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Positive Emotional Arousal Increases Persistence of Memory Traces: Possible Mechanisms.

Cicle XXV

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Abstract

We compared the ability of high negative and positive emotional arousal to increase the persistence of consolidated memory traces. Negative emotional arousal was modulated by manipulating shock levels in a step-through inhibitory avoidance (IA). Mice trained with either low (Low mice,0.35 mA) or high (High mice, 0.7 mA) shock intensities showed increased step-through latencies when tested 24 hours after training, but only mice trained with the higher shock intensity showed retention of the IA learning 1 week after training. Moreover, we analyzed the expression of the immediate early gene c-fos induced by IA training to investigate the activation of brain regions (hippocampus, amygdala and rostral anterior cingulate cortex) known to play a role in emotional memory. c-fos expression did not increase depending on shock intensity. Positive emotional arousal was modulated by manipulating emotional salience of the testing cage of an object recognition test (ORT). Mice trained in either a high (chocolate-associated, High) or a low (inedible piece of plastic-associated, Low) emotionally arousing cage showed discrimination of a novel object 24 hours after training, but only High mice showed retention 96 hours after training. Furthermore, we investigated the effects of post-trial administration of the dopamine (DA) D1 receptor antagonist SCH23390 and the β-adrenergic receptor antagonist Propranolol on retention of this version of ORT. The lower dose of the SCH23390 (0.01 mg/kg) was effective on object discrimination expressed by Low mice tested 24 hrs post-training and by High mice tested

96 hours after training. A higher dose (0.025 mg/kg) reduced object discrimination in High mice tested either 24 or 96 hrs post-training. Either a high (2 mg/kg) or a low (1 mg/kg) dose of Propranolol reduced object discrimination in High mice tested 24 hours post-training, whereas neither one was effective in Low mice. Only the higher dose affected memory of sampled object in High mice tested 96 hours post-training. In a final experiment we observed induction of c-fos expression by ORT training. Training with high levels of positive emotional arousal did not promote high levels of c-fos expression in hippocampus but it did promote high c-fos expression in BLA and rACC. Together these data suggest that a memory trace consolidated in a state of high emotional arousal is more persistent than one consolidated in a state of low emotional arousal regardless of the hedonic valence of experience and that involve plasticity in BLA and rACC.

INTRODUCTION.

Memory is the ability to store acquired informations that is common to many forms of life from the most simple to the more complex and has a huge adaptive advantage. In most species, including humans, the ability to select and prioritize what information is important to remember, relative to less salient or peripheral information, is an essential skill for the eficient use of memory. Indeed, many theories suggest that human memory system may be “tuned” to remember information that is necessary for survival.

Clinical evidence that cerebral trauma induces loss of recent memory was reported in the past. A possible explanation of this kind of amnesia, namely retrograde, seems to be the consolidation hypothesis. The consolidation hypothesis was proposed by Müller and Pilzecker in 1900 they found that memory of newly learned information was disrupted by the learning of other information shortly after the original learning and suggested that processes underlying new memories initially persist in a fragile state and consolidate over

time. This process seems to be the main mechanism of animal and human learning.

Long-term memory, long-lasting memory and memory consolidation.

Memory consolidation hypothesis is also supported by the observation that the memory is not unitary process. Indeed, it is divided in two phases: short-term (STM) that lasts minutes to few hours, and long-term memory (LTM) a protein (and mRNA) synthesis-dependent phase that lasts several hours to days, weeks or even lifetime. Therefore, a definite property of LTM is its sensitivity to protein synthesis inhibitors. During memory formation, protein synthesis is thought to be required to transform newly learned information into stable synaptic modifications. Therefore the phenomenon is commonly termed “synaptic consolidation”. Synaptic consolidation involves post-translational modification of synaptic proteins, activation of transcription factors, modulation of gene

expression at synapses and cell body, including Immediate Early Genes (IEGs) such as c-fos, reorganization of synaptic protein including membrane receptors and cytoskeletal elements, all together culminating in synaptic remodeling, which is assumed to make the trace stable. These processes are similar to those observable during devolpment. In both cases, gene expression is regulated by extracellular signals, and in both cases, similar ubiquitous intracellular signaling cascades, such as the ciclic adenosine monophoshate/protein kinase A (cAMP/PKA) and the mitogen-activated protein kinase (MAPK) cascades, are recruited, and cellular remodeling and growth occur. Thus, by definition, memory consolidation is progressive post-acquisition stabilization of memory. Hence, several decades after Müller and Pilzecker proposal, at the beginning of this new millennium, the consolidation hypothesis still guides research investigating the time-dependent involvement of neural systems and cellular processes enabling lasting memory (McGaugh, 1966; Dudai, 1996, 2002, 2004; Lechner et al., 1999; Dudai and Eisenberg, 2004). During synaptic

consolidation memory traces are temporarely vulnerable to disruption. The use of treatments administered shortly or immediately after training to impair or enhance memory provides a highly effective and extensively used method of influencing memory consolidation without affecting either acquisition or memory retrieval (McGaugh, 1966; Davis & Squire, 1984; Dudai, 1996).

Another type of process of memory stabilization takes weeks, months, or even years to be accomplished. It is believed to involve reorganization over time of the brain circuits that moves memory trace to brain areas not involved in acquisition nor stablization of LTM. This type of process is termed “system consolidation”.

As an example the hippocampus is necessary for encoding and consolidation of memory traces. However, ones consolidated, traces reorganize and move to the neocortex, so that ultimately the neocortex can independently maintain the specific internal representation and actualize it in retrieval. It is not yet known what triggers system consolidation, but the most parsimonious account is that over time, upon recurrent activation of the hippocampal trace either in explicit

recall or in implicit processing (e.g., sleep or emotinal arousal), the hippocampal formation and related structures send synaptic messages to neocortical neurons, and these messages trigger synaptic consolidation locally.

Emotional arousal and memory consolidation.

Emotionally significant experiences tend to be well remembered (Bradley et al., 1992). Significant experiences such as birthdays, graduation ceremonies, or the loss of a loved one typically leave lasting and vivid memories. Therefore, it has been suggested that persistence of consolidated memory traces is modulated by the level of emotional arousal associated with experience.

The findings that post-acquisition drug treatments can enhance memory consolidation suggested the possibility that endogenous processes activated by experiences may serve to regulate the consolidation of

experiences (McGaugh, 1983; McGaugh and Gold, 1989). Indeed, a large body of evidence indicates that emotional arousal induced by certain experiences enhances LTM duration by converting into a persistent one acting on memory consolidation (for review see McGaugh, 2006). In line with this view, many studies suggest that the learning and memory processes are strongly influenced by evaluation mechanisms of input information. These mechanisms controls local synaptic plasticity processes influencing the possibility that the new information is stored in LTM. This modulatory effect act on those informations that are relevant for organisms and depends on emotional state of learned experience. Recently, Bekinschtein and coworkers (2007; 2008a; 2008b) trained rats in inhibitory avoidance (IA) with one of two foot-shock intensities (0.4 and 0.7 mA), then tested memory retention 24, 48 hours or 1 week afterward. Both trainings produced LTM (i.e., lasting 24 hrs), but only the stronger one produced LLM (i.e., lasting 1 week in rodents). This indicates that persistence of LTM is increased by emotional arousal of experiences and that enhancement

is proportional to level of emotional arousal.

The brain ability to render more relevant informations more persist has a high adaptive value because it fosters persistent avoidance of potentially harmful situations. On the other hand pathlogical persistence of emotional memory is associated with post-traumatic stress disorder (PTSD). According to the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) of American Psychiatric Association, PTSD is a severe anxiety disorder that can develop after exposure to any event that results in psychological trauma. This event may involve the threat of death to oneself or to someone else, or to one's own or someone else's physical, sexual, or psychological integrity, overwhelming the individual's ability to cope: in other words situation characterized by extreme levels of negative emotional arousal. Finally, the main symptoms of PTSD are recurrent, intrusive and persistent memories of the traumatic event.

Neurobiological bases of PTSD are investigated by the use of single trial memory tests involving high level of negative emotional arousal. The results of these

tests indicate that stress hormones and cathecolamines are determinant for consolidation of persistent memories under high level of emotional arousal. Hormones of the adrenal medulla (epinephrine) and adrenal cortex (cortisol in human and corticosterone in rodents or glucocorticoids) are relased during and immediately after emotionally arousing training experiences, such as IA and fear conditioning in laboratory animals (McCarty and Gold, 1981; McGaugh and Gold, 1989) and there is extensive evidence that these hormones modulate memory consolidation (Dornelles et al., 2007; McGaugh and Roozendaal, 2002; Oitzl and de Kloet, 1992; Roozendaal, et al., 1999; Roozendaal, et al., 2008). The effects of stress hormone on memory consolidation are not linear. Moderate doses of epinephrine or glucocorticoids enhance memory consolidation but lower or higher doses are less effective or may even impair memory consolidation (Atsak, 2011; Gold and van Buskirk, 1975; Okuda et al., 2004; Roozendaal et al., 2006, 2009). Posttraining injections of moderate doses of corticosterone or dexamethasone, a synthetic ligand, enhance memory consolidation in a water-maze

spatial task (Sandi et al, 1997). However, the same glucocorticoid treatment impairs memory consolidation when the task is made more aversive by lowering the water temperature (Sandi et al, 1997; Akirav et al., 2004). Similarly, epinephrine and glucocorticoids are known to enhance memory of IA when administered after a mild or low-arousing foot shock, but to impair memory consolidation when given after a strong, highly aversive foot shock that produces robust memory in control animals (Gold et al., 1975). The memory-enhancing effect of adrenal stress hormones involves release of norepinephrine (NE) and activation of β-adrenoceptors in specific brain areas (McGaugh, 2000; 2004; McGaugh and Roozendaal, 2002; Quirarte, et al 1998). The activation of β-adrenoceptors by released NE could result in facilitation of synaptic transimission by increasing intracellular cAMP concentration and new protein synthesis, thus contributing to memory consolidation (Liang et al., 1995; Ferry et al., 1999; Roozendaal et al., 2002). In fact, antagonists or agonists of these receptors, administered after exposure to emotionally motivated tasks, modulate memory

consolidation (Introini-Collison et al., 1991; Cahill et al., 1994 and 2000; Ferry et al., 1999; Hatfield and McGaugh, 1999; Quirarte et al, 1998;, Roozendaal et al., 2006 and 2008). This hypothesis is supported by the evidence that systemically and in loco post-training administration of Propranolol, a β-adrenoceptor antagonist, impair consolidation of many emotionally arousing tasks (Hatfield et al., 1999; Cahill, 2000; Dornelles et al., 2007; Miranda et al., 2003; Roozendaal et al., 2002, 2006, 2008).

Consolidation processes are also susceptible to treatments that influence dopamine (DA) transimission. There are two main classes of dopamine receptors that are categorized by their ability to stimulate (D1-like) or inhibit (D2-like) the adenyl cyclase/cAMP/protein kinase A (PKA) pathway. Many studies show that post-acquisition blockade or stimulation of either D1 or D2, reduces and facilitates, respectively, memory consolidation in different tasks (Castellano, 1991; LaLumiere, 2004; Managò, 2009; Moncada, 2011). In a study by Castellano and cooworkers (1991), for example, immediately post-training administration of the

DA D1 receptor antagonist SCH23390 prevented retention of IA 24 hours later. In a study of 2010 deLima and cooworkers showed increased retention of object memory by selective D1 receptor aagonist.

Studies of the influence of emotional arousal on memory support a major role of the baso-lateral complex of amygdala (BLA). It has been proposed that BLA modulates consolidation of emotional memories in a number of learning systems (McGaugh, 2000; 2004 McGaugh and Roozendaal, 2002). Glucocorticoid receptors are densely located in the hippocampus, infusion of glucocorticoid agonists in dorsal hippocampus after training enhance memory. BLA lesions or infusion of β-adrenoceptor antagonists into BLA block the effect of glucocorticoids either administered sistematically or infused directely into dorsal hippocampus (Roozendaal et al., 1999).

Moreover, a number of studies suggest that the persistence of emotional memory involves activation of β-adrenoceptor within the BLA (McGaugh, 2000; 2004 McGaugh and Roozendaal, 2002). In addition,

LaLumiere and cooworkers (2004) investigated the influence of DA infused into the BLA immediately after training enhanced retention of IA. The opposite effect was promoted by post-training intra-BLA infusions of the D1 receptor antagonist SCH 23390. Finally, blockade of local β-adrenergic receptor prevented the facilitatory effect of DA in BLA on memory consolidation. These findings support a major role of DA receptor and β-adrenergic receptors in the BLA in the consolidation of emotional memories. Neuroimaging studies have revealed that during the processing of emotional information, there are correlations between the strength of activity in the amygdala and in the hippocampus and the strength of these correlations can correspond with the magnitude of the mnemonic boost for emotional information (Kensinger & Corkin, 2004).

The rostral anterior cingulate cortex (rACC) is involved in early formation of fear memory and storage of long-lasting emotional memories (Frankland, 2004). Post-training stimulation of rACC selectively enhanced retentiona of footshock in a modifeid version of IA that allowed to separate context from foot-shock training

(Malin et al., 2005, 2007). Interestly, in the same studies, the authors demonstrated a selective role of hippocampus for the context and the involvment of BLA in consolidation of all components of the task (Malin, 2005).

The activity-dependent expression of IEGs is thought to be a crucial step in the formation of LTM. Zhang and cooworkers (2011) analyzed the expression of c-fos and Arc genes following a IA training. Indeed, as prevously discussed IEGs are part of the early transcriptional phase associated with memory consolidation. Moreover, Arc and c-fos are required for formation of fear memories (Fleischmann et al., 2003) and are targets of CREB, which is required for the consolidation of LTM (Bourtchouladze et al., 1994; Oike et al., 1999; Kida et al., 2002; Pittenger et al., 2002; Kozus et al., 2004). This study described IA training-induced c-fos activation in several brain areas including the hippocampus and the BLA but no activation in ACC and Arc expression in the same brain areas with the addition of ACC (Zhang et al., 2011).

Moreover, in the same study infusion of

anysomicin into ACC impaired LT-IA memory without affecting ST-IA memory.

Aims and rationale of the experiments.

As discussed so far, emotional arousal increases persistence of memory traces by influencing consolidation processes. These hypotheses are supported by the results obtained using experimental procedures that modulate negative emotional arousal, such as IA or fear conditioning. These procedures mimic the impact of traumatic events thus modeling PTSD in experimental animals. However, emotional arousal can to be promoted by positive experiences and positive emotional arousal renders associated memories more persistent too.

The general aim of this study is to investigate whether persistence of memories associated with positive emotional arousal is modulated by the same mechanisms involved by negative emotional arousal. To this aim, I contributed to develop a modified version of the Object Recognition Test (ORT) that allows to vary the amount of positive emotional arousal during the object sampling (training) phase. The procedure was developed in outbred mice to be exploited in genetic

studies.

Therefore, the first set of experiments (Part 1) presented here evaluated behavioral and central effect of IA training with different shock intensities in mice from the same outbred strain to collect comparable data on negative emotional arousal. IA retrieval 24 hours or one week after training was used as behavioral measure of memory persistence. Moreover, expression of c-Fos induced by IA training was evaluated by immunohistochemistry to map brain areas involved in consolidation of memories under different levels of negative emotional arousal.

A second set of experiments (Part 2) tested the validity of the modified version of ORT to evaluate the impact of positive emotional arousal on memory consolidation. Experimental mice explored sample objects in a test cage previously associated with a palatable food (chocolate) following 15 hours of food deprivation whereas control mice explored sample objects, always following food deprivation, in a test cage previously associated with a small piece of plastic. Discrimination of a novel object tested 24 or 96 hrs later

was used as behavioral measure of memory persistence. The relative contribution of food deprivation and chocolate feeding was tested in different groups pf animals. Finally, the involvement of consolidation and emotional arousal were tested by immediately post-training administration of the DA D1 antagonist SCH23390 and of the ß-adrenergic antagonist Propanolol.

A final experiment evaluated expression of c-Fos induced by object sampling to map brain areas involved in consolidation of memories under positive emotional arousal. The results were compared with those obtained with IA training.

EXPERIMENTS.

Part 1: Effects of shock intensity on persistence of inhibitory avoidance (IA) memory and on induction of c-Fos expression by IA training.

Materials and Methods.

Subjects

Male CD1 mice (Harlan Laboratories, Udine, Italy) were purchased at 6 weeks of age and housed four to a cage on a 12 hours light-dark cycle (lights on at 7:00 am). Food and water were available *ad libitum.* Mice were left undisturbed for two weeks before behavioral testing. Experiments were carried out in accordance with

the Italian national law (DL 116/92) on the use of animals in research.

Behavioral procedures

Inhibitory avoidance

Mice were trained and tested in a step-through IA task. The apparatus consisted of a trough-shaped alley (91 cm long, 15 cm deep) divided into two compartments by a retractable door: an illuminated safe compartment (31 cm long) and a dark shock compartment (60 cm long). Mice were not habituated to the dark compartment before the training trial. On the training trial, each mouse was placed in the lit start compartment facing away from the shock chamber. After the mouse stepped with all four paws into the dark compartment, the retractable door was closed and an inescapable foot-shock (0, control mice, 0.35 Low-trained mice: or 0.7 mA High-trained mice, 50 Hz, 2.0 sec) was administered. The mouse was removed from the dark compartment 30 sec after termination of the foot-shock. The lowest shock intensity was chosen on

the basis of previous studies showing that it is sufficient to induce IA retention for at least 24 hours in mice (Baarendse et al., 2008). The highest shock intensity was chosen on the basis of a previous study in mice showing that it induces IA retention beyond 24 hours (Boccia et al., 2004). On the retention trial, each mouse was placed into the light compartment with the retractable door open and allowed to explore the box freely. The latency to enter the dark compartment with all four paws was recorded with a timer by a nearby experimenter as a measure of retention. Retention test ended either if the mouse stepped into the dark compartment with all four paws or if it failed to cross within 180 sec. In the latter case, mouse was assigned a score of 180 sec. Foot-shock was omitted on retention test. Each mouse was tested only once.

Tissue preparation.

One hour after training, animals were sacrificed via rapid cervical dislocation, the brains were removed from skull in approximately 90 seconds and then post-

fixed in 4% formaldehyde (Sigma) diluted in 0.1 M (pH 7.4) phosphate buffer (PB) overnight at 4° C. Then brains were immersed for 48-72 hours at 4 °C in PB/sucrose 30% for cryoprotection. Then brains were blocked in the coronal plane at the level of the optic chiasm and their anterior parts were frozen with dry ice and cut in 40-µm coronal sections with a sliding microtome. Three adjacent series of 8–10 sections sampling the dorsal hippocampus, amygdala and rostral anterior cingulate cortex were selected from each brain on the basis of Mouse Brain Atlas of Paxinos and Franklin 2001 and immunostained for c-fos as shown in figure 2.

Imunohistochemistry.

Floating sections were processed at room temperature in 24-well microplates on gentle agitation. The steps for imunohistochemistry procedure were as follows:

1. 3 times for 10 minutes in Phosphate

Buffer + 0.3% Triton X-100 (PBTX)

2. 30 minutes incubation in PBTX + H_2O_2 (3% for non perfused brains)
3. 3 times for 10 minutes in PBTX (or PB 0.1M)
4. 30 minutes incubation in Avidin blocking solution [Vector Laboratories] (2 drops/5ml) diluted in PBTX
5. 3 times for 10 minutes in PBTX (or PB 0.1M)
6. 30 minutes incubation in Biotin blocking solution [Vector Laboratories] (2 drops/5ml) diluted in PBTX
7. 3 times for 10 minutes in PBTX (or PB 0.1M)
8. Overnight incubation in primary rabbit anti c-fos polyclonal antibody (Oncogene, AB-5) diluted 1:20000 in PBTX + 2% Normal Goat Serum (Vector Laboratories)

9. 3 times for 10 minutes in PBTX (or PB 0.1M)
10. 2 hours incubation secondary antibody anti-Rabbit (Vector Laboraories) diluted 1:1000 in PBTX
11. 3 times for 10 minutes in PBTX (or PB 0.1M)
12. 1 hour incubation Avidin-Biotin Complex (Vector Laboratories) diluted 1:1000 in PBTX (prepare 30 min before use)
13. 3 times for 10 minutes in PB 0.1M
14. 4 minutes incubation in DAB solution diluted 1:2 compared to manufacturer instructions (sections to be compared must be treated simultaneously, i.e. using Costar® Netwells, 15 mm diameter, 500 µm Mesh)
15. 3/4 times for 10 minutes in PB 0.1M

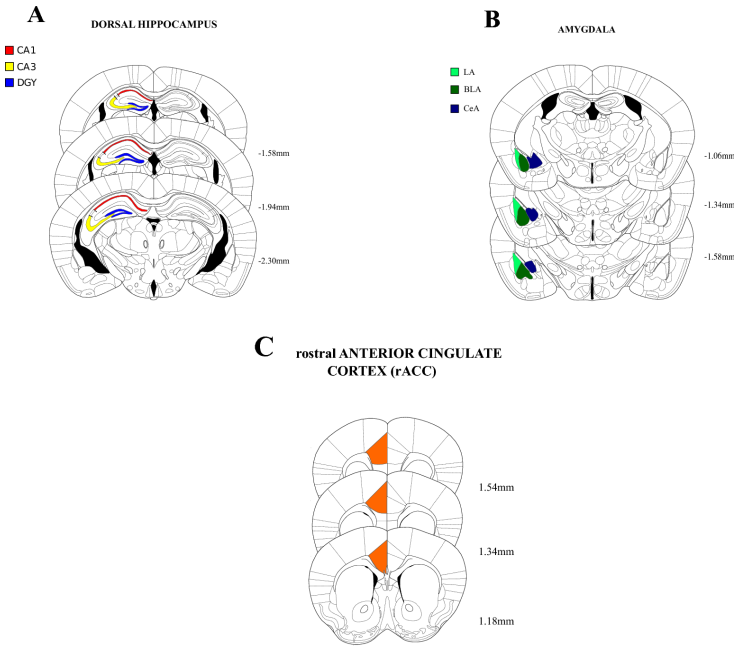

Figure 2. Approximate levels of dorsal hippocampus, amygdala and rostral anterior cingulate cortex selected on the basis of Mouse Brain Atlas of Paxinos and Franklin 2001.

Data analysis.

Statistical analyses were performed on a Debian GNU/Linux workstation using the R free software environment for statistical computing and its graphical interface R-Commander (Fox, 2005). An error probability level of $p < 0.05$ was accepted as statistically significant. Because a cut-off of 180 seconds was imposed during test sessions, the step-through latency was expressed as median and inter-quartile range and analyzed with non-parametric tests in experiment 1 Kruskal–Wallis ANOVA was used when comparing more than two groups. Two-sample Wilcoxon test (two-tailed) was used as *post hoc* test following a significant overall Kruskal–Wallis test.

For experiment 2 the brain regions were acquired at 4x objective magnification and ImageJ 1.46p free software from http://imagej.nih.gov/ij was used to quantify c-fos immunostaining cells. Because no cut-off was imposed to analyze the c-fos expression, in thi case parametric one-way ANOVA was used when comparing

all groups in different brain regions. Tukey was used as *post hoc* test following a significant overall ANOVA test.

Experiment 1.

This experiment was aimed to test in mice the effect of magnitude of negative arousal on LTM duration (Bekinschtein et al., 2007). Ninety-six mice were randomly assigned to this experiment. One-third of mice were pseudo-trained by omitting the shock (control mice, cont), one- third were trained with the 0.35 mA shock (low-trained mice, Low), and the remainder were trained with the 0.7 mA shock (high-trained mice, High). Half of the mice for each shock condition (n = 16) were tested for retention 24 hours after training, the other half were tested 1 week after training.

Experiment 2.

This experiment was aimed to evaluate c-Fos expression induced by IA training in brain areas involved in consolidation of memories under different levels of negative emotional arousal. We first identified brain regions, such as amygdala, dorsal hippocampus and rostral anteior cingulate cortex, where gene expression is activated after learning inhibitory avoidance (IA) by analyzing the expression of the immediately early genes c-fos as marker. Three groups of mice were trained under both experimental conditions from Experiment 1: 0.35 (Low) or 0.7 mA (High) foot-shock and an additional group of mice were taken directly from their home cage (naïve group, cont). All the mice for each condition (n = 8) were sacrificed one hour after training and the brains were processed for c-fos immunostaining (see above in material and methods paragraph).

Results.

