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DOI: https://doi.org/10.1016/j.foodres.2023.113808

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Food Research International 176 (2024) 113808

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Hypothalamic inflammation and the development of an obese phenotype induced by high-fat diet consumption is exacerbated in alpha7 nicotinic cholinergic receptor knockout mice

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ARTICLE INFO

Keywords:
Alpha7 nicotinic acetylcholine receptor
Obesity
High-fat diet
Inflammation
Hypothalamus
Cholinergic pathway

ABSTRACT

Hypothalamic inflammation and metabolic changes resulting from the consumption of high-fat diets have been linked to low grade inflammation and obesity. Inflammation impairs the hypothalamic expression of α7 nicotinic acetylcholine receptor (α7nAChR). The α7nAChR is described as the main component of the anti-inflammatory cholinergic pathway in different inflammation models. To assess whether the reduction in α7nAChR expression exacerbates hypothalamic inflammation induced by a high-fat diet (HFD), were used male and female global α7nAChR knockout mouse line in normal or high-fat diet for 4 weeks. Body weight gain, adiposity, glucose homeostasis, hypothalamic inflammation, food intake, and energy expenditure were evaluated. Insulin sensitivity was evaluated in neuronal cell culture. Consumption of an HFD for 4 weeks resulted in body weight gain and adiposity in male Chrna7-/- mice and the hypothalamus of male Chrna7-/- mice showed neuroinflammatory markers, with increased gene expression of pro-inflammatory cytokines and dysregulation in the nuclear factor kappa B pathway. Moreover, male Chrna7-/- mice consuming an HFD showed alterations in glucose homeostasis and serum of Chrna7-/- mice that consumed an HFD impaired insulin signalling in neuronal cell culture experiments. In general, female Chrna7-/- mice that consumed an HFD did not show the phenotypic and molecular changes found in male mice, indicating that there is sexual dimorphism in the analysed parameters. Thus, receptor deletion resulted in increased susceptibility to hypothalamic inflammation and metabolic damage associated with HFD consumption in male mice.

1. Introduction

The hypothalamus plays a pivotal role in the control of energy homeostasis. The activity of neurons located in the hypothalamus is modulated by hormones and nutrient signals (Mehay, Silberman, & Arnold, 2021). Leptin and insulin bind to their receptors and induce the expression of anorexigenic neuropeptides (pro-opiomelanocortin [POMC] and cocaine-and amphetamine-regulated transcript [CART]), increasing energy expenditure and inhibiting food intake (Friedman, 2019; Kwon, Kim, & Kim, 2016). On the other hand, in fasting conditions the expression of orexigenic neuropeptides (neuropeptide Y [NPY] and agouti-related protein [AgRP]) is increased, stimulating food intake and reducing energy expenditure (Friedman & Halaas, 1998; Mantzoros, 1999).

Studies with experimental models of diet-induced obesity (DIO) have shown the close relationship between the high consumption of diet rich in saturated fatty acids (HFD) and the impairment of homeostasis (Cesar & Pisani, 2017; Li, Wu, Liu, & Yang, 2020; Milanski et al., 2009; Razolli et al., 2020; Thaler et al., 2012). In the hypothalamus, HFD stimulate pro-inflammatory pathways that lead to activation of microglia and the increased expression of inflammatory cytokines. These proteins impair hypothalamic leptin and insulin signalling, leading to energy imbalance and obesity (Milanski et al., 2009; Moraes et al., 2009; Souza et al., 2019a, Souza et al., 2019b; Thaler et al., 2012). The alpha7 nicotinic

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https://doi.org/10.1016/j.foodres.2023.113808
Received 14 September 2023; Received in revised form 27 November 2023; Accepted 2 December 2023
Available online 3 December 2023
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acetylcholine cholinergic receptor (α7nAChR) has been implicated in the reduction of inflammatory cytokine expression in different tissues and cells (Wang et al., 2003; Xie et al., 2020). Thus, it has been investigated as a potential target to treat diseases associated with inflammatory pathway activation, such as sepsis (Souza et al., 2019a), rheumatoid arthritis (van Maanen, Stoof, LaRosa, Vervoordeldonk, & Tak, 2010), inflammatory bowel disease (Ruzafa, Cedillo, & Hone, 2021), diabetes and obesity (Gupta, Lacayo, Greene, Leahy, & Jetton, 2018; Li et al., 2018; Nishio et al., 2017). However, there is little information on the function of α7nAChR in the control of energy homeostasis in a DIO model. Therefore, considering the role of alpha7 in reducing the inflammatory process, it can play an important role in preventing inflammation in homeostatic control mechanisms in the hypothalamus, such as leptin and insulin signaling.

α7nAChR is the most abundant nicotinic receptor in the central nervous system. It plays a critical role in the cholinergic anti-inflammatory pathway (CAP), inhibiting the expression of inflammatory cytokines (Piovesana, Intriago, Dini, & Tata, 2021; Wang et al., 2003). Although α7nAChR is a cation channel of the Cys-loop receptor family (Piovesana et al., 2021), it is also responsible for activating the

