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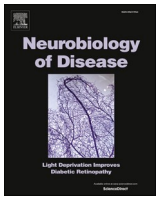

# Role of CB2 cannabinoid receptor in the development of food addiction in male mice

A. García-Blanco [a,1], Á. Ramírez-López [a,1], F. Navarrete [b,c,d], M.S. García-Gutiérrez [b,c,d], J. Manzanares [b,c,d], E. Martín-García [a,e,f,*,2], R. Maldonado [a,e,*,2]

[a] Laboratory of Neuropharmacology-Neurophar, Department of Medicine and Life Sciences, Universitat Pompeu Fabra (UPF), Barcelona, Spain
[b] Neurosciences Institute, University Miguel Hernández-CSIC, Avda de Ramón y Cajal s/n, San Juan de Alicante, Alicante 03550, Spain
[c] Research Network in Primary Care of Addictions, Health Institute Carlos III, MICINN and FEDER, Madrid 28029, Spain
[d] Instituto de Investigación Sanitaria y Biomédica de Alicante (ISABIAL), Alicante, Spain
[e] Hospital del Mar Medical Research Institute (IMIM), Barcelona, Spain
[f] Departament de Psicobiologia i Metodologia de les Ciències de la Salut, Universitat Autònoma de Barcelona (UAB), Cerdanyola del Vallès, Barcelona, Spain

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ABSTRACT

The endocannabinoid system plays an important role in multiple behavioral responses due to its wide distribution in the central nervous system. The cannabinoid CB1 receptor was associated to the loss of behavioral control over food intake occurring during food addiction. The cannabinoid CB2 receptor (CB2R) is expressed in brain areas canonically associated with addictive-like behavior and was linked to drug-addictive properties. In this study, we evaluated for the first time the specific role of the CB2R in food addiction by using a well-validated operant mouse model of long-term training to obtain highly palatable food. We have compared in this model the behavioral responses of wild-type mice, mutant mice constitutively lacking CB2R, and transgenic mice overexpressing CB2R. The lack of CB2R constitutes a protective factor for the development of food addiction and the impulsive and depressive-like behavior associated. In contrast, the overexpression of CB2R induces a vulnerable phenotype toward food addiction after long-term exposure to highly palatable chocolate pellets. Relevant transcriptomic changes were associated to resilience and vulnerability to food addiction depending on the genotype, which provides a mechanistic explanation for these behavioral changes. Therefore, CB2R may constitute a potential therapeutic target for the loss of eating control and the comorbid emotional effects associated to food addiction.

## 1. Introduction

Food addiction is a multifactorial disorder characterized by loss of control over food intake (Randolph, 1956). The concept of food addiction is still controversial, and according to a well-accepted diagnosis tool, the Yale Food Addiction Scale 2.0 (Gearhardt et al., 2016), food addiction affects from 2% to 12% of healthy body mass index individuals. The prevalence rises among people suffering from obesity (18–24%), and eating disorders (50%), reaching the highest value in bulimia nervosa (85%) (Fernandez-Aranda et al., 2018). The exposure and accessibility to food with high palatability and caloric content in western society are leading to an increase of the socio-economic burden associated with food addiction, impaired by the lack of effective treatments. Therefore, there is a need to understand the neurobiological mechanisms underlying food addiction in order to identify novel possible therapeutic approaches.

Food intake is regulated by homeostatic mechanisms that control energy intake and expenditure to maintain a metabolic balance (Onaolapo and Onaolapo, 2018). In parallel, allostatic mechanisms control food intake, regardless of energetic requirements, through the reward circuits under the influence of food palatability and environmental factors, among others (Caron and Richard, 2017). Both the homeostatic

* Corresponding authors at: Laboratory of Neuropharmacology-Neurophar, Department of Medicine and Life Sciences, Universitat Pompeu Fabra (UPF), Barcelona, Spain.
*E-mail addresses:* elena.martin@upf.edu (E. Martín-García), rafael.maldonado@upf.edu (R. Maldonado).
[1] These authors contributed equally.
[2] These authors equally supervised this work.

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and the allostatic systems regulate food intake, although in pathological conditions, the hedonic system can override the homeostatic control (Caron and Richard, 2017). The hypothalamus is the main brain area involved in the homeostatic control, whereas the allostatic mechanisms are mainly under the control of the mesocorticolimbic system, which includes the ventral tegmental area (VTA) and its projections to the nucleus accumbens (NAc) and cortical areas including the medial prefrontal cortex (mPFC). Misbalances in this brain reward system are commonly shared across food and drug addiction (Maldonado et al., 2021). Common neuroanatomical and neurophysiological alterations have been proposed to underlie the neurobiological substrate of both disorders that share multiple behavioral alterations including loss of control over intake, enhanced compulsivity and motivation, and altered reward sensitivity.

Genetically modified mouse models lacking (CB2KO) or overexpressing CB2R (CB2xP) have allowed important advances in understanding the physiological role of this receptor (García-Gutiérrez et al., 2010; García-Gutiérrez and Manzanares, 2011; Navarro et al., 2022). Previous studies reported reduced cocaine-reinforcing effects in CB2xP mice (Aracil-Fernández et al., 2012) and in WT mice receiving a CB2R agonist (Xi et al., 2011), although CB2R antagonists also decreased cocaine-reinforcing effects (Adamczyk et al., 2012). Alcohol-reinforcing effects were enhanced in CB2KO mice (Ortega-Álvaro et al., 2015), although alcohol consumption was also increased by CB2R agonists (Onaivi et al., 2008). CB2KO mice showed protection against nicotine-reinforcing effects and withdrawal (Ignatowska-Jankowska et al., 2013; Navarrete et al., 2013) and the same effects were obtained by CB2R antagonist (Ignatowska-Jankowska et al., 2013; Navarrete et al., 2013) linking CB2R with increased vulnerability to nicotine abuse. Therefore, the modulation of CB2R produces differential effects on the addictive properties of drugs depending on the model and the substance of abuse under study. Targeting CB2R for addictive processes represents an interesting approach considering the absence of several centrally mediated side effects that have limited the use of CB1R ligands, such as psychoactive, motor, and cognitive side effects (Cabañero et al., 2021a).

This study aims to elucidate the involvement of CB2R in food addiction. We hypothesize that the modification in the expression of CB2 receptors will impact the development of food addiction. For this purpose, we used genetically modified mice either lacking or overexpressing CB2R trained in a well-established operant food addiction model that evaluates 3 addiction criteria and 4 addiction-related phenotypic traits. At the end of the operant protocol, mice were evaluated for anxiety and depression-like behavior. Several genes of interest were also investigated in different brain areas involved in food intake control and reward. The results obtained support the relevance of the endocannabinoid system in food addiction and highlight the role of CB2R as a novel potential therapeutic target for this disorder.

## 2. Methods

### 2.1. Animals

CD1 wild-type (WT) mice ($n = 42$) were purchased from Charles River (France). Transgenic mice overexpressing cannabinoid receptor 2 (CB2xP) ($n = 28$) and cannabinoid receptor 2 knockout (CB2KO) mice ($n = 25$) were kindly supplied by J. Manzanares laboratory (Instituto de Neurociencias, Universidad Miguel Hernández-CSIC Alicante, Spain). Homozygote CB2KO mice were initially generated on a C57BL/6 J congenic background (provided by Nancy E. Buckley, Cal State Polytechnic University, Pomona, CA, USA), and the CB2KO founders were crossed with outbred CD1 (Charles River, France) background (Buckley et al., 2000) for eight generations. Mice overexpressing CB2R were on a CD1 congenic background (Racz et al., 2008b). All the mice were male, two months old and weighed 40 ± 3 g at the beginning of the experiment. The male sex was chosen considering the previous literature that has validated the operant food addiction model only in male, but not in female mice (Domingo-Rodriguez et al., 2020; García-Blanco et al., 2022; Mancino et al., 2015; Martín-García et al., 2020). All the behavioral experiments were conducted in the animal facility at Universitat Pompeu Fabra-Barcelona Biomedical Research Park (UPF-PRBB; Barcelona, Spain). Mice were housed individually and maintained in a controlled temperature (21 ± 1 °C) and humidity (55 ± 10%) with food and water available ad libitum. All the experiments were performed during the dark phase of a reverse light/dark cycle (light on at 8:00 pm, light off at 8:00 am). All behavioral experiments were approved by the local ethical committee (Comitè Ètic d'Experimentació Animal-Parc de Recerca Biomèdica de Barcelona) and were performed in accordance with the European Communities Council Directive (2010/63/EU). All the experiments were performed under blind and randomized conditions.

CB2 receptor overexpression in CB2xP mice was previously described in detail (García-Gutiérrez et al., 2010). These studies showed the comparative CB2R relative gene expression profile between WT and CB2xP mice in several brain regions by real-time PCR. CB2R was significantly overexpressed in the caudate putamen (CPu), nucleus accumbens (NAcc), cingulate cortex (Cg Ctx), amygdala (Amy), hippocampus (Hipp; CA2, CA3, and DG subregions), ventromedial nucleus (VMN), arcuate nucleus (Arc), substantia nigra (SN), ventral tegmental area (VTA), and dorsal and medial raphe (DR and MnR, respectively). In addition, the cell type distribution of CB2 receptor in CB2xP was also previously described (Racz et al., 2008a, 2008b; Aracil-Fernández et al., 2012). The expression of transgenic CB2R was pronounced in both microglial cells and neurons of CB2xP (Racz et al., 2008a). Finally, (Aracil-Fernández et al., 2012) immunohistochemistry studies revealed that CB2R is overexpressed in neurons and astrocytes of CB2xP mice in the NAcc and VTA (Aracil-Fernández et al., 2012).

### 2.2. Operant behavior apparatus

Operant responding maintained by chocolate-flavored pellets was performed in mouse operant chambers (Model ENV-307A-CT, Med Associates, Georgia, VT, USA). The chambers were equipped with two retractable levers, one randomly assigned as the active lever and the other as the inactive for the entire experimental protocol. Pressing on the active lever resulted in a food pellet delivery paired with a stimulus-light (cue-light) located above the active lever, whereas pressing on the inactive lever had no consequences. A food dispenser equidistant between the two levers allows the delivery of food pellets when pertinent. The operant chambers were made of aluminum and acrylic and were housed inside soundproof boxes equipped with fans to provide ventilation and white noise. The chambers' floor consists of a metal sheet with holes. The floor was changed in the shock test sessions by a grid floor made of metal bars able to conduct electrical current, which was also used as a contextual cue for the aversive cue reactivity test the day after the shock test allowing mice to discriminate between different contexts.

