests, and these effects are blocked by the administration of the CB antagonist rimonabant (Bortolato et al., 2006; Patel and Hillard, 2006). Nevertheless, it should be noted that in an another study Moreira and co-workers (Moreira et al., 2007) found that co-administration of anandamide and AM404 in the rat periaqueductal gray (a brain structure related to aversive response) elicited anxiolytic-like responses in the elevated plus maze test, whereas AM404 alone did not. In the present study, we found that administration of AM404 did not influence the emotional parameters taken onto consideration in the Spatial Open Field test, like the time spent in the central part of the arena during the first session of the task. However, it is important to note that, while AM404 administration did not influence the investigation of the objects through session

2–6 in a context characterized by a low-level of emotional activation, rats treated with the lower dose of AM404 and exposed to a stressful environment spent less time investigating objects, whereas the higher doses re-established the investigation activity at similar level of the vehicle-treated rats. The inhibition or the maintenance of the investigative behavior can be related to an anxiogenic or an anxiolytic phenotype, respectively (Crawley, 1985). It is possible to speculate that this biphasic effect may depend on a differential regulation activity on both GABAergic and gutamatergic neurons mediated by different doses of the Endocannabinoid transport inhibitor (Foldy et al., 2007; Hashimotodani et al., 2007).

Regarding the cognitive performance, here we show for the first time that a pharmacologically-induced enhancement of endocannabinoid tone differentially modulates memory recognition in rats depending on different emotional states and different nature of the considered cognitive parameters (e.g., either spatial or novel object discrimination). Concerning the object displacement, although the results did not reach any statistical significance it could be important to note that the treatment effect profile resemble a trend of a typical U-shaped dose response curve, in accordance with other results showing a similar dose-dependent biphasic response induced by cannabinoids, particularly by anandamide (Sulcova et al., 1998) and by the psychoactive constituent of Cannabis sativa preparation △9-tetrahydrocannabinol (Onaivi et al., 1990; Valjent et al., 2001). Concerning the object substitution, the lower doses of AM404 disrupted the ability to recognize a novel object in a stressful condition (HA) but not in a low arousal context (LA). It is well-established that the capability to recognize a new setting of the environment is important for species survival, but also that the impact of the object novelty ismore salient than a spatial rearrangement with the same objects (Mumby et al., 2002). However, the capability to discriminate a novel object in the arena can be lost under particular circumstances such as in a more stressful context, after repeated exposure to an aversive environment and experimental manipulation of the endocannabinoid tone as in the present study (Save et al., 1992;Mumby et al., 2002; Hebda-Bauer et al., 2010). These data confirm previous findings showing similar effects in humans and laboratory animals where acute or chronic exposure to the psychoactive

constituent of cannabis, △9-tetrahydrocannabinol, induces impairment in cognitive function (Egerton et al., 2006; Ranganathan and D'Souza, 2006; Solowij and Battisti, 2008; Campolongo et al., 2009c, 2011; D'Souza et al., 2009; Sofuoglu et al., 2010). In rodents, cannabinoid direct agonists induce impairment in several cognitive performances such as spatial learning, working memory, and attentional processes (Presburger and Robinson, 1999;Hampson and Deadwyler, 2000; Verrico et al., 2004; Robinson et al., 2007; Boucher et al., 2009, 2011). It is possible to speculate that these effects derive from cannabinoid-mediated disruption of cortical and hippocampal activity, crucially involved in encoding of the stimulus and making cognitive associations (Robbe et al., 2006; Deadwyler et al., 2007; Robbe and Buzsaki, 2009). The present results confirm the hypothesis that cannabinoid drugs, depending on the dose tested and the emotional state of the subject, could induce different effects on short-term memory parameters. The dissimilar effects induced by exposure to a different emotional state could depend on the activation of the hypothalamic-pituitary-adrenal (HPA) axis triggered by a HA context and to the subsequent release of stress hormones, such as glucocorticoids. It is well known that this axis plays a crucial role in the stress response and that these hormones differentially modulate cognitive functions (Roozendaal and McGaugh, 1997; Mizoguchi et al., 2004; Atsak et al., 2011). In particular, de Quervain and co-workers (2009) reported that elevated glucocorticoid levels, elicited by aversive contexts, impair memory retrieval, and working memory.Moreover, further studies, conducted by our group, shed light on the crucial role of endocannabinoid signaling in the basolateral complex of the amygdala in modulating consolidation of aversive memory by an interaction with the glucocorticoid system (Campolongo et al., 2009a,b; Hill et al., 2010; Atsak et al., 2011).

Taken together, the present findings support the hypothesis of a fundamental role of the environment in influencing both the behavioral and cognitive outcomes in the Spatial Open Field task. Most importantly, it emerges that drugs that enhance endocannabinoid signaling by interfering with endocannabinoid deactivation induce different effects on short-term memory performance depending on the level of emotional arousal induced by different environmental settings.

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Chapter 3

Double Dissociation of the Requirement for GluN2B- and GluN2A-Containing NMDA Receptors in the Destabilization and Restabilization of a Reconsolidating Memory

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Abstract

Signaling at NMDA receptors (NMDARs) is known to be important for memory reconsolidation, but while most studies show that NMDAR antagonists prevent memory restabilization and produce amnesia, others have shown that GluN2B-selective NMDAR antagonists prevent memory destabilization, protecting the memory. These apparently paradoxical, conflicting data provide an opportunity to define more precisely the requirement for different NMDAR subtypes in the mechanisms underlying memory reconsolidation and to further understand the contribution of glutamatergic signaling to this process. Here, using rats with fully consolidated pavlovian auditory fear memories, we demonstrate a double dissociation in the requirement for GluN2B-containing and GluN2A-containing NMDARs within the basolateral amygdala in the memory destabilization and restabilization processes, respectively. We further show a double dissociation in the mechanisms underlying memory retrieval and memory destabilization, since AMPAR antagonism prevented memory retrieval while still allowing the destabilization process to occur. These data demonstrate that glutamatergic signaling mechanisms within the basolateral amygdala differentially and dissociably mediate the retrieval, destabilization, and restabilization of previously consolidated fear memories.

Introduction

Memory reconsolidation is the process by which previously consolidated memories become destabilized at retrieval and require restabilization to persist in the brain (Lewis, 1979; Nader, 2003). The neurochemical basis of reconsolidation has been intensively studied, particularly the requirement for signaling at the NMDA subtype of glutamate receptor (NMDAR). However, although NMDAR-mediated signaling is required for the reconsolidation (restabilization) of conditioned stimulus (CS)–drug (Sadler et al., 2007; Brown et al., 2008; Itzhak, 2008; Milton et al., 2008; Milton et al., 2012), CS–spatial (Przybyslawski and Sara, 1997), and CS–fear (Pedreira et al., 2002; Lee et al., 2006) memories, antagonism at the GluN2B subtype of NMDAR has been shown to prevent the destabilization of CS–fear memories, thereby protecting them from the effects of amnestic agents (Ben Mamou et al., 2006). These paradoxical, apparently conflicting data provide the opportunity to better understand the contribution of signaling at glutamatergic receptors to the reconsolidation process.

NMDARs exist as tetramers, typically composed of two GluN1 and two GluN2 subunits (Dingledine et al., 1999). The GluN2 subunits consist of four different types (GluN2A–D), of which GluN2A and GluN2B have been the most studied. In addition to differences between GluN2A-containing (GluN2A-NMDARs) and GluN2B-containing NMDARs (GluN2B-NMDARs) in their sensitivity to glutamate and their activation kinetics, these subtypes of receptor also couple to different proteins within the postsynaptic density, activating divergent intracellular signaling pathways (Kim et al., 2005; Ivanov et al., 2006; Zhang et al., 2008). For example, the C-terminal domain of GluN2B-NMDARs suppresses CREB and activates the ubiquitin–proteasome system (UPS), while GluN2A-NMDAR activation promotes CREB phosphorylation and is neuroprotective (Hardingham et al., 2002; Martel et al., 2012). These differences at the molecular level may have important functional implications; activation of GluN2B-NMDARs promotes long-term depression (LTD), while activation of GluN2A-NMDARs promotes long-term potentiation (LTP) in the hippocampus (Liu et al., 2004).

The basolateral amygdala (BLA) is required for both CS–fear memory consolidation (Campeau and Davis, 1995; Killcross et al., 1997; Koo et al., 2004) and reconsolidation (Nader et al., 2000). Furthermore, NMDARs within the BLA have been implicated in both memory destabilization (Ben Mamou et al., 2006) and restabilization (Milton et al., 2008) processes. Thus, we hypothesized that memory destabilization and restabilization may be mediated through the different subtypes of NMDAR within the BLA, GluN2B-NMDARs being required for destabilization and GluN2A-NMDARs being required for restabilization. Furthermore, since AMPARs are required for memory retrieval (Day et al., 2003; Bast et al., 2005; Winters and Bussey, 2005) and because memory reconsolidation can only occur when a memory is retrieved (Lewis, 1979; Nader, 2003), we further hypothesized that AMPARs would be necessary for the destabilization process. Finally, we investigated the effects of reducing presynaptic glutamate release by treatment with an agonist at metabotropic 2/3 glutamate receptors (mGlu2/3Rs) on the balance of these mnemonic processes. We hypothesized that the memory should neither be retrieved nor destabilized, and therefore restabilization of the memory would not be required for it to persist.

Materials and Methods

Subjects.

Subjects were 93 male Lister–Hooded rats (Charles River Laboratories) housed in pairs in a vivarium on a reversed light-dark cycle (lights on at 1900 h). Subjects were food restricted, although not deprived, being fed 25 g per rat of lab chow after training or testing each day. Access to water was *ad libitum* except for when inside the conditioning chambers. All procedures were conducted in accordance with the UK Animals (Scientific Procedures) Act 1986.

Surgery.

Rats were implanted with bilateral guide cannulae (16 mm, 24 gauge; Coopers Needle Works) located just dorsal to the basolateral amygdala (Fig. 1) as described previously (Milton et al., 2008). The coordinates for cannula implantation were anteroposterior − 2.6

mm and mediolateral ± 4.5 mm (relative to bregma) and dorsoventral − 5.6 mm (relative to dura). A recovery period of 7 days was given before behavioral training and testing began.