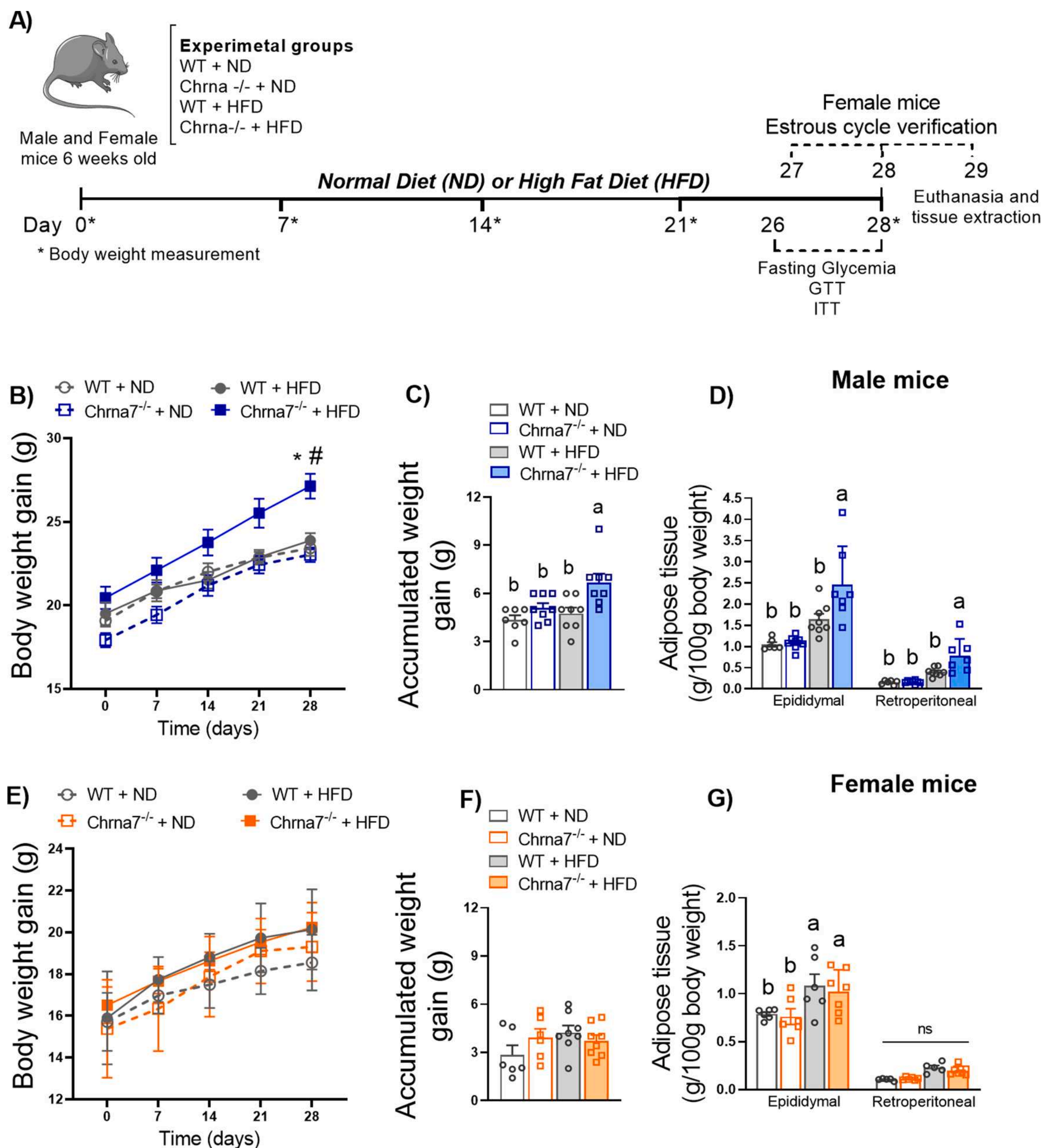

Fig. 1. Deletion of α7nAChR (Chrna7$^{-/-}$) contributes to body weight gain and adiposity in male mice fed a hig-fat diet (HFD) for 4 weeks. (A) Schematic illustrating the experimental timeline of the wildtype (WT) and knockout (Chrna7$^{-/-}$) mice fed the normal diet (ND) or the HFD for 4 weeks. **(B)** Body weight gain in male Chrna7$^{-/-}$ mice. Two-way analysis of variance (ANOVA) with the post hoc Bonferroni test (*$p < 0.05$ for Chrna7-/- + ND vs. Chrna7-/- + HFD; #$p < 0.05$ for Chrna7-/- + ND vs. Chrna $^{-/-}$ + HFD). **(C)** Accumulated weight (final weight – initial weight) in male Chrna7$^{-/-}$ mice. One-way ANOVA with the post hoc Bonferroni test. Different letters show statistical differences. **(D)** Adiposity in male Chrna7$^{-/-}$ mice. One-way ANOVA with post hoc Bonferroni test. Different letters show statistical differences. **(E)** Body weight gain in female Chrna7$^{-/-}$ mice. Two-way ANOVA with post hoc Bonferroni test (*$p < 0.05$ for Chrna7-/- + ND vs. Chrna7-/- + HFD; #$p < 0.05$ for Chrna7-/- + ND vs. Chrna $^{-/-}$ + HFD). **(F)** Accumulated weight (final weight – initial weight) in female Chrna7$^{-/-}$ mice. One-way ANOVA with post hoc Bonferroni test. Different letters show statistical differences. **(G)** Adiposity in female Chrna7$^{-/-}$ mice. One-way ANOVA with the post-hoc Bonferroni test. Different letters show statistical differences. $p < 0.05$ and $n = 6–8$ mice per group as indicated by dots. The data are presented as mean ± standard error of mean.

Janus kinase 2 (JAK2)–signal transducer and activator of transcription 3 (STAT3) pathway and modulates neuropeptide and cytokine expression (de Jonge et al., 2005; Souza et al., 2019b). Deletion of α7nAChR in mice (Chrna$^{-/-}$) does not impair the hypothalamic control of energy homeostasis and liver metabolism by itself. However, Chrna7-/- mice fed a high-fat diet (HFD) present significantly compromised liver metabolism (Li et al., 2018). Furthermore, we recently showed that short-term HFD consumption reduces the expression of hypothalamic α7nAChR in mice and potentiates the inflammatory response to lipopolysaccharide (LPS) (Souza et al., 2019a; Martins et al., 2021). However, the role of α7nAChR in preventing damage induced by HFD consumption to the hypothalamic leptin signalling and control of homeostasis are not known. In the present study, we hypothesised that α7nAChR deletion potentiates the harmful effect of HFD consumption on the central control of homeostasis. To test the hypothesis, we used genetically modified (Chrna$^{-/-}$) and wildtype (WT) mice to evaluate hypothalamic inflammation, leptin signalling and energy expenditure in mice fed a normal diet (ND) and an HFD.

2. Methods

2.1. Experimental animal model

For this study, male and female mice (C57BL/6J background, B6.129S7-Chrna7tm1Bay/J, #003232), heterozygous (Chrna$^{+/-}$) and mutant homozygous (Chrna$^{-/-}$) for α7nAChR were provided by the State University of Campinas Animal Breeding Center. The Chrna7-/- and WT mice used in our experiments were derived from heterozygous breeding pairs. The genotype of interest was confirmed by genotyping and gene expression tests in different tissues (Supplementary Fig. 1). They were maintained in individual cages with a controlled temperature (22–24 °C), a 12-h photoperiod and water and food provided *ad libitum*. The experimental procedures involving mice were performed in accordance with the guidelines of the Brazilian Society of Laboratory Animal Science (SBCAL) and were approved by the Ethics Committee on Animal Use (CEUA) (ID protocol 5386-1) of the University of Campinas (UNICAMP).

2.2. Experimental design and experimental diet protocol

Six-week-old male and female mice were randomly divided into four groups for the preliminary metabolic analyses: WT mice fed with a ND (WT + ND), Chrna7-/- mice fed a ND (Chrna7-/- + ND), WT mice fed an HFD (WT + HFD) and Chrna7-/- mice fed an HFD (Chrna7-/- + HFD). The experiment lasted for 4 weeks (Fig. 1A). The ND was NUVILAB® Cr-1, (Nuvital, PR, Brazil). The HFD was modified to have 45 % lipid content and adapted according to the AIN-93 M, as previously described (Simino et al., 2017, Simino et al., 2017). Body weight was recorded every week during the experiment. After euthanising the mice with an intraperitoneal injection containing ketamine 100 mg kg^{-1} and xylazine 5 mg kg^{-1}, tissues were isolated and frozen in liquid nitrogen for further analyses.

2.3. Indirect calorimetry

Energy expenditure was assessed by using the Comprehensive Lab Animal Monitoring System (CLAMS, Columbus Instruments, Columbus, OH, USA). At the end of experimental period, mice were kept in the individual boxes they had been adapted to at 22 °C, a 12-h photoperiod and with free access to the HFD and water. Measurements were recorded for the next 24 h.

2.4. Intraperitoneal glucose tolerance test (ipGTT) and intraperitoneal insulin tolerance test (ipITT)

Blood samples were collected from the tail vein to determine serum glucose levels with an electronic glucometer (Accu-Chek Active, Roche, Brazil). The ipGTT was performed in mice that had been fasted overnight (for 12 h), refed over 2 h and then fasted again (for 4 h). The animals were challenged with an intraperitoneal injection of glucose solution (1 g kg^{-1} body weight). Blood was collected from the tail vein to measure glucose levels at 0, 15, 30, 60 and 120 min. The ipITT was performed in mice that had been fasted (for 12 h), refed over 2 h and then fasted again (for 4 h). The mice were challenged with an intraperitoneal injection of insulin (1.5 IU kg^{-1} body weight). Blood was collected from the tail vein to measure glucose levels at 0, 5, 10, 15 and 30 min. The area under the curve (AUC) was used to calculate the glucose (ipGTT) and insulin (ipITT) responses. The tests were established according to the protocol described by Virtue and Vidal-Puig (Virtue & Vidal-Puig, 2021).