### 2.3. Food pellets

During the operant conditioning sessions, animals received after pressing the active lever a 20 mg chocolate-flavored pellet consisting in a highly palatable isocaloric food (TestDiet, Richmond, IN, USA). These pellets had a similar caloric value (3.44 kcal/g: 20.6% protein, 12.7% fat, 66.7% carbohydrate) to the standard maintenance diet provided to mice in their home cage (3.52 kcal/g: 17.5% protein, 7.5% fat, 75% carbohydrate) with some slight differences in their composition: chocolate flavor (2% pure unsweetened cocoa) and enhanced sucrose content (8.3% standard diet food vs 50.1% highly palatable pellets). These pellets were presented only during the operant behavior sessions, and animals were maintained on standard chow for their daily food intake.

### 2.4. Experimental design

#### 2.4.1. Operant training

A total of 95 mice were trained for 118 days to obtain chocolate-flavored pellets. In the operant conditioning sessions, mice were under an FR1 schedule of reinforcement for 6 days (1 lever-press resulted in 1 pellet delivery) followed by 112 days of FR5 (5 lever-presses resulted in 1 pellet delivery) (Fig. 1a). The beginning of each session was signaled by turning on a house light placed on the chamber's ceiling during the first 3 s. Daily operant training sessions maintained by chocolate-flavored pellets lasted 1 h and were composed of 2 pellet periods (25 min each) separated by a pellet-free period (10 min). During the pellet periods, pellets were delivered contingently after an active response paired with a stimulus light (cue light). A time-out period of 10 s was established after each pellet delivery, where the cue light was off, and no reinforcer was provided after responding on the active lever. Responses on the active and inactive lever performed during the time-out periods were recorded. In contrast, the pellet-free period was signaled by the illumination of the entire operant box, and no pellet was delivered after responding on any lever. Mice were returned to their home cages after each session.

As previously described (Martín-García et al., 2011), the operant response was acquired when all the following conditions were achieved: (1) mice maintained a stable response with <20% deviation from the mean of the total number of reinforcers earned in 3 consecutive sessions (80% of stability); (2) at least 70% responding on the active lever; and (3) a minimum of 10 reinforcers per session.

#### 2.4.2. Three addiction criteria

The food addiction criteria were evaluated at three different time points as previously described (García-Blanco et al., 2022): early (5–18 training days), middle (48–65 training days), and late (95–112 training days). The food addiction criteria gathered the main hallmarks of addiction based on DSM-IV (Deroche-Gamonet et al., 2004), DSM-5 and now included in the food addiction diagnosis through the YFAS 2.0 (Gearhardt et al., 2016) and therefore, are used to classify mice as nonaddicted (0–1 criteria) and addicted (2–3 criteria) (Fig. 1a).

Persistence of response: persistent desire or unsuccessful efforts to cut down displayed by continuous food-seeking behavior even if the food reward is signaled as not available. It is measured by the number of non-reinforced active responses during the pellet-free period (10 min) on the 3 consecutive days before the progressive ratio (PR).

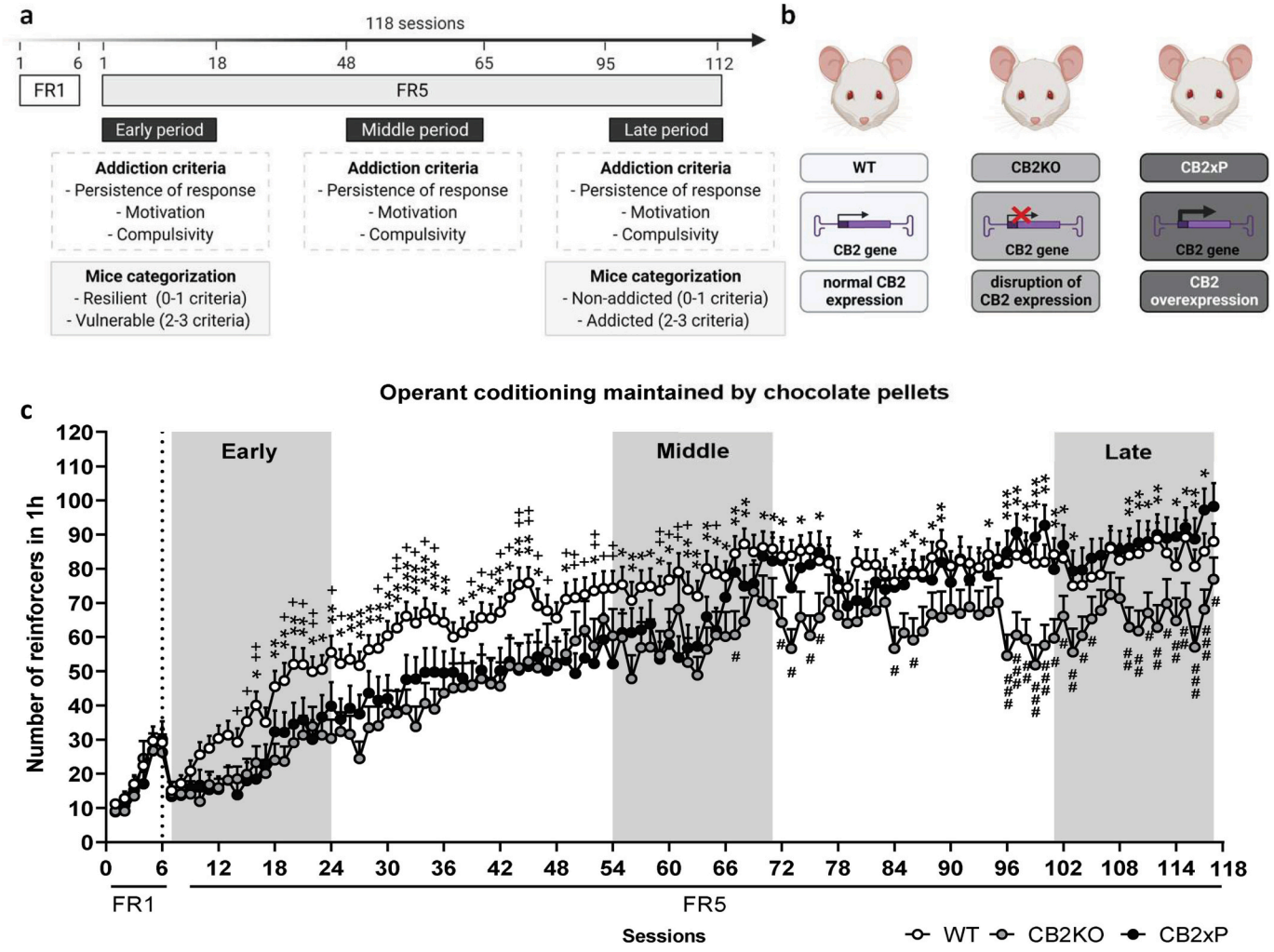


**Fig. 1.** The reinforcement for chocolate-flavored pellets was reduced in CB2R-lacking mutants suggesting a resistant phenotype. a Experimental timeline of the protocol. b Schematic representation of the three mice genotypes used in this study. WT mice that express CB2R constitutively, mutant mice that do not express CB2R (CB2KO) and mutant mice that overexpress CB2R (CB2xP). c Number of chocolate-flavor pellets obtained in each daily operant training session (repeated-measures ANOVA; session effect $p < 0.001$ and interaction genotype/session $p < 0.001$). In the early period, both CB2xP (*post-hoc* LSD test ++$p < 0.01$) and CB2KO (*post-hoc* LSD test ***$p < 0.001$) mice obtained less reinforcers than WT mice. CB2KO mice maintained this difference over the entire protocol (*post-hoc* LSD test WT vs CB2KO ***$p < 0.001$) while CB2xP mice reached the levels of WT mice in later stages of the protocol (*post-hoc* LSD test WT vs CB2xP NS) and therefore were different to CB2KO (*post-hoc* LSD test CB2KO vs CB2xP ###$p < 0.001$).

Motivation: considerable effort and time spent in obtaining the reward measured by the PR schedule of reinforcement. The response required to earn one single pellet escalated according to the following series: 1, 5, 12, 21, 33, 51, 75, 90, 120, 155, 180, 225, 260, 300, 350, 410, 465, 540, 630, 730, 850, 1000, 1200, 1500, 1800, 2100, 2400, 2700, 3000, 3400, 3800, 4200, 4600, 5000, and 5500. The maximal number of responses that the animal is willing to perform to obtain one pellet is referred to as the breaking point. The maximum duration of the PR session was 5 h or until mice did not respond on any lever during 1 h.

Compulsivity: continued use despite negative consequences evaluated as the resistance to punishment when chocolate-flavored pellets intake is coupled with an aversive stimulus. Mice were placed in an operant box without the metal sheet with holes and consequently with the grid floor exposed (contextual cue). During this session, mice underwent an FR5 schedule in which they received an electric foot-shock (0.18 mA, 2 s) after 4 responses and received another electric foot-shock (0.18 mA, 2 s) and a pellet paired with the cue light after the 5th response. The schedule was reinitiated after time-out period (10 s after pellet delivery) and after the fourth response if mice did not perform the fifth response within 60 s. The total number of shocks performed in 50 min was used to evaluate compulsivity-like behavior, previously described as resistance to punishment (Deroche-Gamonet et al., 2004; Mancino et al., 2015).

#### 2.4.3. Establishment of mice subpopulations

After performing the three behavioral tests to measure the food addiction behavior, mice were categorized as food addicted or non-addicted depending on the number of positive criteria they achieved. A mouse was considered positive for a particular addiction criterion when the score of the specific behavioral test was above the 75th percentile of the normal distribution of the WT control group. Mice that achieved 2 or 3 addiction criteria were considered addicted animals, and mice that achieved 0 or 1 addiction criteria were considered nonaddicted animals, as previously reported (Domingo-Rodriguez et al., 2020; Mancino et al., 2015).

#### 2.4.4. Behavioral tests to evaluate addiction-like phenotypic traits

Four additional phenotypic traits were also evaluated as factors of vulnerability to addiction in each period (early, middle and late):

Impulsivity: Considered as motor disinhibition, is defined as the inability to stop a response once it is initiated (Dalley et al., 2011). Impulsivity was measured as the number of non-reinforced active responses during the time-out period (10 s) after each pellet delivery, as previously described (Domingo-Rodriguez et al., 2022; García-Blanco et al., 2022; Mancino et al., 2015; Martín-García et al., 2020). The three consecutive days before the progressive ratio test are considered for this criterion (Martín-García et al., 2020). In the cycle of addiction, there is a transition from impulsivity in the early stages to compulsivity in the later stages (Everitt et al., 2008). This transition has its neurobiological correlation in the shift from ventral to dorsal striatum control of this behavioral hallmark (Belin et al., 2008).

