MicroRNAs signatures associated with vulnerability to food addiction in mice and humans

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Abstract

Food addiction is characterized by a loss of behavioral control over food intake and is associated with obesity and other eating disorders. The mechanisms underlying this behavioral disorder are largely unknown. We aim to investigate the changes in miRNAs expression promoted by food addiction in animals and humans and their involvement in the mechanisms underlying the behavioral hallmarks of this disorder. Sharp similitudes were found between the miRNAs signatures in the medial prefrontal cortex (mPFC) of our animal cohort and the miRNAs circulating levels in our human cohort allowing to identify several miRNAs of potential interest for the development of this disorder. TuD inhibition of miRNA-29c-3p in the mouse mPFC promotes persistence of response and enhances the vulnerability to develop food addiction, whereas miRNA-665-3p inhibition promotes compulsive-like behavior and also enhances food addiction vulnerability. In contrast, miRNA-137-3p inhibition in the mPFC does not affect the development of food addiction. Therefore, miRNA-29c-3p and miRNA-665-3p could be acting as protective factors towards food addiction. The elucidation of these novel epigenetic mechanisms provides advances toward innovative biomarkers and possible future interventions for food addiction and related disorders based on the strategies now available to modify miRNA activity and expression.

Introduction

Food addiction is a multifactorial complex disorder characterized by loss of control over food intake and has increased its prevalence in recent years (1), particularly during the current COVID-19 pandemic (2). This behavioral alteration is related to obesity and eating disorders and lacks effective treatments leading to high socio-economical costs worldwide. Despite the early definition of this concept (3), the 5th edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) does not include the construct of food addiction due to its controversy and lack of strong scientific evidence. However, a widely accepted instrument is currently used in the clinic to measure food addiction, the Yale Food Addiction Scale (YFAS) (4) and a new YFAS 2.0 has been recently designed to apply the DSM-5 criteria for substance use disorder to eating behaviors. The YFAS 2.0 food addiction criteria can be summarized in three behavioral hallmarks also used in rodent models to mimic this disorder: persistent food-seeking, high motivation to obtain food, and compulsive-like behavior (5). Studies in animals and humans have discovered important similitudes in the neurobiological substrate underlying substance use disorders and food addiction. Indeed, animal studies revealed similar brain networks involved in both disorders (6–10), whereas human neuroimaging studies also showed similar neuroadaptations within the reward circuits in the mesocorticolimbic system in both groups of patients (11, 12).

Eating behavior is controlled by complex networks of signals leading to homeostatic mechanisms that promote intake directly depending on energy requirements and an allostatic control that stimulates food intake independently of energy needs to accumulate energy for possible deficits of food supplies (13). The hypothalamus mainly controls homeostatic mechanisms within the central nervous system (13), whereas the main pathway for the allostatic eating control is the

mesocorticolimbic system (6, 8, 10). Notably, the cortico-striatal pathway regulates the reward circuitry, and the prefrontal cortex (PFC) is responsible for behavioral self-control (7, 14). Therefore, we focused our study on the epigenetic signatures of food addiction in the medial prefrontal cortex (mPFC) since we have recently pointed out the crucial role of this cortical area in the development of eating addictive-like behavior in mice (8). Indeed, the chemogenetic inhibition of the glutamatergic projections of the prelimbic (PL) area of the mPFC enhanced food addiction vulnerability in mice (8). Furthermore, the modulation of the endocannabinoid and the dopaminergic signaling systems in this pathway confers vulnerability to food and cocaine addiction-like behavior (15). In agreement, synaptic deficits in the PL to nucleus accumbens (NAc) core projections have been associated with addiction-like behavior towards highly palatable food (7). Consistently, mPFC hypoactivity has been associated with obesity and drug addiction in neuroimaging studies in humans (14), further supporting the interest of the mPFC as the targeted area to investigate the epigenetic signatures of food addiction.

The interactions between genes and the environment seem crucial in the vulnerability to develop food addiction. Epigenetics is essential in the interplay between these two factors to understand how the environment controls gene function changes without modifying gene sequence. Epigenetic mechanisms are mainly mediated by DNA methylation, histone modification, and microRNAs (miRNAs) (16). MiRNAs are small non-coding RNA molecules that regulate gene expression by binding to target messenger RNAs (mRNAs) to inhibit translation or promote mRNA degradation. Each miRNA can regulate up to hundreds of downstream targets, and every mRNA can be targeted by several miRNAs, creating a dynamic system that allows the cells to fine-tune gene expression (17). MiRNAs play a crucial role in metabolic alterations leading to obesity (16)

and in several psychiatric disorders and physio-pathological processes related to drug addiction, such as reward, synaptic plasticity, learning, withdrawal, and relapse (18–20). However, the role of miRNAs in the development of food addiction has not been yet investigated. Considering the close overlap between drug and food addiction and the relationships between food addiction and obesity, miRNAs could also play a crucial role as key mediators between environments and genetic vulnerability in the development of these disorders (21). Furthermore, miRNAs could represent potential biomarkers of interest for the early detection of physiopathological alterations that may lead to food addiction.

In this study, we have obtained and characterized extreme subpopulations of addicted and non-addicted male mice to identify the differential miRNAs expression in the mPFC associated with vulnerability to food addiction. Using similar food addiction-like criteria, we have applied the YFAS 2.0 score to classify a cohort of patients of both genders to assess the possible association of this behavioral disorder with circulating miRNAs. Finally, we have functionally validated the involvement of selected miRNAs differentially expressed in addicted mice and food addict patients in specific phenotypes of this addictive-like behavior.

Results

Selection of extreme subpopulations of addicted and non-addicted mice. To study the miRNAs signatures underpinning the susceptibility to develop food addiction-like behavior towards palatable food, C57Bl/6J mice (n=58) underwent a long operant food addiction protocol (98 days) to obtain standard or palatable pellets (Fig. 1A and Fig. S1A-B). We have already used a similar operant protocol to identify vulnerable and resilient mice to develop this behavior in previous

studies (8, 22). The different sample sizes of mice trained with standard (n=7) or palatable (n=51) pellets were based on the power analysis calculation (see methods) that has considered the results of our previous studies in order to obtain a significant percentage of addicted mice. The male sex was chosen considering the previous literature in food (8, 10, 23) and drug (24, 25) addiction models. In spite of all these studies previously performed in male rodents, further studies will be necessary to validate these models in female mice and rats. During FR1, mice trained with standard or palatable pellets had the same intake. However, as expected, the palatable-pellet trained group showed a higher number of responses than the standard-pellet trained group in the FR5 period (Fig. 1B). Indeed, we have reported similar differences in operant responding for standard and palatable pellets in our previous studies showing the high reinforcing value of this palatable food (10). The three hallmark criteria of food addiction, persistence of response, motivation, and compulsive-like behavior, were evaluated during the early (day 1 to 15), medium (day 42 to 55), and late (day 78 to 92) training period. In the early and medium periods, significant differences between mice trained with palatable and standard pellets were shown only in the persistence of response and motivation criteria, respectively (Fig. S1C-H). In the late period, chocolate-trained mice presented higher persistence of response, motivation, and compulsive-like behavior compared to mice trained with standard pellets (Fig. 1C-E), revealing the requirement of a long training period to fully develop all the manifestations of food addiction in this mouse model. In this late period, mice were categorized considering the three addiction-like criteria previously used in our food addiction mouse model (8, 10) based on the DSM-5 for substance use disorders and YFAS 2.0 food addiction diagnosis: persistence of response, motivation and compulsive-like behavior (26, 27). Mice that achieved 2 or 3 addiction-like criteria were considered addicted, and mice that achieved 0 or 1 criteria were considered

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non-addicted in agreement with our previous studies (8, 10) and the requirement of 55% achievement of the total criteria for severe diagnosis of the substance use disorder in the DSM-5. We obtained 25.5% of mice trained with chocolate pellets reaching 2-3 criteria (addicted mice) compared with 0% of standard pellets trained mice (Fig. 1F), confirming that only palatable food, but not standard chow, was able to trigger the addicted behavior (10). Consequently, further analyses of behavioral and miRNAs signatures of addiction were performed only in mice trained with chocolate-flavored pellets, without any further analysis in mice trained with standard pellets. In agreement, significant positive correlations between the number of criteria achieved and the results obtained in each specific criterion confirmed that addicted mice performed high values in all criteria (Fig. S1I-K). We also evaluated in our operant paradigm four well-recognized phenotypic traits related to addiction (22, 28, 29). First, impulsivity that considers the inability to change the course of an action once it is initiated (30). Second, cognitive inflexibility that indicates the incapacity to shift responding to stimuli that have previously predicted the availability of reward (31). Third, appetitive cue-reactivity, which refers to the strength shown in the association of the appetitive stimuli with the cue (32). Finally, aversive cue-reactivity that indicates the value of the aversive cue in controlling the behavior (33). We found higher impulsivity, cognitive flexibility impairment, appetitive cue reactivity, and lower aversive cue reactivity in addicted animals compared with resilient mice (Fig. 1G-J, Fig. S2A-J). The number of pellets intake and the body weight were similar in addicted and non-addicted mice (Fig. 1K-L). Significant positive correlations were also found between the number of criteria achieved and each phenotypic trait (Fig. S2C-F) underlying the relevance of these behavioral traits for the food addiction phenotype. Furthermore, levels of impulsivity, cognitive flexibility, and appetitive cue-reactivity increased over time in addicted mice, whereas they remained stable in non-addicted

mice (Fig. S2G-I). In contrast, aversive cue reactivity was stable across time in both groups (Fig. S2J). Therefore, we have identified in our operant behavioral model a particular subgroup of mice vulnerable to develop food addiction after a long operant training, and we have characterized the main behavioral traits of these mice.