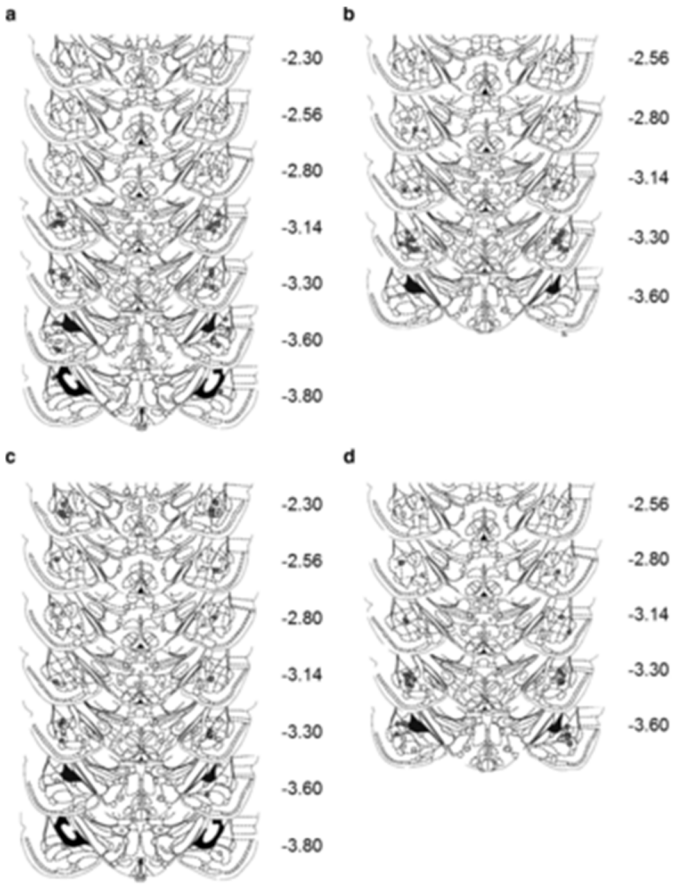

Figure 1. Cannulae placements. All cannulae placements were within the BLA. The placements for individual experiments are shown separately. For each placement the white circles represent the vehicle/vehicle group, the white squares the vehicle/anisomycin group, the gray circles the drug/vehicle group, and the gray squares the drug/anisomycin group where the drugs were IFEN (***a***), NVP-AAM077 (***b***), LY293558 (***c***), and LY317206 (***d***). Coordinates are given from bregma. This figure was modified, with permission, from Paxinos and Watson (2004).

Intracerebral drug administration.

Infusions were carried out using a syringe pump (Harvard Apparatus) and 5 µl Hamilton syringes connected to injectors (28 gauge, projecting 2 mm beyond the guide cannulae; Plastics One) by polyethylene tubing. The rats received two infusions: one immediately before the memory reactivation session, and one immediately afterward. All infusions were begun 30 s after the insertion of the injectors and performed over 2 min at a rate of 0.25 µl min^{-1} (total volume of 0.5 µl $side^{-1}$). One minute of waiting time was imposed from the end of the infusion to the removal of the injectors to allow diffusion of the solution away from the infusion site.

Drugs.

Rats received either the protein synthesis inhibitor anisomycin or its vehicle as their second (post-reactivation) infusion. Anisomycin (125 µg $µl^{-1;}$ Sigma-Aldrich) was dissolved in equimolar HCl and then pH balanced to pH 7.4 with NaOH. This dose of anisomycin has previously been shown to disrupt memory reconsolidation (Ben Mamou et al., 2006).
Prior to memory reactivation, rats received infusions of drugs targeting the glutamatergic signaling system or the appropriate vehicle. The GluN2B diheteromeric receptor-selective (Williams, 1993) NMDAR antagonist ifenprodil (Ascent Scientific) was dissolved in PBS at a concentration of 2 µg $µl^{-1}$; this dose has previously been shown to disrupt memory destabilization (Ben Mamou et al., 2006). The GluN2A-preferring (Auberson et al., 2002) NMDAR antagonist NVP-AAM077 (Sigma-Aldrich) was dissolved in PBS at a concentration of 5 µg $µl^{-1}$; this dose has been shown to reduce the expression of fear-potentiated startle (Walker and Davis, 2008). The mGlu2/3R agonist LY317206 (Doherty et al., 1999) and the AMPAR antagonist LY293558 (Ornstein et al., 1993) were both generous gifts from Eli Lilly. LY317206 was dissolved in PBS at a concentration of 2 µg $µl^{-1}$, and LY293558 in ddH_2O at a concentration of 1.33 µg $µl^{-1}$. This dose of LY317206 has been shown to reduce fear-potentiated startle (Walker et al., 2002), and the dose of LY293558 is higher than the ineffective doses used previously in the amygdala (Di Ciano and Everitt, 2004).

Behavioral procedures

Testing took place in four conditioning chambers (Med Associates) with the assignment of individual conditioning chambers counterbalanced across experimental groups within each experiment. Rats were first habituated to the context in a 2 h session in which neither the clicker CS nor the shock unconditioned stimulus (US) were presented. Following the end of this habituation session, they were returned to the home cage. Twenty-four hours later, they were placed back into the same experimental chamber for the fear conditioning session; during this time, they were first exposed to the context (with no CS or US) for 35 ± 1 min, then exposed to a single CS–US pairing of a clicker (10 Hz, 80 dB, 60 s) CS and a 0.5 mA, 1 s scrambled footshock US. For the experiment investigating the requirement of AMPARs in memory reconsolidation, a different brand of experimental chamber (Paul Fray Limited) was used, but it was set up in the same configuration as the Med Associates chambers, other than the shock duration (0.5 s). To ensure comparability in the strength of learning, five CS-shock pairings were used in this experiment. All other aspects of the behavioral procedures remained the same.

Twenty-four hours following the end of training, the rats were returned to the same conditioning chambers for a memory reactivation session. After 1 min of context exposure, the clicker CS was presented for 60 s. The session was recorded through a CCTV system onto a DVD to allow for offline manual scoring of behavior. Two CS–fear memory tests were conducted following the memory reactivation session; these test sessions, conducted 24 h and 8 d following reactivation, had the same format as the memory reactivation session, except that no drug infusions were given.

Briefly, and as before (Ben Mamou et al., 2006), if memory destabilization was prevented by the pre-reactivation infusion, then anisomycin would not have an amnestic effect when it was subsequently infused, since the memory would not be in a destabilized and labile state when anisomycin was applied. If memory restabilization was prevented by the pre-reactivation infusion, then all experimental groups treated with the drug would be predicted to show amnesia at subsequent test, as would a group that had received an infusion of vehicle followed by anisomycin. If neither destabilization nor restabilization

were prevented by the pre-reactivation infusion, then only the two groups that had received post-reactivation anisomycin would be predicted to show amnesia.

Histology

At the end of the experiments, the rats were killed with an overdose of anesthetic (Dolethal, Vétoquinol) and transcardially perfused through the ascending aorta with 0.01 m PBS, followed by 4% paraformaldehyde (PFA). The brains were removed and stored in 4% PFA for at least 24 h before being transferred to a 20% sucrose solution for cryoprotection before sectioning. The brains were sectioned at 60 µm and stained with cresyl violet. Cannulae placements (Fig. 1) were verified using light microscopy (Leica).

Data collection and statistical analysis

Data for the reactivation and test sessions were scored offline and blind to treatment by A.L.M. Data from the conditioning session were not recorded because there is no measure of conditioned freezing when animals receive only one pairing of the CS with shock. However, shock delivery was recorded by the experimenter observing the unconditioned response in the conditioning session. Freezing was defined as a cessation of movement apart from respiration and was measured instantaneously at 5 s intervals. Freezing during the first minute of the session was assessed to provide a measure of fear to the context, and the second as a measure of fear to the CS. All data were converted to percentages before analysis. Data were analyzed by repeated-measures ANOVA with CS (context vs cue) and Session (reactivation vs 24 h test vs 8 d test) as within-subject factors, and Drug 1 [vehicle (VEH) vs drug] and Drug 2 [VEH vs anisomycin (ANI)] as between-subjects factors. For clarity, these are reported in the text using the name of the drug used in the experimental group (e.g., Drug 2 is shown as ANI). Where the data violated the assumption of sphericity as assessed using Mauchly's test, a correction was applied; the Greenhouse–Geisser correction if $\varepsilon < 0.75$, and the Huynh–Feldt correction if $\varepsilon > 0.75$, as recommended by Cardinal and Aitken (2006). Where appropriate, further ANOVAs or pairwise comparisons were conducted; all pairwise comparisons were adjusted using the

Šidák correction, which is a mathematically accurate form of the Bonferroni estimation (Cardinal and Aitken, 2006).

Results

GluN2B-NMDARs are required for memory destabilization, not restabilization

Administration of the GluN2B-selective NMDAR antagonist ifenprodil (IFEN) directly into the BLA before memory reactivation prevented the destabilization of the CS–fear memory. This was shown by the prevention of anisomycin-induced amnesia when IFEN was given before reactivation, but not when the vehicle was given before reactivation (Fig. 2*b*). All groups had previously conditioned to the CS, as all rats showed greater freezing to the CS than the context in the reactivation and the test sessions [$F_{1, 25}$ = 33.0, p < 0.001, η^2 = 0.57]. While the VEH/ANI group showed less freezing than the VEH/VEH group at the 24 h test [$F_{(1, 14)}$ = 5.8, p = 0.031, η^2 = 0.29], the IFEN/ANI group froze comparably to the IFEN/VEH group [F < 1, p = 0.66]. Analysis of the 8 d test was complicated by an overall reduction in conditioned freezing [CS × session: $F_{1, 25}$ = 4.7, p = 0.04, η^2 = 0.16] most likely attributable to the gradual extinction that occurred after the last nonreinforced retrieval test. However, while the VEH/VEH and VEH/ANI groups showed equivalent conditioned freezing at the 8 d test [$F_{1, 14}$ = 2.2, p = 0.16], so did the IFEN/VEH and IFEN/ANI groups [$F_{1, 11}$ = 3.4, p = 0.09], consistent with the prevention of destabilization of the CS–fear memory by IFEN.

IFEN did not acutely affect the retrieval of the conditioned fear memory (Fig. 2*a*); collapsing across groups for the first infusion (VEH, n = 16; IFEN, n = 13) there was no difference in the level of conditioned freezing shown during the memory reactivation session [F < 1, p = 0.95]. These findings confirm the previous report (Ben Mamou et al., 2006) that GluN2B-NMDARs are required for memory destabilization.

