



THE UNIVERSITY *of* EDINBURGH

Edinburgh Research Explorer

## **Prolactin maintains transient melaninconcentrating hormone expression in the medial preoptic area during established lactation**

**Citation for published version:**

Kokay, IC, Grattan, DR & Murray, JF 2020, 'Prolactin maintains transient melaninconcentrating hormone expression in the medial preoptic area during established lactation', *Journal of Neuroendocrinology*, vol. 32, no. 2, e12827. <https://doi.org/10.1111/jne.12827>

**Digital Object Identifier (DOI):**

[10.1111/jne.12827](https://doi.org/10.1111/jne.12827)

**Link:**

[Link to publication record in Edinburgh Research Explorer](#)

**Document Version:**

Peer reviewed version

**Published In:**

Journal of Neuroendocrinology

**General rights**

Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

**Take down policy**

The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact [openaccess@ed.ac.uk](mailto:openaccess@ed.ac.uk) providing details, and we will remove access to the work immediately and investigate your claim.



1 **TITLE:**

2 Prolactin maintains transient MCH expression in the mPOA during established  
3 lactation

4

5 **AUTHORS:**

6 IC Kokay<sup>1</sup>, DR Grattan<sup>1</sup>, JF Murray<sup>2</sup>

7 **ADDRESSES:**

8 1. Centre for Neuroendocrinology, Department of Anatomy, School of Biomedical  
9 Sciences, University of Otago, Dunedin, New Zealand

10 2. Faculty of Science and Technology, University of Westminster, London, UK and  
11 now Centre for Discovery Brain Science, University of Edinburgh, Edinburgh, UK.

12 Email: [jmurra19@exseed.ed.ac.uk](mailto:jmurra19@exseed.ed.ac.uk)

13

14 **KEYWORDS:** melanin concentrating hormone, prolactin, pSTAT5, medial preoptic  
15 area, late lactation

16

17 The authors of the manuscript have no conflicts of interest to declare.

18 The data that support the findings of this study are available from the corresponding  
19 author upon reasonable request.

20 **Abstract**

21 A population of neurones in the medial part of the medial preoptic area (mPOA)  
22 transiently express melanin concentrating hormone (MCH) in mid to late lactation in  
23 the rat, and this expression disappears on weaning. Prolactin is known to mediate  
24 many of the physiological adaptations that occur within the dam associated with  
25 lactation and the mPOA is well endowed with prolactin receptors (Prlr) hence we  
26 hypothesized that these transiently MCH-expressing cells may be regulated by  
27 prolactin. By *in situ* hybridization we show that approximately 60 % of the cells  
28 expressing prepro-MCH (*Pmch*) mRNA in the medial part of the mPOA on Day 19 of  
29 lactation also express *Prlr* mRNA. To demonstrate that these transiently MCH-  
30 expressing cells can acutely respond to prolactin, dams were treated with  
31 bromocriptine on the morning of Day 19 of lactation and then given vehicle or  
32 prolactin 4 h later. In the prolactin-treated animals, over 80 % of the MCH-  
33 immunopositive cells were also immunopositive for phosphorylated signal  
34 transducer and activator of transcription 5 (pSTAT5), an indicator of prolactin  
35 receptor activation: double immunopositive cells were rare in vehicle-treated  
36 animals. Finally, the effect of manipulating the circulating concentrations of  
37 prolactin on Days 17, 18 and 19 on the number of MCH-immunopositive cells on  
38 Day19 was determined. Reducing circulating concentrations of prolactin over Days  
39 17, 18 and 19 of lactation with or without a suckling stimulus resulted in a reduction  
40 ( $p < 0.05$ ) in the number of MCH-immunopositive cells in the medial part of the  
41 mPOA on Day 19 of lactation. Further research is required to determine the  
42 functional role(s) of these prolactin-activated transiently MCH-expressing neurones

43 however we suggest the most likely role involves adaptations in maternal

44 metabolism to support the final week of lactation.

45 *285 words*

46 **Introduction**

47 Melanin concentrating hormone (MCH) is a neuropeptide predominately expressed  
48 in the incerto-hypothalamus and lateral hypothalamus and has roles in a wide  
49 variety of physiological functions including energy balance and reproduction  
50 (reviewed by both: 1, 2). Additional expression of prepro-MCH (*Pmch*) in the medial  
51 part of the medial preoptic area (mPOA) and the paraventricular nucleus (PVN)  
52 during lactation was first described by Knollema and colleagues (3). In virgin or  
53 pregnant rats, *Pmch* expression is not detected in these regions (3, 4). The *Pmch*/  
54 MCH-immunopositive cells first appear in the medial part of the mPOA and PVN in  
55 mid-lactation (Days 8-14) in the rat and the highest number have been reported on  
56 Days 15-21 (3, 4). There is no MCH-immunopositive staining in these cell bodies  
57 after weaning (3). A decrease in the number of MCH-immunopositive cells within  
58 the mPOA between Days 15 and 21 of lactation independent of the suckling stimulus  
59 has been reported (5). However others have reported that the number of suckling  
60 pups was positively correlated with the number of MCH-immunopositive cells within  
61 the mPOA on Days 12, 15 and 19 of lactation and that the number of cells increased  
62 as lactation progressed (6).

63

64 The mPOA of the lactating dam is associated with changes in maternal behaviour (7,  
65 8, 9; reviewed by 10, 11, 12, 13). To date however the emphasis has been on  
66 understanding the role of the mPOA in establishing both maternal behaviours and  
67 maternal physiological responses to the initiation and maintenance of early  
68 lactation. There is a paucity of research investigating the period of late lactation

69 even though it is recognised that there are changes in maternal behaviour (14) as  
70 well as changes in both the dam's food intake and body weight (15) and, if she is not  
71 already pregnant, the activity of her reproductive axis in anticipation of oestrus  
72 following weaning (16). The function of the cells transiently expressing MCH in the  
73 medial part of the mPOA are unknown but it is not unreasonable to speculate that  
74 they may be involved in one or more of these changes in maternal behaviour and  
75 physiology in late lactation. The role of prolactin in stimulating the initiation of  
76 maternal behaviours during lactation is well established (reviewed by 10, 13, 17).  
77 The mPOA is a brain region rich in prolactin receptors (Prlr: 13, 18, 19, 20, 21) and  
78 the number of receptors increases with lactation (22, 23). Prolactin concentrations  
79 remain high through lactation, stimulated by the suckling stimulus (25, 26). We  
80 therefore hypothesized that the transient expression of MCH in the medial part of  
81 the mPOA in late lactation is regulated by prolactin.

82

83 Whilst transient expression of MCH has been reported in cells in both the mPOA and  
84 the PVN, in the present study we have exclusively investigated the population found  
85 in the medial part of the mPOA. The first aim of this study, was to determine if *Pmch*  
86 and *Prlr* co-express in the mPOA on Day 19 of lactation. Having demonstrated co-  
87 expression, the second aim was to demonstrate if these MCH-immunopositive cells  
88 on Day 19 of lactation would acutely respond to prolactin by expressing  
89 immunoreactive phosphorylated signal transducer and activator of transcription 5  
90 (pSTAT5), an indicator of prolactin receptor activation. The majority of the MCH-  
91 immunopositive cells in the mPOA did acutely respond to prolactin. Finally, the

92 effect of reducing circulating concentrations of prolactin in late lactation on the  
93 number of MCH-immunopositive cells within the medial part of the mPOA on Day 19  
94 of lactation was determined. Two methods of reducing circulating prolactin  
95 concentrations were employed to control for any suckling stimulus effects. Lactating  
96 rats suckling 8 pups were administered with a dopaminergic agonist, bromocriptine,  
97 to inhibit prolactin release on Days 17, 18 and 19. The suckling stimulus was  
98 maintained in these animals through continuous cross-fostering. Another group of  
99 lactating rats suckling 8 pups had their pups removed on Day 17 resulting in both a  
100 decrease in prolactin release and the removal of the suckling stimulus.

