Attenuated hypothalamic responses to α-melanocyte stimulating hormone during pregnancy in the rat

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Key points

- Increased appetite and weight gain occurs during pregnancy, associated with development of leptin resistance, and satiety responses to the anorectic peptide α-melanocyte stimulating hormone (α-MSH) are suppressed.
- This study investigated hypothalamic responses to α-MSH during pregnancy, using c-fos expression in specific hypothalamic nuclei as a marker of neuronal signalling, and *in vivo* electrophysiology in supraoptic nucleus (SON) oxytocin neurons, as a representative α-MSH-responsive neuronal population that shows a well-characterised α-MSH-induced inhibition of firing.
- While ICV injection of α-MSH significantly increased the number of c-fos-positive cells in the paraventricular, supraoptic, arcuate and ventromedial hypothalamic nuclei in non-pregnant rats, this response was suppressed in pregnant rats. Similarly, SON oxytocin neurons in pregnant rats did not demonstrate characteristic α-MSH-induced inhibition of firing that was observed in non-pregnant animals.
- Given the known functions of α-MSH in the hypothalamus, the attenuated responses are likely to facilitate adaptive changes in appetite regulation and oxytocin secretion during pregnancy.

Abstract During pregnancy, a state of positive energy balance develops to support the growing fetus and to deposit fat in preparation for the subsequent metabolic demands of lactation. As part of this maternal adaptation, the satiety response to the anorectic peptide α-melanocyte stimulating hormone (α-MSH) is suppressed. To investigate whether pregnancy is associated with changes in the response of hypothalamic α-MSH target neurons, non-pregnant and pregnant rats were treated with α-MSH or vehicle and c-fos expression in hypothalamic nuclei was then examined. Furthermore, the firing rate of supraoptic nucleus (SON) oxytocin neurons, a known α-MSH responsive neuronal population, was examined in non-pregnant and pregnant rats following α-MSH treatment. Intracerebroventricular injection of α-MSH significantly increased the number of c-fos-positive cells in the paraventricular, arcuate and ventromedial hypothalamic nuclei in non-pregnant rats, but no significant increase was observed in any of these regions in pregnant rats. In the SON, α-MSH did induce expression of c-fos during pregnancy, but this was significantly reduced compared to that observed in the non-pregnant group. Furthermore, during pregnancy, SON oxytocin neurons did not demonstrate the characteristic α-MSH-induced inhibition of firing rate that was observed in non-pregnant animals. Melanocortin receptor mRNA levels during pregnancy were similar to non-pregnant animals, suggesting that receptor down-regulation is unlikely to be a mechanism underlying the attenuated responses to α-MSH during pregnancy. Given the known functions of α-MSH in the hypothalamus, the attenuated responses will facilitate adaptive changes in appetite regulation and oxytocin secretion during pregnancy.

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Abbreviations   AgRP, agouti-related peptide; α-MSH, α-melanocyte stimulating hormone; CCK, cholecystokinin; MC3-R, melanocortin 3 receptor; MC4-R, melanocortin 4 receptor; MRAP, melanocortin receptor accessory protein; POMC, pro-opiomelanocortin; PVN, paraventricular nucleus of the hypothalamus; SON, supraoptic nucleus; VMH, ventromedial nucleus of the hypothalamus.

Introduction

During pregnancy, it is physiologically appropriate to gain weight. This allows the mother to supply the energy required for the growth and development of the fetus, as well as preparing adequate energy reserves for the subsequent, metabolically demanding period of lactation. In non-pregnant adults, body weight is maintained at a relatively stable level due to complex interactions between short- and long-term regulators of energy balance. The adipocyte-derived hormone leptin plays a vital role in this system, providing a long-term negative feedback signal to the hypothalamus to suppress appetite and increase metabolic rate, thus maintaining relatively stable body fat levels. Despite elevated leptin concentrations, hyperphagia is maintained throughout pregnancy (Ladyman & Grattan, 2004). In addition, central administration of exogenous leptin, which significantly decreases food intake in non-pregnant rats, fails to do so in day 14 pregnant rats (Ladyman & Grattan, 2004). This indicates that pregnancy is a physiological state of leptin resistance, allowing a period of positive energy balance and weight gain. Pregnant rats also become insensitive to the acute satiety effects of cholecystokinin (CCK), a short-term regulator of appetite (Ladyman *et al.* 2011), indicating that both long- and short-term regulation of appetite are modulated during pregnancy to facilitate a state of positive energy balance.

The melanocortin system is a key downstream pathway mediating the actions of both leptin (Balthasar *et al.* 2005) and CCK (Fan *et al.* 2004; Blevins *et al.* 2009), and involves activation of pro-opiomelanocortin (POMC) neurons in the arcuate nucleus. Alpha-melanocyte stimulating hormone (α-MSH) is a derivative of the POMC precursor protein and, once released in response to leptin or CCK, acts on melanocortin 3 (MC3-R) and melanocortin 4 receptors (MC4-R) in the hypothalamus (Mountjoy, 2010) to inhibit food intake and increase energy expenditure. Melanocortin receptors are also targeted by an endogenous antagonist, agouti-related peptide (AgRP), which is produced in neuropeptide-Y (NPY) neurons of the arcuate nucleus. AgRP promotes food intake, and AgRP mRNA is upregulated in response to fasting (Mizuno & Mobbs, 1999; Korner *et al.* 2001). Using c-fos as a neuronal marker to assess cellular responses, the paraventricular nucleus of the hypothalamus (PVN) has been identified as a key site of action of α-MSH (Thiele *et al.* 1998; McMinn

et al. 2000). The PVN, in particular, has a high density of MC4-R and receives neuronal inputs from POMC and AgRP neurons located in the arcuate nucleus (Bagnol *et al.* 1999; Cowley *et al.* 1999). Indeed, MC4-Rs within the PVN are critical for MC4-R-mediated regulation of appetite (Balthasar *et al.* 2005; Garza *et al.* 2008). Within the hypothalamus, MC3-R-expressing neurons are found in nuclei associated with energy homeostasis, including the arcuate nucleus and the ventromedial nucleus of the hypothalamus (VMH) (Roselli-Rehfuss *et al.* 1993), although the exact nature of the role of MC3-R in energy homeostasis remains to be determined (Butler *et al.* 2000; Begriche *et al.* 2013).

While leptin resistance induced by a high fat diet can be bypassed by administration of the downstream regulator α-MSH (Pierroz *et al.* 2002; Hansen *et al.* 2005), we have shown that pregnant rats do not show a satiety response to α-MSH (Ladyman *et al.* 2009). Given the critical role of the melanocortin system in the anorectic response to leptin (Marsh *et al.* 1999), this suggests that the downstream impairment of the melanocortin response may underlie pregnancy-induced leptin resistance. Therefore, the aim of the current study was to examine hypothalamic responses to α-MSH during pregnancy to determine if the lack of satiety response to α-MSH during pregnancy is associated with an impaired activation of hypothalamic cells by α-MSH, using two independent markers of cellular response: (1) α-MSH-induced expression of c-fos in key hypothalamic nuclei (McMinn *et al.* 2000), and (2) the well-characterised inhibitory action of α-MSH on the firing rate of oxytocin neurons *in vivo* (Sabatier *et al.* 2003). To address the possible mechanism behind a loss of response to α-MSH, the expression of mRNA for melanocortin receptors and levels of the endogenous antagonist AgRP were examined during pregnancy.

