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STAT5 ablation in AgRP neurons increases female adiposity and blunts food restriction adaptations

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Abstract

AgRP neurons are important players in the control of energy homeostasis and are responsive to several hormones. In addition, STAT5 signalling in the brain, which is activated by metabolic hormones and growth factors, modulates food intake, body fat and glucose homeostasis. Given that, and the absence of studies that describe STAT5 function in AgRP cells, the present study investigated the metabolic effects of *Stat5a/b* gene ablation in these neurons. We observed that STAT5 signalling in AgRP neurons regulates body fat in female mice. However, male and female STAT5-knockout mice did not exhibit altered food intake, energy expenditure or glucose homeostasis compared to control mice. The counter-regulatory response or glucoprivic hyperphagia induced by 2-deoxy-D-glucose treatment were also not affected by AgRP-specific STAT5 ablation. However, under 60% food restriction, AgRP STAT5-knockout mice had a blunted upregulation of hypothalamic *Agrp* mRNA expression and corticosterone serum levels compared to control mice, suggesting a possible role for STAT5 in AgRP neurons for neuroendocrine adaptations to food restriction. Interestingly, *ad libitum* fed knockout male mice had reduced *Pomc* and *Ucp-1* mRNA expression compared to control group. Taken together, these results suggest that STAT5 signalling in AgRP neurons regulates body adiposity in female mice, as well as some neuroendocrine adaptations to food restriction.

Key Words

- ▶ neuroendocrine adaptation
- ▶ energy expenditure
- ▶ food intake
- ▶ POMC

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Introduction

The arcuate nucleus of the hypothalamus (ARH) is an important sensory relay from peripheral signals to the central nervous system (CNS) receiving information about the availability of fuel sources. As a consequence, ARH neurons coordinate appropriate adaptive responses, for example, regulating food intake and energy expenditure (Könner *et al.* 2009). Recently, single-cell analysis

performed in the hypothalamus revealed an important heterogeneity of ARH cells indicating that additional investigations are necessary to fully characterize hypothalamic regulations of body energy homeostasis (Lanfray & Richard 2017, Romanov *et al.* 2017). Among the approximately 50 transcriptionally distinct ARH cell populations, several agouti-related protein (AgRP),

neuropeptide Y (NPY) and proopiomelanocortin (POMC) subtypes were identified. AgRP and POMC neurons are classically described as orexigenic and anorexigenic cells, respectively, and are known components of the central melanocortin system (Cone 2005).

The proximal position to the median eminence, where the blood–brain barrier (BBB) is more permeable than other brain regions, turns ARH neurons more responsive to many peripheral signals including leptin, insulin, glucocorticoids, ghrelin, thyroid hormones (Coppola & Diano 2007), growth hormone (GH) (Furigo *et al.* 2017), prolactin (PRL) (Furigo *et al.* 2014) as well as many other circulating factors (Andermann & Lowell 2017). Some of these hormones, such as leptin, GH and PRL, act in the central control of metabolism and are ligands of cytokine receptors, which after hormone binding, induce activation of the Janus Kinase-Signal transducer and activator of transcription (JAK/STAT) pathway. The JAK/STAT signalling pathway plays a critical role in the regulation of several organs that control metabolism, including the brain, both under energy homeostasis or obesogenic conditions (Gurzov *et al.* 2016), suggesting that this pathway is an important component of the mechanisms that regulate food intake (Ladyman & Grattan 2013). Therefore, altered JAK/STAT signalling may contribute to failures in the mechanisms of body weight maintenance observed in the obesity (Ladyman & Grattan 2013).

STAT5 is a transcription factor from the STAT family activated by hormones that control metabolism. Liver-specific deletion of the *Stat5a* and *Stat5b* genes aggravate fatty liver in response to high-fat diet feeding mice (Baik *et al.* 2016). Additionally, muscle-specific STAT5 deletion augments lipid accumulation in skeletal muscle and liver of mice in response to high-fat diet (Baik *et al.* 2017). In the CNS, *Stat5a* and *Stat5b* genes are highly expressed in specific populations of hypothalamic neurons that mediate the actions of cytokines in the regulation of energy balance (Lee *et al.* 2008). Mice with a conditional deletion of *Stat5a* and *Stat5b* genes in the CNS develop severe obesity and hyperphagia (Lee *et al.* 2008), and a conditional deletion of *Stat5a* and *Stat5b* genes in the CNS develop severe obesity and hyperphagia (Lee *et al.* 2008).

A strong candidate for STAT5 activation in the control of metabolism is leptin. Although it has been demonstrated the role of leptin receptor (LEPR) subunits in the activation STAT5 pathway *in vitro* (Gong *et al.* 2007), the studies *in vivo* can be controversial. While it has been reported that systemic treatment with leptin increases the number of nuclear STAT5 signals in the hypothalamus,

particularly in the ARH, the ventral premammillary nucleus (PMV), and the supraoptic nucleus (SO) (Mütze *et al.* 2007), other research group showed no effect on the number of cells labelled to phospho-STAT5 (pSTAT5) within the ARH, ventromedial nucleus (VMH) or the paraventricular nucleus (PVH) of the hypothalamus after leptin treatment (Ladyman *et al.* 2012). It has been shown that the ablation of STAT5 from LEPR-expressing cells failed to alter energy balance, suggesting that STAT5 plays little role in the control of energy balance by leptin (Pan *et al.* 2019).

On the other hand, as mentioned earlier, since STAT5 is also recruited by PRL and GH, it is likely that metabolic effects of STAT5 may be triggered by these hormones, that present similarities both in their structural conformations and receptors (Nicoll *et al.* 1986). The relevance of both hormones controlling metabolism is well established. Prolactin has been considered a diabetogenic hormone since hyperprolactinaemic patients have decreased glucose tolerance and hyperinsulinemia in a glucose tolerance test (Landgraf *et al.* 1977, Tuzcu *et al.* 2009). Bromocriptine, which suppresses PRL, improves glucose tolerance and decreases insulin levels in humans (Landgraf *et al.* 1977, Cincotta *et al.* 1999) and in leptin-deficient mice (Cincotta *et al.* 1997, Furigo *et al.* 2019a). These observations are corroborated by direct antagonism of PRL receptor with G129R-hPrl treatment, which improves the insulin sensitivity of leptin-deficient mice (Furigo *et al.* 2019a). Also, the absence of PRLRs is accompanied by reduction in body weight and fat deposition in male and female mice (Bole-Feysot *et al.* 1998, Freemark *et al.* 2001).

In accordance, GH has been reported as an important hormone with metabolic actions either in peripheral organs, such as muscle and liver (Møller & Jørgensen 2009) or in the CNS (Furigo *et al.* 2017, 2019b). Recently, it has been demonstrated that GH-dependent STAT5 signalling plays an important role in hepatic lipid metabolism (Barclay *et al.* 2011). In the CNS, it has been revealed an important GH action specifically in the AgRP neurons regulating neuroendocrine responses to weight loss (Furigo *et al.* 2019b). However, the intracellular signalling involved in this function remains unknown.

