OPERANT BEHAVIOR TO OBTAIN PALATABLE FOOD MODIFIES ERK ACTIVITY IN THE BRAIN REWARD CIRCUIT

Thomas Guegan[1], Laura Cutando[1], Giuseppe Gangarossa[2#], Emanuela Santini[2], Gilberto Fisone[2], Albert Martinez[3], Emmanuel Valjent[2#], Rafael Maldonado* [1]& Miquel Martin*[1]

[1] Laboratori de Neurofarmacologia, Univeristat Pompeu Fabra, PRBB, Barcelona, Spain.

[2] Department of Neuroscience, Karolinska Institutet, Stockholm, Sweden.

[3] Departament de Biologia Cel.lular, Universitat de Barcelona/ Institut de Recerca en Biomedicina, Barcelona/CIBERNED, Spain.

Current address: IGF. Department of Neurobiology Inserm U661, UMR 5203 CNRS, University Montpellier I & II ; 141, rue de la Cardonille, 34094 Montpeller Cedex 05-France

* Corresponding authors:

Rafael Maldonado and Miquel Martín.

Laboratori de Neurofarmacologia.

Universitat Pompeu Fabra, Parc de Recerca Biomedica de Barcelona

C/ Dr. Aiguader 88

08003, Barcelona, Spain.

Telf: +34 93 316 08 24 FAX: +34 93 316 09 01

E-mail address: rafael.maldonado@upf.edu and miquel.martin@upf.edu

Abstract

Food palatability produces behavioral modifications that resemble those induced by drugs of abuse. Palatability-induced behavioral changes require the activation of the endogenous cannabinoid system and changes in structural plasticity in neurons of the brain reward pathway. The ERK intracellular pathway is activated by CB1 receptors (CB1-R) and plays a crucial role in neuroplasticity. We investigated the activation of the ERK signaling cascade in the mesocorticolimbic system induced by operant training to obtain palatable food and the involvement of the CB1-R in these responses. Using immunofluorescence techniques, we analyzed changes in ERK intracellular pathway activation in the mesocorticolimbic system of wild-type and CB1 knockout mice (CB1-/-) trained on an operant paradigm to obtain standard, high caloric or high palatable food. Operant training for high palatable food, but not for standard or high caloric food, produced a robust activation of the ERK signaling cascade in the same brain areas where this training modified structural plasticity. These changes induced by the operant training were absent in CB1-/-. We can conclude that the activation of the ERK pathway is associated to the neuroplasticity induced by operant training for palatable food and might be involved in CB1-R mediated alterations in behavior and structural plasticity.

Keywords: CB1 cannabinoid receptor, ERK-intracellular pathway, food-seeking, mesocorticolimbic, neuroplasticity, palatability, immunofluorescence.

1. Introduction

Several studies have suggested that food palatability can trigger the development of seeking behavior and binge-eating, which can promote overeating and obesity (Olszewski et al., 2011; Johnson and Kenny, 2010; Gearhardt et al., 2011). Food induced seeking behavior and binge-eating has been reported to be associated to a deregulation in the activity of the mesocorticolimbic system (Johnson and Kenny, 2010; Wang et al., 2004). Similar neurobiological modifications have also been described in drug addicts. Thus, brain imaging studies have shown a reduction in D2 receptor availability in the striatum of obese patients, a change that has also been described in cocaine addicts (Wang et al., 2004). Moreover, obese subjects also present behavioral responses that resemble those observed in drug addicts, such as loss of control, craving and seeking behavior (Corsica and Pelchat, 2010).

In our previous study (Guegan et al., back to back publication), we have demonstrated that behavioral alterations that resembles addictive-like behavior produced by the operant training for palatable food depends on the activation of the CB1-R and are mediated by changes in structural plasticity in neurons from the nucleus accumbens (NAc) shell and medial prefrontal cortex (mPFC). Different intracellular pathways have been shown to be activated by CB1-R and might be involved in mediating some of the behavioral and neuroplastic changes induced by palatable food. Interestingly, the ERK signaling cascade is activated by CB1-R at the level of the mesocorticolimbic system (Corbille et al., 2007). Moreover, ERK activation in the mesocorticolimbic system plays a crucial role in mediating behavioral responses related to the addictive properties of drugs of abuse by a mechanism that suggests changes in neuroplasticity (Girault et al., 2007). In agreement, pharmacological inhibition of ERK attenuates cocaine-induced changes in dendritic branching and dendritic spine densities in medium spiny neurons in

the NAc shell and striatum (Ren et al., 2010). However, little is known about the role of the ERK pathway in mediating the reinforcing effects of natural rewards, and its possible involvement in the behavioral alterations promoted by food.