Experiment 1. Figure 3 a and b show step-through latency measured, respectively, 24 hours and 1 week after training as a function of shock intensity. For 24 hours test (figure 2a), Kruskal–Wallis ANOVA revealed a significant difference between groups [$H_{(2)}$ = 23.25; $p<0.001$]. *Post hoc* analysis revealed that groups trained with either shock intensities had a higher step-through latency respect to pseudo-trained group (cont) but did not differ one another (Low vs cont: W = 26.5, $p<0.001$; High vs cont: W = 11, $p<0.0001$). For 1 week test, Kruskal–Wallis ANOVA revealed a significant difference between groups [$H_{(2)}$ = 13.44; $p<0.01$]. *Post hoc* analysis revealed that the group trained with the high shock had a higher step-through latency respect to both pseudo- and low shock-trained groups (High vs cont: W = 41.5, $p<0.001$; High vs cont: W = 197.5, $p<0.01$) and that the latter groups did not differ one another. These results indicate that duration of fear LTM depends on the magnitude of negative arousal experienced during training.

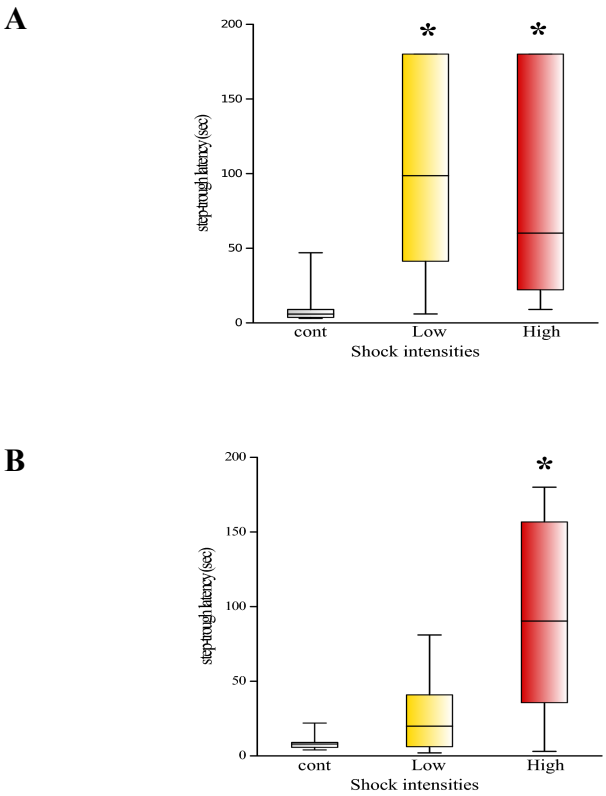

Figure 3. (A) Step-through latency (median and inter-quartile range) as a function of foot-shock intensity (Low vs High) measured 24 hrs post-training (*vs 0, $p < 0.001$); (B) Step-through latency (median and inter-quartile range) as a function of foot-shock intensity (Low vs High) measured 1 week post-training (*vs 0, $p < 0.001$).

Experiment 2. Figure 4, 5 and 6 show brain layers, microphotographs examples and the pattern of c-fos expression induced by training of step-through IA with the two shocks condition compared with naïve group of brain regions here reported. One-way ANOVA revealed a significant difference between groups in all dorsal hippocampus subfields investigated (CA1: $F_{2,21} = 12.91$, $p < 0.001$ CA3: $F_{2,21} = 12.65$ $p < 0.001$; DGY: $F_{2,21} = 13.00$, $p < 0.001$). Tukey's *post hoc* analysis revealed that Low-trained group had higher c-fos expression in CA1, CA3 and DGY respect to the High-trained and naïve groups which not differ from each other (figure 4b) Moreover, one-way ANOVA revealed a significant difference between groups in basolateral (BLA) and lateral (LA) amygdala but not difference in central (CeA) nuclei of amygdala (BLA: $F_{2,21} = 6.96$, $p < 0.01$; LA: $F_{2,21} = 3.61$, $p < 0.05$). Tukey's *post hoc* analysis revealed that all shocked groups had higher c-fos expression in BLA respect to naïve group whereas only low shock-trained mice show higher c-fos response respect to control group in LA but did not differ one another in

both brain areas (figure 5b). Finally, one-way ANOVA analysis revealed significant effect in rostral anterior cingulate cortex (rACC: $F_{2,21} = 52.22$, $p < 0.001$). Tukey's post hoc shows that both Low and High mice differ to controls and also differ from each other in rACC (figure 6b).

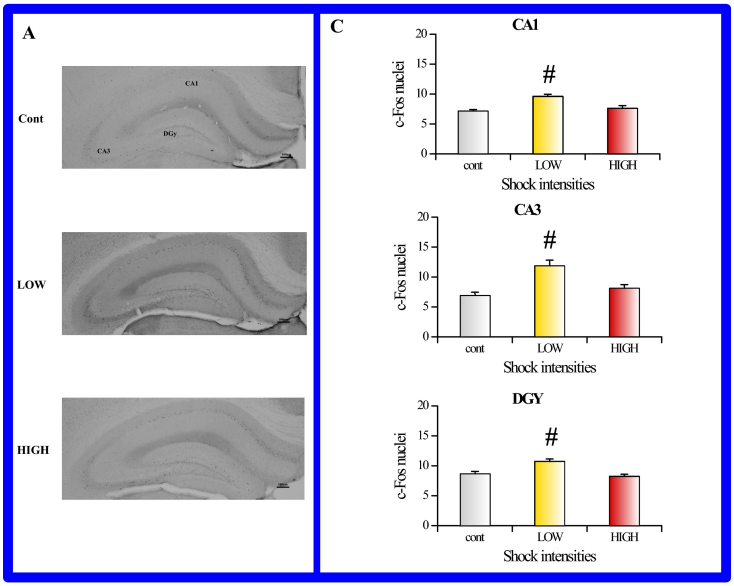

Figure 4. Induction of c-fos expression promoted by different intensities of foot-shock after IA training in dorsal hippocampus (CA1, CA3 and dentate gyrus). **A.** Representative microscope photogarphs of brain regions of animals exposed to different shocks in IA training. **B.** Means (± SEM) of density of c-fos activation in CA1, CA3 and DGy from all groups exposed to both foot-shocks intensities in IA training and control. # vs all, $p < 0.05$. Calibration bar 100µm.

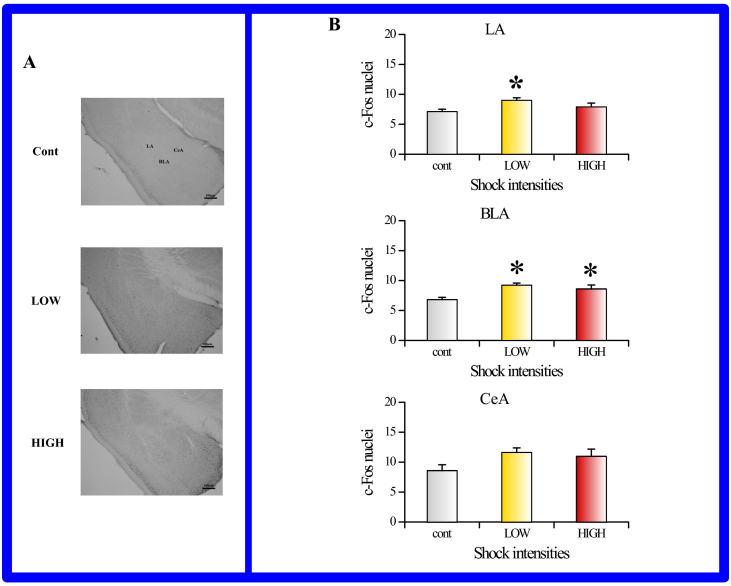

Figure 5. Induction of c-fos expression promoted by different intensities of foot-shock after IA training in amygdala (lateral, LA; basolaterlal, BLA; and central, CeA amygdala). **A.** Representative microscope photogarphs of brain regions of animals exposed to different shocks in IA training. **B.** Means (± SEM) of density of c-fos activation in LA, BLA and CeA from all groups exposed to both foot-shocks intensities in IA training and control. * vs cont, $p < 0.05$. Calibration bar 100µm.

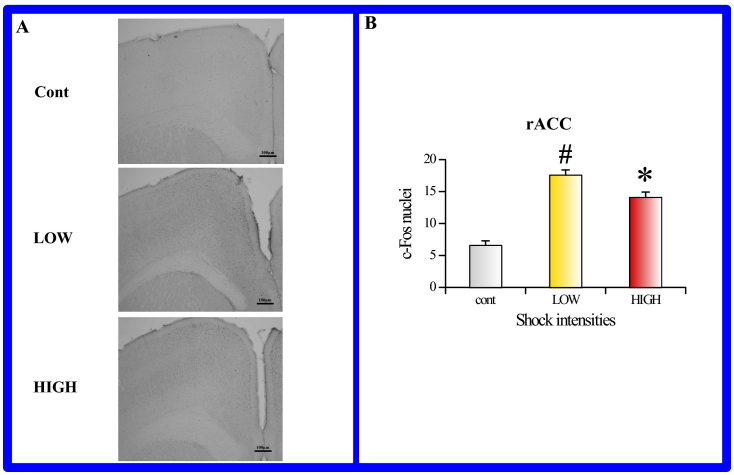

Figure 6. Induction of c-fos expression promoted by different intensities of foot-shock after IA training in rostral Anterior Cingulate Cortex. **A.** Representative microscope photogarphs of brain regions of animals exposed to different shocks in IA training. **B.** Means (± SEM) of density of c-fos activation in CA1, CA3 and DGy from all groups exposed to both foot-shocks intensities in IA training and control. * vs cont, $p < 0.05$; # vs all, $p < 0.05$. Calibration bar 100µm.

Discussion.

IA training under both shock intensities promoted memory consolidation of the experience as shown by expression of similar levels of IA step-through latencies 24 hours after training. Nonetheless, only High mice showed IA one week after training. These results replicate, in mice, those obtained with step-down IA training in rats (Bekinnschtein et al., 2007; 2008 a; b; Katche et al; 2009). Therefore they offer support to the view that moderate emotional arousal is sufficient to promote LTM but insufficient to establish LLM.

IA training increased c-Fos expression in all sampled areas but not in the CeA. However, training with high shock promoted c-Fos expression only in BLA and rACC. These findings are in line with evidence that both BLA and rACC are involved in modulating the storage of emotional events and that BLA interact with rACC to consolidate IA under high emotional arousal (Malin et al., 2005, 2007). The lack of activation of c-Fos expression in hippocampus CA1 and CA3 by training with the high shock is in contrast with results

obtained in rats (Katche et al; 2009). Discrepant results could be explained by differences in the species and/or the task used step-through for the present results and step-down IA for results obtained by the rat study. However, is worth pointing out that the results of the Katche study did not support any relationship between IEGs expression early postraining and persistence of established LTM.

Part 2: Validation of an experimental procedure that modulates the amount of positive emotional arousal during object sampling (training) in an object recognition test (ORT)

Material and Methods.

Subjects

Male CD1 mice (Harlan Laboratories, Udine, Italy) were purchased at 6 weeks of age and housed four to a cage on a 12 hours light-dark cycle (lights on at 7:00 am). Food and water were available *ad libitum* except when food deprivation was required by design (see methods). Mice were left undisturbed for two weeks before behavioral testing. Experiments were carried out in accordance with the Italian national law (DL 116/92) on the use of animals in research.

Drugs

All drugs were injected intraperitoneally (i.p.). The dopamine D1 receptor antagonist SCH 23390 (Schering Corporation, USA) and the β-adrenergic receptor antagonist dl-Propranolol hydrochloride (Sigma-Aldrich, Milano, Italy) were dissolved in 0.9% saline solution at a volume of 10 ml/kg at doses of 0, 0.01 or 0.025 mg/kg and 0, 1 or 2 mg/kg respectively. The doses were chosen on the basis of previous findings that they do affect memory consolidation for the dopamine D1 receptors antagonist (Castellano et al. 1991; 1997) and reduce noradrenergic activation (Cahill, 2000; Lee et al., 2001; Zhang et al., 2008) for β-adrenergic receptor antagonist. All drugs were administered immediately or 120 minutes post-training.

Behavioral procedures.

Object recognition.

Mice underwent object recognition test in a custom made apparatus consisting of a square box (60 x 60 x 30 cm) made of black Plexiglas® subdivided in four equal arenas. The floor of the arenas was made of textured transparent Plexiglas® surmounting a plate of opalescent Plexiglas® thick 1 cm. The apparatus was dimly illuminated by halogen light sources placed below it (Nilsson et al., 2007). Light sources (four) were carefully positioned approximately 50 cm under each corner of the apparatus and directed toward the room floor. This allowed an homogeneous illumination throughout all arenas (~10 lux, measured with a PCE-EM882 multimeter from http://www.pce-italia.it). The apparatus was placed in a ventilated sound-attenuated cabinet. Objects to discriminate were four types, one type for arena: white wooden cube, gray plastic cylinder, gray plastic sphere and silver metallic parallelepiped (Fig. 7). They were available in triplicate copy and,

based on pilot studies, all aroused comparable levels of exploration in CD1 mice (data not shown). For training phase, objects were placed at two opposite corners of the arenas (6.5 cm from the object center to the corner). To prevent mice from displacing objects during testing, they were temporarily fixed to the floor of the arenas with repositionable adhesive pastels (UHU Patafix White).

On the training trial, mice were allowed to freely explore two identical objects (Sample objects: A1 and A2) for 15 minutes. On the test trial, the third copy of the familiar object (A3) and a novel object (B) were placed in the same location of training and mice were allowed to freely explore them for 10 minutes. Cohorts of four mice belonging to the same cage and to the same experimental group were tested simultaneously. Each mouse was tested only once. All combinations and locations of objects were counterbalanced to reduce potential biases because of preference for particular locations or objects. Between each session, apparatus and objects were thoroughly cleaned with 70% ethanol to remove urine and fecal boli and to homogenize olfactory trails. Mice behavior was recorded with a

camera connected to a Debian GNU/Linux (http://www.debian.org) workstation equipped with hardware MPEG encoding capabilities. MPEG videos were blindly analyzed by experienced observers. Object exploration was defined as pointing the nose to the object at a distance of 1 cm and/or touching it with the nose. Turning around, climbing or sitting on an object was not considered as exploration.

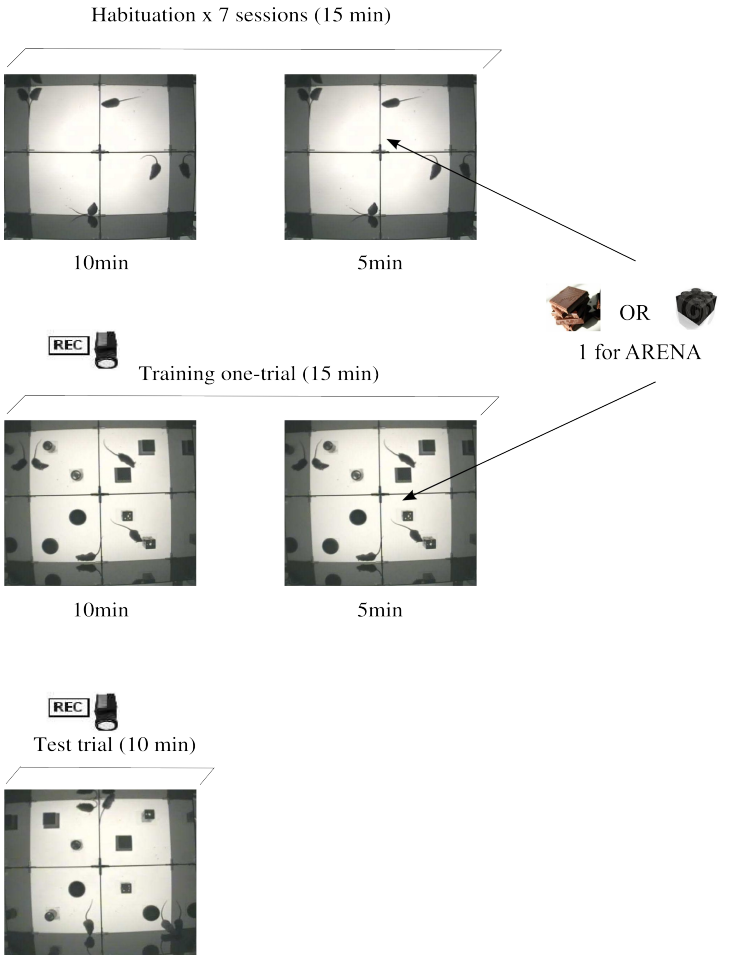

Figure 7. Phases of modified version of one-trial Object Recognition Task. Upper panel shows habituation phase, intermediate pannel training trial and lower pannel test trial.

Data analysis.

Statistical analyses were performed on a Debian GNU/Linux workstation using the R free software environment for statistical computing and its graphical interface R-Commander (Fox, 2005). An error probability level of $p < 0.05$ was accepted as statistically significant. Data from experiment 1 were analyzed using: (1) two-way analysis of variance (ANOVA) for independent factors to compare High and Low mice either at 24 or 96 hours post-training; (2) two-way ANOVA for independent factors to compare High mice free-fed (FF) or food-deprivated (FD) either at training or at test; (3) one-way ANOVA to compare mice that, except for initial pre-exposure, received chocolate only during conditioning phase (PRE), only after training (POST), both during conditioning and after training (PRE + POST). In case of significant two-way interaction simple effect analysis was performed with the Student's t-test for independent samples (Welch variant, two-tailed). Data from experiment 2 were analyzed using: (1) one-way ANOVA to compare to

compare either High mice treated with different SCH 23390 doses (0, 0.01 or 0.025 mg/kg) and Propranolol (0, 1, or 2 mg/kg) and to compare Low mice treated with all doses of Propranolol. (2) Student's t-test for independent samples (Welch variant, two-tailed) to compare Low mice treated with vehicle or 0.01 mg/kg SCH immediately post-training. Welch two-tailed test was also used to compare High mice treated with vehicle or 0.025 mg/kg SCH or 2 mg/kg Propranolol 120 min post-training. In case of significant overall one-way F ratio, ANOVA was followed by Tukey's post hoc tests.

For all experiments, one sample t-tests (two-tailed) were used to determine whether the discrimination index (D.I.) of each experimental group was different from 0 (chance level). Mice showing less than 5 s of total object exploration during ORT test were excluded from analysis (Sik et al., 2003).

Experiment 1.

This experiment was aimed to set-up a model to evaluate the effect of positive emotional arousal on LTM duration in mice and to evaluate the relative role of acute food deprivation and of the caloric and/or fat properties of chocolate in that model.

Effect of positive arousal on long-term memory duration. Sixty-four mice were randomly assigned to this experiment. Mice were preexposed for 15 hours to milk chocolate (Lindt, two pieces for cage, each weighing approximately 3 grams) mixed to standard food in the home-cage from 7:00 pm of the day before the starting of behavioral testing. Then, mice underwent a delay conditioning phase lasting 4 days that we call habituation. This phase comprised two daily sessions on day 1-3 (10:00 am and 2:00 pm) and a single session on day 4 (10:00 am). Sessions were as follows: mice were allowed to freely explore the empty arenas for 10 minutes then, depending on the experimental group, chocolate pieces (high emotional arousing experience,

High, n = 16) or black Lego ® blocks (low emotional arousing experience Low, n = 16), approximately of the same form and size of chocolate pieces, were dropped in the arenas by the experimenter (one stimulus for arena). Mice were allowed to interact with these stimuli for additional 5 minutes (total habituation session duration: 15 minutes). At 7:00 pm of day 3 all mice were food-deprived. On day 4 at 2:00 pm all mice underwent ORT training. Training session differed from habituation sessions only for the presence of sample objects in the arenas. Immediately after training, mice were returned to their home cages and they were given *ad libitum* food. Half mice from each group were tested for sample object recognition 24 hours after training, the remainder were tested 96 hours after training. At 7:00 pm of the day before test session all mice were food-deprived.

Control of the role of acute food deprivation. Sixty-four mice were randomly assigned to this experiment. Mice were submitted to the same protocol as the High group except for the following differences: a first group (n = 16, FF-FF) was never food-deprived neither before

training nor test session; a second group (n = 16, FF-FD) was food-deprived 15 hours before training but not before test; a third group (n = 16, FD-FF) was food-deprived 15 hours before test but not before training. These groups were compared with the High group of previous experiment, here renamed FD-FD. Sample object recognition was tested 96 hours after training.

Control of the role of schedule of chocolate exposure. Forty-eight mice were randomly assigned to this experiment. Mice were submitted to the same protocol as the High group but, half (PRE n=16) received chocolate only during conditioning phase, the remainder (POST n=16) received chocolate only during the last 5 minutes of training. Such groups were compared with the High group of previous experiment, here renamed PRE+POST (n=16). Mice were tested for sample object recognition 96 hours after training.

Experiment 2.

The following set of experiments aim at pharmacological validation of the test.

Effects of post-training administration of SCH23390 on retention of a sampled object 24 or 96 hours after ORT training. One hundred forty-four mice were used in this set of experiments. Two groups of mice (n = 12) were trained in the same protocol of Low group of experiment 3 and received vehicle or the low dose of SCH immediately post-training and were tested for object discrimination 24 hours after training. Six groups of mice (n = 12) were treated identically to the High group of Experiment 3 and administered 0, 0.01 or 0.025 of SCH 23390 (SCH) immediately after training. Four groups of mice (n = 12) received an injection of the highest dose of SCH or vehicle 120 minutes after training. Half of the groups from each treatments were tested for object discrimination 24 hours after training, the reminders were tested 96 hours after training, excpet for Low-trained mice that were tested only for 24 hours

after training.

Effects of post-training administration of Propanolol on retention of a sampled object 24 and 96 hours after ORT training. One hundred fifty-six mice were used in this set of experiments. Three groups of mice (n = 12) were trained in the same protocol of Low group of experiment 3 and received 0, 1 or 2 mg/kg injection of Propranolol immediately post-training and were tested for object discrimination 24 hours after training. Six groups of mice (n = 12) were treated identically to the High group of Experiment 3 and administered 0, 1 or 2 mg/kg of Propranolol immediately after training. Four groups of mice (n = 12) received an injection of the highest dose of Propranolol or vehicle (0 mg/kg) 120 minutes after training. Half of the groups from each treatments were tested for object discrimination 24 hours after training, the reminders were tested 96 hours after training, excpet for Low-trained mice that were tested only for 24 hours after training.

Results.

Experiment 1. *Effect of positive arousal on long-term memory duration.* Two-way ANOVA for total exploration time did not reveal significant effects nor interaction for factors "emotionally arousing experience" and "inter-trial interval". Figure 8 shows the discrimination index (D.I.) measured 24 and 96 hours after training as a function of both high and low emotional situation. Two-way ANOVA for D.I. revealed a significant interaction between aroused situation and inter-trial interval [$F_{1,60}$=5.06, $p<0.05$]. For 24 hours test, simple effect analysis did not reveal a significant D.I. difference between High and Low groups and one sample t-tests revealed that D.I. from both groups was significantly above chance level [High: t_{15}=3.56, $p<0.005$; Low: t_{15}=5.46, $p<0.0001$]. For 96 hours test, simple effect analysis revealed that Low group had a lower D.I. respect to High group [t_{30}=3.02, $p<0.01$]. Moreover, simple effect analysis for each level of stimulus paired with test context revealed that only the D.I. of Low group at 96 hours test was lower than that of

24 hours test [t_{30}=2.42, $p<0.05$]. One sample t-tests revealed that only D.I. from Low group was significantly above chance level [t_{15}=7.24, $p<0.0001$].

Control of the role of acute food deprivation. Two-way ANOVA did not reveal significant effects nor interaction between factors "feeding at training" and "feeding at test" in total exploration time. Figure 9 shows the D.I. measured 96 hours after training as a function of feeding state (FF vs FD) before ORT sessions (training vs test). Two-way ANOVA revealed a significant effect of "feeding before training" [$F_{1,60}$=5.06, $p<0.05$] and a significant interaction between "feeding before training" and "feeding before test" [$F_{1,60}$=4.93, $p<0.05$]. Simple effects analysis revealed that: D.I. of mice food-deprived before both training and test (FD-FD) was higher than that of mice food-deprived before training but not before test (FD-FF) [t_{30}= -2.50, $p<0.05$]; D.I. of mice food-deprived before both training and test (FD-FD) was higher than that of mice free-fed before training but not before test (FF-FD) [t_{30} = -3.11, $p< 0.005$]; D.I. of mice free-fed before both training and test (FF-FF) did not

differ from that of mice free-fed before training but not before test (FF-FD); D.I. of mice free-fed before both training and test (FF-FF) did not differ from that of mice free-fed before test but not before training (FD-FF). One sample t-tests revealed that D.I. was significantly above chance level for all groups [FD-FD: $t_{15}=7.25$, $p<0.0001$; FD-FF: $t_{15}=3.74$, $p<0.005$; FF-FD: $t_{15}=2.86$, $p<0.05$; FF-FF: $t_{15}=3.92$, $p<0.005$].

