

Original Paper

JAK2/STAT3 Pathway is Required for $\alpha 7$ nAChR-Dependent Expression of POMC and AGRP Neuropeptides in Male Mice

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Key Words

$\alpha 7$ nAChR • nAChR • Hypothalamus • Energy homeostasis • Mice

Abstract

Background/Aims: Cholinergic signalling mediated by the activation of muscarinic and nicotinic receptors has been described in the literature as a classic and important signalling pathway in the regulation of the inflammatory response. Recent research has investigated the role of acetylcholine, the physiological agonist of these receptors, in the control of energy homeostasis at the central level. Studies have shown that mice that do not express acetylcholine in brain regions regulating energy homeostasis present with excessive weight gain and hyperphagia. However, it has not yet been well-described in the literature which cholinergic receptor subunits are involved in this response; moreover, the signalling pathways responsible for the observed effects are not fully delineated. The hypothalamus is the regulating centre of energy homeostasis, and the $\alpha 7$ subunit of the nicotinic acetylcholine receptor ($\alpha 7$ nAChR) is highly expressed in this region. When active, $\alpha 7$ nAChR recruits proteins such as JAK2/STAT3 to mediate its signalling; the same intracellular components are required by leptin, an anorexigenic hormone. The aim of the present study was to evaluate the role of the hypothalamic $\alpha 7$ nAChR in the control of energy homeostasis. **Methods:** The work was performed on Swiss male mice. Initially, using immunofluorescent staining on brain sections, the presence of $\alpha 7$ nAChR in hypothalamic cells regulating energy homeostasis was evaluated. Animals were submitted to stereotaxis in the lateral ventricle and intracerebroventricular stimulation (ICV) was used for the administration of an agonist (PNU) or antagonist (α -bungarotoxin) of $\alpha 7$ nAChR. Metabolic parameters were evaluated and the expression of neuropeptides was evaluated in the hypothalamus by real-time PCR and western blot. The expression of hypothalamic neuropeptides was evaluated in mice treated with siRNA or inhibitors of JAK2/STAT3 (AG490 and STATTIC) proteins. We also evaluated food intake

in $\alpha 7$ nAChR knockout animals ($\alpha 7$ KO). Additionally, in mouse hypothalamic cell culture (the mypHoA-POMC/GFP lineage), we evaluated the expression of neuropeptides and pSTAT3 after stimulation with PNU. **Results:** Our results indicate co-localisation of $\alpha 7$ nAChR with α -MSH, AgRP and NPY in hypothalamic cells. Pharmacological activation of $\alpha 7$ nAChR reduced food intake and increased hypothalamic POMC expression and decreased NPY and AgRP mRNA levels and the protein content of pAMPK. Inhibition of $\alpha 7$ nAChR with an antagonist increased the mRNA content of NPY and AgRP. Inhibition of $\alpha 7$ nAChR with siRNA led to the suppression of POMC expression and an increase in AgRP mRNA levels. $\alpha 7$ KO mice showed no changes in food intake. Inhibition of proteins involved in the JAK2/STAT3 signalling pathway reversed the effects observed after PNU stimulation. POMC-GFP cells, when treated with PNU, showed increased POMC expression and nuclear translocation of pSTAT3. **Conclusion:** Thus, selective activation of $\alpha 7$ nAChR is able to modulate important markers of the response to food intake, suggesting that $\alpha 7$ nAChR activation can suppress the expression of orexigenic markers and favour the expression of anorexics using the intracellular JAK2/STAT3 machinery.

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Introduction

The control of food intake is a widely studied mechanism of key importance for elucidating the events that take place during metabolic disorders, such as obesity [1, 2]. The hypothalamus is the major regulatory centre of energy homeostasis and integrates peripheral information from several metabolic and hormonal signals, including insulin, leptin and ghrelin [3, 6]. Acetylcholine also appears to be involved in the regulation of feeding behaviour, mostly on account of its structural similarity with nicotine, an important appetite suppressor [7]. Acetylcholine has been shown to exert neuromodulatory effects on energy homeostasis by depolarising and activating anorexigenic pro-opiomelanocortin (POMC) neurons while inhibiting the synaptic excitatory activity of orexigenic NPY neurons in the hypothalamic arcuate nucleus [8].

Nicotinic acetylcholine receptors (nAChRs) are calcium-selective ion channels. To date, eleven neuronal subunits of nAChRs that assemble as homopentamers ($\alpha 7$ and $\alpha 9$) or heteropentamers ($\alpha 4\beta 2$, $\alpha 7\beta 2$, $\alpha 2\beta 2$, $\alpha 9\alpha 10$, $\alpha 4\beta 2$) have been identified [9]. The $\alpha 7$ nAChR subunit is highly expressed in the hypothalamus and studies show that POMC and NPY neurons in the arcuate nucleus receive direct stimulation from cholinergic axon terminals [10].

The $\alpha 7$ nAChR subunit has been extensively studied in the context of the cholinergic anti-inflammatory pathway [11]. The activation of $\alpha 7$ nAChR in monocytes and macrophages triggers the recruitment of the cytosolic protein JAK2, leading to the formation of a heterodimer complex that initiates STAT3 signal transduction [12]. Apart from negatively regulating the inflammatory response, the JAK2/STAT3 signalling pathway is involved in the regulation of a variety of biological processes, including the hypothalamic suppression of food intake in response to leptin [13, 14].