Methods

Animals

Ten-week-old female Sprague Dawley rats were obtained from our colony at the University of Otago and housed under a 14:10 h light–dark cycle (lights on at 05.00 h) for anatomical studies or a 12:12 h light–dark cycle (lights on at 07.00 h) for electrophysiology studies. Temperature was maintained at 21 ± 1°C. All animals had free access to food and water, except during fasting when only water was

available. The oestrous cycle was monitored by daily cytological examination of vaginal smears. To generate timed pregnancies, pro-oestrous females were housed overnight with a male rat and impregnation was confirmed by the presence of sperm in the vaginal smear the following morning (day 0 of pregnancy). All experimental protocols were approved by the University of Otago Animal Ethics Committee.

α-MSH induced c-fos in the hypothalamus during pregnancy

To assess the c-fos response of hypothalamic nuclei to intracerebroventricular (ICV) administration of α-MSH, non-pregnant and pregnant animals (day 6 or 7 of pregnancy) underwent stereotaxic surgery to implant guide cannula (Plastics One, Roanoke, VA, USA) into the right lateral ventricle as previously described (Ladyman & Grattan, 2004). After surgery, rats were housed individually and food intake, water intake and weight gain were monitored daily for approximately 1 week. To determine correct cannula placement, rats were given a 2 μl ICV injection of angiotensin-II (25 ng μl^{-1}; Bachem, Torrance, CA, USA). Cannula placement was considered correct if rats consumed more than 5 ml of water in the 30 min after injection.

On day 13 of pregnancy or metoestrus for the non-pregnant rats, food was removed from the cages 1 h prior to the start of the dark phase (5–6 per group). The following morning, between 09.00 and 11.00 h, rats received a 2 μl injection of either 10 μg α-MSH (Bacham), or saline into the right lateral ventricle. Ninety minutes after ICV injection, animals were anaesthetised with sodium pentobarbitone (17 mg/100 g body weight for pregnant rats or 6 mg/100 g for dioestrous rats, IP injection) and transcardially perfused with heparinised saline, followed by 4% paraformaldehyde in 0.1 M PBS. Brains were removed and post-fixed in the same fixative overnight at room temperature. Brains were then immersed in 30% sucrose in 0.1 M PBS until they sank, then rapidly frozen and stored until further processing. Serial coronal sections (30 μm) from the hypothalamus were cut on a cryostat then processed for immunohistochemistry using a c-fos-specific antibody (1:10,000, Calbiochem, San Diego, CA, USA) as previously described (Ladyman *et al.* 2011).

In vivo electrophysiology

On the day of electrophysiology, rats were anaesthetised by intraperitoneal injection of urethane (ethyl carbamate; 1.25 g kg^{-1}). The pituitary stalk and the right supraoptic nucleus (SON) were exposed by the transpharyngeal approach. Following removal of the meninges, a U-shaped microdialysis probe (in-house design, supplied by Prof. M.

Ludwig, University of Edinburgh; total membrane length 2.0 mm, permeable to 10 kDa; Spectra/Por RC Hollow Fibres, Spectrum Medical Inc., Houston, TX, USA) was bent (between 100 and 200 deg) and the loop of the membrane was positioned to lie flat over the exposed ventral surface of the brain over the SON. A glass recording pipette (15–40 MΩ, filled with 0.9% NaCl) was lowered into the SON through the centre of the microdialysis loop. A side-by-side SNEX-200 bipolar stimulating electrode (Science Products GmbH, Hofheim, Germany) was placed on the pituitary stalk to elicit antidromic action potentials in SON neurons. The SON was continuously dialysed with artificial cerebrospinal fluid (composition in mM: NaCl 138, KCl 3.36, NaHCO$_3$ 9.52, Na$_2$HPO$_4$ 0.49, urea 2.16, CaCl$_2$ 1.26, MgCl$_2$ 1.18) at 3 μl min^{-1} and the dialysate was changed to include α-MSH [2.5 μg μl^{-1} (1.5 mM) for 30–60 min] during recording. At the end of experiments, rats were killed by an overdose of intravenous urethane.

SON neuronal activity was recorded onto a personal computer using Spike2 software (Cambridge Electronic Design, Cambridge, UK) and analysed off-line. Neurons were characterised as oxytocin neurons on the basis of a transient excitation following intravenous CCK injection (20 μg kg^{-1}, 0.5 ml kg^{-1} in 0.9% saline; Sigma) (Brown *et al.* 1996).

Melanocortin receptor expression in the hypothalamus during pregnancy

The hypothalamic location of MC3-R mRNA and MC4-R mRNA expression was determined by *in situ* hybridization. Female rats were anaesthetised with sodium pentobarbital and transcardially perfused with 2% paraformaldhyde in 0.1 M PBS. Brains were post-fixed in the same fixative overnight at room temperature then transferred to 30% sucrose in 0.1 M PBS until they sank (<5 days) and stored at −80°C until coronal sections (16 μm) were collected through the hypothalamus. Antisense and sense riboprobes complementary to fragments of human MC4-R were transcribed from a cloned cDNA template (donated by Julian G. Mercer, Rowett Research Institute, Aberdeen). MC3-R riboprobes were synthesized from RT-PCR products generated using an oligonucleotide primer set (F: TCTTCCTGTGCAGCCTGGCTA, R: TGCTGTGGTAACGGAGGGCA, gene accession number NM_001025270.3) that contained SP6 (forward) and T7 (reverse) RNA polymerase promoter sequences ligated to the 5′ ends.

In situ hybridization for MC4-R was carried out as previously described (Ladyman *et al.* 2009). Briefly, slides were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer and acetylated as described. Sections were then hybridized overnight at 58°C using [^{35}S] UTP radioactive riboprobes for MC4-R mRNA (1.2×10^7 c.p.m. per section). Sections were then treated with RNase A,

desalted and given a final high stringency wash in $0.1\times$ SSC. Following dehydration and drying, sections were exposed to Kodak BioMas MR film (Radiographic Supplies Ltd, Christchurch, New Zealand) for 3 weeks to generate autoradiographs.

For MC3-R mRNA, *in situ* hybridization using [^{35}S] UTP radioactive riboprobes was carried out as previously described (Phillipps *et al.* 2013). Briefly, slides were fixed with 2% paraformaldehyde in 0.1 M phosphate buffer, treated with proteinase K ($2\,\mu g\,ml^{-1}$, Roche, Manneheim, Germany), and acetylated by incubation in 0.25% (v/v) acetic anhydride in TEA buffer. Sections were dehydrated, immersed in chloroform and then rehydrated. Sections were then hybridized overnight at 55°C using [^{35}S] UTP radioactive riboprobes for MC3-R mRNA (1.2×10^6 c.p.m. per section). Slides were treated with RNase A (Sigma-Aldrich, St Louis, MO, USA), and desalted, then given a final high stringency wash in $0.1\times$ SSC (containing 10 mM β-mercaptoethanol and 1 mM EDTA). Following dehydration and drying, sections were exposed to Kodak BioMas MR film (Radiographic Supplies Ltd) for 2 weeks to generate autoradiographs.