Control of the role of schedule of chocolate exposure. One-way ANOVA for total exploration did not reveal significant group differences. Figure 10 shows the D.I. measured 96 hours after training as a function of High emotionally arousing experience. One-way ANOVA for D.I. revealed a significant group effect [$F_{2,45}=7.30$, $p<0.01$]. Tukey post-hoc analysis revealed that POST group had a lower D.I. respect to both PRE and PRE+POST groups which did not differ each other. One sample t-tests revealed that D.I. was significantly above chance level only for PRE [$t_{15}=4.46$, $p<0.001$] and PRE+POST [$t_{15}=7.30$, $p<0.0001$].

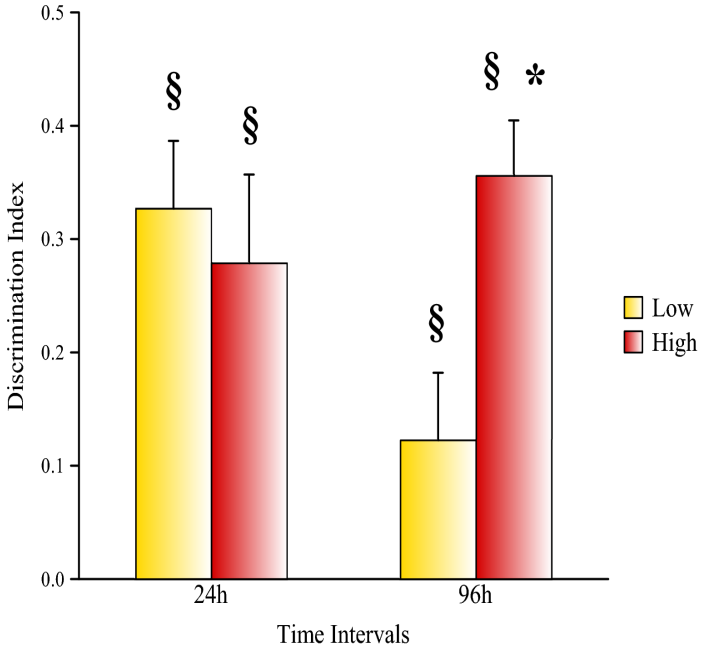

Figure 8. Discrimination index (mean ± SEM) as a function of stimulus paired with test context measured 24 or 96 hrs post-training (*vs High, $p < 0.01$; # vs 24 hrs, $p < 0.05$; § vs 0, $p < 0.0001$).

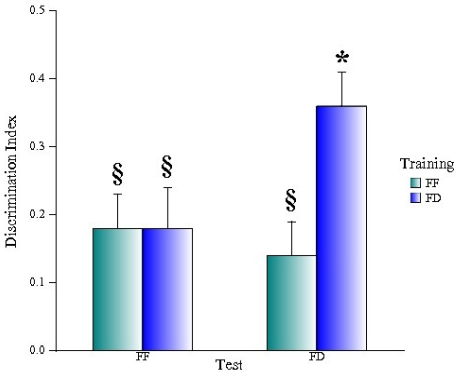

Figure 9. Discrimination index (mean ± SEM) as a function of feeding state before behavioral testing measured 96 hrs post-training (*vs FD–FF $p < 0.05$; # vs FF–FD, $p < 0.005$; § vs 0, $p < 0.0001$).

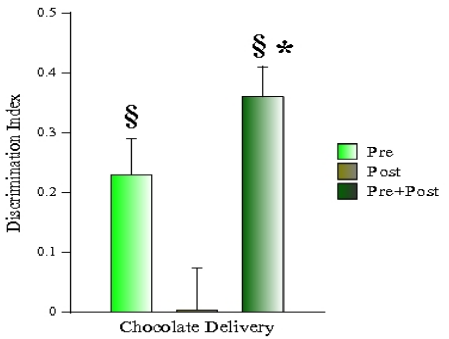

Figure 10. Discrimination index (mean ± SEM) as function of chocolate administration protocol measured 96 h post-training (*vs all, $p < 0.05$; § vs 0, $p < 0.01$).

Experiment 2. *Effects of post-training administration of SCH23390 on retention of a sampled object 24 or 96 hrs after ORT training.* Figure 11a shows the D.I. measured 24 hours after training in Low mice treated with vehicle or low dose of SCH23390. Two-sample t-test revealed that SCH-treated mice had lower D.I. respect to vehicle-treated mice ($t_{16.68}$= 2.306, $p<0.05$). One sample t-test revealed that D.I. was significantly above chance level only in the vehicle-treated group (t_{10} = 5.88 $p<0.001$). Data collected in High mice tested 24 or 96 hours post-training are shown in figure 11 (b and c). One-way ANOVA did not reveal significant group differences for total exploration time. Post-trial administration of the dopamine D1 antagonist SCH 23390 dose-dependently decreased discrimination of a novel object in a test performed 24 hours after training (Fig. 11b). One-way ANOVA revealed a significant effect of the drug treatment on D.I. between groups ($F_{2,33}$ = 3.621, $p < 0.05$). Tukey's post hoc revealed that only the group treated with high dose of SCH showed significantly lower D.I. than the vehicle-treated group. One sample t-tests

revealed that D.I. of both saline-treated mice and of mice treated with the low dose of SCH significantly above chance level (0 mg/kg $t_{11} = 4.997$ $p < 0.001$; 0.01 mg/kg $t_{10} = 2.463$ $p<0.05$). Post-trial administration of the dopamine D1 antagonist SCH dose-dependently decreased discrimination of a novel object tested 96 hrs after training (Fig. 11c). One-way ANOVA revealed a significant effect of the treatment on D.I. ($F_{2,33}= 16.307$, $p<0.001$). Tukey's *post hoc* revealed a significant differences between D.I. expressed by different groups. One sample t-tests revealed that D.I. was significantly above chance level in the vehicle-treated group as well as in the group treated with low dose of SCH (0 mg/kg $t_{11} = 11.86$ $p<0.0001$; 0.01 mg/kg $t_{11} = 6.929$ $p<0.0001$). Table 1 shows the D.I. presented by High mice receiving pharmacological treatments 120 min post-training. For SCH-treated and vehicle-treated mice two sample t-test did not reveal significant differences between groups in mice tested either 24 and 96 hrs after training. One sample t-test revealed that D.I. was significantly above chance level for all groups 24 and 96 hrs after training.

Effects of post-training administration of Propanolol on retention of a sampled object 24 or 96 hours after ORT training. Figure 12a shows the D.I. measured 24 hours after training in Low mice. One-way ANOVA did not revealed a significant effect of the drug treatment on D.I. between groups. One sample t-test revealed that D.I. was significantly above chance level in all groups of mice (0 mg/kg $t_{11} = 5.88$ $p < 0.001$; 1 mg/kg $t_{11} = 4.059$ $p < 0.05$; 2 mg/kg $t_{11} = 4.658$ $p < 0.001$). Data collected in High mice tested 24 or 96 hours post-training are shown in figure 12 (b and c). One-way ANOVA did not reveal significant group differences for total exploration. Post-trial administration of the β-adrenergic receptor antagonist Propranolol dose-dependently decreased discrimination of a novel object tested 24 hours after training (figure 12b). One-way ANOVA revealed a significant effect of the drug treatment on D.I. between groups ($F_{2,33} = 3.987$, $p<0.05$). Tukey's post hoc revealed that the groups treated with both doses of Prorpranolol showed significantly lower D.I. than the vehicle-treated group. One sample t-tests revealed that D.I. is significantly above chance level in all groups (0 mg/kg

t_{11} = 4.997 $p<0.001$; 1 mg/kg t_{11} = 2.333 $p<0.05$; 2 mg/kg t_{11} = 3.23 $p<0.01$).

Post-trial administration of the β-adrenergic receptor antagonist Propranolol dose-dependently decreased discrimination of a novel object tested 96 hours after training (Fig. 12c). One-way ANOVA revealed a significant effect of the treatment on D.I. ($F_{2,33}$ = 8.524 $p < 0.01$). Tukey's post hoc revealed that Propranolol only the group treated with high dose of Propranolol had a lower D.I. than the vehicle-treated group. One sample t-tests revealed that D.I. was significantly above chance level only for vehicle-treated group and low dose Propranolol-treated group (0 mg/kg t_{11} = 11.86 $p<0.0001$; 1 mg/kg t_{11} = 7.23 $p<0.0001$). Table 1 shows the D.I. presented by High mice receiving pharmacological treatments 120 min post-training. For Propranolol-treated and vehicle-treated mice two sample t-test did not reveal significant differences between groups in mice tested 24 amd 96 hours after training. One sample t-test revealed that D.I. was significantly above chance level for all groups 24 and 96 hours after training (see Table 1).

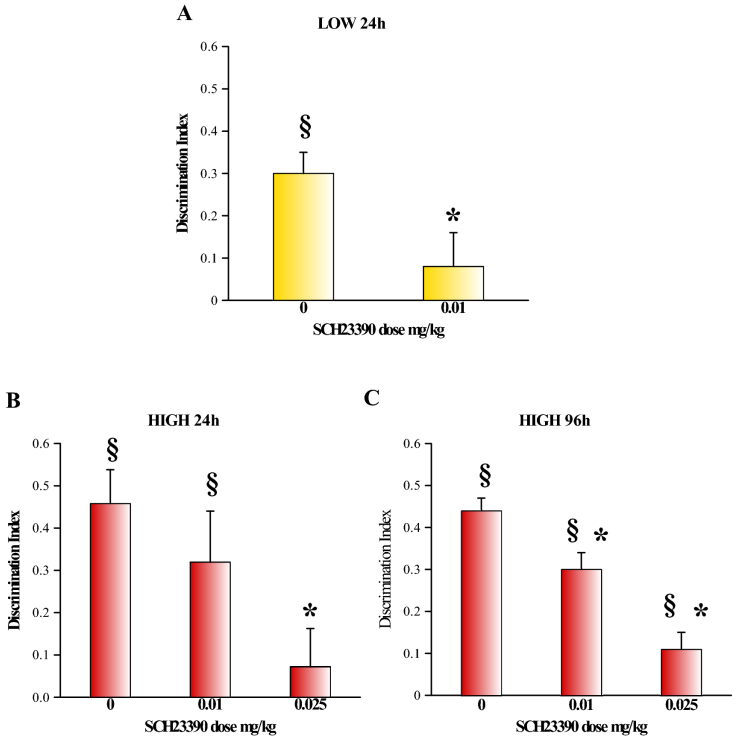

Figure 11. A. Discrimination index (mean ± SEM) as a function of SCH23390 dose (0 and 0.01 mg/kg) injected immediately after training in the low arousing version of ORT measured 24 hrs post-training. * vs saline, $p<0.05$; § vs zero, $p<0.05$. (**B** and **C**) Discrimination index (mean ± SEM) as a function of SCH23390 dose (0, 0.01 and 0.025 mg/kg) injected immediately after training in the high arousing version of ORT measured 24 (**B**) or 96 (**C**) hrs post-training.

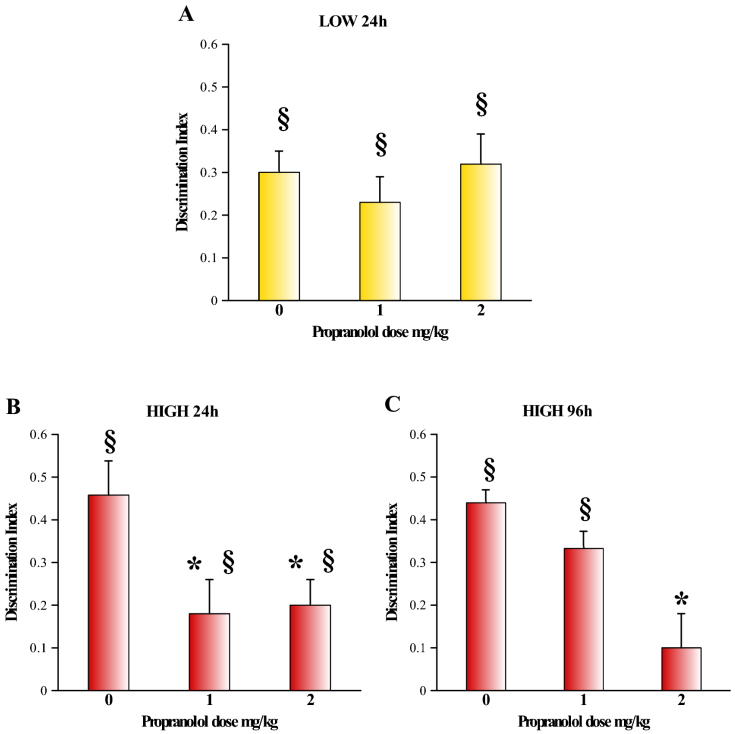

Figure 12. A Discrimination index (mean ± SEM) as a function of Propranolol dose (0, 1 and 2 mg/kg) injected immediately after training in the low arousing version of ORT measured 24 hrs post-training. * vs vehicle, $p<0.05$; § vs zero, $p<0.05$. (**B** and **C**) Discrimination index (mean ± SEM) as a function of Propranolol dose (0, 1 and 2 mg/kg) injected immediately after training in the high arousing version of ORT measured 24 (**B**) or 96 hrs (**C**) post-training.

Drug (mg/kg)	**120 post (24h)**		**120 post (96h)**	
Vehicle	0.27 ± 0.05	t_{10}= 4.884 p<0.001	0.29 ± 0.05	t_{11}= 4.12 p<0.05
SCH (0.025)	0.19 ± 0.06	t_{11} = 2.98 p<0.05	0.29 ± 0.05	t_{11}=6.18 p<0.0001
Prop (2)	0.3 ± 0.05	t_{11}=5.922 p<0.0001	0.26 ± 0.05	t_{11}=6.56 p<0.01

Table 1. Mean (± SEM) and one sample t-tests for chance level of D.I. on the testing trial 24 or 96 hours after training of SCH23390 and Propranolol groups injected with highest doses 120 min after training (120 post).

Discussion.

The main result of this set of experiments is that long-term object memory is enhanced when sampling is performed under positive emotional arousal. Indeed, in the present experiments we demonstrated that mice remember a sampled object for 24 hours but this memory can last 96 hours if object sampling is performed in high positive emotional arousal.

The present study used a modified version of the widely used ORT that allowed modulation of the memory trace acquired during sampling by positive emotional arousal. To this aim we associated the experimental context with chocolate and then increased the motivational salience of the unconditioned stimulus by submitting the mice to a period of food deprivation immediately before ORT training and testing phases. According to several studies on reward anticipation, animals respond with a positive emotional arousal when they are returned into contexts that predictst the availability of highly palatable, caloric, and fat foods (Alcaro et al., 2007; Boissy et al., 2007; Alcaro and

Panksepp, 2011). Mice trained and tested in a context associated with an inedible plastic (Low) stimulus were used as controls.

Both Low and High mice discriminated the novel object when tested 24 hours after training demonstrating intact memory of the previously sampled object. However, 96 hours after training only mice trained and tested in high positive emotional situation, the chocolate-associated context (High), were still able to discriminate the novel object. These data support the view that experiences associated with a state of positive emotional arousal are consolidated in more lasting memories as reported for those associated with negative ones. Control experiments reported by the present work indicate that the LTM of the sampled object is modulated by the chocolate associated context rather than by the chocolate feeding immediately after sampling. Indeed, mice that did receive chocolate on the object sampling session without previous chocolate-context pairing did not show discrimination of a novel object 96 hours later, whereas mice that did not receive chocolate but were trained in a context previously associated with chocolate

did. These findings rule out the influence of sugar or fat (Campolongo et al., 2009; Smith et al., 2011) on memory consolidation in our experimental conditions. Moreover, in our standard experimental condition chocolate was made available at the end of the sampling session to prevent negative emotional arousal associated with frustration (for review see Flaherty,1996) as well as contingency between sample object and reward (Hughes, 2007).

Moreover, the data suggest that the positive emotionally arousing experience was capable to promote a lasting memory of the sampled object also in free-feeding mice; however, food-deprived mice showed a D.I. significantly higher than free-fed mice. This result supports the hypothesis that imposing 15 hours of food deprivation before objects sampling increased the positive emotional arousal promoted by chocolate-associated context in line with the hypothesis that a physiological depletion enhances the incentive value of the unconditioned and conditioned goal stimuli (Berridge, 2004). Mice food-deprived before training but not before test showed lower discrimination than mice

trained and tested in food deprivation strongly supporting a state-dependent learning. Finally, mice food-deprived only on test day (96 hours) did not show any improvement of object discrimination in comparison with free-fed mice. The latter observation indicates a selective effect of the motivational state on memory formation and/or consolidation rather than on retrieval. Taken together, these findings support the view that acute food deprivation increased emotional arousal promoted by the context associated with chocolate, it is tempting to speculate that the condition of feeding deprivation invested the experience of re-entering into the reward-predicting context of a flashbulb-like quality (Brown and Kulik, 1977).

The main result of the pharmacological experiments is the observation that LTM of the sampled object was impaired by post-training administration of propanolol only when sampling was performed under positive emotional arousal. Lack of effect of Propanolol in Low mice is in apparent contrast with previous results showing that Propanolol infused in the BLA of rats immediately following object sampling interferes with

discrimination of a novel object tested 24 hrs later (Roozendaal et al., 2008). However, in the cited experiments rats were exposed to the testing cage for the first time during ORT training, whereas in the present experiments mice had 7 previous experiences with the test cage. It is reasonable to hypothesize that the level of emotional arousal promoted by the training situation in the rat experiment (Rozendaal et al., 2008) was much higher than that experienced by mice from the Low group in the present study. Finally, the impairing effects of Propanolol on discrimination of a novel object were observable in mice treated immediately but not 120 minutes post-sampling. These results support the hypothesis that activation of ß-adrenergic receptors mediates the impact of emotional arousal on memory consolidation regardless of the hedonic sign (positive or negative) of the experience.

In sharp contrast with the effects of Propanolol, the dopamine D1 receptor antagonist SCH23390, administered post-trial decreased 24 hours retention performance in both High and Low mice. In addition, the lower dose of the D1 antagonist was effective in Low

but not in High mice. Previous work has demonstrated the DA dependence of learning for step-down inhibitory avoidance, the radial maze, and water maze (Packard and White, 1991; Gasbarri et al., 1996; Bernabeu et al., 1997; O'Carroll et al., 2006). However, pre-training SCH23390 administration was reportedly ineffective in ORT (deLima et al.,2010). The discrepancy between this previous and the present results is difficult to explain although the two studies differed for the species (mice in the present rats in the previous), drug dosages (the highest dose used in the present experiments was one half of the lowest used in the previous), and time of treatment (pre-training vs post-training). The effects observed in the 24 hours test suggested reduced sensitivity to D1 receptors blockade in mice trained under high emotional arousal. In previous experiments we have demonstrated that whereas retention of High and Low mice do not differ on the 24 hours test, only High mice show discrimination of a novel object when tested 96 hours after training (experiment 1). Therefore, three additional groups (0 and 0.01, 0.25 mg/kg doses of SCH23390) of High mice were tested 96 hours after

object sampling. The results, showing that both doses were effective in reducing retention expressed at this time point, did not support the hypothesis that ORT under high emotional arousal is less dependent on D1 receptor stimulation. Finally, our effects on retention induced by the dopamine D1 receptor antagonists seem to be due to an effect on memory consolidation. Indeed, these effects were observed when drugs were given immediately, but not 120 minutes, post-training when the memory trace is susceptible to modulation (Gold et al., 1975). These results support the hypothesis that activation of dopamine D1 receptors mediates memory consolidation regardless of the level and the hedonic sign (positive or negative) of the experience.

Part 3: Effects of different levels of positive emotional arousal on the induction of c-Fos expression by ORT training.

Materials and Methods.

Subjects.

Male CD1 mice (Harlan Laboratories, Udine, Italy) were purchased at 6 weeks of age and housed four to a cage on a 12 hours light-dark cycle (lights on at 7:00 am). Food and water were available *ad libitum* except when food deprivation was required by design (see methods). Mice were left undisturbed for two weeks before behavioral testing. Experiments were carried out in accordance with the Italian national law (DL 116/92) on the use of animals in research.

Tissue preparation.

One hour after training, animals were sacrificed via rapid cervical dislocation, the brains were removed from skull in approximately 90 seconds and then post-fixed in 4% formaldehyde (Carlo Erba) diluted in 0.1 M (pH 7.4) phosphate buffer (PB) overnight at 4° C. Then brains were immersed for 48–72 h at 4 °C in PB/sucrose 30% for cryoprotection. Brains were blocked in the coronal plane at the level of the optic chiasm and their anterior parts were frozen with dry ice and cut in 40-μm coronal sections with a sliding microtome. Three adjacent series of 8–10 sections sampling the dorsal hippocampus (figure 12a), amygdala (figure 13a) and rostral anterior cingulate cortex (figure 14a) were selected from each brain on the basis of Mouse Brain Atlas of Paxinos and Franklin 2001 and immunostained for c-fos as shows figure 2.

Imunohistochemistry.

Floating sections were processed at room temperature in 24-well microplates on gentle agitation. The steps for imunohistochemistry procedure were as follows:

1)3 x 10 min in Phosphate Buffer + 0.3% Triton X-100 (PBTX)

16. 30 min incubation in PBTX + H_2O_2 (3% for non perfused brains)
17. 3 x 10 min in PBTX (or PB 0.1M)
18. 30 min incubation in Avidin blocking solution [Vector Laboratories] (2 drops/5ml) diluted in PBTX
19. 3 x 10 min in PBTX (or PB 0.1M)
20. 30 min incubation in Biotin blocking solution [Vector Laboratories] (2 drops/5ml) diluted in PBTX
21. 3 x 10 min in PBTX (or PB 0.1M)

22. Overnight incubation in primary rabbit anti c-fos polyclonal antibody (Oncogene, AB-5) diluted 1:20000 in PBTX + 2% Normal Goat Serum (Vector Laboratories)
23. 3 x 10 min in PBTX (or PB 0.1M)
24. 2 hours incubation secondary antibody anti-Rabbit (Vector Laboraories) diluted 1:1000 in PBTX
25. 3 x 10 min in PBTX (or PB 0.1M)
26. 1 hour incubation Avidin-Biotin Complex (Vector Laboratories) diluted 1:1000 in PBTX (prepare 30 min before use)
27. 3 x 10 min in PB 0.1M
28. 4 min incubation in DAB solution diluted 1:2 compared to manufacturer instructions (sections to be compared must be treated simultaneously, i.e. using Costar® Netwells, 15 mm diameter, 500 µm Mesh)

29. 3 x 10 min in PB 0.1M

Data analysis

For this experiment the brain regions were acquired at 4x objective magnification and ImageJ 1.46p free software from http://imagej.nih.gov/ij was used to quantify c-fos immunostaining cells. Statistical analyses were performed on a Debian GNU/Linux workstation using the R free software environment for statistical computing and its graphical interface R-Commander (Fox, 2005). An error probability level of $p < 0.05$ was accepted as statistically significant. One-way ANOVA was used when comparing all groups separately in different brain regions. In case of significant overall one-way F ratio, ANOVA was followed by Tukey's post hoc tests.

Experiment.

This experiment was aimed to evaluate c-Fos expression induced by object sampling to map brain areas involved in consolidation of memories under positive emotional arousal. We first identified brain regions, such as amygdala, dorsal hippocampus and rostra antyerior cingulate cortex, where gene expression is activated after our version of ORT by analyzing the expression of the immediately early genes c-fos as marker. Forty mice were randomly assigned to this experiment. One group of mice (n = 8), which were control group, were trained in the same conditions of experiment 4 but neither chocolate piece nor black Lego® block was dropped in the arenas by the experimenter and have not been introduced the sample objects into the arenas during the training phase (control mice, control). Sixteen mice were trained as in experiment 4 half of mice (n = 8) was High group and the other half (n = 8) was Low group. Two additional groups of mice (n = 8) were trained as control group except for piece of chocolate wich was dropped after 10

minutes and mice were allowed to interact with this stimulus for additional 5 minutes (only chocolate-paired mice, CHOC). The remaining mice were trained in the same condition of High and Low group of the experiment 4 but did not receive neither piece of chocolate nor lego block (mice only trained to object recognition, ORT). All the mice for each condition were sacrificed one hour after training and the brains were processed for c-fos immunostaining (see above in material and methods paragraph).

Results.