The ablation of cholinergic neurons in the basal forebrain leads to hyperphagia and severe obesity in mice [15], whereas $\alpha 7$ nAChR activation in a mouse model of diabetes improves metabolic parameters by reducing food intake [16]. Furthermore, studies show that the activation and inhibition of nicotinic receptors can modulate neuronal excitability in POMC and NPY neurons [17, 18].

Despite its crucial importance for the hypothalamic suppression of food intake, the activation of the JAK2/STAT3 signalling pathway in this context still requires further investigation. Our hypothesis is that neurons expressing NPY/AGRP and POMC neuropeptides also express $\alpha 7$ nAChR receptor that controls eating behavior by activating the JAK2/STAT3 pathway and the expression of neuropeptides in the hypothalamus. Thus, this study aimed to investigate the selective role of $\alpha 7$ nAChR in the control of food intake through the activation of the JAK2/STAT3 signalling pathway.

Materials and Methods

Ethics statement and animal model

All the experiments were done in accordance with Brazilian College of Animal Experimentation (COBEA) regulations and after approval by the Ethics Committee on Animal Use at UNICAMP (Protocol 4787-1/2018). Swiss male mice were provided by the Animal Breeding Centre at the University of Campinas (Campinas, SP, Brazil) and housed in a 12 h light/dark cycle in a temperature-controlled environment.

A total of ninety eight Swiss male mice were selected for the present work and randomly distributed in the experimental groups. Four mice were used for the perfusion technique. All the others were submitted cannula implantation surgery. Twenty mice were used for PNU and saline stimulation (ten mice for respirometry and ten mice for mRNA analysis). Two mice died during surgery recovery. Twenty animals were selected for α -btX and saline stimulation (ten for ingestion analysis and 10 for mRNA analysis). Four mice died during surgery. Twenty mice were selected for mRNA analyses of si-RNA and sc-RNA treatment (six mice died during the protocol to ICV injection). Fifteen animals were selected for the experimental group with antagonist AG490. They were divided in saline, PNU, AG490 + PNU groups. Fifteen animals were selected for the experimental group with STATTC antagonist. They were divided in saline, PNU, STATTC + PNU. Four mice were selected for qualitative analysis of protein content of pSTAT3 after treatment with PNU. Four $\alpha 7$ KO mice ($\alpha 7$ nAChR^{-/-}C57BL/6 background, Chrna7tm1Bay, Jax stock no. 003232) that were kindly provided by Dr. Hosana Gomes Rodrigues (UNICAMP) and 4 wild type (WT C57BL/6) mice were used for food intake analysis.

Cannula implantation

Mice were continually instrumented with an ICV (lateral ventricle) cannula and kept under controlled temperature ($22 \pm 1^\circ\text{C}$) and light-dark conditions (12 h) in individual cages. Surgery was performed under anaesthesia with isoflurane (induction 5%, 2% maintenance) [19], and all efforts were made to minimise animal suffering. The cannula was implanted into the lateral cerebral ventricle at pre-established coordinates from Bregma, i.e. anteroposterior -0.34 mm, lateral -1.0 mm and depth -2.2 mm, according to a previously described method [20]. The correct position of the cannula was confirmed after recovery by assessing the dipsogenic response after the administration of 2 μL of 10^{-6} M angiotensin II (Sigma, MO, USA). Animal recovery was monitored for 1 week and none showed signs of distress.

Intracranial microinfusions

The infusion rate was controlled by a manually operated microsyringe (Hamilton, Bonaduz, Switzerland). Polyethylene tubing connected the syringe to 28 gauge injectors (Plastics One) projecting 5 mm below the guide cannulae. Drugs or saline were infused, and thereafter the cannulae were left in place for an additional 30 sec to allow for diffusion of the saline or drug into the tissue before removing the injector. After the stimulus time described in Table 1, mice received a mixture containing ketamine (100 mg/kg bw) and xylazine (10 mg/kg bw) as an anaesthetic protocol and decapitation was used to cull the mice that had been starved for 16 h.

Table 1. Specifications of drug treatments, dose, injected volume, stimulus time, drug label and reference

Drug	Dose	Stimulus time for evaluation of protein content	Stimulus time for evaluation of gene expression	Reference
PNU- 282987 sigma	450 picomoles/mouse	20 min	40 min	BRUNZELL; MCINTOSH, 2011
α - Bungarotoxin Cayman Chemical	2 μg /mouse	-	6 h	DAMAJ; et al,2005
AG490 Cayman Chemical	10 nmol/mouse	20 min	20 min	DAMM, et al, 2013
STATTC Cayman Chemical	60 picomoles/mouse	20 min	20 min	
Saline	3 μL (0,9%)	20 min	20 min	-

Food intake and energy expenditure measurement

Food intake (g) was monitored for 24 h after the overnight fast, when mice were refed. Indirect calorimetry heat (kcal/body weight) and food intake were measured using an indirect calorimetry system (Oxymax, Columbus Instruments) installed under a constant environmental temperature (22°C) and light/dark cycle (12 h). For this assay, mice were previously adapted to individual chambers with free access to food and water.

Food intake was measured after ICV injection of the respective compound or control vehicle, and intake data were collected 1, 2, 4, 8, and 24 h after PNU, α -bungarotoxin (α Btx) or control vehicle administration. In $\alpha 7$ KO ($\alpha 7$ nAChR^{-/-} C57BL/6 background, Chrna7tm1Bay, number 003232; kindly provided by Profa. Dra. Hosana Gomes Rodrigues) and wild type (WT C57BL/6) mice, food intake was measured for 24 h after an overnight fast at 2, 4, 8 and 24 h after refeeding.