In view of this information, and the lack of studies in the literature about which neuronal population is relevant for the action of STAT5 signalling in the CNS to control metabolism, in the present study, we have analysed the importance of the STAT5 in the AgRP neurons by investigating the metabolic consequences in mice carrying an AgRP-specific inactivation of *Stat5a/b* genes.

Materials and methods

Animals

Cell-specific ablation of the *Stat5a/b* genes was achieved by breeding mice carrying *loxP*-flanked *Stat5a/b* alleles (Cui *et al.* 2004, Buonfiglio *et al.* 2015, Furigo *et al.* 2018) with the AgRP-IRES-Cre mouse (*AgRP^{tm1(Cre)}Low1/J*, The Jackson Laboratory). Control mice were homozygous for the *loxP*-flanked *Stat5a/b* alleles, whereas their littermates carrying the Cre alleles were considered the conditional knockout mice (named as AgRP STAT5). We used male and female mice for *in vivo* experiments and validation experiments. Mice were in the C57BL/6 background, weaned at 3–4 weeks of age and their mutations confirmed by genotyping the DNA that had been previously extracted from the tail tip (REDEExtract-N-Amp™ Tissue PCR Kit, Sigma). Mice were maintained in standard conditions of 12-h light/dark cycles (lights on at 8:00 h) and controlled temperature (22–24°C) and received potable water and regular chow *ad libitum* (2.99 kcal/g; 9.4% calories from fat; Quimtia, Brazil), unless described during experiments. All experiments were carried out in compliance with NIH guidelines for the care and use of laboratory animals and were previously approved by the Ethics Committee on the Use of Animals of the Institute of Biomedical Sciences at the University of São Paulo (protocol # 73/2017).

Validation of STAT5 ablation

To demonstrate the STAT5 ablation, we used micropunches to collect specifically the ARH and then determine *Stat5a* and *Stat5b* mRNA levels, as described in previous publication (Silveira *et al.* 2017). Briefly, adult AgRP STAT5-knockout and control mice (20 weeks) were anesthetized and decapitated, and the entire brain was removed. Brain coronal sections (500 µm) were cut using a vibratome. ARH micropunches were obtained using 18-gauge needle. Bilateral punches were taken from a coronal section, obtained at –1.70 to –2.18 mm relative to bregma according to the mouse brain atlas (Franklin & Paxinos 2008). Total RNA from the punches was extracted using the Pico Pure RNA isolation kit according to the manufacturer's instructions (ThermoFisher Scientific).

STAT5 ablation in AgRP neurons was further determined by treating control and AgRP STAT5-knockout mice with an acute i.p. injection of pbs (control group, *n*=5) or porcine pituitary GH (pGH; 20 µg/g, from Dr A F Parlow, National Hormone and Peptide Program, National Institute of Diabetes and Digestive and Kidney Diseases;

control pGH, *n*=5 and AgRP STAT5, *n*=4). We have used GH to induce pSTAT5 in AgRP neurons since 95% of AgRP neurons express GH-induced pSTAT5 (Furigo *et al.* 2019b). Ninety minutes after GH injection, mice were deeply anesthetized with isoflurane and perfused transcardially with saline, followed by a 10% buffered formalin solution (~150 mL per mouse). Brains were collected and post-fixed in the same fixative for 30–60 min and cryoprotected overnight at 4°C in 0.1 M PBS containing 20% sucrose. Brains were cut in 30-µm thick sections using a freezing microtome. Brain slices were rinsed in 0.02 M potassium PBS, pH 7.4 (KPBS), followed by pre-treatment in water solution containing 1% hydrogen peroxide and 1% sodium hydroxide for 20 min. After rinsing in KPBS, sections were incubated in 0.3% glycine and 0.03% lauryl sulphate for 10 min each. Next, slices were blocked in 3% normal donkey serum for 1 h, followed by incubation in anti-pSTAT5^{Tyr694} primary antibody (1:1000; Cell Signaling) for 40 h. After incubation in the primary antibody, slices were subjected to fluorescence reaction, when sections were rinsed in KPBS and incubated for 90 min in AlexaFluor⁴⁸⁸-conjugated secondary antibody (1:500, Jackson ImmunoResearch). Sections were mounted onto gelatine-coated slides and the slides were covered with Fluoromount G mounting medium (Electron Microscopic Sciences, Hatfield, PA, USA). Photomicrographs of the ARH (Bregma: –1.46 mm) and the paraventricular nucleus of the hypothalamus (as a negative control; Bregma: –0.94 mm) were acquired with a Zeiss AxioCam HRc camera coupled to a Zeiss Axioimager A1 microscope (Zeiss). Images were digitized using Axiovision software (Zeiss), and the ImageJ Cell Counter software (<http://rsb.info.nih.gov/ij/>) was used to manually count the number of pSTAT5-responsive cells in the ARH and the PVH.

To evaluate the innervation density of AgRP fibres, brain sections of control and AgRP STAT5-knockout mice were subjected to an immunofluorescent reaction as described earlier, using the primary antibody anti-AgRP (raised in rabbit; 1:2000; Phoenix Pharmaceuticals; #H-003-53). The integrated optical density of one representative rostro caudal level of the PVH and ARH was obtained using the ImageJ software and subtracted by the integrated optical density assessed in adjacent areas with low staining (background).

Metabolic evaluation

Body weight was monitored weekly from 9 to 20 weeks of age. Then, 5-month-old mice were single housed, and their food intake was recorded daily for 1 week. Body fat

mass and body lean mass were measured by time-domain NMR (TD-NMR) using the LF50 body composition mice analysed (Bruker, Germany). Oxygen consumption (VO_2), carbon dioxide production (CO_2), respiratory exchange ratio (RER; VCO_2/VO_2) and ambulatory activity were analysed using the Comprehensive Lab Animal Monitoring System (CLAMS; Columbus Instruments, Columbus, OH, USA). After an adaptation period of 3 days inside the CLAMS, these metabolic parameters were evaluated for 4 consecutive days. Therefore, the results presented were the average of this period.

Evaluation of glucose homeostasis and counter-regulatory response to hypoglycaemia

After 4 h of fasting and 5 h of lights on, mice were subjected to a glucose tolerance test (GTT; 2 g glucose/kg b.w.; i.p.). After 3–4 days of recovery, mice were subjected to the insulin tolerance test (1 IU/kg insulin; i.p.) under the same fasting and lights conditions. In both tests, glycaemia was measured using a glucose metre (One Tough Ultra; J&J) via blood samples collected at intervals of 20–30 min from the tail. To evaluate the counter-regulatory response (CRR), mice received an i.p. injection of 0.5 g/kg 2-deoxy-D-glucose (2DG; Sigma), which causes glucopaenia and consequently induces a robust CRR, and their glycaemia was determined in intervals during 180 min. To evaluate hypoglycaemia-induced hyperphagia, *ad libitum*-fed mice received i.p. injections of either saline or 2DG (0.5 g/kg) and the food intake was recorded 2, 3 and 4 h afterwards.