Cognitive inflexibility: defined as the incapacity to shift responding to stimuli that have previously predicted the availability of reward. It is measured by the ability to modify the operant behavior when the active and the inactive levers were reversed in a single training session without previous learning. The errors are the number of active-reversed responses (previous inactive lever in a typical training session) performed in 1 h.

Appetitive cue-reactivity: The cue-induced food-seeking test, which consisted of a 90-min session, assessed the conditioning to an appetitive stimulus (cue-light). In the first 60 min, all active and inactive lever-presses were recorded but produced no consequences. In the next 30 min, the cue light associated with pellet delivery during a typical operant training session was illuminated with no contingent pellet reinforcement. To signal the change in the schedule, the cue light was presented twice non-contingently and for 4 s.

Aversive cue-reactivity: To study the conditioning to an aversive stimulus (grid floor), non-reinforced active responses during the following session after the shock test were measured. Mice were placed in the operant box for 1 h with the same grid floor used during the shock test. However, during this session, pressing the active lever had no consequences: no shock, no chocolate-flavored pellets, and no cue light.

### 2.5. Anxiety-like behavior

Anxiety-like behavior was evaluated by the elevated plus-maze test once the long-term operant training protocol was finished. A black Plexiglas apparatus consisting of 4 arms (29 cm long x 5 cm wide), 2 open and 2 closed, set in a cross from a neutral central square (5 × 5 cm) elevated 40 cm above the floor was used. Light intensity in the open and closed arms was 45 and 5 lx, respectively. Mice were placed in the central square facing one of the closed arms and tested for 5 min. The time spent in the open and closed arms of the maze was determined as a measure of anxiety-like behavior, whereas the total entries in the open and closed arms were considered a measure of locomotor activity, as previously reported (La Porta et al., 2015).

### 2.6. Depressive-like behavior

Depressive-like behavior was evaluated at the end of the long-term operant training protocol using the forced swimming test (Porsolt et al., 1978). Briefly, mice were individually placed into a glass cylinder (17.5 × 12.5 cm) filled 15 cm high with water (22 ± 1 °C). Mice were subjected to forced swimming for 6 min, and the total duration of immobility, disregarding small hind limb movements to keep the head above water, was measured during the last 4 min when mice showed a sufficiently stable level of immobility.

### 2.7. Statistics

#### 2.7.1. Statistical analysis of behavioral data

IBM SPSS 19 (SPSS Inc., Chicago, USA) was used to analyze all the data. Normality was determined by Kolmogorov-Smirnov's test. Parametric tests were performed if normality criteria were met. Repeated measures ANOVA was used to test the evolution over time. One-way ANOVA or two-way ANOVA were used when required for comparisons between groups followed by subsequent post hoc analysis (Fisher's Least Significant Difference “LSD” test) when required. Non-parametric tests were performed if normality criteria were not meet. Kruskal Wallis or Friedman test was applied followed by Mann-Whitney's *U* test when required. Chi-square analyses were performed to compare the percentage of addicted and nonaddicted mice, considering the observed frequencies with those obtained in the control WT group. Results were expressed as individual values with the median and the interquartile range. A probability of 0.05 or less was considered statistically significant.

#### 2.7.2. Principal component analysis

The principal component analysis (PCA) technique was used to evaluate the multidimensional data obtained in mice chronically trained with chocolate-flavored pellets. PCA and orthogonal varimax rotation were conducted using the 3 addiction-like criteria and the 4 phenotypic traits considered as vulnerability factors of addiction and were dimensionality reduced to the minimum number of components that best explain and maximize the variance present in the data set. An eigenvalue >1 was set as selecting components criterion according to the Kaiser criterion. PCA was used to explain the maximum total variance in a correlation matrix by transforming the original variables into linear components (Field, 2018). Factor loadings of the principal component 1 (PC1) and principal component 2 (PC2) in all variables were studied. Individual mice clustering according to addiction or non-addiction in the space yielded by the 2 PCA components, which accounted for the

maximum data variance, were represented. The order of factor loading of the different variables in PC1 and PC2 was considered, and loading >0.7 were considered as mainly contributing to the component.

### 2.8. Gene expression studies by real-time PCR

Relative gene expression analyses of cannabinoid receptor 1 (cnr1) in the NAc, prelimbic (PL), orbitofrontal (OFC) and infralimbic (IL) areas, opioid receptor (Oprm1) in the NAc, tyrosine hydroxylase (TH) in the VTA, corticotropin-releasing factor (Crf) in the paraventricular nucleus (PVN), dopamine receptor D1 (Drd1) and dopamine receptor D2 (Drd2) in PL, OFC and IL areas, were carried out. At the end of the behavioral procedure, mice were sacrificed by dislocation, and brains were removed from the skull and frozen over dry ice. Coronal sections (500 μm) containing the regions of interest were cut in a cryostat (−10 °C) according to Paxinos and Franklin atlas (Franklin and Paxinos, 2001), mounted onto slides, and stored at −80 °C. Sections were microdissected following the method described by Palkovits (Palkovits, 1983). Total RNA was obtained from brain micropunches using TRI extraction reagent (Applied Biosystems, Madrid, Spain). Reverse transcription to complementary DNA (cDNA) was carried out following the manufacturer's instructions (Applied Biosystems). The relative abundances of Cnr1 (Mm00432621_s1), Oprm1 (Mm01188089_m1), Crf (Mm01293920_s1), and Th (Mm00447546_m1) gene expressions were quantified in a StepOne Plus Sequence Detector System (Life Technologies, Madrid, Spain) and the relative abundances of Drd1 (5'-AGATTGACCAGGAAGAGGCC-3′ and 5'-GCAATCCAAGCCATACCAGG-3′) and Drd2 (5'-CCATCTCTTGCCCACTGCTCTTTGG-3′ and 5'-GGTGACGATGAAGGGCACGTAGAAC-3′) were quantified in a QuantStudio 12 K System by Applied Biosystems. Each assay was undertaken in technical triplicate to ensure the reliability of single values and the average calculated for data analyses. All reagents were obtained from Life Technologies, and the manufacturer's protocols were followed. The reference genes used were 18S rRNA (Mm03928990_g1) and β-Actin (5'-CACAGCC1GGATGGCTACGT-3′ and 5'-CGTGAAAAGATGACCCAGATCA-3′). All primer-probe combinations were optimized and validated for relative quantification of gene expression. Data for each target gene was normalized to the endogenous reference gene, and the fold change in target gene expression was determined using the 2-ΔΔCt method (Livak and Schmittgen, 2001).

## 3. Results

### 3.1. The reinforcement for chocolate-flavored pellets was reduced in CB2KO mice suggesting a resistant phenotype

WT ($n = 42$), CB2KO ($n = 25$), and CB2xP ($n = 28$) mice were trained in the operant chambers under FR1 schedule of reinforcement during 6 sessions followed by 112 sessions under FR5 to acquire an operant responding maintained by chocolate-flavored pellets (Fig. 1a, b). During FR1, all groups increased the number of reinforcements across sessions without significant differences between genotypes (repeated measures ANOVA, genotype $F_{(2,92)} = 0.24$, $p = 0.78$; session $F_{(5,460)} = 37.30$, $p < 0.001$; genotype/session interaction $F_{(10,460)} = 0.64$, $p = 0.65$, Fig. 1c). When the effort was increased to 5 lever presses (FR5), all genotypes showed a progressive increase in the number of reinforcements across time (repeated measures ANOVA, genotype $F_{(2,92)} = 4.29$, $p = 0.17$; session $F_{(111,10,212)} = 82.15$, $p < 0.001$; genotype/session interaction $F_{(222,10,212)} = 2.89$, $p < 0.001$ Fig. 1c). Interestingly, the number of reinforcements was significantly reduced in CB2KO compared to WT mice over the whole FR5 period (*post-hoc* LSD test $p < 0.001$). In contrast, CB2xP mice showed a reduced number of reinforcements compared to WT mice only in the early period, but no differences were found from the middle period onwards (*post-hoc* LSD test, NS, Fig. 1c). In the late period, CB2xP mice also showed a significantly higher number of reinforcements compared to CB2KO mice (*post-hoc* LSD test, $p < 0.001$, Fig. 1c). These results reveal that chocolate-flavored pellets were less reinforcing for CB2KO mice since the early period suggesting that the loss of CB2R may be a preexisting protective factor.

### 3.2. The impulsive-like behavior was decreased in CB2KO mice suggesting a resilient phenotype

As a measure of impulsive-like behavior, active lever presses during the 10 s of the time out period were recorded over each operant training session (repeated measures ANOVA, genotype $F_{(2,92)} = 3.69$, $p = 0.029$; session $F_{(111,10,212)} = 16.78$, $p < 0.001$; genotype/session interaction $F_{(222,10,212)} = 1.31$, $p = 0.0014$). CB2KO mice showed a reduced number of non-reinforced active responses compared to WT mice among all FR5 sessions (*post-hoc* LSD test, $p < 0.001$ Fig. 2a), suggesting that CB2KO mice are less impulsive. In contrast, CB2xP mice exhibited a similar number of active lever presses in the time-out period compared to WT mice (*post-hoc* LSD test, NS, Fig. 2a).

Most of the WT (91.30%), CB2KO (96.5%), and CB2xP (90.32%) mice achieved the acquisition criteria during the FR5 after an average of 17.36 ± 2.56, 26.72 ± 4.87, and 29.68 ± 5.40 sessions, respectively (Fig. 2b). No significant differences in the day of acquisition among genotypes were revealed indicating similar acquisition levels of the operant conditioning learning driven by chocolate-flavored pellets (Kruskal-Wallis, $H = 2.76$, $p = 0.25$, Fig. 2b). Therefore, the differences observed were not induced by a differential acquisition of the operant training.

WT and CB2KO mice progressively gained weight over time (repeated measures ANOVA, genotype $F_{(2,92)} = 12.60$, $p = 0.029$; week $F_{(12,1104)} = 197.51$, $p < 0.001$; genotype/week interaction $F_{(24,1104)} = 15.94$, $p < 0.001$; *post-hoc* LSD test, $p = 0.63$, Fig. 2c) without significant differences between them. In contrast, CB2xP mice showed significantly lower body weight than WT (*post-hoc* LSD test, $p < 0.001$) and CB2KO (*post-hoc* LSD test, $p < 0.001$) mice (Fig. 2c).