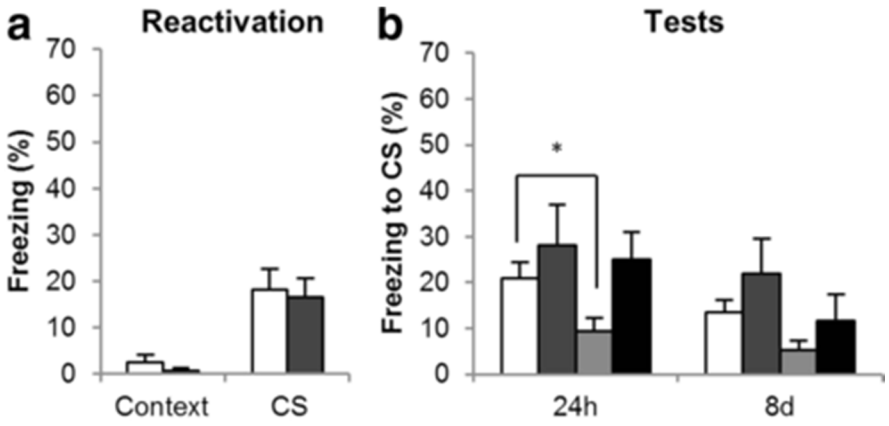

Figure 2. Effects of the GluN2B-selective NMDAR antagonist on CS–fear memory reconsolidation. Administration of the GluN2B-selective NMDAR antagonist had no effect on the retrieval of the CS–fear memory at reactivation (***a***), but it did prevent the destabilization of the CS–fear memory (***b***). Data are presented as means ± SEM. Group sizes (and colors in ***b***) were VEH/VEH, $n = 8$ (white); IFEN/VEH, $n = 8$ (mid-gray); VEH/ANI, $n = 8$ (pale gray); and IFEN/ANI, $n = 5$ (dark gray). In ***a*** the groups are collapsed with the white bar representing the average of the VEH/VEH and VEH/ANI groups, and the gray bar the IFEN/VEH and IFEN/ANI groups. Asterisk (*) denotes $p < 0.05$.

GluN2A-NMDARs are required for memory restabilization, not destabilization

Administration of the GluN2A-preferring NMDAR antagonist NVP-AAM077 (NVP) before the memory reactivation session reduced conditioned freezing at subsequent tests conducted 24 h and 8 d later (Fig. 3*b*). All rats had conditioned to the CS, as all groups showed greater freezing to the CS than the context in the reactivation and the test sessions [$F_{1, 15} = 12$, $p = 0.003$, $\eta^2 = 0.45$]. Administration of NVP reduced conditioned freezing in the subsequent test sessions [CS × Session × NVP: $F_{2, 30} = 4.8$, $p = 0.016$, $\eta^2 = 0.24$], but not during the reactivation session. Analyses of individual sessions showed that there were no differences between experimental groups during reactivation [CS × NVP: $F_{1, 15} = 2.3$, $p = 0.15$; CS × ANI: $F < 1$, $p = 0.52$; CS × NVP × ANI: $F < 1$, $p = 0.87$], but NVP-treated animals froze less than VEH-treated rats during the test at 24 h [CS × NVP: $F_{1, 15} = 8.5$, $p = 0.011$, $\eta^2 = 0.36$]. The effect of NVP was persistent in that there was no overall reduction in freezing between the 24 h and the 8 d test [$F< 1$], although there was a reduction in the VEH-treated groups [CS × Session × NVP: $F_{1, 15} = 9.75$, $p = 0.007$, $\eta^2 = 0.39$;

pairwise comparisons showed a reduction in conditioned freezing between the 24 h and 8 d test in the VEH-treated groups, $p = 0.024$, but not in the NVP-treated groups, $p = 0.22$]. This is consistent with extinction of the CS–US association in the VEH/VEH group, but not in the other experimental groups, which could not reduce freezing any further. Pairwise comparisons also revealed that the NVP-treated groups did not differ from the VEH/ANI group [all p values > 0.41]. Thus, administration of NVP produced amnesia regardless of whether anisomycin was also administered. Furthermore, the amnesia shown by the NVP/ANI group supports the view that NVP did not block destabilization, because if it had then this group would have shown intact memory.

NVP did not acutely affect the retrieval of the conditioned fear memory during the reactivation session (Fig. 3*a*); collapsing across groups for the first infusion (VEH, $n = 9$; NVP, $n = 10$), despite the apparent numerical reduction in freezing in the NVP-treated group, there was no statistical difference between freezing levels in the two experimental groups [$F_{1, 17} = 2.4$, $p = 0.14$]. However, the NVP-treated group showed levels of freezing that did not significantly differ from zero [$p = 0.34$] consistent with the previous observation that this dose of NVP reduces the expression of fear-potentiated startle (Walker and Davis, 2008). This result supports our hypothesis that GluN2A-NMDARs are required for memory restabilization while not being required for memory destabilization.

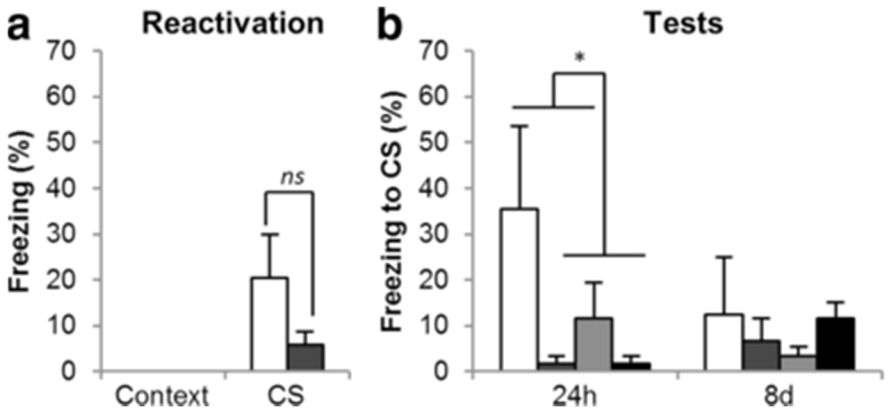

Figure 3. Effects of the GluN2A-preferring NMDAR antagonist on CS–fear memory reconsolidation. Administration of the GluN2A-preferring NMDAR antagonist had no effect on the retrieval of the CS–fear memory at reactivation (***a***), but it prevented the restabilization of the CS–fear memory (***b***). Data are presented as means ± SEM. Group sizes (and colors in ***b***) were: VEH/VEH, $n = 4$ (white); NVP/VEH, $n = 5$ (mid gray); VEH/ANI, $n = 5$ (pale gray); and NVP/ANI, $n = 5$ (dark gray). In ***a*** the groups are collapsed, with the white bar representing the average of the VEH/VEH and VEH/ANI groups, and the gray bar the NVP/VEH and NVP/ANI groups. Asterisk (*) denotes $p < 0.05$; *ns* denotes nonsignificant differences

AMPARs are not required for destabilization or restabilization

Administration of the AMPAR antagonist LY293558 before memory reactivation did not reduce conditioned freezing during the test sessions and did not prevent the post-reactivation anisomycin infusion from inducing amnesia (Fig. 4*b*). All rats had conditioned to the CS, as they showed greater freezing to the CS than the context following training [$F_{1, 21} = 37.7$, $p < 0.001$, $\eta^2 = 0.64$]. LY293558 did not affect freezing in the 24 h test session [CS × LY293558: $F < 1$, $p = 0.45$], but anisomycin infusion resulted in amnesia [CS × ANI: $F_{1, 21} = 16$, $p = 0.001$, $\eta^2 = 0.44$]. Indeed, the group that received anisomycin following LY293558 froze less than the group that received vehicle before reactivation [CS × ANI: $F_{1, 9} = 6.0$, $p = 0.037$, $\eta^2 = 0.40$], demonstrating that LY293558 did not prevent the destabilization of the memory. This anisomycin-induced amnesia was also observed at the 8 d test [$F_{1, 21} = 7.82$, $p = 0.011$, $\eta^2 = 0.27$], even though there was extinction of conditioned freezing at the 8 d test compared to the 24 h test [$F_{1, 21} = 10.1$, $p = 0.005$, $\eta^2 =$

0.32]. This is consistent with a previous report in which the AMPA/kainate receptor antagonist CNQX was shown to leave memory destabilization and restabilization intact when given before memory reactivation (Ben Mamou et al., 2006).

LY293558 acutely reduced conditioned freezing during the memory-reactivation session (Fig. 4*a*); collapsing across groups for the first infusion (VEH, $n = 14$; LY293558, $n = 11$), the groups given LY293558 before the memory reactivation froze to the CS less than groups that had received the infusion of vehicle [CS × LY293558: $F_{1, 23} = 6.8$, $p = 0.016$, $\eta^2 = 0.23$]. Thus, AMPARs are required for memory retrieval but not memory destabilization.

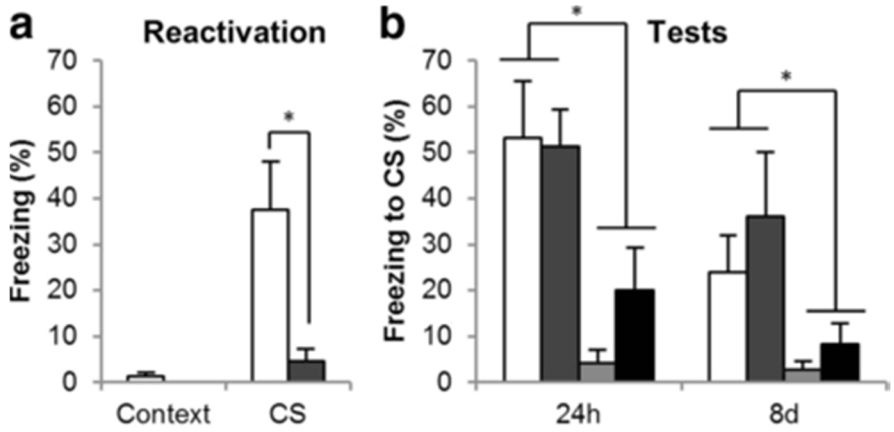

Figure 4. Effects of the AMPAR antagonist on CS–fear memory reconsolidation. The AMPAR antagonist LY293558 acutely impaired the retrieval of the CS–fear memory at reactivation (***a***), but it affected neither the destabilization nor the restabilization of the CS–fear memory (***b***). Group sizes (and colors in ***b***) were VEH/VEH, $n = 8$ (white); LY293558/VEH, $n = 6$ (mid gray); VEH/ANI, $n = 6$ (pale gray); and LY293558/ANI, $n = 5$ (dark gray). In ***a*** the groups are collapsed, with the white bar representing the average of the VEH/VEH and VEH/ANI groups and the gray bar the LY293558/VEH and LY293558/ANI groups. Asterisks (*) denote $p < 0.05$.