Food restriction tests

Male and female mice were subjected to a 60% food restriction protocol, in which each mouse received 40% of their normal intake 2 h before lights off for 5–7 consecutive days, as recently described (Furigo *et al.* 2019b). During this period, the metabolic parameters of male mice were continuously assessed by the CLAMS. The body weight, body composition (by TD-NMR) and glycaemia were monitored at the time the food was provided for male and female groups. Changes in VO_2 (energy expenditure) during food restriction were reported as percentage of the values obtained from baseline (typically 2–3 days of recordings before food restriction). In addition, subgroups of 12-week-old control and AgRP STAT5-knockout male and female mice were killed at the beginning of the light cycle (8:00 h) on the second day of food restriction. Mice with *ad libitum* access to food were killed at the same time.

The hypothalamus, the interscapular brown adipose tissue (iBAT) and trunk blood were collected for subsequent gene expression and hormonal analyses.

Gene expression analysis

To validate the mice model or to analyse whether the ablation of *Stat5a/b* caused changes in the gene expression of orexigenic, anorexigenic and thermogenic modulators, total RNA from the micro-dissected ARH, the entire hypothalamus or the iBAT was extracted with TRIzol (Invitrogen). Assessment of RNA quantity and quality was performed with an Epoch Microplate Spectrophotometer (Biotek). Total RNA was incubated in DNase I RNase-free (Roche Applied Science). RT was performed with 2 μg of total RNA with SuperScript II Reverse Transcriptase (Invitrogen) and random primers p(dN)6 (Roche Applied Science). Real-time PCR was performed using the 7500TM Real-Time PCR System (Applied Biosystems) and Power SYBR Green PCR Master Mix (Applied Biosystems). Relative quantification of mRNA was calculated by $2^{-\Delta\Delta\text{Ct}}$. Data were normalized to the geometric average of *Actb* and *Ppia* and reported as fold changes compared to values obtained from the control group (set at 1.0). The following primers were used: *Agrp* (forward: ctttggcggaggtgctagat; reverse: aggactcgtgcagccttacac), *Actb* (forward: gctccggcatgtgcaaag; reverse: catcacaccctggtgccta), *Ppia* (forward: tatctgcactgccaagactgagt; reverse: ctctctgctggtcttgcattcc), *Npy* (forward: cagatactactccgctctgcg; reverse: gggctggatctcttgcata), *Pomc* (forward: tagatgtgtggagctggtgc; reverse: ccagcgagaggtcgagtttg), *Stat5a* (forward: gacgtggcctctcacactga; reverse: cgctggactccatgcttctc), *Stat5b* (forward: tccatcgtgtcttccagatcg; reverse: ggactccgtccttgataccg), *Ucp1* (forward: gaggtgtggcagtggttcattg; reverse: ggcttgcatctgcacctca).

Hormonal analysis

Commercially available ELISA kits were used to determine the serum concentrations of corticosterone (Arbor Assays, #K014-H1) and T4 (Calbiotech, # T4044T-100). Corticosterone and T4 ELISA kits have a limit of detection determined as 16.9 pg/mL and 25 $\mu\text{g}/\text{dL}$, and an intra- and inter-assay coefficients of variability $\leq 6\%$ and $\leq 8\%$, respectively.

Statistical analysis

The data were analysed by different statistical tests according to the experiment: the unpaired two-tailed

Student's *t* test, one-way ANOVA followed by Tukey's multiple comparison test or two-way ANOVA, followed by Sidak's multiple comparisons test, when applicable. Statistical analyses were performed using the GraphPad Prism software. We considered *P* values <0.05 to be statistically significant. All results are expressed as mean $\pm$ s.e.m., as well as the *F* (DFn, DFd) values were shown in the figure legends.

Results

Ablation of STAT5 in AgRP-expressing neurons

AgRP-expressing cells are limited to the ARH (Broberger *et al.* 1998). Thus, to demonstrate the efficacy of our target gene deletion, we assessed *Stat5a* and *Stat5b* mRNA expression from the ARH. AgRP STAT5-knockout mice showed reduced *Stat5a* and *Stat5b* mRNA expression compared to control (Fig. 1A). STAT5 is a key transcription factor recruited by numerous growth factors and cytokines, including PRL and GH (Grimley *et al.* 1999, Furigo *et al.* 2017). Previous studies have shown that 95% of AgRP neurons either express *Ghr* mRNA (Kamegai *et al.* 1996) or STAT5 phosphorylation (pSTAT5) after an acute GH injection (Furigo *et al.* 2019b). On the other hand, AgRP neurons do not express prolactin receptors or prolactin-induced pSTAT5 (Ladyman *et al.* 2017). Thus, GH-injected control mice should express a large amount of pSTAT5-positive cells in the ARH, whereas AgRP STAT5-knockout mice were expected to show a reduction. Pbs-injected mice exhibited few pSTAT5-positive cells in the ARH (Fig. 1B and C) and other brain regions, represented in the photomicrography of the paraventricular nucleus of hypothalamus (PVH; Fig. 1F). As previously shown (Furigo *et al.* 2017, 2019b), an acute GH injection induced a large amount of pSTAT5 in the ARH of control mice (Fig. 1B and D). In contrast, AgRP STAT5-knockout mice showed reduced pSTAT5 expression in the ARH (Fig. 1B and E). A similar pSTAT5 expression between control (Fig. 1B and G) and AgRP STAT5 mice was observed in other brain areas, including the PVH (Fig. 1B and H), confirming that the reduction of pSTAT5 was restricted to the ARH. Thus, our gene ablation was efficient in reducing the expression of *Stat5a/b* mRNA and the amount of GH-induced pSTAT5-positive cells in the ARH.

In order to analyse whether the STAT5 deletion affected AgRP projections, we performed an immunofluorescent reaction to label AgRP in control and AgRP STAT5 knockout

Figure 1

Validation of the experimental mice model by mRNA expression of *Stat5a/b* and immunofluorescence to detect phosphorylated STAT5 (pSTAT5) in the hypothalamus of control and AgRP STAT5-knockout mice. *Stat5a* and *Stat5b* mRNA expression in the ARH of control (*n* = 5) and AgRP STAT5-knockout (*n* = 4) mice (A). pSTAT5-responsive cells quantification 90 min after an i.p. injection of pbs in control or GH (20 μ g/g) in control and AgRP STAT5-knockout mice (B). (C, D, E, F, G and H) Epifluorescence photomicrographs showing the distribution of pSTAT5 after pbs treatment in the control (C and F), and pGH in the control (D and G) and AgRP STAT5-knockout mice (E and H). (I, J, K, L, M and N) Quantification of integrated optical density (IOD) of AgRP fibres (I and L) and epifluorescence photomicrographs showing the AgRP fibres in the ARH (J and K) and PVH (L, M and N) of control and AgRP STAT5-knockout mice. ARH (arcuate nucleus of hypothalamus), PVH (paraventricular nucleus of hypothalamus) Scale Bar C-H = 50 μ m, J-N = 100 μ m. The unpaired two-tailed Student's *t*-test was used for comparisons in the graphs A, I and L, while the one-way ANOVA followed by Tukey's multiple comparison test was used in the graph B. Mean $\pm$ s.e.m. #*P* = 0.067; **P* < 0.05. A full colour version of this figure is available at <https://doi.org/10.1530/EJE-19-0158>.

mice. We observed no differences in the integrated optical density of AgRP fibres in the ARH (Fig. 1L, J and K) or PVH (Fig. 1L, M and N).