Cell culture analysis

The mypHoA-POMC/GFP cell line was used, kindly provided by Dr. Denise Belsham (University of Toronto) and Dr. Marciane Milanski. mypHoA-POMC/GFP corresponds to a cell line of immortalised neurons from the hypothalamus of C57BL/6J-Tg (POMC EGFP) 1Low/J mice. The cells were cultured in Dulbecco's Modified Eagle's Medium (Invitrogen) culture medium supplemented with 5% foetal bovine serum (Invitrogen) and 1% penicillin (100 U/mL)/streptomycin (100 μ g/mL) (Invitrogen) and incubated at 37°C with 5% CO₂ and 95% humidity. PNU 282987 1 μ M (Sigma), a selective $\alpha 7$ nAChR agonist, was added to the culture medium for 15 minutes and the content of $\alpha 7$ nAChR and the phosphorylation of STAT3 were evaluated.

RT-PCR analysis

Cell culture or whole hypothalamic RNA was extracted using TRIzol reagent (Invitrogen Corporation, CA, USA) according to the manufacturer's recommendations. Total RNA was quantified on a Nanodrop ND-2000 spectrophotometer (Thermo Electron, WI, USA). Reverse transcription was performed with 3 ng of total RNA and a High Capacity cDNA Reverse Transcription kit (Life Technologies Corporation, Carlsbad, CA, USA). All oligonucleotide primers and fluorogenic probe sets for TaqMan real-time PCR were obtained from Applied Biosystems: POMC (Mm00435874_m1), NPY (Mm01410146_m1), AgRP (Mm00475829_g1) and ACTB (4351315). Each PCR reaction contained 60 ng of complementary DNA. Gene expression was quantified by real-time PCR performed on an ABI Prism 7500 Fast platform. Data were analysed using Sequence Detection System 2.0.5 (Life Technologies Corporation, Carlsbad, CA, USA), and expressed as relative values determined by the comparative threshold cycle (Ct) method (2^{-DDCt}) according to the manufacturer's recommendations.

Tissue extraction and western blot

Hypothalamic samples were frozen in liquid nitrogen and stored at -80°C until processing. The tissues were homogenised in freshly prepared ice-cold buffer [1% (v/v) Triton X-100, 0.1 M Tris, pH 7.4, 0.1 M sodium pyrophosphate, 0.1 M sodium fluoride, 0.01 M EDTA, 0.01 M sodium vanadate, 0.002 M PMSF and 0.01 M aprotinin]. Insoluble material was removed by centrifugation at 12,000 rpm for 30 min at 4°C. The protein concentration in the supernatant was determined using the Bradford dye-binding method. The supernatant was resuspended in Laemmli sample buffer and boiled for 5 min before separation by SDS-PAGE using a miniature slab gel apparatus (BioRad, Richmond, CA, USA). Electrotransfer of proteins from the gel to a nitrocellulose membrane was performed for 120 min at 120 V. Nitrocellulose membranes were probed overnight at 4°C with specific antibodies as described: phospho-STAT3 (#9145S), β -actin (#4967S) and phospho-AMPK (#2535S) from Cell Signaling (Danvers, MA, USA), NPY (sc133080) from Santa Cruz Technology (Dallas, Texas, USA) and α -MSH (bs1848R) from Bioss Antibodies (Woburn, Massachusetts USA). Membranes were incubated for 2 h at room temperature with secondary antibodies (KPL, Gaithersburg, MD, USA). Proteins recognised by the secondary antibodies were detected by chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate, ThermoFisher Scientific, MA, USA). Band intensities were quantified by optical densitometry of the developed autoradiographs using Scion Image software (ScionCorp, MD, USA) and the intensities of the bands were normalised to those of total protein or loading control (β -actin).

Immunofluorescence

Mice were perfused with 4% paraformaldehyde (PFA; Sigma-Aldrich, St. Louis, MO, USA) in PBS at pH 7.4. Brains were extracted and fixed in 4% PFA. Then, the tissue was embedded in Tissue-Tek (Sakura, Torrance, CA, USA), frozen and sectioned into 15 μ m-thick coronal sections. Slides were blocked with 1% albumin for 120 min. After that, they were incubated with specific antibodies overnight. The primary antibodies used were: NPY (1:200, sc-133080) and AgRP (1:200, sc-133080) from Santa Cruz Biotechnology, α -MSH (1:200, bs1848R) from Bioss Antibodies (Woburn, Massachusetts USA), anti- $\alpha 7$ nAChR (1:50, ab23832) from Abcam (Cambridge MA, USA) and an α -bungarotoxin CF® 488A conjugate (1:25, 300005) from Biotium (Fremont, CA, USA). Slides were incubated with secondary antibodies for 90 min. The secondary antibodies used were goat anti-mouse AlexaFluor 594 (1:200, A-11032) and donkey anti-rabbit AlexaFluor 488 (1:500 A-21206) from Invitrogen, and donkey anti-goat IgG-R (1:100, sc-2094) from Santa Cruz Biotechnology. TO-PRO3 iodine was used for nuclear labelling (1:1000; Life Technologies, Carlsbad, CA, USA). Slides were visualised and imaged using a TCS SP5 II Leica confocal microscope (Leica Microsystems, Wetzlar, Hesse, Germany).