Data on c-fos expression of defined mice groups in investigated brain regions are reported in figure 13, 14 and 15. Statistical analysis did not revealed significant difference between groups in dentate gyrus (DGy), whereas analysis revelead significant difference between groups in CA1 ($F_{2,21} = 9.97$, $p<0.001$) and CA3 ($F_{2,21} =$

4.88, $p<0.05$) (Fig. 12b). Tukey's post-hoc showed that both High- and Low-trained mice, wich did not differ each other, had higher c-fos expression respect to control group in CA1 and CA3 subfields. Analysis of dentate gyrus of dorsal hippocampus did not revealed stattistical difference between groups. Furthermore, one-way ANOVA of the comparison between cont, CHOC and ORT groups revealed a significant effect in CA1 and CA3 subfields of dorsal hippocampus (CA1, $F_{2,20} = 7.53$, $p < 0.01$; CA3, $F_{2,20} = 6.59$, $p < 0.01$). Tukey's post hoc showed that only the chocolate increased CA3 c-fos expression whereas in CA1 c-fos expression was higher both in ORT and CHOC group respect to control group of mice (figure 12c). Statistical analysis to compare control (cont), Low and High groups of mice revealed significant differences between groups in basolateral (BLA, $F_{2,21} = 3.80$, $p < 0.05$), lateral (LA, $F_{2,21} = 4.83$, $p < 0.05$) and central (CeA, $F_{2,21} = 6.71$, $p < 0.01$) nuclei of amygdala (figure 13b). Tukey's post-hoc revealed that in all subfields of amygdala investigated c-fos expression was significant higher in High respect to control mice (cont) and only in CeA c-fos expression was higher in

High-trained mice also respect to Low group. In another one-way ANOVA analysis, that compare ORT and CHOC group with control, did not revealed significant difference between groups in basolateral amygdala (BLA) (figure 13c) whereas in the same group statistical analysis revelead significant difference between groups in lateral amygdala (LA, $F_{2,20} = 5.32$, $p < 0.05$), central amygdala (CeA, $F_{2,20} = 6.08$, $p < 0.01$). Tukey's post-hoc analysis showed that CHOC group had higher c-fos immunoreactivity respect to control group in LA and CeA.

Finally, statistical analysis revelead significant difference between groups in rostral anterior cingulate cortex (rACC, $F_{2,21} = 3.74$, $p < 0.05$). Tukey's post-hoc analysis showed that only High group had higher c-fos immunoreactivity respect to control group (figure 15b). In another one-way ANOVA analysis, that compare ORT and CHOC group with control, did not revealed significant difference between groups in rACC (figure 15c).

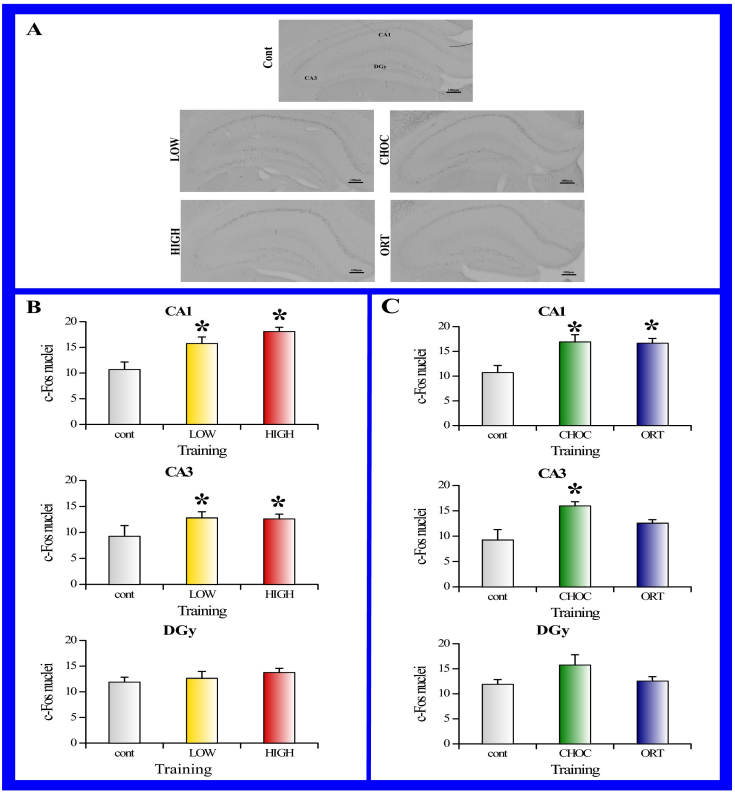

Figure 13. Induction of c-fos expression promoted by stimulus-test cage pairing (**B**) and trained without stimuli and not-trained with chocolate (**C**) compaired with control group in dorsal hippocampus (CA1, CA3 and dentate gyrus). **A.** Representative microscope photographs of brain regions of animals of all groups from both comparisons. **B.** Means (± SEM) of density of c-fos activation in CA1, CA3 and DGy from the groups exposed to both emotionally arousing situations (Low and High) and control group. **C.** Means (± SEM) of density of c-fos activation in CA1, CA3 and Dgy from the groups that controls for stimulus (CHOC) and training (ORT) and control group. * vs Cont, $p < 0.05$. Calibration bar 100µm.

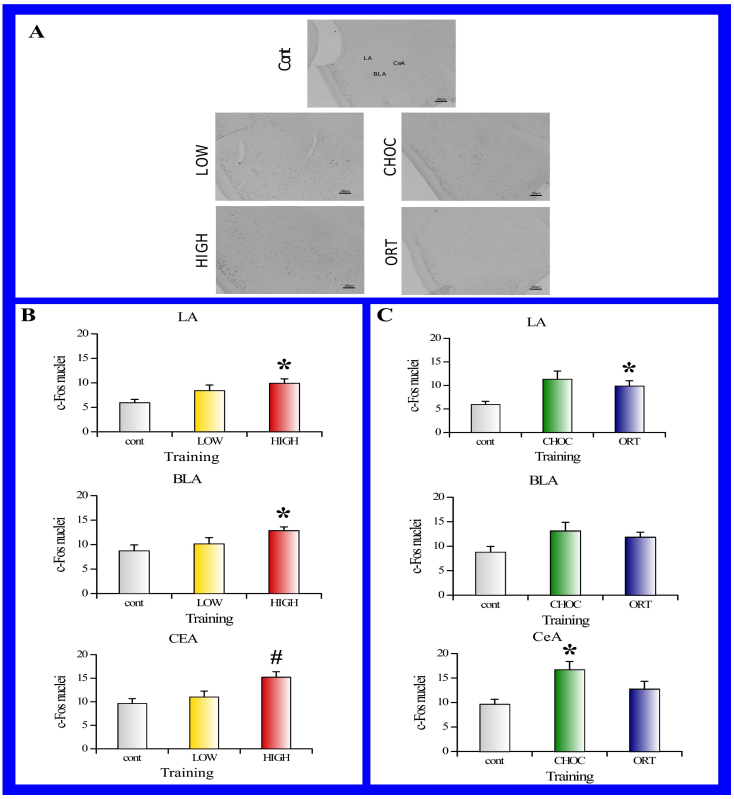

Figure 14. Induction of c-fos expression promoted by positive emotional arousal situation in amygdala (lateral, LA; basolateral, BLA and central amygdala). **A.** Representative microscope photographs of brain regions of animals of all groups from both comparisons. **B.** Means (± SEM) of density of c-fos activation in LA, BLA and CeA from the groups exposed to both emotionally arousing situations (Low and High) and control group. **C.** Means (± SEM) of density of c-fos activation in LA, BLA and CeA from the groups that controls for stimulus (CHOC) and training (ORT) and control group. # vs all, $p < 0.05$; * vs Cont, $p < 0.05$. Calibration bar 100µm.

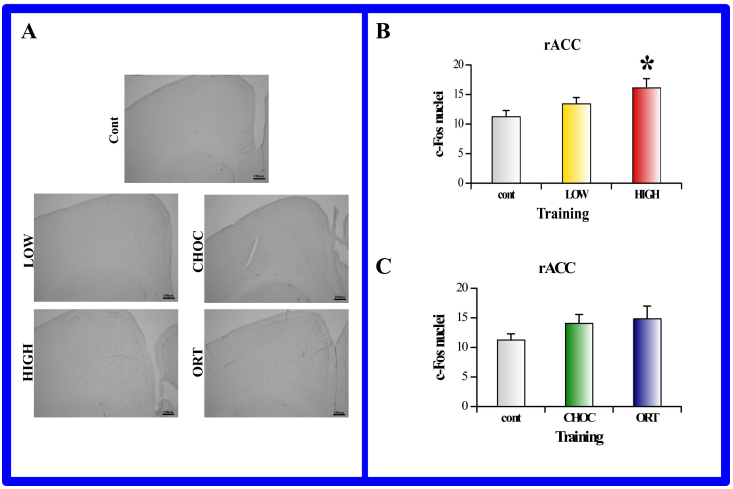

Figure 14. Induction of c-fos expression promoted by positive emotional arousal situation in rostral Anterior Cingulate Cortex (rACC). **A.** Representative microscope photographs of brain regions of all groups from both comparisons. **B.** Means (± SEM) of density of c-fos activation in LA, BLA and CeA from the groups exposed to both emotionally arousing situations (Low and High) and control group. **C.** Means (± SEM) of density of c-fos activation in rACC from the groups that controls for stimulus (CHOC) and training (ORT) and control group. * vs Cont, $p < 0.05$. Calibration bar 100µm.

Discussion.

The results of this final set of experiments reveal a strong homology between the neurobiological mechanisms involved in the modulation of memory consolidation by positive and negative emotional arousal.

Indeed, training for ORT in a state of high positive arousal is associated with a selective increase of c-Fos expression in the BLA and rACC. This conclusion is supported by the observation that only in these brain areas ORT training in the chocolate-associated environment, but neither ORT training per se (ORT group) nor exposition to chocolate per se (CHOC group), enhanced c-Fos expression. Instead, enhanced c-Fos expression in CA1 and CA3 of the hippocampus, LA and CeA was observable in mice from ORT and CHOC groups too.

The observation that both BLA and rACC were the only brain areas showing increased c-Fos expression following IA training with high shock levels offers

further support to the hypothesis that these two brain areas are involved by plastic changes associated with memory consolidation in condition of high emotional arousal.

Another interesting finding of this set of experiments is the selective activation of c-Fos expression in CeA by the exposure to chocolate and chocolate-associated stimuli. This conclusion is supported by the significant difference increase of c-Fos expression between the High and Low groups and between CHOC and ORT group and is in line with recent results supporting a main role of CeA in controlling the target and intensity of appetitive–consummatory behaviors in food-intake tests (Mahler et al., 2009).

GENERAL CONCLUSIONS

The aim of this study was to test the hypothesis that positive and negative emotional arousal influence the same consolidation processes. The results obtained support this hypothesis. Indeed either high shock during IA training or expectation of a palatable food by food deprivation during object sampling for ORT training promoted more persistent memories and c-Fos expression in both the BLA and rACC.

The procedure used to modulate positive emotional arousal during object sampling in ORT is novel. Thus, we tested its sensitivity to post-trial pharmacological manipulations known to interfere with consolidation processes. The results obtained by these experiments support a main role of consolidation mechanisms in promoting both LTM and LLM of the sampled object. Moreover they supported a role of activation of ß-adrenergic receptors in memory consolidation in ORT when training is performed under

high but not low emotional arousal. A wealth of previous evidences support the view that consolidation of memory associated with negative emotional arousal involves activation of ß-adrenergic receptors in the BLA and BLA interaction with cortical areas, with the notable inclusion of rACC (Malin et al., 2005, 2007). Therefore, we suggest that these mechanisms are implicated in consolidation of memory acquired under high positive emotional arousal too.

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My Pubblications (in attachment)

1) "Association between striatal accumulation of FosB/ΔFosB and lon-term psychomotor sensitization to amphetamine in mice depends on the genetic background." Conversi D, Orsini C, Colelli V, **Cruciani F**, Cabib S. Behav Brain Res. 2011 Feb 2;217(1):155-64. Epub 2010 Oct 23. PMID: 20974185.

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3) "In vivo catecholaminergic metabolism in the medial prefrontal cortex of ENU2 mice: an investigation of the cortical dopamine deficit in phenylketonuria." Pascucci T, Giacovazzo G, Andolina D, Conversi D, **Cruciani F,**

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Research report

Association between striatal accumulation of FosB/ΔFosB and long-term psychomotor sensitization to amphetamine in mice depends on the genetic background

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ABSTRACT

Previous results demonstrated association between increased FosB/ΔFosB immunostaining in the ventromedial striatum and behavioral sensitization to amphetamine promoted by repeated stress or by repeated pairings of the psychostimulant and the testing cage in mice of the C57BL/6J strain. The present experiments tested this association in an additional protocol, its stability following the end of the sensitizing procedure and its generalization to mice from a different inbred strain. Eleven days after repeated administration of amphetamine within their home-cages, mice of the C57BL/6J strain expressed sensitization to the psychomotor effects of the psychostimulant when tested in a novel cage. At this time-point the same mice showed increased FosB/ΔFosB immunostaining in the ventromedial striatum. Instead, mice of the genetically unrelated DBA/2J inbred strain expressing robust sensitization in the same protocol did not show changes in FosB/ΔFosB immunostaining throughout the striatal complex. Lack of effects in FosB/ΔFosB immunostaining was also observed in DBA/2J mice behaviorally sensitized by repeated pairings of amphetamine with the test cage. These results demonstrate that mice, depending on the genetic background, can develop robust and long-lasting behavioral sensitization to amphetamine in the absence of striatal ΔFosB accumulation.

1. Introduction

Behavioral sensitization, the enhanced behavioral response to addictive drugs promoted by repeated administration, has been extensively used as a behavioral index of drug-induced brain plasticity in genetic mouse models [1–3]. In most cases presence or absence of behavioral sensitization is taken to support or rule out, respectively, a major role of the targeted genetic product in drug-induced brain plasticity. However, there is evidence that different neural mechanisms are involved in the development and expression of this behavioral phenotype depending on the inducing procedure, the drug used, and the genetic background of the tested animals [4–7].

We have previously reported that mice of the inbred strain C57BL/6J (C57), show increased striatal FosB/ΔFosB immunostaining when sensitized to the locomotor effects of amphetamine [8]. In the ventromedial caudate enhanced FosB/ΔFosB immunostaining was observed in mice sensitized either by repeated stress (10 days of 2 h restraint) or by repeated amphetamine pairings with the test cage (1 daily injection of 2.5 mg/kg over 4 consecutive days), strongly supporting the association between this phenotype and the outcome (sensitization) rather than the procedure or the drug used. Moreover, in both experimental conditions changes in FosB/ΔFosB immunostaining were produced by the repeated rather than the acute treatments, suggesting a major contribution of ΔFosB: the highly stable splice product of the immediate early gene FosB [9].

The latter hypothesis is supported by results obtained by a different group demonstrating increased striatal ΔFosB, measured by Western blots, in C57 mice sensitized by repeated amphetamine [10]. However, a different study did not find accumulated ΔFosB in the accumbens or striatum of adult outbred CD1 mice exposed to 7 daily amphetamine injections within the home cage [11]. Contrasting findings could be explained by differences in the protocols used for repeated amphetamine treatments or with strain differences. Indeed, although the C57 strain is the preferred background for genetic models, mice from this strain, as mice from any other inbred strain, share the specific genetic make-up inherited from their single ancestral pair.

Finally, the most relevant characteristic of behavioral sensitization or ΔFosB accumulation is a relative stability after the end of the

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sensitizing procedures. However, in our previous experiments both locomotor sensitization and FosB/ΔFosB immunostaining were evaluated shortly (24 h) after the end of the sensitizing treatment.

The present experiments were designed to evaluate: (1) the association between increased striatal FosB/ΔFosB and locomotor sensitization to amphetamine induced by an additional protocol in C57 mice; (2) the persistence of this association after the end of the sensitizing treatment; and (3) its generalization to mice of a different and genetically unrelated inbred strain. In a first set of experiments we tested locomotor sensitization to amphetamine expressed by mice of the C57 strain in a novel cage at different time-intervals following the last of repeated injections of the psychostimulant within the home-cages, and evaluated striatal FosB/ΔFosB immunostaining 11 days after the end of drug-pretreatment, when expression of the sensitized response was maximal. In a second set of experiments we tested mice of the inbred strain DBA/2J (DBA) in the same protocol.

The results obtained demonstrated association between long-term locomotor sensitization to amphetamine and enhanced FosB/ΔFosB immunostaining only in C57 mice. However, the lack of association between the behavioral and neural phenotypes in DBA mice could have been specific for the sensitizing procedure used or due to a strain-specific rapid decay of the ΔFosB signal in these mice. Therefore, we performed a third set of experiments and evaluated FosB/ΔFosB immunostaining shortly (24 h) as well as 11 days after ceasing a treatment with repeated amphetamine within the test cage.

2. Materials and methods

2.1. Experimental subjects

Male mice of the inbred strains DBA/2JICo (DBA) and C57BL/6JICo (C57) were purchased from Charles River (Calco, Italy) at six weeks of age and housed four to a standard cage (Technoplast, no. 1144b, Italy). Food and water were available ad libitum. Animals were maintained on a 12/12 h light–dark cycle (lights on 7:00 am) for 2 weeks before testing and then they were randomly assigned to the different treatment groups. All experiments were conducted in strict accordance with the Italian national law (DL 116/92) on laboratory animals based on the European Communities Council Directive of November 24, 1986 (86/609/EEC). All efforts were made to minimize the number of animals used and their suffering.

2.2. Drugs

D-Amphetamine (Research Biochemicals Inc., USA) was dissolved in saline solution (0.9% NaCl) and administered intraperitoneally (i.p.) in a volume of 1 ml/100 g at the dose of either 1.0 or 2.5 mg/kg.

2.3. Apparatus for behavioral data collection

Behavioral data were collected and analyzed by the "EthoVision" (Noldus Information Technology, Wageningen, The Netherlands), a fully automated video tracking system that allows the simultaneous acquisition from up to 16 independent arenas. The acquired video tracks were processed by the software to extract the variable "distance traveled" (cm) as an estimate of locomotor activity. In all experiments locomotor activity was measured in test cages made of gray PVC (10 cm × 40 cm × 16 cm) and covered by transparent Plexiglas. Behavioral recording sessions lasted 1 h.

2.4. Behavioral experiments

A schematic presentation of the time line of the different experiments is presented in Table 1.

2.4.1. Experiment 1

Five groups (n = 4) of C57 mice were used for this set of experiments. Two groups of mice were injected twice daily for 4 consecutive days within their home cages with saline, while the other groups received amphetamine (2.5 mg/kg) according to the same protocol. All saline-pretreated mice and mice from one of the amphetamine-pretreated groups were tested for locomotor activity in a previously unexplored cage 11 days after the end of the pretreatment. Half of the saline-pretreated mice received a saline injection (Saline) and the other half a challenge with 1 mg/kg of amphetamine (Acute) immediately before testing. The amphetamine-pretreated mice were challenged with 1 mg/kg of amphetamine (Repeated +11) immediately before testing. The other two groups of amphetamine-pretreated mice were challenged with 1 mg/kg of amphetamine 5 (Repeated +5) or 17 days (Repeated +17) after the end of the drug pretreatment.

2.4.2. Experiment 2

Five groups (n = 4) of DBA mice were used for these experiments. Treatments and protocols were the same as described in Experiment 1.

2.4.3. Experiment 3

This set of experiments involved 14 groups (n = 4) of DBA mice. Mice from all groups were injected twice daily for 4 consecutive days: once in the home cage within the colony room and once in the test cage located in a different room. Four groups of DBA mice received saline in both home and test cages (Saline). Six groups of mice received saline in the home cage and amphetamine (2.5 mg/kg) in the test cage (Paired). Four groups of mice received amphetamine (2.5 mg/kg) in the home cage and saline in the test cage (NPaired).

Each day at 9.00 am, mice were transported, within their home cages, to the testing room. After 1 h, they received the first injection (either saline or amphetamine, depending on the group) and were immediately placed in the test cages where locomotor activity was recorded as described. Then, they were returned to the colony room within their home cages. At 5.00 pm, mice received the second injection in their home cage (saline or amphetamine, depending on the group).

Twenty-four hours after the last injection two groups of DBA mice from each of the 3 pretreatment conditions received a challenge injection of saline or amphetamine (1 mg/kg) and were immediately introduced in the test cage where locomotor activity was recorded. Other two groups from each condition were tested, following the same procedure, 10 days later (day 11). The remaining two groups of DBA mice from the Paired condition were tested under amphetamine challenge (1 mg/kg) 5 or 17 days after the end of the pre-treatment.

2.5. Immunohistochemestry

2.5.1. Experimental groups

Experiments 1 and 2 were aimed at associating long-term effects of repeated amphetamine on behavior with stable FosB/ΔFosB immunostaining. Therefore, in both experiments we used tissue collected from Saline and Acute groups to test for the acute effects of the drug and compared results obtained from these samples with those from the group expressing maximal long-term locomotor sensitization to amphetamine challenge (pretreated with amphetamine and challenged with the drug 11 days after the end of the pretreatment: Repeated +11).

As for Experiment 3, it aimed at testing the ability of a different sensitizing treatment to induce an increase of striatal FosB/ΔFosB immunostaining in DBA mice. The sensitizing paradigm chosen was previously shown to induce both locomotor sensitization to amphetamine and increased ventral striatal FosB/ΔFosB immunostaining in C57 mice [8]. The protocol requires experimental groups exposed to repeated pairings of amphetamine with a test cage (Paired), two groups controlling for the acute effects of the (repeated saline in both home and test cages: Acute), and two groups controlling for the associative effect of drug pairing with the test cage (treated with amphetamine in the home cages but repeatedly exposed to the test cage under saline: NPaired). In this paradigm animals express sensitized response in the Paired but not in the NPaired condition, due to the excitatory influence of the drug-associated context in the former and the inhibitory influence of the explicitly non-associated context in the latter condition [12,13].

FosB/ΔFosB immunostaining was evaluated in tissue samples from mice of the Acute, Paired and NPaired groups challenged with amphetamine 24 h after the end of the drug pretreatment and from mice of the Paired +11 group which were challenged with amphetamine 10 days later. Observation of high FosB/ΔFosB immunostaining in both Paired groups and in the NPaired group was to suggest stable accumulation of ΔFosB by the repeated drug administration; lower FosB/ΔFosB immunostaining in the samples from the Paired +11 group was to suggest a rapid decay of the signal after the end of the sensitizing procedure; and lower FosB/ΔFosB immunostaining in the samples from the NPaired group was to suggest the main contribution of FosB possibly elicited by the drug-associated context.

2.5.2. Tissue preparation

All mice were killed and their brains collected at the end of the 60 min test. Specimens were prepared as previously described [8,14]. Brains were excised and placed in chilled 10% neutral buffered formalin. Two hours later, they were trimmed on the coronal plane approximately between the frontal pole and the bregma. The resulting brain slices (~3 mm) were re-immersed in freshly prepared formalin solution and stored overnight at 4 °C on gentle agitation. Fixed slices were stored in 30% sucrose at 4 °C until they sank, and then they were frozen with dry ice and cut in 40μ transverse sections with a sliding microtome. Three adjacent series of sections were collected from each brain approximately between 2.34 and 0.74 mm from bregma and immunostained for FosB/ΔFosB and dopamine transporter proteins.