In this study, we have investigated the ability of operant training to obtain palatable food, that was associated to enhanced-seeking behavior and neuroplastic alterations (Guegan et al., back to back publication) to promote changes in the activity of the ERK intracellular pathway and the possible involvement of CB1-R in these modulatory actions. For this purpose, CB1-/- and wild-type littermates (CB1+/+) were trained to lever-press to obtain standard, high caloric or high palatable pellets and the changes in the activation of different downstream proteins of the ERK signaling cascade (p-ERK, p-H3 and p-rS6) were evaluated. We found that operant behavior to obtain high palatable food produced an increase in the activation of the ERK signaling cascade in the same areas of the mesocorticolimbic system where this training produced changes in structural plasticity. These modifications induced by the operant training were absent in CB1-/-.

2. Experimental procedures

2.1. Animals

The experiments were carried out in male CB1-/- and CB1+/+ littermates from 8–12 weeks old at the beginning of the experiments. The generation of CB1-/- and CB1+/+ was described previously (Zimmer et al., 1999). Briefly, homozygous CB1+/+ and CB1-/- were bred by back-crossing of chimeric and heterozygous animals to C57BL6/J and interbreeding of heterozygous animals for at least 10 generations in order to obtain a pure C57BL6/J background. The animals were individually housed and maintained in a controlled temperature (21±1°C) and humidity (55±10%) room with a 12:12-h reversed light/dark cycle (on at 8 p.m and off at 8 a.m.). All the experiments were performed during the dark phase of the dark/light cycle. Animals were habituated to the experimental room and handled for one week before starting the experiments with *ad libitum* access to standard chow and water. All animal procedures were conducted in accordance with the standard ethical guidelines (European Communities Directive 86/60-EEC, Animal Welfare Assurance #A5388-01, IACUC Approval Date 06/08/2009) and approved by the local ethical committee (Comitè Ètic d'Experimentació Animal-Institut Municipal d'Assistència Sanitària-Universitat Pompeu Fabra).

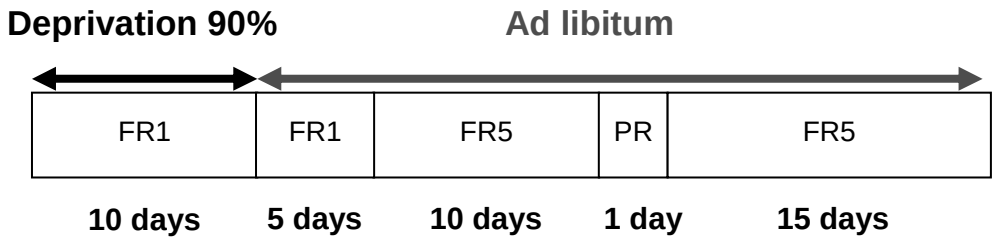
2.2. Acquisition of operant responding maintained by food

Operant responding maintained by food was evaluated in mouse operant chambers (Model ENV-307A-CT, Med Associates, Georgia, VT, USA). The chambers were made of aluminium and acrylic, had grid floors (EVV-414, Med. Associates Inc., St Albans, USA), and were housed in sound and light-attenuated boxes equipped with fans to provide ventilation and white noise. The chambers were equipped with two retractable

levers, one randomly selected as the active and the other as the inactive. Pressing on the active lever resulted in a pellet delivery (standard, fat-enriched pellet or high palatable) together with a stimulus-light during two sec (associated-cue), while pressing on the inactive lever had no consequences. A food dispenser equidistant between the two levers permitted delivery of food pellets when required. The beginning of the each operant responding session was signaled by turning on a house light placed on the ceiling of the box for three sec that was then turn off during the remaining duration of the session. The side of the active and inactive lever was counterbalanced between animals. Each session started with a priming delivery of one pellet. A time-out period of 10 sec was established after each pellet delivery. During this period, the cue-light was off and no reinforcer was provided after responding on the active lever. Responses on the active lever and all the responses performed during the time-out period were also recorded. The session was terminated after 100 reinforcers were delivered or after one hour, whichever occurred first. One hour daily sessions were conducted seven days per week during a period of 41 days. The animals were food deprived five days before starting sessions to maintain their weight at 90 % of their *ad libitum* initial weight adjusted for growth, and this food restriction regime was maintained during the first 10 sessions of the operant behavior training to permit the appropriate acquisition of the task. Additional standard- and high palatable-yoked groups were included. These groups were subjected to the same experimental conditions except that no reinforcer and no cue-light were presented after pressing in any of the two levers exposed in the operant chamber. However, standard- and high palatable-yoked groups received passively the same amount of pellets and with the same frequency as the CB1+/+ that were trained to obtain them.