Short interfering RNA (siRNA) experiments

Mice were assigned to $\alpha 7$ nAChR siRNA or control siRNA treatment. $\alpha 7$ nAChR siRNA (sc-42533), and control siRNA (sc-37007) were obtained from Santa Cruz Biotechnology. The siRNAs (1.5 pmol) were infused into the lateral ventricle for 5 days.

Statistical analysis

Data are expressed as mean $\pm$ SD. Student's unpaired t-tests were used to compare the differences between two groups. A one-way ANOVA was used to compare three or more categorical groups to establish whether there was a difference between them. Statistical significance for all analysis was set at $p < 0.05$. All statistical comparisons were performed using GraphPad Prism 6.01 (<http://www.graphpad.com/scientific-software/prism/>).

Results

Orexigenic (NPY/AgRP) and anorexigenic (α -MSH) neurons express $\alpha 7$ nAChR protein in the hypothalamus

Immunofluorescent analysis of the hypothalamus showed that, in most neurons in the ARC nucleus, $\alpha 7$ nAChR colocalised with NPY/AgRP neurons in the hypothalamus, indicated by red arrows (Fig. 1A and B). However, there were some cells that expressed $\alpha 7$ nAChR but not NPY/AgRP neuropeptides (green arrows). To detect the colocalisation of $\alpha 7$ nAChR and α -MSH neuropeptide, we used α Btx, which binds, to $\alpha 7$ nAChR with high affinity. As can be observed there were cells expressing both proteins (red arrows) and cells expressing only $\alpha 7$ nAChR (green arrows) (Fig. 1C).

$\alpha 7$ nAChR activation reduced food intake and modulated hypothalamic neuropeptide expression: Feeding behaviour and energy expenditure after ICV injection of PNU was monitored. The energy expenditure was not different between saline and PNU mice (Fig. 2A), but eating time showed a tendency to reduce in PNU compared to saline mice (Fig. 2B). Food intake was monitored for 24 h after ICV injection. One hour after injection, food intake was significantly reduced (60%) in PNU compared to saline mice. This effect was observed until 8 h after PNU injection. There was no difference between the groups 24 h after PNU administration (Fig. 2C-G). These effects were accompanied by an altered expression profile of neuropeptides. POMC expression was significantly higher (5.5-fold) and α -MSH showed a tendency ($p=0.06$) to increase in mice injected with PNU than saline mice (Fig. 3A and B). On the other hand, NPY and AgRP expression in PNU mice was reduced (45% and 63%, respectively) compared to saline mice (Fig. 3C and D). Additionally, the pAMPK level was significantly reduced in PNU (40%) compared to saline mice (Fig. 3E).

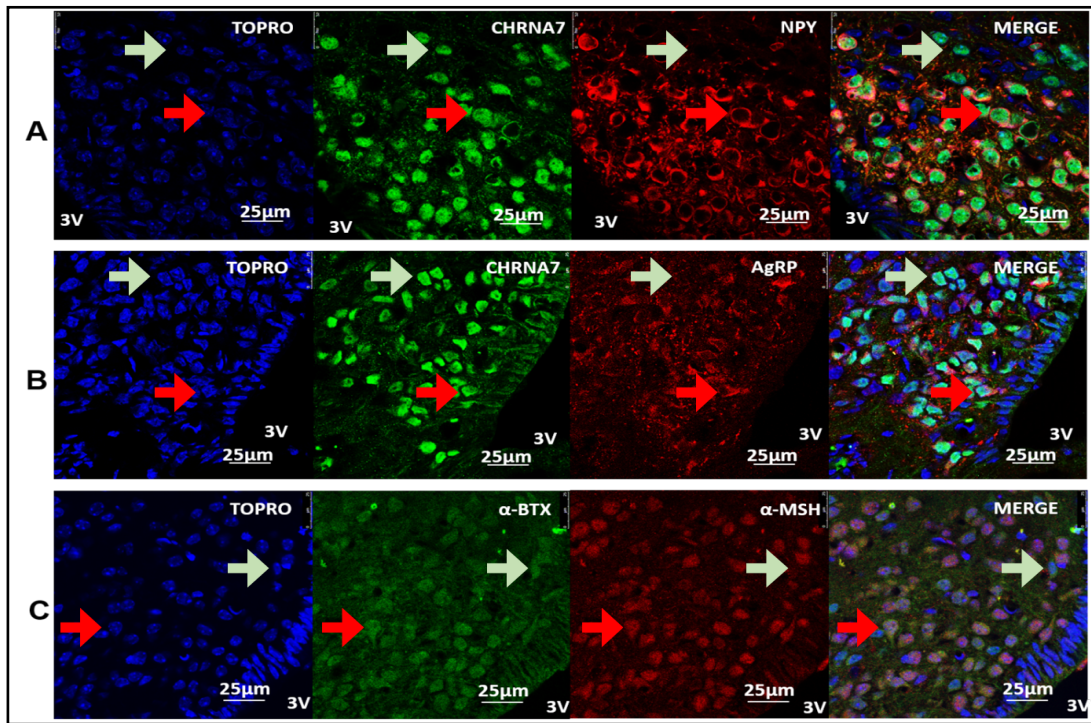


Fig. 1. AchR $\alpha 7$ and NPY/AgRP/ α -MSH neurons are colocalised in the hypothalamus. Confocal images illustrating (A) NPY neurons (red) and AchR $\alpha 7$ (green), (B) AgRP neurons (red) and AchR $\alpha 7$ (green), (C) α -MSH neurons (red) and α Bgt (green); nuclear labelling was done with TO-PRO3 (blue) and images were merged. Staining was performed on coronal sections of the brain (adult male mouse, 7 weeks old). 1000x magnification. V3, third ventricle (n=4).