2.5.3. Immunostaining

Immunostaining was performed as previously described [8,14]. Briefly, FosB/ΔFosB antigens were detected with a rabbit polyclonal antiserum (sc-48, Santa Cruz Biotechnology, Santa Cruz, CA USA, diluted 1:1000) raised against an internal region of FosB protein that is present also in ΔFosB isoforms [15]. Dopamine

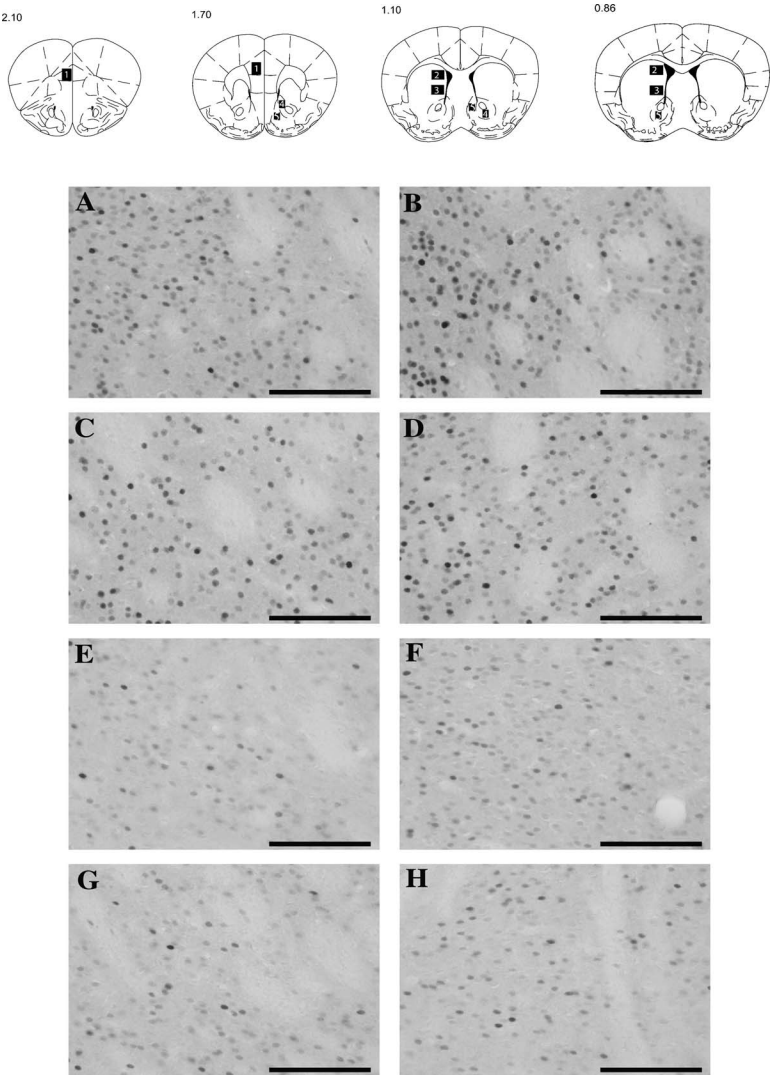

Fig. 1. Upper panel: schematic representation of sample areas subjected to image analysis. (1) Medial prefrontal cortex; (2) dorso-medial caudate-putamen; (3) ventro-medial caudate-putamen; (4) accumbens core and (5) accumbens shell. Numbers above drawings indicate distance from bregma. Drawings were adapted from Franklin and Paxinos [18]. Lower panel: representative photomicrograph of FosB/ΔFosB-immunoreactive cell nuclei from dorso-medial (A, B, E and F) or ventro-medial (C, D, G and H) caudate-putamen of C57 (A–D) and DBA (E–H) mice repeatedly treated with saline (A, C, E and G) or amphetamine (B, D, F and H) before amphetamine challenge in a novel test cage. See Section 2 for more detail. Scale bar = 100 μm^2.

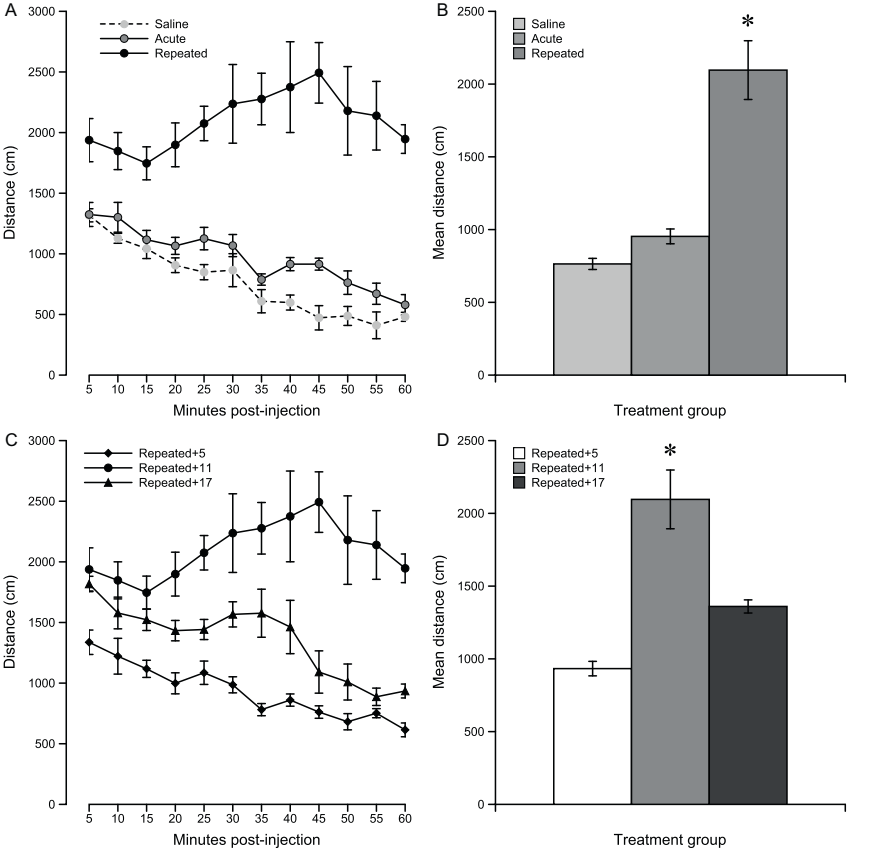

Fig. 2. Locomotor activity (mean cm ± S.E.; (A and C) 5 min blocks; (B and D) 60 min) expressed by C57 mice. (A and B) Effects of saline (Saline) or amphetamine (Acute) challenge in mice repeatedly injected with saline and effects of amphetamine challenge in mice pretreated with the psychostimulant 11 days before test (Repeated +11). (C and D) locomotor effects of amphetamine challenge in mice pretreated with the psychostimulant 5 (Repeated +5), 11 (Repeated +11) or 17 (Repeated +17) days before amphetamine challenge. *$p < 0.01$ vs. all other groups.

transporter protein was detected with a monoclonal rat antibody raised against the N-terminus of human dopamine transporter (mab369, Chemicon International, Inc., Temecula, CA, USA, diluted 1:10,000). Secondary immunodetection was performed with biotinylated antibodies (goat anti-rabbit, Vector Laboratories Inc., Burlingame, CA, USA, or donkey anti-rat, Jackson Immunoresearch Europe Ltd., Cambridgeshire, UK; both diluted 1:500). Peroxidase labeling was obtained by standard avidin–biotin procedure (Vectastain ABC elite kit, Vector Laboratories, diluted 1:500). Metal-enhanced diaminobenzidine (FastDAB, Sigma) was the chromogen. Sections from each experiments were processed in a single batch of immunostaining allowing quantitative comparisons within experiment.

2.5.4. Microscopy and image analysis

Visual examination and digital imaging of immunoreacted sections were performed with a Zeiss Axiophot light transmission microscope equipped with a CoolSnap CCD camera (Fig. 1).

Quantitative image analysis was performed as previously described, measures were taken bilaterally from four to six rostrocaudal levels per region, depending on the rostrocaudal extension of the brain region, and then averaged to obtain a single value per subject [8,14,16]. Briefly, immunoreactive nuclei were automatically counted and expressed as no. of nuclei/mm^2 in selected brain regions using the public domain image analysis software ImageJ (http://rsb.info.nih.gov/ij/; [17]) running on a GNU/Linux operating system (Debian 5.0, www.debian.org). The brain regions quantitatively analyzed were the medial prefrontal cortex (mpFC), the dorso-medial caudate-putamen (dmCP), the ventromedial caudate-putamen (vmCP), the Core (NAc Core) and the Shell (NAc Shell) of Nucleus Accumbens. Brain regions were identified according to "The Mouse Brain in stereotaxic coordinates" [18]. Dopamine transporter immunostaining of adjacent slides was used to better discriminate core and shell subdivisions of nucleus accumbens [14,16,19]. The lateral extent of caudate was not quantitatively analyzed because, in accordance with similar studies [20,21], it was virtually devoid of immunoreactive nuclei in all groups from both experiments.

Table 1

Experiment	Group	Pretreatment		Test		Immunohistochemistry
		Home cage	Test cage	Withdrawal (days)	Challenge	
1, 2	Saline	SAL	–	11	SAL	C57, DBA
1, 2	Acute	SAL	–	11	AMPH	C57, DBA
1, 2	Repeated +11	AMPH	–	11	AMPH	C57, DBA
1, 2	Repeated +5	AMPH	–	5	AMPH	–
1, 2	Repeated +17	AMPH	–	17	AMPH	–
3	Saline	SAL	SAL	1	SAL	
3	Acute	SAL	SAL	1	AMPH	DBA
3	Paired	SAL	AMPH	1	SAL	
3	Paired	SAL	AMPH	1	AMPH	DBA
3	NPaired	AMPH	SAL	1	SAL	
3	NPaired	AMPH	SAL	1	AMPH	DBA
3	Saline +11	SAL	SAL	11	SAL	–
3	Acute +11	SAL	SAL	11	AMPH	–
3	Paired +11	SAL	AMPH	11	SAL	
3	Paired +11	SAL	AMPH	11	AMPH	DBA
3	NPaired +11	AMPH	SAL	11	SAL	–
3	NPaired +11	AMPH	SAL	11	AMPH	–
3	Paired +5	SAL	AMPH	5	AMPH	–
3	NPaired + 17	AMPH	SAL	17	AMPH	–

2.6. Statistical analyses

Locomotor activity data from Experiments 1 and 2 were evaluated by mixed model analyses of variance (ANOVA) with Treatment (3 levels = Saline, Acute, Repeated +11) or Elapsed time (3 levels = Repeated +5; Repeated +11; Repeated +17) as between factor, and Minutes as repeated measure (1–12 bins, 5 min each). As for locomotion data from Experiment 3, two independent analyses were performed on data collected 24 h or 11 days after the end of the drug pretreatment by mixed model three-way ANOVAs with Challenge (Saline, Amphetamine 1 mg/kg) and Pretreatment (Saline, Paired and NPaired) as the between factors, and Minutes (1–12 bins, 5 min each) as the within factor. Finally, a mixed model ANOVA with Elapsed time (3 levels = 5 days after pretreatment: Paired +5; 11 days after pretreatment: Paired +11; 17 days after pretreatment: Paired +17) as between factor and Minutes as repeated measure (1–12 bins, 5 min each) tested the stability of the sensitized locomotor response to amphetamine challenge in mice from the Paired condition. Locomotion data of the group Paired +11 were those collected in the group pretreated with amphetamine in the test cage and challenged with the drug 11 days after the end of pretreatment.

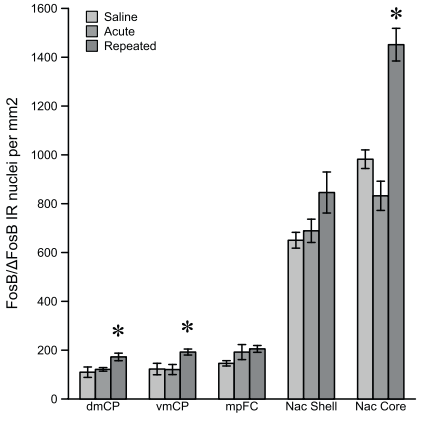

Fig. 3. FosB/ΔFosB-immunoreactive cell nuclei (mean number of nuclei/1 mm^2 ± S.E.) in different brain areas of C57 mice challenged with saline (Saline) or amphetamine (Acute) following saline pretreatment or challenged with amphetamine following pretreatment with the psychostimulant, 11 days after the end of pretreatments (Repeated +11). *$p < 0.05$ vs. all other groups.

Immunostaining data were analyzed independently for each brain structure by one-way ANOVA for independent measures (3 levels = Saline, Acute, and Repeated +11) in Experiments 1 and 2; and 4 levels: Acute, Paired, Npaired, Paired +11 in Experiment 3).

ANOVAs were followed by Fisher PLSD (protected least significant difference) tests when appropriate. For all analyses $\alpha = p < 0.05$.

3. Results

3.1. Experiment 1

Mice of the C57 strain pretreated with amphetamine in their home cages showed sensitization to the psychomotor effects of amphetamine challenge in a novel cage 11 days after the end of pretreatment (Fig. 2A and B). Statistical analysis of locomotion data revealed a main effect of Treatment ($F(2,9) = 34.763$; $p < 0.0001$), a main effect of the repeated measure ($F(2,11) = 6.136$; $p < 0.0005$), and a significant interaction between the two factors ($F(22,99) = 6.738$; $p < 0.0005$). Post hoc comparisons performed on the mean distance traveled over the 60 min test (Fig. 2B) indicated that mice from the group Repeated +11 were significantly more active than either drug-naïve mice (Saline) or mice acutely challenged with the psychostimulant (Acute).

The locomotor response to amphetamine challenge expressed by mice repeatedly exposed to the drug was maximal 11 days after the end of the drug pretreatment (Fig. 2C and D). Statistical analysis of locomotion data revealed a main effect of the time elapsed from the end of the pretreatment ($F(2,9) = 22.926$; $p < 0.0001$), a main effect of the repeated measure ($F(2,11) = 5.035$; $p < 0.0005$), and a significant interaction between factors ($F(22,99) = 4.819$; $p < 0.0005$). Post hoc comparisons performed on the mean distance traveled over the 60 min test (Fig. 2D) indicated that mice tested 11 days after the end of the drug pretreatment (Repeated +11) were significantly more active than mice challenged with the same dose of amphetamine 5 (Repeated +5) or 17 days (Repeated +17) after drug pretreatment.

C57 mice sensitized by repeated amphetamine within the home cages and challenged with amphetamine in a novel cage 11 days after the end of the drug pretreatment (Repeated +11) showed increased FosB/ΔFosB immunostaining in the dorsomedial ($F(2,11) = 6.71$; $p < 0.05$) and ventromedial ($F(2,11) = 5.12$; $p < 0.05$) caudate and in the NAc Core ($F(2,11) = 32.92$; $p < 0.0001$). In all cases, post hoc comparisons revealed that FosB/ΔFosB immunostaining in mice from this group was significantly different

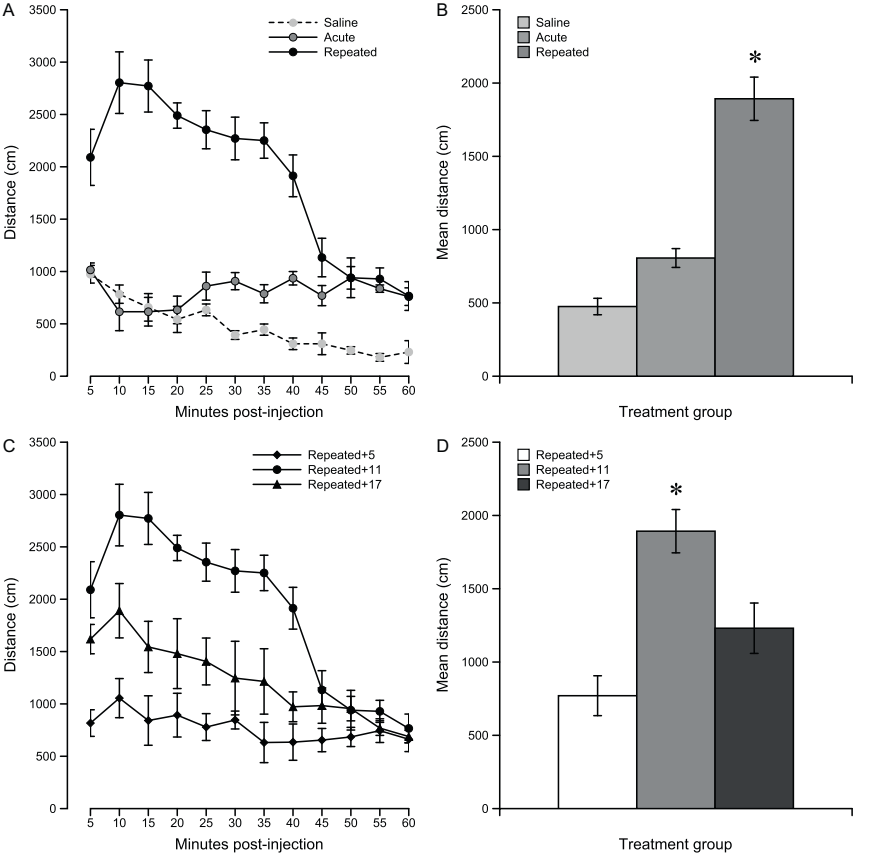

Fig. 4. Locomotor activity (mean cm ± S.E.; (A and C) 5 min blocks; (B and D) over 60 min) expressed by DBA mice. (A and B) Effects of saline (Saline) or amphetamine (Acute) challenge in mice repeatedly injected with saline and effects of amphetamine challenge in mice pretreated with the psychostimulant 11 days before test (Repeated +11). (C and D) Locomotor effects of amphetamine challenge in mice pretreated with the psychostimulant 5 (Repeated +5), 11 (Repeated +11) or 17 (Repeated +17) day before amphetamine challenge. *$p < 0.01$ vs. all other groups.

in comparison with that of drug-naïve mice (Saline) or mice acutely challenged with the psychostimulant (Acute) (Fig. 3).

3.2. Experiment 2

Mice of the DBA strain pretreated with amphetamine in their home cages showed a significant increase of the locomotor response to amphetamine challenge in a novel cage when tested 11 days after the end of the drug pretreatment (Fig. 4A and B). Statistical analysis of locomotion data revealed a main effect of Treatment ($F(2,9) = 56.669$; $p < 0.0001$), a main effect of the repeated measure ($F(2,11) = 26.054$; $p < 0.0001$), and a significant interaction between factors ($F(22,99) = 17.080$; $p < 0.0001$). Post hoc comparisons performed on the mean distance traveled over the 60 min test (Fig. 4B) indicated that mice of the Repeated +11 group were more active than drug-naïve mice (Saline) or mice acutely challenged with the psychostimulant (Acute).

The locomotor response to amphetamine challenge expressed by sensitized DBA mice was maximal 11 days after the end of the drug pretreatment (Fig. 4C and D). Statistical analysis of locomotion data revealed a significant effect of the time elapsed from the end of the pretreatment ($F(2,9) = 13.669$; $p < 0.005$), a main effect of the repeated measure ($F(2,11) = 30.439$; $p < 0.0001$), and a significant interaction between factors ($F(22,99) = 6.480$; $p < 0.0005$). Post hoc comparisons performed on mean distance traveled over the 60 min test (Fig. 4D) indicated that mice tested 11 days after the end of the drug pretreatment (Repeated +11) were significantly more active than mice challenged with the same dose of amphetamine 5 (Repeated +5) or 17 days (Repeated +17) after drug pretreatment.

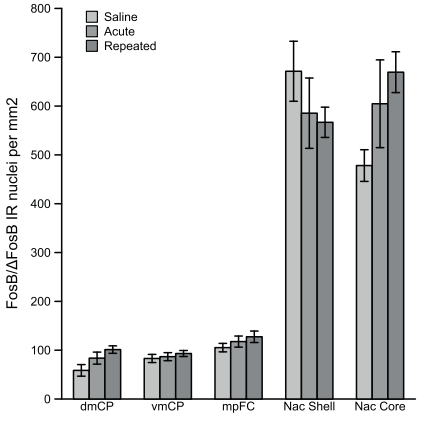

Fig. 5. FosB/ΔFosB-immunoreactive cell nuclei (mean number of nuclei/1 mm^2 ± S.E.) in different brain areas of DBA mice challenged with saline (Saline) or amphetamine (Acute) following saline pretreatment or challenged with amphetamine following pretreatment with the psychostimulant, 11 days after the end of pretreatments (Repeated +11). Statistical results are reported in Table 1.

DBA mice sensitized by repeated amphetamine within the home cages did not show increased FosB/ΔFosB immunostaining in any of the sampled brain areas 11 days after the end of drug pretreatment (Fig. 5). Statistical analyses of FosB/ΔFosB immunostaining data did not show significant differences between experimental croups in dmCP ($F(2,11) = 3.98$; $p = 0.06$), vmCP ($F(2,11) = 0.45$; $p = 0.65$), mpFC ($F(2,11) = 1.09$; $p = 0.37$), NAc Shell ($F(2,11) = 0.94$; $p = 0.42$) or NAc Core ($F(2,11) = 2.61$; $p = 0.12$).

3.3. Experiment 3

No sign of locomotor sensitization was observable in mice challenged with amphetamine in the drug-paired test-cage 24 h after the end of the pretreatment (Table 2). Statistical analysis revealed a main effect of Challenge ($F(1,18) = 4.846$; $p < 0.05$) but neither effect of Pretreatment ($F(2,18) = 1.603$; $p = 0.228$) nor interaction between factors ($F(1,18) = 0.447$; $p = 0.646$). The analysis revealed a significant main effect of the repeated measure ($F(11,198) = 16.905$; $p < 0.0001$) and a significant interaction between the independent factors and the repeated measure ($F(22,198) = 2.746$; $p < 0.0001$).

DBA mice tested 11 days after the end of pretreatment showed a sensitized locomotor response to amphetamine in the drug-associated testing cage (Fig. 6A and B). Statistical analyses revealed a main effect of Pretreatment ($F(2,18) = 19.057$; $p < 0.005$), a main effect of Challenge ($F(1,18) = 46.146$; $p < 0.0001$), and a significant interaction between Pretreatment and Challenge ($F(2,18) = 10.084$; $p < 0.005$). The analysis also revealed a significant main effect of the repeated measure ($F(11,198) = 42.311$; $p < 0.0001$) and a significant interaction among all factors ($F(22,198) = 2.67$; $p < 0.0005$); but neither a significant interaction between Minutes and Pretreatment ($F(22,198) = 1.079$; $p = 0.372$), nor between Minutes and Challenge ($F(11,198) = 0.403$; $p = 0.953$). Individual between-groups comparisons performed on the mean distance traveled over the 60 min test (Fig. 6B) indicated that mice challenged with amphetamine in the drug-associated cage (Paired) were more active than either mice acutely challenged with the drug (Acute) or mice challenged in the test cage associated with saline (NPaired). No significant differences were found among groups challenged with saline following identical pretreatment.

Table 2
Mean locomotion expressed over the 60 min test 24 h after the last day of pretreatment.

Pretreatment	Challenge	
	Saline	Amphetamine
Saline	355 ± 25	588 ± 45
Npaired	362 ± 26	575 ± 42
Paired	408 ± 50	835 ± 150

Data are expressed as mean cm (±S.E.).

Finally, in the group pretreated with amphetamine in the test cage the sensitized response to the drug challenge peaked 11 days after the end of the pretreatment and decreased thereafter (Fig. 7A and B). Statistical analysis of locomotion data revealed a main effect of the time elapsed from the end of the pretreatment ($F(2,9) = 54.153$; $p < 0.0001$), a main effect of the repeated measure ($F(11,99) = 13,223$; $p < 0.0001$); and a significant interaction between the two factors ($F(22,99) = 2.65$; $p < 0.001$). Post hoc comparisons performed on the mean distance traveled over the 60 min test (Fig. 7B) indicated that mice tested 11 days after the end of the drug pretreatment (Paired +11) were significantly more active than mice tested 5 (Paired +5) or 17 days (Paired +17) after drug pretreatment.

In Fig. 8 are presented data on FosB/ΔFosB immunostaining. No differences between groups were observed in the dmCP ($F(3,15) = 0.69$; $p = 0.58$), vmCP ($F(3,15) = 1.81$; $p = 0.19$), mpFC ($F(3,15) = 1.87$; $p = 0.18$), and the NAc Core($F(3,15) = 1.64$; $p = 0.23$). Significant difference among groups were found in the NAc Shell ($F(3,15) = 4.16$; $p < 0.05$). Post hoc comparisons revealed a significant reduction of FosB/ΔFosB immunostaining in mice of the Paired group challenged with amphetamine 11 days after the end of the pretreatment (Paired +11) in comparison with mice acutely challenged with the drug (Acute).

4. Discussion

The main finding of the present study is the demonstration that amphetamine can promote robust long-lasting locomotor sensitization in mice without inducing striatal ΔFosB accumulation.

Levels of FosB/ΔFosB immunostaining in dmCP, vmCP, NAc Core and Shell or mpFC of DBA mice repeatedly treated with amphetamine within their home cages and challenged with the psychostimulant in a novel test cage or repeatedly treated with amphetamine within the test cage did not differ from levels observable in saline-pretreated mice. However, both pretreatments increased the psychomotor stimulant effect of amphetamine challenge up to 11 days after the end of the drug pretreatment. In the present experiments, FosB/ΔFosB immunostaining was induced by an antiserum that recognizes both ΔFosB and full-length FosB [15], therefore the results indicate lack of effects of repeated amphetamine on both splice variants of the transcription factor.