STAT5 ablation in AgRP neurons alters the energy balance of female, but not of male mice

AgRP neurons are classically described as important players in the control of energy homeostasis (Morton & Schwartz 2001). In addition, STAT5 signalling in the brain modulates several metabolic aspects, including food intake, body fat and glucose homeostasis (Lee *et al.* 2008, Patterson *et al.* 2012). Thus, we investigated whether STAT5 ablation in AgRP neurons leads to metabolic changes in male and female mice. AgRP STAT5-knockout male mice exhibited similar weight gain along time compared to control mice (Fig. 2A). Additionally, 5-month-old AgRP STAT5-knockout male mice exhibited no differences in body weight (Fig. 2B), body fat (Fig. 2C), lean body mass (Fig. 2D), food intake (Fig. 2E), energy expenditure (VO_2 ; Fig. 2F), RER (Fig. 2G) and ambulatory activity (Fig. 2H) compared to control mice. These results indicate that

Figure 2

AgRP STAT5-knockout male mice showed no evidence of metabolic imbalances. Body weight change of control and AgRP STAT5-knockout male mice ($n = 10$ – 20 depending on age) (A). Body weight (B), body fat mass (C), body lean mass (D), food intake relative to body weight (E), energy expenditure (VO_2) (F) and respiratory quotient in the light, dark and 24-h cycles (G) of 5-month-old male mice ($n = 16$ – 18). The unpaired two-tailed Student's *t*-test was used for comparisons. Mean ± S.E.M.

Figure 3

AgRP STAT5-knockout female mice showed increased body weight and body fat mass. Body weight change of control and AgRP STAT5-knockout female mice ($n = 8$ – 15 depending on age) (A). Body weight (B), body fat mass (C), body lean mass (D), food intake relative to body weight (E), energy expenditure (VO_2) (F) and respiratory quotient in the light, dark and 24-h cycles (G) of 5-month-old female mice ($n = 11$ – 13). The unpaired two-tailed Student's *t*-test was used for comparisons. Mean ± S.E.M. * $P < 0.05$.

STAT5 ablation did not cause metabolic imbalances in male mice. In contrast, female AgRP STAT5-knockout mice exhibited higher body weight than control mice throughout the evaluation period (Fig. 3A and B). The increased body weight of AgRP STAT5-knockout mice was predominantly due to higher fat mass (Fig. 3C). Body lean mass (Fig. 3D), food intake (Fig. 3E), VO_2 (Fig. 3F), RER (Fig. 3G) and ambulatory activity (Fig. 3H) were similar between groups.

STAT5 ablation in AgRP neurons does not affect glucose homeostasis or the counter-regulatory response to hypoglycaemia

AgRP STAT5-knockout male mice presented normal glycaemic response during the GTT (Fig. 4A and B) and ITT (Fig. 4C and D). Both groups presented the expected increases in glycaemia after 2DG treatment (Fig. 4E and F), as well as similar food intake increases at 2, 3 and 4 h after 2DG injection (Fig. 4G). As seen in males, AgRP STAT5-knockout female mice displayed a similar response

Figure 4

Glucose homeostasis and counter-regulatory response in control and AgRP STAT5-knockout male mice. Blood glucose levels during a glucose tolerance test (GTT; (main effect of STAT5 ablation ($F(1,180) = 1.88$, $P = 0.1720$), main effect of time ($F(5,180) = 69.08$, $P < 0.001$) and interaction ($F(5,180) = 0.14$, $P = 0.9822$); $n = 14-18$) (A). Area under the curve (AUC) of the GTT ($P = 0.4607$) (B). Blood glucose levels during an insulin tolerance test (ITT; (main effect of STAT5 ablation ($F(1,166) = 2.37$, $P = 0.1255$), main effect of time ($F(6,166) = 38.99$, $P < 0.001$) and interaction ($F(6,166) = 0.39$, $P = 0.8857$); $n = 13-14$) (C). AUC of the ITT ($P = 0.3029$) (D). Blood glucose levels during a counter-regulatory response induced by 2-deoxy-D-glucose (2DG) infusion (main effect of STAT5 ablation ($F(1,161) = 0.65$, $P = 0.9502$), main effect of time ($F(6,161) = 92.34$, $P < 0.001$) and interaction ($F(6,161) = 0.27$, $P = 0.9502$); $n = 12-13$) (E). AUC of the 2DG test ($P = 0.7113$) (F). Food intake after intraperitoneal (i.p.) injection of either phosphate-buffered saline (PBS) or 2DG (0.5 mg/kg body weight: time 2 h (main effect of STAT5 ablation ($F(1,56) = 0.42$, $P = 0.52$), main effect of 2DG ($F(1,56) = 29.59$, $P < 0.0001$), interaction ($F(1,56) = 0.23$, $P = 0.63$); time 3 h (main effect of STAT5 ablation ($F(1,56) = 0.001$, $P = 0.97$), main effect of 2DG ($F(1,56) = 40.39$, $P < 0.0001$), interaction ($F(1,56) = 0.164$, $P = 0.69$); time 4 h (main effect of STAT5 ablation ($F(1,56) = 0.176$, $P = 0.67$), main effect of 2DG ($F(1,56) = 54.13$, $P < 0.0001$), interaction ($F(1,56) = 0.53$, $P = 0.47$); $n = 14-16$) (G). The effect of mutation in the glycaemic tests were analysed by two-way ANOVA, and the contrasts were identified by Sidak's multiple comparisons test. The unpaired two-tailed Student's *t*-test was used for comparisons of AUC. All results were expressed as mean $\pm$ s.e.m.

to GTT (Fig. 5A and B), as well as a normal increase in the glycaemia and food intake after 2DG injection (Fig. 5E, F and G), compared to control females. However, AgRP STAT5-knockout female mice showed higher glycaemia at the time point 180 min of the ITT and a non-significant increase ($P = 0.15$) in the area under curve (Fig. 5C and D).