Fig. 2. ICV injection of PNU reduces food intake in the male mouse. PNU (450 picomol/mouse) was administered by ICV injection; after 40 minutes the mouse was submitted to a respirometric evaluation of (A) heat, (B) eating time, and food intake in 1 h (C), 2 h (D), 4 h (E), 8 h (F) and 24 h (G). (saline n = 5, PNU n = 4). * p < 0.05 ** p < 0.01. HYPO= Hypothalamus.

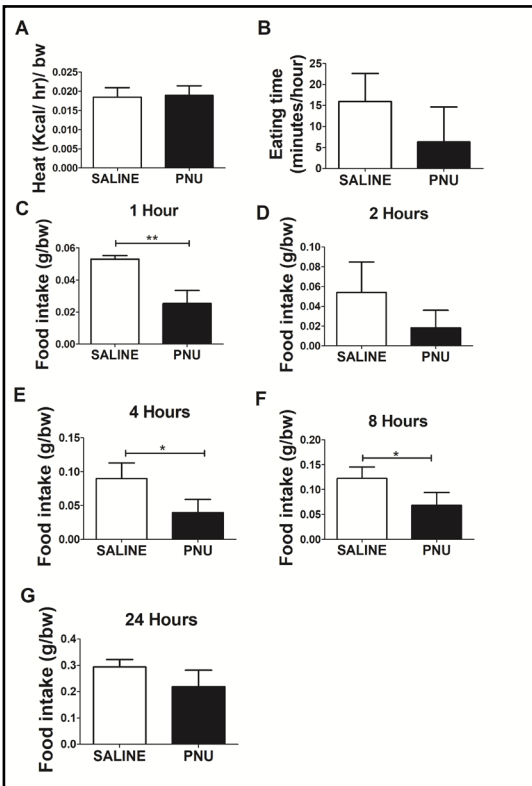


Fig. 3. ICV injection of PNU modulated hypothalamic neuropeptides involved in the modulation of food intake. PNU (450 picomol/mouse) was administered by ICV injection; after 40 minutes, the hypothalamus was removed. (A) POMC mRNA (saline n = 5, PNU n = 4) (B) α -MSH (saline n = 4, PNU n = 4) (C) NPY mRNA (saline n = 5, PNU n = 4) (D) AgRP mRNA (saline n = 5, PNU n = 4) (E) pAMPK (saline n = 4, PNU n = 4). For relative gene expression analysis, ACTB was used as the endogenous control. Student's t-test analysis was used * p < 0.05, *** p < 0.001. HYPO=Hypothalamus.

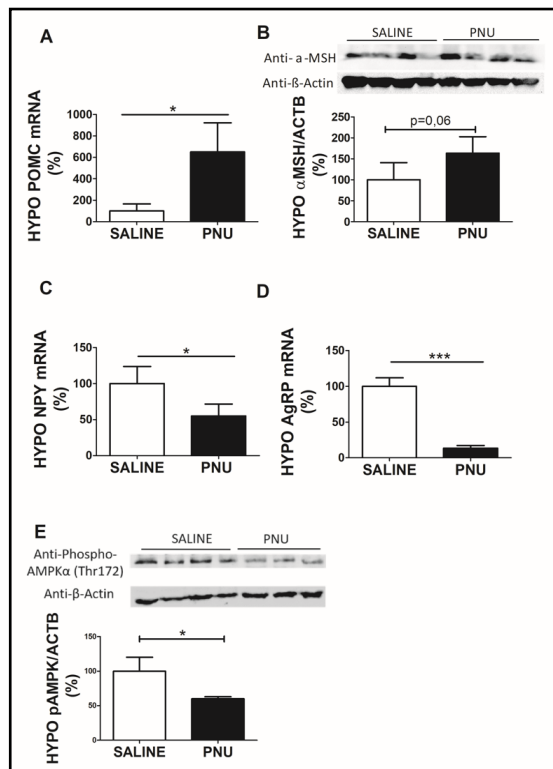
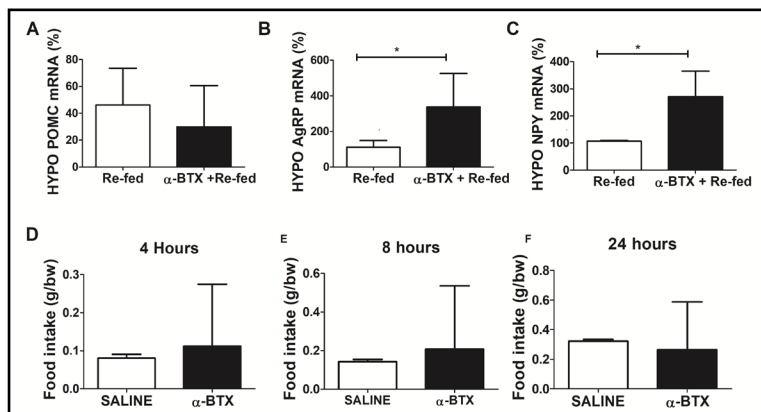


Fig. 4. ICV injection of α Bgt decreases hypothalamic neuropeptides involved in food intake. Male mice were fasted for 24 h and re-fed for 24 h. α Bgt (2 μ g/mouse) was administered by ICV injection; after 6 h of refeeding, the hypothalamus was removed. (n re-fed=3, α Bgt re-fed=4) (A) POMC mRNA, (B) AgRP mRNA, (C) NPY mRNA. Cumulative food intake is shown at (D) 4 h, (E) 8 h and (F) 24 h after refeeding (saline=5, n= α Bgt=4). For gene expression analysis, ACTB was used as the endogenous control. Student's t-test analysis was used to test differences between groups. * p < 0.05.