2.5. Ovariectomy of female Chrna7-/- mice

Female Chrna7-/- mice underwent ovariectomy to remove the ovaries as reported to da Silva Dias et al. (2019). At the age of 5 weeks, female Chrna7-/- mice were anaesthetised with isoflurane inhalation. A small abdominal incision was made, the oviduct was sectioned, and the ovary removed. For the pseudo-surgery (Sham) control, bilateral incisions were made, reaching only the skin and subcutaneous tissue, without locating and removing the ovaries. After surgery, the animals received local analgesia with carprofen for 3 days and allowed to recover for 7 days.

2.6. STAT3 phosphorylation stimulated by leptin in male Chrna7-/- mice

Male mice (the WT + ND, WT + HFD, Chrna7-/- + ND and Chrna7-/- + HFD groups) were fasted overnight (for 12 h) and intraperitoneally injected with saline or leptin (2.5 μg g^{-1} body weight, Sigma-Aldrich, St. Louis, MO, USA) at 08:00 h. Thirty minutes after injections, the mice were euthanised and the hypothalamus collected to protein analysis by western blotting. Tests were established according to Garcia-Galiano and colleagues (Garcia-Galiano et al., 2017).

2.7. Protein kinase B (AKT) phosphorylation stimulated by insulin in vitro

The murine hypothalamic neuronal cell line mHypoA-2/29 (RRID: CVCL_D394) was cultured in Dulbecco's Modified Eagle's Medium (DMEM) High Glucose (supplemented with 10 % FBS and 1 % penicillin–streptomycin solution [100 U mL^{-1}; 100 μg mL^{-1}]) (all from Sigma-Aldrich). The cells were treated for 24 h with 2 % serum from male Chrna7-/- or WT mice that had been fed an HFD. Some of the wells received insulin at 100 nM and the controls received no stimulation. After 10 min, the medium was collected, and the protein content was extracted for western blotting. The tests were established according to protocol described by Amaral and colleagues (Amaral et al., 2022).

2.8. mRNA quantification

Total RNA was extracted using TRIzol Reagent (Life Technologies Corporation, Carlsbad, CA, USA) according to the manufacturer's recommendations and quantified on a Nanodrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Relative expression was determined by using the TaqMan PCR Master Mix (Applied Biosystems, Massachusetts, EUA) and primers for target genes (Applied Biosystems): *Tnfa* (Mm00443258m1), *Il1b* (Mm00434228m1), *Il6* (Mm00446190m1), *Il10* (Mm01288386_m1), *Cx3cl1* (Mm00436454m1), *Pomc* (Mm00435874_m1), *Npy* (Mm01410146_m1), *Agrp* (Mm00475829_g1), *Cartpt* (Mm04210469_m1) and *Chrna* (Mm01312230_m1). *Gapdh* (Mm99999915_g1) was used as a housekeeping gene. The data are expressed as relative values determined by the comparative threshold cycle (Ct) method ($2^{-\Delta\Delta Ct}$). The data were

analysed using the sequence detection system 2.0.5 (Applied Biosystems).

2.9. Protein quantification and western blotting

To extract protein, fragment of tissue was homogenised in freshly prepared ice-cold buffer (1 % [v/v] Triton X-100, 0.1 mol/L Tris, pH 7.4, 0.1 mol/L sodium pyrophosphate, 0.1 mol/L sodium fluoride, 0.01 mol/L EDTA, 0.01 mol/L sodium vanadate, 0.002 mol/L PMSF and 0.01 mg/mL aprotinin). Insoluble material was removed by centrifugation 10.000*g*/30 min at 4 °C. The protein concentration (supernatant) was determined using the Bradford method. The supernatant was resuspended in Laemmli sample buffer and boiled (5 min) before separation by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) using a miniature slab gel apparatus (Bio-Rad, Hercules, CA, USA). Electrotransfer of proteins from the gel to a nitrocellulose membrane was performed for 120 min at 120 V (constant) in transfer buffer containing methanol. The membranes were subsequently blocked with a solution containing 5 % free-fat milk in Tris-buffered saline with Tween 20 (TBST; 10 mM Tris, 150 mM NaCl, and 0.5 % Tween 20) for 2 h at room temperature. After blocking, the membranes were washed three times with TBST for 5 min each and then incubated overnight at 4 °C with specific primary antibodies from Cell Signaling Technology (Danvers, MA, USA): p-IkappaB kinase (IKK) α/β (#2697), IkappaB (IκB) (#9242), p-nuclear factor kappa B (NF-κB) (#3033S) and GAPDH (#T5168) as an endogenous control or vinculin (ab130007) from Abcam Plc (Cambridge, United Kingdom). Next, the membranes were washed three times with Tris-buffered saline with Tween 20 (TTBS; 10 mM Tris, 150 mM NaCl, and 0.5 % Tween 20) and incubated for 2 h at room temperature with secondary antibodies with goat peroxidase-conjugated secondary antibodies (KPL, Gaithersburg, MD, USA) diluted in TTBS (containing 3 % dry non-fat milk). Proteins were detected by chemiluminescence (Super Signal West Pico Chemiluminescent Substrate, Thermo Fisher Scientific). The band intensity were evaluated by densitometry using Scion Image software (Scion Corp, MD, Washington, USA) and band intensities were normalised to loading control (GAPDH or β-actin).

2.10. Statistical analyses

The data were analysed using GraphPad Prism 8.0.1 (GraphPad Software Inc., San Diego, CA, USA). First, the Kolmogorov–Smirnov and F-test to determine whether the data had a normal distribution and equal variance, respectively. Then, the data were analysed with unpaired two-tailed Student's *t*-test, two-way analysis of variance (ANOVA) or repeated measured ANOVA when appropriate. Post hoc comparisons were performed by using the Bonferroni test, $p < 0.05$ was considered statistically significant and the data are presented as the mean ± standard error of the mean.

3. Results

3.1. Male Chrna7-/- mice fed an HFD are susceptible to body weight gain and adiposity

After consuming an HFD for 4 weeks, male Chrna7-/- mice showed an increase in body weight (Fig. 1B and C) and adiposity (Fig. 1D) at the end of 4 weeks compared with male WT mice. However, female Chrna7-/- mice showed similar body weight gain (Fig. 1E and F) and adiposity (Fig. 1G) to female WT mice. In ND fed there were no differences for either sex (Fig. 1B–G).

3.2. Male and female Chrna7-/- mice fed an HFD showed discrete changes regarding glucose homeostasis

Male Chrna7-/- mice (Fig. 2A and B) but not female Chrna7-/- mice (Fig. 2G and H) fed an HFD presented reduced tolerance to glucose compared with WT mice. Furthermore, male but not female Chrna7-/- mice presented higher fasting glucose levels than WT mice (Fig. 2C and I). The sensitivity to insulin evaluated by the ipITT and the decay in glucose concentration (kITT) was not different in Chrna7-/- mice compared with WT mice (Fig. 2D and J).

3.3. Chrna-/- mice exposed to HFD consumption resulted in increased inflammatory markers in male but not in female mice

Hypothalamic *Tnfa*, *Il1b* and *Il6* gene expression was upregulated in Chrna7-/- male mice compared with WT mice after HFD fed (Fig. 3A). There were no differences in *Il10* and *Cx3cl1* gene expression (Fig. 3A). On the other hand, there were no differences in hypothalamic gene expression between female Chrna7-/- and WT mice fed an HFD for 4 weeks (Fig. 3E). We measured the expression of IκB and the phosphorylation level of IKK and NF-κB by western blot (Fig. 3B–D and F–H). IκB expression in the hypothalamus of male Chrna7-/- mice was reduced compared with male WT mice after consuming an HFD for 4 weeks (Fig. 3C), while the phosphorylation of IKK and NF-κB was not different between male Chrna7-/- and WT mice. In female Chrna7-/- mice, phosphorylation of NF-κB was reduced compared with female WT mice after consuming an HFD for 4 weeks (Fig. 3H), but phosphorylation of IKK and IκB expression were not different (Fig. 3F and G).