101

## 102 **Methods**

### 103 *Animals and experimental treatments*

104 Female Sprague-Dawley rats aged 10 weeks were obtained from the Hercus Taieri  
105 Research Facility at the University of Otago. Animals were group-housed (n=6 per  
106 cage), unless stated otherwise, and maintained under a 14:10 h light:dark cycle with  
107 an ambient temperature of  $22 \pm 1^\circ\text{C}$ . Food and water were available *ad libitum*  
108 throughout the duration of the experiments. All experimental procedures were  
109 approved by the University of Otago Animal Ethics Committee.

110

111 To generate lactating rats, the stage of the oestrous cycle was monitored daily by  
112 collection of vaginal smears and on the day of a positive proestrous smear, individual  
113 rats were housed overnight with a male and mating confirmed by the presence of

114 spermatozoa in the vaginal smear the following morning. Pregnant females were  
115 individually housed from around day 16 of pregnancy. On Day 2 postpartum (day of  
116 birth=Day 0 postpartum), litters were normalized to 8 pups each and then circulating  
117 prolactin concentrations were manipulated on Days 17-19 of established lactation as  
118 described next.

119

120 One group of lactating rats (vehicle plus suckling, n=6) received vehicle (250 µl saline  
121 in 10% ethanol) sub-cutaneously at 8 am and 6 pm on Days 17 and 18 of lactation  
122 and at 8 am on Day 19 of lactation. Pups were cross-fostered every 12 h. Two hours  
123 following the last vehicle injection, rat dams were deeply anaesthetized with sodium  
124 pentobarbitone (300 mg/kg) and transcardially perfused with 50 ml of ice-cold saline  
125 followed by 250 ml of 4 % paraformaldehyde in 0.1M phosphate buffer (pH 7.4). A  
126 second group of lactating rats (bromocriptine plus suckling, n=6) received 500 µg of  
127 bromocriptine (500 µg/250 µl saline in 10% ethanol) sub-cutaneously at 8 am and 6  
128 pm on Days 17 and 18 of lactation and at 8 am on Day 19 of lactation to suppress  
129 production of endogenous prolactin. Pups were cross-fostered every 12 h to ensure  
130 pups were fed, enabling maintenance of the suckling-stimulus despite inhibition of  
131 milk synthesis in bromocriptine-treated dams. No differences in the number of pups  
132 latched to the nipples or the strength of that latching were noted every 12 h when  
133 the pups were removed and replaced from either the vehicle- or bromocriptine-  
134 treated dams every 12 h. In between cross-fostering time points, no behavioural  
135 changes between the pups suckling the bromocriptine-treated dams and those not  
136 treated with bromocriptine were noted. Rats were perfused 2 h following the final



137 bromocriptine injection. A third group of rats (vehicle and no suckling, n=4) received  
138 vehicle injections as above on lactation days 17 and 18 and 19 but on day 17 of  
139 lactation the pups were removed. Rats were perfused 2 h after the final vehicle  
140 injection on day 19.

141

142 Finally, to examine the acute response to prolactin, a group of lactating rats (n=9)  
143 were injected with bromocriptine at 8 am on day 19 of lactation only as described  
144 above. Four hours later these same animals were injected intra-peritoneally with  
145 either 1 mg/kg body weight ovine prolactin (n=6; Sigma, St. Louis MO) dissolved in  
146 sterile saline or given vehicle alone (n=3). Rats were anaesthetised 45 min later and  
147 perfused as described above. Sections from these rats were processed to determine  
148 the number of MCH-immunopositive cells that were also immunopositive for  
149 pSTAT5.

150

151 Following transcardial perfusion, all brains were removed, post-fixed overnight in  
152 the same fixative solution, then cryoprotected in a 30% sucrose solution in 0.1 M  
153 phosphate buffer till the brains sank. Brains were then frozen on powdered dry ice  
154 and stored at -80°C until sectioned using a cryostat. A series of coronal sections  
155 (alternatively, 16 µm thick for *in situ* hybridization and 30 µm thick for free-floating  
156 immunohistochemistry) were cut through the mPOA from approximately bregma -  
157 0.24 mm to bregma -1.32 mm. An additional series of 16 µm and 30 µm thick  
158 sections were cut at the level of the incerto-hypothalamic and lateral hypothalamic  
159 areas (approximately bregma -1.30 mm to bregma -3.24 mm). Sections for *in situ*

160 hybridization were mounted onto APS-coated slides and stored at -20°C with  
161 dessicant. Sections for immunohistochemistry were collected into cryoprotectant  
162 solution in 12-well plates and stored at -20°C.

163

164 *Double-label in situ hybridization for Pmch and the long form of the prolactin*

165 *receptor (Prlr) mRNA*

166 Preliminary single label *in situ* hybridization experiments comparing the distribution  
167 and abundance of *Pmch*-mRNA expressing cells detected using isotopically labelled  
168 probes with the distribution detected using non-radioactively labelled probes  
169 (digoxigenin), established that with our protocol digoxigenin-labelled probes were  
170 sufficiently sensitive and specific to label *Pmch* mRNA in the mPOA. To  
171 simultaneously detect mRNA for both *Pmch* and the long form of the prolactin  
172 receptor (*Prlr*) in tissue sections, double-label *in situ* hybridizations were performed.  
173 Template cDNA was prepared by PCR using primer pairs designed from GenBank  
174 (Bethesda, MD) mRNA sequences for pro-melanin-concentrating hormone (*Pmch*:  
175 Accession number NM-012625.1, nucleotides 294-527) and the long form of the  
176 prolactin receptor (*Prlr*: Accession number NM\_001034111, nucleotides 1344-1644).  
177 T7 and SP6 promotor sequences were incorporated onto the ends of the primer  
178 sequences and the resulting cDNA templates then used to directly transcribe RNA  
179 hybridization probes. The specificities of the cDNA templates were confirmed by  
180 Sanger sequencing. Antisense and sense probes were synthesized using a  
181 digoxigenin RNA-labeling kit for *Pmch* (Roche Diagnostics GmbH, Mannheim) and  
182 antisense and sense probes labelled with <sup>35</sup>S-UTP were generated for *Prlr* using an *in*

183 *vitro* transcription kit (Promega, Madison, WI). Unincorporated nucleotides were  
184 removed by running the probes through mini Quick spin RNA columns (Roche  
185 Diagnostics GmbH, Mannheim).

186

187 Sections containing either the mPOA or the incerto-hypothalamic and lateral  
188 hypothalamic areas from lactating animals either treated with bromocriptine or  
189 vehicle on Days 17, 18 and 19 were thawed for 5 min at 55°C, then immersed in 2 %  
190 paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 5 min, washed in sodium  
191 citrate buffer (SSC: 150 mM NaCl, 15 mM sodium citrate, pH 7.0), then permeablized  
192 with proteinase K (2 µg/ml) followed by acetylation with 0.1M triethanolamine HCl  
193 (pH 8) containing 0.25% acetic anhydride for 10 min. Following washes with SSC,  
194 sections were subjected to a series of graded alcohol/chloroform steps, before being  
195 air-dried for 2-3 h. A total volume of 90 µl hybridization buffer (100 mM DDT, 0.3 M  
196 NaCl, 20 mM Tris pH 8, 5 mM EDTA, 1 X Denhardt's solution, 10 % dextran sulphate,  
197 50 % formamide) containing 25 ng of digoxigenin-labeled *Pmch* antisense or sense  
198 probe/100 µl of hybridization buffer and approximately 833,300 cpm of <sup>35</sup>S-labelled  
199 *Prlr* antisense or sense probes/100 µl of hybridization buffer were applied to each  
200 slide and the slides coverslipped with Hybrislips.

