Maternal obesity programs hypothalamic miR-505-5p expression in mouse offspring and impacts hypothalamic fatty acid sensing

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Abstract

In utero exposure to maternal obesity programs a susceptibility to develop obesity. Animal models have shown that offspring obesity is often preceded by increased food intake, however, the mechanisms that mediate these changes are not understood. Using a mouse model of maternal diet-induced obesity we observed increased intake specifically of a high-fat pellet in adult offspring of obese mothers. Through small RNA sequencing, we identified programmed overexpression of miR-505-5p in the hypothalamus of offspring of obese mothers that is established in the fetus, and confirmed *in vitro* that fatty acid exposure increases expression of miR-505-5p. Pulsed SILAC analysis demonstrated protein targets of miR-505-5p are enriched in pathways involved in fatty acid metabolism. These include key components of neuronal fatty acid sensing, such as Cpt1a, that are implicated in BMI regulation in human genetic studies. Over-expression of miR-505-5p decreased neuronal fatty acid uptake and metabolism *in vitro*. Importantly, intra-cerebroventricular injection of a miR-505-5p mimic in mice resulted in increased high-fat pellet intake. Collectively these data suggest that maternal obesity induces over-expression of miR-505-5p in offspring hypothalamus, resulting in altered fatty acid sensing and increased intake of high-fat diet. This represents a novel mechanism by which maternal obesity programs obesity in offspring.

1. Introduction

The rapid rise in metabolic disorders observed worldwide represents a significant health challenge. A strong influence of genetic predisposition to develop metabolic disease is undisputed, but the rapid increase in obesity prevalence suggests a robust interplay between genetic factors and the environment [1]. It is now well established that the phenotype of an individual can be influenced by *in utero* and early post-natal environmental conditions, such as the nutritional state of the mother, giving rise to the notion of "developmental programming" [2]. Numerous studies in humans and animal models have highlighted the association between maternal obesity and a number of long-term adverse health outcomes in the offspring, including risk of obesity, type 2 diabetes, and cardiac dysfunction [3]. Growing evidence from animal models shows that exposure to maternal obesity can cause disruption of the hypothalamic circuits responsible for maintenance of energy homeostasis, which may contribute to increased offspring susceptibility to obesity later in life [4].

The hypothalamus is the key region of the brain controlling metabolism, containing specialized neurons that sense nutrients and hormones. The exposure to increased fat and/or sugar during critical windows of development has been shown to program increased food intake in animal models [4], including increased preference for high- fat and high- sugar foods in rats [5,6]. Little is known about the molecular mechanisms by which maternal overnutrition programs permanent changes in feeding behaviour in offspring, except for research from the Bruning and Bouret groups showing that maternal obesity alters development of hypothalamic neuronal projections [7,8] and parts of the dopaminergic circuitry [9].

The stable nature of metabolic phenotypes in offspring exposed to maternal obesity suggests lasting changes to gene profiles across the lifespan. There is accumulating evidence that miRNAs, which are small non- coding RNAs that regulate gene function through degradation of mRNAs and/or inhibition of protein translation [10], may reflect an important mechanism by which maternal environment can alter long-term phenotypes in the offspring [3,11]. We have previously reported that programmed cell-autonomous up-regulation of miR-126 in adipose tissue of offspring of obese mothers is responsible for downregulation of the genes *Irs1* and *Lnpk*, contributing to both insulin resistance and endoplasmic reticulum stress respectively in

these animals [12,13]. Furthermore, another study has demonstrated that up regulation of miR-204-5p expression in brown adipose tissue of offspring born to obese mothers leads to enhanced white adipogenic commitment [14]. Few studies to date have explored the role of miRNAs in the regulation of hypothalamic energy sensing [15-18], and none have examined the potential contribution of miRNAs to programming of long-term hypothalamic function by the perinatal environment.

A growing body of research suggests that neurons within the hypothalamus are able to sense and respond to changes in fatty acid (FA) levels in the circulation [19-22], in a manner similar to that previously reported for glucose-sensing neurons [23]. Circulating FAs, particularly long chain fatty acids, may act as cellular messengers, which inform specific neuronal subtypes in the hypothalamus about whole body nutrient status allowing the regulation of feeding behaviour, hepatic glucose production and insulin secretion in order to maintain energy homeostasis [20,24,25]. Studies in rodents and humans have shown that oleic and palmitic acid alter hypothalamic expression of neuropeptide Y (NPY) and pro-opiomelanocortin (POMC) respectively, [26-28], and are associated with a reduction in food intake. Furthermore, both oleic [29] and octanoic acid [30] can reduce food intake and increase energy expenditure via regulation of POMC neuron excitability, confirming that several FAs can act centrally to regulate food intake. Notably, some studies have suggested that neuronal responses to FAs are blunted in diet- induced obese animals [31,32], and may contribute to their lack of energy homeostasis. Whether the nutritional environment *in utero* impacts on neuronal FA sensing- as has been reported for glucose sensing [33,34]- remains unclear, although one study has suggested that maternal HFD consumption during pregnancy alters the sensitivity of offspring ventromedial hypothalamic neurons to oleic acid [33].

The aim of this study was therefore to explore the contribution of hypothalamic miRNAs in mediating the effects of maternal obesity on offspring obesity risk. We profiled miRNA expression in the offspring hypothalamus to identify miRNAs that could mediate increased high-fat diet intake and adopted a combination of *in vitro* and *in vivo* approaches to demonstrate the causal effects of altered levels of a specific miRNA on hypothalamic function and feeding behaviour.

2. Methods

2.1. Mouse model

We utilised a mouse model of maternal diet-induced obesity previously published by our group [35]. Briefly, female C57BL/6J mice were randomly assigned (by a technician not involved in subsequent phenotyping) prior to mating to either a control chow diet (RM1; Special Dietary Services, Witham UK) or a highly palatable obesogenic high-fat diet (45% total calories from fat, Special Dietary Services, Witham UK) supplemented with condensed milk (Nestle, UK) fortified with mineral mix AIN93G (Special Dietary Services, Witham UK). After approximately three weeks on the respective diets, females were mated with chow-fed males for their first pregnancy. Dams remained on their respective diets throughout pregnancy and lactation and the first litter was culled after weaning. After first pregnancy, proven breeders from the obese group with a body composition around 30–35% fat and body weight exceeding 35g were mated for second pregnancy along with age matched lean dams. A subset of dams was sacrificed on embryonic day 13 (E13) to collect fetal hypothalamus. For studies requiring adult offspring, on post-natal day 2 litters were reduced to 6 pups to ensure standardized milk supply. One male and female mouse per litter was included in each separate experiment, therefore the statistical number relates to the dam.

2.2. Food intake and body composition analyses in offspring

At weaning, male and female mice from control or obese mothers were randomly assigned to either a control chow (as above) or high-fat diet (as above), creating 4 experimental offspring groups: offspring born to control mothers weaned onto a chow diet (Off-C Chow), offspring born to control mothers weaned onto a HFD (Off-C HFD), offspring born to obese mothers weaned onto a chow diet (Off-Ob Chow), offspring born to obese mothers weaned onto a HFD (Off-Ob HFD) (illustrated in Figure 1A). *Ad libitum* food intake was measured twice a week at 09:00 from the age of 3 weeks to 8 weeks. Animals were weighed weekly, and body composition determined in fed animals using Time-Domain Nuclear Magnetic Resonance imaging (TD-NMR; Minispec Plus, Bruker, Billerica, MA, USA).