The observation of higher levels of FosB/ΔFosB immunostaining in the NAc Shell of DBA mice challenged with amphetamine 24 h after the end of repeated saline in comparison with mice challenged with the psychostimulant 11 days (but not 24 h) after the end of repeated amphetamine suggests a modest and short-lasting effect of repeated handling associated with the experimental procedure. Such an effect would indicate a specific inability of amphetamine to increase FosB/ΔFosB in DBA mice that does not extend to non-pharmacological stimulations. In line with this hypothesis, increased FosB/ΔFosB immunostaining has been observed in the

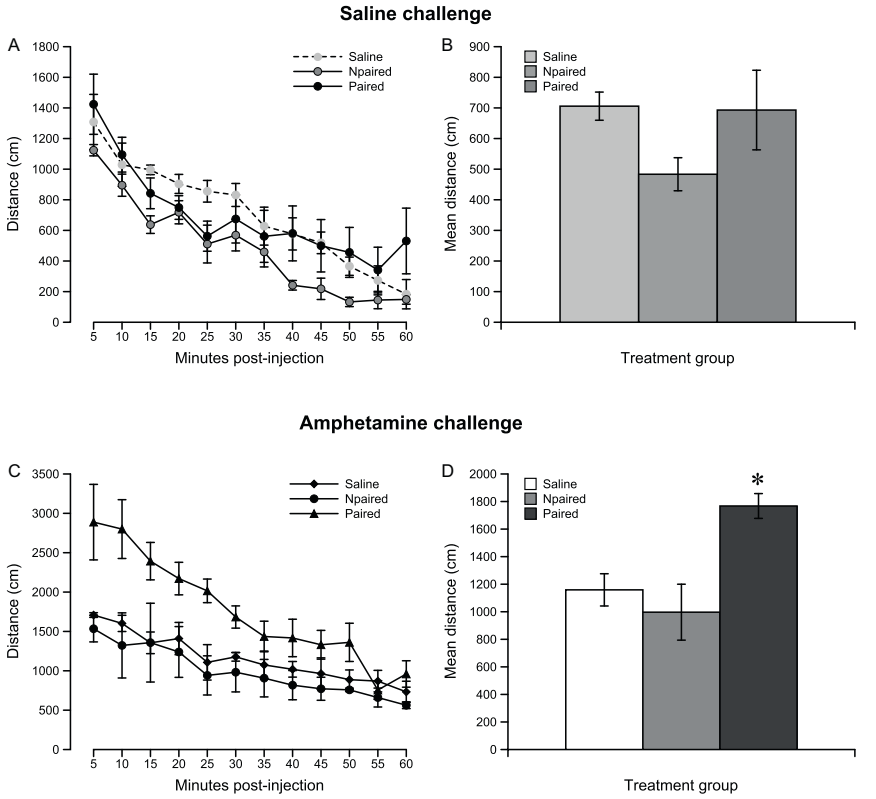

Fig. 6. Locomotor activity (mean cm ± S.E.; (A and C) 5 min blocks; (B and D) over the 60 min test) in DBA mice tested 11 days after the end of pretreatments. The upper panel shows data collected in mice challenged with saline following saline pretreatment in both the home and the test cage (Acute); amphetamine in the home cage and saline in the test cage (NPaired); saline in the home cage and amphetamine in the test cage (Paired). The lower panel shows data collected in mice from the 3 pretreatment groups challenged with amphetamine before test. *$p < 0.01$ vs. all other groups.

mpFC of mice of this strain repeatedly exposed to a novel environment in comparison with mice exposed to the same environment for the first time [14].

Identical protocols of amphetamine pretreatment induce long-lasting increase of striatal FosB/ΔFosB immunostaining in mice of the C57 inbred strain. The present results demonstrated increased FosB/ΔFosB immunostaining in both dmCP and vmCP as well as in the Core of the NAc of mice behaviorally sensitized by repeated amphetamine administration within their home cages 11 days after the end of the drug pretreatment. In a previous study [8] we observed increased FosB/ΔFosB immunostaining in the vmCP of C57 mice behaviorally sensitized by repeated amphetamine pairing with the test cage using the same protocol adopted for DBA mice in Experiment 3. The effect was evident 24 h after the end of the sensitizing procedure. Finally, increased striatal FosB/ΔFosB immunostaining is observable in the vmCP of mice of the C57 strain sensitized to the locomotor effect of amphetamine by repeated restraint stress [8].

These data indicated a consistent association between increased FosB/ΔFosB in the vmCP and behavioral sensitization to amphetamine induced by very different procedures in mice of the C57 strain.

The increase in FosB/ΔFosB immunostaining observed in C57 mice challenged with amphetamine following pre-treatment with the psychostimulant or exposure to repeated restraint stress in comparison with mice challenged with the same dose of the psychostimulant after pre-treatment with saline or no manipulation suggests that ΔFosB accumulation contributes to the phenomenon. Indeed, it has been shown that full-length FosB is induced by acute challenges, is short-lived, and is reduced by repeated treatments while ΔFosB is barely observable in acutely challenged preparations, is relatively stable, and accumulated though repeated treatments [9]. Moreover, acute amphetamine was unable to increase FosB/ΔFosB immunostaining beyond levels promoted by saline in any of the sampled areas of mice from both strains (Figs. 3 and 5). This same dose of amphetamine promotes signif-

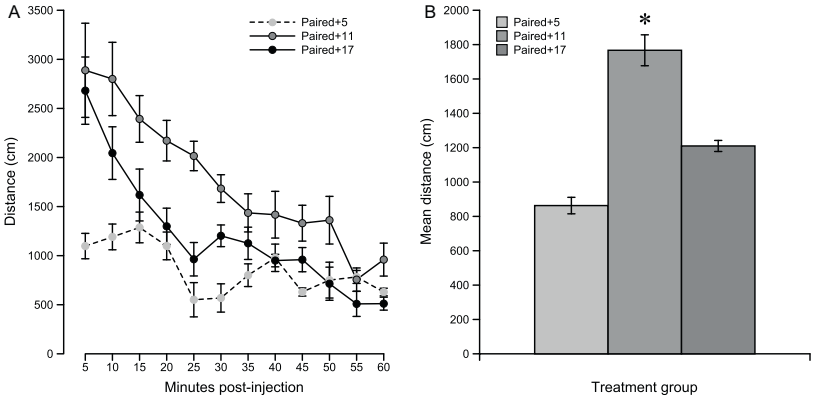

Fig. 7. Locomotor activity (mean cm ± S.E.; (A) 5 min blocks; (B) over the 60 min test) by DBA mice repeatedly treated with amphetamine in the test cage and challenged with the psychostimulant 5 (Paired +5), 11 (Paired +11) or 17 (Paired +17) after the end of the pretreatment. *$p < 0.01$ vs. all other groups.

icant c-Fos expression in most of these brain areas of C57 as well as DBA mice [14]. Therefore, full length FosB appears to be rather insensitive to acute stimulation by amphetamine in mice.

Comparison between results obtained in mice of the DBA and C57 inbred strains offer strong support to the hypothesis that accumulation of ΔFosB in the striatal complex by repeated amphetamine is a strain-specific phenotype in mice. It should be pointed out that although mice from the two inbred strains show marked differences in stress-induced behavioral sensitization to amphetamine [22] they show comparable amphetamine-induced sensitization (Figs. 2 and 4). Therefore, the absence of increased striatal ΔFosB does not seem to reduce development or expression of a sensitized response to the psychostimulant in mice.

Thus, the present results offer support the hypothesis that mice from the two strains develop behavioral sensitization through different and independent mechanisms. In line with this hypothesis, it has been shown that in DBA but not in C57 mice cocaine sensitization requires a corticosterone-dependent mechanisms involving strain-specific neuroadaptations within the midbrain dopamine (DA) system [6].

On the other hand, the absence of amphetamine-induced FosB/ΔFosB accumulation within the striatal complex of mice from the DBA strain could depend on the strain-specific pharmacological effects of the drug. Previous studies have demonstrated that while amphetamine-induced DA release in the ventral striatum is impulse-independent in mice of the DBA strain, in C57 mice it is impulse-dependent and mediated by norepinephrine transmission within the mpFC [19,23].

In conclusion, the results obtained by the present study demonstrate that mice can develop robust and long-lasting behavioral sensitization to amphetamine in the absence of ΔFosB accumulation in striatal compartments. Moreover, they support the involvement of strain-specific neural mechanisms in the development of sensitization to the behavioral effects of psychostimulants and suggest cautious generalization of causal relationships between neural and behavioral phenotypes observed in specific genetic backgrounds.

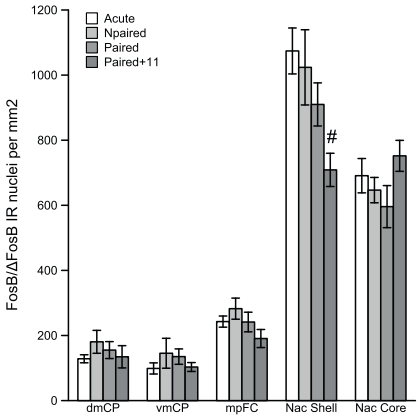

Fig. 8. FosB/ΔFosB-like immunoreactivity (mean number of nuclei/mm^2 ± S.E.) in different brain areas of DBA mice challenged with amphetamine (Acute) following saline pretreatment in both the home and the test cage (Acute); amphetamine in the home cage and saline in the test cage (NPaired); saline in the home cage and amphetamine in the test cage (Paired) 24 h after the end of pretreatment and of mice of the Paired group challenged with amphetamine 11 days after the end of pretreatment (Paired +11). #$p < 0.05$ vs. Acute.

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Positive and negative emotional arousal increases duration of memory traces: common and independent mechanisms

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We compared the ability of positive and negative emotional arousal to increase the duration of consolidated memory traces. Positive arousal was modulated by manipulating the motivational salience of the testing cage of an object recognition test. Negative emotional arousal was modulated by manipulating shock levels in a step-through inhibitory avoidance (IA). Mice trained in either a high (chocolate-associated) or a low (inedible object-associated) emotionally arousing cage showed discrimination of a novel object 24 h after training, but only mice trained in the more arousing cage showed retention 96 h after training. Mice trained with either low (0.35 mA) or high (0.7 mA) shock intensities showed increased step-through latencies when tested 24 h after training, but only mice trained with the higher shock intensity showed retention of the IA learning 1 week after training. Administration of the phosphodiesterase type IV inhibitor Rolipram immediately after training in the two low arousing conditions increases duration of both responses.

Keywords: long-term memory, persistence, emotional arousal, hedonic valence, object recognition, inhibitory avoidance, cAMP, Rolipram

INTRODUCTION

The establishment of long-term memory (LTM) requires the stabilization of learning-induced synaptic changes. This process is referred to as memory consolidation (McGaugh, 1966; Dudai, 1996, 2002, 2004; Lechner et al., 1999; Dudai and Eisenberg, 2004). However, not all long-term "consolidated" memories last equally. Indeed, everyday experience shows that duration of LTM is highly variable. The behavioral and brain factors underlying this variability are poorly understood.

A large body of evidence indicates that emotional arousal enhances LTM duration by acting on memory consolidation (for review see McGaugh, 2006). However, whether this effect is independent of the hedonic valence (positive or negative) of arousing experience is unknown. Indeed, testing this hypothesis is hampered by the lack of one-trial tasks specifically designed to model the effects of positive arousal on LTM duration. Thus, the first aim of the present study was to develop a behavioral protocol able to induce differentially persistent LTM traces in mice, depending on the magnitude of positive emotional arousal induced by the testing cage during a single learning trial. To this aim, the novel object recognition test (ORT) was used as a one-trial, non-aversive, learning task (Ennaceur and Delacour, 1988; Ennaceur, 2010) and the emotional salience of the testing cage was modulated by repeatedly pairing it with a highly palatable food (chocolate).

Recently, it has been observed that intra-hippocampal infusion of the 8-bromoadenosine-3′, 5′-cyclic monophosphate (8Br-cAMP) analog of cyclic adenosine monophosphate (cAMP) immediately after a weak (low arousing) inhibitory avoidance (IA) training mimics the effect of a strong (high arousing) training by converting a rapidly decaying LTM into a long-lasting one (Rossato et al., 2009). This finding suggests that the enhancement of LTM duration induced by a high emotional arousal may be mediated by cAMP-dependent signaling around the time of memory formation. However, it is unknown whether cAMP-dependent signaling is involved in arousal-induced enhancement of LTM duration regardless of the hedonic valence of the learning experience. Thus, the second aim of the present study was to comparatively evaluate the role of cAMP-dependent signaling in the modulation of LTM duration in both the positive task here reported and in a negative standard one (IA). To this aim, the phosphodiesterase type IV inhibitor Rolipram, known to enhance intracellular cAMP concentration, was administered in a single peripheral injection after low arousing training conditions that induce, in both tasks, only non-persistent LTM traces (i.e., lasting 24 h but not beyond 96 h).

MATERIALS AND METHODS

SUBJECTS

Male CD1 mice (Harlan Laboratories, Udine, Italy) were purchased at 6 weeks of age and housed four to a cage on a 12-h light–dark cycle (lights on at 7:00 a.m.). Food and water were available *ad libitum* except when food deprivation was required by design (see Materials and Methods). Mice were left undisturbed for 2 weeks before behavioral testing. Experiments were carried out in accordance with the Italian national law (DL 116/92) on the use of animals in research.

DRUGS

Rolipram (Sigma-Aldrich, Milano, Italy) was freshly suspended in 5% ethanol and 95% saline solution on every experimental day (Rutten et al., 2006). Rolipram was injected intraperitoneally

(i.p.) in a volume of 10 ml/kg at the following doses: 0, 3, and 10 mg/kg. These doses were chosen because they have been shown to be rapidly effective to elevate cAMP concentration in the mouse brain (Randt et al., 1982). For either IA or object recognition experiments, Rolipram was administered immediately or 120 min post-training.

BEHAVIORAL PROCEDURES

Object recognition

Mice underwent ORT in a custom made apparatus consisting of a square box (60 cm × 60 cm × 30 cm) made of black Plexiglas® subdivided in four equal arenas. The floor of the arenas was made of textured transparent Plexiglas® surmounting a plate of opalescent Plexiglas® thick 1 cm. The apparatus was dimly illuminated by halogen light sources placed below it (Nilsson et al., 2007). Light sources (four) were carefully positioned approximately 50 cm under each corner of the apparatus and directed toward the room floor. This allowed an homogeneous illumination throughout all arenas (∼10 lux, measured with a PCE-EM882 multimeter from http://www.pce-italia.it). The apparatus was placed in a ventilated sound-attenuated cabinet. Objects to discriminate were four types, one type for arena: white wooden cube, gray plastic cylinder, gray plastic sphere, and silver metallic parallelepiped. They were available in triplicate copy and, based on pilot studies, all aroused comparable levels of exploration in CD1 mice (data not shown). For training phase, objects were placed at two opposite corners of the arenas (6.5 cm from the object center to the corner). To prevent mice from displacing objects during testing, they were temporarily fixed to the floor of the arenas with repositionable adhesive pastels (UHU patafix white). On the training trial, mice were allowed to freely explore two identical objects (sample objects: A1 and A2) for 15 min. On the test trial, the third copy of the familiar object (A3) and a novel object (B) were placed in the same location of training and mice were allowed to freely explore them for 10 min. Cohorts of four mice belonging to the same cage and to the same experimental group were tested simultaneously. Each mouse was tested only once. All combinations and locations of objects were counterbalanced to reduce potential biases because of preference for particular locations or objects. Between each session, apparatus and objects were thoroughly cleaned with 70% ethanol to remove urine and fecal boli and to homogenize olfactory trails. Mice behavior was recorded with a camera connected to a Debian GNU/Linux[1] workstation equipped with hardware MPEG encoding capabilities. MPEG videos were blindly analyzed by experienced observers. Object exploration was defined as pointing the nose to the object at a distance of 1 cm and/or touching it with the nose. Turning around, climbing, or sitting on an object was not considered as exploration.

Experiment 1

This experiment was aimed to set-up a model to evaluate the effect of positive emotional arousal on LTM duration in mice and to evaluate the relative role of acute food deprivation and of the caloric and/or fat properties of chocolate in that model.

[1]http://www.debian.org

Effect of positive arousal on long-term memory duration. Sixty-four mice were pre-exposed for 15 h to milk chocolate (Lindt, two pieces for cage, each weighing approximately 2 g) mixed to standard food in the home-cage from 7:00 p.m. of the day before the starting of behavioral testing. Then, mice underwent a delay conditioning phase lasting 4 days. This phase comprised two daily sessions on days 1–3 (10:00 a.m. and 2:00 p.m.) and a single session on day 4 (10:00 a.m.). Sessions were as follows: mice were allowed to freely explore the empty arenas for 10 min then, depending on the experimental group, chocolate pieces (CHOC, $n = 16$) or black Lego® blocks (OBJ, $n = 16$), approximately of the same form and size of chocolate pieces, were dropped in the arenas by the experimenter (one stimulus for arena). Mice were allowed to interact with these stimuli for additional 5 min (total session duration: 15 min). At 7:00 p.m. of day 3 all mice were food-deprived. On day 4 at 2:00 p.m. all mice underwent ORT training. Training session differed from previous ones only for the presence of sample objects in the arenas. Immediately after training, mice were returned to their home cages and they were given *ad libitum* food. Half mice from each group were tested for sample object recognition 24 h after training, the remainder were tested 96 h after training. At 7:00 p.m. of the day before test session all mice were food-deprived.

Control of the role of acute food deprivation. Forty-eight mice were submitted to the same protocol as the CHOC group except for the following differences: a first group ($n = 16$, FF–FF) was never food-deprived; a second group ($n = 16$, FD–FF) was food-deprived 15 h before training but not before test; a third group ($n = 16$, FF–FD) was food-deprived 15 h before test but not before training. CHOC group was renamed FD–FD when compared to FF–FF, FF–FD, and FD–FF groups. Mice from these groups were tested for sample object recognition 96 h after training.

Control of the role of schedule of chocolate exposure. Thirty-two mice were treated identically to the CHOC group but, half (PRE) received chocolate only during conditioning phase, the remainder (POST) received chocolate only during the last 5 min of training. CHOC group was renamed PRE + POST when compared to PRE and POST groups. Mice from these groups were tested for sample object recognition 96 h after training.

Experiment 2

This experiment was aimed to evaluate the ability of Rolipram to enhance LTM duration in the OBJ condition. Thirty-two mice were randomly assigned to this experiment. Mice were treated identically to the OBJ group of Experiment 1. Three groups of mice ($n = 8$) were administered, respectively, vehicle, 3 or 10 mg/kg immediately after training. One group ($n = 8$) were administered 3 mg/kg of Rolipram 120 min after training. Mice were tested for sample object recognition 96 h after training. Lowest effective Rolipram dose has been previously determined in pilot studies (data not shown).

INHIBITORY AVOIDANCE

Mice were trained and tested in a step-through IA task. The apparatus consisted of a trough-shaped alley (91 cm long, 15 cm deep) divided into two compartments by a retractable door: an

illuminated safe compartment (31 cm long) and a dark shock compartment (60 cm long). Mice were not habituated to the dark compartment before the training trial. On the training trial, each mouse was placed in the lit start compartment facing away from the shock compartment. After the mouse stepped with all four paws into the dark compartment, the retractable door was closed and an inescapable foot-shock (0, 0.35, or 0.7 mA, 50 Hz, 2.0 s) was administered. The mouse was removed from the dark compartment 30 s after termination of the foot-shock. The lowest shock intensity was chosen on the basis of previous studies showing that it is sufficient to induce IA retention for at least 24 h in mice (Baarendse et al., 2008). The highest shock intensity was chosen on the basis of a previous study in mice showing that it induces IA retention beyond 24 h (Boccia et al., 2004). On the retention trial, each mouse was placed into the light compartment with the retractable door open and allowed to explore the box freely. The latency to enter the dark compartment with all four paws was recorded with a timer by a nearby experimenter as a measure of retention. Retention test ended either if the mouse stepped into the dark compartment or if it failed to cross within 180 s. In the latter case, mouse was assigned a score of 180 s. Foot-shock was omitted on retention test. Each mouse was tested only once.

Experiment 3

This experiment was aimed to test in mice the effect of magnitude of negative arousal on LTM duration (Bekinschtein et al., 2007). Ninety-six mice were randomly assigned to this experiment. One-third of mice were pseudo-trained by omitting the shock, one-third were trained with the 0.35 mA shock, and the remainder were trained with the 0.7 mA shock. Half of the mice for each shock condition ($n = 16$) were tested for retention 24 h after training, the other half were tested 1 week after training.

Experiment 4

This experiment was aimed to evaluate the ability of Rolipram to enhance LTM duration in the low shock condition (0.35 mA). Sixty mice were randomly assigned to this experiment. Four groups of mice ($n = 12$) were trained with the 0.35 mA shock, then they were administered vehicle, 3 or 10 mg/kg of Rolipram immediately post-training or they were administered 10 mg/kg of Rolipram 120 min post-training. In order to rule out a non-specific drug effect a fifth group ($n = 12$) was pseudo-trained and it was administered 10 mg/kg of Rolipram immediately after being removed from the dark compartment. All mice were tested for retention 1 week post-training. Lowest effective Rolipram dose has been previously determined in pilot studies (data not shown).

DATA ANALYSIS

Statistical analyses were performed on a Debian GNU/Linux workstation using the R free software environment for statistical computing[2] and its graphical interface R-Commander (Fox, 2005). An error probability level of $p < 0.05$ was accepted as statistically significant.

[2]http://www.r-project.org/

Object recognition

Data from Experiment 1 were analyzed using: (1) two-way analysis of variance (ANOVA) for independent factors to compare CHOC and OBJ mice either at 24 or 96 h post-training; (2) two-way ANOVA for independent factors to compare CHOC mice free-fed (FF) or food-deprivated (FD) either at training or at test; (3) one-way ANOVA to compare mice that, except for initial pre-exposure, received chocolate only during conditioning phase (PRE), only after training (POST), both during conditioning and after training (PRE + POST). In case of significant two-way interaction simple effect analysis was performed with the Student's *t*-test for independent samples (Welch variant, two-tailed). In case of significant overall one-way *F* ratio, ANOVA was followed by Tukey's *post hoc* tests.

Data from Experiment 2 were analyzed using: (1) one-way ANOVA to compare mice treated with different Rolipram doses (0, 3, and 10 mg/kg); (2) Student's *t*-test for independent samples (Welch variant, two-tailed) to compare vehicle-treated mice and mice treated with 3 mg/kg Rolipram 120 min post-training). For all experiments, one sample *t*-tests (two-tailed) were used to determine whether the discrimination index (D.I.) of each experimental group was different from 0 (chance level). Mice showing less than 5 s of total object exploration during ORT test were excluded from analysis (Sik et al., 2003).

Inhibitory avoidance

Because a cut-off of 180 s was imposed during test sessions, the step-through latency was expressed as median and inter-quartile range and analyzed with non-parametric tests. Kruskal–Wallis ANOVA was used when comparing more than two groups. Two-sample Wilcoxon test (two-tailed) was used either when comparing two groups or as *post hoc* test following a significant overall Kruskal–Wallis test.

RESULTS

OBJECT RECOGNITION

Experiment 1

Effect of magnitude of positive arousal on long-term memory duration. Two-way ANOVA for total exploration time did not reveal significant effects nor interaction for factors "stimulus paired with test context" and "inter-trial interval". **Figure 1A** shows the D.I. measured 24 and 96 h after training as a function of stimulus paired with test context. Two-way ANOVA for D.I. revealed a significant interaction between stimulus paired with test context and inter-trial interval ($F_{1,60} = 5.06$, $p < 0.05$). For 24 h test, simple effect analysis did not reveal a significant D.I. difference between CHOC and OBJ groups and one sample *t*-tests revealed that D.I. from both groups was significantly above chance level (CHOC: $t_{15} = 3.56$, $p < 0.005$; OBJ: $t_{15} = 5.46$, $p < 0.0001$). For 96 h test, simple effect analysis revealed that OBJ group had a lower D.I. respect to CHOC group ($t_{30} = 3.02$, $p < 0.01$). Moreover, simple effect analysis for each level of stimulus paired with test context revealed that only the D.I. of OBJ group at 96 h test was lower than that of 24 h test ($t_{30} = 2.42$, $p < 0.05$). One sample *t*-tests revealed that only D.I. from CHOC group was significantly above chance level ($t_{15} = 7.24$, $p < 0.0001$). These results indicate that long-term duration of object memory is enhanced when

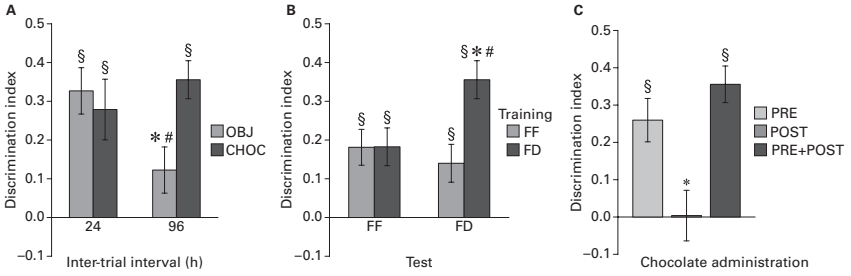

FIGURE 1 | (A) Discrimination index (mean ± SEM) as a function of stimulus paired with test context measured 24 or 96 h post-training (*vs CHOC, $p < 0.01$; #vs 24 h, $p < 0.05$; §vs 0, $p < 0.0001$); **(B)** Discrimination index (mean ± SEM) as a function of feeding state before behavioral testing measured 96 h post-training (*vs FD–FF, $p < 0.05$; #vs FF–FD, $p < 0.005$; §vs 0, $p < 0.0001$); **(C)** Discrimination index (mean ± SEM) as a function of chocolate administration protocol measured 96 h post-training (*vs all, $p < 0.05$; §vs 0, $p < 0.01$).

sampling is performed in an environment previously associated to a rewarding stimulus.