Principal component analysis revealed differential patterns of behavioral factor loadings in food addiction-like behavior in mice. We have evaluated the links between the different behavioral addiction-like criteria and phenotypic traits defined in our behavioral paradigm in order to obtain objective criteria to select mice vulnerable and resilient to develop food addiction (Fig.2A). For this purpose, we have performed a principal component analysis (PCA), and the percentage of variance explained by the two principal components (PC) was 33.7% (PC1) and 24.0% (PC2) (Fig 2B). The main addiction criteria loading in PC1 were persistence and motivation, whereas compulsive-like behavior was the predominant criterion in PC2 (Fig. 2C-D). Among the four phenotypic traits, impulsivity and cognitive flexibility had the highest loading in PC1, whereas aversive and appetitive cue-reactivity showed the main loading in PC2. PCA allowed classifying two extreme phenotypes of mice vulnerable (n=6) or resistant (n=6) to develop food addiction-like behavior based on these criteria and phenotypic traits (Fig. 2A-B). These extreme subgroups of mice trained with chocolate pellets were selected to identify brain miRNAs signatures of vulnerability to food addiction (Fig. S1A). Significant differences in all behavioral addiction hallmarks (Fig. S1L-N) and phenotypic traits (Fig. S1Q-T) were confirmed between these extreme sub-groups of addicted and non-addicted mice. In contrast, no differences between groups were reported in pellet intake, indicating that the possible differential epigenetic changes are due to the addictive-like phenotype and not to distinct food intake or body weight (Fig. S1O-P).

MiRNA signatures of vulnerability to addiction in mice. We performed small RNAseq of the mPFC, an area critically engaged in loss of eating control (34), to characterize miRNA expression signatures for food addiction vulnerability. We compared miRNA profiles of extreme subpopulations of resilient (n=6) and vulnerable (n=6) mice in a discovery sample and replicated the results in independent cohorts of resilient (n=6) and vulnerable (n=6) mice of a replica sample (Fig. 2D). For this purpose, we used a quantitative gradual addiction scale to order mice in their degree of food addiction severity in the inverted U-shape curve of the normal distribution (Fig. S1A). The discovery sample was composed of animals with the 6 most extreme values of this inverted U-shape curve, and the 6 animals with the following extreme values in the curve formed the replica sample. The comparison of the extreme resilient and vulnerable cohorts identified 11 miRNAs showing significant differential expression in the mPFC both in the discovery and replica samples (Table 1-2 and Tables S1-2): nine were underexpressed (mmu-miR-29c-3p, -124-3p, -137-3p, -211-5p, -544-3p, -665-3p, -876-5p, -3072-3p and -3085-3p) and two overexpressed (mmu-miR-100-5p and -192-5p) in the addicted group compared to resilient mice (Table 1 and Tables S1-2). Significant overlap between discovery and replica was observed only for the downregulated miRNAs, but not for the upregulated ones that were then excluded for potential functional validation studies (Table 2). Network analysis revealed that downregulated miRNAs and their target genes are highly interconnected (Fig. 3A).

Target genes regulated by the miRNAs altered in addicted mice.

Many of those miRNAs co-regulate target genes involved in several pathways relevant to cognitive function and addiction, including long-term depression, glutamatergic synapse,

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cholinergic synapse, mTOR, cAMP, MAPK, oxytocin, and neurotrophin signaling pathways. These target genes are also involved in several pathways related to morphological changes in the nervous system, such as axon guidance, focal adhesion, actin cytoskeleton, adherens junction and gap junction, as well as pathways related to metabolism, including insulin resistance, lipolysis, adipocytokine, and thyroid hormone pathway (Fig. 3B, Fig. S3A-F, and Table S4).

We then explored if the expression of the targets of these miRNAs in the mPFC was altered. For this purpose, we performed RNAseq of the samples of the same addicted and non-addicted mice used to analize the miRNA profile. We observed an enrichment of altered target genes for 9 out of 11 miRNAs, either in the discovery or the replica samples. Interestingly, these enrichments were revealed for the target genes of all the miRNAs underexpressed in the addicted group compared to resilient mice: mmu-miR-29c-3p, -124-3p, -137-3p, -211-5p, -544-3p, -665-3p, -876-5p, -3072-3p and -3085-3p (Table 1). Thus, our results indicate that these particular miRNAs found differentially expressed affect the expression of their regulated transcripts in the mPFC of these addicted mice, affecting the above-mentioned pathways potentially relevant for food addiction and related processes.

MicroRNA signatures in humans. YFAS 2.0 score was used in a cohort of patients (n=51) to identify possible signatures of circulating miRNAs associated with food addiction. First, we evaluated whether the three addiction-like criteria measured in our food addiction mouse model recapitulate the principal features of the food addiction properly in our human cohort selected (Table 3) using the 35-item self-report YFAS 2.0. For this purpose, the human data extracted from the YFAS 2.0 questionnaire was analyzed, considering that several YFAS 2.0 questions can be grouped under these three addiction-like criteria used in mice. As expected, the sum of the YFAS

2.0 questions under the criteria of persistence of response, motivation, and compulsive-like behavior was much higher in participants diagnosed with food addiction than in non-addicted subjects in both genders (Fig. 4A-C). Notably, the severity of the food addiction diagnosis in humans (2-3 criteria: mild, 4-5 criteria: moderate, and 6-11: severe) positively correlated with the total question score in the three addiction criteria of persistence of response, motivation and compulsive-like behavior in women, whereas persistence of response and compulsive-like behavior also positively correlated in men, indicating that greater severity of the disease means higher rates of score in the three hallmarks of addiction (Fig. 4D-F).

Interestingly, similitudes were found in the association between these behavioral hallmarks of addiction and circulating miRNAs signatures in humans with those previously revealed in our animal studies. In agreement, the circulating levels of hsa-miR-29c-3p were negatively correlated with persistence of response and compulsive-like behavior in men, and hsa-miR-665-3p levels were also negatively correlated with motivation in this gender (Fig. 4G-I). Indeed, the circulating levels of hsa-miR-29c-3p were negatively associated with both the YFAS 2.0 (Fig. 4J) and the sensitivity to reward (Fig. 4K) scores in men, in exact agreement with the findings in our mouse model of food addiction. Circulating levels of hsa-miR-29c-3p and hsa-miR-665-3p did not correlate with these reward related behavioral responses in women. Conversely, the circulating levels of hsa-miR-192-5p were positively associated with the sensitivity to reward score in women, but not in men (Fig. 4L). The circulating levels of hsa-miR-29c-3p and hsa-miR-665-3p were also correlated negatively with the body mass index (BMI) in individuals with high YFAS 2.0 scores (data not shown). We then inspected these miRNAs in a large genome-wide association study (GWAS) of BMI, including more than 700,000 subjects, and found that MIR-665 gene was significantly associated with BMI (p=0.02). Interestingly, target genes for hsa-miR-29c-3p and

hsa-665-3p were significantly enriched among those genes significantly associated with BMI in this large GWAS cohort (Table S5), further suggesting that both miRNAs are relevant candidates to be involved in food addiction.

Functional validation of candidate miRNAs. Considering the results in these mouse and human cohorts, we performed a functional validation of the most promising candidate miRNAs identified in the mPFC of addicted mice and plasma in our human cohort. The selection of the miRNAs candidates was based on the expression level and enrichment of target genes showing differential expression (Fig. 3A, Table 1) and the previous literature about the functional role of these miRNAs. We first selected the miRNA most striking in common in our animal and human cohorts, mmu-miR-29c-3p, which was previously reported as an epigenetic marker related to methamphetamine addiction (35). Target genes of this miRNA are enriched on several pathways relevant to the addictive process (dopaminergic synapse, MAPK, and neurotrophin signaling pathways), neuronal morphological changes (axon growth, axon guidance, cellular adhesion and focal adhesion) and pathways related to digestion and metabolism (carbohydrate digestion and absorption, insulin signaling pathway, and insulin resistance) (Table S6). We aimed to mimic the underexpression of mmu-miR-29c-3p observed in the mPFC of addicted mice, which is in agreement with the negative correlation with the YFAS 2.0 score found in our human cohort. Thus, we used a Tough-Decoy inhibitor (TuD) with an adeno-associated virus (AAV)-anti-mmu-miR-29c-3p TuD-GFP, previously validated (35), which was stereotaxically microinjected into the PL mPFC to selectively inhibit mmu-miR-29c-3p function (Fig. 5A-B). We have previously demonstrated the crucial role of this subregion of the mPFC in the food intake loss of control (8). After TuD bilateral microinjection, mice underwent an operant conditioning schedule as

previously described (8, 22) to evaluate a possible early development of food addiction after short operant training due to these epigenetics manipulations (Fig. 5A). All the mice included in our behavioral analysis were correctly microinjected in this subregion of the mPFC, as revealed by GFP detection (Fig. 5C). Inhibition of mmu-miR-29c-3p in the PL area significantly increased persistence of response and enhanced motivation during a short operant training period of 29 sessions, and the percentage of mice achieving addiction-like criteria with palatable pellets was 50.0% compared to 15.8% of mice injected with a control TuD (Fig. 5E-H). In contrast, no significant differences in palatable food reinforcement during FR1 and FR5, compulsive-like behavior, and other behavioral phenotypic traits were obtained (Fig. 5D, Fig. S5A-D). A positive correlation between the number of criteria reached and the values obtained in each criterion was found (Fig. 5I-K). No differences were revealed after mmu-miR-29c-3p underexpression in other variables, such as body weight or food intake (Fig. 5L-M). These results demonstrate a crucial role of miR-29c in the mPFC for the development of two particular hallmarks of food addiction, persistence of response and motivation.