Blockade of Glu release left destabilization and restabilization intact

LY317206, an agonist at presynaptic class II/III metabotropic glutamate receptors (mGlu2/3Rs), affected neither the destabilization nor the restabilization of the CS–fear memory (Fig. 5*b*). All groups had conditioned to the CS during training, as shown by increased freezing during the CS compared to the context [$F_{1, 16} = 29$, $p < 0.001$, $\eta^2 = 0.64$].

However, although anisomycin produced amnesia as assessed at the 24 h test [CS × ANI: $F_{1, 16} = 7.2$, $p = 0.016$, $\eta^2 = 0.31$], there was no effect of LY317206 on conditioned freezing [CS × LY317206: $F < 1$, $p = 0.74$], indicating that it did not prevent the restabilization of the CS–fear memory. Furthermore, as the LY317206/ANI group showed less freezing to the CS than the LY317206/VEH group at the 24 h test [CS × ANI: $F_{1, 10} = 11$, $p = 0.008$, $\eta^2 = 0.52$], the memory destabilized during the reactivation session, and this process was not prevented by agonism at mGlu2/3Rs. The anisomycin-induced amnesia observed at 24 h persisted at the trend level in the 8 d test [$F_{1, 16} = 4.14$, $p = 0.059$, $\eta^2 = 0.21$], although the data from the 8 d test were compromised by the extinction of conditioned freezing in the VEH/VEH group [pairwise comparisons revealed reduced conditioned freezing at the 8d test in this group, $p = 0.035$, but no differences in the other groups, all p values > 0.49]. LY317206 did not acutely affect the retrieval of the conditioned fear memory during the reactivation session (Fig. 5*a*); collapsing across groups for the first infusion (VEH, $n = 8$; LY317206, $n = 12$), there was no statistically significant difference between freezing levels in the two groups [$F_{1, 18} = 2.4$, $p = 0.14$] despite the numerical reduction in conditioned freezing. Thus, agonism at presynaptic mGlu2/3Rs did not affect the destabilization or restabilization and did not produce a statistically significant reduction in the retrieval of the CS–fear memory.

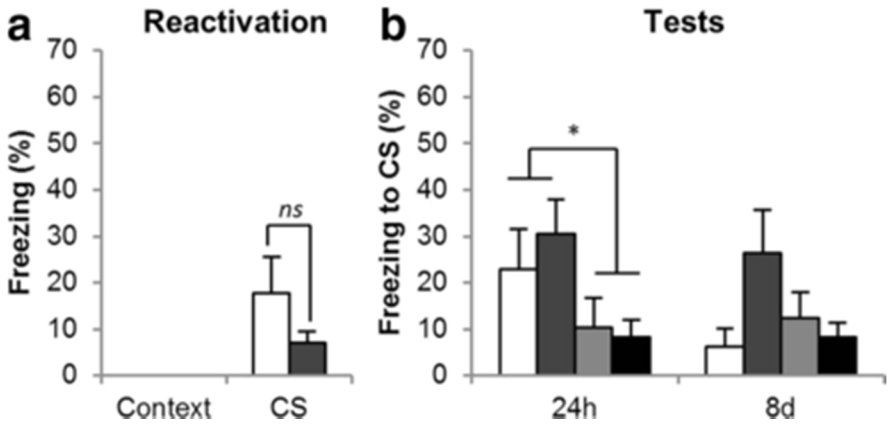

Figure 5. Effects of the mGlu2/3R agonist on CS–fear memory reconsolidation. The mGlu2/3R agonist LY317206 did not prevent retrieval of the CS–fear memory at reactivation (***a***), and it prevented neither destabilization nor restabilization of the CS–fear memory (***b***). Group sizes (and colors in ***b***) were VEH/VEH, $n = 4$ (white); LY317206/VEH, $n = 6$ (mid gray); VEH/ANI, $n = 4$ (pale gray); and LY317206/ANI, $n = 6$ (dark gray). In ***a*** the groups are collapsed, with the white bar representing the average of the VEH/VEH and VEH/ANI groups and the gray bar the LY317206/VEH and LY317206/ANI groups. Asterisk (*) denotes $p < 0.05$; *ns* denotes nonsignificant differences.

Discussion

The present data support the view that GluN2B-NMDARs and GluN2A-NMDARs within the BLA are required for memory destabilization and restabilization, respectively. This is the first demonstration of a double dissociation in the function of these two subtypes of NMDAR in memory reconsolidation. Furthermore, we also demonstrate a double dissociation between memory retrieval (dependent upon AMPARs) and the induction of memory lability (dependent upon GluN2B-NMDARs).

The doubly dissociable involvement of different NMDAR subtypes in destabilization and restabilization enables resolution of the apparent discrepant findings in the literature that antagonism at GluN2B-NMDARs prevents memory destabilization (Ben Mamou et al., 2006), but that nonselective NMDAR antagonism with d-APV (Milton et al., 2008) or MK-801 (Przybyslawski and Sara, 1997; Pedreira et al., 2002; Sadler et al., 2007; Brown et al., 2008; Itzhak, 2008; Lee and Everitt, 2008; von der Goltz et al., 2009) prevents the restabilization of memories. We hypothesize (Fig. 6) that the differential effects of

nonsubtype-selective NMDAR antagonists on these mnemonic processes depend upon the balance between signaling at GluN2B-NMDARs and GluN2A-NMDARs, since only after the brief destabilization process is there a requirement for the longer-lasting restabilization process to be engaged. The differences in duration of the two processes, subserved by different subpopulations of NMDAR expressed within BLA neurons (Müller et al., 2009), would suggest that the nonsubtype-selective NMDAR antagonists tested in previous studies appear to be sufficient to prevent GluN2A-dependent signaling, but fail significantly to affect GluN2B-dependent signaling.

Thus, we hypothesize that nonselective NMDAR antagonists exert their amnestic effects primarily through GluN2A-containing NMDARs. Consistent with this view are the findings that GluN2A-preferring NMDAR antagonists prevent the acquisition of spatial memory (Hu et al., 2009) and conditioned fear (Dalton et al., 2012), as do nonsubtype-selective NMDAR antagonists (Morris et al., 1986; Davis et al., 1992; Fanselow and Kim, 1994). Furthermore, the effects of nonselective NMDAR antagonists on other processes, such as cortical oscillations, are more similar to the effects of GluN2A-preferring NMDAR antagonists than GluN2B-selective NMDAR antagonists (Kocsis, 2012). Thus, the glutamate transmission-dependent destabilization and restabilization processes would be predicted to engage parallel, independent molecular mechanisms mediated by the two subtypes of NMDAR. This perspective can be integrated with already known mechanisms underlying memory destabilization; for example, GluN2B-containing NMDARs recruit the ubiquitin-proteasome system via CaMKII (Bingol et al., 2010), thus allowing the protein degradation that is required for the induction of memory lability (Lee et al., 2008)

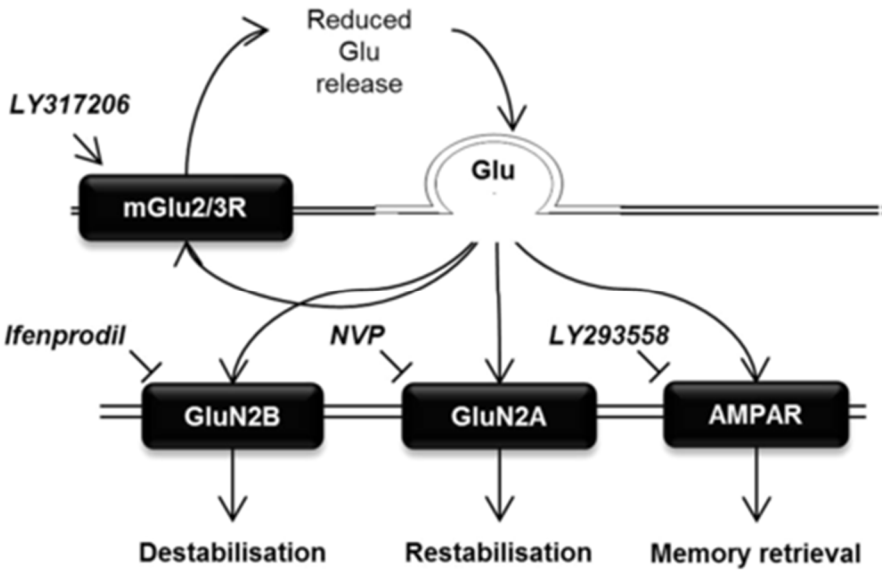

Figure 6. Hypothesized glutamatergic mechanisms underlying memory stability. GluN2B-containing NMDARs are required for memory destabilization, and IFEN prevents this process. GluN2A-containing NMDARs are required for memory restabilization, which is prevented by NVP-AAM077. The AMPAR antagonist LY293558 reduces memory retrieval but has no effect on destabilization or restabilization. The presynaptic mGlu2/3R agonist LY317206 reduces glutamate release, reducing memory retrieval via AMPARs but leaving the balance of GluN2A and GluN2B activity intact (thus having no effect on destabilization or restabilization).

Surprisingly, we found a further dissociation in the mechanisms underlying memory retrieval (dependent upon AMPARs) and destabilization (dependent upon GluN2B-NMDARs). These data refute our original hypothesis concerning AMPARs, although they are consistent with previous findings that signaling via AMPARs is necessary for memory expression and retrieval (Day et al., 2003; Bast et al., 2005; Winters and Bussey, 2005). It is perhaps surprising, from a theoretical perspective, that memory retrieval and destabilization might be disrupted independently, since reconsolidation theories maintain that memories must be retrieved to become once again susceptible to disruption with amnestic agents (Lewis, 1979; Nader, 2003). We therefore propose that the processes of memory retrieval and memory destabilization are doubly dissociable, but that behavioral

procedures used to induce memory destabilization often induce memory retrieval as well. This hypothesis clearly warrants further investigation.

As predicted, the LY317206-induced reduction in glutamate release (Doherty et al., 1999) resulted in decreased activity at AMPARs, GluN2B-NMDARs, and GluN2A-NMDARs and hence no observable effect on memory retrieval, destabilization, or restabilization. Although this is consistent with the mechanisms shown in Figure 6, these data also indicate that such drugs are unlikely to have utility in the treatment of maladaptive memories that characterize some neuropsychiatric disorders. There has been interest in indirectly modulating signaling at NMDARs by reducing glutamate release presynaptically since, as has been noted previously (Milton and Everitt, 2010), systemic NMDAR antagonists are unlikely to be used clinically because of their problematic psychotomimetic side effects. However, our data do support the view that selectively targeting GluN2A-NMDARs may provide a useful therapeutic strategy; the psychotomimetic effects of systemic NMDAR antagonism are likely mediated through GluN2B-NMDARs (De Vry and Jentzsch, 2003), and so it may be possible to develop GluN2A-NMDAR-selective therapies that are appropriate for clinical use.