Control of the role of acute food deprivation. Two-way ANOVA did not reveal significant effects nor interaction between factors "feeding at training" and "feeding at test" in total exploration time. **Figure 1B** shows the D.I. measured 96 h after training as a function of feeding state (FF vs FD) before ORT sessions (training vs test). Two-way ANOVA revealed a significant effect of "feeding before training" ($F_{1,60} = 5.06$, $p < 0.05$) and a significant interaction between "feeding before training" and "feeding before test" ($F_{1,60} = 4.93$, $p < 0.05$). Simple effects analysis revealed that: (1) D.I. of mice food-deprived before both training and test (FD–FD) was higher than that of mice food-deprived before training but not before test (FD–FF; $t_{30} = -2.50$, $p < 0.05$); (2) D.I. of mice food-deprived before both training and test (FD–FD) was higher than that of mice free-fed before training but not before test (FF–FD; $t_{30} = -3.11$, $p < 0.005$); (3) D.I. of mice free-fed before both training and test (FF–FF) did not differ from that of mice free-fed before training but not before test (FF–FD); (4) D.I. of mice free-fed before both training and test (FF–FF) did not differ from that of mice free-fed before test but not before training (FD–FF). One sample *t*-tests revealed that D.I. was significantly above chance level for all groups (FD–FD: $t_{15} = 7.25$, $p < 0.0001$; FD–FF: $t_{15} = 3.74$, $p < 0.005$; FF–FD: $t_{15} = 2.86$, $p < 0.05$; FF–FF: $t_{15} = 3.92$, $p < 0.005$). These results indicate that acute food deprivation magnifies the enhancement of LTM duration promoted by sampling objects in the chocolate-associated context.

Control of the role of schedule of chocolate exposure. One-way ANOVA for total exploration did not reveal significant group differences. **Figure 1C** shows the D.I. measured 96 h after training as a function of chocolate administration protocol. One-way ANOVA for D.I. revealed a significant group effect ($F_{2,45} = 7.30$, $p < 0.01$). Tukey's *post hoc* analysis revealed that POST group had a lower D.I. respect to both PRE and PRE + POST groups which did not differ each other. One sample *t*-tests revealed that D.I. was significantly above chance level only for PRE ($t_{15} = 4.46$, $p < 0.001$) and PRE + POST ($t_{15} = 7.30$, $p < 0.0001$). These results indicate that the improvement of object memory observed in mice trained in the chocolate-associated context does not depend on the caloric and/or fat properties of chocolate.

Experiment 2

One-way ANOVA for total exploration did not reveal significant group differences. **Figure 2A** shows the D.I. measured 96 h after training in the object-paired environment as a function of the Rolipram dose injected immediately post-training. One-way ANOVA revealed a significant difference in D.I. between groups

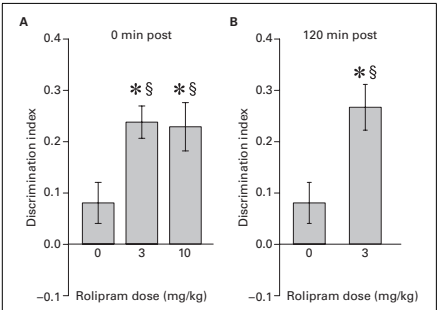

FIGURE 2 | (A) Discrimination index (mean ± SEM) as a function of Rolipram dose (0, 3, and 10 mg/kg) injected immediately after training in the object-paired context measured 96 h post-training (*vs vehicle, $p < 0.05$; §vs 0, $p < 0.01$); **(B)** Discrimination index (mean ± SEM) as a function of Rolipram dose (0 vs 3 mg/kg) injected 120 min after training in the object-paired context measured 96 h post-training (*vs vehicle, $p < 0.01$; §vs 0, $p < 0.001$).

($F_{2,20} = 4.51$, $p < 0.05$). Tukey's *post hoc* revealed that Rolipram enhanced D.I. respect to vehicle at both doses which did not differ one another. One sample *t*-tests revealed that D.I. was significantly above chance level only for Rolipram-treated groups [3 mg/kg ($t_7 = 7.65$, $p < 0.001$); 10 mg/kg ($t_7 = 4.82$, $p < 0.01$)]. **Figure 2B** shows the D.I. measured 96 h after training in object-paired environment in group injected 120 min post-training with 3 mg/kg of Rolipram and in the vehicle group. Two-sample *t*-test revealed that Rolipram-treated mice had higher D.I. respect to vehicle-treated mice ($t_{13} = -3.02$, $p < 0.01$). One sample *t*-test revealed that D.I. was significantly above chance level only for Rolipram-treated mice ($t_7 = 5.91$, $p < 0.001$). These results indicate that Rolipram enhances LTM duration either when injected immediately or 120 min post-training.

INHIBITORY AVOIDANCE

Experiment 3

Figures 3A,B show step-through latency measured, respectively, 24 h and 1 week after training as a function of shock intensity. For 24 h test, Kruskal–Wallis ANOVA revealed a significant difference between groups [$H_{(2)} = 23.25$; $p < 0.001$]. *Post hoc* analysis revealed that groups trained with either shock intensities had a higher step-through latency respect to pseudo-trained group but did not differ one another (0.35 vs 0: $W = 26.5$, $p < 0.001$; 0.7 vs 0: $W = 11$, $p < 0.0001$). For 1 week test, Kruskal–Wallis ANOVA revealed a significant difference between groups [$H_{(2)} = 13.44$; $p < 0.01$]. *Post hoc* analysis revealed that the group trained with the 0.7 shock had a higher step-through latency respect to both pseudo- and 0.35-trained groups (0.7 vs 0: $W = 41.5$, $p < 0.001$; 0.7 vs 0.35: $W = 197.5$, $p < 0.01$) and that the latter groups did not differ one another. These results indicate that duration of fear LTM depends on the magnitude of negative arousal experienced during training.

Experiment 4

Figure 4A shows step-through latency measured 1 week after training with the 0.35 shock as a function of the Rolipram dose injected immediately post-training. Kruskal–Wallis ANOVA revealed a significant difference between groups [$H_{(2)} = 7.55$; $p < 0.05$]. *Post hoc* analysis revealed that only the group treated with 10 mg/kg of Rolipram had a step-through latency higher than vehicle-treated group ($W = 23.5$; $p < 0.01$). **Figure 4B** shows step-through latency measured 1 week after training with the 0.35 shock in group injected with vehicle or 10 mg/kg Rolipram 120 min post-training. Two-sample Wilcoxon test did not reveal significant difference between groups. **Figure 4C** shows step-through latency measured 1 week after training in groups injected immediately after pseudo-training with vehicle or 10 mg/kg Rolipram. Two-sample Wilcoxon test did not reveal significant difference between groups. Taken together, these results indicate that Rolipram enhances fear LTM only at the highest dose and only when injected immediately after training. Moreover, Rolipram has no effect on LTM duration in absence of shock.

DISCUSSION

Two are the main findings of the present study. The first is that long-term object memory is enhanced when sampling is performed under positive emotional arousal. Indeed, in the present experiments we demonstrated that mice remember a sampled object for 24 h but this memory can last 96 h if object sampling is performed in an environment previously associated with a rewarding stimulus. The second finding is that pharmacological stimulation of cAMP cascade enhances the duration of low emotional memories regardless of hedonic valence. Thus, we found that mice treated with Rolipram post-trial showed discrimination of an object sampled under low emotional arousal 96 h earlier and show IA of a context associated 1 week before with a low intensity shock.

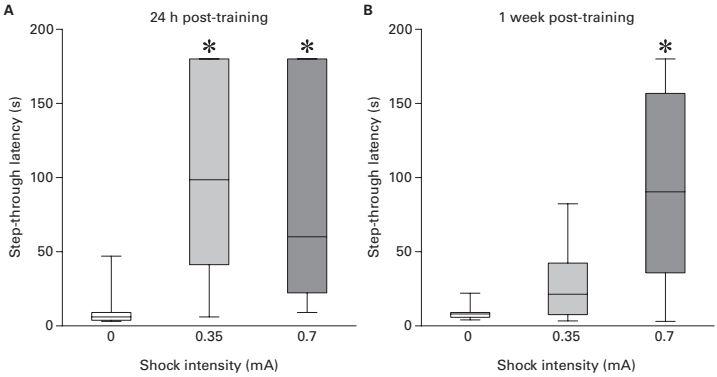

FIGURE 3 | (A) Step-through latency (median and inter-quartile range) as a function of foot-shock intensity (0.35 vs 0.7 mA) measured 24 h post-training (*vs 0, $p < 0.001$); **(B)** Step-through latency (median and inter-quartile range) as a function of foot-shock intensity (0.35 vs 0.7 mA) measured 1 week post-training (*vs 0, $p < 0.001$).

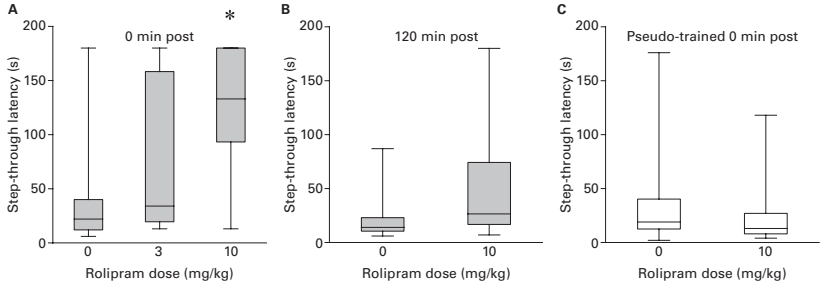

FIGURE 4 | (A) Step-through latency (median and inter-quartile range) as a function of foot-shock intensity (0.35 vs 0.7 mA) measured 1 week post-training (* vs all, $p < 0.01$); **(B)** Step-through latency (median and inter-quartile range) as a function of Rolipram dose (0, 3, and 10 mg/kg) injected immediately after training with the 0.35 mA foot-shock measured 1 week post-training (*vs all, $p < 0.05$); **(C)** Step-through latency (median and inter-quartile range) as a function of Rolipram dose (0 vs 10 mg/kg) injected 120 min after training with the 0.35 mA foot-shock measured 1 week post-training.

POSITIVE EMOTIONAL MODULATION OF LONG-TERM MEMORY

The present study used a modified version of the widely used ORT that allowed modulation of the memory trace acquired during sampling by positive emotional arousal. To this aim we associated the experimental context with chocolate and then increased the motivational salience of the unconditioned stimulus by submitting the mice to a period of food deprivation immediately before ORT training and testing phases. According to several studies on reward anticipation, animals respond with a positive emotional arousal when they are returned into contexts that predicts the availability of highly palatable, caloric, and fat foods (Alcaro et al., 2007; Boissy et al., 2007; Alcaro and Panksepp, 2011). Mice trained and tested in a context associated with an inedible plastic stimulus were used as controls.

Both experimental and control mice discriminated the novel object when tested 24 h after training demonstrating intact memory of the previously sampled object. However, 96 h after training only mice trained and tested within the chocolate-associated context were still able to discriminate the novel object. These data support the view that experiences associated with a state of positive emotional arousal are consolidated in more lasting memories as reported for those associated with negative ones.

Control experiments reported by the present paper indicate that the LTM of the sampled object is modulated by the chocolate-associated context rather than by the chocolate feeding immediately after sampling. Indeed, mice that did receive chocolate on the object sampling session without previous chocolate-context pairing did not show discrimination of a novel object 96 h later, whereas mice that did not receive chocolate but were trained in a context previously associated with chocolate did. These findings rule out the influence of sugar or fat (Campolongo et al., 2009; Smith et al., 2011) on memory consolidation in our experimental conditions. Moreover, in our standard experimental condition chocolate was made available at the end of the sampling session to prevent negative emotional arousal associated with frustration (for review see Flaherty, 1996) as well as contingency between sample object and reward (Hughes, 2007).

Moreover, the data suggest that the chocolate-associated context was capable of promoting a lasting memory of the sampled object also in free-feeding mice; however, food-deprived mice showed a D.I. significantly higher than free-fed mice. This result supports the hypothesis that imposing 15 h of food deprivation before objects sampling increased the positive emotional arousal promoted by chocolate-associated context in line with the hypothesis that a physiological depletion enhances the incentive value of the unconditioned and conditioned goal stimuli (Berridge, 2004). Mice food-deprived before training but not before test showed lower discrimination than mice trained and tested in food deprivation strongly supporting a state-dependent learning. Finally, mice food-deprived only on test day (96 h) did not show any improvement of object discrimination in comparison with free-fed mice. The latter observation indicates a selective effect of the motivational state on memory formation and/or consolidation rather than on retrieval. Taken together, these findings support the view that acute food deprivation increased emotional arousal promoted by the context associated with chocolate, It is tempting to speculate that the condition of feeding deprivation invested the experience of re-entering into the reward-predicting context of a flashbulb-like quality (Brown and Kulik, 1977).

NEGATIVE EMOTIONAL MODULATION OF LONG-TERM MEMORY

Results from IA experiment confirm and extend to the mouse those of Bekinschtein et al. (2007, 2008a,b) in rats. Indeed, we found that training with both foot-shock intensities promoted LTM (i.e., IA at 24 h post-training) but only training with the stronger one induced a persistent LTM (1 week). The facilitating effect of negative arousal on LTM duration is in line with the above described effect of positive arousal, strongly suggesting that emotional arousal enhances LTM duration regardless of hedonic valence.

PHARMACOLOGICAL STIMULATION OF CAMP CASCADE ENHANCES PERSISTENCE OF LOW EMOTIONAL MEMORIES

Pharmacological experiments evaluated the effects of single post-trial administration of Rolipram on retention of low emotional memories. We used two different tests: ORT and step-through IA. For both tests we considered protocols capable of promoting LTM traces of moderate persistence. In the case of ORT, we used mice trained and tested in the context associated with the plastic stimulus because these animals show retention 24 h but not 96 h after training. In the case of IA, we performed a preliminary experiment using 0.35 and 0.70 mA and chose the lower shock intensity that was effective in increasing step-through latencies 24 h but not 1 week post-trial.

The effects of Rolipram were tested at the time-points at which no retention was observable (96 h for ORT and 1 week for IA) in vehicle-treated mice. In both cases we found retention in Rolipram-treated mice. Rolipram is a phosphodiesterase type IV inhibitor that, at the doses used in the present experiments, promotes a rapid rise of cAMP concentration in the mouse brain (Randt et al., 1982). The treatment, in both cases, was performed immediately after a single training session, when the memory traces are under consolidation and susceptible of manipulation (McGaugh, 1966). Therefore, the present results suggest that an increase of brain cAMP concentrations during consolidation mimics the effects of high emotional arousal on persistence of long-term memories.

Nonetheless, major differences were found in the effects of Rolipram on ORT and IA retention. Thus, only the high dose of the phosphodiesterase inhibitor was effecting in enhancing step-through latencies 1 week after IA training, whereas the lower dose was already capable to enhance D.I. to the level of the high dose in mice tested 96 h after ORT training. The most economic explanation of this difference could be that step-through latencies and D.I. were tested at different time points after training. Indeed, if the pharmacological manipulation is increasing duration of the memory traces, a lower dose could be sufficient to produce a 96-h duration whereas only larger doses would induce 1 week-lasting memories. Another difference between the effects of Rolipram on retention in the two conditions was that the drug improved object memory when administered 120 min post-trial, whereas it improved IA retention only when administered immediately post-trial. It should be noted that Rolipram improves object memory in rats if injected 3 h, but not immediately, after sampling (Rutten et al., 2007). However, the dose administered in the present study is much more higher than the one used by Rutten et al. (2007). Thus, the observed efficacy of immediate post-sampling administration could depend on the half-life of Rolipram extending up to 120 min. On the other hand, such hypothesis fails to explain why an even higher dose (10 mg/kg) enhances IA retention if administered immediately, but not 120 min, after training. This may suggest that newly formed non-aversive memories remain susceptible to cAMP stimulation for a longer time respect to aversive ones.

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ORIGINAL ARTICLE

In vivo catecholaminergic metabolism in the medial prefrontal cortex of ENU2 mice: an investigation of the cortical dopamine deficit in phenylketonuria

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Abstract
Objective Phenylketonuria (PKU) is an inherited metabolic disease characterized by plasma hyperphenylalaninemia and several neurological symptoms that can be controlled by rigorous dietetic treatment. The cellular mechanisms underlying impaired brain functions are still unclear. It has been proposed, however, that phenylalanine interference in cognitive functions depends on impaired dopamine (DA) transmission in the prefrontal cortical area due to reduced availability of the precursor tyrosine. Here, using Pah^{enu2} (ENU2) mice, the genetic murine model of PKU, we investigated all metabolic steps of catecholamine neurotransmission within the medial preFrontal Cortex (mpFC), availability of the precursor tyrosine, synthesis and release, to find an easy way to reinstate normal cortical DA neurotransmission.
Methods and results Analysis of blood and brain levels of tyrosine showed reduced plasma and cerebral levels of tyrosine in ENU2 mice. Western blot analysis demonstrated deficient tyrosine hydroxylase (TH) protein levels in mpFC of ENU2 mice. Cortical TH activity, determined *in vivo* by measuring the accumulation of l-3,4-dihydroxyphenylalanine (L-DOPA) in mpFC after inhibition of L-aromatic acid decarboxylase with NSD-1015, was reduced in ENU2 mice. Finally, a very low dose of L-DOPA, which bypasses the phenylalanine-inhibited metabolic steps, restored DA prefrontal transmission to levels found in healthy mice.
Conclusion The data suggests that a strategy of using tyrosine supplementation to treat PKU is unlikely to be effective, whereas small dose L-DOPA administration is likely to have a positive therapeutic effect.

Introduction

Phenylketonuria (PKU; McKusick 2610600) is an inherited metabolic disease caused by a deficiency of the enzyme phenylalanine hydroxylase, which is necessary to convert phenylalanine to tyrosine. This results in accumulation of phenylalanine (> 20 mg/dl), known as hyperphenylalaninemia, and reduction of tyrosine concentrations in the blood and brain. Treatment of PKU requires maintaining blood phenylalanine within an acceptable range (between 2 and 10 mg/dl) by restricting phenylalanine from the diet. If the disease is untreated, patients develop severe mental retardation and neuropathological signs. Compliance with a rigid low phenylalanine diet is difficult (Giovannini et al 2007; MacDonald 2000), and it is still unknown when or if the diet can be safely interrupted (De Roche and Welsh 2008; Stemerdink et al 2000; Diamond et al 1994). Indeed, evidence indicates that even mildly elevated blood phenylalanine levels induce deficits in cognitive functions involving the prefrontal cortical area (Brumm et al 2004: Channon et al 2004; De Roche and Welsh 2008; Diamond et al 1994; Huijbregts et al 2002; Leuzzi et al 2004; Schmidt et al 1994;

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Smith et al 2000; White et al 2002; Stemerdink et al 2000), in particular executive abilities. This suggests that excess phenylalanine interferes with cortical functioning. The medial prefrontal cortex (mpFC) is widely innervated by biogenic aminergic neurons, which have a major role in emotion and cognitive functions (Arnsten and Robbins 2002; Lapiz and Morilak 2006; Aston-Jones and Cohen 2005; Goldman-Rakic 1999; Clarke et al 2004, 2005, 2006; Walker et al 2009). Moreover, there are reports of reduced levels of biogenic amines in *post mortem* brain tissue (McKean 1972), low levels of biogenic amine metabolites in cerebrospinal fluid of patients with hyperphenylalaninemia (Bonafé et al 2001; Butler et al 1981) and reduced brain amine levels and metabolism in PAH^{enu2} mice (ENU2) (Puglisi-Allegra et al 2000; Pascucci et al 2002, 2008), that is, the genetic murine model of PKU. Dopamine (DA) is the most studied among cerebral biogenic amines (Diamond 2007; Joseph and Dyer 2003). Although reduction of cerebral DA metabolism has been reported in PKU patients (Diamond et al 1994; Hanley et al 2000; Krause et al 1985; Guttler and Lou 1986; Lou et al 1987; Luciana et al 2004; Lykkelund et al 1988; McKean and Peterson 1970; Paans et al 1996) and ENU2 mice (Joseph and Dyer 2003; Pascucci et al 2009; Puglisi-Allegra et al 2000; Smith and Kang 2000), DA metabolism in mpFC of PKU organisms has not been investigated until now. Therefore, it is difficult to determine the mechanisms by which high blood phenylalanine levels reduce cortical DA biosynthesis (De Groot et al 2010; Martynyuk et al 2010).

ENU2 mice represent a qualified model for clarifying neurochemical deficits in pFC of PKU organisms, because they are characterized by a biochemical phenotype that closely resembles untreated human PKU, as well as by reduced enzymatic activity of phenylalanine hydroxylase, high blood phenylalanine levels, hypomyelination, biochemical and behavioural deficits (Andolina et al 2010; Cabib et al 2003; Embury et al 2007; Glushakov et al 2005; Joseph and Dyer 2003; Martynyuk et al 2005; Pascucci et al 2002, 2008, 2009; Puglisi-Allegra et al 2000; Smith and Kang 2000; Zagreda et al 1999). In particular, previous data showed deficits in DA and norepinephrine (NE) metabolism in the mpFC of ENU2 mice (Joseph and Dyer 2003; Puglisi-Allegra et al 2000; Pascucci et al 2009). Since DA availability in mpFC is very important in executive functions, the elucidation of the mechanism by which phenylalanine reduces cortical DA metabolism is essential. Thus, the present study was aimed at investigating catecholaminergic metabolism in the mpFC of ENU2 mice and at suggesting strategy to reinstate normal cortical catecholamine levels.

First, we investigated tyrosine blood and brain levels in order to evaluate the influence of excess phenylalanine on brain availability of catecholamine precursor. Second, we evaluated expression and *in vivo* activity of the tyrosine hydroxylase (TH) enzyme in mpFC of ENU2 mice. Finally, we evaluated the effect of l-3,4-dihydroxyphenylalanine (L-DOPA), the direct DA precursor, on activation of the frontal cortical catecholaminergic transmission in the presence of high circulating phenylalanine levels. Since restraint stress is known to induce a clear-cut increase of amine outflow in the mpFC of rodents (Cuadra et al 2001; Matuszewich et al 2002; Pascucci et al 2007), and phenylketonuric mice are unable to activate catecholamine release under stress (Pascucci et al 2009), we assessed the effect of L-DOPA on cortical catecholamine release of restrained ENU2 mice.

Materials and methods

Animals

Homozygous (-/-) Pah^{Enu2} (ENU2) and (+/+) Pah^{Enu2} (WT) male mice of the background strain (BTBR) were obtained from heterozygous mating. Genetic characterization was performed on DNA prepared from tail tissue using the Easy DNA Kit (Invitrogen, Carlsbad, CA, USA). The enu2 mutation was detected after PCR amplification of exon 7 of the Pah gene and digestion with Alw261 restriction enzyme (Promega corporation, Madison, Wi, USA) as described (Pascucci et al 2008). At postnatal day 28, animals (sex matched) were housed 2-4 per standard breeding cage with food and water *ad libitum* on a 12:12h dark: light cycle (light on 07.00 am -07.00 pm h). Experiments started when animals reached 8 weeks of age. All mice were housed individually 24 h before surgery for microdialysis. Naive animals were used for each experiment.

All experiments were conducted in accordance with European legislation (EEC no. 86/609), Italian national legislation (DL no. 116/92) governing the use of animals for research, and the guidelines of the National Institutes of Health on the use and care of laboratory animals.

Drugs

Chloral hydrate, NSD-1015, and L-DOPA were purchased from Sigma-Aldrich (St. Luis, MO, USA). NSD-1015 was dissolved in artificial CSF and perfused through the microdialysis probe. Chloral hydrate (450 mg/kg) and L-DOPA (0.5, 1, 2.5 mg/kg) were dissolved in saline (0.9 % NaCl) and injected i.p. in a volume of 10 ml/kg.

Brain and blood assay

For brain and blood phenylalanine and tyrosine assay, WT (n=6) and ENU2 (n=6) mice were sacrificed by decapitation. Brains and blood were prepared for biochemical analysis.

First, the brains were removed, frozen and stored in liquid nitrogen until the day of biochemical assay. Frozen whole brains were weighed and homogenized in 0.05M HClO4 (1:100 ml/mg). The homogenates were centrifuged at 10000 x g for 20 min at 4 °C.