To quantify levels of MC3-R and MC4-R mRNA, brains from dioestrous, non-pregnant ($n = 6$) and day 14 pregnant rats ($n = 6$) underwent laser capture micro-dissection and quantitative RT-PCR (qPCR) as previously described (Phillipps *et al.* 2013). Based on the *in situ* data, the PVN was selected for analysis of MC4-R, while the VMH was selected to assess MC3-R. Coronal cryosections (10 μm thickn) were taken at 20 and 40 μm intervals through the PVN and VMH, respectively. Slides were placed upside down in a Leica laser capture micro-dissector (LMD) (Leica, Wetzlar, Germany) and the entire PVN or VMH isolated from appropriate sections within 1 h. Cells were collected into lysis buffer (with 1%, v/v, β-mercaptoethanol) (Qiagen RNeasy Micro kit, Valencia, CA, USA) and total RNA extracted with an on column DNase digestion step according to the manufacturer's instructions for the Qiagen RNeasy Micro kit. RNA quality and quantity was determined using an Agilent Bioanalyser (Agilent Technologies, Inc., Waldbronn, Germany). Only RNA samples with distinct 18S and 28S ribosomal bands and RNA integrity number (RIN) values >6.00 were used for qPCR analysis.

Total RNA (1–4 ng) was reversed transcribed using the Superscript III First Strand Synthesis System (Invitrogen New Zealand Limited, Victoria, Australia) in accordance with the manufacturer's instructions. Resultant cDNA was stored at -20°C prior to PCR amplification. Oligonucleotide primer/probe sets were designed using PrimerQuest software (Integrated DNA Technologies, Coralville, IA, USA) and purchased from Integrated DNA Technologies: MC3-R (accession no.NM_001025270.3): F: 5′-CCACAGCATCATGACGGTTAGGA-3′; R: 5′-TCTTG CTCTCGGAGTAGACGATGAAC-3′; P: 5′-/56FAM/AGC CCTCTC/ZEN/CTTGATCGTGGCCATCTGGGT/3IABk FQ/-3′; and MC4-R (accession no. NM_013099.2): F: 5′-TGGCTGTGGCGGACATG-3′; R: 5′-TGTTTAGCAG GGTGATGACGAT-3′; P: 5′-/56-FAM/TGGTGAGCG/ ZEN/TTTCGAACGGGTCA/3IABkFQ/-3′. B-actin (accession no. NM_031144) was used as a reference gene: F: 5′-AGATGACCCAGATCATGTTTGAGA-3′; R: 5′-ACCAG AGGCATACAGGGACAA-3′; P: 5′-/56FAM/TCAACAC CC/ZEN/CAGCCATGTACGTAGCC/3IABkFQ/-3′.

Single-plex qPCRs (20 μl) were set up by addition of PCR-grade water, optimized final concentrations of primers (600 nM: β-actin forward and reverse primers, MC3-R reverse primer, MC4-R reverse primer, 900 nM: MC3-R forward primer, 300 nM: MC4-R forward primer), and probe (200 nM for all genes) and LightCycler 480 Probes Master (1× concentration, Roche Applied Science, Mannheim, Germany). Template cDNA (2 μl) was added to appropriate wells in duplicate. Each plate contained negative wells in which RNase-free water was added in place of template cDNA.

All plates were run using the LightCycler 480 Instrument II Real time PCR system (Roche Applied Science). The following protocol was utilised: initial preincubation at 95°C for 10 min, followed by 45 cycles of 95°C for 10 s, 60°C for 30 s and 72°C for 1 s, and a final cooling step at 40°C for 10 s. The raw data were run through an absolute quantification/2nd derivative maximum analysis using the associated LightCycler 480 software to calculate Cq values for each reaction. Relative gene expression analysis was undertaken using the comparative Cq method as described previously (Phillipps *et al.* 2013).

AgRP immunoreactivity in the PVN during pregnancy

Day 14 pregnant ($n = 3$) and non-pregnant rats in the dioestrous phase of the oestrous cycle ($n = 3$) were perfused with 4% paraformaldehyde in 0.1 M PBS and brains processed as described for c-fos immuno-histochemistry above. Coronal sections (35 μm) were sliced throughout the PVN and collected in three series. Sections were washed five times with PBS/0.1% triton for 10 min per wash and then incubated in blocking solution (PBS/0.1% triton/5% normal goat serum/0.25% BSA) for 1 h. Following this, sections were incubated for 48 h at 4°C in block solution containing the guinea pig poly-conal anti-AgRP antibody (dilution 1:10,000; Antibodies Australia, Clayton, Australia). Sections were then washed in PBS/0.1% triton as described above, then incubated for 2 h at room temperature with a goat anti-guinea pig Alexa fluor 555 antibody (dilution 1:500). Following further washes, sections were mounted on slides and cover-slipped using vectamount. Sections were viewed and photographed on an Olympus AX70 research microscope (Olympus, Tokyo, Japan). The extent of fibre staining in

the PVN was determined using ImageJ/Fiji software in at least two sections per animal and the mean for each animal was used for statistical comparisons. Briefly, the region of interest was selected, images were binarized and then skeletonized, and total voxels were counted as a measure of the area of region of interest stained positive for AgRP (with background levels subtracted).

Statistics

For the c-fos immunohistochemistry analysis, at least three sections per area from each animal were counted and all analysis was performed blind to treatment group. The number of cells positive for c-fos immunoreactivity was assessed in the PVN and SON (approximately -1.30 to -2.12 mm, relative to Bregma), as well as the arcuate nucleus and the VMH (approximately -2.12 to -3.60 mm relative to bregma) (Paxinos & Watson, 2005). For each rat, the average number of positive cells per section was calculated and used for statistical comparisons between treatment groups. For both c-fos immunohistochemistry data and electrophysiological data, differences between treatment groups were analysed by two-way ANOVA, and where a significant difference was obtained, *post hoc* comparisons were made using Holm–Sidak analysis. To determine the presence of any significant difference in MC3-R and MC4-R mRNA expression a Student's *t* test was used, while to compare AgRP fibre density in PVN between non-pregnant and day 14 pregnant rats a non-parametric Mann–Whitney *U* test was used. All data are presented as mean $\pm$ SEM and $P < 0.05$ was considered statistically significant. Graphpad Prism (GraphPad Software Inc., La Jolla, CA, USA) was used for statistical analyses.

Results

α-MSH induced c-fos in the hypothalamus during pregnancy

The number of c-fos-positive cells after α-MSH or vehicle treatment was determined in the PVN (Fig. 1), arcuate nucleus (Fig. 2), VMH (Fig. 2) and SON (Fig. 3) in both pregnant and non-pregnant rats. Statistical analysis by two-way ANOVA revealed significant interactions between reproductive state and treatment (PVN $P = 0.016$, arcuate nucleus $P = 0.001$, VMH $P = 0.018$, SON $P = 0.025$). In all four hypothalamic regions examined, α-MSH treatment significantly increased the expression of c-fos-positive cells in the dioestrous rats (Figs 1–3). In contrast, there was no significant increase in c-fos-positive cell number after α-MSH treatment in the PVN (Fig. 1), arcuate nucleus (Fig. 2) or VMH (Fig. 2) in the pregnant group. In the SON, α-MSH treatment significantly increased c-fos-positive

cell number in the pregnant animals, but the increase was significantly attenuated compared to the α-MSH-treated dioestrous group (Fig. 3).

Effect of α-MSH on electrical activity of oxytocin neurons during pregnancy

In vivo extracellular single-unit recordings were made from 12 oxytocin neurons from nine female rats during microdialysis application of α-MSH (1.5 mM for 30–60 min). Similar to previous findings (Sabatier *et al.* 2003), the mean spontaneous firing rate of all six oxytocin neurons recorded from non-pregnant rats decreased progressively by approximately 50% during microdialysis application of α-MSH (Fig. 4). In contrast to this inhibitory effect in non-pregnant rats, on average oxytocin neuron firing rate was not affected by α-MSH administration in mid-pregnant (pregnancy day 10–16) rats, with most neurons showing no response and two cells even showing an increase in firing after α-MSH (Fig. 4). Overall, the effect of α-MSH on the firing rate of oxytocin neurons was significantly different in pregnant compared with non-pregnant rats ($P < 0.05$).