201

202 Hybridizations were carried out overnight at 55°C then slides were washed with SCC  
203 buffer (all post-hybridization SSC washes also had 10 mM B-mercaptoethanol and 1  
204 mM EDTA added to the solution) and treated with Ribonuclease A (20 µg/l) for 30

205 min at room. Following additional SSC washes (most stringent wash, 0.1 X SSC at 64  
206 °C for 2 h), sections were washed in a solution of 100 mM Tris/150mM NaCl (pH 7.5)  
207 and then incubated for 48 h with anti-digoxigenin antibody conjugated to alkaline  
208 phosphatase (diluted 1:2000). Sections were washed 3 times, then incubated in  
209 levamisole (1 mg/ml) solution. The digoxigenin-labeled probes were detected by  
210 incubation with NBT/BCIP (nitroblue tetrazolium chloride/5-bromo-4 chloro-3-  
211 indolyl-phosphate) substrate for 24 h followed by four, consecutive 30 min washes in  
212 buffer (150 mM NaCl, 10 mM NaH<sub>2</sub>PO<sub>4</sub>, 1 mM EDTA) to eliminate residual NBT and  
213 BCIP. Sections were then dipped briefly in distilled water followed by 70% ethanol  
214 and dried at RT. Slides were exposed to scientific imaging film for 7 days to generate  
215 autoradiograms and subsequently coated with LM-1 Hypercoat emulsion (Amersham  
216 Biosciences), placed in light-proof slide boxes containing desiccant and stored at 4°C  
217 for 5 weeks. Slides were developed in Kodak D19, fixed with Ilford Hypan,  
218 dehydrated through graded ethanols and cleared in xylene before coverslipping with  
219 VectaMount™ mounting medium.

220

### 221 *Double-label immunohistochemistry for MCH and pSTAT5*

222 Sections taken from the mPOA and the incerto-hypothalamic and lateral  
223 hypothalamic areas of the lactating animals given bromocriptine and prolactin or  
224 vehicle on Day 19 only were dual-labelled for MCH and pSTAT5. Sections were  
225 treated as for single-label immunohistochemistry (described below) with the  
226 addition of an antigen-retrieval step (for pSTAT5: 27): sections were incubated with  
227 0.01M Tris (pH10) for 10 min at 90°C immediately following 6 x washes in 0.05M TBS

228 to remove cryoprotectant. Sections were then incubated in blocking solution (0.05 M  
229 TBS, 0.25% Triton-X-100, 2 % BSA) for 1 h and endogenous peroxidases quenched in  
230 a 1% hydrogen peroxide/ 40% methanol solution for 10 min. After 3 x 10 min washes  
231 the tissue was incubated for 48 h at 4 °C in blocking solution containing 1.5% normal  
232 goat serum and rabbit anti-pSTAT5 (Tyr694, Cell Signalling Technology, Beverly, MA;  
233 RRID:AB\_2315225) at a 1:2,000 dilution. After further rinses, and incubation in  
234 biotinylated secondary goat anti-rabbit antibody (Vector, BA-1000;  
235 RRID:AB\_2313606) diluted 1:500 for 90 min at RT, sections were incubated for 1 h at  
236 RT in avidin-biotin-complex solution (ABC Elite kit; Vector Laboratories, Burlingame,  
237 CA, USA). Staining for pSTAT5 was then visualized using nickel-enhanced 3-3'-  
238 diaminobenzidine solution catalyzed with glucose oxidase to produce black nuclear  
239 staining. This step was followed by a second peroxidase activity quenching step  
240 before incubating tissue in blocking solution containing 1.5% normal goat serum and  
241 anti-MCH (M8440, Sigma-Aldrich; RRID:AB\_260690) diluted 1:80,000 for 24-48 h at  
242 4°C. After a TBS wash, sections were incubated with secondary antibody horseradish  
243 peroxidase-conjugated goat anti-rabbit IgG (DAKO) at 1:400 dilution for 90 min.  
244 Sections were then reacted with non-nickel-enhanced 3-3'-diaminobenzidine  
245 solution resulting in brown cytoplasmic staining of MCH-immunopositive cells.  
246 Sections were mounted onto slides and processed as for single label  
247 immunohistochemistry (below). In addition, each run included negative control  
248 sections in which the primary antibody was omitted from the wells. No staining was  
249 observed in these sections.

250

251 *Single-label immunohistochemistry for MCH*

252 To identify the number of MCH-immunopositive cells in the mPOA following  
253 manipulation of prolactin release, free-floating sections containing either the mPOA  
254 or the incerto-hypothalamic and lateral hypothalamic area were washed 6 x in 0.05  
255 M TBS (50 mM Tris, 150 mM NaCl, pH 7.6) to remove cryoprotectant, followed by  
256 incubation in blocking solution (0.05 M TBS, 0.25% Triton-X-100, 2 % BSA) for 1 h.  
257 Endogenous peroxidases were quenched in a 1% hydrogen peroxide/ 40% methanol  
258 solution for 10 mins, washed again then incubated for 48 h at 4°C in blocking  
259 solution containing 1.5% normal goat serum and anti-MCH (M8440, Sigma-Aldrich;  
260 RRID:AB\_260690) at a 1:80,000 dilution. Following further washes in 0.05M TBS, the  
261 tissue was incubated for 2-3 h with biotinylated secondary goat anti-rabbit antibody  
262 diluted 1:500. Next, sections were incubated with avidin-biotin-complex (ABC Elite  
263 kit; Vector Laboratories, Burlingame, CA, USA) for 1 h at RT. Finally, MCH  
264 immunoreactively was visualized by incubation in nickel-enhanced 3-3'-  
265 diaminobenzidine solution catalyzed with glucose oxidase to produce black  
266 cytoplasmic staining. Sections were then mounted onto gelatin-coated slides, dried  
267 overnight before dehydration in a series of graded alcohols, cleared in xylene and  
268 coverslipped under DPX-mounting medium.

269

270 *Analyses*

271 The total number of *Pmch* mRNA-expressing cells and the number of *Pmch* mRNA-  
272 expressing cells that co-expressed *Prlr* mRNA within the mPOA on both sides of the

273 third ventricle were analysed in two sections per animal (n=4 vehicle-treated  
274 animals; n=4 bromocriptine-treated animals). Sections were photographed under  
275 brightfield illumination at 400 x magnification. *Pmch* mRNA-expressing cells were  
276 identified by the presence of purple/red cytoplasmic staining. To identify double-  
277 labelled cells, the number of silver grains (representing *Prlr* mRNA) overlying each  
278 *Pmch* mRNA-expressing cell within the mPOA, was quantified using the particle  
279 counting function of ImageJ software. For each section, five background  
280 measurements of silver grain densities also were made over adjacent areas of the  
281 section. Cells were considered positively labeled for *Prlr* mRNA if the signal to  
282 background ratio was greater than 3 times mean background values. In sections of  
283 the incerto-hypothalamic and lateral hypothalamic areas, no cells were double-  
284 labelled for *Pmch* mRNA and *Prlr* mRNA, therefore, cell numbers were not quantified  
285 in these areas.