Considering the preferential involvement of mmu-miRNA29c-3p only in these specific hallmarks of addiction, we extended our validation studies to two additional miRNAs, mmu-miR-665-3p and mmu-miR-137-3p. The mmu-miR-665-3p was previously associated with the regulation of the expression of cannabinoid receptors in patients with severe heart failure and with changes in microbiota composition (36). The mmu-miR-137-3p was selected for its involvement in psychiatric disorders, including schizophrenia and impaired sociability (37). We used the same TuD strategy to selectively inhibit mmu-miR-655-3p and mmu-miR-137-3p in the PL mPFC to evaluate its involvement in food addiction development (Fig. 5A-B). Inhibition of mmu-miR-665-3p in the PL mPFC enhanced compulsive-like behavior after short-operant training, without

modifying the other hallmarks of addiction (Fig. 6C-E), the reinforcement for chocolate-flavored pellets during FR1 and FR5, or other behavioral phenotypic traits (Fig. 6B, Fig. S6A-D). Accordingly, a subset of 36.8% of mice with inhibition mmu-miR-665-3p in this brain area reached addiction-like criteria in this short training period, a percentage significantly higher than control mice (16.7%) (Fig. 6F). All the mice included in the study were correctly microinjected in this mPFC subregion, as revealed by GFP detection (Fig. 6A). Positive correlations between the number of criteria reached and the values obtained in each criterion were found (Fig. 6G-I), without significant differences in body weight or food intake (Fig. 6J-K).

Finally, functional validation of mmu-miR-137-3p was also performed using TuD in the PL mPFC (Fig. 5A-B). All the mice included in the study were correctly microinjected in this mPFC subregion, as revealed by GFP detection (Fig. 7A). Inhibition of this miRNA in mice did not yield significant differences compared to the control group in any of the three addiction-like criteria, reinforcement for palatable food during FR1 and FR5 or behavioral phenotypic traits, whereas positive correlations between the number of criteria and the values obtained in each criterion were found, as expected (Fig. 7B-I and Fig. S7). No significant differences in body weight or food intake were revealed after mmu-miR-137-3p inhibition (Fig. 7J-K).

We have validated that our TuD strategy efficiently inhibits the function of the target miRNAs by assessing the expression of related target genes in the PL mPFC. Thus, the expression of mmu-miR-665-3p target genes *Ncam1* and *Rbfox1* were significantly upregulated in AAV-anti mmu-miR-665-3p TuD, and the expression of these genes was also higher in addicted TuD mice than non-addicted mice (Fig. S4A-B). Therefore, the inhibition of mmu-miR-29c-3p and mmu-miR-665-3p expression in the PL mPFC enhanced the vulnerability to develop food addiction, whereas mmu-miR-137-3p inhibition in this brain area did not modify food addictive-like behavior.

Target genes regulated by miR-29c-3p, miR-665-3p and miR-137-3p

To further understand the regulatory mechanisms of these three miRNAs, we selectively inspected their differentially expressed target genes using the RNAseq data of the discovery and replica samples (Table S3). We found a small overlap between differentially expressed targets of these three miRNAs, suggesting that most were regulated specifically by each corresponding miRNA (Fig. 8A). This is in line with the findings observed in the functional validation using the TuD approach, in which mmu-miR-29c-3p and mmu-miR-665-3p were found to affect different behavioral hallmarks of addiction. The differentially expressed targets of mmu-miR-29c-3p were involved in interesting KEEG pathways such as focal adhesion, insulin resistance, axon guidance, PI3-Akt, and relaxing signaling pathways (Fig. 8B and table S7), and neurite out-growth signalling and extracellular matrix organization from the Reactome pathways (Table S8). In the case of mmu-miR-665-3p, differentially expressed target genes were enriched in the metabolism of lipids, according Reactome patways (Table S8). Among the enriched GO biological processes in the differentially expressed targets (Table S9), we find mRNA destabilization, extracellular matrix organization and cell adhesion (for mmu-miR-29c-3p), cellular lipid metabolic process (for mmu-miR-665-3p), and adult walking behavior (for mmu-miR-137-3p) (Table S9). Interestingly, *Trp53inp2* gene, a target of mmu-miR-29c-3p and mmu-miR-665-3p, was differentially expressed in the discovery and replica samples, and its encoded transcript participates in axonal growth by interacting with the NGF receptor TrkA and it is involved in processes of morphological changes in the nervous system (38). Also, two differentially expressed genes, *Luzp1* and *Mtmr4*, were targets for all three miRNAs (mmu-miR-29c-3p, mmu-miR-665-3p and mmu-miR-137). *Luzp1* encodes a protein involved in the actin cytoskeleton that participates in neuronal morphological

changes. Remarkably *Mtmr4*, involved in dephosphorylation, was found in a GWAS of eating behavior in pigs as highly associated with feeding behavior (39).

Discussion

We have investigated the changes in miRNAs expression promoted by food addiction in animals and humans and their involvement in the mechanisms underlying the behavioral hallmarks of this disorder. For this purpose, we have used similar food addiction-like criteria in male mice and humans of both genders to select extreme addicted and non-addicted sub-populations. MiRNAs expression was evaluated in the mPFC in the mouse cohort, and plasma circulating miRNAs were assessed in humans. Two sub-groups of extreme phenotypes of mice vulnerable and resilient to develop food addiction were selected based on the three addiction-like criteria and the main related phenotypic traits. Our behavioral characterization has taken into account each specific endophenotype that integrates this complex behavioral disease. PCA analysis of the mouse behavioral responses revealed a predominant load of two hallmarks of addiction, persistence of response and motivation, in a first component defining these extreme phenotypes, whereas the major weight in the second component corresponded to the third addiction criterion, compulsive-like behavior. Persistence of response is associated with the difficulty of stopping reward-seeking due to habit formation or disruption of extinction learning and has been reported to involve the participation of the mPFC (5, 40), whereas the compulsive-like behavior has been directly related to the network activity of the mPFC glutamatergic projections to the NAc (8). Motivation is linked to reward processing, and the mPFC has also been associated with the decision-making process evaluated in our motivation paradigm (41).

We have revealed that the extreme phenotype groups of mice showed a particular miRNAs signature in the mPFC, an area closely involved in addictive behavior (5, 8, 28, 40, 41). This signature was characterized by a predominant down-regulation of various relevant miRNAs in addicted mice, and altered expression of their specific target genes. Subsequent analysis of the target genes of these down-regulated miRNAs showed enrichment in gene pathways related to cognitive processes, which are essential for the main behavioral components of addictive behavior, including persistence of response, motivation, compulsive-like behavior, craving, and relapse (28). The miRNAs downregulated also target genes involved in other pathways relevant for addiction, such as the glutamatergic and cholinergic transmission, MAPK, and neurotrophin signaling pathways that play a crucial role in compulsive-like behavior, craving, relapse and neuronal morphological changes (28). Also, the oxytocin signaling pathway regulated by these miRNAs is relevant in addiction for its role with the mesocorticolimbic dopaminergic pathway modulating reward-related processes (42). A key gene in the oxytocin signaling pathway is Oxtr, encoding for oxytocin receptor, and is a target of miR-29c-3p differentially expressed in the discovery and replica samples. Several of the targeted genes of these miRNAs are also involved in pathways related to metabolism and obesity, including insulin resistance, lipolysis, adipocytokines, and thyroid hormone pathway. The loss of behavioral control that characterizes the hallmarks of addiction and the related behavioral phenotypic traits frequently occurs in obese patients (12, 41), underlying the relevance of these common pathways regulated by the targeted genes. Interestingly, mmu-miR-29c-3p and mmu-miR-665-3p targeted genes are also involved in the majority of these pathways, highlighting the potential relevance of these particular miRNAs in neuronal functions that are essential for the development of food addiction.

Similar addiction-like criteria to those employed in our animal studies were used to classify our cohort of patients using the YFAS 2.0 in order to evaluate the correlation of these human behavioral alterations with circulating miRNAs for a translational comparison of animal and human findings. We first verified that the three addiction criteria measured in our mouse model recapitulate the principal features of food addiction in our human cohort to ensure the translational value of the behavioral parameters evaluated. As expected, participants diagnosed with food addiction showed the highest score of the YFAS 2.0 questions under the criterion of persistence of response, motivation, and compulsive-like behavior. Taking into the previously reported sexual dimorphism in all kinds of reward-related behaviors (43, 44), including the transcriptomic profiles associated to addiction (45) and stress vulnerability (44), we have investigated this possible dimorphism in the behavioral responses evaluated in our human cohort. Both genders of addicted subjects showed similar highest and significantly correlated scores of persistence of response, motivation, and compulsive-like behavior, although men did not reach the significance in motivation probably due to the limited number of subjects of this gender reaching the YFAS 2.0 score required for food addiction. Interestingly, sharp similitudes were identified in the miRNAs signatures in our animal and human cohorts. The most striking correlations were found in men with peripheral circulating levels of hsa-miR-29c-3p. Interestingly, the circulating levels of this miRNA in men correlated with the YFAS 2.0 score, sensitivity to reward, persistence of response, compulsive-like behavior, and BMI in the same negative direction as its PFC expression was observed in our male mouse cohort, pointing hsa-miR-29c-3p as a potential biomarker for this eating disorder. Hsa-miR-665-3p also correlated with motivation and BMI in our men human cohorts in the same direction than this miRNA was correlated with the vulnerability to develop food addiction in male mice. Women did not show

any significant correlation of hsa-miR-29c-3p and hsa-miR-665-3p with the different behavioral responses evaluated. Indeed, only hsa-miR-192-5p correlated with sensitivity to reward in women, but in the opposite direction than in men, further underlying the existence of sexual dimorphism in these reward-related behavioral responses.