2.3. Small RNA sequencing in the offspring hypothalamus

Small RNA sequencing was conducted in 8-week-old male offspring of control and obese mothers fed a chow diet (Off-C Chow and Off-Ob Chow groups), to avoid confounding factors of HFD consumption on hypothalamic gene expression. After culling by cervical dislocation, brains were immediately frozen and stored at -80°C until sectioning at 12 µm on a cryostat (Leica Biosystems, Wetzlar, DE). PVH and ARC tissue was collected from brain sections using laser capture microdissection with a PALM Robot Microbean (Carl-Zeiss, Gottingen, DE). RNA was extracted using a miRNeasy Micro Kit (Qiagen Sciences), following the manufacturer's protocol, to preserve total RNA including miRNA. Library preparation and small RNA sequencing of the PVH and ARC samples was performed as described previously [36] using a TruSeq® Small RNA Library Preparation kit (Illumina, Cambridge, UK). Libraries were sequenced using a HiSeq 4,000 platform. Raw reads were mapped to the mouse genome through bowtie v1.2.3. Reads per Kilobase of transcript per Million mapped reads were produced from RNA Sequencing raw output and statistically analysed through likelihood ratio test using edgeR version 3.36.0. A P value cut off of <0.05 was used to determine significantly regulated miRNAs.

2.4. Cell culture and in vitro treatments

For neural progenitor cell culture, hypothalamus was collected from fetuses on E13. Following rapid dissection of the fetal hypothalamus under a dissecting scope, neural progenitor cells were harvested as described previously [37] and cultured in Neurocult ™ proliferation media (Stem Cell Technologies, Cambridge UK) containing recombinant human EGF (Stem Cell Technologies, Cambridge UK) according to manufacturer's instructions. Cells were cultured for 8 days *in vitro* before extraction of RNA as described below. mHypoE-N46 cells (generously donated by Dr. Clemence Blouet) cultured in growth medium (High glucose Dulbecco's modified Eagle Medium supplemented with 5% fetal bovine serum, 1% Penicillin-Streptomycin, 2% L-glutamine) were treated for 24h with glucose (20 mM), oleic acid (250 µM), linoleic acid (250 µM), palmitic acid (250 µM) or stearic acid (250 µM) (all purchased from Sigma- Aldrich, Saint Louis, Missouri, USA). After 24h incubation with respective treatments, RNA was extracted as described below. To analyse the gene expression of miR-505-5p targets, mHypoE-N46 cells were transfected with a miR-505-5p mimic (miRCURY LNA miRNA mimic, Qiagen) or negative control, using Lipofectamine-3000 transfection kit

(Invitrogen, Waltham, Massachusetts, USA). After 48h of transfection, RNA was extracted for gene expression analyses.

2.5. Pulsed stable isotope labelling by amino acids in cell culture (pSILAC)

pSILAC proteomic methodology was performed as described previously [12]. Briefly, GT1-7 cells were cultivated in regular DMEM media containing light lysine (Lys 0) in two cell culture dishes for 24h and then transfected with miR-505-5p mimic or negative control. After 8h, normal growth medium from both cell populations was replaced with SILAC media containing either heavy lysine [L-lysine-$^{13}C_6$-$^{15}N_2$ (Lys8)] or medium-heavy lysine [L-Lysine-$^{2}H_4$ (Lys4)] for the miR-505-5p mimic cells and miRNA negative control cells, respectively. After 24h, protein extracts from both cell populations were combined and subjected to SDS-PAGE separation, digested with trypsin and submitted to high performance liquid chromatography tandem mass spectrometry (HPLC/MS/MS). The ratio of heavy and medium-heavy peptides obtained reflected differences in the translation of the corresponding protein induced by the miRNA mimic. Raw signal data were processed in Maxquant 1.5.2.8 using a Uniprot Mus musculus database (downloaded on 29 Jan 2016, 24,802 sequences). Results were merged with predicted targets for miR-505-5p obtained from Target Scan (http://www.targetscan.org/vert_72/). After bioinformatic analysis, differentially expressed transcripts were validated by qPCR analysis. Pathway enrichment analysis was performed in Ingenuity® Pathway Analysis (IPA – Qiagen). A P- value cut-off of 0.01 was used to determine genes to be mapped to IPA networks.

2.6. RNA extraction and gene expression analysis

All RNA extraction from tissue and cultured cells was performed using the miRNeasy Micro Kit (Qiagen) to preserve total RNA including small RNAs. cDNA was reverse transcribed using a RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) or miRCURY LNA RT Kit (Qiagen Sciences) for analysis of mRNA or miRNA respectively. Gene expression was analysed using a StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific, USA) and either Power SYBR Green PCR Master Mix or Taqman Universal PCR Master Mix (both Thermo Fisher Scientific, USA). Relative quantification of mRNA and miRNA was calculated by the $2^{-\Delta\Delta Ct}$ method. Data were normalized to the geometric average of housekeeping genes *Snord68* and *Unisp6* when

miRNA was analysed or to *Sdha* when target genes were analysed (the expression of housekeepers did not change between groups) and values were reported as fold changes compared to values obtained from the control group (set at 1.0). Primers for SYBR Green-based PCR of mRNA were obtained from Sigma Aldrich, miRCURY LNA assays for qPCR of miRNA were purchased from Qiagen, and TaqMan probes were obtained from ThermoFisher Scientific. All primer sequences and assay IDs are listed in Supplementary table 1.

2.7. Fatty acid uptake

mHypoE-N46 cells were cultured in a 24 well plate and transfected with miR-505-5p mimic or negative control as described above. After 48h, cells were treated with 1 µM [1-^{14}C]-oleic acid (PerkinElmer) diluted in high glucose DMEM containing 0.05% BSA for 1h. Cells used for protein quantification were incubated in uptake medium not containing the radionuclide. After the incubation, media from wells were removed and transferred to 4 ml of scintillation fluid. Cells were carefully washed three times in 1 ml ice cold 0.1% fatty acid free BSA in PBS. After washing, cells were scraped into 200 µl RIPA buffer, transferred to microcentrifuge tubes and centrifuged at 16,000 g for 10 min. 100 µl of lysate was added to 4 ml of scintillation fluid and a beta scintillation counter used to measure concentration of radionuclide. The fatty acid uptake rate was calculated using the concentration of radionuclide, incubation time and total protein amount (pmol/min/mg).

2.8. Fatty acid metabolism

A fatty acid oxidation protocol was adapted from Pantaleao *et al.* [36]. Briefly, mHypoE-N46 cells were cultivated in a 24 well cell culture dish and transfected with miR-505-5p mimic or negative control in triplicate. After 48h of transfection, cells were washed with warm PBS and incubated in 500 µl of High-glucose DMEM containing 12.5 mM HEPES, 0.3% fatty acid-free BSA, 1 mM L-carnitine, 100 µM oleic acid and 0.4 µCi/ml [1-^{14}C]-oleic acid. 200 µl of concentrated hydrochloric acid was injected into the wells, and the acid solution was left to incubate for an extra hour. The acid solution was then centrifuged at maximum speed (≥ 14,000 × g) for 10 min at 4 °C and 500 µl supernatant transferred to a scintillation vial for radioactivity measurement of acid soluble metabolites from fatty acid metabolism (Acetyl-CoA, acyl-carnitines etc).