Inhibition of $\alpha 7$ nAChR stimulates NPY and AgRP neurons

α Btx plays an antagonistic role when bound to $\alpha 7$ nAChR. The ICV injection of α Btx did not modify POMC expression (Fig. 4A) but significantly increased the expression of NPY and AgRP (63% and 225%, respectively) (Fig. 4B and C). Feeding behaviour after ICV injection of α Btx (4, 8 and 24 h) was not modified (Fig. 4D-F). Food intake was assessed in $\alpha 7$ nAChR knockout animals during the transition from the fasting state to the fed state. Supplementary Fig. S1 A-E shows that there were no differences in food intake between $\alpha 7$ nAChR $^{-/-}$ and WT animals. For all supplemental material see www.cellphysiolbiochem.com.

Reduction in hypothalamic $\alpha 7$ nAChR expression reduces POMC and increases AGRP expression

To evaluate the contribution of hypothalamic $\alpha 7$ nAChR expression to the expression of neuropeptides (POMC, AGRP and NPY), mice were treated (ICV) with a siRNA against $\alpha 7$ nAChR, which resulted in an inhibition (40%) of hypothalamic $\alpha 7$ nAChR (Fig. 5A). This reduction of $\alpha 7$ nAChR expression resulted in diminished (96%) POMC expression (Fig. 5B) and increased (45%) AGRP expression (Fig. 5C), but the NPY level was not altered by siRNA injection (Fig. 5D).

Additionally, we evaluated the capacity of $\alpha 7$ nAChR activation to induce POMC expression in cell culture. To perform this analysis, mypHoA-POMC/GFP cells were used. These cells express $\alpha 7$ nAChR protein as demonstrated by immunofluorescence and western blot analysis (Fig. 6A and Fig. 1SB). PNU treatment induced STAT3 phosphorylation and migration to the nucleus (Fig. 6C), which was accompanied by a significant increase in POMC expression (Fig. 6B).

Fig. 5. ICV injection of AChR $\alpha 7$ siRNA for 5 days decreased POMC expression in the hypothalamus. Protein content of (A) CHRNA7 in the hypothalamus of male mice after injection of sc-siRNA control solution for 5 days, or injection of AChR $\alpha 7$ siRNA for 3 days and 5 days (n = 3 for all groups). Expression of mRNA in the hypothalamus of mice after 5 days of injection of AChR $\alpha 7$ siRNA (B) mRNA Chrna7 mRNA POMC (C) AgRP (D) NPY (scRNA-A n = 4, AChR $\alpha 7$ siRNA n = 7). For the evaluation of gene expression, ACTB was used as the endogenous control. In panel A, one-way ANOVA was used. In B, C and D Student's t-test was used ** p < 0.01. HYPO=Hypothalamus.

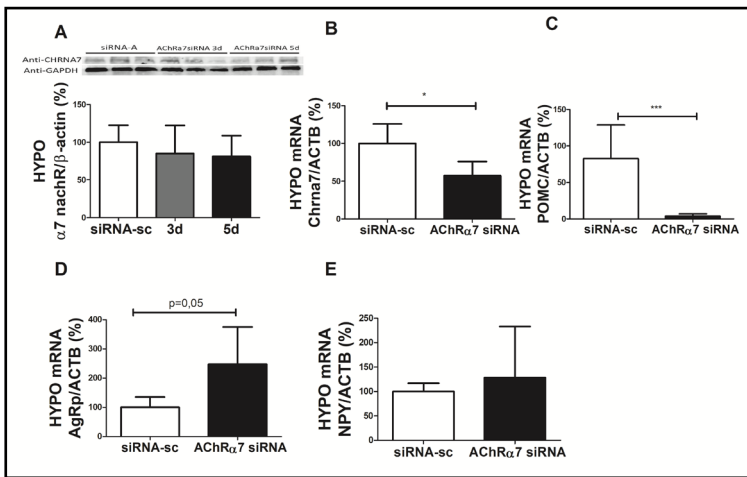


Fig. 6. Response to PNU of the mypHoA-POMC/GFP neuronal lineage. A) Confocal images illustrating TOPRO (blue), labelling of the $\alpha 7$ nAChR with the CHRNA7 antibody (red) B) POMC mRNA. D) Protein content of pSTAT3 cytoplasmic (cyto) and nuclear extract (Nuc). ** p < 0.01.