The results of these experiments reveal the complexity of the glutamatergic mechanisms underlying CS–fear memory reconsolidation within the BLA. In summary, GluN2A-containing and GluN2B-containing NMDARs have dissociable roles in memory restabilization and destabilization, respectively. While AMPARs are required for memory retrieval, they do not appear necessary for memory destabilization, suggesting that these two processes are independently regulated. Furthermore, agonism at presynaptic mGlu2/3Rs has no overall effect on the strength of a CS–fear memory. In addition to further elucidating the glutamatergic mechanisms underlying the reconsolidation of fear memories, these data also further support the possible utility of modulating specific glutamate receptors in the clinical treatment of anxiety disorders to disrupt persistent maladaptive and intrusive memories.

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Chapter 4

The CB1 receptor antagonist AM251 impairs reconsolidation of pavlovian fear memory in the rat basolateral amygdale

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In preparation

Introduction

Memory reconsolidation refers to a process through which a previously consolidated and recalled memory turns into a labile state and become susceptible to being manipulated. Thus, this instability could result in newly strengthened and/or integrated or disrupted memories. For example, pharmacological manipulation before or immediately after retrieval, could prevents (Debiec *et al*, 2002; Milton *et al*, 2013; Nader *et al*, 2000; Taubenfeld *et al*, 2009; Wang *et al*, 2009) or enhances the expression of conditioned fear response (Lee *et al*, 2006b). That is, manipulation of reconsolidation process could be used as a novel tool to disrupt maladaptive memories in neuropsychiatric disorders, such as post-traumatic stress disorder, in humans (Brunet *et al*, 2008; Debiec and LeDoux, 2006; Oyarzun *et al*, 2012; Schiller *et al*, 2010).

Growing evidence indicates a fundamental involvement of the endocannabinoid system in regulation of memory processing of emotionally salient events (Atsak *et al*, 2012; Campolongo *et al*, 2009b; Campolongo and Trezza, 2012b; Hauer *et al*, 2011). This is consistent with the localization of cannabinoid receptors (CB) throughout the cortico-limbic system, in brain regions involved in regulation of learning and memory such as hippocampus, basolateral amygdala (BLA) and prefrontal cortex. Recently it has been shown that bilateral intra-BLA infusion of the direct cannabinoid receptor agonist WIN55,212-2 immediately after inhibitory avoidance training enhances memory consolidation in rats (Campolongo *et al*, 2009b). However, the use of drugs that directly bind and activate brain cannabinoid receptors may be limited by their abuse liability. Indirect cannabinoid agonists, that increase endocannabinoid signalling by interfering with endocannabinoid degradation/transport, are emerging as a new pharmacological tool. The enhancing effect on memory consolidation has been confirmed recently by the enhancement of memory consolidation, induced by potentiation of the endocannabinoid tone, through inhibition of the enzyme responsible of endogenous cannabinoid degradation Fatty Acid Amide Hydrolase (FAAH). The indirect cannabinoid agonist URB597, a FAAH-inhibitor agent, bilaterally infused in the BLA, enhanced consolidation for aversive memory and this effect is prevented by blocking CB1 receptors with infusion of the CB1 receptor antagonist AM251 (Ratano *et al*, 2011).

However, the role of endocannabinoid system in memory reconsolidation is still poorly understood. Indeed, evidence indicates that the activation or inactivation of endocannabinoid receptors bidirectionally modulate memory reconsolidation of aversive events (de Oliveira Alvares L. *et al*, 2008; Kobilo *et al*, 2007; Lin *et al*, 2006; Suzuki *et al*, 2008). However, while the CB1 receptor agonist, WIN 55,212-2 was reported to impair reconsolidation of fear-potentiated startle after CS re-exposure (Lin *et al*, 2006), the CB1 receptor antagonist rimonabant did not enhance memory or produce amnesia, suggesting that CB1 receptors are not required for memory reconsolidation (Suzuki *et al*, 2004). In a more recent study, Stern and co-workers demonstrate that the phytocannabinoid cannabidiol is able to disrupt contextual fear memories when systemically administered immediately after memory reactivation (Stern CA *et al*, 2012) suggesting that the effect induced by cannabidiol is possibly dependent on cannabinoid type-1 receptor-mediated signaling mechanisms.

However these poor and contrasting findings do not help in clarifying how the endocannabinoid system is involved in reconsolidation process. In order to better understand how the endocannabinoid system influence memory reconsolidation, in the present study we aimed to evaluate the effects of the endocannabinoid system manipulation directly within the basolateral amygdala on reconsolidation of pavlovian fear memory in an auditory fear conditioned paradigm, as well as the mechanisms underlying memory processing after cue re-exposure during memory recall. Moreover, as it is known that CB1 receptors localize presynaptically in the BLA on a distinct subpopulation of GABAergic interneurons (Katona *et al*, 2001), interaction between endocannabinoid and GABAergic neurotransmission was evaluated in order to investigate the mechanisms underlying the effects on memory reconsolidation after pharmacological manipulation of CB1 receptors.

Materials and Methods

Subjects.

112 Male Lister-Hooded rats (300-320 at the time of surgery, Charles River) were housed in pairs in a *vivarium* on a reversed light-dark cycle (lights on at 1900hrs). All subjects

were food restricted, but not deprived, being fed 25 g per rat of lab chow after training or testing each day starting from the day of surgery. Access to water was *ad libitum* except for when inside the conditioning chambers and during infusion procedure. All procedures were conducted in accordance with the UK Animals (Scientific Procedures) Act 1986 and the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes.

Surgery.

Rats were anesthetized with intramuscular injection of a mixture of ketamine (80mg/kg) and xylazine (10mg/kg) and implanted with bilateral guide cannulae (16mm, 24 gauge; Coopers Needle Works Ltd) located just dorsal to the basolateral amygdala as described previously (Milton *et al*, 2008a). The co-ordinates for cannula implantation were AP - 2.6 mm and ML ± 4.5 mm (relative to bregma) and DV – 5.6 mm (relative to dura). Stainless steel obdurators were inserted into both cannulae to maintain patency. A recovery period of at least 7 days was given prior to behavioral testing.

Drug infusion.

Intra-BLA administration was carried out using a syringe pump (Harvard Apparatus) and 5 µl Hamilton syringes, connected to injectors (28 gauge, projecting 2 mm beyond the guide cannulae; Plastics One Inc.) by polyethylene tubing. All infusions were begun 30 seconds after the insertion of the injectors and performed over 2 minutes at a rate of 0.25 µl min^{-1} (total volume of 0.5 µl $side^{-1}$). One minute of waiting time was imposed from the end of the infusion to the removal of the injectors to allow the drugs to diffuse from the injection site. The CB1 receptor agonist URB597 (Cyclohexylcarbamic acid 3´-carbamoyl-biphenyl-3-yl ester , Sigma-Aldrich ,30 ng per 0.5 µL per side), the CB1 receptor antagonist AM251 (N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3 carboxamide, Tocris, 300 ng per 0.5 µL per side) and $GABA_A$ receptor antagonist 1(S),9(R)-(−)-Bicuculline methiodide (bic, Sigma, 50 ng per 0.5 µL per side) were dissolved in a vehicle (veh) containing 5% polyethylene glycol, 5% Tween-80 and 90% saline. All doses

were chosen based on previous studies (Koya *et al*, 2009) and on data from pilot experiments.

Histology

After completion of behavioral testing, the rats were killed with a dose of anaesthetic and transcardially perfused with 0.01 M PBS, followed by 4% paraformaldehyde (PFA). Brains were collected and stored in 4% PFA for at least 24 hours, before being transferred to 20% sucrose solution for cryoprotection prior to sectioning. Subsequently, the brains were cut to produce 60 µm coronal sections, and stained with Cresyl Violet. The cannulae placement assessment was conducted under light microscopy (Leica), and subjects were only included in the statistical analysis if the injectors were located bilaterally within the BLA, and there was no bilateral damage to the amygdala or any other area of the brain.

Behavioural procedures.

Auditory Fear Conditioning was performed in four operant chambers (Med Associates Inc.). On day 1 (habituation session), the rats were habituated to the experimental chamber for 2 hours and allowed to freely explore the context. On day 2, for fear conditioning (conditioning session), the rats were placed in the same experimental context as in the habituation session, and exposed to two CS-US pairings. The CS was an auditory clicker (10 Hz, 80 dB, 60 s) and the US a mild electric footshock (0.5 mA, 1 s). The first CS-US pairing was presented after 35 ± 1 minute from the start of the session, followed by a 5± 1 minute interval when a second CS-US pairing was given. The conditioning session terminated 5 minutes after the last footshock delivery. On day 3, for memory reactivation (reactivation session), the rats were exposed during a brief 2 min session to a single presentation of the 60 s CS after 60 s of context exposure. All rats received an intra-BLA infusion of the drugs before or immediately after the memory reactivation session to evaluate the effect of the drugs on memory reconsolidation process. As control, an additional group of non-reactivated rats were habituated and conditioned following the same behavioral procedure except for receiving drug injections in the holding room on day 3 without being exposed to the reactivation session. The

conditioned freezing was tested during a single presentation of the 60 s CS after 60 s of context exposure (testing sessions). Testing took place 24 h [post-reactivation long-term memory (PR-LTM24h), day 4], and 8 days (PR-LTM8d, day 8) after memory reactivation to test long-term memory retention. Freezing behaviour was video-recorded, the behavioural outcome subsequently scored offline by an observer unaware of drug treatment, and analyzed for freezing. Freezing is defined as the lack of movement except for breathing at 5 s intervals to give the percentage time freezing during the CS. Freezing during the first minute of the testing session was assessed as measure of fear reaction to the experimental context, and during the second as measure of fear to the CS.

Statistical Analysis

Data were analyzed by repeated measures ANOVA, with CS (Context *vs*. Cue) and Session (Reactivation *vs*. PR-LTM24h*vs*. PR-LTM8d) as within-subject factors, and Treatment (VEH *vs*. URB597 *vs*. AM251) as between-subjects factors. Where the data violated the assumption of sphericity as assessed using Mauchly's test, a correction was applied; the Greenhouse-Geisser correction if $\varepsilon < 0.75$, and the Huynh-Feldt correction if $\varepsilon > 0.75$, as recommended by Cardinal & Aitken (2006). Where appropriate, further ANOVAs or pairwise comparisons were conducted; all pairwise comparisons were adjusted using the Šidák correction, which is a mathematically accurate form of the Bonferroni estimation (Cardinal *et al*, 2006).