### 3.3. Differences between genotypes in the classification of addicted and nonaddicted mice

Considering the results of the 3 food addiction criteria in the late period, mice were individually categorized as nonaddicted (covering 0–1 criteria) or addicted (covering 2–3 criteria), as previously reported (Domingo-Rodriguez et al., 2020; García-Blanco et al., 2022; Mancino et al., 2015). Only 4.00% of CB2KO mice during the early training were classified as addicts, revealing a phenotype more resilient to develop food addiction compared to the WT control group (21.43% addicted mice) (Chi-square, $\chi^2 = 4.51$, $p = 0.034$, Fig. S1a). With regards to CB2xP, only 7.14% of mice were classified as addict in the early training (Chi-square, $\chi^2 = 3.39$, $p = 0.065$, Fig. S1a). During the middle training, the percentage of mice classified as addicts increased in all genotypes compared to the early period (WT = 28.57%, CB2KO = 20.00%, CB2xP = 17.86% animals covering 2–3 criteria). Neither CB2KO (Chi-square, $\chi^2 = 0.90$, $p = 0.343$) nor CB2xP (Chi-square, $\chi^2 = 1.58$, $p = 0.209$) differed from WT mice at this period (Fig. S2b). In the late period, similar percentage of CB2KO (28.00%) and WT mice (19.05%) were classified as addicts (Chi-square, $\chi^2 = 1.30$, $p = 0.254$, Fig. 3a). In contrast, CB2xP mice reached a higher percentage of addicts (35.71%) than WT mice (Chi-square, $\chi^2 = 5.04$, $p = 0.025$, Fig. 3a) suggesting that CB2R overexpression constitutes a risk factor to develop food addiction after long-term training.

### 3.4. Evolution over time of training of the three-addiction criteria in the different genotypes

WT mice presented an stable persistence across the periods (Friedman's test, $\chi2 = 4.84$, $p = 0.089$), while CB2KO (Friedman's test, $\chi2 = 11.76$, $p = 0.003$) and CB2TG (Friedman's test, $\chi2 = 19.50$, $p < 0.001$) mice increased their persistence over the periods (Fig. 3b). CB2KO did

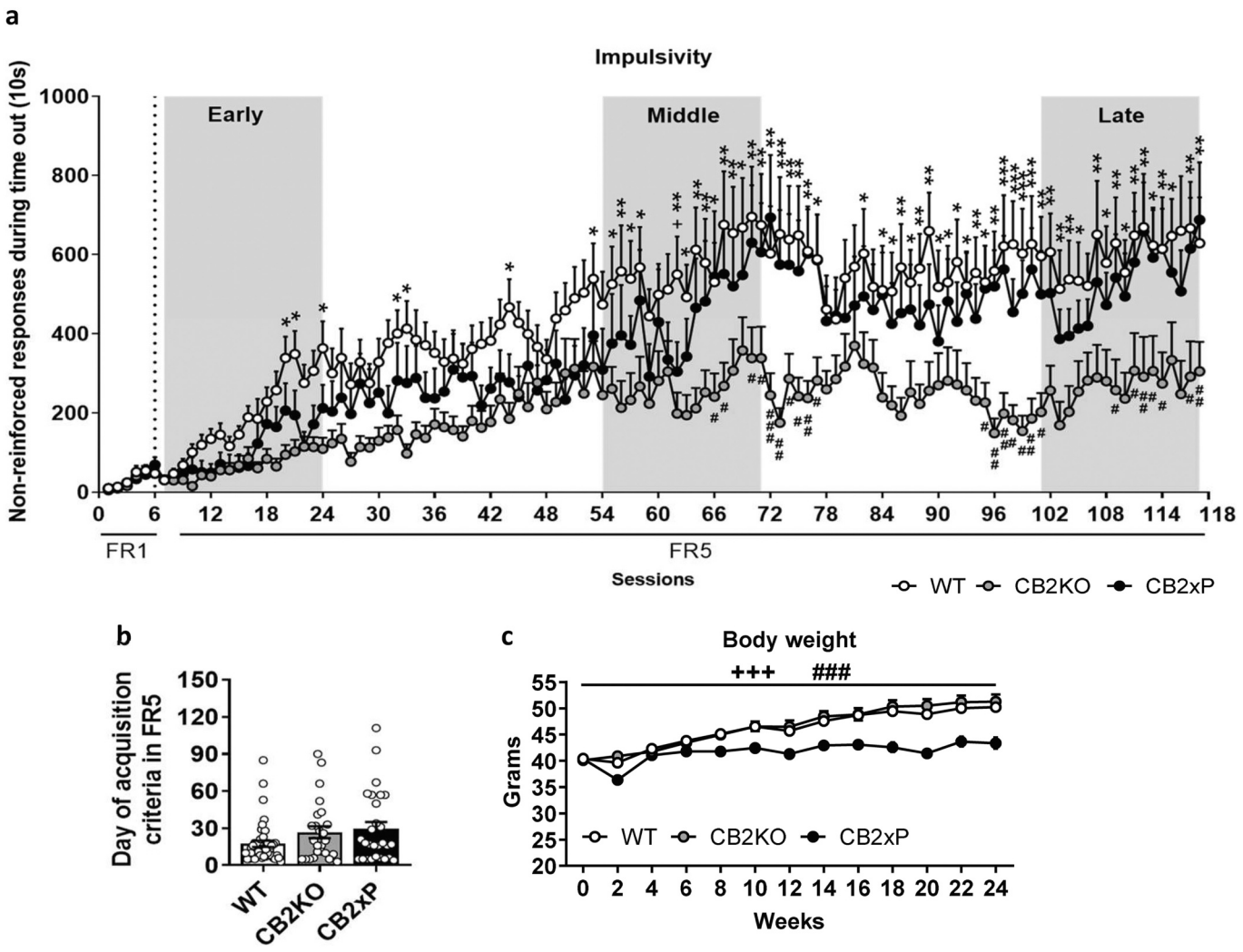


**Fig. 2.** The impulsive-like was decreased in CB2R-lacking mice suggesting a resilient phenotype. a Regarding the impulsivity across the protocol (repeated-measures ANOVA; session effect $p < 0.001$ and interaction genotype/session $p < 0.01$). CB2KO mice showed a reduced impulsivity compared to WT mice (*post-hoc* LSD test WT vs CB2KO ***$p < 0.001$), whereas CB2xP and WT mice exhibited similar responses (*post-hoc* LSD test WT vs CB2xP NS). b Day of acquisition of the operant conditioning behavior during the FR5 period (Kruskal-Wallis, NS). c Weekly measurements of body weight in grams. CB2xP mice showed a reduced body weight compared to WT and CB2KO mice (repeated measures ANOVA, genotype effect, $p < 0.001$; *post-hoc* LSD test WT vs CB2xP +++$p < 0.001$, *post-hoc* LSD test CB2KO vs CB2xP ###$p < 0.001$). (Data are expressed as mean ± SEM, WT $n = 42$, CB2KO $n = 25$, CB2xP $n = 28$, Statistical details are included in Table S2).

not differ from WT mice in any period, while CB2TG mice present lower persistence than WT mice in the early period (Mann-Whitney's U, U = 374.5, $p = 0.010$) and higher persistence than WT mice in the late period (Mann-Whitney's U, U = 382, $p = 0.014$) (Fig. 3b). The three genotypes increased their motivation across the protocol (WT mice Friedman's test, $\chi2 = 26.91$, $p < 0.001$, CB2KO mice Friedman's test, $\chi2 = 19.49$, $p < 0.001$, CB2xP mice Friedman's test, $\chi2 = 22.06$, $p < 0.001$) (Fig. 3c). CB2KO mice showed decreased motivation than WT mice both in the early (Mann-Whitney's U, U = 372.5, $p = 0.047$) and middle (Mann-Whitney's U, U = 374, $p = 0.048$) periods. The same was observed in CB2xP mice, which also showed decreased motivation than WT mice both in the early (Mann-Whitney's U, U = 404.5, $p = 0.027$) and middle (Mann-Whitney's U, U = 383.5, $p = 0.013$) periods (Fig. 3c). Regarding the compulsivity-like behavior, the three genotypes decreased their compulsivity across the protocol (WT mice Friedman's test, $\chi2 = 31.14$, $p < 0.001$, CB2KO mice Friedman's test, $\chi2 = 15.52$, $p < 0.001$, CB2xP mice Friedman's test, $\chi2 = 6.88$, $p = 0.032$) (Fig. 3d). CB2KO mice did not showed different compulsivity compared to WT mice, while CB2xP showed increased compulsivity compared to WT mice in the middle (Mann-Whitney's U, U = 356, $p = 0.004$) and increased compulsivity compared to CB2KO mice in the middle (Mann-Whitney's U, U = 194.5, $p = 0.005$) and late (Mann-Whitney's U, U = 243, $p = 0.049$) periods (Fig. 3d).

Regarding the nonaddicted mice subgroups, the most relevant results were the increased persistence of nonaddicted CB2xP mice compared to nonaddicted WT mice in the late period (Mann-Whitney's U, U = 203, $p = 0.048$) (Fig. S2a). Nonaddicted CB2KO (Mann-Whitney's U, U = 189, $p = 0.023$) and nonaddicted CB2xP (Mann-Whitney's U, U = 192, $p = 0.027$) mice showed a reduced motivation in the late period compared to nonaddicted WT mice (Fig. S2b). Nonaddicted CB2xP mice showed increased compulsivity in the middle period compared to nonaddicted WT (Mann-Whitney's U, U = 157, $p = 0.003$) and to nonaddicted CB2KO (Mann-Whitney's U, U = 77.5, $p = 0.006$) mice (Fig. S2c).