286

287 For the analysis of co-localization of MCH-immunopositive cells activated by pSTAT5,  
288 digital images were captured under brightfield at 200 X magnification using an  
289 Olympus AX70 microscope and QImaging Micropublisher digital camera. The total  
290 number of MCH-immunopositive cells and the total number of MCH-immunopositive  
291 cells displaying clear nuclear pSTAT5 staining were counted in 3 sections  
292 (systematically selected since the anatomical distribution of the MCH-  
293 immunopositive cells is very consistent and first cells appear at the same rostro-  
294 caudal level) of the mPOA per animal (n=6 prolactin-treated animals; n=3 vehicle-  
295 treated animals). The region of the mPOA was outlined and the area overlaid with a

296 grid using ImageJ. The number of single-labelled and double-labelled cells within  
297 each square of the grid that fell within the area of the mPOA were then identified,  
298 categorized and counted. Only cells that clearly contained brown cytoplasm (MCH-  
299 positive cells), or both brown cytoplasmic staining and a distinct black nucleus  
300 (double-labelled cells) were counted. Only the brightness and contrast of the images  
301 were adjusted. As there was no evidence of double-labelled MCH- and pSTAT5-  
302 immunopositive cells in the incerto-hypothalamic and lateral hypothalamic areas  
303 sections, cell numbers were not quantified in these sections. Data are presented as  
304 the mean percentage of double labelled cells of all the MCH-immunopositive cells  
305 per rat. Experimental differences were analysed by Student's t-test.

306

307 For the analysis of the single-label immunohistochemistry, sections were imaged  
308 as for dual-label immunohistochemistry. The total number of MCH-immunopositive  
309 cells present within the boundaries of the mPOA on both sides of the third ventricle  
310 of 3-4 sections per animal were counted. Data are presented as mean number of  
311 immunopositive cells counted per rat  $\pm$  SEM. Differences between groups were  
312 analysed by one way analysis of variance followed by Tukey-Kramer *post-hoc*  
313 analysis.

314

315 All analyses were performed using GraphPad Prism Software and differences  
316 between groups were considered significant if  $p < 0.05$ .

317



318 **Results**

319 Prepro-MCH (*Pmch*) mRNA was detected in cells within the medial part of the mPOA,  
320 as well as in a few cells located more medially to these within the periventricular  
321 nucleus, of the hypothalamus of the rat dam on day 19 of lactation (Figure 1a).  
322 Double-label *in situ* hybridization for both *Pmch* and *Prlr* mRNA demonstrated co-  
323 localization of the two transcripts in some but not all *Pmch* mRNA-positive cells  
324 (Figure 1b). When rats were administered with a dopaminergic agonist,  
325 bromocriptine, on Days 17, 18 and 19, the number of *Pmch* mRNA-positive cells on  
326 Day 19 was markedly decreased compared to vehicle-treated animals (Figure 1c;  $p <$   
327 0.05). In both vehicle- and bromocriptine-treated animals, although the total  
328 number of positive cells was different, the proportion of *Pmch* mRNA positive cells  
329 co-labelled with *Prlr* mRNA was similar (Figure 1d: 61% of vehicle-treated group  
330 versus 60% of bromocriptine-treated group). Within the medial mPOA, there were  
331 also many cells that were *Prlr* mRNA-positive but negative for *Pmch* mRNA: it was  
332 however not possible to robustly quantify these cells. Many cells in sections  
333 containing the incerto-hypothalamic and lateral hypothalamic areas strongly  
334 expressed mRNA for *Pmch* but no co-expression with *Prlr* mRNA was observed (see  
335 Figure 1e). No positive labelling was detected in negative control sections incubated  
336 with sense probes (data not shown).

337

338 To determine if the MCH-immunopositive cells found in the mPOA on Day 19 of  
339 lactation were acutely responsive to prolactin, a group of lactating rats were treated  
340 with bromocriptine on the morning of Day 19 only. Four hours later these rats

341 received either prolactin or vehicle and 45 min later were killed. By dual-label  
342 immunohistochemistry, sections from these animals were stained for both MCH  
343 (DAB: brown cytoplasmic staining) and pSTAT5 (nickel-enhanced DAB: black nuclear  
344 staining). Within the medial part of the mPOA, cells were identified that were either  
345 MCH-immunopositive, both MCH and pSTAT5 immunopositive as well as pSTAT5  
346 immunopositive alone (Figure 2a, b and c). Of all the MCH-immunopositive cells, the  
347 majority (87.3 %) were also immunopositive for pSTAT5 after the animals were  
348 treated with prolactin (Figure 2d). When animals were treated with vehicle, the  
349 identification of both MCH- and pSTAT5-immunopositive cells was low because of  
350 their rarity within the mPOA (Figure 2d). Within the incerto-hypothalamic and the  
351 lateral hypothalamic areas, no cells were identified that were both MCH- and  
352 pSTAT5-immunopositive (Figure 2e and f).

353

354 MCH-immunopositive cells were found in the mPOA on Day 19 of lactation (Figure  
355 3a) and the distribution of the immunopositive cells was very similar to that of cells  
356 expressing *Pmch* (Figure 1a). Immunopositive staining for MCH was confined to the  
357 cytoplasm of cells (Figure 3a). In the absence of prolactin, induced either by  
358 administering bromocriptine on Days 17, 18 and 19 or by withdrawing pups on Day  
359 17, the number of immunopositive cells was markedly reduced on Day 19 (Figure 3b,  
360 c and d: 1 way ANOVA,  $p < 0.05$ ).

361

362 **Discussion**

363 By both *in situ* hybridization and immunohistochemistry, we have confirmed the  
364 induction of MCH expression in a specific population of cells within the medial part  
365 of the mPOA in established lactation. For the first time we have demonstrated that  
366 the majority of these MCH-positive cells also express the long form of the prolactin  
367 receptor, and are prolactin responsive during lactation, as indicated by prolactin-  
368 induced expression of pSTAT5. Finally, we have shown that suppression of prolactin  
369 secretion during established lactation markedly reduced expression of MCH, even in  
370 the presence of the ongoing suckling stimulus. These data demonstrate that  
371 expression of MCH in the medial part of the mPOA during lactation is dependent on  
372 prolactin action.

373

374 As others before, we have confirmed that some cells within the medial part of the  
375 mPOA express *Pmch*/MCH on day 19 of lactation (3, 4, 5, 6). We hypothesized that  
376 this transient expression of MCH in the mPOA in late lactation is regulated by  
377 prolactin. To exert its biological effects, prolactin binds to the long form of the  
378 prolactin receptor (*Prlr*). Expression of the long form of the receptor has been  
379 previously demonstrated in the mPOA of both pregnant and early lactating rats (18,  
380 22, 28). Using double *in situ* hybridization, we have now demonstrated that the long  
381 form of the *Prlr* is also expressed in the mPOA of the late lactating rat and that a sub-  
382 population of these *Prlr* mRNA-positive cells also express *Pmch*.