Results

Pre-reactivation infusion of URB597 and AM251 did not affect retrieval and reconsolidation of pavlovian fear memory.

To evaluate the role of the endocannabinoid system in the BLA on the modulation of fear memory reconsolidation, the CB1 indirect agonist URB597 or the CB1 antagonist AM251 was bilaterally infused into the BLA 30 min before the reactivation session. All experimental groups had previously conditioned to CS, as all rats showed a greater fear response to the CS than to the context during the reactivation session ($F_{1,25}$=41.59; p<0.001). As shown in Fig. 1, there were no differences in conditioned freezing during the

test sessions between animals given URB, AM or vehicle ($F_{2,25}$=0.98; p=0.39), and though conditioned freezing reduced across the test sessions ($F_{2,50}$=5.83; p<0.005), it did so in a similar way across all experimental groups ($F_{4,50}$=0.06; p=0.99). Pairwise comparisons revealed that there was a statistically significant difference in levels of freezing of all treated rats between the reactivation session and PR-LTM8d (reactivation *vs* PR-LTM8d, p=0.012) and between PR-LTM24h and PR-LTM8d (PR-LTM24h *vs* PR-LTM8dPR-LTM8d, p=0.02) sessions. This might indicates that repeated exposure without reinforcing could activate an extinction process inducing a reduction of the fear response. Moreover, ANOVA on the single reactivation session did not reveal a treatment effect on freezing levels ($F_{2,25}$=0.41; p=0.67) showing that the pre-infused drugs did not acutely affect the expression of conditioned freezing. Therefore, neither URB597 nor AM251, at the doses used, affected retrieval or memory reconsolidation, as all groups showed the same level of freezing response over the reactivation and test sessions.

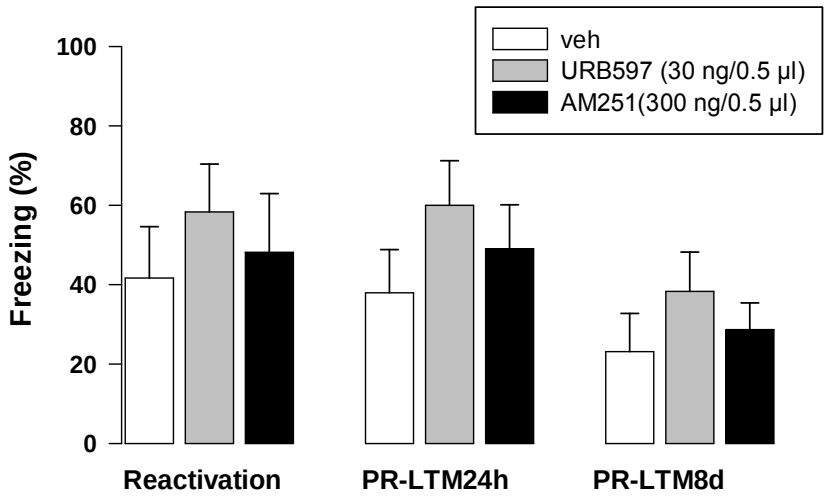

Fig. 1 Effects of the indirect CB1 agonist URB597 (30ng/0.5ul) and the CB1 antagonist AM251 (300ng/0.5ul) on CS–fear memory reconsolidation. Administration of URB597 (30ng/0.5ul) or AM251 (300ng/0.5ul) before reactivation session had no effect on the retrieval of the CS–fear memory at reactivation and did not alter expression of freezing response 24h or 8d post-reactivation. Data are presented as means ± SEM. Group sizes were veh, *n* = 9; URB597 (30ng/0.5ul), *n* = 10; AM251 (300ng/0.5ul), *n* = 9

Immediately post-reactivation infusion of the CB1 antagonist AM251 disrupted reconsolidation of pavlovian fear memory

The CB1 antagonist AM251 infused in BLA immediately post-reactivation session disrupted memory reconsolidation of pavlovian fear memory. All rats had previously conditioned to CS, as all groups showed a greater fear response to the CS than to the context during the reactivation session ($F_{1,29}$=5.52; p<0.001). As shown in Fig. 2, there were differences in conditioned freezing during the test sessions between animals given URB597, AM251 or vehicle ($F_{2,29}$=6.00; p=0.007) and across both test sessions ($F_{2,29}$=4.78; p=0.012) and all experimental groups ($F_{4,58}$=5.53; p=0.001) Pairwise comparisons revealed that rats infused with AM251 immediately after the reactivation session froze less when re-exposed to the context 24h later (PR-LTM24h *vs* reactivation p=0.011) and 8 days later (PR-LTM7d *vs* reactivation p=0.001) when compared with the same rats exposed to the CS prior to the treatment. Additionally, pairwise comparisons showed that rats administered with URB597 had higher levels of freezing when re-exposed to the CS 24h after memory reactivation (PR-LTM24h *vs* reactivation, p=0.041) when compared with the same group prior to the treatment. However this fear response was not a persistent effect over the time as when re-exposed to the CS 8 days post-reactivation URB597-treated rats showed the same freezing response as the same group prior to the infusion (PR-LTM8d *vs* reactivation, p=0.96). Moreover, analyses of single test sessions showed a significant main effect of treatment for both PR-LTM24h ($F_{2,29}$=12.033, p<0.001) and PR-LTM8d ($F_{2,29}$=6.819, p=0.004). Post hoc analysis showed that AM251-treated rats had freezing levels significantly lower when compared with vehicle and URB597-treated rats after both 24h (p=0.005; p<0.001 respectively) and 8d (p=0.038; p=0.004, respectively) the reactivation session. Thus, intra-BLA infusion of the indirect CB1 agonist URB597 seems to potentially enhance memory reconsolidation while the antagonist AM251 had an opposite effect and persistently disrupted reconsolidation of pavlovian fear memory.

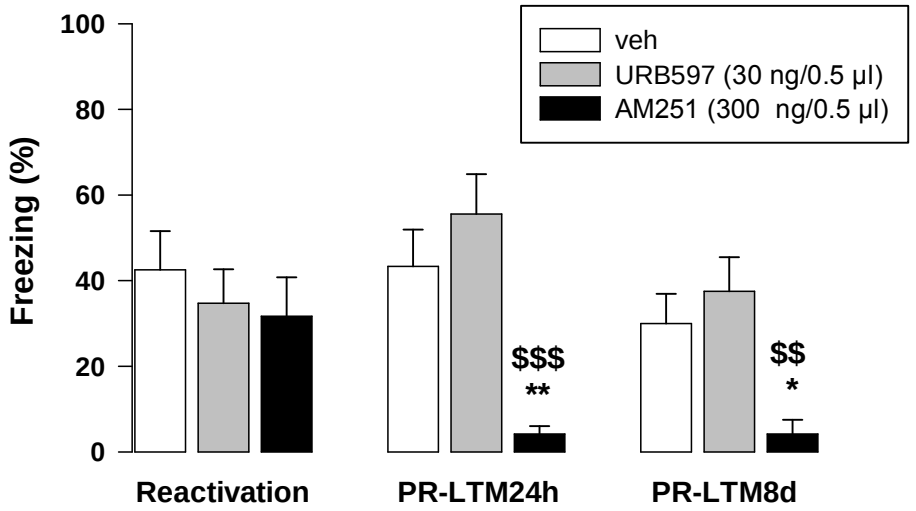

Fig. 2 Effects of the indirect CB1 agonist URB597 (30ng/0.5ul) and the CB1 antagonist AM251 (300ng/0.5ul) on CS–fear memory reconsolidation. Administration of URB597 (30ng/0.5ul) immediately after the reactivation session had no effect on CS–fear memory reconsolidation both 24h and 8d after reactivation. AM251 (300ng/0.5ul) persistently impaired memory reconsolidation when compared with vehicle and URB597 (30ng/0.5ul)-treated rats after both 24h and 8d after the reactivation session (**p<0.01 *vs* veh; **$$$** p<0.001 *vs* URB597 (30ng/0.5ul)) (*p<0.05 *vs* veh; **$$** p<0.01 *vs* URB597 (30ng/0.5ul)). Data are presented as means ± SEM. Group sizes were veh, *n* = 10; URB597 (30ng/0.5ul), *n* = 12; AM251 (300ng/0.5ul), *n* = 10

Disruption of memory reconsolidation induced by intra-BLA AM251 administration was dependent on memory reactivation.

To establish that the disruption of memory reconsolidation is dependent on the reactivation process, different groups of rats were infused with the same doses of URB597 or AM251 or vehicle, but were not exposed to the memory reactivation session. All rats had previously conditioned to CS, as all groups froze more during the CS presentation than to the context during the reactivation session ($F_{1,21}$=23.454; p<0.001). ANOVA did not reveal a significant main effect of treatment ($F_{2,21}$=0.024; p=0.976) but showed a significant main effect of session (PR-LTM24h *vs* PR-LTM8d, $F_{1,21}$=5.931; p=0.024) and a significant interaction session x treatment effect ($F_{2,21}$=4.572; p=0.022) (Fig. 3). Pairwise

comparisons showed a statistically significant decrease of freezing levels between test sessions for both vehicle (PR-LTM24h *vs* PR-LTM8d, p=0.023) and AM251 treated rats (PR-LTM24h *vs* PR-LTM8d, p=0.010). However, analysis of single sessions did not show a statistically significant differences in freezing response among groups 48h or 8d after conditioning (veh *vs* URB597 *vs* AM251, $F_{2,21}$=0.414; p=0.666 and $F_{2,21}$=1.563; p=0.233, respectively). Thus, 48h and 7d after conditioning the fear memory is still consolidated and was not altered by drug administrations. Subsequently, the decreasing effect of AM251 previously observed on freezing response is dependent on stimulus re-exposure during the memory reactivation session.