Melanocortin receptor expression in the hypothalamus during pregnancy

MC3-R mRNA expression within the hypothalamus was predominantly confined to the VMH and the arcuate nucleus (Fig. 5). MC4-R mRNA expression was localized to the PVN, with the SON and arcuate nucleus displaying a weaker signal for this riboprobe (Fig. 5). Additional non-hypothalamic sites also showed strong signal for MC4-R with the medial amygdala (Fig. 5) and caudate putamen (not shown) displaying dark staining on the autoradiogram. Within the VMH, positive hybridization of MC3-R was limited to the dorsomedial areas of this nucleus (Fig. 5). There was no significant difference between levels of MC3-R mRNA in the VMH ($P = 0.4$) or MC4-R mRNA in the PVN ($P = 0.073$) in day 14 pregnant and non-pregnant rats, as determined by qPCR following laser microdissection of these nuclei (Fig. 5).

AgRP immunoreactivity in the PVN during pregnancy

AgRP fibre immunoreactivity was observed within the PVN (Fig. 6), as described previously (Broberger *et al.* 1998; Bagnol *et al.* 1999). There was no significant difference in the extent of fibre coverage in the PVN of pregnant and non-pregnant rats ($P = 0.4$).

Discussion

This study has documented a profound change in the hypothalamic response to α-MSH during pregnancy. In all hypothalamic areas investigated, there was a marked decrease in the ability of α-MSH to induce c-fos expression in cells. As a representative example of a known population of α-MSH-responsive neurons, we examined the electrophysiological response to α-MSH in supraoptic oxytocin neurons *in vivo*. In non-pregnant female rats, α-MSH decreased oxytocin neuron firing rate, as described previously (Sabatier *et al.* 2003). In contrast, there was an overall loss of α-MSH-induced inhibition of firing rate of SON oxytocin neurons in mid-pregnancy, consistent with the loss of α-MSH-induced c-fos expression, with some neurons even showing an opposite response. These data, coupled with our prior demonstration that the satiety response to α-MSH is lost during pregnancy, indicate a widespread insensitivity to α-MSH in the hypothalamus during pregnancy.

Several studies have previously documented that α-MSH induces c-fos expression in the PVN and SON (Thiele *et al.* 1998; McMinn *et al.* 2000; Olszewski *et al.* 2001; Sabatier *et al.* 2003; Kawasaki *et al.* 2009; Rowland *et al.* 2010). As elevated c-fos expression is widely thought to reflect neuronal activity, many of these studies were interpreted to suggest that α-MSH excited neurons within these nuclei. As discussed in several reviews (Hoffman & Lyo 2002; Kovacs 2008), however, it is important to recognise that c-fos is activated by several signal transduction pathways, and it is possible to dissociate changes in these 2nd messenger systems from depolarisation of
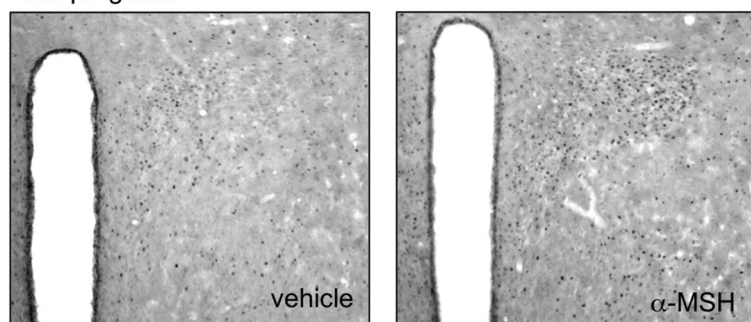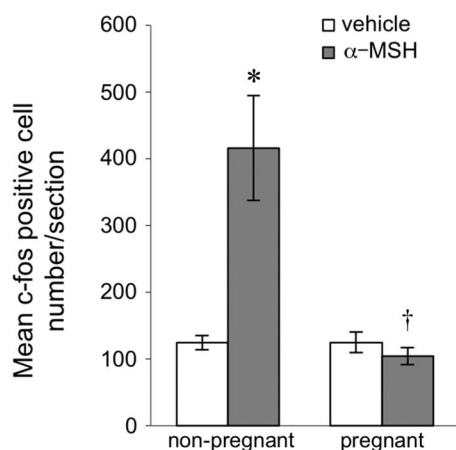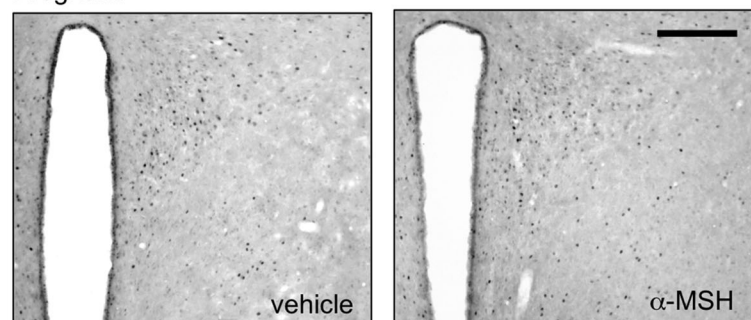

Figure 1. Expression of c-fos in representative coronal sections of the paraventricular nucleus 90 min after ICV injection of α-MSH or vehicle in non-pregnant and day 14 pregnant rats

Scale bar = 200 μm. Bars represent the mean $\pm$ SEM number of c-fos-positive cells in the paraventricular nucleus of the hypothalamus after α-MSH or vehicle treatment in non-pregnant and day 14 pregnant rats (n = 5–6 per group). *Significant with respect to vehicle-treated group of the same physiological state ($P < 0.05$). †Significant with respect to α-MSH-treated non-pregnant group ($P < 0.05$).