Considering the close similitudes between animal and human results on miR-29c-3p in terms of correlations with food addiction scores, we first validated in mice the functional relevance of this miRNA in the mPFC. The PL sub-region of the mPFC was chosen for this validation considering its crucial role in the vulnerability to develop food addiction (8). Mmu-miR-29c-3p inhibition in this brain area dramatically enhanced the development of food addictive-like behavior in mice. Indeed, half of the mice exposed to this epigenetic modification reached the addiction-like criteria in a short training period compared to the low percentage of control mice that reached these criteria. Interestingly, this epigenetic change mainly affected two particular hallmarks of addiction, persistence of response and enhanced motivation of mice. Persistence of response and motivation were the hallmarks of addiction with the main load in the first component identified by PCA to define this behavioral alteration. However, mmu-miR-29c-3p inhibition had no consequences on the third addiction-like criteria, compulsive-like behavior. Taking into account that the inhibition of this first miRNA has a partial effect on this addictive behavior, we also selected two additional miRNAs, mmu-miR-665-3p and mmu-miR-137-3p, for functional validation considering their role in responses related to metabolic (24, 25) and psychiatric disorders (28) and the common changes found in our mouse and human cohorts with regards to mmu-miR-665-3p. Inhibition of mmu-miR-665-3p in the PL mPFC also significantly enhanced the development of food addictive-like behavior. Interestingly, this epigenetic change mainly altered compulsive-like behavior, a criterion unaffected by mmu-miRNA29c-3p inhibition that represents

the main addiction hallmark in the second PCA component of this behavioral disorder. In contrast, inhibition of mmu-miRNA-137-3p in this brain area had no impact on the development of food addictive-like behavior. In agreement with the results of this behavioral validation, enrichment of the differentially expressed gene targets of mmu-miR-29c-3p and mmu-miR-665-3p was found in the discovery and replication samples, but in the case of mmu-miR-137-3p enrichment was only observed in the replication sample, which could explain in part the results obtained. It is remarkable that miR-29c-3p and miR-665-3p contribute to food addiction by differently and selectively affecting each one of the two main behavioral components that define this disorder, possibly mediating together an important interplay between genes and environment relevant for the development of food addiction. Furthermore, in humans BMI is associated with genetic risk variants present in the MIR-665 gene and also in the target genes of both hsa-miR-665-3p and hsa-miR-29c-3p, providing additional support for the important role of these miRNAs in the vulnerability to develop food addiction described in our study.

Finally, the expression of mmu-miR-29c-3p, mmu-miR-665-3p and mmu-miR-137-3p target genes was also evaluated in our mouse cohort to provide insights into the possible regulatory mechanisms involved in the behavioral responses modified by down-regulating these miRNAs. In agreement with the different behavioral responses obtained after the down-regulation of these miRNAs, a small overlap in their differentially expressed targeted genes was found in our cohort of mice. The target genes of mmu-miR-29c-3p and mmu-miR-665-3p differentially expressed are mainly involved in several pathways related to morphological changes in the nervous system, such as axon growth and guidance as well as cellular and focal adhesion. These changes suggest the possible involvement of morphological and/or neuronal connectivity changes in the behavioral effects on food addiction promoted by these miRNAs, in agreement with the

morphological (46, 47) and functional connectivity changes previously reported during drug addiction and obesity (12). Therefore, our validation studies have allowed identifying the specific role of miR-29c-3p and miR-665-3p in each main behavioral component of food addiction in a crucial cortical area for the development of this disorder, the PL mPFC (8), and suggest a possible involvement of morphological and/or neuronal connectivity changes in these mechanisms.

The identification of a differential miRNAs signature in mice and humans vulnerable to develop food addiction and of the functional role of miR-29c-3p and miR-665-3p in specific behavioral components of this disorder represents a relevant advance in the understanding of the epigenetic mechanisms underlying food addiction. The similitudes between our animal and human results provide an important translational value that supports the applicability of our findings. This novel understanding of the role of miRNAs in the development of food addiction may open new approaches for the identification of possible biomarkers for the early diagnosis of this disorder. Our findings may also be helpful for the development of future innovative therapies for food addiction and related eating disorders based on the novel strategies now available to modify miRNA activity and expression.

Methods

Detailed methods are provided in the online supplemental material. Animal procedures were conducted in strict accordance with the guidelines of the European Communities Council Directive 2010/63/E.U. and approved by the local ethical committee (Comitè Ètic d'Experimentació Animal-Parc de Recerca Biomèdica de Barcelona, CEEA-PRBB, agreement

N°9213). In agreement, maximal efforts were made to reduce the suffering and the number of mice used.

The three addiction-like criteria considered in the food addiction mouse model were evaluated using three specific behavioral tests. These criteria summarized the hallmarks of addiction based on the DSM-5 for substance use disorders and the food addiction diagnosis through the YFAS 2.0 (26, 27).

1. Persistence of response is the criterion that measures persistent desire for the reward. It is measured during three consecutive sessions of FR5 before the progressive ratio test of motivation (Figure S1B). Considers the non-reinforced active responses during the pellet-free period (10 min) of an FR5 session, when the box is illuminated and signaling the unavailability of pellet delivery, as persistence of food-seeking behavior or unsuccessful efforts to cut down.

2. Motivation is measured with the progressive ratio (PR) schedule of reinforcement to evaluate the effort to obtain the chocolate-flavored pellets (8). 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 performs to obtain one pellet was the last event completed, referred to as the breaking point. The maximum duration of the progressive ratio session was 5 h or until mice did not respond on any lever within 1 h.

3. Compulsive-like behavior is measured as the total number of shocks in the session of shock test (50 min). Each pellet delivery was associated with an electric footshock as a punishment in this test. In this shock session, mice were under an FR5 schedule of reinforcement during

50 min with two scheduled changes: at the fourth active lever-response mice received only an electric footshock (0.18 mA, 2 s) without pellet delivery, and at the fifth active lever-response, mice received another electric footshock with a chocolate-flavored pellet paired with the cue light. The schedule was reinitiated after 10 s pellet delivery (time-out period) and after the fourth response if mice did not perform the fifth response within 1 min.

The addiction-like criteria were attributed after the performance in these three behavioral tests. Then, mice were categorized as addicted or non-addicted depending on the number of positive criteria they had achieved. A mouse was considered positive for an addiction-like criterion when the score of the specific behavioral test was above the 75th percentile of the normal distribution of palatable food-trained mice of the control group. Mice that achieved 2 or 3 addiction-like criteria were considered addicted, and mice that achieved 0 or 1 addiction-like criteria were considered non-addicted in agreement with our previous studies that considered addict mice those that reached 2-3 criteria of addiction (8, 10). Similarly, the severe diagnosis in the DSM-5 of the substance use disorder does not require 100% of criteria accomplished since it is given when 6 or more criteria out of 11 are reached (55% of the total). Therefore, in our mouse model, we have also considered a similar value of 66% of the total criteria to define the addicted phenotype (2 or more out of 3 being positive).

Statistics

All statistical comparisons were performed with SPSS software (IBM, version 25). The comparison between two groups was analyzed by Student t-test or U Mann-Whitney, depending on the distribution defined by the Kolmogorov-Smirnov normality test. ANOVA with repeated measures was used when required to test the evolution over time with the subsequent post hoc analysis

(Fisher L.S.D.). The relationship between the individual values of the 3 addiction-like criteria or the 4 addiction-related phenotypic traits and the final addiction criteria achieved were analyzed by Pearson's correlation coefficient. The percentage of addicted mice compared with the non-addicted ones was tested by the chi-square analyses, the observed frequencies with the frequencies obtained in the control group were compared. A p-value <0.05 was used to determine statistical significance. The sample sizes were similar to those reported in previous publications, with a power analysis superior to 80% (8).

Author contributions

E.M-G. and R.M. conceived and designed the experimental approaches in animal studies. J.M.F.-R. conceived and designed the experimental approaches in humans. B.C. and N.F.-C conceived the characterization of miRNA signatures; A.B., A.G.-B and L.D-R. performed the behavioral phenotype characterization with the supervision of E.M.-G. and R.M. A.G.-B, J.C.-D. and L.D-R. performed statistical analyses and graphs with the supervision of E.M.-G and R.M.; J.C.-D. and L.P.-C. performed the RNA extractions, small RNA sequencing and the bioinformatic analyses supervised by B.C. and N.F.-C.; S.A. performed the DNA extractions, DNA library preparation for sequencing and analyses supervised by C.S.; A.B. and S.A. contributed to data analysis and result interpretation. J-L. and J.M-P. performed the studies in humans with the supervision of J.M.F.-R.; J.E.-C. contributed to bioinformatic analysis of behavioral data and miRNAs expression in mice. E.M.-G., and R.M. wrote the manuscript and prepared the figures and tables with the support of A.-G.-B.; B.C., J.C-D., J.M.F.-R. and N.F.C. provided a critical review of the manuscript with inputs from all the other authors.

Acknowledgments

Disclosures

The authors report no biomedical financial interests or potential conflicts of interest.

Data and materials availability

All data are available in the main text or supplementary materials. Correspondence and requests for materials should be addressed to RM. The RNA sequencing data used in this study is available at the public repository "Sequence Read Archive" (SRA) under accession number SUB11199383.

Supplementary materials

Supplementary material for this article is available. Figures S1 to S7 and tables S1 to S14 are available online.