The neuroendocrine adaptations to food restriction are attenuated by STAT5 ablation in AgRP neurons

A recent study demonstrated a key role of GH action in AgRP neurons regulating the neuroendocrine adaptations to food restriction (Furigo et al. 2019b). In the present study we investigated whether AgRP STAT5-knockout mice show metabolic alterations during this condition. Thus, control and AgRP STAT5-knockout male and female mice were studied in *ad libitum*-fed conditions or during 60% food restriction (F.R.). Two days of F.R. were sufficient to increase hypothalamic *Npy* and *AgRP* gene expression in control mice of both sexes (Fig. 6A, B and D, E). Notably, *Stat5a/b* ablation in AgRP neurons prevented F.R.-induced increase in *AgRP* mRNA levels in the hypothalamus of male mice (Fig. 6A) and a similar tendency was observed

in female mice ($P = 0.067$, Fig. 6D). *Npy* mRNA levels were upregulated during F.R. in AgRP STAT5-knockout mice as in control mice in both sexes (Fig. 6B and E). Interestingly, *Pomc* mRNA expression was suppressed in AgRP STAT5-knockout male mice during fed condition, compared to control animals (Fig. 6C), while this difference was not observed in the female group (Fig. 6F). In addition, F.R. reduced hypothalamic *Pomc* mRNA expression similarly in both groups and sexes, disappearing the difference found in the male group at the fed state. In order to investigate the effects of food restriction in parameters related to thermogenesis, we analysed the mRNA expression of *Ucp1* in the iBAT of control and AgRP STAT5-knockout male mice (Fig. 6G). Both groups had this mRNA expression decreased after F.R., but, interestingly, AgRP STAT5-knockout mice had a tendency ($P = 0.051$) of lower *Ucp1* mRNA expression at *ad libitum* condition compared to control mice. Next, we assessed whether AgRP STAT5-knockout male mice exhibit a normal endocrine response to weight loss. Serum corticosterone levels were increased in mice under F.R. compared to *ad libitum*. Notably, the increase of serum corticosterone levels was prevented in AgRP STAT5-knockout mice (Fig. 6H). Serum T4 levels were not altered by F.R. or STAT5 ablation (Fig. 6I).

Figure 5

Glucose homeostasis and counter-regulatory response in control and AgRP STAT5-knockout female mice. Blood glucose levels during a glucose tolerance test (GTT; (main effect of STAT5 ablation ($F(1,132) = 0.00$, $P = 0.9963$), main effect of time ($F(5,132) = 32.94$, $P < 0.001$) and interaction ($F(5,132) = 0.55$, $P = 0.7365$); $n = 11-13$) (A). Area under the curve (AUC) of the GTT ($P = 0.9468$) (B). Blood glucose levels during an insulin tolerance test (ITT; (main effect of STAT5 ablation ($F(1,154) = 6.63$, $P = 0.0110$), main effect of time ($F(6,154) = 71.45$, $P < 0.001$) and interaction ($F(6,154) = 0.50$, $P = 0.8078$); $n = 11-13$) (C). AUC of the ITT ($P = 0.1578$) (D). Blood glucose levels during a counter-regulatory response induced by 2-deoxy-D-glucose (2DG) infusion (main effect of STAT5 ablation ($F(1,154) = 1.62$, $P = 0.2054$), main effect of time ($F(6,154) = 99.95$, $P < 0.001$) and interaction ($F(6,154) = 0.36$, $P = 0.9037$); $n = 11-13$) (E). AUC of the 2DG test ($P = 0.5997$) (F). Food intake after intraperitoneal (i.p.) injection of either phosphate-buffered saline (PBS) or 2DG (0.5 mg/kg body weight): time 2 h (main effect of STAT5 ablation ($F(1,44) = 1.77$, $P = 0.19$), main effect of 2DG ($F(1,44) = 32.69$, $P < 0.0001$), interaction ($F(1,44) = 0.90$, $P = 0.35$); time 3 h (main effect of STAT5 ablation ($F(1,44) = 0.76$, $P = 0.39$), main effect of 2DG ($F(1,44) = 116.1$, $P < 0.0001$), interaction ($F(1,44) = 0.002$, $P = 0.96$); time 4 h (main effect of STAT5 ablation ($F(1,44) = 0.10$, $P = 0.74$), main effect of 2DG ($F(1,44) = 173.8$, $P < 0.0001$), interaction ($F(1,44) = 0.12$, $P = 0.73$); $n = 11-13$) (G). The effects of mutation in the glycaemic tests were analysed by two-way ANOVA. The unpaired two-tailed Student's *t*-test was used for comparisons of AUC. All results were expressed as mean $\pm$ S.E.M. * $P < 0.05$.

Since our former results indicated that STAT5 ablation in AgRP neurons causes an attenuation in the neuroendocrine adaptations to food restriction, we then studied if AgRP-specific STAT5 ablation leads to metabolic changes during this condition. F.R. caused a progressive reduction in the energy expenditure (Fig. 7A), but this response was similarly observed between control and AgRP STAT5-knockout male mice. The respiratory quotient (Fig. 7B) and the weight loss during F.R. were also not different between the groups (Fig. 7C). However, a greater body fat loss was observed in AgRP STAT5-knockout mice after 5 days of food restriction (Fig. 7D). No differences in body lean mass (Fig. 7E) and glycaemia (Fig. 7F) were observed between groups during F.R.

The effects of 60% F.R. were also evaluated in female mice. AgRP STAT5-knockout and control females had no difference in weight loss (Fig. 8A). However, AgRP STAT5 knockout presented higher body fat mass at the third day of F.R. (Fig. 8B) and higher lean mass at the days 4 and 5 of F.R. (Fig. 8C), compared to control animals. The blood glucose levels during F.R. were not different between groups (Fig. 8D).

Discussion

While some studies recognize the importance of STAT5 signalling in the whole brain or in specific peripheral organs for the regulation of metabolism (Lee *et al.* 2008, Baik *et al.* 2016, 2017), the information about the specific areas of the CNS in which STAT5 action is required for the energy homeostasis is limited. Given the importance of AgRP neurons for the control of food intake and energy expenditure, together with the fact that these neurons are subject to hormonal inputs that activate the STAT5 signalling pathway; in the present study, we analysed the importance of the STAT5 in the AgRP neurons via cell-specific inactivation of *Stat5a/b* genes.