neurons. The most direct demonstration of this was provided by Luckman *et al.* (1994), who showed that cholinergic receptor activation increased both action potential firing and c-fos expression in oxytocin neurons, but that electrical stimulation of action potential firing using the pattern recorded during cholinergic receptor activation did not induce c-fos expression. Hence, c-fos expression was mediated by receptor-mediated signal transduction, rather than by neuronal firing, *per se*. There are many examples where strong activation of neuronal firing is not accompanied by c-fos expression (Hoffman & Lyo 2002; Kovacs 2008). For example, oxytocin neurons do not show elevated c-fos during lactation, despite robust increases in neuronal activity (Fenelon *et al.* 1993). In particular, it is known that c-fos does not provide a marker of activity in neurons experiencing a net synaptic inhibition (Chan *et al.* 1993; Kovacs 2008). While less common, there are also examples where expression of c-fos can be induced in the absence of an increase in neuronal firing rate or, indeed, in the presence of inhibition of firing. For example, in the striatum, dopamine induces c-fos expression through an increase in cAMP, and at the same time inhibits neuronal firing via D1 receptors (Berreta *et al.* 1992). α-MSH action on oxytocin neurons provides
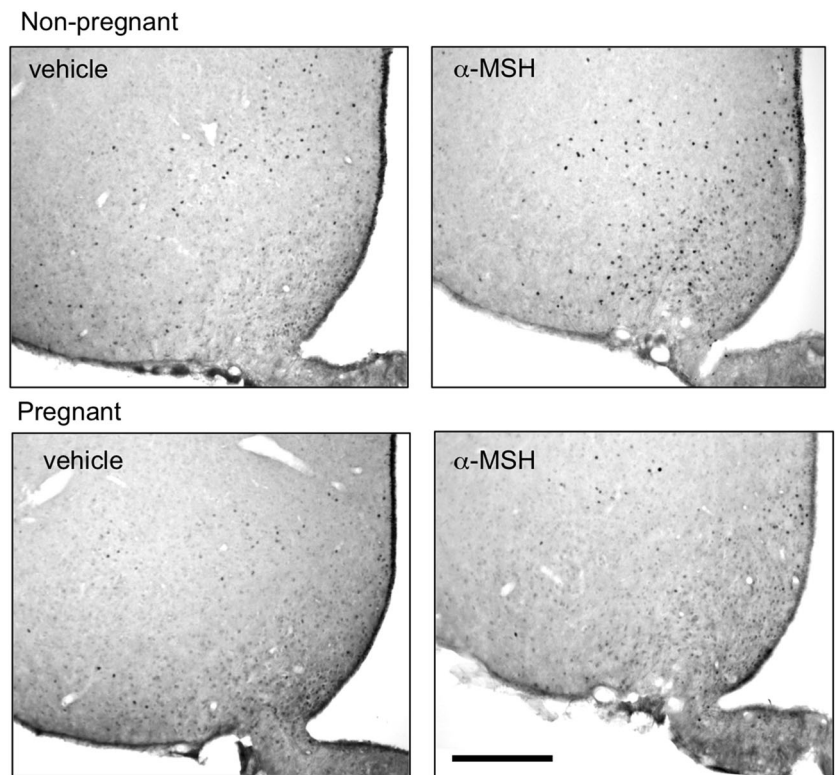

Figure 2. Expression of c-fos in representative coronal sections of the arcuate nucleus and ventromedial nucleus of the hypothalamus (VMH) 90 min after α-MSH or vehicle ICV injection in non-pregnant and day 14 pregnant rats
Scale bar = 200 μm. Bars represent the mean ± SEM number of c-fos-positive cells in the arcuate nucleus and the VMH after α-MSH or vehicle treatment in non-pregnant and day 14 pregnant rats (n = 5–6 per group). *Significant with respect to vehicle-treated group of the same physiological state (P < 0.05). †Significant with respect to α-MSH-treated non-pregnant group (P < 0.05).
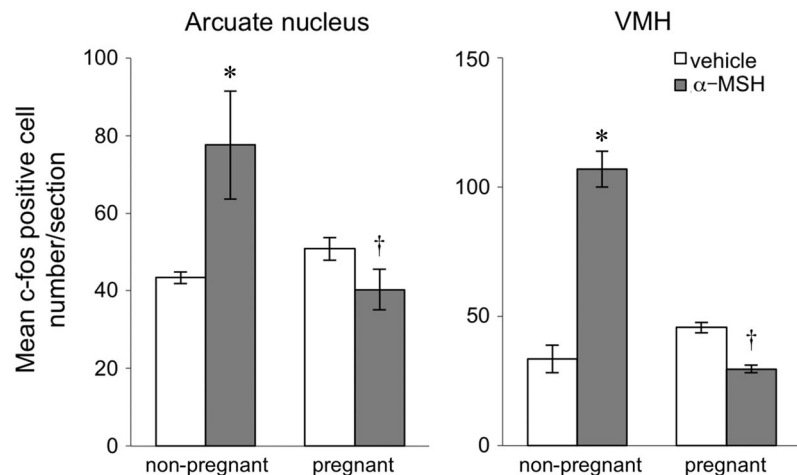
another similar example, with α-MSH administration directly into the SON shown to stimulate c-fos expression in oxytocin neurons, associated with an α-MSH-induced decrease in firing rate of these cells (Sabatier *et al.* 2003). Our data in non-pregnant animals replicate these observations, and confirm that the normal response of oxytocin neurons to α-MSH is an increase in c-fos expression together with an inhibition of neuronal firing rate. The physiological consequences of this complex response have been nicely reviewed (Sabatier, 2006), and it potentially provides a mechanism to allow differential secretion of oxytocin from different compartments within the neurons. The induction of c-fos probably involves activation of cAMP in the oxytocin neurons, as the MC4-R predominantly acts via Gs to activate adenylate cyclase (Tao, 2010), and cAMP represents one of the major pathways involved in activating c-fos expression (Hoffman & Lyo 2002). Alternatively, some recent evidence from amygdala neurons suggests that activation of presynaptic MC4-Rs can induce an inhibition of N-type calcium channels, potentially reducing inhibitory inputs to the postsynaptic neurons to induce an increase in c-fos expression (Agosti *et al.* 2014). Whether a similar mechanism is involved in oxytocin neurons is not known. In contrast, the inhibition of firing appears to involve α-MSH-induced increases in intracellular calcium in oxytocin neurons, which triggers a release of endocannabinoids to act in a retrograde manner to inhibit
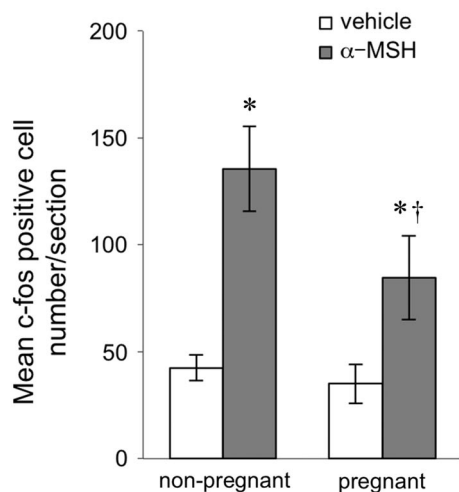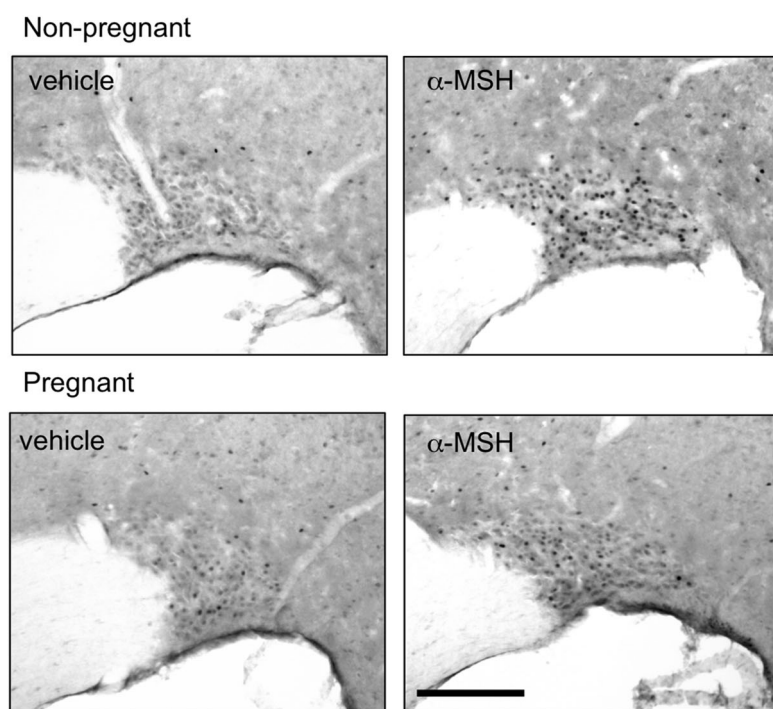

Figure 3. Expression of c-fos in representative coronal sections of the supraoptic nucleus 90 min after α-MSH or vehicle ICV injection in non-pregnant and day 14 pregnant rats
Scale bar = 200 μm. Bars represent the mean ± SEM number of c-fos-positive cells in the supraoptic nucleus after α-MSH or vehicle treatment in non-pregnant and day 14 pregnant rats (n = 5–6 per group). *Significant with respect to vehicle-treated group of the same physiological state. †Significant with respect to α-MSH treated non-pregnant group ($P < 0.05$).

presynaptic inputs to these cells (Sabatier & Leng, 2006). Remarkably, both the increase in c-fos expression and the inhibition of firing of oxytocin neurons in response to α-MSH are lost during pregnancy.