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Figures and legends
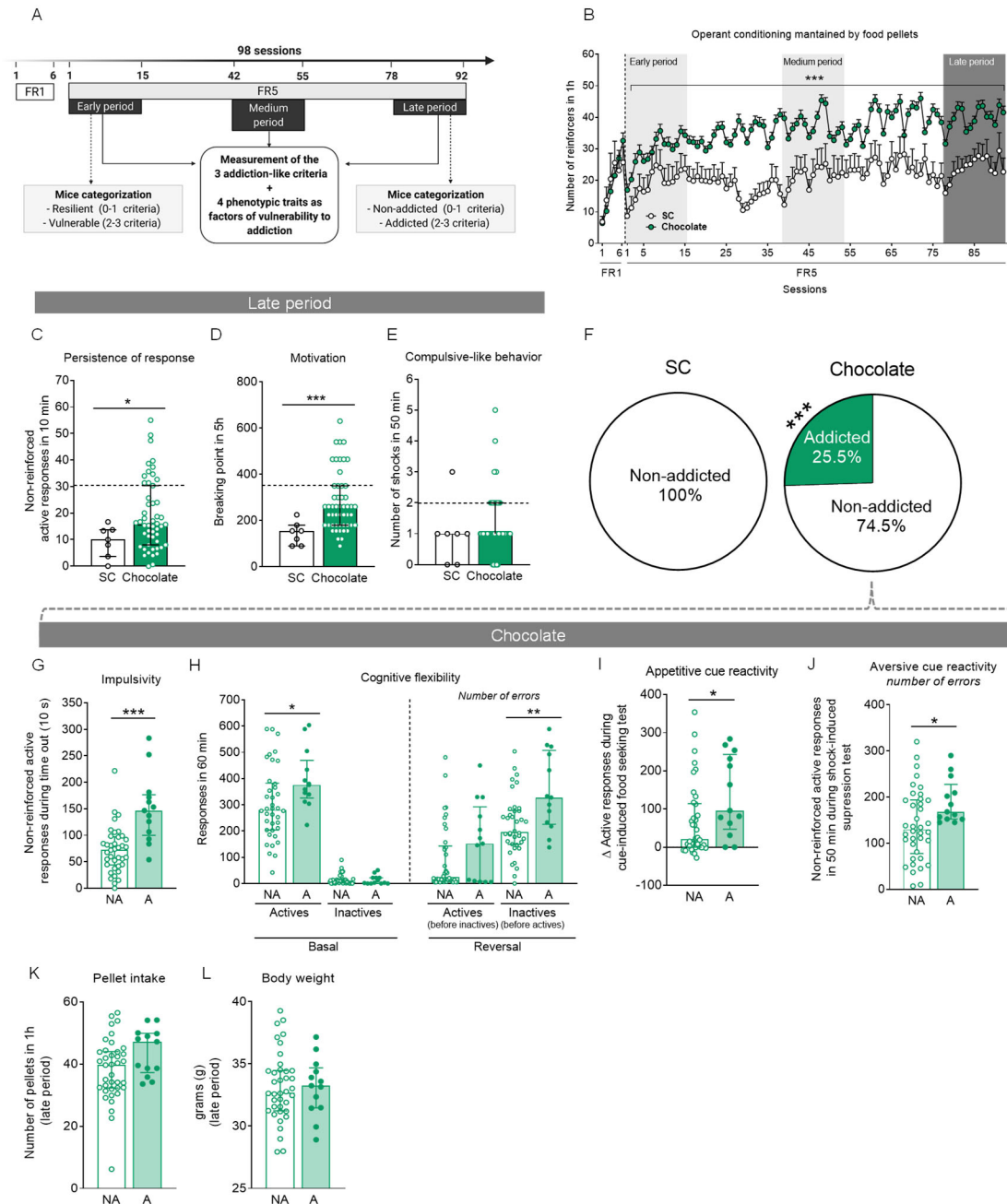
Fig. 1. Extreme subpopulations of addicted and non-addicted mice were obtained from mice trained with chocolate-flavored pellets. (A) Timeline of the experimental sequence. **(B)** Operant conditioning maintained by chocolate-flavored or standard chow (SC) pellets. Mice trained with chocolate increased the number of reinforcers in 1 h FR5 daily sessions compared to mice trained with SC (mean ± SEM; repeated measures

ANOVA; pellets effect *** P<0.001, pellets x sessions P<0.001). **(C-E)** The three addiction-like criteria for the SC and chocolate groups in the late period. **(C)** Persistence of response (U Mann-Whitney *P<0.05). **(D)** Motivation (U Mann-Whitney ***P<0.001). **(E)** Compulsive-like behavior. The dashed horizontal lines indicated the 75th percentile of the distribution of the chocolate group. It is used as a threshold to consider a mouse positive for one criterion. **(F)** Percentage of addicted and non-addicted mice trained with chocolate and SC pellets classified at the late period (Chi-square ***P<0.001). (n=51 mice trained with chocolate pellets, n=7 mice trained with ST). **(G-J)** Tests for the four phenotypic traits in the late period for the chocolate group divided into addicted (A) and non-addicted mice (NA). **(G)** Impulsivity (t-test ***P<0.001). **(H)** Cognitive flexibility (U Mann Whitney *P<0.05; **P<0.01. **(I)** Appetitive cue-reactivity. Increased active response after the presentation of the cue-light, U Mann-Whitney P<0.05). **(J)** Aversive cue-reactivity. The number of non-reinforced active responses after the shock-test with the same discriminative stimulus (grid floor) as shock-test. Pressing the active lever had no consequences: no shock, no pellets, and no cue-light (t-test, *P< 0.05). **(K)** Pellets intake and **(L)** body weight for those mice classified as A and NA of mice trained with chocolate pellets. (n=38 mice A and n=13 as NA mice trained with chocolate pellets). Data is represented with individual values with the interquartile range. Statistical details are included in Table S10.
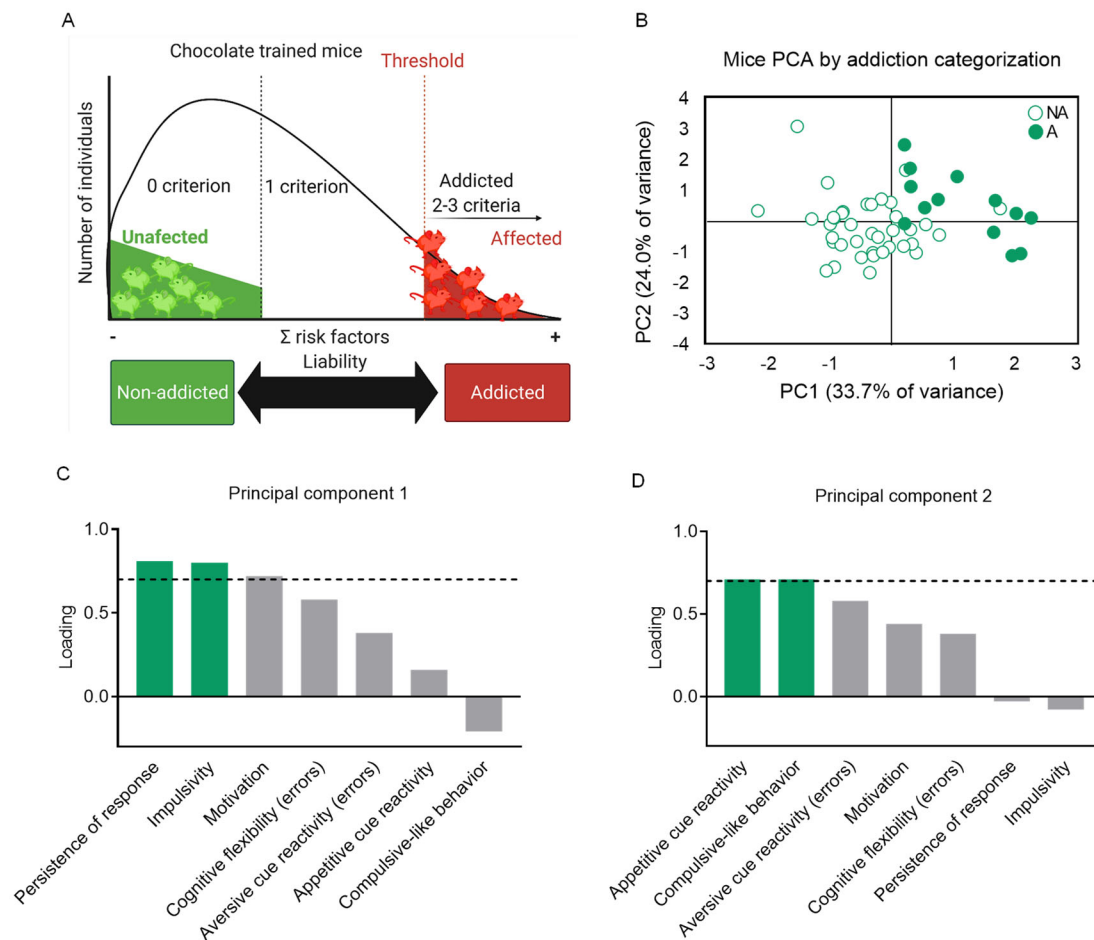
Fig. 2. Principal components analysis (PCA) of the three addiction criteria and the four phenotypic traits. (A) Inverted U-shaped curve showing that operant training with chocolate-flavored pellets allows distinguishing extreme subpopulations of addicted and non-addicted mice. **(B)** Mice subjects clustered by addicted or non-addicted on the space yielded by two components of the PCA that account for the maximum data variance with factor loadings of principal component (PC) 1 (33.7%) and PC2 (24%). **(C-D)** The order of factor loading of the different variables in the PC1 and PC2 is represented. The dashed horizontal line marked loadings >0.7, mainly contributing to the component. With respect to the addiction criteria, a dissociation between persistence and motivation for one side and compulsive-like behavior for the other is observed. Impulsivity and