Initially, we presented evidence that *Stat5a/b* gene ablation in AgRP neurons was satisfactory by the mRNA expression analyses in the ARH. The decreased, but not complete ablation of *Stat5a/b* mRNA expression observed in AgRP STAT5-knockout mice is explained by the heterogeneity of ARH nucleus, that contemplates not only AgRP neurons, but a variety of cell types (Broberger *et al.* 1998, Romanov *et al.* 2017). Additionally, by

Figure 6

Gene expression and hormonal alterations during food restriction of male and female mice. (A, B and C) Male mice mRNA expression of *AgRP* (main effect of STAT5 ablation ($F(1,14) = 6.54$, $P = 0.0229$), main effect of food intervention ($F(1,14) = 51.31$, $P < 0.0001$) and interaction ($F(1,14) = 6.533$, $P = 0.0229$); $n = 4-6$) (A), *Npy* (main effect of STAT5 ablation ($F(1,15) = 0.07480$, $P = 0.7882$), main effect of food intervention ($F(1,15) = 25.54$, $P = 0.0001$) and interaction ($F(1,15) = 0.8307$, $P = 0.3765$); $n = 4-6$) (B) and *Pomc* (main effect of STAT5 ablation ($F(1,17) = 3.291$, $P = 0.0874$), main effect of food intervention ($F(1,17) = 60.71$, $P < 0.0001$) and interaction ($F(1,17) = 8.078$, $P = 0.0113$); $n = 4-6$) (C) in the hypothalamus of control and AgRP STAT5-knockout male mice at *ad libitum* fed or after 2 days of food restriction. (D, E and F) Female mice mRNA expression of *AgRP* (main effect of STAT5 ablation ($F(1,19) = 2.86$, $P = 0.11$), main effect of food intervention ($F(1,19) = 72.96$, $P < 0.0001$) and interaction ($F(1,19) = 2.15$, $P = 0.16$); $n = 4-6$) (D), *Npy* (main effect of STAT5 ablation ($F(1,20) = 1.09$, $P = 0.30$), main effect of food intervention ($F(1,20) = 18.24$, $P = 0.0004$) and interaction ($F(1,20) = 0.012$, $P = 0.92$); $n = 4-8$) (E) and *Pomc* (main effect of STAT5 ablation ($F(1,15) = 0.75$, $P = 0.398$), main effect of food intervention ($F(1,15) = 31.9$, $P < 0.0001$) and interaction ($F(1,15) = 0.0046$, $P = 0.95$); $n = 3-7$) (F) in the hypothalamus of control and AgRP STAT5-knockout female mice at *ad libitum* fed or after 2 days of food restriction. G, male mice mRNA expression of *Ucp-1* (main effect of STAT5 ablation ($F(1,16) = 4.46$, $P = 0.0508$), main effect of food intervention ($F(1,16) = 8.50$, $P = 0.01$) and interaction ($F(1,16) = 1.86$, $P = 0.19$); $n = 4-6$) in the interscapular brown adipose tissue of control and AgRP STAT5-knockout male mice at *ad libitum* fed or after 2 days of food restriction. (H and I) Serum concentration of corticosterone (main effect of STAT5 ablation ($F(1,15) = 6.934$, $P = 0.0188$), main effect of food intervention ($F(1,15) = 22.66$, $P = 0.0003$) and interaction ($F(1,15) = 4.079$, $P = 0.0616$); $n = 3-6$) (H) and T4 (main effect of STAT5 ablation ($F(1,17) = 0.3860$, $P = 0.5426$), main effect of food intervention ($F(1,17) = 0.04861$, $P = 0.8281$) and interaction ($F(1,17) = 0.06449$, $P = 0.8026$); $n = 4-6$) (I) of control and AgRP STAT5-knockout male mice at *ad libitum* and 2 days of food restriction. The two-way ANOVA test was used, followed by Sidak's multiple comparisons test. All results were expressed as mean $\pm$ S.E.M. * $P < 0.05$.

immunohistochemistry we have shown that the *Stat5a/b* deletion was limited to the neurons in the ARH and did not cause changes in the AgRP projections since we demonstrated no altered AgRP fibres density in AgRP STAT5-knockout mice.

As earlier mentioned, AgRP neurons are classically described as important players in the control of energy

homeostasis (Morton & Schwartz 2001, Zagmutt *et al.* 2018), modulating both energy expenditure (Morselli *et al.* 2018) and food consumption (Andermann & Lowell 2017). Recently, it was demonstrated an important role of AgRP neurons in the control the neuroendocrine adaptations to starvation regulated by GH (Furigo *et al.* 2019b). However, the intracellular pathways responsible

Figure 7

AgRP STAT5-knockout male mice showed normal response to food restriction (F.R.). (A, B, C, D, E and F) Changes in energy expenditure during 4 days of F.R. relative to baseline (main effect of STAT5 ablation ($F(1,105) = 1.44$, $P = 0.2335$), main effect of days of F.R. ($F(3,105) = 28.24$, $P < 0.001$) and interaction ($F(3,105) = 0.16$, $P = 0.9253$; $n = 15-16$) (A). Changes in respiratory quotient during F.R. baseline (main effect of STAT5 ablation ($F(1,76) = 0.00$, $P = 0.9551$), main effect of days of F.R. ($F(3,76) = 3.71$, $P = 0.0192$) and interaction ($F(3,76) = 0.95$, $P = 0.4224$; $n = 10-11$) (B). Changes, during F.R., in body weight (main effect of STAT5 ablation ($F(1,183) = 5.54$, $P = 0.0196$), main effect of days of F.R. ($F(5,183) = 379.58$, $P < 0.001$) and interaction ($F(5,183) = 0.83$, $P = 0.5287$; $n = 10-11$) (C), body fat mass (main effect of STAT5 ablation ($F(1,180) = 10.43$, $P = 0.0015$), main effect of days of F.R. ($F(5,180) = 86.71$, $P < 0.001$) and interaction ($F(5,180) = 0.86$, $P = 0.5067$; $n = 10-11$) (D), lean body mass (main effect of STAT5 ablation ($F(1,186) = 0.24$, $P = 0.6245$), main effect of days of F.R. ($F(5,186) = 134.30$, $P < 0.001$) and interaction ($F(5,186) = 0.16$, $P = 0.9780$; $n = 10-11$) (E) and blood glucose (main effect of STAT5 ablation ($F(1,120) = 0.25$, $P = 0.6157$), main effect of days of food restriction ($F(4,120) = 18.86$, $P < 0.001$) and interaction ($F(4,120) = 0.76$, $P = 0.5502$; $n = 11-13$) (F). The two-way ANOVA test was used, followed by Sidak's multiple comparisons test. All results were expressed as mean $\pm$ S.E.M. * $P < 0.05$.

for this response were not elucidated. The fact that STAT5 is a signalling factor that is responsible for the intracellular action of several hormones, including GH, opens the possibility of investigating this signalling pathway in AgRP neurons. With this purpose, AgRP STAT5-knockout mice were studied either in *ad libitum*-fed conditions or under metabolic challenges such as food restriction and hypoglycaemia.