The training sessions started with a fixed-ratio 1 (FR1) schedule of reinforcement during 15 days (during the first 10 days mice were food deprived) where one active nose-poke resulted in one food pellet. A minimum of 14 CB1+/+ on each experimental group (included yoked-high palatable animals) were sacrificed after the exposure to the 10^{th} session, to evaluate the effects of short term exposure to different types of food on structural plasticity and molecular changes.

The rest of the animals were then feed *ad libitum* during the remaining operant behavior training. Operant training on FR1 was followed by a period of 10 days with a fixed ratio 5 (FR5) schedule (five active nose-pokes were required to obtain one pellet). Mice were then exposed to a progressive ratio (PR) schedule in which the response requirement to earn one pellet escalated according to the following series: 1-2-3-5-12-18-27-40-60-90-135-200-300- 450-675-1000. The PR session lasted for four hours or until mice stopped responding for at least one hour, and was performed only once. The breaking point was determined in each animal as the last response ratio completed. Finally, mice were exposed to a FR5 schedule for 15 additional days. After each session, mice were returned to their home-cages. The chambers were cleaned at the end of each session to prevent the presence of odor of the previous mouse. Therefore, the schedule of the experiment is summarized as follows:



As previously described (Barbano et al., 2009), the criteria for the achievement of the operant responding were acquired when all of the following conditions were met: (i) mice maintained a stable responding with less than 20 % deviation from the mean of the total number of reinforcers earned in three consecutive sessions (80 % of stability); (ii) at least 75 % responding on the active hole, and (iii) a minimum of 10 reinforcers per session. All the mice included in this study have achieved these criteria in all the experimental phases.

2.3. Food pellets

During the operant experimental sessions, animals were presented with 20 mg dustless precision standard pellets (TestDiet, Richmond, IN, USA), high palatable pellets (TestDiet, Richmond, IN, USA) or fat-enriched pellets (Bio-serv, Frenchtown, NJ, USA). The standard pellet formula was similar to the standard maintenance diet provided to mice in their home cage (24.1 % protein, 10.4 % fat, 65.5 % carbohydrate, with a caloric value of 3.30 kcal/g). High palatable pellets presented similar caloric value to standard pellets (20.5 % protein, 12.7 % fat, 66.8 % carbohydrate, with a caloric value of 3.48 kcal/g) with some differences in their composition. Thus, high palatable pellets were modified by the addition of chocolate flavor (2% pure unsweetened cocoa), and although the carbohydrate content was similar in standard (65.5%) and high palatable pellets (66.8%), the proportion of sugars within this carbohydrate content was different: sucrose content in high palatable pellets was 50.11 % of the total carbohydrates, whereas in standard pellets was only 3.09 %. This different composition together with the cocoa content made high palatable pellets much more palatable than standard pellets. Finally, we also used a high caloric fat-enriched formula (14% protein, 60% fat, 26% carbohydrate, with a caloric value of 5.32 kcal/g).

These pellets were presented only during the operant behavior sessions. Otherwise, animals were maintained on standard chow for their daily food intake.

2.4. Tissue preparation and immunofluorescence

Immunoflurorescence studies were performed as previously described (Bertran-Gonzalez et al., 2008). Briefly, thirty min after the beginning of the last training session, mice were deeply anesthetized by intraperitoneal injection (0.2 ml/10 g body weight) of a mixture of ketamine (100 mg/kg) and xylazine (20 mg/kg) prior to rapid intracardiac perfusion with 120 ml of 4 % PFA in 0.1 M PBS, pH 7.5, delivered with a peristaltic pump at 30 ml/min for 4 min. Brains were post-fixed overnight in the same solution and stored at 4°C. Thirty µm-thick sections were cut with a vibratome (Leica, VT 1000 S, Nussloch, Germany) and stored at -20°C in a solution containing 30 % (vol/vol) ethylene glycol, 30 % (vol/vol) glycerol and 0.1 M sodium phosphate buffer, until they were processed for immunofluorescence. Brain regions were identified using a mouse brain atlas (Paxinos and Franklin, 2001) as described above, and sections equivalent to the following bregma coordinates were taken (mm): 1.80 for mPFC and 1.32 for NAc. Free-floating sections were rinsed in Tris-buffered saline (TBS; 0.25 M Tris and 0.5 M NaCl, pH 7.5), incubated for 5 min in TBS containing 3 % H_2O_2 and 10 % methanol, and then rinsed three times for 10 min each in TBS. After 20 min incubation in 0.2 % Triton X-100 in TBS, sections were rinsed three times in TBS again. Finally, they were incubated overnight at 4°C with the different primary antibodies. For detection of phosphorylated proteins, 0.1 mM NaF was included in all buffers and incubation solutions. Phosphorylation of several proteins was analyzed using rabbit polyclonal antibodies against phospho-Thr202/Tyr204-ERK1/2 (1:400, Cell Signalling Technology, Beverly, MA), phospho-Ser235/236-S6 (1:500, Cell Signaling