2.9. Characterisation of miR-505-5p targets in human datasets

miR-505-5p fatty acid metabolism related target genes were mapped to orthologous genes in the human genome using ENSEMBL and integrated with common variant genome-wide association study (GWAS) data for body mass index (BMI), from the GIANT BMI study [38]. Each of the miR-505-5p target genes was annotated based on proximity to genome-wide significant signals ($p<5x10^{-8}$), in 1Mb windows; 500kb up- or downstream of the gene's start or end site. For genes with proximal GWAS signals, we calculated genomic windows of high linkage disequilibrium (LD; $R^2>0.8$) for each given signal and mapped these to the locations of known enhancers for the target genes, using the activity-by-contact (ABC) enhancer maps [39]. We also performed a gene-level MAGMA analysis [40], which collapses all observed genomic variants within a given gene and calculates aggregate gene-level associations to the phenotypic trait. Genes exhibiting an FDR-corrected MAGMA p-value <0.05 were considered significant. Finally, for genes in loci containing at least a suggestive level of GWAS significance ($p<5x10^{-5}$), we performed SMR and HEIDI tests (v1.02; [41]) using blood expression quantitative trait loci (eQTL) data from the eQTLGen study [42]. For the eQTL analyses, we considered gene expression to be associated with BMI if the FDR-corrected p-value for the SMR-test was p<0.05 and the p-value for the HEIDI test was >0.001.

2.10. Intra-cerebroventricular injection of miR-505-5p mimic

Adult C57BL/6J male mice were implanted with an intra-cerebroventricular (ICV) canula (Plastics One, Inc.) in the lateral ventricle (coordinates from Bregma: anteroposterior -0.4; mid-lateral -1.0 and dorsoventral -2.3 mm). One week prior to the ICV surgery, mice were exposed to a high fat diet (60% fat) for familiarization and for basal food intake measurement. After 5 days of recovery from surgery, mice received a 0.5µM dose of a miR-505-5p mimic (Qiagen) or negative control combined with HiPerfect transfection reagent (Qiagen). The ICV treatment was repeated once every 24h for 3 days through the cannula and *ad lib* food intake was measured once every 24h.

2.11. Statistics

Data were analysed using Prism 8 (Graphpad, La Jolla, USA) by different tests according to the numbers of groups and time points: unpaired or paired t-test (as specified) for experiments with two treatment groups, one-way ANOVA for experiments with 3 or more groups at one time point, two-way ANOVA for experiments with 3 or more groups over time. Tukey multiple comparisons test were performed after ANOVA. Outliers were detected by Grubb's method and excluded, as described in the figure legends. For pSILAC, statistical analysis of normalized labelled: unlabelled protein ratios was performed using multiple linear models with Bayesian correction using limma package (v.2.16.0) for R (v.4.1.2). All data are shown as mean $\pm$ SEM. In each case n refers to the number of different litters represented.

2.12. Study approval

The animal studies performed in this manuscript were licensed by the UK Home Office under project license P5FDF0206, the University of Cambridge Animal Welfare and Ethical Review Body and by the Ethics Committee of Animal Welfare from Instituto de Ciencias Biomedicas-Universidade de Sao Paulo (protocol number 72/2017).

3. Results

3.1. Offspring born to obese mothers show increased intake of a high-fat diet and subsequent increased adiposity

Maternal diet-induced obesity during pregnancy has been suggested to program increased food intake and subsequently adiposity in the offspring. We recorded food intake, body weight and adiposity of offspring born to obese and control mothers, who were themselves exposed to either a normal chow or an obesogenic high fat diet (HFD) after weaning (Figure 1A). The male offspring of obese mothers (Off-Ob chow) did not consume more calories during *ad lib* feeding when weaned onto a chow diet compared to the offspring of lean mothers fed a chow diet (Off-C chow) (Figure 1B). However, when weaned onto an HFD, offspring born to obese mothers (Off-Ob HFD) showed higher daily caloric intake from 6 weeks of age compared to offspring of lean mothers weaned onto the same HFD (Off-C HFD) (Figure 1C). When exposed to an HFD from weaning the offspring of both lean and obese mothers showed increased body weight from 6 weeks of age compared to their siblings weaned onto a chow diet (Figure 1D), but with no effect of the maternal diet. Longitudinal adiposity measurements showed increased adiposity in animals weaned onto a HFD compared to chow fed animals; interestingly, this increase in adiposity occurred earlier and was greater in the offspring of obese mothers (Figure 1E). An increase in HFD pellet intake was also found to precede the increase in adiposity in female offspring of obese mothers (Suppl. Figure 1).