383

384 When prolactin binds to the long form of the Prlr the JAK/STAT intracellular  
385 signalling pathway is activated and as a result STAT5 is phosphorylated.  
386 Phosphorylated STAT5 (pSTAT5) acts a transcription factor to elicit the biological  
387 effects of prolactin (29). The detection of immunopositive pSTAT5 is used as a  
388 surrogate marker of the long form of the Prlr because the available antibodies to the  
389 long form of the Prlr are often not sensitive enough (30). Low numbers of cells  
390 immunopositive for pSTAT5 have previously been detected in the mPOA of virgin  
391 mice with the number increasing in both pregnant and early lactating mice (27, 31).  
392 We have now demonstrated that a significant proportion of the immunopositive  
393 MCH cells found in the mPOA are also immunopositive for pSTAT5 in response to  
394 prolactin administration, but not vehicle, four hours after treatment with  
395 bromocriptine on the morning of Day 19 of lactation only. Hence, in late lactation a  
396 high proportion of the transiently-expressing MCH cells in the mPOA are being  
397 activated by prolactin.

398

399 The pattern and number of *Pmch*/ MCH-immunopositive cells has been studied  
400 during lactation in both the incerto-hypothalamus and lateral hypothalamic areas, by  
401 several groups, but usually as a single entity rather than as two distinct areas. It has  
402 been reported as being decreased (32), unchanged (3, 4, 5) or increased by lactation  
403 (33). In our study we noted no alteration in pattern of MCH-immunopositive cells in  
404 the incerto-hypothalamus and found no MCH-immunopositive cells co-localized with  
405 pSTAT5 suggesting that prolactin had not acted on any of these cells.

406

407 Expression of MCH in cells within the medial part of the mPOA in late lactation  
408 requires prolactin, but not suckling *per se*, and we have demonstrated this in two  
409 ways. On Day 19 of lactation, the number of MCH-immunopositive cells was  
410 reduced after prolactin release was inhibited either by treatment with bromocriptine  
411 on Days 17, 18 and 19 or pup withdrawal on Day 17 compared with the vehicle  
412 treated controls; that is, with or without the maintenance of a suckling stimulus,  
413 respectively. We have demonstrated that these MCH-immunopositive cells are also  
414 pSTAT5 immunopositive and hence prolactin activated. Others have demonstrated  
415 that the number of suckling pups in late lactation determines the number of cell  
416 bodies within the medial part of the mPOA expressing MCH (6). This would suggest  
417 that neuronal stimulation may stimulate transient MCH expression however it is  
418 likely that this is an indirect effect. Alvisi and colleagues (5) found no co-localization  
419 of suckling-induced c-Fos expression with MCH in the medial part of the mPOA on  
420 days 15 to 21 of lactation and central administration of prolactin does not induce c-  
421 Fos expression in the mPOA of rats (24). There were still some cells in the mPOA  
422 transiently expressing MCH after the bromocriptine treatment or pup withdrawal  
423 suggesting that either a yet to be identified factor is also involved in their  
424 maintenance or that the time period of the withdrawal of maintenance was  
425 insufficient to extinguish all expression. Based on our data it would appear therefore  
426 that the suckling stimulus *per se* is not maintaining the expression of the MCH but  
427 rather that it is the suckling-induced release of prolactin that is responsible (25).  
428

429 Although we have demonstrated that prolactin is involved in the maintenance of  
430 these transiently expressing MCH cells within the mPOA in late lactation, it is unlikely  
431 that prolactin initiates this transient expression. Others have reported that the  
432 transiently expressing MCH cell bodies first appear on Days 8 to 12 of lactation (3, 4).  
433 At this time circulating concentrations of prolactin, albeit not as high as in the early  
434 days of lactation, are still very high compared to a non-lactating female (26). It is  
435 possible that progesterone may be the primary initiating signal. The secretion of  
436 progesterone from the corpora lutea of lactation has reached a maximum by Day 8  
437 and started to decline at Day 12 (35). There are progesterone receptors in the  
438 mPOA at this time: one group reports that the number of oestrogen-induced  
439 progesterone receptors in the mPOA appear to increase in response to increased  
440 circulating oestrogen concentrations in late lactation (day 15 compared to day 20  
441 post-partum) (36) whilst another reports that the number of progesterone receptors  
442 in the mPOA appears to have stabilized at pre-pregnancy numbers by Day 7 of  
443 lactation (34). Progesterone induces changes in maternal behaviour in the second  
444 half of lactation (34) and these effects may be mediated by the transiently  
445 expressing MCH cells since administration of MCH into the mPOA in early lactation  
446 suppresses maternal behaviours (48). Another possible trigger may be the mid-  
447 lactation decrease in circulating leptin concentrations (37, 38, 39; reviewed by 40).  
448 Leptin receptors have been detected in the mPOA of female non-pregnant, non-  
449 lactating mice and rats (41, 42). In the *ob/ob* mouse, MCH expression within the  
450 incerto-hypothalamic and lateral hypothalamic areas is increased (43) hence the  
451 mid-lactation nadir in leptin may be responsible for up-regulating MCH expression in  
452 the medial part of the mPOA and then prolactin maintains expression. It is also

453 possible that another as yet unidentified trigger could be responsible for initiating  
454 the transient expression of MCH in these cells within the mPOA hence further  
455 research is required.

456

457 The role of the MCH-expressing cells within the medial part of the mPOA remains  
458 uncertain, and we have not directly addressed this in the present study. As they are  
459 maintained by prolactin, then it can be assumed that they are mediating some of the  
460 known roles of prolactin in late lactation. In late lactation, the possible roles for the  
461 transient prolactin-induced expression of MCH in the mPOA fall broadly into three  
462 areas: adaptive reduction of maternal behaviour preceding weaning; regulation of  
463 the dam's return to reproductive activity; and modulation of maternal energy  
464 homeostasis.

465

466 A role for MCH in stimulating maternal behaviours (nest building, pup retrieval and  
467 maternal aggression) in early lactation has been suggested (44, 45), but such effects  
468 precede the transient expression of MCH in the medial part of the mPOA and are  
469 likely to involve incerto-hypothalamic MCH neurones. In late lactation, the dam's  
470 behaviour to her pups changes as she begins to encourage their increasing  
471 independence as weaning approaches. These changes in behaviour include a  
472 reduction in pup retrieving and nest building (14) as well as a reduction in the dam's  
473 aggression in response to intrusion by strange male conspecifics (46, 47). In contrast  
474 to the stimulatory actions of incerto-hypothalamic MCH neurones, MCH  
475 administration into the mPOA of dams inhibits the normal pup retrieval and nest

476 building behaviours of early lactation, hence mimicking the behaviours seen in late  
477 lactation (48). Prolactin is thought to be involved in the onset of maternal behavior  
478 at birth, but is not required for ongoing expression of maternal behaviour. Whether  
479 there is an active role for prolactin, through its action on the transient MCH-  
480 expressing cells of the medial mPOA, in termination of maternal behavior during late  
481 lactation has not been evaluated.

482

483 It has long been proposed that in late lactation, lactational anoestrus is primarily  
484 maintained by prolactin (49). The mPOA is a site of kisspeptin and GnRH cell bodies  
485 that are known to be activated as part of the LH surge (50, 51, 52). Bilateral  
486 administration of MCH into the mPOA of ovariectomized, oestradiol benzoate-  
487 primed rats results in the generation of LH surge-like release of LH (53, 54, 55). It is  
488 therefore possible that the transiently expressing MCH cells are involved in  
489 stimulating the LH surge that occurs on Day 20 post-partum in the absence of post-  
490 partum mating and in the presence of lactation (16). As for the maternal behaviour  
491 effect, it is difficult though to conceive a role for prolactin in the regulation of the  
492 dam's return to reproductive activity given the well established inhibitory effects of  
493 prolactin on the hypothalamic-pituitary-gonadal axis. Recent data suggests that the  
494 inhibitory actions of hyperprolactinemia on the hypothalamic-pituitary-gonadal axis  
495 are exerted through the kisspeptin neurones of the arcuate (56) and therefore  
496 further work is required to understand prolactin's role(s) in other hypothalamic  
497 areas.