cognitive flexibility weighted more in the PC1, and both cue reactivities weighted more in the PC2.
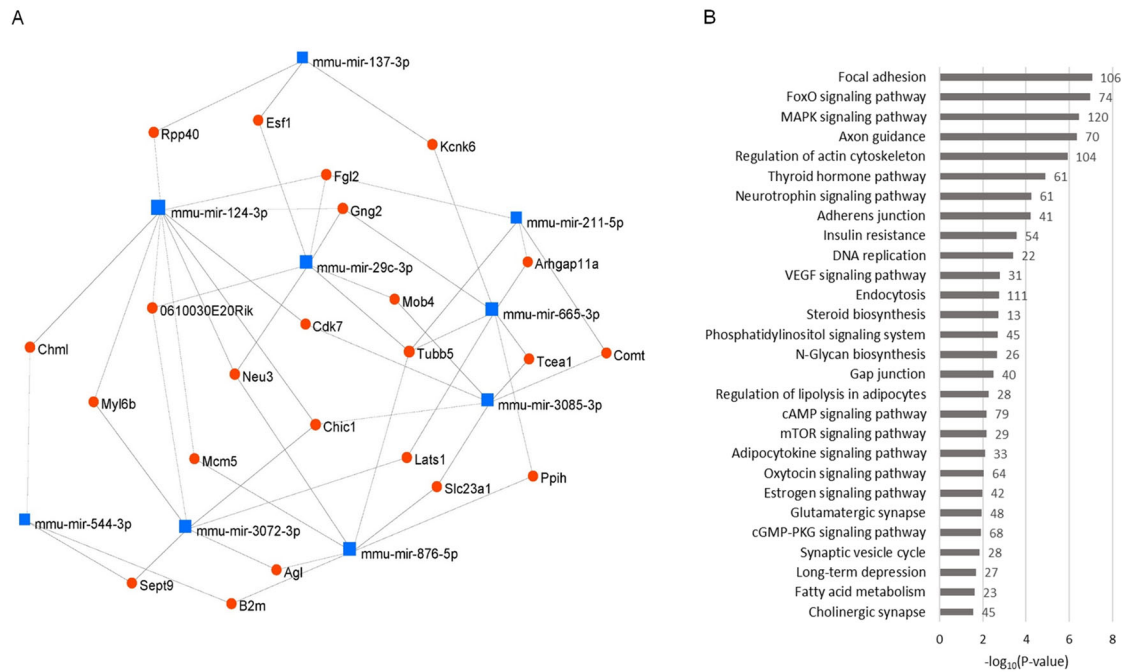
Fig. 3. MiRNAs differentially expressed in mPFC of addicted mice. (A) Network from miRNet analysis based on the 9 downregulated miRNAs and their target genes filtered to retain more nodes with more connections. **(B)** Selection of enriched KEGG pathways identified in the target genes of the downregulated miRNAs.
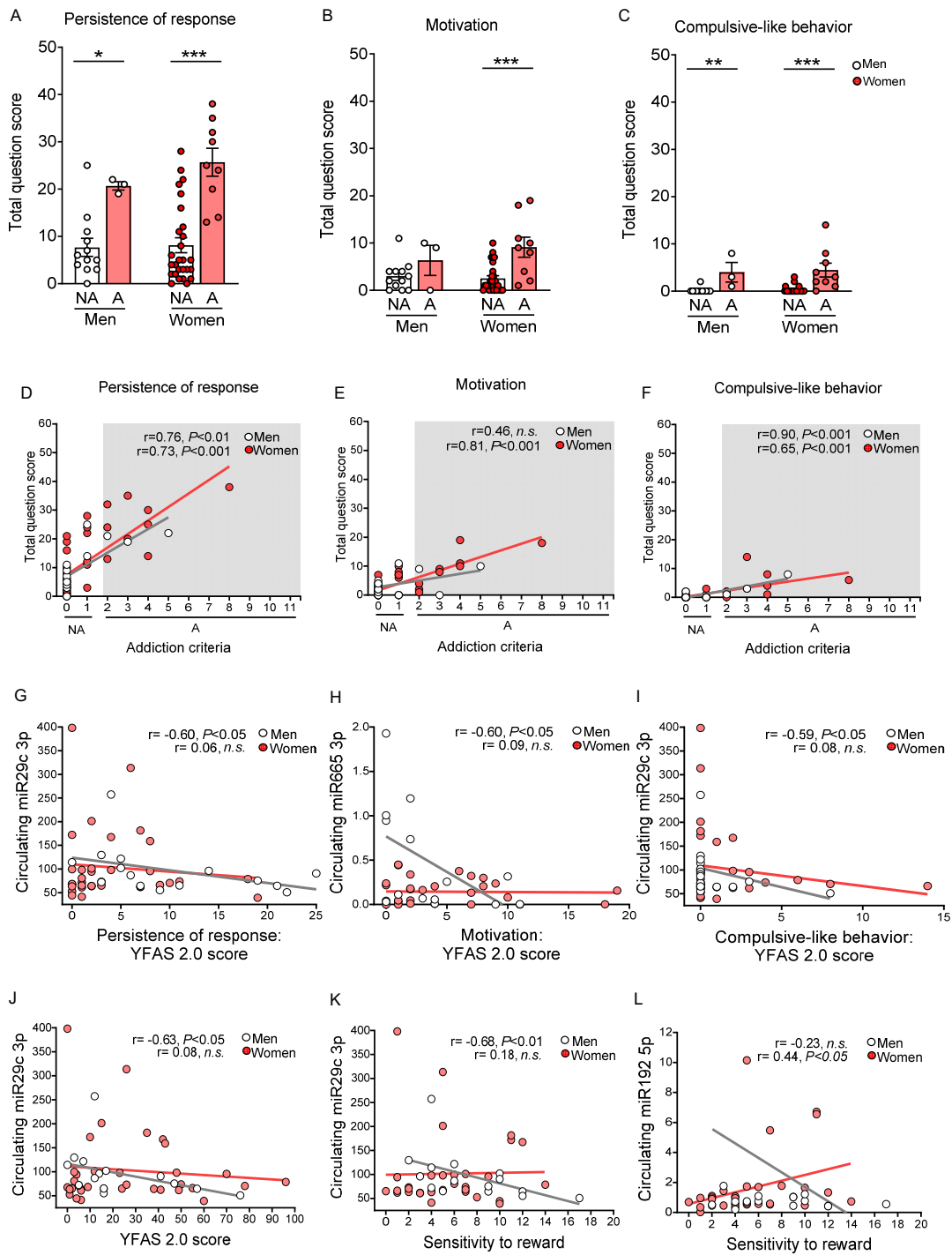
Fig. 4. Behavioral results of the three hallmarks of addiction in a human cohort comparing non-addicted ("NA") and addicted ("A") individuals. (A) in persistence of response, **(B)** motivation, and **(C)** compulsive-like behavior (median with the interquartile range), (*p<0.05,**p<0.01,***p<0.001). **(D-F)** Pearson correlations between addiction-like criteria reached and the total question score obtained in **(D)**