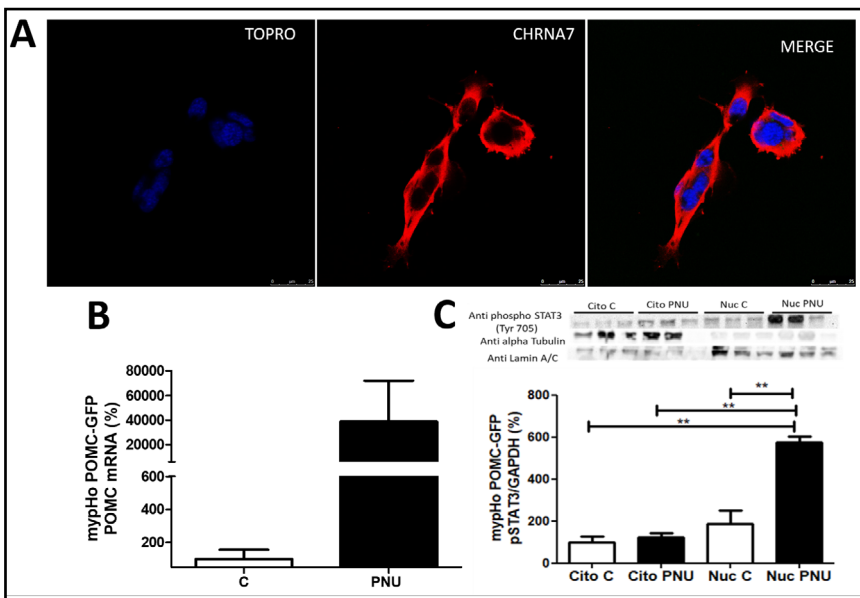
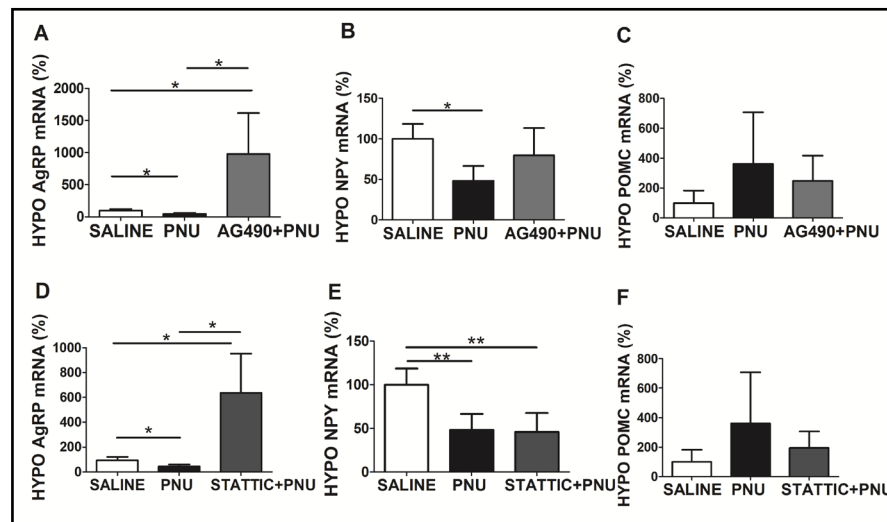


Fig. 7. PNU anorexigenic action depends on JAK2/STAT3 signalling. 5 mM AG490 (2 μ L/mouse), a specific and potent inhibitor of JAK2, was administered by ICV injection. PNU (450 picomol/mouse) was administered by ICV injection 20 minutes later; the hypothalamus was extracted



after 40 minutes (saline n = 5, PNU n = 4, AG490+PNU n = 5). (A) AgRP mRNA, (B) NPY mRNA (saline n = 5, PNU n = 4), (C) POMC (saline n = 4, PNU n = 4). STATIC 30 μ M (2 μ L/mouse), an inhibitor of STAT3, was administered by ICV injection. PNU (450 picomol/mouse) was administered by ICV injection 20 minutes later; the hypothalamus was extracted after 40 minutes (saline n = 5, PNU n = 4, STATIC+PNU n = 5). (D) AgRP mRNA, (E) NPY mRNA (saline N = 5, PNU n = 4), (F) POMC (saline n = 4, PNU n = 4). For gene expression analysis, ACTB was used as the endogenous control. One-way ANOVA was used to test differences between groups * p < 0.05, ** p < 0.01. HYPO=Hypothalamus.

$\alpha 7$ nAChR activation modulates food intake in a STAT3/JAK2-dependent manner

PNU induced STAT3 (Tyr705) phosphorylation and the inhibitors of JAK2 (AG490) and STAT3 (Sttatic) prevented this effect (Supplementary Fig. S2). To evaluate the role of JAK2 and STAT3 activation on feeding behaviour modulated by $\alpha 7$ nAChR, we administered inhibitors of JAK2 (AG490) or STAT3 (Sttatic) by ICV injection. Both inhibitors efficiently abrogated the effect of PNU on AgRP expression. While PNU injection reduced hypothalamic AgRP expression, the prior injection of AG490 or Sttatic led to a significant increase in AgRP expression compared to control and PNU treated mice (931% and 592%, respectively) (Fig. 7A and D). Neither AG490 or Sttatic significantly modified NPY (Fig. 7B and E) or POMC (Fig. 7C and F) expression.