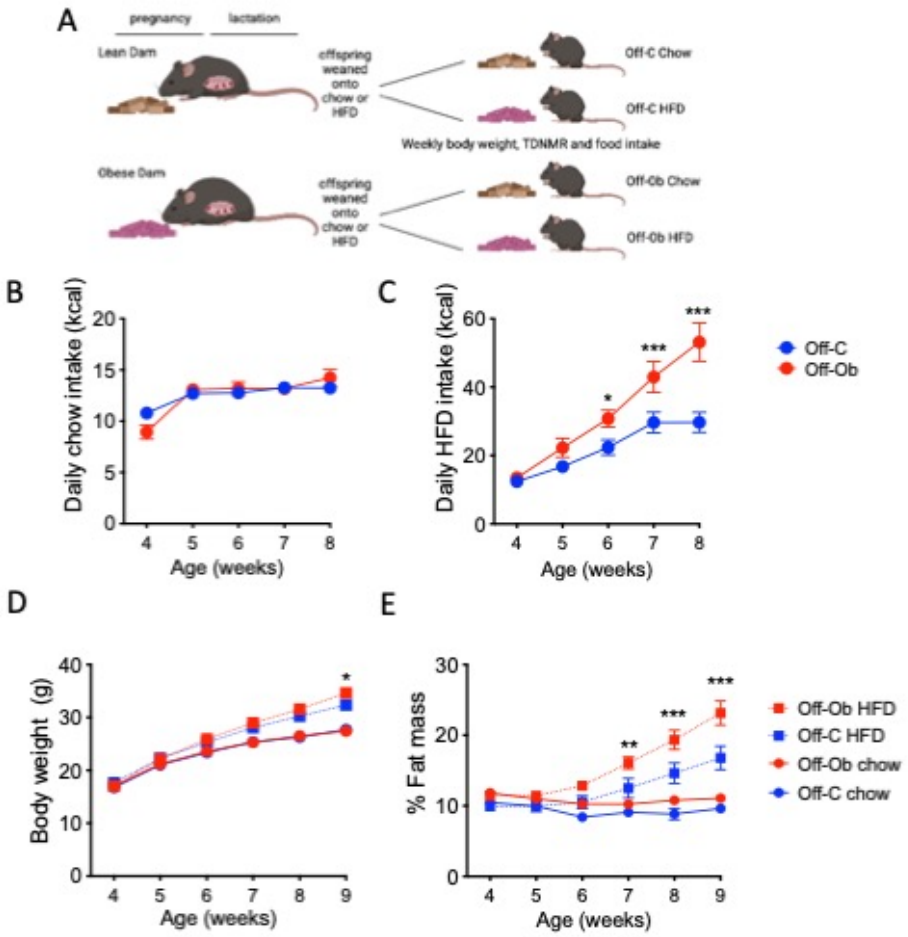

Figure 1. Offspring of obese mothers eat more of a high- fat pellet and develop increased adiposity compared to offspring of lean mothers fed the same diet. A) Diagram depicting mouse model of maternal obesity. At weaning offspring are weaned onto either a chow or HFD, and food intake, body weight and adiposity are measured weekly. **B)** Normal chow daily kilocalorie intake of male offspring born to control (Off-C; n=21) and obese mothers (Off-Ob; n=15). **C)** High fat diet (HFD) pellet daily kilocalorie intake of male offspring born to control (HFD-C; n=23) and obese mother (HFD-O; n=19). **D)** Body weight and **(E)** fat mass from 4 to 9 weeks of age in Off-C chow (n=15), Off-C HFD (n=21), Off-Ob chow (n=12) and Off-Ob HFD (n=13). * P<0.05, **P<0.01, calculated using two way ANOVA with Tukey post-hoc test.

3.2. Maternal obesity programs increased expression of miR-505-5p in the offspring hypothalamus from fetal to adult life

In order to investigate the effect of exposure to maternal obesity on miRNA expression profile in the offspring hypothalamus, we analysed the miRNA profile of two key nuclei, the paraventricular nucleus (PVH) and arcuate nucleus (ARC) of the hypothalamus, in young adult

male mice born to obese mothers or lean control mothers. Small RNA sequencing analysis highlighted 4 miRNA that were dysregulated in the PVH (Suppl. Table 2) and 14 in the ARC (Suppl. Table 3) of offspring exposed to maternal obesity. The miRNA miR-505-5p showed a striking increased expression in both the PVH (Figure 2A) and ARC (Figure 2B) of offspring born to obese mothers in comparison to offspring of control mothers. The result of the small RNA sequencing analysis was confirmed by qPCR for miR-505-5p in a separate cohort of young adult mice (Figure 2C).

In order to ascertain how early in life the over-expression of miR-505-5p is established, we analysed miR-505-5p expression in fetal hypothalamic samples and found a significant upregulation of the miRNA in E13 fetuses exposed to maternal obesity (Figure 2D). To determine if this overexpression is maintained outside the intrauterine environment, which would suggest a cell-autonomous programmed effect, we analysed hypothalamic NPC from E13 offspring and found overexpression of miR-505-5p in NPC after 8 days grown *in vitro* (Figure 2E). miR-505-5p sits in the 3' untranslated region of the gene *Atp11c*, a type IV P-type ATPase. The expression of *Atp11c* was also increased (though to a lesser extent than the miRNA) in the hypothalamus of fetuses exposed to maternal obesity, showing that miR-505-5p is at least in part regulated along with the host gene (Figure 2F). These data show that miR-505-5p overexpression is permanently programmed following exposure to maternal obesity and is therefore a potential candidate to mediate the long-term effects of maternal overnutrition on offspring hypothalamic function.

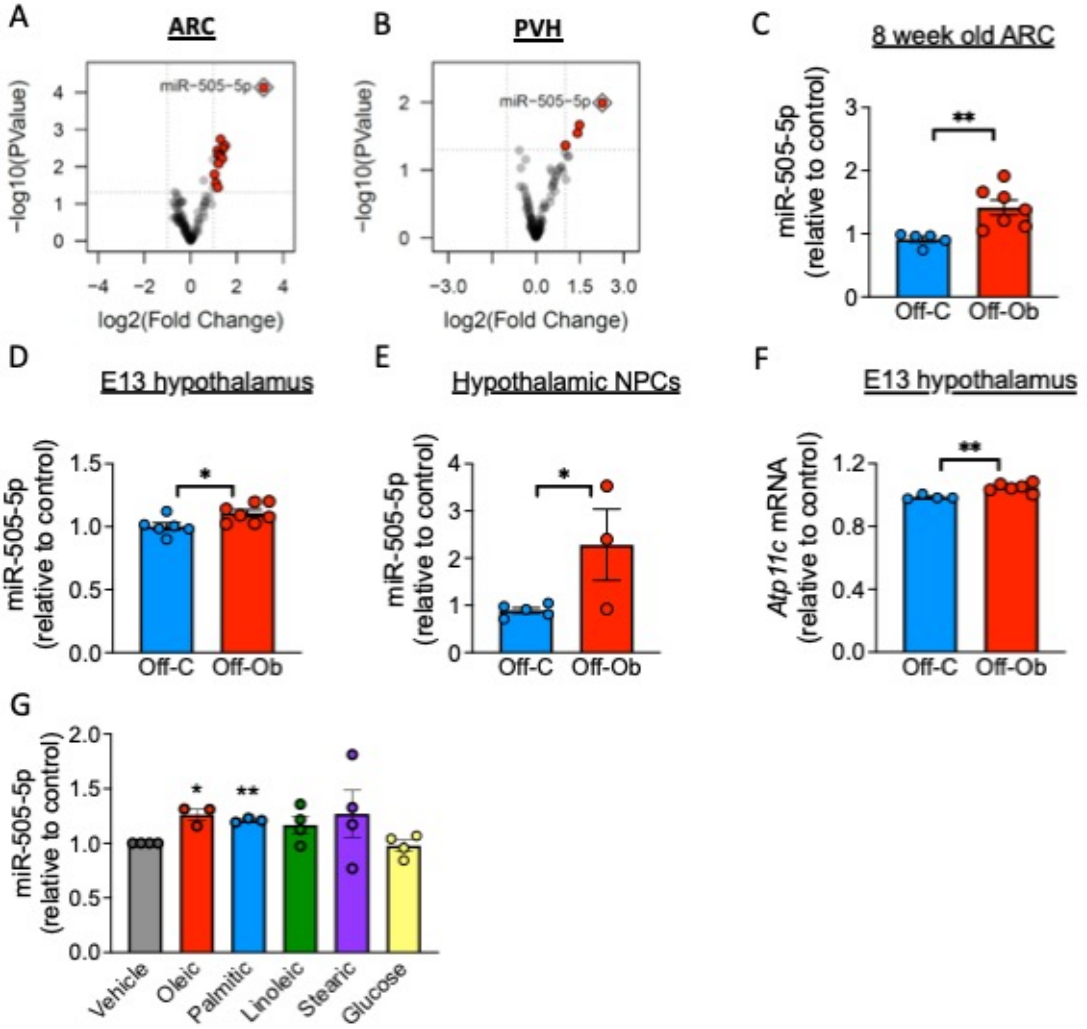

Figure 2. **Hypothalamic miR-505-5p expression is increased in offspring of obese mothers from fetal to adult life, and *in vitro* by exposure to fatty acids. A-B)** Volcano plot of small RNA sequencing results showing significant overexpression of miR-505-5p (red dot surrounded by blue diamond) in the ARC **(A),** and PVH (**B**) of 8-week-old offspring born to obese mothers compared to offspring of lean mothers. **C-E)** qPCR analysis of miR-505-5p expression in **(C)** ARC at 8 weeks of age; **(D)** in whole hypothalamus at E13; **(E)** in hypothalamic neural progenitor cells extracted at E13 and cultivated as neurospheres for 8 days *in vitro*. **F)** *Atp11c* (miR-505-5p host gene) mRNA levels in E13 whole hypothalamus. **G)** miR-505-5p relative expression of mHypoE-N46 cells exposed to oleic acid, palmitic acid, stearic acid, linoleic acid and glucose for 24h. * P<0.05, **P<0.01, calculated using unpaired (C-F) or paired (G) t-test. Outliers were excluded from 8 weeks old analysis for control group (Grubb's method, alpha=0.2, G=1.889, outlier excluded value 1.449); from hypothalamic neural progenitor cells for control group (Grubb's method, alpha=0.2, G=1.673, outlier excluded value 1.661); and from host gene analysis for control group (Grubb's method, alpha=0.2, G=1.679, outlier excluded value 1.056) and obese group (Grubb's method, alpha=0.2, G=2.102, outlier excluded value 0.878). Outliers were excluded from oleic group (Grubb's method, alpha=0.2, G=1.438, outlier excluded value 0.77) and palmitic group (Grubb's method, alpha=0.2, G=1.471, outlier excluded value 1.061).