3.4. Chrna7 deletion in male HFD-mice (Chrna7-/-) result in decreased POMC gene expression, increased food intake and energy expenditure

Male Chrna7-/- mice showed a decrease in *Pomc* gene expression compared with male WT mice (Fig. 4A); there were no differences between female Chrna7-/- and WT mice (Fig. 4B). The gene expression of the other neuropeptides analysed was not altered in males or females (Fig. 4A and B). We analysed whole-body energy homeostasis by measuring the oxygen volume (VO_2), the carbon dioxide volume (VCO_2), the respiratory exchange ratio (RER), heat generation, locomotor activity and food intake (Figs. 5 and 6). HFD consumption increased VO_2 (Fig. 5A and B), food intake (Fig. 6A and B) and heat generation (Fig. 5D), but not locomotor activity (Fig. 6C) in male Chrna7-/- compared with WT mice. In female Chrna7-/- mice, there were no differences in VO_2 (Fig. 5C and D) and VCO_2 (Fig. 5G and H) compared with WT mice, but HFD consumption increased food intake (Fig. 6E and F), heat (Fig. 6H) and locomotor activity in the dark cycle (Fig. 6C). Male Chrna7-/- mice (Fig. 5I and J) and female Chrna7-/- mice (Fig. 5K and L) presented reduced RER compared with WT mice.

3.5. mHypoA cells treated with the serum of male Chrna7$^{-/-}$ fed an HFD showed reduced AKT phosphorylation after insulin stimulation

mHypoA cells previously treated with serum from male WT or Chrna7$^{-/-}$ mice fed an HFD for 4 weeks were challenged with insulin. mHypoA cells treated with serum from male WT mice fed an HFD showed an increase in AKT phosphorylation after insulin stimulation. mHypoA cells treated with serum from male Chrna7$^{-/-}$ mice fed an HFD presented lower AKT phosphorylation than mHypoA cells treated with serum from male WT mice fed an HFD (Fig. 7B). On the other hand, when performing intraperitoneal leptin stimulation, both male WT or Chrna7$^{-/-}$ mice showed an increase in STAT3 phosphorylation (Fig. 7A).

4. Discussion

The activation of inflammatory pathways has been associated with many pathologies such as diabetes and obesity (Gupta et al., 2018; Li et al., 2018; Nishio et al., 2017). Inflammatory cytokines are involved in metabolic disorders and dysregulation of energy homeostasis, changes that are associated with obesity (Rohm, Meier, Olefsky, & Donath, 2022; Shivappa et al., 2017). It is known that inflammatory cytokines impair