Technology, Beverly, MA) and phospho-Ser10- H3 (1:500, Upstate Ltd., UK). In the double labeling experiments, polyclonal antibodies against diphospho-Thr-202/Tyr-204-ERK1/2 (1:400, Cell Signaling Technology, Beverly, MA), phospho-Ser235/236-S6 (1:500, Cell Signaling Technology, Beverly, MA) and phospho-Ser10-H3 (1:500, Upstate Ltd., UK) were used in combination with a monoclonal antibody against dopamine- and cAMP-regulated phosphoprotein Mr~32,000 (DARPP-32, 1:1000, gift from Dr. Paul Greengard, The Rockefeller University, NY, USA) or against tyrosine hydroxylase (1:1000, Chemicon International, Temecula, CA, USA). NeuN was detected by a specific antibody (1:1000, Chemicon International, Temecula, CA, USA). Following incubation with the primary antibodies, sections were rinsed three times for 10 min in TBS and incubated for 45 min with goat Cy3- or Cy2-coupled (1:400, Jackson Lab) secondary antibodies. Sections were rinsed for 10 min twice in TBS and twice in Tris-buffered (TB, 0.25 M Tris) before mounting in 1, 4 – diazabicyclo-[2. 2. 2]-octane (DABCO, Sigma).

2.5. Immunofluorescence Analysis

Single- and double-labeled images from each region of interest were obtained bilaterally using sequential laser scanning confocal microscopy (Zeiss LSM 510, Germany). Neuronal quantification was performed by an observer unaware of genotype and kind of food by counting Cy2- or Cy3- immunoreactive cells immunofluorescence for each marker analyzed. For the mPFC, 2 photos/animal were taken at 20x. For the NAc core, 4 photos/animal were obtained at 40x. For the NAc shell, 6 photos/animal were taken at 40x.

2.6. Immunoblot Analysis

To verify that CB1+/+ and CB1-/- present the same total ERK-positive cells in the NAc shell, NAc shell were extracted to perform an immunoblot analysis. Frozen NAc shell tissues were processed as previously reported (Puighermanal et al., 2009). For immunoblotting, we used antibodies to ERK (1:5000, Cell Signaling Technology, Beverly, MA). Blots containing equal amounts of NAc protein samples (10 μg of protein) to compare in each specific experiment were cut horizontally. Bound primary antibodies were detected with horseradish peroxidase–conjugated antibodies to rabbit or mouse antibodies (Pierce, diluted 1:5.000) and visualized by enhanced chemiluminescence detection (West-Femto-SuperSignal, Pierce). The optical density of the immunoreactive bands was quantified after acquisition on a ChemiDoc XRS System (Bio-Rad) controlled by The Quantity One software v4.6.3 (Bio-Rad). Representative cropped immunoblots for display were processed with Adobe Photoshop 7.0. For quantitative purposes, the optical density values of the specific antibodies were normalized to the detection of specific antibodies to GAPDH values in the same sample and expressed as a percentage of control treatment.

2.7. Statistical analysis

Immunosfluorescent data were analyzed by a two-way ANOVA with genotype and kind of food as between subject factors of variation, followed by corresponding post-hoc analysis when required (Dunnett's test). An additional one-way ANOVA between subjects was used to compare yoked CB1+/+ receiving high palatable food non-contingently with other groups of CB1+/+ followed by the corresponding post-hoc Dunnett's analysis when required. Immunoblot data were analyzed by one-way

ANOVA between subjects. All data were analyzed with SPSS software and are expressed as mean ± SEM. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Operant training to obtain palatable food enhanced ERK phosphorylation in the NAc shell and mPFC

We have demonstrated that operant training during 41 days to obtain high palatable food produced behavioral alterations and structural plasticity changes in the NAc shell and mPFC in mice that resemble those observed during drug addiction (Guegan et al., back to back publication). These behavioral and neuroplastic changes were dependent on CB1-R activation and were not observed in animals trained to obtain standard or high caloric food under similar experimental conditions (Guegan et al., back to back publication). The ERK pathway participates in the molecular mechanisms underlying synaptic plasticity during drug addiction (Girault et al., 2007). We then examined the phosphorylation of ERK in CB1+/+ and CB1-/- trained to lever-press to obtain standard, high caloric or high palatable food (Figs. 1 and 5). Single-fluorescence analysis of the brain slices of mice sacrificed and perfused 30 min after the beginning of the last training session revealed that p-ERK is strongly increased in the NAc shell and mPFC, but not in the NAc core, in CB1+/+ trained to obtain high palatable pellets compared with mice that worked for standard pellets (Figs. 1A, B, C and D and 5A) or that received high palatable pellets non-contingently (Fig. 6A). Moreover, no differences in p-ERK in any of the brain areas studied were observed between CB1+/+ trained to lever-press for standard pellets and their yoked-standard counterparts (Fig. 6A). These data demonstrate that the operant training and associated locomotor responses were not responsible for the activation of p-ERK observed in CB1+/+ (Fig. 1). p-ERK was detected in neuronal nuclei and perikarya as well as surrounding neuropil (Fig. 1A and B). Moreover, p-ERK immunoreactivity was found in the medium-sized spiny neurons as shown by the co-localization with DARPP-32, a specific marker of these neurons