The melanocortin system is a vital neuronal network involved in the regulation of energy balance. Leptin acts to increase α-MSH release from POMC neurons in the arcuate nucleus. α-MSH binds to MCR-4, predominantly located on neurons within the PVN, to suppress food intake (Balthasar *et al.* 2005). Leptin also suppresses production of the melanocortin antagonist AgRP from another population of neurons in the
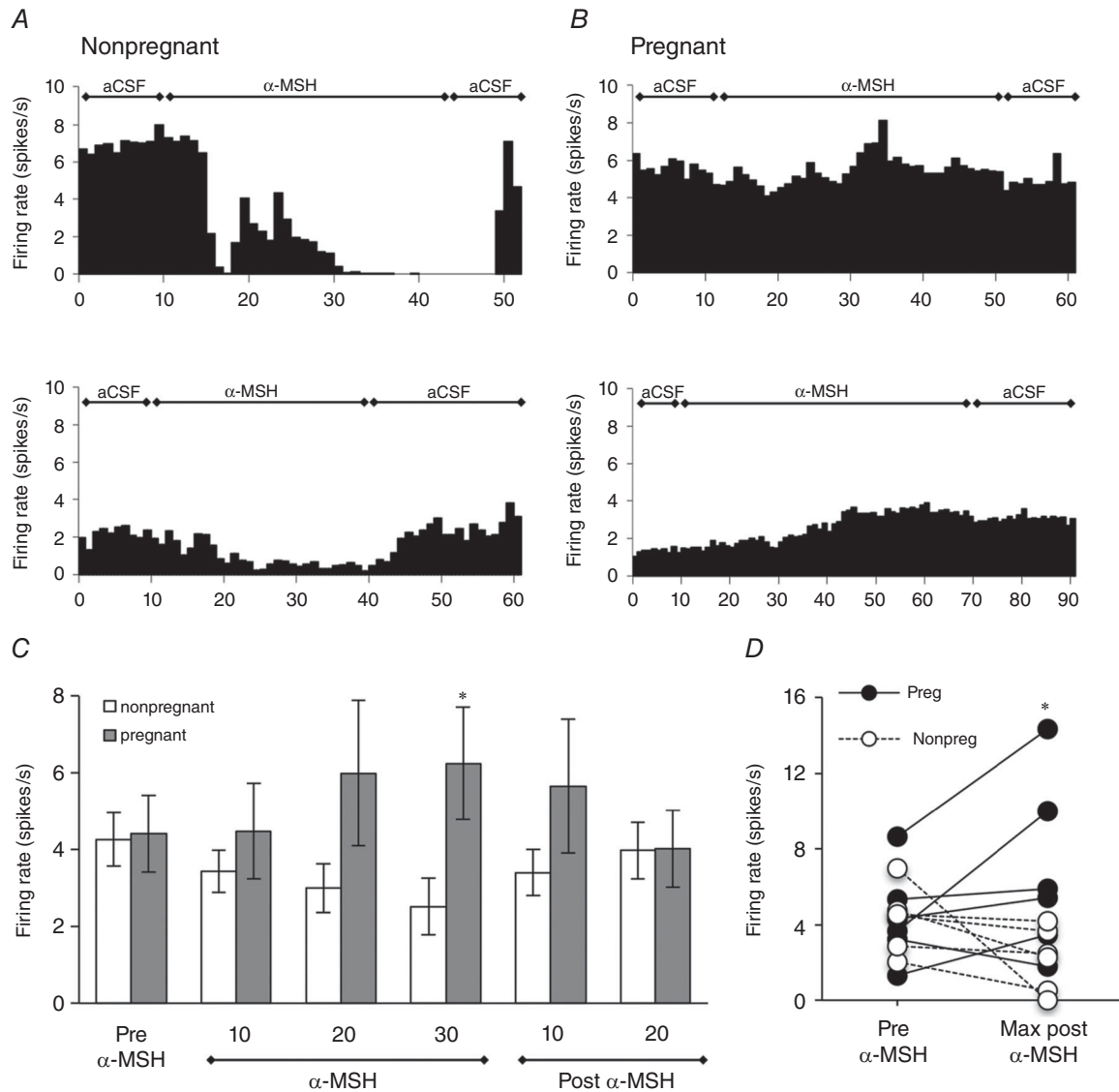

Figure 4. Pregnancy-induced changes in the in vivo electrophysiological response of oxytocin neurons to α-MSH
A and *B*, representative rate-meter records (in 1 min bins) showing changes in firing rate in response to continuous α-MSH of an oxytocin neuron in two representative urethane-anesthetised non-pregnant, virgin (*A*) or pregnant rats (*B*) (please note the x-axis differs for some of these individual recordings, due to variability in basal firing rate of oxytocin neurons *in vivo*). *C*, mean firing rate of identified oxytocin neurons (averaged in 10 min bins) before, during and after continuous dialysis of α-MSH in non-pregnant and pregnant rats (days 10–16). α-MSH decreased firing rate in oxytocin neurons in non-pregnant rats, but had no effect or slightly increased firing rate in pregnancy. *Significant with respect to firing rate in pregnant animals ($P < 0.05$) ($n = 6$ rats per group). *D*. scatter plot illustrating the individual changes in firing from all recorded cells, showing the change from the period prior to α-MSH injection (Pre-αMSH) to the maximum response during administration of α-MSH (Max response αMSH). Note that there is considerable inter-cell variability in firing in both groups, but the α-MSH-induced decrease in firing seen in non-pregnant animals is lost in pregnant animals, with some animals even showing an increase in firing *Response in pregnant animals is significantly different from that in non-pregnant animals ($P < 0.05$).