persistence of response, **(E)** motivation, and **(F)** compulsive-like behavior comparing "NA" and "A" participants. n=51 for human participants (n=39 NA and n=12 A). **(G)** Scatter correlation plots for the association between the expression of the circulating hsa-miR-29c-3p and the Persistence of response YFAS 2.0 score. **(H)** Scatter correlation plots for the association between the expression of the circulating hsa-miR-665-3p and the Motivation to response YFAS 2.0 score. **(I)** Scatter correlation plots for the association between the expression of the circulating hsa-miR-29c-3p and the Compulsive-like behavior YFAS 2.0 score. **(J)** Scatter correlation plot for the association between the expression of the circulating hsa-miR-29c-3p and the YFAS 2.0 score. **(K)** Scatter correlation plot for the association between the expression of the circulating hsa-miR-29c-3p and the Sensitivity to Reward score. **(L)** Scatter correlation plot for the association between the expression of the circulating hsa-miR-192-5p and the Sensitivity to reward score. Statistical details are included in Table S11.
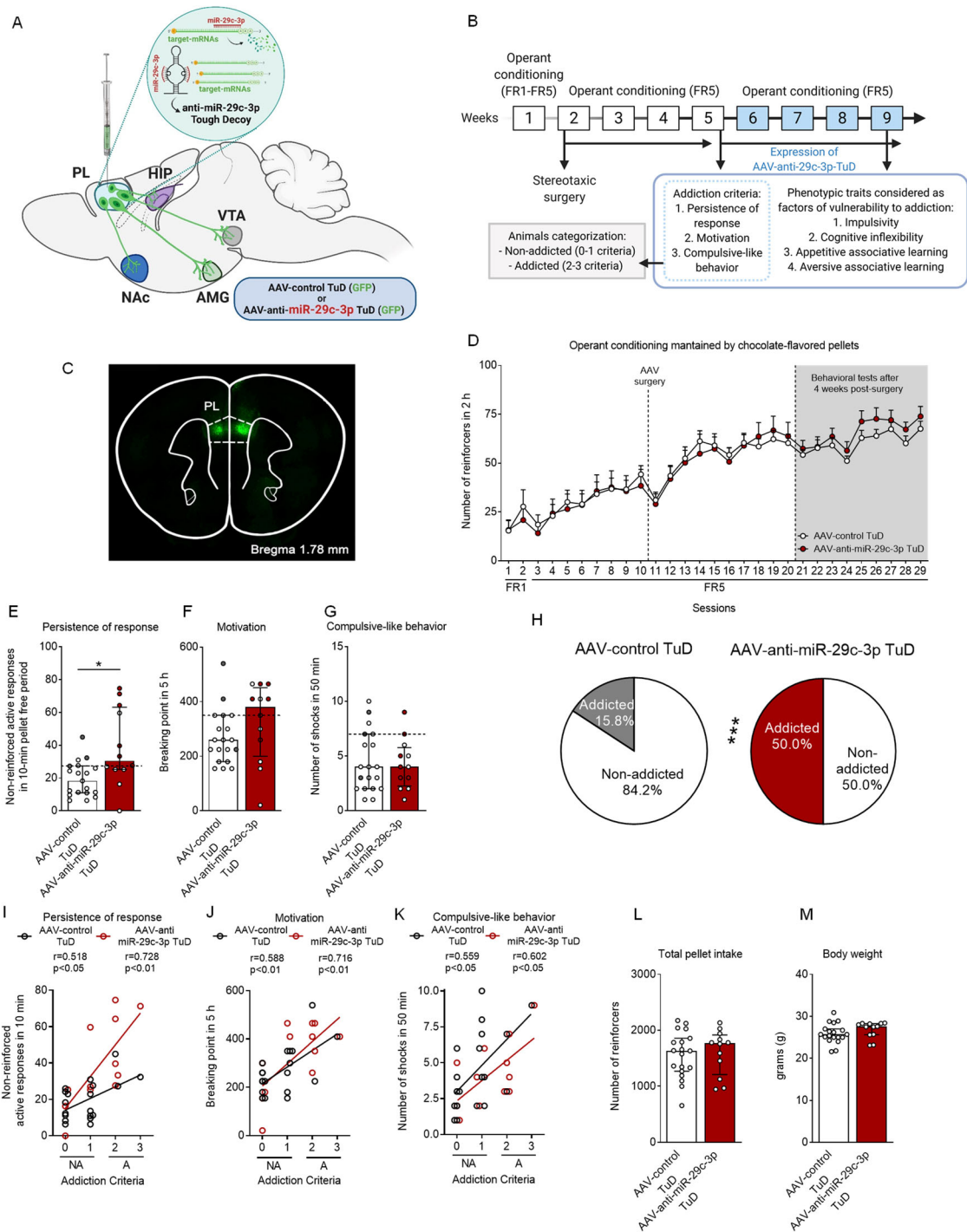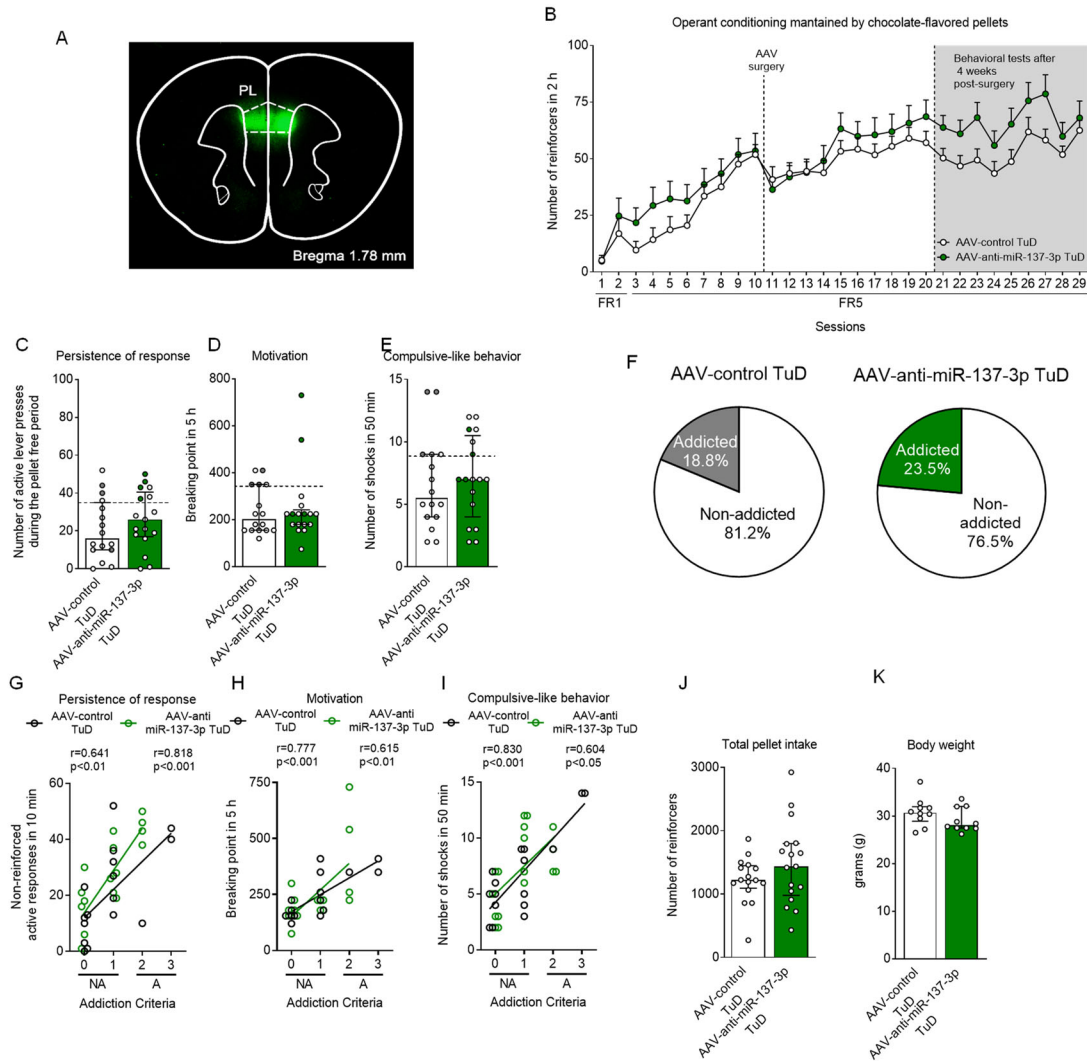
Fig. 5. Functional validation of candidate mmu-miR-29c-3p inhibition. (A) Scheme of viral strategy for inhibiting mmu-miR-29c-3p in PL neurons. Schematic representation of miRNA-mRNA interaction in basal conditions and of miRNA-inhibitor Tough-Decoy mechanism. **(B)** Experimental design. **(C)** Representative fluorescence images showing viral-dependent GFP protein expression at PL injection site. **(D)** Number of reinforcers

during operant training sessions maintained by chocolate-flavored pellets comparing AAV-control TuD mice vs AAV-anti-mmu-miR-29c-3p TuD mice. **(E-G)** Behavioral tests of the three addiction-like criteria showed increased persistence in mice inhibiting mmu-miR-29c-3p (individual values with the median and the interquartile range, t-test, *P <0.05). Addicted mice in grey-filled circles for control and in red-filled circles for mmu-miR-29c-3p mice. **(H)** Increased percentage of mice underexpressing miR-29c-3p classified as food addicted animals (chi-square, ***P < 0.001). **(I-K)** Pearson correlations between individual addiction-like criteria and **(I)** non-reinforced active responses in 10 min, **(J)** breaking point in 5 h, **(K)** number of shocks in 50 min, **(L)** pellet intake and **(M)** body weight (n=19 for AAV-control TuD and n=12 for AAV-anti-mmu-miR-29c-3p-TuD mice). Statistical details are included in Table S12.
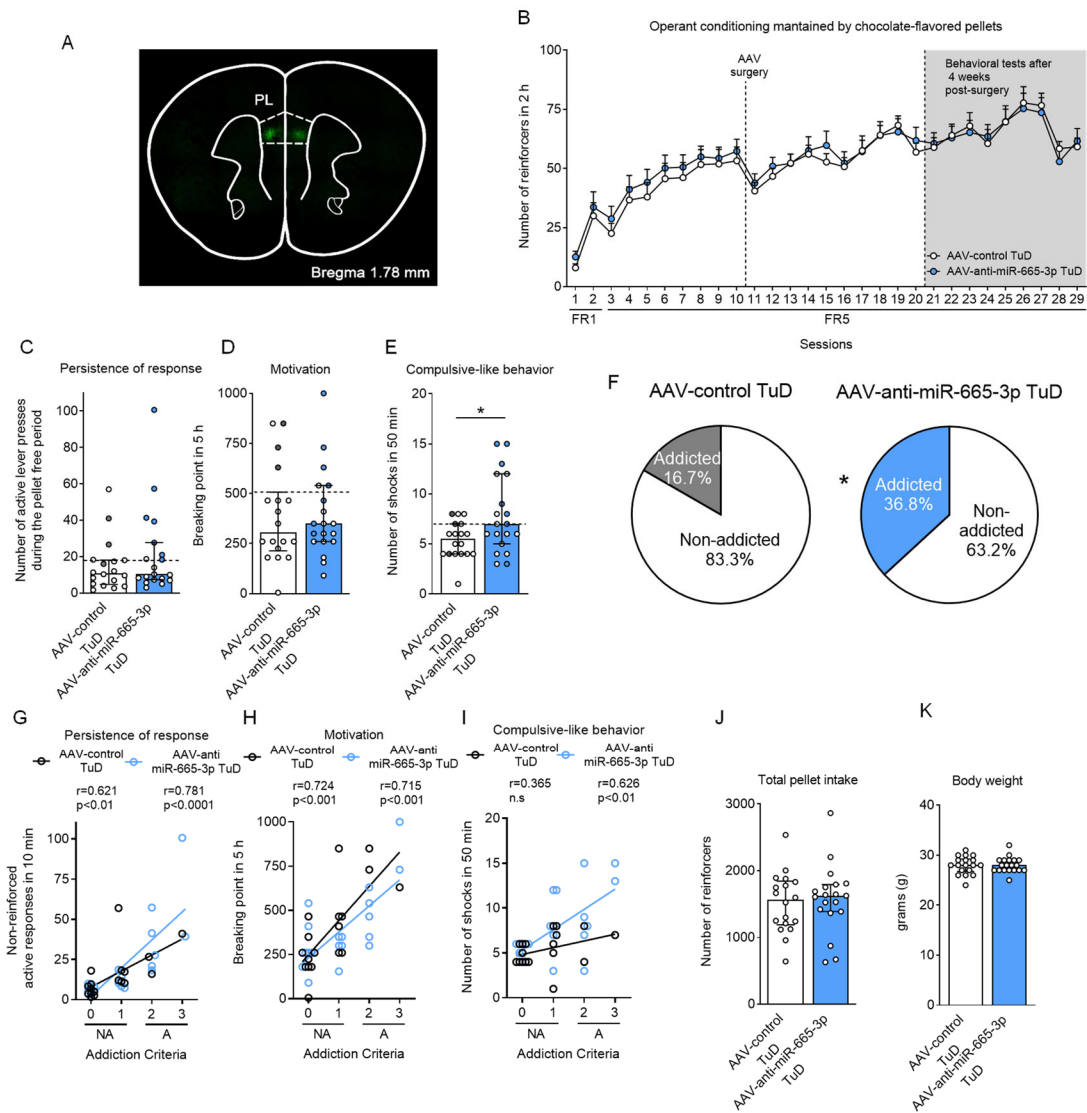
Fig. 6. Functional validation of candidate mmu-miR-665-3p inhibition. (A) Representative fluorescence images showing viral-dependent GFP protein expression at PL injection site. **(B)** Number of reinforcers during operant training sessions maintained by chocolate-flavored pellets comparing AAV-control TuD mice vs AAV-anti-mmu-miR-665-3p TuD mice. **(C-E)** Behavioral tests of the three addiction-like criteria showed increased compulsive-like behavior in mice inhibiting mmu-miR-665-3p (individual values with the median and the interquartile range, t-test, *P <0.05). Addicted mice in grey-filled circles for control and in blue-filled circles for mmu-miR-665-3p mice. (F) Increased percentage of mice with inhibited mmu-miR-665-3p classified as food

addicted animals (chi-square, *P < 0.05). **(G-I)** Pearson correlations between individual

addiction-like criteria and **(G)** non-reinforced active responses in 10 min, **(H)** breaking

point in 5 h, **(I)** number of shocks in 50 min, **(J)** pellet intake and **(K)** body weight (n=18

for AAV-control TuD and n=19 for AAV-anti-mmu-miR-665-3p TuD mice). Statistical

details are included in Table S13.