Regarding the addicted mice subgroups the most interesting results were the decreased persistence of addicted CB2xP mice compared to addicted WT mice in the early period (Mann-Whitney's U, U = 0, $p < 0.001$) and the decreased persistence of addicted CB2KO mice compared to addicted WT mice in the middle period (Mann-Whitney's U, U = 11.5, $p = 0.045$) (Fig. S2d). Regarding the motivation criteria addicted WT mice presented an increased motivation in the middle period compared to CB2KO (Mann-Whitney's U, U = 8.5, $p = 0.021$) and CB2xP (Mann-Whitney's U, U = 16.5, $p = 0.034$) mice (Fig. S2e). Addicted CB2xP mice

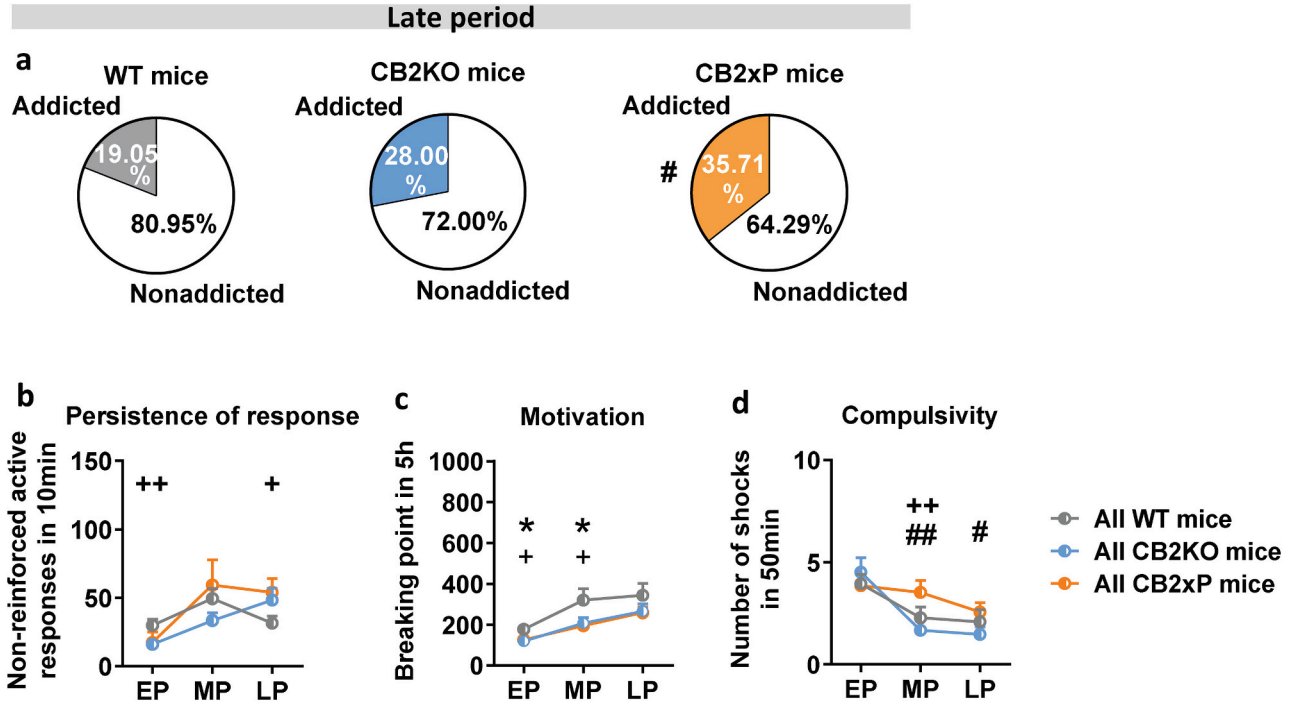


**Fig. 3.** Differences between genotypes in the classification of addicted and nonaddicted mice. a Percentage of mice classified as nonaddicted or addicted based on food addiction-like criteria scoring in the late period (Chi-square, WT vs CB2xP #$p < 0.05$) (WT n = 42, CB2KO n = 25, CB2xP n = 28, Statistical details are included in Table S3). b-d Behavioral evolution in the three addiction criteria tests over the early, middle, and late periods of all mice. Friedman test was used to evaluate evolution over time, statistical details are included in Table S4. Mann-Whitney's *U* test was used to compare groups in each period and significances between WT and CB2KO were represented as *p < 0.05, **p < 0.01 or ***p < 0.001, significances between WT and CB2xP mice were represented as +p < 0.05, ++p < 0.01 or +++p < 0.001 and significances between CB2KO and CB2xP mice were represented as #p < 0.05, ##p < 0.01, ###$p < 0.001$. (Data are expressed as mean ± SEM. WT n = 42, CB2KO n = 25, CB2xP $n = 28$. Statistical details are included in Table S4).

showed decreased compulsivity compared to addicted WT (Mann-Whitney's U, U = 157, $p = 0.003$) only in the early period (Fig. S2f).

### 3.5. *Evolution over time of training of the addiction-like phenotypic traits in the three genotypes*

The statistical analysis of evolution over the early, middle and late periods was performed in the four addiction-related phenotypic traits. The three genotypes increased their impulsivity over the periods (WT mice Friedman's test, $\chi2 = 36.16$, p < 0.001, CB2KO mice Friedman's test, $\chi2 = 27.18$, p < 0.001, CB2TG mice Friedman's test, $\chi2 = 34.83$, p < 0.001) (Fig. S3a). CB2KO presented a decreased impulsivity compared to WT through the whole protocol (early period Mann-Whitney's U, U = 361.5, $p = 0.033$, middle period Mann-Whitney's U, U = 334, $p = 0.013$, late period Mann-Whitney's U, U = 312.5, p = 0.006), while the CB2xP mice showed a decreased impulsivity compared to WT mice only in the early period (Mann-Whitney's U, U = 408.5, $p = 0.031$). CB2xP showed an increased impulsivity compared to CB2KO mice in the late period (Mann-Whitney's U, U = 233, $p = 0.037$) (Fig. S3a).

WT and CB2KO mice showed differences in the evolution of their cognitive inflexibity (WT mice Friedman's test, $\chi2 = 19.19$, $p < 0.001$, CB2KO mice Friedman's test, $\chi2 = 7.48$, $p = 0.024$) while CB2xP results in this phenotypic trait remained stable (CB2xP mice Friedman's test, $\chi2 = 0.87$, $p = 0.646$) (Fig. S3b). Differences between genotypes were only found in the middle period. were CB2xP showed a decreased cognitive inflexibility compared to WT mice (Mann-Whitney's U, U = 387.5, $p = 0.016$) (Fig. S3b).

The appetitive cue reactivity of CB2KO mice showed an evolution (Friedman's test, $\chi2 = 6.32$, $p = 0.042$) while the appetitive cue reactivity of WT and CB2xP mice remained stable (WT mice Friedman's test, $\chi2 = 0.95$, $p = 0.623$, CB2xP mice Friedman's test, $\chi2 = 2.79$, $p = 0.248$) (Fig. S3c). WT mice showed and increased appetitive cue reactivity in the early period compared to CB2KO (Mann-Whitney's U, U = 306.5, $p = 0.005$) and CB2xP (Mann-Whitney's U, U = 405, $p = 0.028$) mice (Fig. S3c).

The aversive cue reactivity of WT and CB2KO mice showed an evolution (WT mice Friedman's test, $\chi2 = 10.02$, $p = 0.007$, CB2xP mice Friedman's test, $\chi2 = 11.84$, p = 0.003) while CB2xP mice remained stable (Friedman's test, $\chi2 = 1.64$, $p = 0.441$) (Fig. S3d). WT mice showed and increased aversive cue reactivity in the early period compared to CB2KO (Mann-Whitney's U, U = 297, p = 0.003) (Fig. S3d).

Regarding the nonaddicted mice subgroups the most relevant results were the decreased impulsivity of nonaddicted CB2KO mice compared to nonaddicted WT (Mann-Whitney's U, U = 136.5, $p = 0.001$) and nonaddicted CB2xP (Mann-Whitney's U, U = 88, $p = 0.019$) mice in the late period (Fig. S3e). No differences were found in the cognitive inflexibility trait between the subgroups of nonaddited mice of the three genotypes (Fig. S3f). Nonaddicted CB2KO mice showed a reduced appetitive cue reactivity in the early period compared to nonaddicted WT mice (Mann-Whitney's U, U = 198, $p = 0.038$) (Fig. S3g). Non-addicted CB2xP mice showed decreased aversive cue reactivity in the middle period compared to nonaddicted WT mice (Mann-Whitney's U, U = 203, $p = 0.049$) (Fig. S3h).

Regarding the addicted mice subgroups the most remarkable results were the decreased impulsivity of addicted CB2xP mice in the early period compared to addicted WT mice period (Mann-Whitney's U, U = 0, $p < 0.001$) and the decreased impulsivity of addicted CB2KO mice compared to addicted WT mice in the middle period (Mann-Whitney's U, U = 9, $p = 0.029$) (Fig. S3i). Cognitive inflexibility was decreased in addicted CB2xP mice compared to WT in the middle period (Mann-Whitney's U, U = 13, p = 0.016) (Fig. S3j). Regarding the appettive cue reactivity, addicted CB2KO mice presented a decreased response in the early period compared to WT mice (Mann-Whitney's U, U = 8.5, $p = 0.021$) (Fig. S3k). Addicted CB2KO mice showed decreased aversive cue

reactivity compared to addicted WT mice in the early (Mann-Whitney's U, U = 0, p < 0.001) and middle (Mann-Whitney's U, U = 9, p = 0.029) period (Fig. S3l).

### 3.6. Principal component analysis revealed differential patterns of behavioral factor loadings in food addiction-like behavior in mice

The links between the different behavioral addiction-like criteria and phenotypic traits were evaluated using PCA.

The percentage of variance explained by the first principal component (PC1) was 41.16% and by the second principal component (PC2) was 17.13%. The distribution of all mice according to these components is shown in Fig. 4a and Fig. 4b. Nonaddicted and addicted animals were distributed in two well-differentiated clusters (Fig. 4a). When representing mice by genotype and addictive phenotype, we can also observe a cluster organization of the different subgroups. The main addiction criterion loading in PC1 was motivation (Fig. 4c), whereas compulsivity was the predominant criterion in PC2 (Fig. 4d). Among the four phenotypic traits, impulsivity and appetitive and aversive cue-reactivities had the highest loading in PC1, whereas cognitive inflexibility showed the lowest loading in both components. Thus, PC1 was the most useful component for discriminating between addicted and non-addicted mice, as it accounts for more variability. Finally, the distribution of the different addiction criteria and phenotypic traits is represented in Fig. 4e, showing the motivation and impulsivity nearest.

Then, the three genotypes were evaluated by PCA separately. In the WT mice group, the percentage of variance explained by the two principal components (PC) was 44.83% (PC1) and 15.21% (PC2). The distribution of WT mice according to these components is shown in Fig. S4a, and the distribution of the different addiction criteria and phenotypic traits in Fig. S4b. The main addiction criterion loading in PC1 for WT mice was motivation (Fig. S4c), whereas compulsivity was the predominant criterion in PC2 (Fig. S4d). Among the four phenotypic traits, impulsivity and both appetitive and aversive cue-reactivities had the highest loading in PC1, whereas cognitive inflexibility showed the lowest loading in PC1. Thus, PC1 was the most useful to discriminate between addicted and nonaddicted mice as it accounts for more variability.