Initially, we have demonstrated that, at *ad libitum*-fed conditions, *Stat5a/b* gene ablation in AgRP neurons increases the adiposity of female mice, while no alterations in food intake, energy expenditure or ambulatory activity were observed. In male mice, we did not find any alteration of body weight, food intake, energy expenditure and ambulatory activity. A previous study that analysed the cytokine-STAT5 signalling in the entire CNS and pituitary gland have shown that the loss of this transcription factor alters energy balance and leads to obesity (Lee *et al.* 2008). Although it was shown that both male and female mutant mice developed severe obesity, characterized by hyperphagia, impaired thermal regulation in response to cold, hyperleptinaemia and insulin resistance (Lee *et al.* 2008), we observed increased body weight just in female-knockout mice, with no association with food intake or energy expenditure. Two issues should be taken into account to understand these results: one is

the fact that our model presents a restricted deletion of *Stat5a/b* to AgRP neuronal population, differently from the deletion of the entire CNS. The other issue is the fact that the ARH is a sexually dimorphic area (Matsumoto & Arai 1980). Concomitantly, although Lee *et al.* (2008) reported alterations in both genders, they also reported a most striking phenotype in females, as we observed in the present study.

Interestingly, the *LepR* ablation in AgRP neurons causes mild obesity in male and female mice, with the most apparent difference in female group, which was confirmed by significantly heavier abdominal fat pads compared with the control group, while no differences were observed in the adiposity of male mice. These findings suggest that the lack of leptin signalling in AgRP neurons may have a greater impact on female mice metabolism (Egan *et al.* 2017). In this study, female-knockout mice also had significant delay in the pubertal onset of oestrous cycles, whereas no significant differences in male puberty onset or adult fecundity were observed (Egan *et al.* 2017). Additionally, female mice overexpressing the subunit *LepR-b* Tyr1077, which has been recognized as displaying a dominant role in the acute phosphorylation of STAT5a and STAT5b (Gong *et al.* 2007) cause impairments in oestrous cyclicity (Patterson *et al.* 2012).

Figure 8

AgRP STAT5-knockout female mice showed no difference during food restriction (F.R.) compared to control mice. (A, B, C and D) Changes, during F.R., in body weight (main effect of STAT5 ablation ($F(1,132) = 6.26$, $P = 0.0136$), main effect of days of F.R. ($F(5,132) = 116.72$, $P < 0.001$) and interaction ($F(5,132) = 0.90$, $P = 0.4862$; $n = 11-13$) (A), body fat mass (main effect of STAT5 ablation ($F(1,116) = 4.24$, $P = 0.417$), main effect of days of F.R. ($F(5,116) = 18.47$, $P < 0.001$) and interaction ($F(5,116) = 0.61$, $P = 0.6957$; $n = 11-13$) (B), lean body mass (main effect of STAT5 ablation ($F(1,132) = 24.43$, $P < 0.001$), main effect of days of F.R. ($F(5,132) = 320.96$, $P < 0.001$) and interaction ($F(5,132) = 1.68$, $P = 0.1449$; $n = 11-13$) (C) and blood glucose (main effect of STAT5 ablation ($F(1,132) = 0.91$, $P = 0.3428$), main effect of days of food restriction ($F(5,132) = 13.97$, $P < 0.001$) and interaction ($F(5,132) = 0.60$, $P = 0.6971$; $n = 11-13$) (D). The two-way ANOVA test was used, followed by Sidak's multiple comparisons test. All results were expressed as mean $\pm$ S.E.M. * $P < 0.05$.

In accordance to that, oestrous cyclicity alteration was observed in female mice model of GHR ablation in LepR cells, which had delayed puberty progression, reduced serum leptin levels, decreased body weight gain and compromised the ovulatory cycle in some individuals, while the lack of GH effects in the entire brain prompted shorter oestrous cycles (Bohlen *et al.* 2019). Considering that LepR cells comprehend the AgRP cells (van de Wall *et al.* 2008), and that STAT5 is the major intracellular effector of GH action (Lanning & Carter-Su 2006, Carter-Su *et al.* 2016, Furigo *et al.* 2017), we could speculate that STAT5 ablation in the AgRP neurons may interfere in oestrus cycle.

Reinforcing this idea, there are reports linking changes in STAT5 signalling with oestrous cyclicity via kisspeptin neurons, although it was shown that STAT5 in these neurons exerts an inhibitory effect in the timing of puberty (Silveira *et al.* 2017). Taking into consideration that AgRP neurons can directly inhibit Kiss1 neurons (Padilla *et al.* 2017), we may speculate that STAT5 can be involved in the AgRP inhibition over kisspeptin neurons,

being therefore another possibility of alteration in oestrus cycle. Therefore, we speculate the possibility of sex hormone alterations in our female mice model, such as oestradiol and/or prolactin serum levels, being related to the body fat increase in females. However, this possibility must be investigated in future studies. Therefore, STAT5 signalling in AgRP neurons seems to regulate the body fat only in female mice, while in males this regulation likely relies on other brain areas.

The role of other intracellular pathways has been investigated in AgRP neurons. In AgRP neuron-specific raptor-knockout mice, it was demonstrated that these animals display normal feeding behaviour and energy homeostasis on both normal and high-fat diet despite a reduced, non-circadian expression of *Agrp* and *Npy*, suggesting that mTORC1 signalling in AgRP neurons controls circadian expression of orexigenic neuropeptides, but it is dispensable for the regulation of feeding behaviour and energy metabolism (Albert *et al.* 2015). However, other study that analysed the mTORC1 signalling pathway in AgRP neurons found that this signalling pathway acts as nutrient sensing in the detection of environmental food cues and internal signals of energy availability and communicates with iBAT to control thermogenesis during nutrient deficiency and excess (Burke *et al.* 2017). Regarding other intracellular signalling in these neurons, Al-Qassab *et al.* (2009) demonstrated an important role of p110 beta subunit of PI3K in AgRP neurons in the control of metabolism since the inactivation of this PI3K catalytic subunit made the mice leaner, with increased leptin sensitivity and resistance to diet-induced obesity. Other study reported that mice expressing a constitutively active version of STAT3 selectively in AgRP neurons are lean and exhibit relative resistance to diet-induced obesity (Mesaros *et al.* 2008). Surprisingly, this phenotype arises from increased locomotor activity, while *Agrp* expression remained unaltered (Mesaros *et al.* 2008). Therefore, other signalling pathways besides the STAT5, such as STAT3 and PI3K seems to trigger a relevant role in the control of energy balance via AgRP neurons, while mTORC-1 signalling in these neurons is related to integration of exteroceptive and interoceptive food-related cues for the modulation of adaptive energy expenditure.

In the present study, we found that STAT5 ablation in AgRP neurons did not affect glucose homeostasis. However, other studies have shown that p70 ribosomal S6 protein kinase-1 (S6K1), a downstream effector protein of the mTORC-1 pathway, is an important protein to regulate glucose homeostasis via AgRP and POMC neurons (Smith *et al.* 2015). It is also interesting that the

tyrosine phosphatase TCPTP, an component of the PI3K pathway, represents another intracellular mechanism in which glucose homeostasis can be controlled due to the suppression of the insulin receptor signalling in AgRP neurons (Dodd *et al.* 2018, Huang *et al.* 2018). In addition, it has been reported that the ablation of AMPK α 2 signalling in AgRP neurons, besides the effects on energy homeostasis that causes age-dependent lean phenotype with increased sensitivity to a melanocortin agonist, abolishes the response of AgRP neurons to glucose (Claret *et al.* 2007). On the other hand, AgRP-specific deletion of Rictor, a key regulatory/structural subunit of the mTORC2 complex did not cause changes in energy balance but led to mild glucose intolerance (Kocalis *et al.* 2014). Therefore, other signalling pathways besides the STAT5, such as mTORC1, mTORC2, PI3K and AMPK α 2, seem to trigger a relevant role in the control of glucose metabolism via AgRP neurons.