(Fig. 4A). In contrast, no p-ERK immunoreactivity was found in dopaminergic terminals identified with a tyrosine hydroxylase antibody (Fig. 4B). Phosphorylation of ERK induced by training to obtain high palatable pellets in the NAc shell and mPFC was not observed in CB1-/- (Figs 1B, C and D and 5A). Operant behavior to obtain high-fat content pellets also produced a significant increase in ERK phosphorylation in the NAc shell and mPFC of CB1+/+, but not of CB1-/- (Figs 1A, B, C and D and 5A).

3.2. CB1+/+ and CB1-/- present the same neuronal content and ERK levels in the NAc shell and core and mPFC

Possible differences in between genotypes in their neuronal content or in their total levels of ERK protein in the NAc shell and core and mPFC could account for the modifications in neuroplasticy and behavior observed in CB1-/- when compared to CB1+/+ (see Guegan et al., back to back publication). To discard these possibilities, we also evaluated the total ERK and neuronal contents in all three brain areas studied. Western-blot analysis revealed no differences in the total ERK content between genotypes (Fig. 7A). Moreover, immunofluorescence analysis showed no differences in the total amount of NeuN positive cells in CB1+/+ when compared to CB1-/-. These data excludes the possibility that neuronal deficits or a diminish level of activation of ERK signaling cascade could explain the differences in the behavioral responses or neuroplasticity between genotypes.

3.3. Operant training to obtain palatable food induces histone H3 and ribosomal subunit S6 phosphorylation in the NAc shell and mPFC

To further characterize molecular events involved in neuronal plasticity induced by palatable food, we next studied the activation of two downstream targets of the ERK pathway, histone H3, an important component of the nucleosomal response (Stipanovich et al., 2008), and the ribosomal protein S6 (rS6), a component of the small 40S ribosomal subunit implicated in protein translation (Ferrari and Thomas, 1994). The phosphorylation of histone H3 and rS6 was strongly increased in the NAc shell of CB1+/+ trained with high palatable pellets, but not with standard or high-fat pellets (Figs. 2A and C and 3A and C). Phospo-H3 and p-rS6 occurred selectively in medium spiny neurons of the NAc shell as demonstrated by the colocalization with DARPP-32 (Fig. 4C and E). Moreover, double immunolabeling of p-ERK/p-H3 or p-ERK/p-rS6 demonstrated an almost complete co-localization in the NAc (Fig. 4D and F). Operant behavior to obtain palatable food, but not other kinds of food, strongly increased rS6 phosphorylation in the mPFC (Fig. 5C). No change in p-H3 was observed in the mPFC after training with palatable food (Fig. 5B). No modification in p-H3 (Fig. 2B and D) and a slightly enhanced p-rS6 (Fig. 3B and D) was observed in the NAc core of mice trained with palatable food. All these changes in histone H3 and rS6 phosphorylation in the NAc shell and mPFC were also prevented in CB1-/- further supporting a causal relationship between ERK activation and the behavioral and neuroplastic changes promoted by palatable food operant training through CB1-R activity. As for ERK, histone H3 and rS6 phosphorylation requires contingent association between the operant behavior and the delivery of the high palatable food, because no effects were observed in non-contingent high palatable yoked group (Fig. 6A). Moreover, no differences in p-H3 and p-rS6 in any of the brain areas studied were observed between CB1+/+ trained

to lever-press for standard pellets and their yoked-standard counterparts (Fig. 6A). These data demonstrate that operant training and associated locomotor responses were not responsible for the activation of p-H3 or p-rS6 observed in CB1+/+ (Fig. 1).