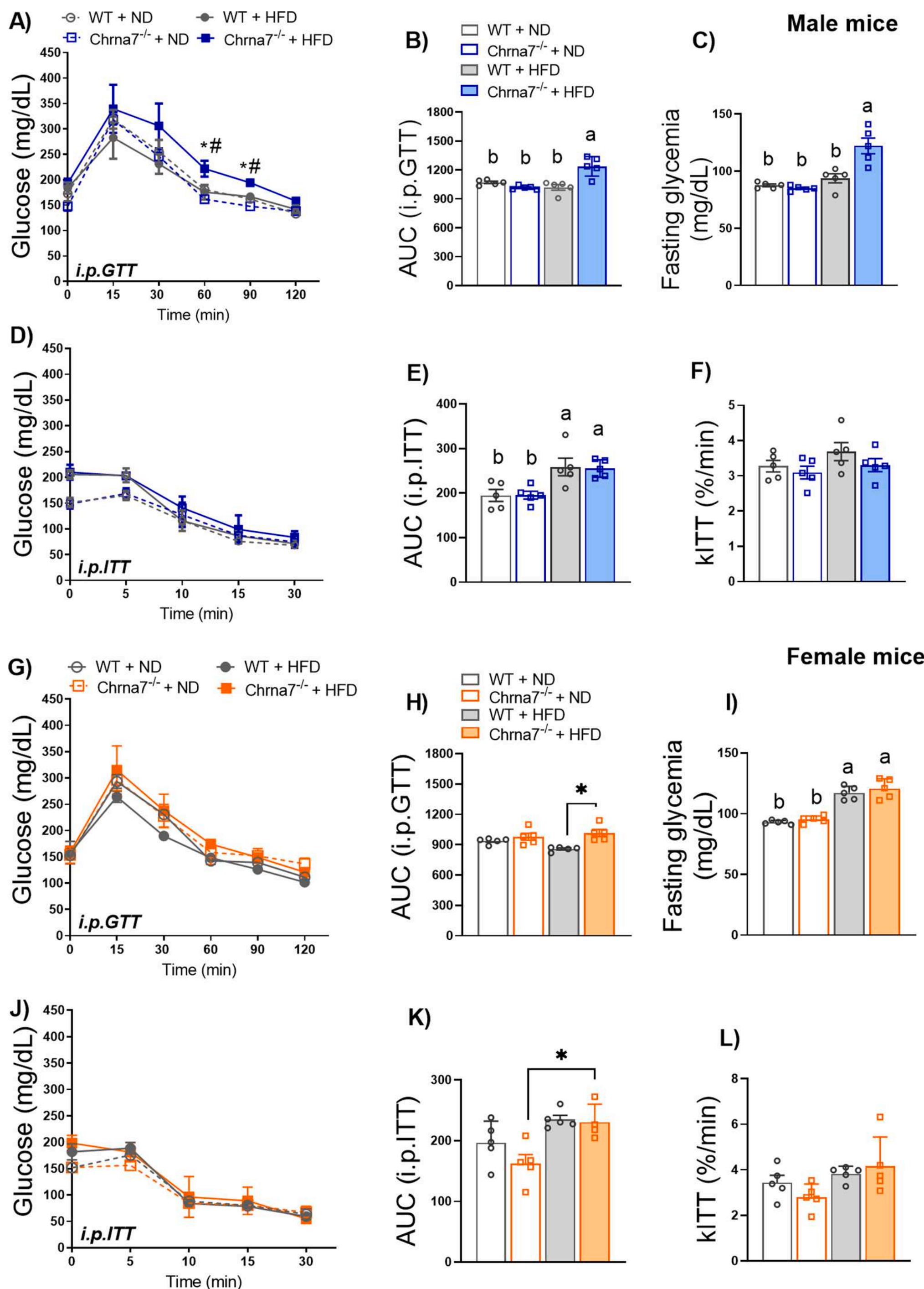

Fig. 2. Fasting blood glucose and peripheral glucose tolerance response are affected in Chrna7$^{-/-}$ mice fed a high-fat diet (HFD) (A) Intraperitoneal glucose tolerance test (ipGTT) in male Chrna7$^{-/-}$ mice. Two-way analysis of variance (ANOVA) with the post hoc Bonferroni test (*$p < 0.05$ for Chrna7-/- + ND vs. Chrna7-/- + HFD; #$p < 0.05$ for Chrna7-/- + ND vs. Chrna $^{-/-}$ + HFD). **(B)** Area under the curve for the ipGTT in male Chrna7$^{-/-}$ mice. One-way ANOVA with the post hoc Bonferroni test. Different letters show statistical differences. **(C)** Blood glucose levels in fasting glycaemia in male Chrna7$^{-/-}$ mice. One-way ANOVA with the post hoc Bonferroni test. Different letters show statistical differences. **(D)** Intraperitoneal insulin tolerance test (ipITT) in male Chrna7$^{-/-}$ mice. Two-way ANOVA with the post hoc Bonferroni test (*$p < 0.05$ for Chrna7-/- + ND vs. Chrna7-/- + HFD; #$p < 0.05$ for Chrna7-/- + ND vs. Chrna $^{-/-}$ + HFD). **(E)** Area under the curve for the ipGTT in male Chrna7$^{-/-}$ mice. One-way ANOVA with the post hoc Bonferroni test. Different letters show statistical differences. **(F)** Decay of the glucose rate (kITT) during the insulin tolerance test in male Chrna7$^{-/-}$ mice. One-way ANOVA with the post hoc Bonferroni test. Different letters show statistical differences. **(G)** ipGTT in female Chrna7$^{-/-}$ mice. Two-way ANOVA with the post hoc Bonferroni test (*$p < 0.05$ for Chrna7-/- + ND vs. Chrna7-/- + HFD; #$p < 0.05$ for Chrna7-/- + ND vs. Chrna $^{-/-}$ + HFD). **(H)** Area under the curve for the ipGTT in female Chrna7$^{-/-}$ mice. One-way ANOVA with post-hoc Bonferroni. Different letters show statistical differences. **(I)** Blood glucose levels in fasted female Chrna7$^{-/-}$ mice. One-way ANOVA with the post hoc Bonferroni test. Different letters show statistical differences. **(J)** ipITT in female Chrna7$^{-/-}$ mice. Two-way ANOVA with the post hoc Bonferroni test (*$p < 0.05$ for Chrna7-/- + ND vs. Chrna7-/- + HFD; #$p < 0.05$ for Chrna7-/- + ND vs. Chrna $^{-/-}$ + HFD). **(K)** Area under the curve for the ipGTT in female Chrna7$^{-/-}$ mice. One-way ANOVA with the post hoc Bonferroni test. Different letters show statistical difference. **(L)** kITT during the insulin tolerance test in female Chrna7$^{-/-}$ mice. One-way ANOVA with the post hoc Bonferroni test. Different letters show statistical difference. $p < 0.05$ and $n = 5$ mice per group as indicated by the dots). The data are presented as mean ± standard error of mean.

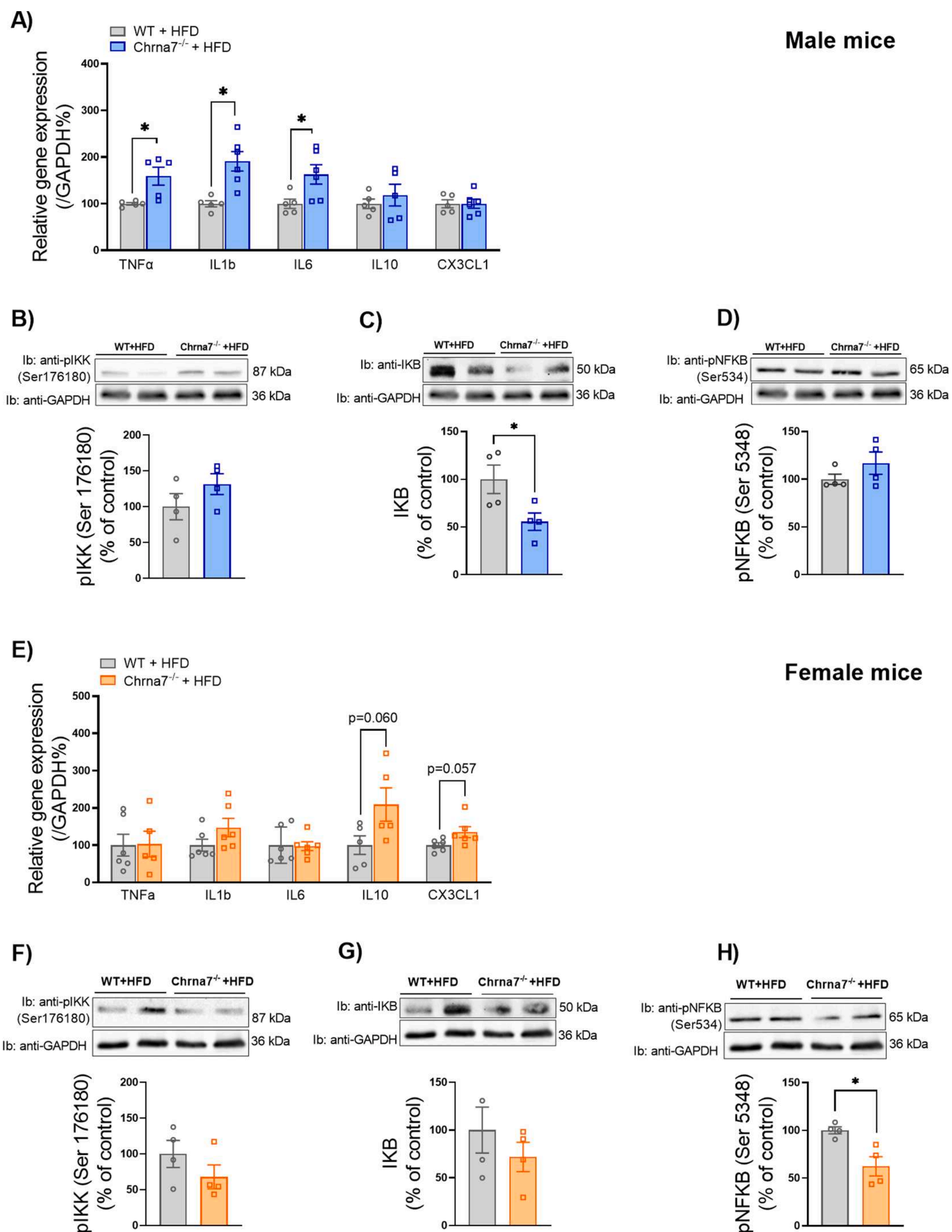

Fig. 3. Chrna7$^{-/-}$ male mice fed a high-fat diet (HFD) showed increased expression of inflammatory markers in the hypothalamus. (A) Relative gene expression of cytokines and chemokines in male Chrna7$^{-/-}$ mice hypothalamus. Student's *t*-test. **(B–D)** Relative protein expression of pIKK, IκB and pNF-κB in male Chrna7$^{-/-}$ hypothalamus. Student's *t*-test. **(E)** Relative gene expression of cytokines and chemokines in female Chrna7$^{-/-}$ hypothalamus. Student's *t*-test. **(F–H)** Relative protein expression pIKK, IκB and pNF-κB in female Chrna7$^{-/-}$ hypothalamus. Student's *t*-test. *$p < 0.05$; n = 4–5 mice per group as indicated by the dots. The data are presented as the mean ± standard error of mean.

hypothalamic leptin and insulin signalling, contributing to energy imbalance and obesity (de Git & Adan, 2015; Münzberg, Flier, & Bjørbæk, 2004). The α7nAChR has been implicated in the reduction of inflammatory cytokine expression in different tissues and cells (Báez-Pagán, Delgado-Vélez, & Lasalde-Dominicci, 2015; Piovesana et al., 2021; Shen & Yakel, 2012; Shytle et al., 2004). Furthermore, agonists of α7nAChR in human and mice cells have shown therapeutic potential for obesity and type 2 diabetes (Xie et al., 2020).