498



499 Both incerto-hypothalamic and lateral hypothalamic MCH play a significant role in  
500 stimulating food intake (43) and MCH expression in these areas are increased by  
501 fasting (57). It is possible that increased MCH in the mPOA during lactation could  
502 contribute to this orexigenic role. Towards the end of the lactation although the  
503 animals are not fasting, *per se*, they are in a state of negative energy balance as they  
504 utilize body reserves to maintain milk production. During the second half of  
505 lactation food consumption is higher (15, 58) and this appears to be regulated by  
506 prolactin, as bromocriptine treatment will reduce food intake while bromocriptine  
507 plus chronic intraventricular infusion of prolactin will restore food consumption to  
508 control amounts (59). The prolactin-activated MCH cell bodies in the medial part of  
509 the mPOA may have a role in increasing food intake. As suggested by Rondini and  
510 colleagues, it is also possible that other neuropeptides related to metabolic control  
511 and/or leptin may be responsible for the increased expression of MCH in the mPOA  
512 (4); that is, that the role of these transiently expressing MCH, prolactin activated  
513 cells in the face of the “increased energy drain” of late lactation is to stimulate  
514 feeding above the already increased levels seen earlier in lactation. Further research  
515 is required to determine if these transiently expressing MCH cell bodies have  
516 projections that extend to the third ventricle and participate in the recently  
517 described stimulation of feeding behaviour by MCH through cerebral ventricular  
518 volume transmission (60).

519

520 We have demonstrated that the majority of MCH-immunopositive cells that are  
521 transiently expressed in the medial part of the mPOA during late lactation express

522 the prolactin receptor and that the maintenance of MCH expression in these cells is  
523 dependent on prolactin action. Further research is required to determine the  
524 functional role(s) of these prolactin-activated transiently MCH-expressing cells  
525 however the most likely role would appear to involve adaptations in maternal  
526 metabolism to support the final week of lactation.

527

### 528 **Acknowledgements**

529 We thank Tony Sapsford and Amanda Wyatt for their excellent technical assistance.  
530 This work was supported by an HRC Programme Grant 14/568 (DRG), a Society for  
531 Reproduction and Fertility Academic Scholarship (JFM) and a British Society for  
532 Neuroendocrinology Research Visit Grant (JFM).

533 **Figure Legends**

534 Figure 1. *In situ* hybridization showing expression of *Pmch* mRNA and *Prlr* mRNA  
535 expressing neurones in sections from a rat dam treated with vehicle (on Days 17, 18  
536 and 19) and suckling pups on Day 19 of lactation (panels a, b and f) and a rat dam  
537 treated with bromocriptine (on Days 17, 18 and 19) and suckling pups on Day 19 of  
538 lactation (panel c): a. Low power dark-field photomicrograph showing the  
539 distribution of *Pmch* mRNA (white clusters of silver grains) in the mPOA; b.  
540 representative brightfield image of *Pmch* mRNA-expressing cells (purple/red  
541 cytoplasmic labeling) dual-labeled for prolactin receptor (*Prlr*) mRNA (black clusters  
542 of silver grains over cells). Top insert shows a high power image of the area outlined  
543 in the black box of a cell expressing *Pmch* mRNA only. Bottom insert shows a high  
544 power image of the area outlined in the black box of two positive dual-labelled cells;  
545 c. representative brightfield image of *Pmch* mRNA-expressing cells (purple/red  
546 cytoplasmic labeling) dual-labeled for prolactin receptor (*Prlr*) mRNA (black clusters  
547 of silver grains over cells). Insert shows a high power image of the area outlined in  
548 the black box of a positive dual-labelled cell; d. quantification of the mean ( $\pm$  SEM)  
549 total number of *Pmch* mRNA-expressing cells per rat in the mPOA of rat dams  
550 suckling pups on day 19 of lactation after receiving either vehicle (n=4 animals) or  
551 bromocriptine treatment (n=4 animals). \*p < 0.05; e. The proportion of *Pmch*-  
552 expressing neurones co-expressing *Prlr* mRNA in the mPOA on day 19 of lactation  
553 following vehicle or bromocriptine treatment; f. representative low power image of  
554 *Pmch* mRNA-expressing cells (purple/red cytoplasmic labeling) dual labelled for *Prlr*  
555 mRNA in the incerto-hypothalamic and lateral hypothalamic areas. In these regions,

556 no *Pmch* mRNA-expressing cells were observed to co-express *Prlr* mRNA. A high  
557 power image of two single-labelled *Pmch* mRNA-expressing cells is shown in the  
558 insert in e. Abbreviations, f, fornix; mPOA, medial preoptic area; och, optic chiasm;  
559 3V, third ventricle. Scale bars: (a) = 150  $\mu\text{m}$ ; (b and c) = 50  $\mu\text{m}$ , inserts in (b and c) =  
560 10  $\mu\text{m}$ ; (e) = 100  $\mu\text{m}$ , insert in (e) = 10  $\mu\text{m}$ .

561 Figure 2. Double label immunohistochemistry for melanin concentrating hormone  
562 (MCH: brown cytoplasmic staining) and phosphorylated Stat5 (pStat5: black nuclear  
563 staining) following prolactin administration, in the medial preoptic area (mPOA) of  
564 dams on Day 19 of lactation: a. representative image of the mPOA (scale bar: 50  
565  $\mu\text{m}$ ); b. and c. higher magnification of the boxed areas in (a) with examples of cells  
566 either immuno-positive for MCH (blue arrow) or pSTAT5 alone (orange arrow) or  
567 immuno-positive for both MCH and pSTAT5 (black arrow) (scale bar: 15  $\mu\text{m}$ ); d.  
568 quantification of the percentage ( $\pm$  SEM) of cell bodies in the mPOA that were co-  
569 labelled for MCH and pSTAT5 following administration of either prolactin or vehicle.  
570 \* $p < 0.05$ ; e. and f. representative images of (e) low and (f) high, magnification of the  
571 lateral hypothalamus of a rat dam on Day 19 of lactation following prolactin  
572 administration showing MCH immuno-labelling (brown cytoplasmic staining). No  
573 MCH-immunopositive cell bodies are also immunopositive for pSTAT5. Arrows  
574 indicate neurones single-labelled for MCH. Scale bars: (e) = 100  $\mu\text{m}$  and (f) = 30  $\mu\text{m}$ .  
575 Abbreviations, f, fornix; ic, internal capsule.