Discussion

Acetylcholine has been associated with the regulation of feeding behaviour. It has an effect on energy homeostasis by depolarising and activating anorexigenic pro-opiomelanocortin (POMC) neurons while inhibiting the synaptic excitatory activity of orexigenic NPY neurons in the hypothalamic arcuate nucleus [7, 8]. Acetylcholine has structural similarity with nicotine, a known inhibitor of appetite [21]. Recently, Calarco and colleagues showed that nicotine can modulate neuronal excitability in POMC and NPY neurons [17]. However, the participation of the $\alpha 7$ nAChR and JAK2/STAT3 pathway had not been described. The relation between $\alpha 7$ nAChR activation and JAK2/STAT3 signalling was first described by de Jonge and colleagues in peritoneal macrophages. These authors showed that the vagal anti-inflammatory pathway acts by $\alpha 7$ subunit-mediated JAK2-STAT3 activation in monocytes and macrophages to reduce inflammatory signals [12]. The JAK2/STAT3 signalling pathway is also involved in the hypothalamic suppression of food intake in response to leptin [13, 14, 22]. Thus, the JAK2/STAT3 pathway could have an important role in the effect of $\alpha 7$ nAChR activation on food intake. Although previous studies have shown the presence of cholinergic

terminals in the hypothalamus, the effects of acetylcholine on food intake has not been associated with a specific subunit in the arcuate nucleus [15, 23]. Moreover, the literature is controversial regarding the identification of the role of cholinergic receptors. Jeong and colleagues have shown that the activation of cholinergic fibres in the ARC stimulates food intake, but this effect was abolished by a muscarinic receptor antagonist [23]. Herman and colleagues showed that enhanced cholinergic signalling decreased food consumption [15].

Corroborating the previous study by Herman and colleagues, we show here that intracerebroventricular injection of PNU, a positive allosteric modulator selective for the $\alpha 7$ nAChR, reduced food intake and hypothalamic AMPK phosphorylation. Hypothalamic AMPK plays a critical role in hormonal and nutrient-derived anorexigenic and orexigenic signals. It has been shown that the inhibition of hypothalamic AMPK is necessary for the effects of leptin on food intake and body weight [24]. Reinforcing the anorexigenic effect of $\alpha 7$ nAChR activation, we show here that hypothalamic $\alpha 7$ nAChR activation is sufficient to reduce NPY and AgRP and increase POMC expression. These data suggest that $\alpha 7$ nAChR activation has a similar effect as leptin in the hypothalamus to control feeding behavior.

In addition to the presence of cholinergic terminals in the hypothalamus [15, 23], Calarco and colleagues recently identified a complex pattern of nAChR expression in the ARC controlling food intake. The authors showed that, in addition to neurons, $\alpha 7$ nAChR mRNA was also enriched in other cell types and could regulate other circuits that control food intake [17]. Here, we show that $\alpha 7$ nAChR is colocalized in NPY and POMC neurons. We also show that the JAK2/STAT3 pathway has an important role mediating the effect of $\alpha 7$ nAChR activation on NPY and POMC expression.

Hypothalamic acetylcholine levels increase significantly soon after the start of a meal, suggesting that it plays an important role in the control of food intake [25]. In this study, we show that the increase in acetylcholine in the hypothalamus was not caused by local detection of nutrients, but by the anticipation of food intake, suggesting the involvement of motivational circuits in the basal forebrain. This mechanism seems counter-intuitive considering that energy deficit activates AgRP and inhibits POMC neurons. However, we found in the present study that the activation of hypothalamic $\alpha 7$ nAChR with PNU resulted in a classic anorectic effect, increasing POMC expression and food intake while reducing NPY expression. However, Chen and colleagues propose that AgRP and POMC neurons detect food rapidly and modulate feeding behaviour, such as foraging, which promotes the discovery of food [26]. AgRP neurons, in addition to the effect of stimulating food intake, induce motivational processes to obtain food [27, 28]. Thus, hypothalamic $\alpha 7$ nAChR could be acting to control the intensity of NPY/AGRP neuron activation. Our data in fasted mice show that the inhibition $\alpha 7$ nAChR using α Btx, a non-competitive antagonist of $\alpha 7$ nAChR [29], resulted in increased expression of NPY and AGRP compared to control mice. Thus, in the hypothalamus, the presence of $\alpha 7$ nAChR seems to control the intensity of the orexigenic response (NPY and AGRP expression) during refeeding in fasted mice by downregulating the transcriptional activity of NPY/AGRP neurons. We believe that $\alpha 7$ nAChR could play an important role in NPY/AGRP neurons to control motivational behaviour regarding food intake. Corroborating this possibility, the time spent feeding was less in mice that received an ICV injection of an $\alpha 7$ nAChR agonist compared to control mice, suggesting that an inhibitory effect on the motivational mechanism could be occurring. To test this possibility, we evaluated feeding behaviour in $\alpha 7$ KO and wild type (WT) mice after fasted period (24 h). However, the food intake of $\alpha 7$ KO was similar to WT mice at all time points evaluated (1, 2, 4, 8 and 24 h). We must consider that there is an interaction between NPY/AGRP and POMC neurons that promotes reciprocal regulation [30, 31]. Although insulin levels in $\alpha 7$ KO mice were similar to those of WT mice [32], serum leptin and ghrelin levels in $\alpha 7$ KO have not been assessed yet. Thus, these hormonal levels could contribute to modulating feeding behaviour and to mitigating the effect of $\alpha 7$ nAChR deletion. Additional experiments employing POMC and AGRP $\alpha 7$ KO mice could provide robust data to help us understand the contribution of this receptor to energy homeostasis and food intake.

Conclusion

It is concluded that $\alpha 7$ nAChR may be one of the main receptors involved in the response to food intake mediated by ACh, since it modulates the activation of essential proteins of the response to food intake in the hypothalamus, such as pAMPK, JAK2 and STAT3. These mediators are involved in the control of the expression of the hypothalamic neuropeptides POMC, AgRP and NPY.

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Animal experiments conform to internationally accepted standards and have been approved by the appropriate institutional review body.

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Disclosure Statement

The authors declare no conflicts of interest.

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