3.4. Changes in ERK intracellular pathway activation were absent in CB1+/+ exposed to the operant training for a short period

We have reported that a short operant training period of 10 days to obtain standard, high caloric or high palatable food did not produce changes in structural plasticity in the NAc shell and mPFC (Guegan et al., back to back). To further characterize the possible relationships between the changes promoted by palatable food operant training and activation of the ERK signaling cascade, we have evaluated the phosphorylation of ERK, H3 and rS6 in CB1+/+ exposed to operant training to obtain standard, high caloric or high palatable food during this short operant training period of 10 days. Interestingly, the changes promoted by operant training to obtain palatable food during 41 days in p-ERK, p-H3 and p-rS6 were not revealed in any of the brain areas investigated in mice exposed to this short period of training of 10 days. Indeed, this short operant training only produced a minor increase in the phosphorylation of H3 in the NAc core and mPFC (Fig. 6B). No changes in the phosphorylation of ERK, H3 or rS6 were revealed in mice trained during this short period to obtain standard or high caloric food in any brain area studied (Fig. 6B). In agreement with our previous results in structural plasticity, these data suggest that changes in ERK signaling pathway activation also require a long period of operant training with palatable food.

4. Discussion

In this study, we revealed for the first time that repeated operant training to obtain palatable food increases activation of the ERK intracellular cascade in specific structures of the corticolimbic system that have been previously related to the behavioral and plasticity alterations promoted by drugs of abuse. Most of these changes in the ERK pathway were selectively produced by palatable food operant training since they were absent in mice trained to obtain standard food or passively receiving palatable food (yoked mice), whereas only moderate changes were revealed in mice trained to obtain high caloric food under similar experimental conditions. We have previously reported that this operant training for palatable food, but not for standard or high caloric food, produces behavioral alterations related to impulsivity and binge-eating disorders and structural plasticity changes in the same corticolimbic structures. Using genetic and pharmacological approaches, we have also demonstrated that CB1-R are necessary to produce these behavioral, morphological and biochemical changes induced by reinforced learning with palatable food (Guegan et al., back to back publication).

Structural plasticity is induced by long-lasting dendritic remodeling that is thought to reflect strong actin cytoskeleton rearrangements, which are controlled by various intracellular signaling pathways including the ERK cascade (Goldin and Segal, 2003). Persistent structural changes induced by palatable food operant training (Guegan et al., back to back publication) were accompanied by a robust ERK activation in the NAc shell and mPFC. ERK activation, as well as the behavioral and morphological changes (Guegan et al., back to back publication) required contingent association between the operant behavior and the delivery of the reward (Fig. 6A). Moreover, CB1-R activation was also necessary, because these changes were absent in the CB1-/-. No changes in total ERK nor in neuronal density were observed between CB1+/+ and CB1-/-,

excluding possible neuronal deficits or a diminish level of activation of the ERK signaling cascade in the CB1-/- that could explain the differences after the operant training (Fig. 7). Moreover, the operant training by self and the associated motor responses do not seem to participate in the changes in ERK activity revealed in CB1+/+ since these behavioral responses were not correlated with the modifications in ERK activation in CB1+/+ . Indeed, standard food yoked CB1+/+ presented the same p-ERK activation in the NAc shell, core and mPFC as their master-control mice, in spite of the different operant performance of these two groups (see Guegan et al., back to back publication).

These results reveal the involvement of the ERK pathway in the neuronal adaptive changes promoted by palatable food operant training through the activation of CB1-R. Drugs of abuse exposure also increase ERK activation in the NAc by a mechanism dependent of the endocannabinoid system (Corbille et al., 2007; Valjent et al., 2004). Moreover, blockade of ERK interferes with long-lasting behavioral changes induced by drugs of abuse (Girault et al., 2007) and cue-elicited reward-seeking has been reported to require the activation of ERK in the NAc (Shiflett et al., 2008). Thus, the present results reveal the important role of ERK in the NAc in the generation of maladaptive responses in the brain reward system produced in this behavioral model by a natural reward.

The phosphorylation of ERK in the NAc shell in mice trained with palatable food was accompanied by increased phosphorylation of histone H3, an important component of nucleosomal response, suggesting that transcriptional mechanisms are involved in the neuroplastic changes promoted by ERK. ERK-dependent phosphorylation of histone H3 regulates specific loci of particular genes encoding transcription factors after cocaine exposure (Brami-Cherrier et al., 2005). Furthermore, several candidates downstream

from the transcriptional regulator ΔFosB, which is regulated by ERK, have been proposed to mediate drug-induced dendritic remodeling in the NAc. Interestingly, a recent study has described the involvement of ΔFosB in regulating responses to natural rewards. Thus, overexpression of ΔFosB in the NAc increases sucrose intake and promotes sexual behavior in rats and it is involved in the development of cross-sensitization between different types of rewards. In this study, the authors also suggest the possibility that Δ-fosB induction in the NAc may mediate not only key aspects of drug addiction, but also in the development of natural addictions involving compulsive consumption of natural rewards (Wallace et al., 2008). Among ΔFosB-regulated downstream effectors, the cdk5-MEF2 module and the transcription factor NFκB have been shown to regulate structural plasticity induced in the NAc by cocaine (Russo et al., 2010; Pulipparacharuvil et al., 2008).