arcuate nucleus, thereby facilitating activation of the melanocortin-sensitive pathways. In diet-induced obesity, these arcuate nucleus neurons become less responsive to leptin, with the resulting reduction in signalling through the melanocortin pathway contributing to increased food intake, despite rising levels of leptin. Bypassing this leptin resistance by administering α-MSH or melanocortin receptor agonists induces a profound suppression of

food intake (Pierroz *et al.* 2002; Hansen *et al.* 2005), demonstrating that the melanocortin system remains responsive. In contrast, pregnant animals are leptin resistant, and also do not show a satiety response to α-MSH (Ladyman *et al.* 2009). It therefore seems likely that the observed suppression of responses to α-MSH during pregnancy is a key mechanism underlying the development of leptin resistance during pregnancy. The
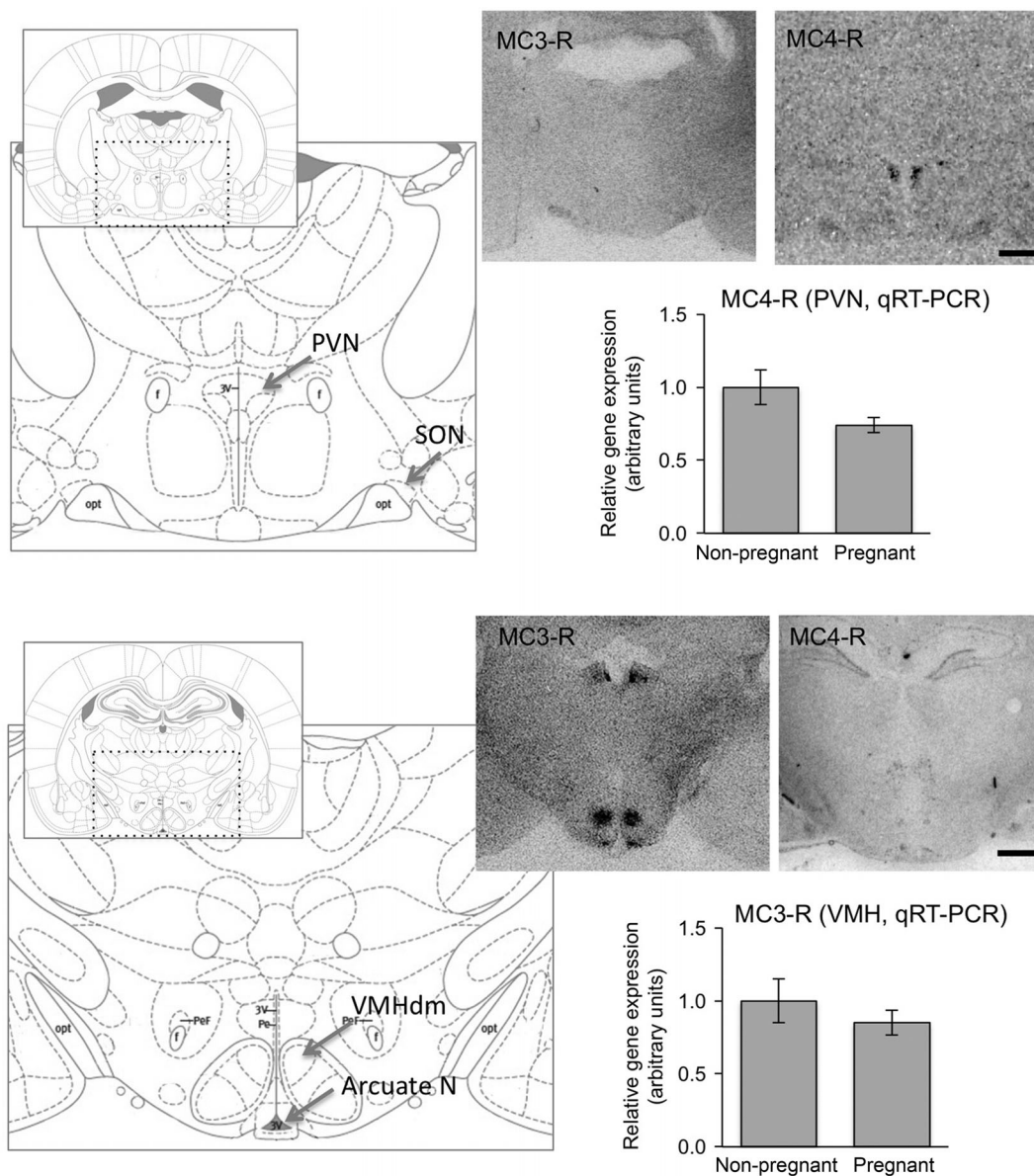

Figure 5. MC3-R and MC4-R mRNA expression in the rat hypothalamus
Representative autoradiographic images of coronal brain sections from a female rat showing expression of MC3-R (left) and MC4-R (right) in the paraventricular nucleus (top) and the arcuate nucleus and ventromedial nucleus of the hypothalamus (VMH) (bottom). Scale bars = 1000 μm. Schematics represent coronal rat brain sections through regions of the hypothalamus −1.56 and −2.40 mm relative to Bregma (Paxinos & Watson, 2005) that correspond to areas shown in the autoradiographic images. Graphs show no significant difference in VMH MC3-R mRNA levels and PVN MC4-R mRNA levels in laser capture microdissected samples from non-pregnant rats ($n = 6$) and day 14 pregnant rats ($n = 6$). Data are presented as relative gene expression with comparison to the control group $\pm$ SEM.

loss of response in these second-order neurons provides an adaptive change in energy homeostasis, facilitating increased appetite in pregnant females and promoting a positive energy balance in preparation for the energy demands of lactation.

The central melanocortin system is also involved in glucose homeostasis, and α-MSH action, particularly within the PVN and VMH, plays a role in stimulating glucose uptake by peripheral tissues (Toda *et al.* 2009). It is possible that suppressed activation of cells by α-MSH in these areas during pregnancy may contribute to the decrease in glucose uptake in maternal peripheral tissues, a pregnancy-specific adaption of glucose homeostasis to reduce insulin sensitivity in the mother and preferentially provide glucose to the fetus (Leturque *et al.* 1980, 1984; Rossi *et al.* 1993).

While our observation of a loss of response to α-MSH during pregnancy seems quite clear, these data are not supported by a previous study using chronic third-cerebral ventricular infusion of SHU9119, a melanocortin-3,4 receptor blocker. That study found that SHU9119 administered from day 4 of pregnancy significantly increased food intake and weight gain during pregnancy, suggesting that there is ongoing melanocortin tone (Heinsbroek & van Dijk, 2009). It seems likely that this apparent contradiction is explained by the different timing in the two experiments. We have not examined responses

to α-MSH earlier than day 14 of pregnancy, but our previous work examining leptin responses has demonstrated that leptin resistance does not develop until between day 7 and day 14 of pregnancy (Ladyman & Grattan, 2004), and it is likely that it is driven by changes in hormone secretion from the placenta around day 11 (Augustine & Grattan, 2008). Given the role of the melanocortin system in mediating the effects of leptin, a similar time frame could be predicted for changes in the melanocortin system during pregnancy. Hence, administration of the antagonist from day 4 may have been inducing an effect prior to the pregnancy-induced change in sensitivity of the melanocortin system.