3.3. Fatty acids, but not glucose, increase the expression of miR-505-5p in a hypothalamic cell line in vitro

We have previously conducted mass spectrometry analysis of the HFD pellet to establish the primary FA components of the obese dam diet, and found the FAs in greatest abundance were palmitic, stearic, oleic and linoleic acid [48]. We therefore sought to identify whether any of these FAs can alter the expression of miR-505-5p in a hypothalamic cell line, and could thus explain the increase in miR-505-5p in the fetal hypothalamus from obese pregnancies. The expression of miR-505-5p was increased after oleic and palmitic acid treatment (Figure 2G). Neither stearic nor linoleic acid treatment caused overexpression of miR-505-5p (Figure 2G). As the obesogenic diet fed to obese dams is also supplemented with condensed milk which is high in sucrose, we also tested to see whether glucose could regulate expression of miR-505-5p *in vitro*. Glucose did not alter the expression of miR-505-5p in hypothalamic cells *in vitro* (Figure 2G). In addition to regulation by nutrients, we also examined whether ARC miR-505-5p levels are modulated *in vivo* by long-term metabolic perturbations. Neither diet- induced obesity or an overnight fast changed the expression of miR-505-5p in the ARC in male mice (Suppl. Figure 2).

3.4. miR-505-5p targets identified by pSILAC are involved in FA uptake and metabolism

miRNAs are known to regulate gene function through degradation of mRNAs and/or via inhibition of protein translation. We performed a proteomic analysis to identify possible targets of miR-505-5p by pSILAC following transfection of a miR-505-5p mimic in a hypothalamic cell line, GT1-7 (Figure 3A). Cumulative fraction analysis showed that proteins encoded from mRNAs containing binding sites for miR-505-5p according to TargetScan (v. 7.2) were downregulated by miR-505-5p mimic transfection, showing that the pSILAC was effective in identifying proteins regulated as a consequence of over-expression of miR-505-5p (Figure 3B). pSILAC data was overlaid with predicted targets containing the miR-505-5p seed sequence identified by TargetScan and miRbase to identify potential direct targets, which were then validated by qPCR (Figure 3C).

IPA revealed that miR-505-5p targets are enriched for processes related to fatty acid metabolism, followed by oxidative stress and mitochondrial processes (Figure 3D). After conducting a manual search in addition to the IPA, we identified 30 proteins related to FA metabolism pathways that were significantly dysregulated in the pSILAC experiment. Twenty-one of these FA related proteins were downregulated as a consequence of miR-505-5p over-expression (Figure 3E), suggesting an overall reduction in FA metabolism processes in the cells. We next investigated the functional consequences of over-expression of miR-505-5p in a hypothalamic cell line on the processes of FA oxidation and FA uptake. We used radio-labelled oleic acid to show that the oleic acid metabolism into acid-soluble metabolites (Figure 3F) and oleic acid uptake (Figure 3F) is decreased in neurons due to miR-505-5p treatment. As FA uptake and metabolism to Acetyl-CoA and TCA cycle intermediates are thought to be key steps through which neurons are able to sense circulating FA levels, these results suggest that altered levels of miR-505-5p *in vivo* may impact hypothalamic fatty acid sensing.

3.5. Targets of miR-505-5p are genetically associated with BMI in humans

To understand the potential contribution of the miR-505-5p FA metabolism related targets to human metabolic health, we mined phenotypic and genetic data from human population studies by assessing data from a common variant GWAS of BMI (N=806,834)[38]. Of the 30 human orthologues to the targets of miR-505-5p, 12 genes were proximal to common variant genome-wide significant signals; when variants were analysed at the gene-level using MAGMA, 7 genes were significantly associated with BMI (Suppl. Table 4). As most common variant GWAS signals are intronic or intergenic, we overlayed these associations with other datasets to understand whether the associated variants can be functionally linked to our target genes (Figure 3G). Genomic variants within 6 of the BMI association signals are within regions of known enhancers[39]. We also observed colocalization between these signals and gene expression levels for 3 target genes.

Across these analyses, our human genetic analyses had the greatest support for CPT1A, HINT1, HSD17B12 and SLC27A4, which had the greatest number of variant to gene predictors (Figure 3G). For CPT1A, the BMI GWAS colocalised with CPT1A eQTLs (FDR-corrected P SMR=5.94×10^{-5} and P HEIDI=0.002, Suppl. Table 4), while signals proximal to HINT1,

HSD17B12 and SLC27A4 all overlapped with known enhancers for these genes. Collectively, these observations suggest that several of the identified genes are functionally associated with BMI in humans.