Our previous studies have demonstrated that consuming a high-fat diet for a short-term lead to a reduction in α7nAChR expression in the hypothalamus, as well as an increase in cytokine expression [2,3]. Furthermore, α7nAChR gene expression is reduced in the white adipose tissue of obese compared with non-obese individuals. However, the expression is restored following a change in lifestyle and and subsequent body weight loss (Cancello et al., 2012), indicating that the inflammatory condition can influence α7nAChR gene expression. This phenomenon may appear paradoxical given that α7nAChR is recognized for its anti-inflammatory properties (Wang et al., 2003). However, we have recently shown that the expression of α7nAChR is impaired by TNF-alpha and microRNA Let7a, further supporting the hypothesis that the

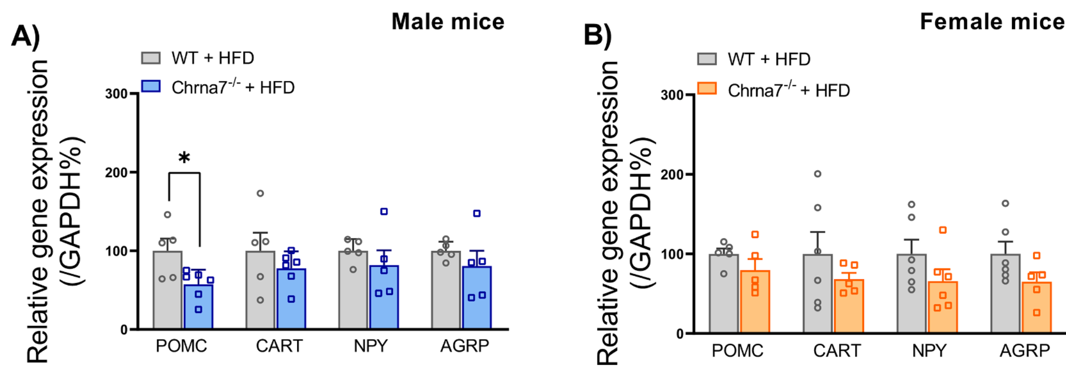

Fig. 4. POMC relative gene expression decrease in the hypothalamus of male Chrna7$^{-/-}$ mice. (A) Relative gene expression of neuropeptides in male Chrna7$^{-/-}$ hypothalamus. Student's *t*-test. **(B)** Relative gene expression of neuropeptides in female Chrna7$^{-/-}$ hypothalamus. Student's *t*-test. Statistical difference *$p < 0.05$; n = 5–6 per group as indicated by the dots). The data are presented as mean ± standard error of mean.

Fig. 5. Altered energy expenditure profile in Chrna7$^{-/-}$ mice fed a high-fat diet (HFD). (A and B) Oxygen volume (VO_2) in the light and dark cycles measured over 24 h in male Chrna7$^{-/-}$ mice. Student's *t*-test. **(C and D)** VO_2 in the light and dark cycles measured over 24 h in female Chrna7$^{-/-}$ mice. Student's *t*-test. **(E and F)** Carbon dioxide volume (VCO_2) in the light and dark cycles measured over 24 h in male Chrna7$^{-/-}$ mice. Student's *t*-test. **(G and H)** VCO_2 in the light and dark cycles measured over 24 h in female Chrna7$^{-/-}$ mice. Student's *t*-test. **(I and J)** The respiratory exchange ratio (RER) in the light and dark cycles measured over 24 h in male Chrna7$^{-/-}$ mice. Student's *t*-test. **(K and L)** RER in the light and dark cycles measured over 24 h in female Chrna7$^{-/-}$ mice. Student's *t*-test. Statistical difference *$p < 0.05$; n = 6–8 mice per group as indicated by the dots). The data are presented as the mean ± standard error of mean.

inflammatory environment can disrupt α7nAChR expression (Simino et al., 2023). Pro-inflammatory cytokine expression and immune cell infiltration in the hypothalamus are observed following short-term HFD consumption (Dorfman & Thaler, 2015; Thaler et al., 2012; Waise et al., 2015). Thus, it appears that the activation of inflammatory pathways in the hypothalamus and the reduction in α7nAChR expression are evident right from the beginning HFD consumption. Therefore, we hypothesize that HFD consumption may impair α7nAChR expression, intensify the hypothalamic inflammatory response triggered by HFD, and worsen metabolic damage in mice.

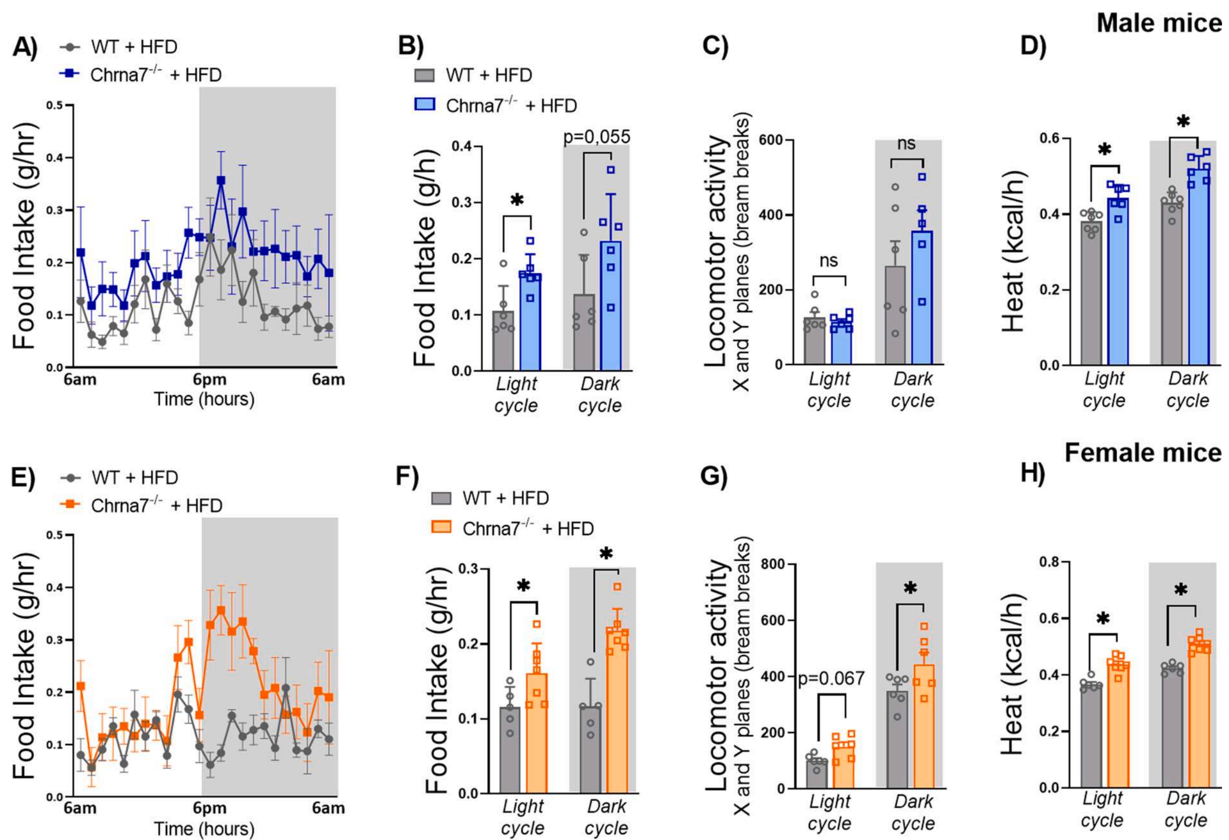

Fig. 6. Increased food intake in male and female Chrna7$^{-/-}$ mice fed a high-fat diet (HFD), but without increased locomotor activity in males. (A and B) Food intake in the light and dark cycles measured over 24 h in male Chrna7$^{-/-}$ mice. Student's *t*-test. **(C)** Spontaneous locomotor activity measured over 24 h in male Chrna7$^{-/-}$ mice. Student's *t*-test. **(D)** Heat production measured over 24 h in male Chrna7$^{-/-}$ mice. Student's *t*-test. **(E and F)** Food intake in light cycle and dark cycle measured over 24 h in female Chrna7$^{-/-}$ mice. Student's *t*-test. **(G)** Spontaneous locomotor activity measured over 24 h in female Chrna7$^{-/-}$ mice. Student's *t*-test. **(H)** Heat production measured over 24 h in male Chrna7$^{-/-}$ mice. Student's *t*-test. Statistical difference *$p < 0.05$; n = 5 mice per group as indicated by the dots. The data are presented as the mean ± standard error of mean.