With reference to the representative example of oxytocin neurons specifically investigated in this study, recent evidence suggests that oxytocin neurons represent the primary output neurons in the PVN mediating action of melanocortins to regulate appetite (Atasoy *et al.* 2012). Both peripheral (Morton *et al.* 2012; Ho *et al.* 2014) and central (Arletti *et al.* 1990; Olson *et al.* 1991; Richard *et al.* 1991) oxytocin is anorectic, involved in both reducing both meal duration and overall food intake. While central administration of an oxytocin antagonist induces a significant increase in food intake in non-pregnant rats, it has no effect in pregnant females (Douglas *et al.* 2007), suggesting that central oxytocin secretion is reduced. This is consistent with our observation of a loss of α-MSH action in the hypothalamus. While we have not specifically looked at PVN oxytocin neurons, we have observed that pregnancy was associated with a loss of α-MSH-induced inhibition of firing rate of SON oxytocin neurons. Oxytocin neurons in the SON are activated during feeding (Johnstone *et al.* 2006), although the exact nature of their role in regulating appetite (as opposed to PVN neurons) is unclear (Sabatier *et al.* 2013). They would certainly contribute to peripheral oxytocin levels, but there is also some evidence that axons from these magnocellular neurons in the SON branch and provide innervation of pre-autonomic neurons (Knobloch *et al.* 2012), thereby potentially contributing to the oxytocinergic regulation of appetite. In non-pregnant rats, central α-MSH inhibits the firing rate of oxytocin neurons in the SON (Sabatier *et al.* 2003), via endocannabinoids (Sabatier & Leng, 2006), along with decreasing oxytocin secretion from the pituitary gland, but increases dendritic release of oxytocin within the hypothalamus (Sabatier *et al.* 2003). Hence, it is also possible that oxytocin released from the dendritic parts of these neurons could influence pre-autonomic neurons elsewhere in the hypothalamus, as has been shown for dendritically released vasopressin (Son *et al.* 2013). Whether the loss of effect of α-MSH on these SON oxytocin neurons during pregnancy contributes to the overall lack of α-MSH-induced satiety or indeed the hyperphagia of pregnancy is unknown, but it is certainly consistent with this hypothesis.
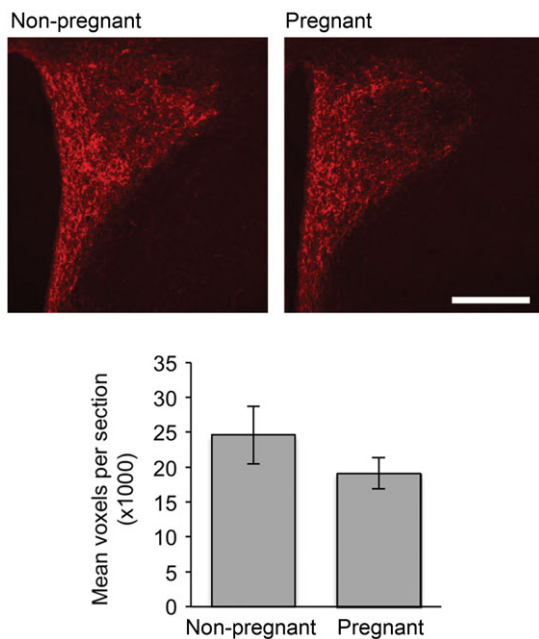

Figure 6. AgRP immunoreactivity
AgRP-positive immunoreactivity in the paraventricular nucleus in representative images from non-pregnant (top) and pregnant (bottom) rats. Scale bar = 200 μm. Graph shows no significant difference in AgRP fibre density in the PVN of non-pregnant and pregnant rats (n = 3 per group).

In addition to a possible role in appetite regulation, oxytocin has numerous other functions, including critical roles both in parturition and in milk ejection during lactation. To enable these functions, the magnocellular oxytocin neurons undergo significant adaptive modifications during pregnancy and lactation resulting in the novel capacity to fire intermittent, co-ordinated, high-frequency bursts of action potentials that result in pulses of oxytocin secreted into the blood at regular intervals (Russell *et al.* 2003; Brown *et al.* 2013). The adaptive changes that prepare these neurons for this new pattern of secretion are initiated during pregnancy, when oxytocin neurons are strongly inhibited and their responses to many stimuli are attenuated (Douglas *et al.* 2007). The loss of an inhibitory effect of α-MSH during pregnancy might be anticipated to induce elevated oxytocin secretion, which would be potentially counter-productive to pregnancy. There are multiple pregnancy-specific factors contributing to the inhibition of peripheral oxytocin secretion at this time, however, including the progesterone metabolite allopregnenalone (Brunton *et al.* 2014), nitric oxide (Srisawat *et al.* 2000), endogenous opioids (Douglas *et al.* 1995) and prolactin (Kokay *et al.* 2006). Together these factors are thought to restrain activity of oxytocin neurons to prevent premature birth. Hence, the change in response of these neurons to α-MSH during pregnancy may be part of the broader adaptive changes in this neuronal system that enable successful parturition and lactation.

Our attempts to elucidate the mechanism underlying the loss of response to α-MSH during pregnancy have not been completely successful. Within the hypothalamus, α-MSH acts through the MC3-R and MC4-R subtypes. Similar to previous studies, MC4-R mRNA was detected in the PVN and more weakly in the SON and arcuate nucleus, while MC3-R mRNA was detected in the arcuate nucleus and dorsomedial region of the VMH (Roselli-Rehfuss *et al.* 1993; Mountjoy *et al.* 1994; Xia & Wikberg, 1997; Kishi *et al.* 2003; Mountjoy, 2010). While we observed slightly lower levels of MC3-R and MC4-R mRNA during pregnancy in the VMH and PVN, respectively, these differences were not statistically significant. Hence, these data suggest that it is unlikely that a down-regulation of melanocortin receptor may underlie the decrease in α-MSH-induced expression of c-fos in hypothalamic cells that was observed during pregnancy. This finding is supported by previous work using RT-PCR techniques, showing that MC4-R mRNA levels in the whole hypothalamus were not significantly different between late pregnant and non-pregnant rats (Rocha *et al.* 2003). We also investigated whether the endogenous melanocortin receptor antagonist AgRP might be upregulated during pregnancy to oppose the actions of α-MSH. There is some inconsistency in prior reports regarding AgRP mRNA during pregnancy.

Our previous work, examining AgRP mRNA by *in situ* hybridization, suggested that levels remain stable in the arcuate nucleus during pregnancy, although dramatically increasing during lactation (Ladyman *et al.* 2009). In contrast, others, using RT-PCR on RNA extracted from the whole hypothalamus, have suggested that levels of AgRP mRNA may be increased during pregnancy (Rocha *et al.* 2003; Szczepankiewicz *et al.* 2009). In the present study, we observed that AgRP fibre density in the PVN was similar in pregnant and non-pregnant animals. Taken together with our previous *in situ* hybridization study (Ladyman *et al.* 2009), these data suggest that it is unlikely that increased AgRP could provide a mechanism to explain the pregnancy-induced loss of α-MSH responses, or the hyperphagia of pregnancy. It is possible that some post-receptor mechanism may impair responses to α-MSH during pregnancy. For example, melanocortin receptor accessory proteins (MRAPs) are expressed in the brain, including the PVN (Chan *et al.* 2009), and at least *in vitro*, these are required for normal melanocortin signalling (Chan *et al.* 2009). Hence, pregnancy-induced changes in the expression or function of MRAPs might contribute to the lack of response to α-MSH.

Overall, the results from the present study show a marked suppression of α-MSH-induced c-fos expression in multiple regions of the hypothalamus during pregnancy compared with non-pregnant controls. In addition, we observed that the α-MSH-induced inhibition of firing rate in SON oxytocin neurons seen in non-pregnant animals was lost during pregnancy in the rat. These data suggest a widespread insensitivity to the actions of α-MSH in the hypothalamus during pregnancy. These studies provide further insight into the complex adaptive changes occurring in the maternal brain during pregnancy, altering the neural networks regulating appetite to favour positive energy balance.

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Additional information

Competing interests

None declared.

Author contributions

Experiments were conceived and designed by S.R.L., R.A.A., H.R.P., C.H.B. and D.R.G. Collection, analysis and interpretation of immunohistochemistry experiments was performed by S.R.L., E.S. and D.R.G.; collection, analysis and interpretation of mRNA experiments was performed by H.R.P., R.A.A. and D.R.G.; collection, analysis and interpretation of electrophysiological experiments was performed by C.H.B. and R.A.A. The manuscript was written by S.R.L., with critical revisions by D.R.G., C.H.B. and R.A.A.

Funding

Acknowledgements