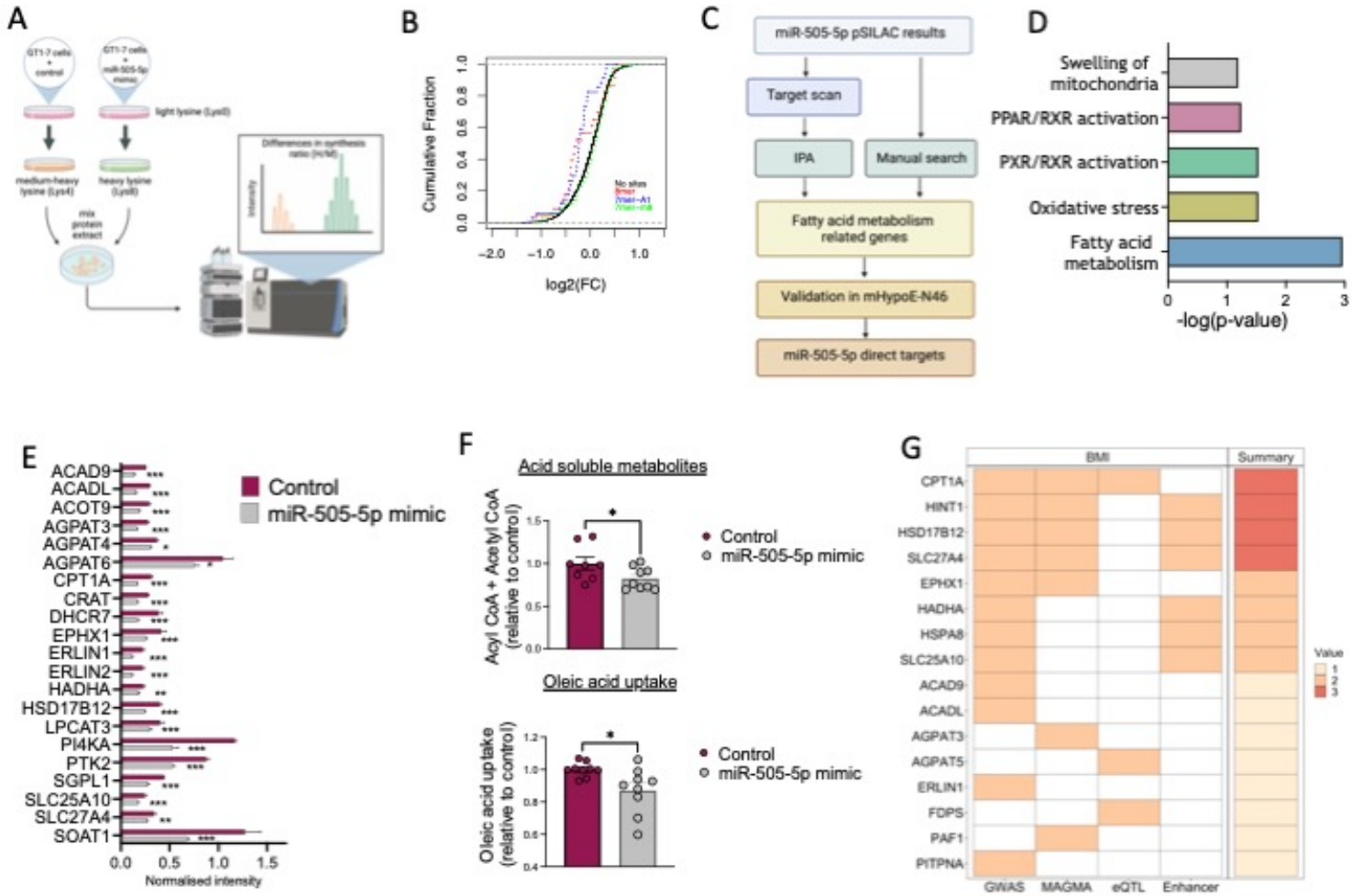

Figure 3. miR-505-5p targets are involved in fatty acid metabolism and genetically associated with BMI in humans. A) Diagram of pulsed SILAC experiment in GT1-7 cells to identify targets of miR-505-5p B) Cumulative fraction analysis of lys8-labelled: lys4-labelled proteins ratio according to occurrence and type of mRNA binding site for miR-505-5p, as predicted by TargetScan (v. 7.2) **C)** Workflow used to identify direct targets of miR-505-5p. **D)** Top regulated pathways among miR-505-50 direct targets as from Ingenuity Pathway Analysis. **E)** miR-505-5p target proteins of interest identified through IPA and manual search of pSILAC data. **F)** Radiolabelled Oleic acid metabolism into acid-soluble metabolites and oleic acid uptake in mHypoE-N46 following transfection with either a miR-505-5p mimic or control for 48h. **G)** Heatmap showing the overlap between human orthologs of identified miR-505-5p fatty acid metabolism related target genes in mice and human genetic datasets. Target genes were annotated on the basis of (i) proximity to GWAS signals, (ii) aggregate gene-level associations to the trait, (iii) colocalization between the GWAS and eQTL data, and (iv) the presence of known enhancers within the GWAS signals. A count of the observed concordant predictors (out of 4) is displayed in Summary (right panel). Expanded results can be found in Supplementary Table 4. *p<0.05, ** p<0.01, *** p<0.001 calculated using unpaired T-test.

3.6. miR-505-5p overexpression *in vitro,* as well as *in utero* exposure to maternal obesity, causes downregulation of genes related to FA sensing in hypothalamic cells.

A manual search combined with the IPA from Figure 4 identified 30 genes linked to FA metabolism (Figure 3E). Among the genes related to FA metabolism that were downregulated by miR-505-5p were genes related to fatty acid transport (*Slc25a10* and *Slc27a4* (that encodes the protein FATP4)) and fatty acid metabolism (*Acot9*, *Agpat3*, *Cpt1a* and *Soat1* (also known as *Acat*)). To confirm the downregulation of predicted miR-505-5p targets relevant to FA metabolism (as identified in Figure 3), we measured expression of these genes following transfection of a miR-505-5p mimic in the mHypoE-N46 hypothalamic cell line. miR-505-5p mimic treatment decreased the mRNA expression of *Acot9*, *Agpat3*, *Cpt1a*, *Slc25a10, Slc27a4* and *Soat1* (Figure 4A), compared to cells treated with a vehicle control.

Decreased mRNA expression of the genes *Acot9*, *Agpat3*, *Cpt1a*, *Slc25a10* and *Slc27a4* was also observed in the fetal hypothalamus at E13 of offspring from obese mothers, compared to the offspring of lean mothers (Figure 4B). This is at the same age that we previously observed an increase in hypothalamic miR-505-5p levels (Figure 2D). These data show that both over expression of miR-505-5p in a hypothalamic cell line, and exposure to maternal obesity *in vivo* - which we have shown programs increased miR505-5p in the hypothalamus - downregulate the expression of genes related to FA metabolism.