In CB2KO mice, PC1 explained 47.99% of the variance and PC2 the 20.48% The distribution of CB2KO mice according to these components is represented in Fig. S5e, and the distribution of the addiction criteria and phenotypic traits in Fig. S5f. Similarly to WT mice, the main addiction criterion loading in PC1 of CB2KO mice was motivation, followed by the persistence of response (Fig. S5g). The compulsivity criterion did not meet the proposed loading criterion (0.40) in CB2R mutants (Field, 2018) (Fig. 3g). With regards to the phenotypic traits, impulsivity and appetitive cue-reactivity in CB2KO mice weighted more in PC1, whereas cognitive inflexibility weighted mainly in PC2 (Fig. S5h). PC1 was also the most useful to discriminate between addicted and nonaddicted mice.

In the CB2xP group, the percentage of variance explained by PC1 was 40.96% and 17.77% by PC2. The distribution of CB2xP mice according to the two components is represented in Fig. S6i, where the PC1 was again the most useful to discriminate between addicted and nonaddicted mice. Behavioral tests clustered according to the loading in two components of the PCA are shown in Fig. S6j. The main addiction criterion loading in PC1 for CB2xP mice was the persistence of response, followed by motivation, whereas compulsivity showed the lowest loading in PC1 (Fig. S6k). Among the four phenotypic traits, aversive cue-reactivity and impulsivity had the highest loading in PC1. Appetitive cue-reactivity showed the main loading in PC2 (Fig. S6l). The cognitive inflexibility did not meet the proposed loading criterion (0.40).

### 3.7. Emotional alterations after long-term exposure to palatable pellets

Depressive-like behavior was evaluated at the end of the experimental protocol by the forced swimming test. No significant genotype differences were found in the immobility time shown (one-way ANOVA, NS) (Fig. 5a).

Anxiety-like behavior was evaluated by the elevated plus maze test. Both CB2KO and CB2xP genotypes showed unaltered anxiety-like behavior when compared to the WT group (one-way ANOVA, NS) (Fig. 5c and Fig. 5d).

### 3.8. Alterations in gene expression after long-term exposure to palatable pellets

Molecular analysis was performed by RT-qPCR in two key areas of the mesolimbic system (NAc and VTA), the PVN and three cortical areas (PL, IL, and OFC), which are part of the medial prefrontal cortex, previously related to addiction (Fig. 6c, i) (Blakemore and Robbins, 2012; Diamond, 2013; Miller and Cohen, 2003; Radcliffe Hospital et al., 2005).

In the NAc, the expression levels of the gene encoding CB1R (*Cnr1*) (Fig. 6a) were found to be different between the three genotypes (one-way ANOVA: $F_{(2,45)} = 5.59$, $p = 0.007$). RT-qPCR showed decreased levels of *Cnr1* gene expression in CB2xP compared to WT mice (*post-hoc* LSD test, I-J = 0.15, $p = 0.048$) and CB2KO (*post-hoc* LSD test, I-J = 0.25, $p = 0.002$) in the NAc.

Regarding *Oprm1* gene expression in the NAc (Fig. 6b), one-way ANOVA revealed differences between genotypes ($F_{(2,45)} = 3.74$, $p = 0.032$). CB2xP mice showed lower levels of *Oprm1* gene expression than CB2KO mice (*post-hoc* LSD test, I-J = 0.17, $p = 0.012$).

Concerning the expression of the *Th* gene in the VTA, one-way ANOVA revealed a genotype effect ($F_{(2,40)} = 19.22$, $p < 0.001$), CB2xP displayed lower gene expression levels of *Th* than WT (*post-hoc* LSD test, I-J = 0.41, p < 0.001) and than CB2KO (*post-hoc* LSD test, I-J = 0.41, p < 0.001) (Fig. 6d).

In the RT-qPCR of *Crf* gene in the PVN, one-way ANOVA revealed differences between genotypes ($F_{(2,40)} = 6.79$, $p = 0.003$) (Fig. 6e). The *Crf* mRNA relative expression was significantly increased in CB2xP in comparison to WT (*post-hoc* LSD test, I-J = 0.23, $p = 0.024$) and CB2KO mice (*post-hoc* LSD test, I-J = 0.36, p < 0.001).

Finally, one-way ANOVA analyses of the mRNA relative expression of the *Drd1* gene in the IL, revealed differences between genotypes ($F_{(2,29)} = 3.41$, $p = 0.047$), in which CB2KO mice showed a reduced mRNA expression of *Drd1* gene compared to WT mice (*post-hoc* LSD test, I-J = 0.57, $p = 0.022$).

## 4. Discussion

In this study, we evaluate the role of CB2R in food addiction by dissecting the phenotype of CB2KO and CB2xP mice during the development of this behavioral disorder. Different phenotypic signatures of resilience and vulnerability to develop food addiction were found in CB2KO and CB2xP. Thus, palatable food reinforcement was reduced in CB2KO mice and a low percentage of these mutants developed food addiction during the early training period suggesting a resistant phenotype in agreement with the decreased nicotine self-administration reported in CB2KO mice (Navarrete et al., 2013). In contrast, CB2xP mice showed decreased chocolate self-administration in the early period, but increased responding in the late period suggesting a switch in the reward sensitivity for these mutants. Thus, the reduction in food self-administration between genotypes only occurs in the early period. The length of the operant training seems crucial for these adaptive responses leading to differential behavioral responses. These effects could be related to the dopamine desensitization that has been only reported in long-termed substance abusers (Volkow et al., 2011). Decreased cocaine self-administration was previously exhibited by CB2xP mice during short-operant training, further supporting the relevance of the length of training for revealing this decreased seeking to different rewarding stimuli during short training periods (Aracil-Fernández et al., 2012). However, the percentage of mice reaching addiction criteria in

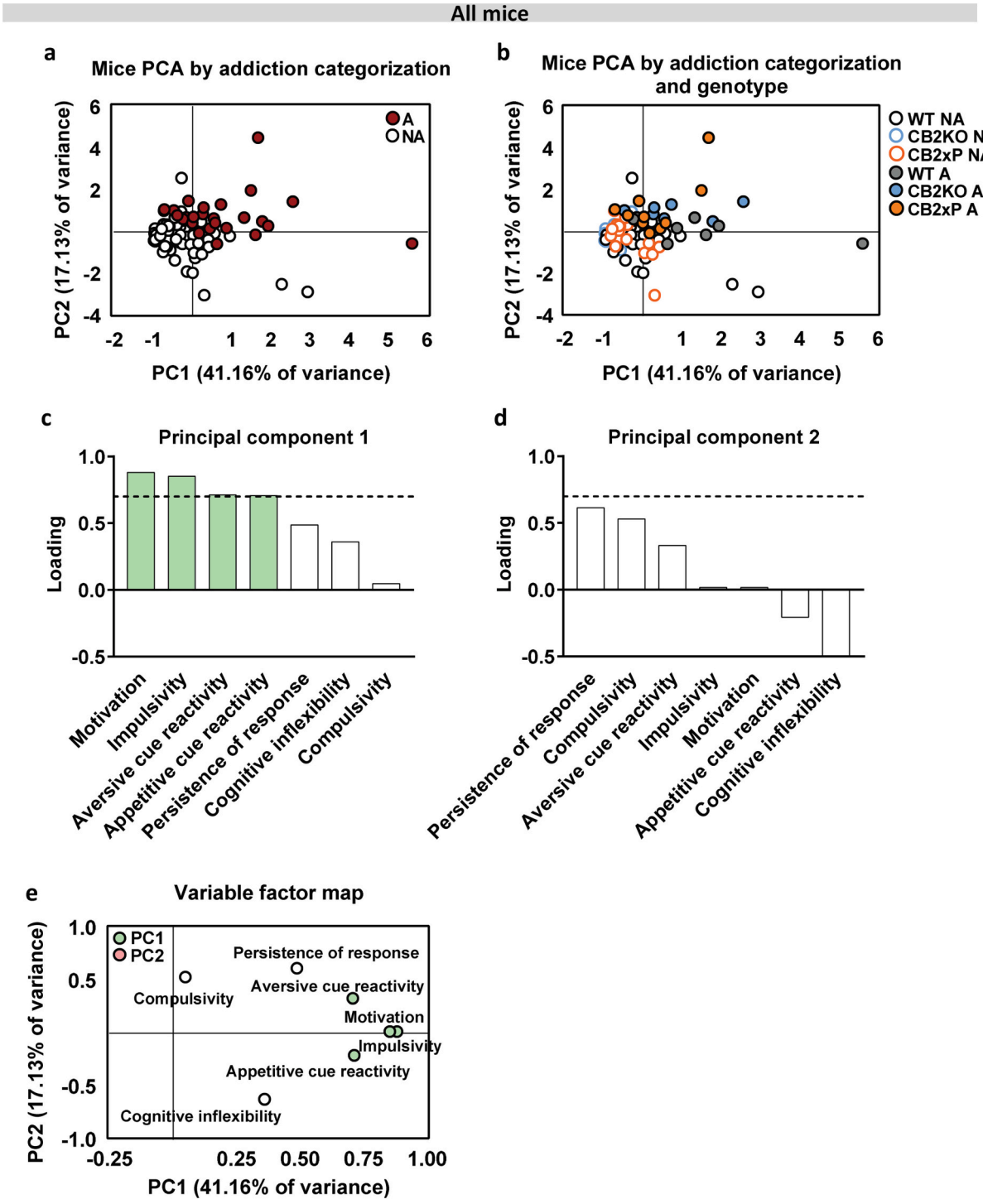


**Fig. 4.** Principal component analysis (PCA) revealed differential patterns of behavioral factor loadings in food addiction-like behavior in mice depending on their addictive phenotype and genotype. a PCA of the three addiction criteria and the four phenotypic traits performed in all mice with factor loadings of principal component 1 (PC1) (41.16%) and principal component 2 (PC2) (17.13%). Mice subjects clustered by addicted or nonaddicted on the space yielded two components of the PCA that account for the maximum variability (n = 25 addicted (A) mice represented in red, $n = 70$ nonaddicted (NA) mice represented in white). b PCA of the three addiction criteria and the four phenotypic traits performed in all mice. Mice subjects clustered by addiction categorization and by genotype on the space yielded two components of the PCA that account for the maximum variability ($n = 34$ nonaddicted WT (WT NA) mice represented as empty grey circles, $n = 18$ nonaddicted CB2KO (CB2KO NA) mice represented as empty blue circles, n = 18 nonaddicted CB2xP (CB2xP NA) mice represented as empty orange circles, $n = 8$ addicted WT (WT A) mice represented as filled grey circles, n = 7 addicted CB2KO (CB2KO A) mice represented as filled blue circles, $n = 10$ addicted CB2xP (CB2xP A) mice represented as filled orange circles). c-d Graphs with the order of factor loading of the different variables in the PC1 and PC2. The dashed horizontal line marked loadings >0.7 mainly contributing to the component. e Behavioral tests clustered according to the loading in two components of the PCA. Green colour represents PC1 and pink colour represents PC2. Results are summarized in Table S1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