We tested our hypothesis by utilizing Chrna7-/- and WT mice. Initially, we observed that the deletion of α7nAChR did not influence molecular and behavioral parameters in mice fed the ND. Indeed, *Chrna7* deletion did not alter body weight gain, retroperitoneal and epididymal adipose tissues mass, fasting glucose and tolerance to glucose and insulin in male and female Chrna7$^{-/-}$ mice fed the ND. Li and colleagues (Li et al., 2018) reported similar findings: the deletion of *Chrna7* had no impact on food intake, plasma insulin levels, or fasting glucose levels when compared to WT mice. Additionally, the authors demonstrated that it was only after HFD consumption that the α7nAChR knockout mice (Chrna7$^{-/-}$) exhibited impairments in energy expenditure, body weight gain, and liver damage compared to WT mice fed HFD. Many studies have shown anti-inflammatory effects through the activation of α7nAChR (Gausserès et al., 2020; Skok & Lykhmus, 2016; Wang et al., 2003). The critical role of α7nAChR in the cholinergic anti-inflammatory pathway depends on the presence of inflammatory mediators that may drive activation of the receptors located in the afferent arm of the vagus nerve leading to acetylcholine release, α7nAChR activation and inhibition of pro-inflammatory cytokine expression (Tracey, 2002). Chrna7$^{-/-}$ mice present elevated pro-inflammatory cytokine production in response to LPS (Tyagi, Agrawal, Nath, & Shukla, 2010), free fatty acids and TNF-α compared with WT mice (X. Wang, Yang, Xue, & Shi, 2011). However, here we demonstrated that when fed the ND, the hypothalamic expression of *Tnfa*, *Il1b* and *Il-6* was not different between Chrna7$^{-/-}$ and WT mice. Thus, in basal conditions (the ND), α7nAChR deletion does not affect the inflammatory pathways.

Dysregulated α7nAChR activity has been associated with neurodegenerative disorders and behavioural disturbances in pro-inflammatory conditions. Nevertheless, the behavioural consequences of α7nAChR deletion seem to be controversial. After analysing several behavioural parameters such as social interaction, hyperactivity, anxiety and depression, Yin and colleagues (Yin, Chen, Yang, Xue, & Schaaf, 2017) did not identify differences between Chrna7$^{-/-}$ and WT mice. On the other hand, many authors have shown that α7nAChR deletion alters cognitive/social behaviour (Nacer et al., 2021) and synaptic function (Ma et al., 2014), increases amyloid precursor protein expression and Aβ levels (Tropea et al., 2021) and promotes metabolic disorders associated with ageing (Gausserès et al., 2020). These behavioural changes may be related to the ion channel function of α7nAChR rather than JAK2--STAT3 activation (de Jonge et al., 2005).

Considering that the hypothalamus is the central region in the control of homeostasis, α7nAChR deletion could result in important metabolic damage. Our group has previously demonstrated that α7nAchR expression is reduced in immortalised hypothalamic neurons directly exposed to inflammatory conditions, such as LPS or pro-inflammatory microglial conditioned medium (Amaral et al., 2022). Additionally, in Swiss mice, we demonstrated that intraperitoneal injection of LPS or consumption of an HFD induced an inflammatory response and reduced the protein content of α7nAchR in the hypothalamus and other tissues (Souza et al., 2019a; Martins et al., 2021).

Importantly, our results showed that consumption of an HFD exacerbated the body weight gain and adiposity in male Chrna7$^{-/-}$ compared with male WT mice. Furthermore, *Tnfa*, *Il1β* and *Il6* gene expression in the hypothalamus increased significantly in male Chrna7-/- compared with male WT mice after consuming an HFD for 4 weeks. Male Chrna7-/- mice also presented reduced IκB expression that could be related to

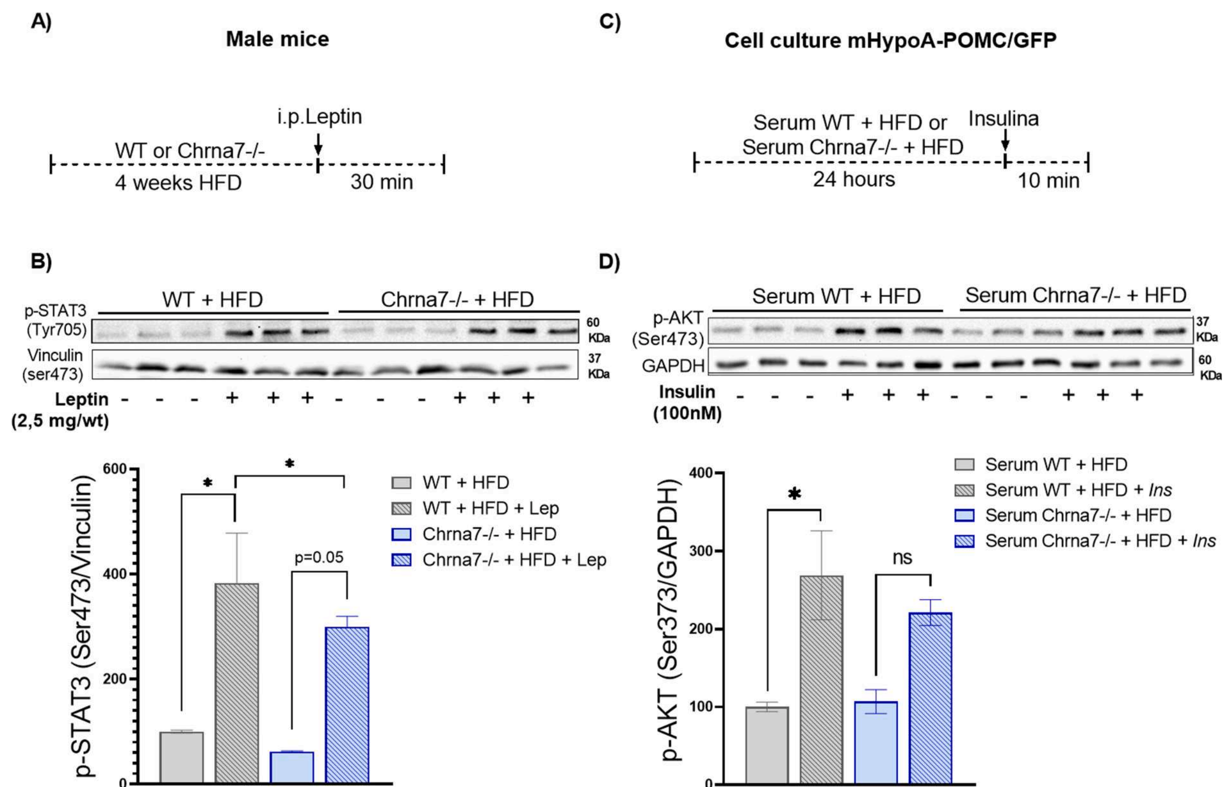

Fig. 7. Deletion of α7nAChR (Chrna7$^{-/-}$) impairs STAT phosphorylation *in vivo* and AKT phosphorylation in response to insulin stimulation *in vitro*. (A) Relative p-STAT protein expression in male Chrna7$^{-/-}$ hypothalamus after consuming an HFD for 4 weeks and intraperitoneal leptin stimulation. One-way analysis of variance (ANOVA) with the post hoc Bonferroni test. **(B)** Relative p-AKT protein expression in mHypoA-POMC/GFP cells incubated for 24 h with serum from male wildtype (WT) or Chrna7$^{-/-}$ mice fed an HFD for 4 weeks and then treated with insulin (100 nM) for 10 min. One-way ANOVA with the post hoc Bonferroni test. Statistical difference *$p < 0.05$; n = 3 mice per group. The data are presented as the mean ± standard error of mean.

nuclear migration and target gene regulation of NF-κB. The expression of pro-inflammatory cytokines such as *Tnfa*, *Il1β* and *Il6* has been linked to NF-κB activity as a transcription factor and the inflammatory response (de Jonge et al., 2005). It is known that JAK2–STAT3 signalling could converge directly and result in NF-κB inhibition (Báez-Pagán et al., 2015). Therefore, the higher expression of inflammatory cytokines seems to be associated with impaired STAT3 activation by α7nAChR-mediated intracellular JAK2–STAT3 signalling.