STAT5 ablation in the AgRP neurons also did not affect food intake or the counter-regulatory response after 2DG treatment in both genders. These data are partially in accordance with a recent study that observed that the ablation of GH receptor in AgRP neurons did not cause metabolic imbalances nor differences in the counter-regulatory response (CRR) triggered by 2DG (Furigo *et al.* 2019b). Nonetheless, in the same work, it was reported a prevention in the hyperphagia induced by 2DG in AgRP GHR KO mice, an effect that was not observed in STAT5 AgRP-knockout mice. The glucopenia induced by 2DG increases the hypothalamic mRNA levels of *Agrp* (Fraley *et al.* 2002) and may induce food intake. However, according to our results, STAT5-independent intracellular pathways are likely involved in the food intake stimulation by glucoprivation in AgRP neurons. Recently, it was demonstrated that ablation of GHR or STAT5 in POMC cells did not affect energy or glucose homeostasis. However, glucoprivic hyperphagia is blunted in male and female GHR-knockout mice, and in male POMC-specific STAT5-knockout mice. Additionally, the absence of GHR in POMC neurons decreased glycaemia during prolonged food restriction in male mice. Thus, GH action in POMC neurons regulates glucoprivic hyperphagia as well as blood glucose levels during prolonged food restriction (Quaresma *et al.* 2019). Likewise, the recovery from hypoglycaemia state may not be regulated by STAT5 in AgRP neurons. In this sense, it is possible that other brain areas or neuronal pathways may be more relevant. Meek *et al.* (2016) have reported that neurons in the lateral parabrachial nucleus (LPBN), a brain area that is implicated in the response to hypoglycaemia,

make synaptic connections with glucoregulatory VMH neurons that project to the anterior bed nucleus of stria terminalis, and established this ascending glucoregulatory circuit. It has also been recently reported that GH receptor expression in the VMH is required for a normal CRR, and these effects are largely independent of STAT5 pathway (Furigo *et al.* 2019c). Therefore, our data suggest that the STAT5 signalling in AgRP neurons does not represent an important component that controls glucose homeostasis.

Recently, it was demonstrated that AgRP neurons play an important role in the neuroendocrine adaptations during weight loss under GH regulation since the capacity of mice carrying AgRP-specific GHR ablation to save energy during food deprivation was impaired (Furigo *et al.* 2019b). At the present study we tested whether STAT5 is a crucial pathway that regulates this response. We have demonstrated that STAT5 is partially involved in the neuroendocrine adaptations to food restriction since AgRP STAT5-knockout male mice had attenuated increase of *Agrp* mRNA levels in the hypothalamus compared to control mice. In the same way, AgRP STAT5-knockout female mice had a tendency to similar results. We also observed attenuation in serum corticosterone upregulation in knockout mice, suggesting that STAT5 regulates these responses via AgRP neurons. No alterations were observed in the energy expenditure at this condition.

On the other hand, the STAT5 ablation did not change the *Npy* and *Pomc* responses to food restriction. Also, STAT5 ablation did not affect the response of iBAT *Ucp-1* mRNA expression to food restriction. However, at *ad libitum* condition, we have observed that AgRP STAT5-knockout male mice presented decreased *Pomc* and *Ucp-1* mRNA expression compared to control mice. It is described in the literature that there is a direct communication by GABA connectivity of AgRP neurons to POMC neurons, which is not required for evoked feeding (Atasoy *et al.* 2012). Therefore, the relevance of AgRP to POMC neuron GABA connectivity depends on the state of AgRP neuron activity, once AgRP neurons can potentially affect POMC neuron function without contributing a significant source of spontaneous GABA input to POMC neurons (Rau & Hentges 2017). Anyway, this alteration did not affect food intake or energy expenditure of AgRP STAT5-knockout mice.

Although a decreased mRNA expression of *Ucp-1* was found in STAT5 AgRP-knockout male mice at *ad libitum* state, this change was not sufficient to change the energy expenditure of this mice model. It is described that AgRP neurons can regulate energy expenditure (Krashes *et al.* 2011, Burke *et al.* 2017), and it has been assigned that

mTORC1 signalling within these neurons modulates iBAT activity (Burke *et al.* 2017). Interestingly, the difference in *Ucp-1* mRNA expression between the groups at *ad libitum* state is abolished under food restriction, suggesting that the food restriction response on iBAT activity is not impaired in STAT5-knockout mice.

Although STAT5 is an important intracellular pathway recruited by GH, the non-replication of all the findings of AgRP-specific GH receptor-knockout mice may be due to the fact that GH activates other intracellular signalling pathways besides STAT5 or may act by non-genomic pathways. Using neuron-specific approaches to measure and manipulate kinase activity specifically within AgRP neurons, previous studies established that fasting increases AMPK activity in AgRP neurons, and this action is necessary and sufficient for fasting-induced changes in spinogenesis and excitatory synaptic activity (Kong *et al.* 2016). In addition, AMPK phosphorylation mediates this plasticity via p21-activated kinase. This provides a signalling and neurobiological basis for both AMPK energy balance regulation and AgRP neuron state-dependent plasticity (Kong *et al.* 2016). Additionally, it was demonstrated that AgRP neuron-mediated feeding was not dependent on suppressing of the melanocortin pathway, indicating that AgRP neurons directly engage feeding circuits (Aponte *et al.* 2011). In another study, it was identified that small-conductance calcium-activated potassium channel 3 is a key intrinsic mediator that coordinates nutritional status with AgRP/NPY neural activities and animals' feeding behaviour and energy metabolism (He *et al.* 2016), demonstrating the importance of the activation of ion channels in the control of metabolism in AgRP neurons. Therefore, other intracellular pathways besides STAT5, or even non-genomic pathways, such as ionic channels, may be involved in the metabolic responses of AgRP neurons.

Taken together, our results indicate that STAT5 ablation in AgRP neurons leads to increased adiposity in female mice and partially regulates the neuroendocrine adaptations to food restriction, such as the *AgRP* gene expression and corticosterone serum levels. The complexity arising from the overlapping and sometimes opposing biological activities of the intracellular pathways, as well as the existence of different hormones that act in the AgRP neurons, highlight the wide range of factors that control the action of these neurons in metabolism. Therefore, it is plausible that the ablation of a single transcription factor, as the STAT5, in a specific neuronal population causes partial alterations in the metabolism, equally important for the elucidation of the complex circuit that controls the energy balance and glucose homeostasis.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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