Further studies will be necessary to identify whether similar transcriptional regulations could mediate drug- and food-induced structural plasticity.

Operant training with palatable food promotes ERK phosphorylation not only in the cell nucleus, but also at dendritic and cytosolic level, suggesting that ERK could also regulate structural plasticity via non-transcriptional mechanisms. In agreement, ERK pathway has been reported to regulate glutamate receptors insertion at synapses or local dendritic protein synthesis, two molecular events involved in dendritic remodeling (Liu et al., 2010). In the present study, ERK activation was accompanied by concomitant phosphorylation of the ribosomal protein S6, an important component of the translational machinery (Roux et al., 2007). Increased S6 phosphorylation may therefore participate in the morphological rearrangements induced at dendritic level by palatable food by controlling several scaffolding proteins associated with the postsynaptic cytoskeleton (Yang et al., 2008). Our findings can not rule out the possible involvement

of other intracellular signaling systems in the changes induced by palatable food operant training in structural plasticity in the corticolimbic system. Indeed, the two downstream proteins of ERK analyzed in this study, p-H3 and p-rS6, are also modulated by other intracellular systems. Thus, the Akt/mTOR intracellular pathway is a direct regulator of the activity of rS6 and participates in the modulation of H3 (Dobashi et al., 2011). In addition, several studies have shown the involvement of this signaling cascade in neuroplasticity (Hoeffer and Klann, 2010) and in the responses induced by the activation of CB1-R (Puighermanal et al., 2009). Further studies will be required to evaluate the possible participation of mTOR in the behavioral and neuroplastic changes promoted by this operant training.

In conclusion, we reveal that repeated operant training to obtain palatable food is associated to the activation of several components of the ERK signaling pathway through CB1-R in restricted areas of the brain reward system. This operant training has been reported to induce behavioral alterations related to addictive-like behavior as well as neuroplastic changes the same brain reward areas (Guegan et al., back to back publication). Taken all these data into consideration, we can postulate a mechanism to explain the involvement of CB1-R in these neuronal adaptive changes promoted by palatable food operant training (Fig. 8). Similar morphological and neurochemical changes have been shown in these brain structures after exposure to drugs of abuse, which play a crucial role in addictive behavior. Therefore, these neural changes induced by palatable food could represent a biological substrate to explain the alterations in food-seeking behavior that promote obesity and eating-related disorders. Our results also provide an important advance in the understanding of the common links between eating disorders and drug addiction.

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FIGURE LEGENDS

Figure 1 Operant training to obtain highly palatable food strongly induces ERK phosphorylation in CB1+/+ in the NAc shell but not in the NAc core. (**A**) Illustration p-ERK in the NAc shell of mice trained for standard, high palatable or high fat pellets. (**B**) Illustration p-ERK in the NAc core of mice trained for standard, high palatable or high fat pellets. (**C**) p-ERK positive cells in the NAc shell and (**D**) NAc core of CB1+/+ and CB1-/- trained for standard, fat-enriched or high palatable pellets (n=5-9 mice per experimental group). Data are expressed as mean ± s.e.m. # $p < 0.05$, # # $p < 0.01$, # # # $p < 0.001$ (CB1+/+ vs CB1–/–);.★ $p < 0.05$, ★★★ $p < 0.001$ (comparison vs standard food).

Figure 2 Operant training to obtain high palatable food strongly increases H3 phosphoylation in CB1+/+ in the NAc shell but not in the NAc core. (**A**) Illustration p-H3 in the NAc shell of mice trained for standard, high palatable or high fat pellets. (**B**) Illustration p-H3 in the NAc core of mice trained for standard, high palatable or high fat pellets. (**C**) p-H3 positive cells in the NAc shell and (**D**) NAc core of CB1+/+ and CB1-/- trained for standard, fat-enriched or high palatable pellets (n=5-8 mice per experimental group). Data are expressed as mean ± s.em. # # # $p < 0.001$ (CB1+/+ vs CB1–/–);★★ ★ $p < 0.001$ (comparison vs standard food).

Figure 3 Operant training to obtain high palatable food strongly increases rS6 phosphorylation in the NAc, mainly in the shell subregion in CB1+/+ mice. (**A**) Illustration p-rS6 in the NAc shell of mice trained for standard, high palatable or high fat pellets. (**B**) Illustration p-rS6 in the NAc core of mice trained for standard, high

palatable or high fat pellets. (**C**) p-rS6 positive cells in the NAc shell and (**D**) NAc core of CB1+/+ and CB1-/- trained for standard, fat-enriched or high palatable pellets (n=5-8 mice per experimental group). Data are expressed as mean ± s.e.m. # $p < 0.05$, # # $p < 0.01$ (CB1+/+ vs CB1–/–); ★★ $p < 0.01$, ★★★ $p < 0.001$ (comparison vs standard food).