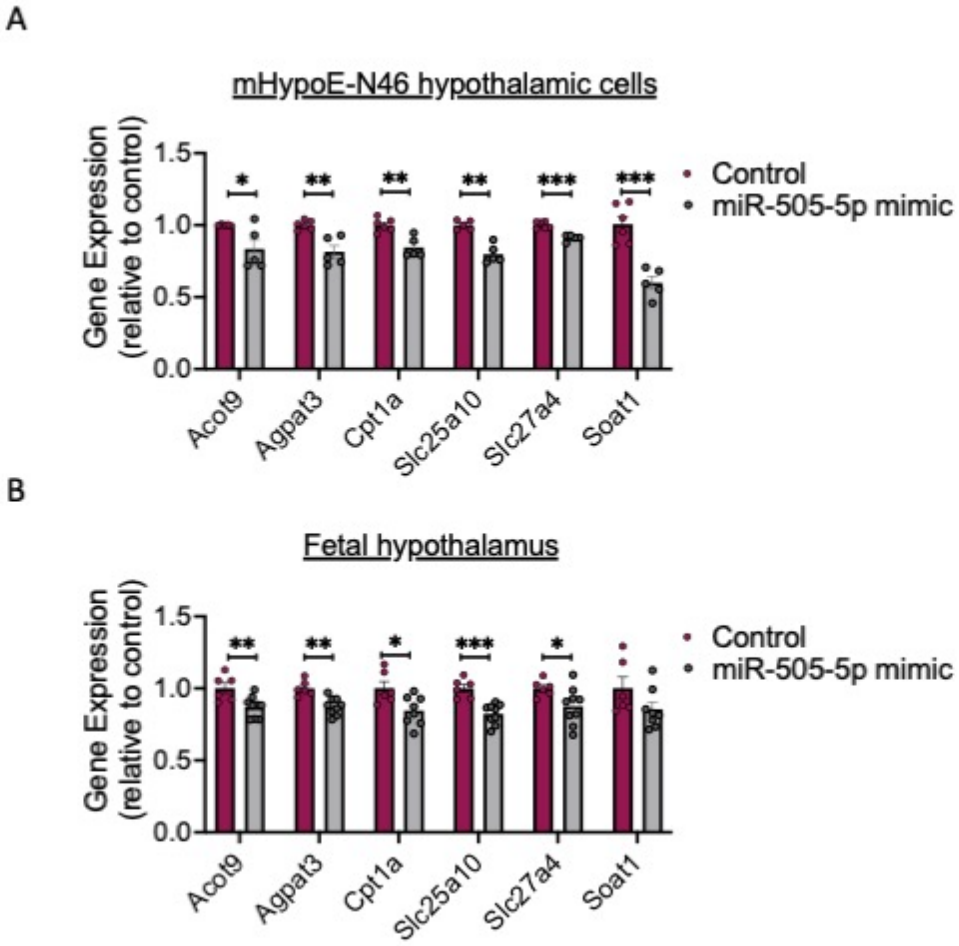

Figure 4. miR-505-5p target genes related to fatty acid sensing are downregulated after treatment with miR-505-5p *in vitro* and in fetal hypothalamus of offspring from obese mothers. A) Relative mRNA expression of genes related to lipid metabolism in mHypoE-N46 cells after treatment with a miR-505-5p mimic and **(B)** in the hypothalamus of fetuses at E13 * P<0.05, ** P<0.01, *** P<0.001, calculated by unpaired T-test.

3.7. Intra-cerebroventricular injection of miR-505-5p increases high-fat- but not chow-pellet intake

Our results demonstrate that targets of miR-505-5p in the hypothalamus include genes involved in neuronal FA uptake and oxidation, and that manipulation of miR-505-5p levels reduces rates of neuronal FA uptake and metabolism. Previous studies have suggested that neuronal FA uptake and metabolism are key processes that allow neurons to sense circulating FA levels and initiate appropriate adjustments to parameters including food intake [20]. We therefore hypothesised that over-expression of miR-505-5p - as seen in the offspring of obese mothers - may alter the ability of hypothalamic neurons to sense circulating FA levels and

result in a change in food intake. In order to verify whether miR-505-5p has a direct effect *in vivo* in the CNS in FA sensing - and therefore food intake - we performed ICV administration of a miR-505-5p mimic or a negative control and measured *ad lib* intake of high- fat diet or chow diet for 3 consecutive days following surgery (Figure 5A). Increased miR-505-5p in the hypothalamus after ICV injection of the miR-505-5p mimic was confirmed on post-mortem dissection (Figure 5B). Mice that received the miR-505-5p mimic ICV had increased high-fat diet intake compared to mice that received a negative control treatment (Figure 5C). The increase in food intake caused by ICV miR-505-5p was exclusively seen when animals were given the high fat diet; ICV miR-505-5p did not increase food intake when animals were fed a chow diet (Figure 5D).

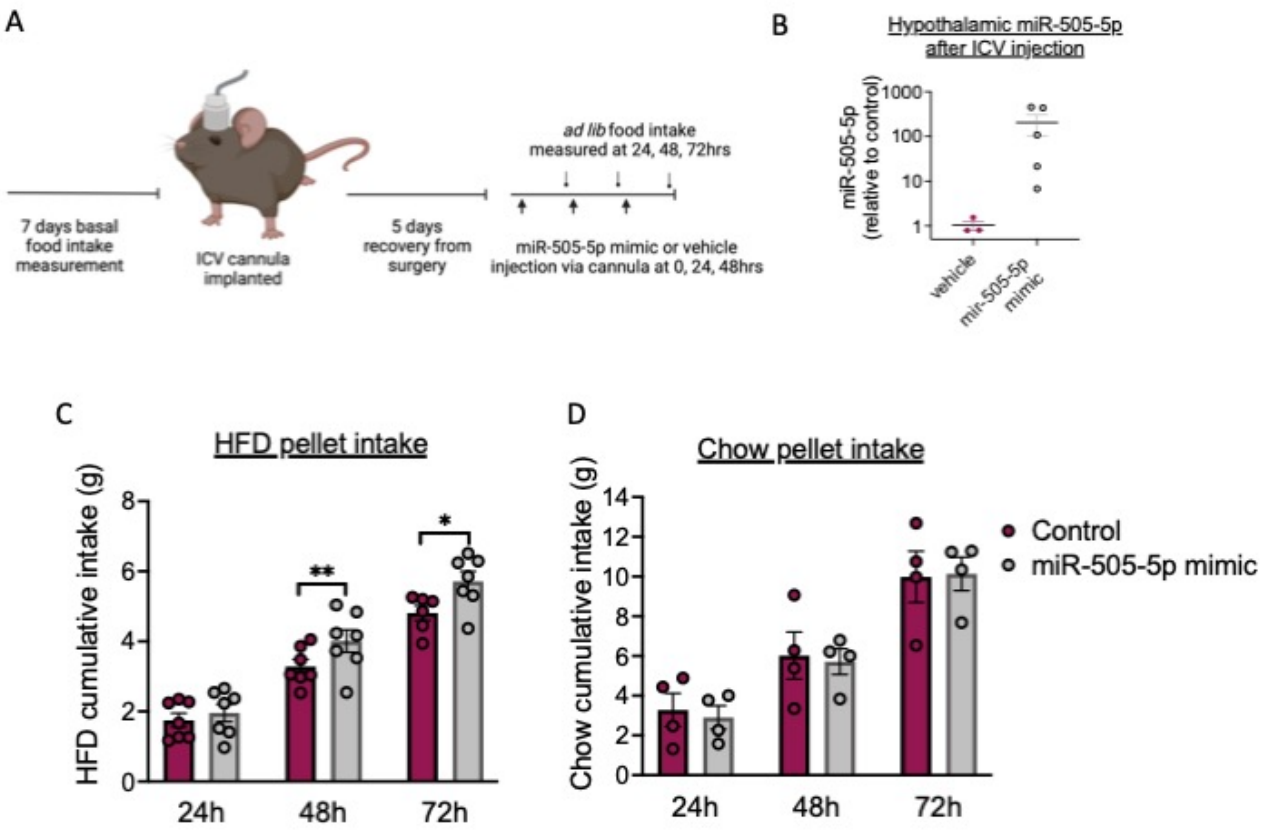

Figure 5. ICV injection of miR-505-5p in adult mice increases intake specifically of a high-fat diet.

A) Diagram depicting surgical schedule. Mice were allowed to recover for 5 days post-implanted of an ICV cannula into the lateral ventricle. Mice received injection of either a vehicle or miR-505-5p mimic via the cannula and food intake was measured over 3 days**. B)** qPCR confirming over-expression of miR-505-5p in the hypothalamus after ICV injection of miR-505-5p mimic. **C)** High-fat diet cumulative food intake after 3 consecutive days of treatment (24- 1 dose, 48- 2 doses and 72 hours- 3 doses- after first treatment). **D)** Normal chow cumulative food intake after 3 consecutive days of treatment (24- 1 dose, 48- 2 doses and 72 hours- 3 doses- after first treatment). * $p<0.05$, ** $p<0.01$, analysed by unpaired T-test

4. Discussion

In the current study, we have used a rodent model to examine the effects of exposure to maternal obesity on offspring food intake, and small RNA sequencing in the hypothalamus to identify underlying mechanisms. Our results showed that maternal obesity alters the miRNA profile in the ARC and PVH, and identified miR-505-5p as a miRNA that is programmed in the

fetal hypothalamus with changed expression maintained through to adulthood. We have shown that targets of miR-505-5p are related to FA sensing processes including FA uptake and metabolism, and that manipulation of miR-505-5p levels in hypothalamic cells *in vitro* alters these processes. Importantly, we have shown that ICV injection of a miR-505-5p mimic increases intake specifically of an HFD. Altered expression of hypothalamic miR-505-5p *in utero* is therefore a novel route by which exposure to maternal obesity programs permanent changes to offspring feeding behaviour, leading to obesity.