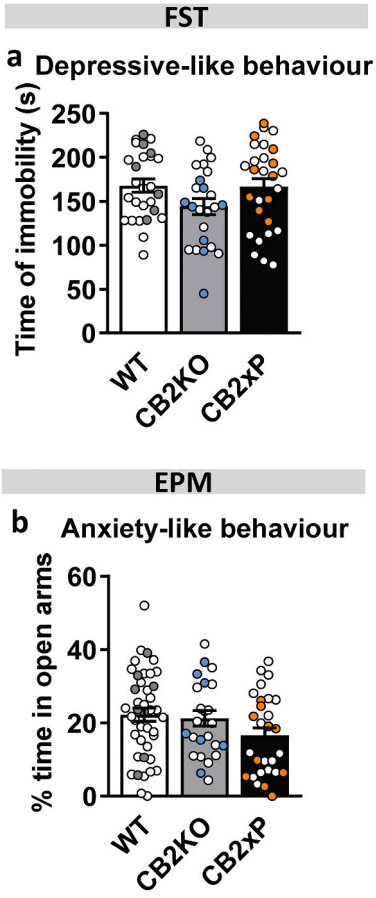


**Fig. 5.** Addicted CB2KO mice present less depressive-like behavior after exposition to long-term operant training maintained by chocolate-favored pellets. Characterization of anxiety-like behavior. a Time of immobility in seconds (s) in the forced swimming test as a measure of depressive-like behavior (one-way ANOVA, NS). b Percentage of time spent in the open arms as a measure of anxiety-like behavior (one-way ANOVA, NS). Data are expressed as mean ± SEM. WT n = 42, CB2KO n = 25, CB2xP n = 28. Statistical details are included in Table S5.

both CB2KO and CB2xP mice was similar in the late period, pointing out the multifactorial nature of this disorder.

Here, the percentages of WT mice classified as vulnerable or resilient in the early and middle periods were equivalent across periods. In the late period, the 19.1% of WT mice were classified as addicted, similar to the prevalence reported in humans (19.9%) (Pursey et al., 2014) and to previous mice studies (22.2–25.5%) (Domingo-Rodriguez et al., 2020; García-Blanco et al., 2022; Mancino et al., 2015). The prolonged exposure to highly palatable food enhanced the percentage of CB2KO mice reaching the addiction criteria in spite of the resilient phenotype observed in these mutants at the early training period. This result highlights that addiction is a multifactorial disease, and knocking out one single gene is not enough to fully revert this phenotype. The protective effect to develop food addiction in the absence of CB2R in a short-operant protocol is in accordance with the lower nicotine self-administration similarly shown by these mutant mice (Navarrete et al., 2013). CB2xP mice revealed an increased vulnerability to develop food addiction after long-term operant training. However, previous studies using these mutants (Aracil-Fernández et al., 2012) or pharmacological CB2R activation (Zhang et al., 2014) showed reduced cocaine self-administration in a short-operant training. These discrepancies can rely on the length of the training protocol or the different properties of the rewarding stimuli.

Phenotypic traits related to vulnerability to food addiction were also evaluated. The lack of CB2R decreased impulsive like-behavior, whereas CB2 overexpression did not modify this behavioral trait. In agreement, previous studies have demonstrated that CB2R blockade decreases impulsivity (Navarrete et al., 2012). Impulsivity has been widely associated with the dopaminergic functioning, and increased impulsivity was correlated with diminished *drd2* availability in the rat NAc (Dalley et al., 2007). Other studies showed that the pharmacological stimulation of CB2R in the VTA and NAc reduced dopaminergic neuron excitability and decreased dopamine extracellular levels in the NAc (Ma et al., 2019; Xi et al., 2011; Zhang et al., 2014). Therefore, CB2R might be involved in impulsive-seeking behavior through modulation of the dopaminergic system. CB2R expression in VTA dopaminergic neurons projecting to NAc was demonstrated to modulate the dopaminergic tone, which has a direct impact on dopamine-related behaviors such as the hedonic effect triggered by abuse of substances and palatable food (Zhang et al., 2014). Furthermore, neurons of the NAc and VTA expressed CB2R, and some of these neurons coexpressed D2R (Aracil-Fernández et al., 2012). Interestingly, endocannabinoids have a role in the dopaminergic modulation of striatal MSNs (André and Gonthier, 2010). Conversely, endocannabinoids and exogenous cannabinoids may also produce aversive effects by activating CB1R on glutamatergic projections from the prefrontal cortex (PFC) to the VTA, whereas the activation of CB2R on dopaminergic neurons from the VTA to the NAc may decrease the rewarding effects (Cabañero et al., 2021b). CB2R localized on glial cells, such as astrocytes or microglia, could reduce dopamine levels by controlling the release of inflammatory cytokines from these cells.

The differences observed in palatable food reinforcement and impulsive-like behavior could not be explained by a learning impairment since the three genotypes acquired the operant behavior in similar timing periods. The body weight of CB2KO mice did not differ from WT mice, and CB2xP mice exhibited reduced body weight gain, although this change did not affect the operant training acquisition. Pellet consumption increased similarly in CB2xP and WT mice independently of the body weight, suggesting that a caloric need did not trigger the intake. In agreement, previous studies showed a lean phenotype of CB2xP and reduced body weight after chronic pharmacological CB2R activation (Ishiguro et al., 2010; Romero-Zerbo et al., 2012; Verty et al., 2015). Previous studies have revealed a selective decrease of food intake in CB2xP mice compared to WT only when measured during 12 h in fasted conditions and during 120 min after food presentation, but not when food intake was evaluated during longer periods (Romero-Zerbo et al., 2012). Furthermore, recent investigations have shown that the pharmacological activation of CB2 with JWH133 decreases sucrose self-administration (Bi et al., 2020), and the use of the CB2 agonist beta-caryophyllene decreases motivational salience and conditioning place preference for palatable food (Dos Santos Barbosa et al., 2023). Conversely, mice lacking CB2R present an age-related obese phenotype with increased food intake (Agudo et al., 2010; Pradier et al., 2015), and CB2R pharmacological blockade stimulates food consumption (Ishiguro et al., 2010). Other studies have reported that CB2KO mice show increased alcohol-drinking behavior and food intake (Pradier et al., 2015). Even though some of this evidence is dependent on the environment, the diet, or the mouse strain (Ishiguro et al., 2010; Pradier et al., 2015), all these results together highlight the interest in targeting CB2R in obesity-associated pathologies in addictive processes closely related to hedonic control systems. In our previous study (Agudo et al., 2010), total food intake, mainly dependent on the homeostatic control of food intake, and several metabolic parameters including insulin sensitivity, adipose tissue inflammation and glucose uptake were evaluated in CB2KO mice. This parameter of food intake under the homeostatic control was increased in CB2KO mice. In our current study, mice were fed ad libitum, fully covering their metabolic needs, and we evaluated the consumption of highly palatable isocaloric pellets during one hour daily operant sessions. Therefore, high palatable pellet seeking under these conditions is mainly dependent on the hedonic control of food intake rather than the homeostatic control (Onaolapo and