576

577 Figure 3. Representative images of cell bodies immuno-positive for melanin  
578 concentrating hormone (MCH) in the medial preoptic area of the rat dam on Day 19

579 of lactation in animals either with circulating concentrations of prolactin  
580 characteristic of lactation (vehicle) or treated to suppress prolactin release  
581 (bromocriptine or pup removal) on Days 17, 18 and 19 of lactation. a. Vehicle was  
582 administered on Days 17, 18 and 19 of lactation: insert shows examples of cells of  
583 interest at 600 fold magnification (indicated by black arrows); b. bromocriptine was  
584 administered on the mornings of Days 17, 18 and 19 of lactation; and c. pups were  
585 removed on the morning of Day 17 and then the dam received vehicle on Days 17,  
586 18 and 19 of lactation. All animals were perfused on the afternoon of Day 19 and  
587 the brains prepared for immunohistochemistry. All low power images taken at 40 x  
588 magnification. Scale bars: (a-c) = 500  $\mu\text{m}$  insert and insert in (a) = 5  $\mu\text{m}$ . mPOA,  
589 medial preoptic area; och, optic chiasm; 3v, third ventricle. d. Quantification of the  
590 number of cell bodies immuno-positive for MCH in the medial preoptic area (mPOA)  
591 of the rat dam on Day 19 of lactation in animals either with circulating  
592 concentrations of prolactin characteristic of lactation (a. vehicle, n = 6) or treated to  
593 suppress prolactin release (b. bromocriptine, n = 6 or c. pup removal, n = 4) on Days  
594 17, 18 and 19 of lactation. Data are presented as the mean  $\pm$  SEM. Differences  
595 between means were analysed by one-way ANOVA followed by Turkey-Kramer test,  
596 ( $F_{2,13} = 18.98$ ): vehicle versus prolactin withdrawal. Bars with different letters are  
597 significantly different ( $p < 0.05$ ).

598

599 **References**

- 600 1. Naufahu J, Cunliffe AD, Murray JF. The roles of melanin-concentrating hormone in  
601 energy balance and reproductive functions: are they connected? *Reproduction*  
602 2013; 146: R141-R150.
- 603 2. Diniz GB, Bittencourt JC. The melanin-concentrating hormone as an integrative  
604 peptide driving motivated behaviors. *Front Syst Neurosci* 2017; 11: 32.
- 605 3. Knollema S, Brown ER, Vale W, Sawchenko PE. Novel hypothalamic and preoptic  
606 sites of prepro-melanin-concentrating hormone messenger ribonucleic Acid  
607 and Peptide expression in lactating rats. *J Neuroendocrinol* 1992; 4: 709-717.
- 608 4. Rondini TA, Donato Jr J, Rodrigues Bde C, Bittencourt JC, Elias CF. Chemical  
609 identity and connections of medial preoptic area neurons expressing melanin-  
610 concentrating hormone during lactation. *J Chem Neuroanat* 2010; 39: 51-62.
- 611 5. Alvisi R, Diniz GB, Da-Silva JM, Bittencourt JC, Felicio LF. Suckling-induced *Fos*  
612 activation and melanin-concentrating hormone immunoreactivity during late  
613 lactation. *Life Sci* 2016; 148: 241-246
- 614 6. Ferreira JGP, Duarte JCG, Diniz GB, Bittencourt JC. Litter size determines the  
615 number of melanin-concentrating hormone neurons in the medial preoptic  
616 area of Sprague Dawley lactating dams. *Physiol Behav* 2017; 181: 75-79.
- 617 7. Numan M. (1974) Medial preoptic area and maternal behavior in the female rat.  
618 *J Compar Physiol Psychol* 1974; 87: 746-759.

- 619 8. Terkel J, Bridges RS, Sawyer CH. Effects of transecting lateral neural connections  
620 of the medial preoptic area on maternal behavior in the rat: nest building, pup  
621 retrieval and prolactin secretion. *Brain Res* 1979; 169: 369-380.
- 622 9. Numan M, Callahan EC. (1980) The connections of the medial preoptic region and  
623 maternal behaviour in the rat. *Physiol Behav* 1980; 25: 653-665.
- 624 10. Dobolyi A, Grattan DR, Stolzenberg DS. Preoptic inputs and mechanisms that  
625 regulate maternal responsiveness. *J Neuroendocrinol* 2014; 26: 627-640.
- 626 11. Bridges RS. Neuroendocrine regulation of maternal behaviour. *Front*  
627 *Neuroendocrinol* 2015; 36: 178-196.
- 628 12. Kohl J, Autry AE, Dulac C. The neurobiology of parenting: A neural circuit  
629 perspective. *Bioessays* 2017; 39: 1-11.
- 630 13. Brown RSE, Aoki M, Ladyman SR, Phillipps HR, Wyatt A, Boehm U, Grattan DR.  
631 Prolactin action in the medial preoptic area is necessary for postpartum  
632 maternal nursing behavior. *Proc Natl Acad Sci U S A* 2017; 114: 10779-10784.
- 633 14. Pereira M, Morrell JI. Changing role of the medial preoptic area in the regulation  
634 of maternal behaviour across the post-partum period: facilitation followed by  
635 inhibition. *Behav Brain Res* 2009; 205: 238-248.
- 636 15. Ota K, Yokoyama A. Body weight and food consumption of lactating rats: effects  
637 of ovariectomy and of arrest and resumption of suckling. *J Endocrinol* 1967;  
638 38: 251-261.

- 639 16. Woodside B. Effects of food restriction on the length of lactational diestrus in  
640 rats. *Horm Behav* 1991; 25: 70-83.
- 641 17. Grattan DR, Bridges RS. Prolactin actions in the brain. In: Pfaff DW, Etgen AM,  
642 Fahrbach SE, Rubin RT eds. *Hormones, brain and behaviour*. New York:  
643 Academic Press; 2009: 2471-2503.
- 644 18. Chui S, Wise PM. Prolactin receptor mRNA localization in the hypothalamus by in  
645 situ hybridization. *J Neuroendocrinol* 1994; 6: 191-199.
- 646 19. Bakowska JC, Morrell JI. (1997) Atlas of the neurons that express mRNA for the  
647 long form of the prolactin receptor in the forebrain of the female rat. *J Comp*  
648 *Neurol* 1997; 386: 161-177.
- 649 20. Pi X, Grattan DR. Distribution of prolactin receptor immunoreactivity in the brain  
650 of estrogen-treated, ovariectomized rats. *J Comp Neurol* 1998; 394: 462-474.
- 651 21. Kokay I, Wyatt A, Phillupps HR, Aoki M, Ectors F, Boehm U, Grattan DR. Analysis  
652 of prolactin receptor expression in the murine brain using a novel prolactin  
653 receptor reporter mouse. *J Neuroendocrinol* 2018; 30: e12634
- 654 22. Pi X, Grattan DR. Increased expression of both short and long forms of prolactin  
655 receptor mRNA in hypothalamic nuclei of lactating rats. *J Mol Endocrinol* 1999;  
656 23: 13-22.
- 657 23. Pi X, Grattan DR. Expression of prolactin receptor mRNA is increased in the  
658 preoptic area of lactating rats. *Endocrine* 1999; 11: 91-98.



- 659 24. Cave BJ, Wakerley JB, Luckman SM, Tortonese DJ. Hypothalamic targets for  
660 prolactin: assessment of c-Fos induction in tyrosine hydroxylase- and  
661 proopiomelanocortin-containing neurones in the rat arcuate nucleus following  
662 acute central prolactin administration. *Neuroendocrinology* 2001; 74: 386-  
663 395.
- 664 25. Grosvenor CE, Mena F, Whitworth NS. The secretion rate of prolactin in the rat  
665 during suckling and its metabolic clearance rate after increasing intervals of  
666 nonsuckling. *Endocrinology* 1979; 104: 372-376.
- 667 26. Grattan DR. The actions of prolactin in the brain during pregnancy and lactation.  
668 *Prog Brain Res* 2001; 133: 153-171.
- 669 27. Brown RS, Kokay IC, Herbison AE, Grattan DR. Distribution of prolactin-  
670 responsive neurons in the mouse forebrain. *J Comp Neurol* 2010; 518: 92-102.
- 671 28. Augustine RA, Kokay IC, Andrews ZB, Ladyman SR, Grattan DR. Quantitation of  
672 prolactin receptor mRNA in the maternal rat brain during pregnancy and  
673 lactation. *J Mol Endocrinol* 2003; 31: 221-232.
- 674 29. Bole-feysot C, Goffin V, Edery M, Binart N, Kelly PA. Prolactin (PRL) and its  
675 receptor: actions, signal transduction pathways and phenotypes observed in  
676 PRL receptor knockout mice. *Endocr Rev* 1998; 19: 225-268.
- 677 30. Sapsford TJ, Kokay IC, Ostberg L, Bridges RS, Grattan DR. Differential sensitivity  
678 of specific neuronal populations of the rat hypothalamus to prolactin action. *J*  
679 *Comp Neurol* 2012; 520: 1062-1077.