The increased intake of an HFD in the offspring of obese mothers precedes the increased adiposity observed in these animals and suggests a causal relationship. Indeed, many studies have now linked changes in food intake to the increased susceptibility to develop obesity, and suggested that altered development and/or function of the hypothalamus plays a key role in this process [4]. To our knowledge, we are the first to conduct small RNA sequencing to identify the full spectrum of miRNAs that are dysregulated in the ARC and PVH following exposure to maternal obesity. A role for hypothalamic miRNAs in maintaining energy homeostasis is largely unexplored, but deletion of the miRNA processing enzyme DICER in POMC neurons results in obesity [49], and hypothalamic miR-29a and miR-103 have been shown to protect against obesity [18,50]. Although a few studies have demonstrated altered hypothalamic miRNA expression in mice in response to nutritional status or leptin administration [16,51,52], whether exposure to altered nutritional status *in utero* programs hypothalamic miRNA profile was previously unknown. Our small RNA sequencing analysis identified miR-505-5p as being highly regulated in both the ARC and PVH of adult offspring after exposure to maternal obesity, and we subsequently demonstrated that increased expression of miR-505-5p is detectable as early as E13 in the fetal hypothalamus. The increased expression of miR-505-5p was maintained *in vitro* in hypothalamic neural progenitor cells after one week, indicating this is a programmed effect in the cell, rather than an immediate effect of the intra-uterine environment on gene expression. Interestingly, the miR-505-5p host gene Atp11c, which encodes a Type IV P-type ATPase, a flippase which is involved in the transport of phospholipids, is also increased in the fetuses of obese mothers. It is intriguing that this apparent nutrient-sensitive miRNA sits within another gene linked to cellular energy levels and that this too is altered by exposure to maternal obesity.

In addition to hypothalamic miR-505-5p expression being increased in the offspring of obese mothers, miR-505-5p was also increased in response to specific exposure to oleic and palmitic acid in a hypothalamic cell line *in vitro*. These fatty acids are abundant in the high-fat pellet that forms part of the obesogenic diet fed to dams in our model [48], and we have shown that oleic acid is increased in the serum of dams fed an obesogenic diet near the end of pregnancy [36]. Our results suggest that increased circulating free- fatty acids may be a key mechanism by which maternal obesity programs changes to the developing offspring hypothalamus. Indeed, recent research by Park *et al* [7] has shown that exposure to high FA concentrations causes reduced neurite outgrowth from the ARC, and this may underlie the widely reported phenotype of reduced ARC to PVH projections in the offspring of obese mothers [7,53].

Unlike the well characterised populations of hypothalamic glucose sensing neurons [23], the presence, location and molecular signature of FA sensing neurons in the hypothalamus is not well defined. FAs are able to cross the blood brain barrier [54,55], and the presence of FA transporters such as FATP1, FATP4 and FAT/CD36, as well as genes required for metabolism of FA [25] support the theory that at least some hypothalamic neuronal populations are FA-sensing. This has led researchers to propose that FA sensing is a mechanism by which the hypothalamus responds to changes in nutrient status, in particular that of an energy surfeit [56]. Current hypotheses suggest neuronal cells use the rate of uptake and/or metabolism of FAs to sense circulating levels, (although there may also be direct regulation of some neurons via electrophysiological effects [29] as is the case for glucose-sensing neurons). For this reason, it is relevant that we have shown that miR-505-5p targets include both FA transporters (FATP4) and key components of FA oxidation pathways (such as CPT1A), and that over-expression of miR-505-5p in a hypothalamic cell line disrupted FA uptake and metabolism. Therefore, altered expression of miR-505-5p caused by *in utero* exposure to high FA levels has the potential to permanently disrupt hypothalamic FA sensing mechanisms. This is supported by the observation that CNS administration of a miR-505-5p mimic results in increased intake of a high-fat diet (but not a chow diet). Thus, our results support the hypothesis that if neurons are unable to sense circulating FA levels due to altered FA uptake and metabolism pathways, animals are not able to maintain energy homeostasis, resulting in increased

consumption of foods high in fat. This is also supported by a previous study showing that maternal HFD consumption during pregnancy reduced the sensitivity of offspring ventromedial hypothalamic neurons to oleic acid [33].

Although we have highlighted only six key genes in our experiments, the pSILAC analysis identified many other targets of miR-505-5p that may contribute to a nutrient sensing phenotype in the offspring of obese mothers. Among these are the SRCAP chromatin remodelling complex, which activates a transcriptional profile that shifts metabolism from anaerobic glycolysis to oxidative metabolism [57]. Interestingly, the pSILAC revealed that many indirect targets of miR-505-5p (i.e. downregulated in pSILAC but do not contain miR-505-5p seed sequence) are related to FA sensing, and many of these also showed genetic associations with BMI in humans. Whilst we have focused on validating a few key direct target genes in the current study, it is unlikely that miR-505-regulates a complex process such as nutrient sensing via only one key target gene, and therefore it is likely that miR-505-5p controls the expression of several master regulators (e.g. transcription factors or chromatin remodelling complexes such as SRCAP) that modulate whole profiles of FA sensing genes. It is particularly notable that many of the proteins we found to be altered following over-expression of miR-505-5p are expressed in mitochondria, so future studies should aim to examine the effects of maternal obesity on offspring mitochondrial dynamics.

One of the limitations of our study is that the small RNA sequencing analysis did not differentiate between different cell types in the hypothalamic nuclei we examined, and given the newly identified role for astrocytic FA metabolism in modulating susceptibility to develop diet- induced obesity [58], the function of miR-505-5p in glial or oligodendrocyte cells should be explored in the future. It is also important to determine whether reduction of hypothalamic miR-505-5p is sufficient to mitigate the effects of maternal obesity on offspring weight gain.

In conclusion, we have identified miR-505-5p as a novel regulator of FA metabolism pathways that is programmed in mice exposed to maternal obesity and could contribute to the increased HFD intake and adiposity seen in the offspring of obese mothers. This is consistent with previous animal studies that have shown a role for hypothalamic FA sensing in

modulating food intake, and is corroborated by our human genetic data analysis. Further defining the mechanisms underlying the programming of miR-505-5p by maternal obesity may hold promise for developing novel interventional strategies to prevent the adverse impacts of maternal obesity on offspring long-term health.

5. Authors contributions

I.C.F., L.D., L.C.P and S.E.O. conceived the project, designed the experiments, interpreted the results. I.C.F., L.D., L.P., J.F, M.C., performed experiments. J.D., L.D., S.E.O. supervised experiments. K.A.K., K.K.O. and J.R.B.P. carried out the genetic analysis. L.D. wrote the manuscript. All authors reviewed and commented on the final version of the manuscript.

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