Figure 4 Double immunostaining in the NAc showing co-localization of p-ERK, p-H3 and p-rS6 in medium spiny neurons. (**A**) Illustration of p-ERK with DARPP-32 double immunostaining showing that ERK phosphorylation occurs specifically in medium spiny neurons. (**B**) Illustration of p-ERK with tyrosin-hydroxylase double immunostaining showing that ERK phosphorylation does not occur in dopaminergic terminals. (**C**) Illustration of p-H3 with DARPP-32 double immunostaining showing that H3 phosphorylation occurs specifically in medium spiny neurons (**D**) Illustration of p-H3 with p-ERK double immunostaining showing co-localization of both proteins. (**E**) Illustration of p-rS6 with DARPP-32 double immunostaining showing that p-rS6 occurs specifically in medium spiny neurons (**F**) Illustration of p-rS6 with p-ERK double immunostaining showing co-localization of both proteins.

Figure 5 Operant training to obtain highly palatable food strongly induces ERK intracellular pathway activation in CB1+/+ in the mPFC. (**A**) p-ERK positive cells in the mPFC of CB1+/+ and CB1-/- trained for standard, fat-enriched or high palatable pellets. (**B**) p-H3 positive cells in the mPFC of CB1+/+ and CB1-/- trained for standard, fat-enriched or high palatable pellets (**C**) p-rS6 positive cells in the mPFC of CB1+/+ and CB1-/- trained for standard, fat-enriched or high palatable pellets CB1+/+ (n=5-9

mice per experimental group). Data are expressed as mean ± s.e.m. # # p < 0.01, # # # p < 0.001 (CB1+/+ vs CB1–/–); ★★★ p < 0.001 (comparison vs standard food).

Figure 6 Molecular adaptations induced by palatable food are dependent on active operant goal-directed behavior learning and occurs only after long-term training. (**A**) Number of p-ERK, pH3 and p-rS6 positive cells in the NAc shell and core, and mPFC of CB1+/+ trained for standard or high palatable pellets and yoked control groups receiving passively standard or high palatable pellets. (B) Number of p-ERK, pH3 and p-rS6 positive cells in the NAc shell and core, and mPFC of CB1+/+ trained for standard, fat-enriched or high palatable pellets for short operant training (10 days). (n=5-9 mice per experimental group). Data are expressed as mean ± s.e.m. ★★ p < 0.01, ★★★ p < 0.001 (contingent high palatable vs yoked high palatable), ★ p < 0.05 (comparison vs standard food).

Figure 7 CB1+/+ and CB1-/- present the same amount of neurons and total ERK in the NAc and mPFC. (A) Western-blot quantification showing total ERK levels in the NAc and mPFC of CB1+/+ and CB1-/-.(B) Immunofluorescence analysis showing NeuN positive cells in the NAc and mPFC of CB1+/+ and CB1-/-.

Figure 8 Hypothetical mechanism to explain the mechanism involved in the adaptive changes promoted by palatable food operant training.

The schema represents a tentative tripartite synapse in the NAc shell among glutamatergic, GABAergic and dopaminergic synapses. Post-synaptic GABAergic medium spiny neurons receive glutamatergic, dopaminergic and GABAergic inputs. Both, glutamatergic and GABAergic presynaptic terminals contain CB1-R, and

subsequently are regulated by endocannabinoids (eCBs). Under physiological conditions, eCBs exert a regulatory modulation of glutamate and GABA release, and subsequently modulate the activation of ERK and downstream signaling cascade at the postsynaptic level (CB1+/+ trained for standard food).

However, when CB1+/+ are long-term trained with high palatable pellets, the rewarding effects induced by this kind of food promote an enhanced dopamine release, together with a possible unbalance between GABA and glutamate release. This unbalance would be the result of the increase of eCBs release at the synapse, which mainly inhibits GABA release, and subsequently lead to a predominant glutamatergic tone. These changes are followed by an increase in the phosphorylation levels of ERK and the subsequent activation of the translational and transcriptional machinery that contribute to the changes in structural plasticity induced by this operant training for palatable food.

Our results show that a down-regulation of CB1-R in the NAc is sufficient to diminish the behavioral and neuroplastic changes promoted by palatable food. We suggest that the inhibitory effects of the eCBs mainly on GABA release are diminished or abolished in CB1-/- or in CB1+/+ treated with AAV9-shCB1. This decrease in CB1-R activity would return the balance between GABAergic and glutamatergic tone to the physiological conditions in spite of the palatable operant training, which leads to a normalization of ERK levels and the downstream signaling cascade. Therefore, no modifications in structural plasticity are observed under these experimental conditions after palatable food operant training.