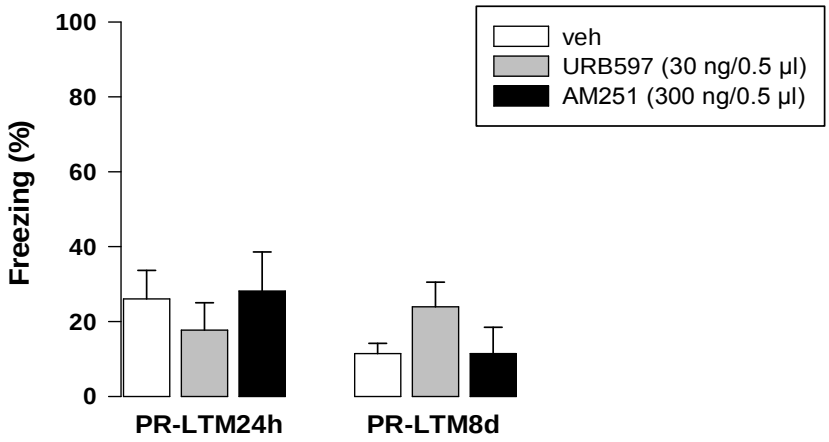

Fig. 3 Effects of the indirect CB1 agonist URB597 (30ng/0.5ul) and the CB1 antagonist AM251 (300ng/0.5ul) on CS–fear memory reconsolidation in rats not exposed to reactivation session. Administration of URB597 (30ng/0.5ul) or AM251 (300ng/0.5ul) in absence of memory reactivation had no effect on the retrieval of the CS–fear memory both 24h and 8d after administration. Data are presented as means ± SEM. Group sizes were veh, *n* = 8; URB597 (30ng/0.5ul), *n* = 8; AM251 (300ng/0.5ul), *n* = 8.

GABAergic neurotransmission was necessary to disruption of memory reconsolidation induced by intra-BLA AM251 administration.

In order to investigate the mechanisms underlying the disruptive effect on memory reconsolidation induced by blocking CB1 neurotransmission, interaction between endocannabinoid and GABAergic neurotransmission was evaluated. All rats had previously conditioned to CS, as all groups froze more during the CS presentation than to the context during the reactivation session ($F_{1,34}$=184.041; p<0.001). ANOVA revealed a significant main effect of treatment ($F_{3,34}$=3.122; p=0.039), a significant main effect of session (reactivation *vs* PR-LTM24h *vs* PR-LTM8d, $F_{3,34}$=75.682; p<0.001) and a significant interaction session x treatment effect ($F_{6,68}$=4.306; p=0.001) (Fig. 4). Pairwise comparisons showed a statistically significant decrease of freezing response between sessions for AM251-treated rats (PR-LTM24h *vs* reactivation, p<0.001; reactivation *vs* PR-LTM8d, p<0.001), for bicuculline infused rats(PR-LTM24h *vs* reactivation, p=0.011; PR-LTM8d *vs* reactivation, p<0.001), and for rats co-administered with AM251 and bicuculline (PR-LTM8d *vs* reactivation, p=0.002). As previously, these differences indicate that the conditioned freezing response is progressively weakened after repeated exposure to the auditory stimuli in absence of the reinforcement. Interestingly, analysis of single sessions revealed that during the reactivation session there was not a statistically significance difference in freezing levels among groups in absence of the treatment ($F_{3,34}$=1.27; p=0.300). However, single test sessions analysis, revealed that freezing response change significantly 24h and 8d after memory reactivation followed by drug infusions ($F_{3,34}$=4.683; p=0.008). Post hoc showed that after 24h AM251 treated rats froze less when compared with vehicle, and bicuculline treated rats (p=0.029; p=0.017) and when compared with rats co-infused with AM251 and bicuculline (p=0.045). Thus, we replicated and confirmed the disruptive effect on memory reconsolidation induced by the CB1 receptor antagonist AM251. Moreover, we discovered that this disruptive effect was reverted by blocking the $GABA_A$ receptor activity. Here we showed that the disruptive effect on reconsolidated memories exerted by CB1 neurotransmission blockade is mediated by the GABAergic system.

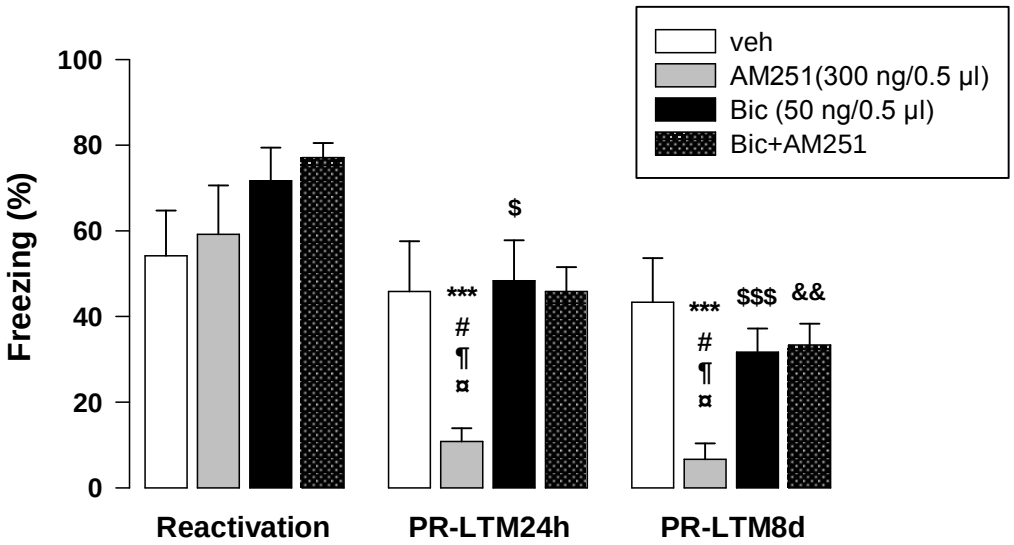

Fig. 4 Effects of the CB1 antagonist AM251 (300ng/0.5ul) or the $GABA_A$ receptor antagonist bicuculline (Bic 50ng/0.5ul) on CS–fear memory reconsolidation Administration AM251 (300ng/0.5ul) immediately after the reactivation session persistently impaired the CS–fear memory both 24h and 8d after the reactivation session ¤p<0.05 *vs* veh; ¶p<0.05 *vs* bic; **#**p<0.05 *vs* AM251+bic; *******p<0.001 PR-LTM24h *vs* reactivation and PR-LTM8d *vs* reactivation; **$$$**p<0.001 PR-LTM8d *vs* reactivation; **$**p<0.05 PR-LTM24h *vs* reactivation; &&p<0.01 PR-LTM8d *vs* reactivation. Data are presented as means ± SEM. Group sizes were veh, *n* = 10; AM251 (300ng/0.5ul), *n* = 10; Bic (50ng/0.5ul), *n* = 10; AM251 (300ng/0.5ul)+ Bic (50ng/0.5ul), *n* = 8

Discussion

In this study we showed for the first time, to our knowledge, that blocking CB1 receptors directly in the BLA disrupted memory reconsolidation of CS-fear memory, based on a classical pavlovian associations between environmental conditioned stimuli (CSs) and negative reinforcer (unconditioned stimuli, or USs). This disruptive effect on memory reconsolidation, which persisted at least 8d after CS-re-exposure, occurred only when the CB1 receptor antagonist AM251 was infused locally in BLA immediately after retrieval. Administration of same drug at the same dose 30 min prior to memory reactivation did not induce an impairing effect on freezing response during retest 24h or 8d later. Local infusion of the indirect CB1 receptor agonist URB597 affected expression of fear memory

reconsolidation when it was administered prior to memory reactivation, on the other hand, it was able to induce only a transient enhancing effect on the freezing response when infused immediately after recall. Moreover, infusion of the same drug in non-reactivated rats did not alter the conditioned freezing response, suggesting that the enhancement of memory reconsolidation is a retrieval-dependent process.

Additionally, we investigated the mechanism underlying endocannabinoid signalling in BLA during memory reconsolidation. Katona and co-workers in 2001 showed that CB1 receptors localize presynaptically in BLA on a distinct subpopulation of GABAergic interneurons suggesting that cannabinoids might alter inhibitory synaptic transmission (Katona *et al*, 2001). In this study, Katona and colleagues demonstrated that CB1 receptor activation inhibited GABA release, and that this effect was reverted by application of CB1 receptor antagonist SR141716 (Katona *et al*, 2001). On the basis of these results, we hypothesised that potentiation of memory reconsolidation through endocannabinoid inactivation in BLA could be mediated by GABAergic signalling. Our hypothesis has been confirmed by local co-infusion of CB1 antagonist AM251 and receptor antagonist bicuculline. Indeed, blocking $GABA_A$ receptor-mediated signalling reverted the erasing effect on memory reconsolidation exerted by AM251, and re-established the expression of conditioned fear.

These findings confirmed our hypothesis that the endocannabinoid system is importantly involved in regulation of memory reconsolidation process. Most importantly, our data suggest that inhibition or activation of GABAergic signalling in a cannabinoid-dependent manner might be a promising neurochemical substrate in modulation of emotional state and a new potential target for treatment of neuropsychiatric disorders, such as post-traumatic stress disorder.

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Chapter 5

General discussion and conclusion

The involvement of the endocannabinoid system in the modulation of emotion and cognition is broadly described in the literature (Abush *et al*, 2010; Akirav, 2011; Bortolato *et al*, 2006; Campolongo *et al*, 2013; Campolongo *et al*, 2012a; Campolongo *et al*, 2009a; Campolongo *et al*, 2009b; de Oliveira Alvares *et al*, 2010). During my PhD program I focused my attention on the investigation of the mechanisms involved in such modulation.

Patel and coworkers (2003) demonstrated that systemic administration of propofol increased the levels of anandamide in mouse brain while administration of benzodiazepines, barbiturates, or volatile anaesthetics did not. Moreover, they showed that the effect of propofol on anandamide levels is mediated by an inhibition of the fatty acid amide hydrolase (FAAH) enzyme, the major degradation enzyme of anandamide and other related compounds. Based on these findings and on the evidence indicating that propofol is also associated with a higher incidence of traumatic memories from perioperative awareness and intensive care unit treatment (Kapfhammer *et al*, 2004b), we hypothesized that propofol administration could modulate the long-term retention of aversive memory. Our findings demonstrate that propofol, in contrast to other commonly used sedatives, enhanced memory consolidation when administered immediately after the exposure to an aversive event. Importantly, we demonstrated that this enhancing

effect on memory consolidation depends on an indirect activation of the endocannabinoid system as propofol effect on memory is blocked by co-administration of the CB1 receptor antagonist rimonabant. These findings indicate that propofol, at anaesthetic doses, enhances emotional memory consolidation when administered immediately after a stressful event by enhancing endocannabinoid signalling. On the other hand, we found that midazolam and pentobarbital, two sedative/anaesthetic drugs that do not increase endocannabinoid signalling (Patel *et al*, 2003), do not enhance the consolidation of memory for inhibitory avoidance training. In accordance with the behavioural data, we also found that propofol administration increases anandamide concentrations in the rat brain 8 min after injection, whereas anandamide plasma concentration remains unaffected. On the whole, our work strongly suggest that propofol should used with caution in individuals during the aftermath of an acute traumatic event and may help to explain the increased incidence of aversive memories from intraoperative awareness seen in patients undergoing total intravenous anaesthesia with propofol.