Blood samples for phenylalanine and tyrosine quantification were placed in heparinized tubes and centrifuged at 2,500 rpm, at +4 °C, for 10 min. An aliquot of supernatant was collected, and transferred to a new tube with 35 % 5-sulfosalicylic acid (10:1 vol/vol), and centrifuged at 8000 rpm, at +4 °C, for 5 min.

Blood and brain samples were allowed to react with the same volume of o-phthaldialdehyde reagent (67.1 mg of o-phthaldialdehyde dissolved in 1.0 ml of methanol plus 50 µl of mercaptoethanol and diluted in 9 ml of borate buffer, 0.4 mM, pH 9.5). After a 2-min reaction time, the sample was transferred to the HPLC system coupled with a fluorescence detector (Waters 474 Model). The excitation and emission wavelengths were set at 330 and 480 nm, respectively. Nova-Pack C18 (3.9 x 150 mm) and Sentry Guard Nova-Pack C18 (3.9 x 20 mm; Waters Assoc.) columns were used. The flow rate was 1.2 ml/min. The mobile phase consisted of 35 % methanol in 0.1 M Na-phosphate buffer, pH 6.5.

Western bolt analysis

Brains of mice from the different groups (WT, n=8; ENU2, n=8) were removed, frozen and then fixed vertically on the freeze plate of a freezing microtome maintained at -10 °C. Punches of mpFC (Fig. 1a) were obtained from frozen brain slices as previously reported (Puglisi-Allegra et al 2000) and stored in liquid nitrogen until the day of assay**.** Each mpFC tissue sample was homogenized at 4 °C in lysis buffer [20 mM Tris (pH 7.4), 1 mM EDTA, 1 mM EGTA, 0,1 % Triton X-100] with protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). Tissue extract was centrifuged at 12000 g at 4 °C for 15 min. The supernatant fluid was removed and stored at – 80. Samples were heated at 95 °C for 3 min and protein (15 mg) was separated by SDS-PAGE (10 % gel).

Membranes were rinsed in Tris- buffered saline (TBS), then blocked in 5 % non-fat milk in TBS with 0.1 % Tween (TBS-T) for 1.5 h at room temperature in TBS-T and incubated overnight in anti-TH antibody (Chemicon, Temecula, CA, USA) (1:3000 dilution) diluted in 3 % BSA, followed by goat anti-rabbit IgG (H+L) AP conjugate (1:2000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) in 2 % non-fat milk in TBS-T, and developed with the ECL-R reaction (Amersham). The film signals were digitally scanned and quantified using densitometric image software (imagej 64), normalized for β-actin level.

In vivo microdialysis

All mice were anesthetized with chloral hydrate, mounted in a stereotaxic frame (David Kopf Instruments, Tujunga, CA) and implanted unilaterally with a guide cannula (stainless steel, shaft outer diameter of 0.38 mm, length 1 mm; Metalant AB, Stockholm, Sweden), fixed with epoxy glue and dental cement, into the mpFC (Fig. 1a; AP, +2.8; L, 0.6; according to the Franklin and Paxinos atlas, 2001).

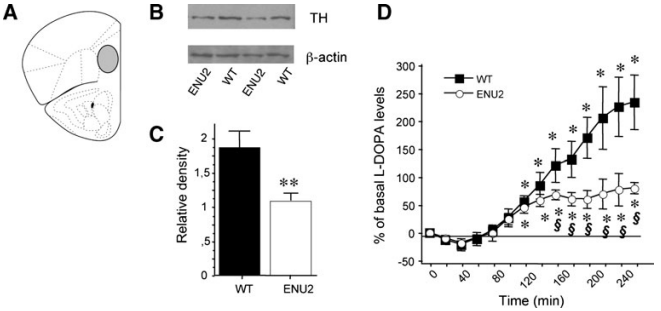

Fig. 1 Reduced expression and *in vivo* activity of TH in mpFC of ENU2 mice. (**a**) Schematic representation of mpFC. (**b**) Western blot of TH protein obtained from mpFC of ENU2 and WT mice and (**c**) quantification of protein using chemiluminescence (mean ± S.E.M.) indicated reduced enzyme availability in mutant mice. Detection of β-actin was used as loading control. (**d**) Enzymatic activity of TH was determined measuring accumulation of transient intermediate L-DOPA *in vivo* during continuous infusion of 20 µM NSD-1015. Dialysates were collected at 20-min intervals. Results are expressed as percent changes (means ± S.E.M.) from basal values. Statistical analyses were performed on raw data. Comparison of time course of changes in extracellular levels of L-DOPA in pFC of ENU2 and WT mice reveals reduction of L-DOPA accumulation in ENU2 mice, significant from 160 min onwards. * $P<0.05$ versus basal values. § $P<0.05$ in comparison with WT

Placement of probes in mpFC was evaluated by methylene blue staining. Only data from mice with correctly placed cannula are reported.

Mice were allowed to recover in their home cage. The probe (2 mm long; MAB 4 cuprophane microdialysis probe, Metelant AB) was introduced 24 h before microdialysis experiments. The mice were lightly anesthetized with chloral hydrate to facilitate manual insertion of the probe into the guide cannula. The dialysis probe was connected to a CMA/100 pump (Carnegie Medicine, Stockholm, Sweden) through PE 20 tubing (Metalant AB) and an ultralow torque dual-channel liquid swivel (model 375/D/22QM; Instech Laboratories, Plymouth Meeting, PA) to allow free movement. Artificial cerebrospinal fluid (147 mM NaCl, 1 mM MgCl, 1.2 mM $CaCl_2$, 4 mM KCl) was pumped through the dialysis probe at a constant flow rate of 2 μl/min. The day of the experiments, each animal was transferred to a Plexiglas cylinder provided with microdialysis equipment (Instech Laboratories, Inc.) and with home cage bedding on the floor. Dialysis perfusion was started 1 h later and mice were left undisturbed for 2 h before baseline samples were collected. Dialysate was collected every 20 min. The mean concentration of the three samples collected immediately before treatment (<10 % variation) was taken as basal concentration. Twenty microliters of each dialysate sample were transferred to HPLC systems for analysis.

In vivo TH activity Cortical TH activity in WT (n=8) and ENU2 (n=8) mice was determined *in vivo* by the accumulation of L-DOPA in mpFC after inhibition of L-aromatic acid decarboxylase with NSD-1015 (Sigma-Aldrich, St. Louis, MO, USA). Ringer solution containing 20 μM of NSD-1015 was pumped through the dialysis probe at a constant flow rate of 2 μl/min, and dialysates were collected at 20-min intervals for 260 min. L-DOPA was assessed by HPLC coupled to an amperometric detector (Decade II model, Antec Leyden, The Netherlands). The detector potential was set at +700 mV against an Ag/AgCl reference electrode. The mobile phase was previously described (Nakahara et al 2000).

L-DOPA treatment Finally, we evaluated the effect of L-DOPA administration on prefrontal cortical DA response to stress in hyperphenylalaninemic mice. DA, DOPAC (3,4-dihydroxyphenylacetic acid), HVA (homovanilic acid) and NE levels were determined simultaneously, utilizing the HPLC system coupled to a coulometric detector (model 5200 °Coulochem II; ESA, Chelmsford, MA). The conditioning cell was set at +400 mV, electrode 1 at +200 mV, and electrode 2 at -250 mV; the mobile phase was previously described (Pascucci et al 2007). A Nova-Pack C18 column (3.9 x 150 mm; Waters) and a Sentry Guard Nova-Pack C18 pre-column (3.9 x 20 mm) maintained at 30 °C were used. The flow rate was 1.1 ml/min. The detection limit of the catecholamines assay was 0.1 pg.

First, we identified a per se ineffective dose of systematically administered L-DOPA by performing a dose-response study. Naive WT (n=6) and ENU2 (n=6) mice were injected i.p. on consecutive days with saline or L-DOPA (0.5, 1, 2.5 mg/kg) and DA and NE in vivo extracellular levels were assessed. Doses were injected in a random order and sufficient time was allowed for neurotransmitter to return to basal levels (no more than 180 min were necessary). No more than two L-DOPA doses were administered daily.

Last, the effect of systemic administration of a per se ineffective dose of L-DOPA on only frontal cortical DA and metabolite response to stress was evaluated, as L-DOPA inability to increase NE frontal cortical extracellular levels. Following collection of baseline samples, animals subjected to the stress experience (WT-sal, n=8; ENU2-sal, n=8; ENU2-L-DOPA 0.5, n=8) were put in a restraint apparatus for 2 h and dialyzate samples were collected every 20 min. The apparatus consisted of an adjustable neck-blocking support mounted on a Plexiglas base and movable U-shaped metal piece that could be fixed to the base at the level of the animal's hips thus preventing it from turning on its back (Cabib and Puglisi-Allegra 1991).

Data analysis

The effect of genotype (WT and ENU2) on phenylalanine and tyrosine brain and blood levels and on brain/blood ratios was evaluated by one-way ANOVAs.

Regarding western blot data, the effect of genotype (WT and ENU2) on TH protein levels in mpFC was evaluated by one-way ANOVA.

For microdialysis data, statistical analyses were always carried out on raw data (concentrations: pg/20 μl). Data were presented in figures as percent changes from baseline levels.

The effect of genotype on L-DOPA accumulation in mpFC was analyzed by repeated-measures ANOVA with one between factor (genotype, two levels, WT and ENU2) and one within factor (time, forteen levels, 0, 20, 40, 60, 80, 100, 120, 140, 160, 180, 200, 220, 240, 260 minutes).

The effect of L-DOPA treatment on DA and NE extracellular levels in mpFC of ENU2 and WT mice was analyzed by repeated-measures ANOVAs with one between factor (treatment, four levels, saline, L-DOPA 0.5, 1, 2.5 mg/kg) and one within factor (time, seven levels, 0, 20, 40, 60, 80, 100 and 120 minutes).

The effect of L-DOPA treatment on DA, DOPAC and HVA extracellular levels in mpFC of ENU2 mice subjected to restraint was analyzed by repeated-measures ANOVAs with one between factor (group, three levels, WT-sal, ENU2-sal and ENU2-L-DOPA 0.5) and one within factor (time, seven levels, 0, 20, 40, 60, 80, 100 and 120 minutes).

Results

Blood and brain levels and brain/blood ratio of phenylalanine and tyrosine in ENU2 and WT mice

To determine whether high phenylalanine levels inhibit tyrosine transport across the blood-brain barrier, we measured phenylalanine and tyrosine blood and brain levels in ENU2 and WT mice (Table 1). The concentration of phenylalanine was significantly higher in ENU2 than in WT mice, both in blood (~2500 %) and in brains (~1700 %). For tyrosine levels, ENU2 showed reduction of blood and brain levels (~40 %) compared with WT mice. Moreover, phenylalanine blood/brain ratio was significantly reduced, and tyrosine blood/brain ratio was not significantly different, in ENU2 compared with WT mice.

Expression and in vivo activity of TH in mpFC of ENU2 and WT mice

Western blot analysis of TH protein (Fig. 1b, c) showed significant difference between the two genotypes ($F_{1,10}$= 11.25, p<.01), revealing a 40 % reduction of TH protein levels in mpFC of ENU2 (1.84±.21) in comparison with WT (1.10±.06) mice.

Figure 1d reports *in vivo* TH activity in mpFC of ENU2 and WT mice. Statistical analysis revealed significant genotype x time interaction ($F_{1,182}$=3.82, p<.05). In WT mice, blockade of aromatic L-amino acid decarboxylase promoted a time-dependent increase of frontal cortical L-DOPA outflow that became significantly higher than basal levels after 120 min, reached maximal levels (233.7 %) at 260 min. ENU2 mice achieved a steady state after 160 min of perfusion, reaching a maximal increase of 80.2 % at 260 min.

In vivo microdialysis

The two genotypes did not differ for DA cortical basal outflow (WT=0.95±.14 pg/20 l; ENU2=0.98±0.15 pg/20 µl), while NE extracellular levels from mpFC of ENU2 mice were significantly reduced (WT=2.12±.23 pg/20 µl; ENU2= 1.24±0.19 pg/20 µl; $F_{1,10}$=8.64, p<.05), as previously reported (Pascucci et al 2009), suggesting that compensatory mechanisms appear to support DA release at the expense of NE in basal conditions.

Dose-response curve of L-DOPA The dose-dependent effect of L-DOPA treatment on catecholaminergic frontal cortical extracellular release was evaluated (Fig. 2a, b). L-DOPA at a dose of 0.5 mg/kg i.p. had no significant effect on DA outflow in either group of mice, but at the dose of 1 and 2.5 mg/kg i.p. produced significant increase of DA cortical extracellular levels (Fig. 2a).

None of these doses of L-DOPA had an effect on extracellular levels of NE in either group, with the exception of a slight increase at 2.5 mg/kg i.p. in ENU2 mice (Fig. 2b).

Effect of L-DOPA treatment on DA cortical neurotransmission in stressed ENU2 mice Because L-DOPA treatment was unable to increase NE frontal cortical extracellular levels, effect of per se ineffective dose of L-DOPA was evaluated on frontal cortical DA outflow and turnover (as measured by their major metabolites, DOPAC and HVA) in ENU2 and WT stressed mice (Fig. 3). We compared frontal cortical DA outflow in WT and ENU2 stressed mice following saline or L-DOPA (0.5 mg/kg i.p.) treatment. Statistical analyses revealed a significant group x time interaction ($F_{18,168}$=3.15, p<.0001). Analyses of DOPAC and HVA extracellular levels in WT, ENU2-sal and ENU2-L-DOPA 0.5 groups revealed significant group x time interactions (DOPAC: $F_{12,126}$=3.77, p<.0001; HVA: $F_{12,126}$=3.90, p<.0001).

As previously reported (Pascucci et al 2009), ENU2 mice did not show the typical increase of prefrontal cortical DA outflow. Indeed, DA decreased below basal levels (Fig. 3). Moreover, stress resulted in augmented DOPAC and HVA extracellular levels in WT but not in ENU2 mice (Fig. 3), although DOPAC (WT-sal=53.34+ 8.87 pg/20 µl; ENU2-sal=59.20+12.61 pg/20 µl; ENU2-L-DOPA 0.5=59.88±17.09 pg/20 µl) and HVA (WT-sal= 118,12±21.72 pg/20 µl; ENU2-sal=154.83±19.24 pg/20 µl; ENU2-L-DOPA 0.5=159.65±27.91 pg/20 µl) basal levels did not differ between groups. Nevertheless, in ENU2 mice (similar to WT-sal) treatment with L-DOPA

Table 1 Blood and brain levels and brain/blood ratios of phenylalanine and tyrosine in WT and ENU2 mice

	WT			ENU2		
	blood	brain	brain/blood ratio	blood	brain	brain/blood ratio
Phenylalanine	92.0 ± 4.8	2.2 ± 0.2	0.023	2334.1 ± 143.7[b]	38.0 ± 4.7[b]	0.016 [a]
Tyrosine	82.8 ± 6.1	3.5 ± 0.4	0.043	34.2 ± 1.7[b]	1.27 ± 0.2[b]	0.039

Amino acids levels (µM) in blood and brain samples and blood/brain ratios in WT and ENU2 mice. Values are expressed as means ± SEM. [a] $p < 0.05$; [b] $p < 0.001$ *vs* WT

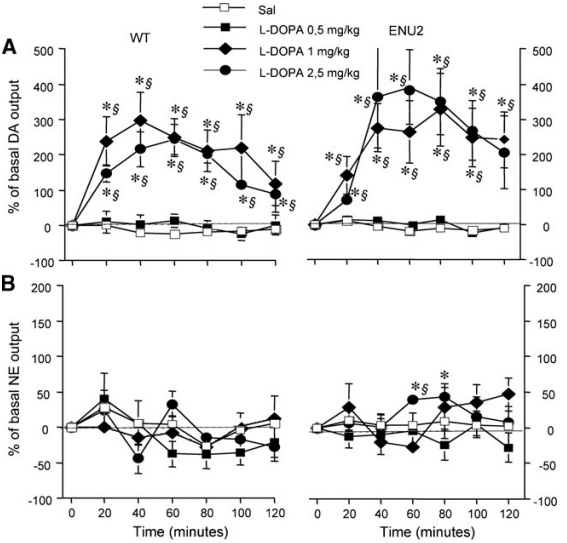

Fig. 2 Dose-dependent effect of L-DOPA on frontal cortical catecholamine extracellular levels. Dose-dependent effect of L-DOPA (0.5, 1.0, 2.5 mg/kg i.p.) on DA (**a**) and NE (**b**) outflow in the mpFC of WT and ENU2 mice. Results are expressed as percent changes (means ± S.E.M.) from basal value during 120-min post-injection. Statistical analyses were performed on raw data. Drug was administered to time 0. * $P<0.05$ vs saline group. § $P<0.05$ in comparison with vehicle-injected mice

caused an immediate significant increase in DA outflow (20-60 min) followed by a return to basal levels as well as partial but significant increase of DOPAC and HVA extracellular levels.

Discussion

This study demonstrates that the reduced DA concentrations reported in prefrontal cortical area of PKU mice are mainly due to reduced cortical expression and activity of the TH enzyme, supporting the use of L-DOPA to treat cortical dopaminergic deficits in phenylketonuric subjects.

It is well known that brain amine levels are reduced in PKU patients and mouse models, and DA is the most extensively studied neurotransmitter. In particular, reduced DA availability in mpFC has been proposed as the biochemical mechanism responsible for reduced cognitive performances observed in PKU patients. It has also been proposed that DA cortical deficits stem from decreased levels of

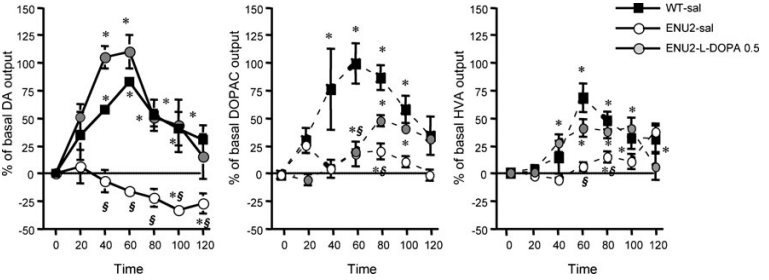

Fig. 3 Effect of L-DOPA on dopaminergic cortical neurotransmission in stressed ENU2 mice. Recovery of DA and metabolite response to stress in mpFC of ENU2 mice submitted to 120 min of restraint following systemic administration of 0.5 mg/kg i.p. of L-DOPA. Dialysates were collected at 20-min intervals. Results are expressed as percent changes (means ± SE) from basal values. Statistical analyses were performed on raw data. Drug was administered to time 0. * $P<0.05$ versus basal values. § $P<0.05$ compared with vehicle-injected WT mice

amino acid tyrosine ("tyrosine/dopamine" theory), the precursor of DA, and that low levels of tyrosine are a consequence of high phenylalanine levels outcompeting other amino acids for transport across the blood-brain barrier (Diamond et al 1994). Although the "tyrosine/dopamine" theory is strong and empirically supported, controversial data have been reported. Indeed, dietary supplements of tyrosine do not improve cognitive performance in PKU patients (Smith et al 1998), and frontal cortical levels of tyrosine do not return to normal levels when PKU mice are placed on the low phenylalanine diet (Joseph and Dyer 2003). These data indicate that reduced tyrosine availability alone cannot explain the DA cortical deficits, suggesting co-existing of several factors. In this study, we investigated full cortical dopaminergic metabolism in ENU2 mice in order to elucidate phenylalanine-induced interferences at each metabolic step and suggest an easy pharmacological way to raise cortical dopaminergic levels.

Synthesis of catecholamines occurs via hydroxylation of tyrosine to L-DOPA by TH. L-DOPA is rapidly decarboxylated by L-aromatic amino acid decarboxylase to DA, which is then metabolized to NE. Thus, we firstly examined blood and brain availability of DA precursors. Although the presence of high phenylalanine and reduced tyrosine blood and brain levels is well known, the evaluation of brain/blood ratios for tyrosine and phenylalanine in this study is not consistent with the hypothesis of phenylalanine-induced inhibition of amino acid transport to the brain, according to previously reported data (Joseph and Dyer 2003). In fact, we observed a significant reduction of blood and brain tyrosine levels, according to the literature, but found no significant difference in the brain/blood ratio between phenylketonuric and normal mice, suggesting that reduced brain levels of tyrosine reflect low tyrosine blood levels more than reduction of access to the brain. Conversely, when we compared the phenylalanine brain/blood ratio in both groups, we found a trend towards reduced phenylalanine access in the brains of PKU mice, which, however, was unable to prevent high brain phenylalanine levels.

Second, based on demonstrations that tyrosine is not the limiting factor on DA biosynthesis (Joseph and Dyer 2003; Pascucci et al 2009), we investigated cortical availability and activity of TH. Analysis of Western blot data confirmed reduced TH protein levels in mpFC of PKU mice (Joseph and Dyer 2003). Although the decreased protein amount of TH could be an adaptive downregulation in response to reduced dopaminergic synthesis, a faster degradation of the TH protein could not be excluded. Moreover, *in vivo* assay of TH cortical activity (measured as accumulation rate of L-DOPA after blockade of the decarboxylating enzyme) showed significant reduction in the rate of DA synthesis in ENU2 vs WT mice. The reduction of L-DOPA accumulation (66 %) was greater than the 40 % reduction seen in TH protein concentration suggesting other mechanisms causing TH inhibition. This most likely involves a direct inhibitory effect of phenylalanine on mpFC TH activity.

So far our data suggestes three complementary factors are able to explain DA reduced biosynthesis in PKU: decrease of precursor availability to the brain and reduction of protein synthesis and activity of TH enzyme. Recently, we demonstrated that deficits of cortical serotonin biosynthesis in PKU mice are due to phenylalanine -induced inhibition of cortical tryptophan hydroxylase activity (Pascucci et al 2009). These results are in agreement with the hypothesis that phenylalanine influences cortical aminergic transmission by inhibiting activity of enzymes hydroxylating amino acid precursors (Curtius et al 1981; McKean 1972; Ogawa and Ichinose 2006).

Third, based on the reduction of prefrontal cortical TH protein and activity levels in ENU2 mice, we were able to identify L-DOPA, the product of tyrosine hydroxylation, as responsible for increasing DA cortical levels. As previously reported (Pascucci et al 2009), when ENU2 mice were subjected to restraint stress, an environmental challenge known to enhance aminergic release in the mpFC (Page and Lucki 2002; Pascucci et al 2007), they showed deficits in the activation of frontal cortical serotoninergic and dopaminergic transmission and altered noradrenergic responses. In particular, no initial increase of DA release followed by decrease below baseline levels was observed compared with WT, although basal frontal cortical outflow of DA was unaffected. Moreover, DA turnover was also affected by hyperphenylalaninaemia, as shown by reduced DOPAC and HVA extracellular levels in ENU2-stressed mice, although DA basal levels were unaffected.These results suggest compensatory mechanisms are involved that maintain suitable DA metabolism necessary to hold basic physiological functions. However, these mechanisms are unable to sustain the activation solicited by stressful experience.

In order to restore cortical dopaminergic response to stress, we administered L-DOPA, the proximal precursor of DA. The L-DOPA dose-response curves obtained in the ENU2 and WT mice were similar suggesting that the cortical DA metabolic pathway following tyrosine hydroxylation step is intact. Nevertheless, an alternative possibility cannot be excluded: i.e. the L-DOPA-induced DA release depends on serotonergic neurons (Carta et al 2007; Navailles et al 2010; Tanaka et al 1999), wherein L-aromatic amino acid decarboxylase is also present. The same L-DOPA doses were unable to increase frontal cortical release of NE, suggesting an impairment of the conversion of DA to NE in cortical neurons. The administration of a *per se* ineffective dose of L-DOPA (0.5 mg/kg i.p.) affected response to stress in mpFC of ENU2 mice, producing activation of DA and metabolite response. These results show that DA metabolism in the mpFC is very sensitive to L-DOPA treatment,

suggesting, under stress challenge, an increase of L-DOPA decarboxylation by DOPA decarboxylase to DA. Although neurological complications related to prolonged treatment with L-DOPA have been reported in Parkinson's disease patients, the dose used here was well below that associated with abnormal movements in human and in animal models.

Altogether, our data suggests that DA cortical deficits in PKU are due to several factors: reduced precursor cerebral availability, reduced cortical TH protein levels and inhibition of TH cortical activity. Thus, our data raises doubts about using tyrosine in PKU patients. A better approach may be the use of low dose L-DOPA which in the PKU mouse is able to increase cortical DA neurotransmission even in the presence of high blood and brain phenylalanine levels.

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Conflict of interest None.

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