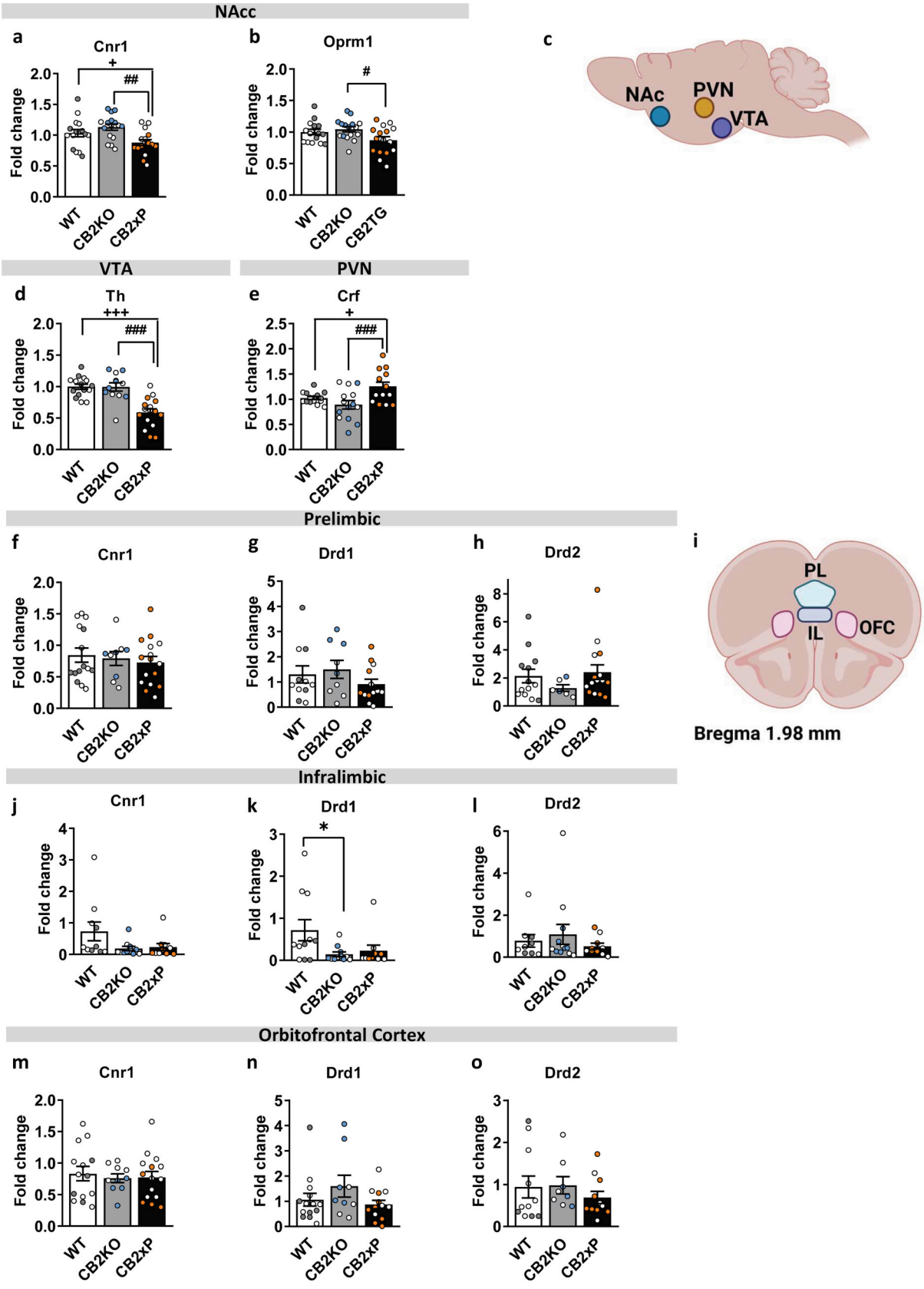


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**Fig. 6.** Alterations in gene expression after long-term exposure to palatable pellets. a Detection of *Cnr1* mRNA in NAc (one-way ANOVA, $p < 0.001$, *post-hoc* LSD test WT vs CB2xP + $p < 0.05$, CB2KO vs CB2xP ##$p < 0.01$). b Detection of *Oprm1* in NAc (one-way ANOVA, $p < 0.05$, *post-hoc* LSD test CB2KO vs CB2xP #$p < 0.05$). c. NAc, PVN and VTA distribution in a sagittal view of the mice brain. d Detection of *Th* mRNA in VTA (one-way ANOVA, $p < 0.001$, *post-hoc* LSD test WT vs CB2xP +++$p < 0.001$, CB2KO vs CB2xP ###$p < 0.001$). e Detection of *Crf* mRNA in PVN (one-way ANOVA, $p < 0.01$, *post-hoc* LSD test WT vs CB2xP + $p < 0.05$, CB2KO vs CB2xP ###$p < 0.001$). f Detection of *Cnr1* mRNA in PL (one-way ANOVA, NS). g Detection of *Drd1* mRNA in PL (one-way ANOVA, NS). h Detection of *Drd2* mRNA in PL (one-way ANOVA, NS). i. PL, IL and OFC distribution in a coronal view of the mice brain. j Detection of *Cnr1* mRNA in IL (one-way ANOVA, NS). k Detection of *Drd1* mRNA in IL (one-way ANOVA, $p < 0.05$, *post-hoc* LSD test WT vs CB2KO *$p < 0.05$). l Detection of *Drd2* mRNA in IL (one-way ANOVA, NS). m Detection of *Cnr1* mRNA in OFC (one-way ANOVA, NS). n Detection of *Drd1* mRNA in OFC (one-way ANOVA, NS). o Detection of *Drd2* mRNA in OFC (one-way ANOVA, NS). Data are expressed as mean ± SEM (WT NA n = 8, CB2KO NA n = 8, CB2xP NA n = 8, WT A n = 8, CB2KO A n = 8, CB2xP A n = 8). Addicted WT mice are represented as grey filled circles, addicted CB2KO mice are represented as blue filled circles and addicted CB2xP mice are represented as orange filled circles, some animals were excluded due to technical limitations. Statistical details are included in Table S6. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Onaolapo, 2018), which was decreased in CB2KO mice. The mesocorticolimbic circuit plays a crucial role in the hedonic system (Onaolapo and Onaolapo, 2018) that is altered in food-addicted individuals. Therefore, CB2R seems to play an opposite role in the homeostatic and hedonic control of food intake.

Differential behavioral signatures with regard to the addiction criteria and the phenotypic traits were revealed in the two subpopulations of addicted and nonaddicted mice in the three genotypes across the early, middle and late periods. Indeed, PCA results revealed an excellent clusterization of addicted and nonaddicted mice total mice and also in each genotype, suggesting that the model is highly predictive and that the addiction criteria and the phenotypic traits explain a sufficient percentage of variance to distinguish the subpopulations.

Neither lack nor overexpression of CB2R had an effect on depressive- nor anxiety-like behavior after the long-term operant training in our food addiction model. Indeed, both CB2KO, and CB2xP mice displayed equivalent despair-induced immobility time in the forced swimming test and exploration in the open arms of the elevated plus maze. Previous studies using the same genetic mouse models showed that CB2R deletion enhanced depressive-like behavior (Ortega-Alvaro et al., 2011) and vulnerability to stress and anxiety-like behavior (Ortega-Álvaro et al., 2015), whereas CB2R overexpression protected from anxiogenic and depressive environmental conditions alteration (García-Gutiérrez et al., 2010; García-Gutiérrez and Manzanares, 2011). On the other hand, the CB2r antagonist AM630 led to anxiolytic and antidepressant effects in naïve mice, while pharmacological activation of CB2r by JWH133 produced anxiety and depressive-like behavior (García-Gutiérrez et al., 2010; García-Gutiérrez and Manzanares, 2011). Thus, genetic and pharmacological manipulations are not comparable in these phenotypes, probably due to the complexity of the inherent neurobiological mechanism involved. In our study, long-term exposure to highly palatable food may interfere with the depressive- or anxiety-like behavior phenotypes of CB2KO and CB2xP mice, probably due to allostatic changes promoted by the repetitive activation of the reward circuits.

Changes in the expression of several genes closely related to reward activity were studied to disentangle the mechanisms involved in the behavioral changes promoted by the modification of CB2R content. CB2KO mice with a food addiction resilient phenotype showed the highest expression levels of the CB1R gene (*cnr1*) in the NAc compared to the other genotypes, and this change was more pronounced in addicted subgroup. In contrast, CB2xP, vulnerable to develop addiction, presented the lowest *cnr1* gene expression level in the NAc. These results are in agreement with previous studies showing that the decrease of CB2R activity by CB2R pharmacological blockade increases CB1R in the NAc, whereas CB1R is decreased in NAc after CB2R agonist administration (Navarrete et al., 2018).

In agreement with the vulnerable phenotype of CB2xP mice, the expression of the mu opioid receptor (*Oprm1*) was lower in these mice. This finding correlates with previous studies reporting that *Oprm1* is reduced in the NAc of CB2xPmice under naïve conditions or after cocaine pre-treatment (Aracil-Fernández et al., 2012) and in WT mice after CB2R pharmacological stimulation (Navarrete et al., 2018). High levels of *Oprm1* were observed in addicted CB2KO mice in agreement with the resistant phenotype of these mutants. Previous studies have also reported increased *Oprm1* in the NAc of CB2KO mice under naïve conditions and after acute ethanol administration (Ortega-Álvaro et al., 2015). Furthermore, pharmacological CB2R blockade increased *Oprm1* in the NAc (Navarrete et al., 2018). Therefore, the interaction between CB2R and Oprm1 seems crucial in food addiction development.

The expression of the gene encoding tyrosine hydroxylase (*th*), the rate-limiting enzyme in dopamine synthesis, was decreased in the VTA of CB2xP mice suggesting a hypodopaminergic state that was independent of the criteria of addiction. Similar results were observed after the pharmacological activation of CB2R (Navarrete et al., 2018). Conversely, previous studies showed that *th* expression in the VTA was decreased in naïve CB2KO mice (Navarrete et al., 2018) and increased in naïve CB2xP mice (Aracil-Fernández et al., 2012), although others did not report differences in CB2KO (Ortega-Álvaro et al., 2015). In our study, the decreased expression of *th* was found in CB2xP mice after a long protocol (4 months) of palatable food self-administration. This decrease in the limiting enzyme in dopamine synthesis in CB2xP mice could correlate with the dopamine desensitization that occurs in the later stages of addictive processes when the disease is fully consolidated (Nimitvilai et al., 2014; Volkow et al., 2011).

In agreement with its resilient phenotype, addicted CB2KO mice displayed lower expression of the corticotropin-releasing factor (CRF) gene (*crf*) in the hypothalamus PVN, which is involved in the homeostatic control of food intake (King, 2005). CRF is a stress hormone implicated in addictive processes and participates in drug-seeking and the manifestations of drug withdrawal (Blacktop et al., 2011). In agreement, we reported a lower expression in food addiction-resistant mice (addicted CB2KO) and increased expression in addiction-vulnerable mice (addicted CB2xP mice). This close relationship between *crf* expression and CB2R content in food addiction vulnerability suggests an important role of this hormone in the mechanisms involved in CB2R-mediated stress-induced vulnerability to food addiction.

Changes in the targeted gene expression were also revealed in the OFC of addicted mice where *cnr1* expression was downregulated independently of the genotype. This dysregulation can be associated with a compensatory mechanism that may appear to try to regulate glutamatergic and gabaergic release. The IL mPFC of CB2KO mice showed a reduced mRNA expression of the *Drd1* gene compared to WT mice, in agreement with a protective factor since recent studies showed upregulation of *Drd1* mRNAs in addicted mice (Domingo-Rodriguez et al., 2022). The expression of the other targeted genes was not modified in the cortical areas in the different genotypes. Several limitations should be taken into account in the present study, such as the heterogeneity of the samples concerning individual differences in behavioral responses; the small fold change in gene fluctuations that could limit the detection of differences in lowly expressed genes.

## 5. Conclusions

In summary, we characterized the role of CB2R in the development of food addiction by dissecting the phenotype of CB2KO and CB2xP mice. We have also identified differential gene expression signatures in

the reward circuit depending on the CB2R content that may underlie the phenotypes of these mutants Different phenotypes with regards to reinforcement, impulsivity, compulsivity, and susceptibility to develop food addiction criteria were revealed in these mutants along our long-termed protocol. Our findings suggest that lack of CB2R activity decreases the reinforcement, protects against impulsivity- and compulsivity-like behavior, and therefore could be a protective factor to develop core features of food addiction in a time-dependent manner. However, it does not influence the final percentage of mice reaching addiction criteria. This phenotype of CB2KO mice was associated with increased *cnr1* in the NAc, and decreased *crf* expression in the PVN. The overexpression of CB2R leads to a food addiction vulnerable phenotype associated with decreased *Cnr1* and *Oprm1* expression in the NAc, reduced levels of *th* in the VTA and increased *crf* expression in the PVN and decreased drd1 mRNA expression in the IL. These alterations in the reward circuit may underline substantial modifications in reward processing due to CB2R overexpression that could explain the enhanced addictive vulnerability of these mice. In summary, our study unravels a new neurobiological mechanism involving CB2Rs in the substrate underlying vulnerability to develop food addiction, which could pave the way toward novel therapeutic approaches for this disorder.

## Authors contribution

E.M-G., J.M. and R.M. conceived and designed the behavioral and gene expression studies, did the funding acquisition, project administration and supervision; A.G.-B. and A.R.-L. performed the behavioral experiments and the statistical analyses and graphs with the supervision of E.M-G. and R.M.; A.G.-B., A.R.-L., F.N. and M.S.G-G: performed the qPCRs. E.M-G., A.G.-B. and R.M. wrote the original draft of the manuscript with review and edits from all the other authors.

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## Declaration of Competing Interest

The authors declare no competing financial interests.

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nbd.2023.106034.

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