Hypothalamic neuroinflammation associated with obesity has been described to affect important mechanisms in the control of homeostasis, such as leptin and insulin signalling (de Jonge et al., 2005). Although our results revealed increased hypothalamic expression of inflammatory cytokines in male Chrna7$^{-/-}$ compared with WT mice fed an HFD, the ability of leptin to induce hypothalamic STAT3 phosphorylation was not different between the groups, suggesting that there was no resistance to leptin (El-Haschimi, Pierroz, Hileman, Bjørbæk, & Flier, 2000; Tropea et al., 2021). A previous study that used C57BL/6J WT mice fed either a normal-fat or an HFD for 4 weeks showed that leptin-induced STAT3 phosphorylation was equivalent in both groups (El-Haschimi et al., 2000). Thus, 4 weeks seems to be insufficient to promote leptin resistance.

The α7nAchR deletion in male mice was accompanied by an increase in the fasting glucose levels and the AUC of the ipGTT. Considering that α7nAChR is also expressed in pancreatic β-cells, the absence of the receptor in these cells may alter insulin secretory mechanisms and glycaemic homeostasis (Gupta et al., 2018; Li et al., 2018; Nishio et al., 2017). Previously, Li and colleagues (Li et al., 2018) demonstrated that male Chrna7-/- mice have greater liver damage and increased inflammatory markers and fibrosis compared with WT mice after HFD consumption.

Interestingly, female mice did not show differences in body weight, adiposity, glucose homeostasis and hypothalamic inflammatory cytokines level associated with α7nAChR deletion and HFD consumption. Female mice fed an HFD are protected from obesity-induced insulin resistance due to oestradiol-mediated reductions in tissue inflammation (Camporez et al., 2019). Oestrogen participates in metabolic homeostasis by regulating lipid and glucose metabolism (Barros & Gustafsson, 2011), and the oestrogen receptor (Erα) is a critical determinant of the anti-inflammatory response (Heine, Taylor, Iwamoto, Lubahn, & Cooke, 2000; Morselli et al., 2014). Thereby, the sexual dimorphic response of Chrna7-/- female mice to HFD consumption could be related to activation of ERα.

Oestrogen increases STAT3 phosphorylation in the hypothalamus and improves leptin sensitivity (Farhadi, Khaksari, Azizian, & Dabiri, 2022). Similarly, α7nAChR activation also stimulates STAT3 phosphorylation (de Jonge et al., 2005) to inhibit food intake and inflammatory cytokine expression (Souza et al., 2019b). Thus, our results suggest that in female Chrna7-/- mice oestrogen signalling can also protect against obesity induced by an HFD. Here we found that ovariectomised female Chrna7-/- mice fed an HFD had increased body weight gain and adiposity compared with non-ovariectomised female Chrna7-/- mice fed an HFD. However, hypothalamic cytokine levels were not affected by ovariectomy. We do not discard the possibility that other factors could be protecting the female mice. For example, a previous study in C57BL/6J mice showed significant differences in the gut microbiota composition and diversity between males and females (Kim, Unno, Kim, & Park, 2020), and mice subjected to ovariectomy showed gut microbiota dysbiosis (Lei et al., 2021). In this sense, hormones generated by the ovaries seem to play important roles in communicating with and regulating the gut microbiota. Interoceptive signals from the intestine seem to be integrated into the central nervous system to regulate feeding behaviour and to influence the homeostatic control of

body weight (Cox, West, & Cripps, 2015; Lei et al., 2021; Moura-Assis, Friedman, & Velloso, 2021). Additionally, it's important to note that the phase of the estrous cycle can influence metabolic and hormonal parameters. We cannot guarantee that all female mice used in the indirect calorimetry protocols were in the same phase of the estrous cycle.

In addition to increased food intake, male Chrna7-/- mice fed a high-fat diet exhibited a decrease in hypothalamic Pomc gene expression compared to WT mice. POMC is an anorectic neuropeptide and studies have suggested that POMC neurons are preferentially affected by changes in hypothalamic inflammation (Razolli et al., 2020). Interestingly, male mice also showed increased energy expenditure with no change in spontaneous activity. We believe that receptor deletion in all tissues may be responsible for systemic metabolic changes. This is a limitation of our study and the specific deletion of α7nAChR in the POMC neurons may be interesting to be investigated in the future.

Using an *in vitro* approach, we evaluated the ability of insulin to induce AKT phosphorylation in a hypothalamic neuronal cell line (mHypoA-2/29) previously conditioned in medium with serum from WT or Chrna7$^{-/-}$ mice fed an HFD. Cells exposed to serum from male Chrna7$^{-/-}$ mice fed an HFD were less responsive to insulin than cells exposed to serum from male WT mice fed an HFD, suggesting the development of insulin resistance. These data provide evidence that α7nAchR deletion is important in exacerbating phenotypic and molecular alterations associated with obesity in adult male mice.

5. Conclusion

The absence of α7nAChR contributes to exacerbating the impairments associated with HFD consumption in male mice but not in females. In male mice, we observed an increase in inflammatory markers in the hypothalamus accompanied by metabolic changes such as body weight gain, adiposity, increased fasting glucose levels, reduced glucose and insulin tolerance, and changes in energy expenditure. Furthermore, *in vitro* assays indicates that such alterations may be accompanied by impaired insulin signalling in POMC neurons.

CRediT authorship contribution statement

Priscilla Karla Fernandes Lopes: Writing – original draft, Data curation, Formal analysis, Investigation, Methodology. **Suleyma de Oliveira Costa:** Data curation, Investigation, Methodology. **Laís A de Paula Simino:** Data curation, Formal analysis, Investigation, Methodology. **Wenicios Ferreira Chaves:** Data curation, Formal analysis, Investigation, Methodology. **Franciely Alves Silva:** Data curation, Investigation. **Caroline Lobo Costa:** Data curation, Investigation. **Marciane Milanski:** Resources, Writing – review & editing. **Leticia Martins Ignacio-Souza:** Resources, Formal analysis, Writing – review & editing. **Adriana Souza Torsoni:** Resources, Writing – review & editing, Conceptualization. **Marcio Alberto Torsoni:** Funding acquisition, Resources, Supervision, Conceptualization, Project administration, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

This work was supported by grants from the Sao Paulo Research Foundation (FAPESP grant numbers 2016/23833-6, 2019/07615-7 and 2021/11772-0) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES, Finance Code 001). Marciane Milanski, Leticia M. Ignacio-Souza, Adriana Souza Torsoni and Marcio Alberto Torsoni are members of the Obesity and Comorbidities Research Center (OCRC) at UNICAMP.

Appendix A. Supplementary materials

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2023.113808.

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