Fig. 7. Functional validation of candidate mmu-miR-137-3p inhibition. (A)

Representative fluorescence images showing viral-dependent GFP protein expression at

PL injection site. **(B)** Number of reinforcers during operant training sessions maintained

by chocolate-flavored pellets comparing AAV-control TuD mice vs AAV-anti-mmu-miR-

137 TuD mice. **(C-E)** Behavioral tests of the three addiction-like criteria do not show an increased addicted phenotype in mice inhibiting mmu-miR-137 (individual values with the median and the interquartile range). Addicted mice in grey-filled circles for control and green-filled circles for miR-137 mice. **(F)** The percentage of mice underexpressing mmu-miR-137 classified as food-addicted animals do not differ from the control group (chi-square, n.s.). **(G-I)** Pearson correlations between individual addiction-like criteria and **(G)** non-reinforced active responses in 10 min, **(H)** breaking point in 5 h, **(I)** number of shocks in 50 min, **(J)** pellet intake and **(K)** body weight (n=16 for AAV-control TuD and n=17 for AAV-anti-mmu-miR-137 TuD mice). Statistical details are included in Table S14.
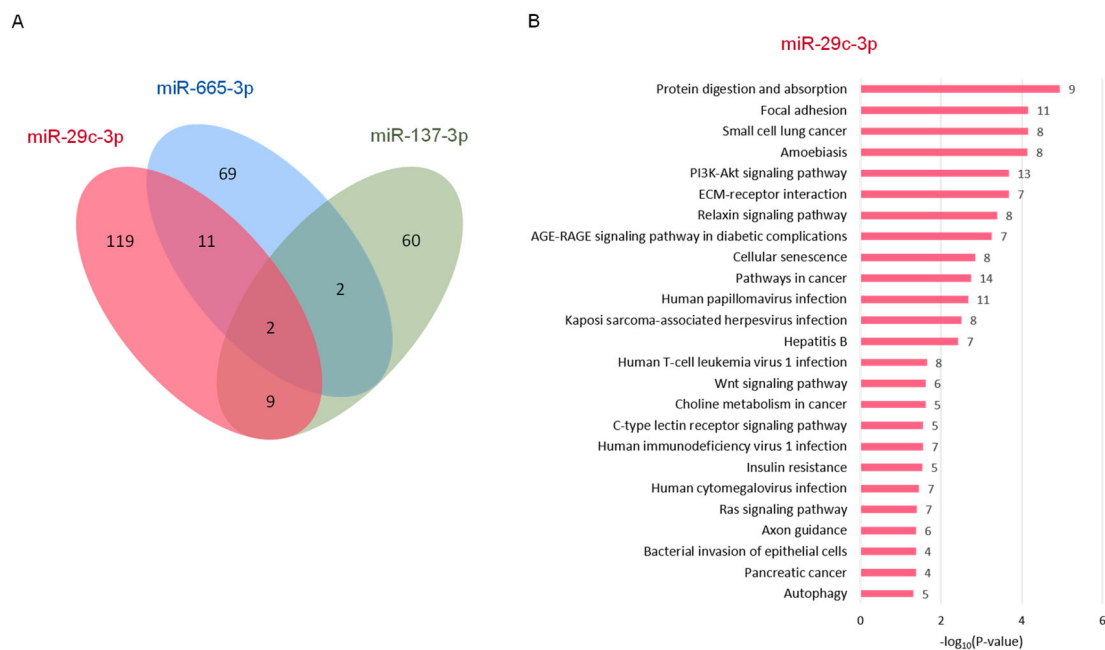
Fig. 8. Differentially expressed targets of miR-29c-3p, miR-665-3p and miR-137-3p.

(A) Venn diagram of the genes found differentially expressed that are targets of these three miRNAs. Genes were considered as differentially expressed only when targets were enriched in the RNAseq data in the discovery sample (statistically significant for miR-29c-3p and miR-665-3p) or in the replication sample (miR-29c-3p, miR-665-3p, and

miR-137-3p; Table 1). **(B)** Enriched KEGG pathways identified in the target genes of the

miR-29c-3p.

Table 1. Summary of the main results from the smallRNAseq and RNAseq analyses and the selection criteria for the functional validation.

| | Results from the smallRNAseq | | | | | | Enrichment analysis of miRNA target genes[A] | | | | | | | | |
| | Discovery | | | Replica | | | Discovery | | | Replica | | | | | |
miRNA	p-value	FC	Expression	p-value	FC	Expression	miRNA targets	DE miRNA targets	Fisher p-value	miRNA targets	DE miRNA targets	Fisher p-value	smallRNAseq significant overlapp	Expression >300	Enrichment of DE targets
mmu-miR-876-5p	0.0003	-1.85	18.51	0.0393	-1.36	15.09	129	18	ns	140	15	1.2E-03	Yes	-	Yes
mmu-miR-211-5p	0.0020	-2.02	74.78	0.0381	-1.40	67.45	511	69	8.9E-03	520	51	7.2E-08	Yes	-	Yes
mmu-miR-3085-3p	0.0073	-1.35	232.02	0.0019	-1.46	239.47	168	26	2.2E-16	187	10	ns	Yes	-	Yes
mmu-miR-665-3p	0.0089	-1.27	337.76	0.0049	-1.39	300.46	380	64	3.7E-05	387	25	0.0350	Yes	Yes	Yes
mmu-miR-3072-3p	0.0174	-1.41	144.61	0.0347	-1.32	115.77	18	2	ns	19	2	ns	Yes	-	-
mmu-miR-124-3p	0.0266	-1.26	81006.63	0.0326	-1.16	77118.81	1253	227	1.7E-04	1270	102	1.9E-09	Yes	Yes	Yes
mmu-miR-29c-3p	0.0294	-1.33	3002.98	0.0139	-1.28	2060.52	650	93	4.4E-04	668	57	2.4E-10	Yes	Yes	Yes
mmu-miR-544-3p	0.0366	-1.44	77.83	0.0374	-1.32	45.85	100	7	ns	101	11	4.7E-03	Yes	-	Yes
mmu-miR-137-3p	0.0383	-1.28	9233.46	0.0315	-1.28	7006.74	579	83	ns	581	73	4.5E-16	Yes	Yes	Yes
mmu-miR-100-5p	0.0405	1.17	91517.51	0.0262	1.22	59293.02	38	2	ns	40	8	3.0E-04	-	Yes	Yes
mmu-miR-192-5p	0.0459	1.16	4450.81	0.0283	1.19	2508.12	93	12	ns	96	7	ns	-	Yes	-

[A]Two-tailed Fisher exact test considering 21.856 mouse protein coding genes and 2.222 differentially expressed protein coding genes in the RNAseq analyses of the discovery and 958 in the replica. In green miRNAs selected for the functional validation with TuD system

Table 2. Comparative analyses examining overlap of differentially expressed miRNAs in mPFC between the discovery and replica samples.

	Nº DE miRNAs discovery	Nº DE miRNAs replica	Common	p-value	OR (95% CI)
Downregulated	44	61	9	<u>5.06E-03</u>	3.34 (1.55-6.78)
Upregulated	28	39	2	4.18E-01	

DE: Differentially expressed; OR: Odds ratio; Underlined: significant p-values (p < 0.05). *Two-tailed Fisher exact test considering the 774 mature miRNAs identified in the smallRNAseq analysis.

Table 3. Demographic data of the human participants included in the study.

	Non-addicted n=39	Addicted n=12	p-value
Age (years)	51,02 ± 9,64	48,95 ± 8,12	0,504
Sex (M/W)	12/27	3/9	0,708
BMI (kg/m^2)	28,47 ± 6,96	39,11 ± 9,71	**1,10E-04**
Fasting glucose (mg/dl)	96,03 ± 8,60	97,92 ± 14,48	0,578
Cholesterol (mg/dl)	202,28 ± 39,32	205,25 ± 32,47	0,813
HDL cholesterol (mg/dl)	65,72 ± 19,23	55,58 ± 10,44	0,088
LDL cholesterol (mg/dl)	123,21 ± 29,48	126,83 ± 24,17	0,700
Triglycerides (mg/dl)	91,31 ± 43,87	114,50 ± 57,31	0,143
GPT (IU l^{-1})	21,62 ± 8,90	17,33 ± 7,22	0,136
GGT (IU l^{-1})	26,87 ± 20,31	22,58 ± 11,73	0,491
Hba1c (%)	5,46 ± 0,21	5,63 ± 0,29	**0,029**
YFAS	13,95 ± 13,19	59,75 ± 17,14	**4,04E-13**
Persistence of response	7,97 ± 7,67	24,42 ± 7,93	**4,76E-08**
Motivation	2,67 ± 2,98	8,42 ± 6,05	**4,43E-05**
Compulsivity	0,23 ± 0,67	4,33 ± 4,03	**1,07E-07**