Drugs that interfere with endocannabinoid degradation increase ongoing endocannabinoid signalling in a temporarily and spatially restricted manner (Janero *et al*, 2009). However, cannabinoid effects on emotionality often exhibit a biphasic profile, as it is also reported by cannabis abusers (Curran *et al*, 2002; Fant *et al*, 1998; Hall and Solowij, 1998). A classical model elucidates this phenomenon referring to the use of different doses of cannabinoid drugs, with low doses generally inducing anxiolytic-like effects and high doses often causing the opposite (Moreira and Wotjak, 2010). However, a wide numbers of studies report evidence that indirect cannabinoid receptor agonists can induce biphasic effects on behaviour, depending on the emotional state of the subject. Haller and co-workers (2009), for instance, have recently demonstrated that the indirect cannabinoid agonist URB597, a FAAH inhibitor responsible of endogenous cannabinoid degradation, did not affect anxiety under mildly stressful circumstances but has strong anxiolytic-like effects in highly aversive testing conditions . Therefore, a new and appealing explanation for the biphasic effects of cannabinoid drugs on behaviour, is now emerging, suggesting that these effects might also depend on previous experiences, the

context of use and the level of emotional arousal at the time of drug administration/consumption (Akirav, 2011; Campolongo *et al*, 2013; Sciolino *et al*, 2011).This finding leaves open the possibility that inhibitors of endocannabinoid transport, which prolong endocannabinoid actions by preventing endocannabinoid access to intracellular hydrolyzing enzymes (Beltramo *et al*, 1997; Kathuria *et al*, 2003), may influence both emotional and cognitive processes depending on the level of environmental aversiveness at the time of drug administration. Thus, we investigated the effect of the prototypical endocannabinoid transport inhibitor AM404 in a non-aversive task, the Spatial Open Field test, under two experimental conditions which differed in the level of emotional arousal at the time of testing (Chapter 2). We found that different levels of environmental aversiveness strongly influence the emotional reactivity of untreated rats without affecting the cognitive performance. On the other hand, we found that AM404 effects on memory recognition strongly depends on the level of emotional arousal.

There is evidence that behavioural responses to the environmental stimuli are strictly dependent on the emotional reactivity induced by the environment itself (Blanchard *et al*, 2001; Haller *et al*, 2009). From an evolutionary point of view, the emotional arousal hailing from environmental context is crucially involved in assessing the novelty and salience of the external stimuli in terms of relevance for the adaptation and survival (Biegler and Morris, 1996; Breivogel *et al*, 1998; Poucet, 1993). Thus, when compared with a previous experience, a novel information recognized as highly relevant is committed to and stored by memory (Lemaire *et al*, 1999). For instance, Mumby and colleagues (2002) suggested that the impact of the object novelty is more salient than a spatial rearrangement with the same objects . That is why, probably, in our study untreated rats exposed to a high or to a low stressful experimental context were equally able to recognize the object substitution but failed to respond to the object displacement. However, we showed that AM404 administration impaired the capability to discriminate between a familiar and a new object in rats exposed to the highly arousal condition. These results strongly support the hypothesis that cannabinoids modulation of cognitive processes depend on the emotional state of the subject at the time of testing. Moreover,

we found that while AM404 administration did not influence object exploration in a context characterized by a low-level of emotional activation, rats treated with a low dose of AM404 and exposed to a more stressful environment spent less time investigating the objects, whereas a higher doses re-established the investigation activity at similar level of the vehicle-treated rats. The inhibition or the maintenance of the investigative behaviour can be related to an anxiogenic or an anxiolytic phenotype, respectively (Crawley, 1985). It is possible to speculate that this biphasic effect may depend on a differential regulation activity on both GABAergic and gutamatergic neurons mediated by different doses of the endocannabinoid transport inhibitor (Foldy *et al*, 2007; Hashimotodani *et al*, 2007). This interpretation seems to be in accordance with previous studies showing that the activation of the CB1 receptor results in a reduction of neurotransmitter release from the pre-synaptic terminal by a retrograde mechanism (Wilson and Nicoll, 2002). Furthermore, Laaris and co-workers (2010) report that direct cannabinoid receptor agonists Δ9-THC inhibits GABA release (Laaris *et al*, 2010). Additionally, other researchers showed a CB1 receptor-mediated inhibition for glutamate release both in rats (Hoffman *et al*, 2010; Wang, 2003) and mice (Kawamura *et al*, 2006). The opposite effects due to GABA and glutamatergic circuits could be explained by a different basal activation of the CB1 receptors expressed on these sub-populations of neurons. The basal activation of the CB1 receptors on glutamatergic synapses, which is lower than the basal CB1 receptors activation on GABA, suggests that the reactivity to an increase in the endocannabinoid tone could make more sensitive glutamatergic neurons than GABAergic neurons (Katona and Freund, 2008). Thus, a minimal increase in endocannabinoid levels could inhibit the release of glutamatergic neurotransmitters and impair the approaching behaviour response, while a higher amount of endocannabinoids could activate the inhibition of the GABA terminal leading to the opposite effect.

It is well established that the hypothalamic-pituitary-adrenal (HPA) axis plays a crucial role in stress response and that the stress hormones, such as glucocorticoids, differentially modulate cognitive functions (Atsak *et al*, 2011; Mizoguchi *et al*, 2004; Roozendaal and McGaugh, 1997). Most importantly, modulation of memory consolidation processes is clearly affected by manipulation of endocannabinoid signalling via a cross-talk with the

glucocorticoid system (Atsak *et al*, 2011; Campolongo *et al*, 2013; Campolongo *et al*, 2009a; Campolongo *et al*, 2009b). These results highlight that drugs enhancing endocannabinoid signalling by interfering with endocannabinoid deactivation induce different effects on short-term memory performance depending on the level of emotional arousal induced by different environmental settings.

In Chapter 3 and 4 it has been evaluated the effect of pharmacological manipulation in the basolateral amygdala on memory reconsolidation. The behavioral paradigm which more than other is well suits for neurobiological analysis in this context is represented by Pavlovian fear conditioning. Fear conditioning is valuable as a neurobiological tool because it involves a specific stimulus, under the control of the experimenter, that reliably elicits a measurable set of behavioural and physiological responses once learning has occurred. In fear conditioning, an emotionally neutral conditioned stimulus, such as a tone, is paired with an emotionally potent, innately aversive unconditioned stimulus (e.g., an electric shock) during a conditioning or acquisition phase.

Considerable evidence indicate the BLA as a critical region for the encoding of associative memories relevant to affective experiences (Aggleton, 2000; Rosenkranz and Grace, 2002).

The first step required for memory reconsolidation is the destabilization induced by retrieval which converted a previously consolidated memory from the 'inactive state' in to the 'active state'. These 'active' memories are newly restabilized back into the 'inactive' state (reconsolidated) through a protein-synthesis dependent process. Thus, disrupting reconsolidation appears to be a valuable target, in terms of therapeutic strategy, in order to reduce the impact of maladaptive memories on behavior.

It is known that NMDAR-mediated signaling is necessary for reconsolidation/restabilization of CS-drug (Brown *et al*, 2008; Itzhak, 2008; Milton *et al*, 2008b; Milton *et al*, 2012; Sadler *et al*, 2007), spatial (Przybyslawski and Sara, 1997) and CS-fear (Lee *et al*, 2006c; Pedreira *et al*, 2002) memories. However, this seems to be in

contrast with other findings showing that pharmacological blockade of GluN2B subtype of NMDAR has been shown to prevent the destabilization of CS-fear memories (Ben Mamou *et al*, 2006). We recently showed that GluN2B-NMDARs GluN2B-NMDARs and GluN2A-NMDARs are differently involved in the regulation of memory reconsolidation where GluN2B-NMDARs is required for memory destabilization while GluN2A-NMDARs is required for memory restabilization (Chapter 3). Our results are supported by previous findings showing that GluN2A-preferring NMDAR antagonists prevent the acquisition of spatial memory (Hu *et al*, 2009) and conditioned fear (Dalton *et al*, 2012).

Increasing clinical evidence links disturbances in endocannabinoid transmission with the etiology of psychopathologies, which are characterized by profound disturbances in emotional regulation disorders (Bangalore *et al*, 2008; Cohen *et al*, 2008; Schneider, 2008). CB1 receptor expression is found in relatively high concentrations in the BLA (McDonald and Mascagni, 2001; Tsou *et al*, 1998), and, most particularly, within the BLA, CB1 receptors are found on inhibitory local GABAergic interneurons (Herkenham *et al*, 1990; Katona *et al*, 2001; Tsou *et al*, 1998). Functionally, activation of BLA CB1 receptors decreases feedforward inhibition via inhibitory interneurons, thereby increasing the activity of BLA projection neurons (Pistis *et al*, 2004). Given that both recall and extinction of conditioned fear memories are correlated with increased release of intra-BLA endocannabinoids (Marsicano *et al*, 2002), this suggests a critical role for intra-BLA CB1 transmission during the processing of emotionally salient information. In chapter 4, we showed that CB1-mediated transmission modulates reconsolidation of pavlovian fear memory and that this neurotransmission is mediated by GABAergic signalling in BLA. Interestingly, we found that pharmacological manipulation of CB1 receptor induced a bidirectional effect on expression of fear response, where the indirect agonist URB597 positively modulates memory reconsolidation, while administration of the CB1-receptor antagonist AM251 disrupt retention of fear memory. These findings are in line with previous observations which showing that the indirect cannabinoid agonist URB597, bilaterally infused in BLA enhanced consolidation for aversive memory and this effect is prevented by blocking CB1 receptors with infusion of the CB1 receptor antagonist AM251 (Ratano *et al*, 2011). Interestingly, we found that the AM251-dependent disruptive effect

is abolished by co-infusion with $GABA_A$receptor antagonist bicuculline, consistently with data revealing the local expression of CB1 receptors on subpopulation of GABAergic neurons in the BLA.

All together these evidence confirm that the endocannabinoid system is crucially involved in the regulation of emotional memory processing. Thus, it is reasonable to hypothesize that targeting cannabinoid neurotransmission could represent a powerful therapeutic tool in treating cognitive disorders linked to emotional distress.

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