- 680 31. Salais-López H, Lanuza E, Agustín-Pavón C, Martínez-García F. Tuning the brain  
681 for motherhood: prolactin-like central signalling in virgin, pregnant, and  
682 lactating female mice. *Brain Struct Funct* 2017; 222: 895-921.
- 683 32. García MC, López M, Gualillo O., Seoane LM, Diéguez C., Señarís RM.  
684 Hypothalamic levels of NPY, MCH, and prepro-orexin mRNA during pregnancy  
685 and lactation in the rat: role of prolactin. *FASEB J* 2003; 17: 1392-1400.
- 686 33. Sun G, Tian Z, Murata T, Narita K, Honda K, Higuchi T. Central and peripheral  
687 immunoreactivity of melanin-concentrating hormone in hypothalamic obese  
688 and lactating rats. *J Neuroendocrinol* 2004; 16: 79-83.
- 689 34. Grieb ZA, Tierney SM, Lonstein JS. Postpartum inhibition of ovarian steroid  
690 action increases aspects of maternal caregiving and reduces medial preoptic  
691 area progesterone receptor expression in female rats. *Horm Behav* 2017; 96:  
692 31-41.
- 693 35. Taya K, Greenwald GS. Peripheral blood and ovarian levels of sex steroids in the  
694 lactating rat. *Endocrinol Jpn* 1982; 29: 453-459.
- 695 36. Barbosa-Vargas E, Pfaus JG, Woodside B. Sexual behavior in lactating rats: Role  
696 of estrogen-induced progesterone receptors. *Horm Behav* 2009; 56: 246-253.
- 697 37. Brogan RS, Grove KL, Smith MS. Differential regulation of leptin receptor but not  
698 orexin in the hypothalamus of the lactating rat. *J Neuroendocrinol* 1997; 12:  
699 1077-1086.

- 700 38. Woodside B, Abizaid A, Jafferli S. Effect of acute food deprivation on lactational  
701 infertility in rats is reduced by leptin administration. *Am J Physiol* 1998; 274  
702 (Regulatory Integrative Comp Physiol 43): R1653–R1658.
- 703 39. Woodside B, Abizaid A, Walker CD. (2000) Changes in leptin levels during  
704 lactation: implications for lactational hyperphagia and anovulation. *Horm*  
705 *Behav* 2000; 37: 353-365.
- 706 40. Woodside B. Mood, food, and fertility: Adaptations of the maternal brain.  
707 *Compr Physiol* 2016; 6: 1493-1518.
- 708 41. Diano S, Kalra SP, Sakamoto H, Horvath TL. Leptin receptors in estrogen  
709 receptor-containing neurons of the female rat hypothalamus. *Brain Res* 1998;  
710 812: 256-259.
- 711 42. Zhang Y, Kerman IA, Laque A, Nguyen P, Faouzi M, Louis GW, Jones JC, Rhodes C,  
712 Münzberg H. Leptin-receptor-expressing neurons in the dorsomedial  
713 hypothalamus and median preoptic area regulate sympathetic brown adipose  
714 tissue circuits. *J Neurosci* 2011; 31: 1873-1884.
- 715 43. Qu D, Ludwig DS, Gammeltoft S, Piper M, Pelleymounter MA, Cullen MJ, Mathes  
716 WF, Przypek R, Kanarek R, Maratos-Flier E. A role for melanin-concentrating  
717 hormone in the central regulation of feeding behaviour. *Nature* 1996; 380:  
718 243-247.
- 719 44. Adams AC, Domouzoglou EM, Chee MJ, Segal-Lieberman G, Pissios P, Maratos-  
720 Flier E. Ablation of the hypothalamic neuropeptide melanin concentrating

721 hormone is associated with behavioral abnormalities that reflect impaired  
722 olfactory integration. Behav Brain Res 2011; 224: 195-200.

723 45. Alachkar A, Alhassen L, Wang Z, Wang L, Onouye K, Sanathara N, Civelli O.  
724 Inactivation of the melanin concentrating hormone system impairs maternal  
725 behaviour. Eur Neuropsychopharmacol 2016; 26: 1826-1835.

726 46. Flannelly KJ, Flannelly L. Time course of postpartum aggression in rats (*Rattus*  
727 *norvegicus*). J Comp Psychol 1987; 101: 101-103.

728 47. Mayer AD, Reisbick S, Siegel HI, Rosenblatt JS. Maternal aggression in rats:  
729 changes over pregnancy and lactation in a Sprague-Dawley strain. Aggress  
730 Behav 1987; 13: 29-43.

731 48. Benedetto L, Pereira M, Ferreira A, Torerolo P. Melanin-concentrating hormone  
732 in the medial preoptic area reduces active components of maternal behavior in  
733 rats. Peptides 2014; 58: 20-25.

734 49. Hansen S, Sodersten P, Eneroth P. Mechanisms regulating hormone release and  
735 the duration of dioestrus in the lactating rat. J Endocrinol 1983; 99: 173-180.

736 50. Lee WS, Smith MS, Hoffman GE. Luteinizing hormone-releasing hormone  
737 neurons express Fos protein during the proestrous surge of luteinizing  
738 hormone. Proc Natl Acad Sci U S A 1990; 87: 5163-5167.

739 51. Porkka-Heiskanen T, Urban JH, Turek FW, Levine JE. (1994) Gene expression in a  
740 subpopulation of luteinizing hormone-releasing hormone (LHRH) neurons prior to  
741 the preovulatory gonadotropin surge. J Neurosci 1994; 14: 5548-5558.

- 742 52. Kinoshita M, Tsukamura H, Adachi S, Matsui H, Uenoyama Y, Iwata K, Yamada S,  
743 Inoue K, Ohtaki T, Matsumoto H, Maeda K. (2005) Involvement of central  
744 metastin in the regulation of preovulatory luteinizing hormone surge and  
745 estrous cyclicity in female rats. *Endocrinology* 2005; 146: 4431-4436.
- 746 53. Gonzalez MI, Baker BI, Wilson CA. Stimulatory effect of melanin-concentrating  
747 hormone on luteinising hormone release. *Neuroendocrinology* 1997; 66: 254-  
748 262.
- 749 54. Murray JF, Adan RAH, Walker R, Baker BI, Thody AJ, Nijenhuis WAJ, Yukitake J,  
750 Wilson CA. Melanin-concentrating hormone, melanocortin receptors and  
751 regulation of LH release. *J Neuroendocrinol* 2000; 12: 217-223.
- 752 55. Murray JF, Hahn JD, Kennedy A, Small C, Bloom SR, Haskell-Luevano C, Coen CW,  
753 Wilson CA. Evidence for a stimulatory action of melanin concentrating  
754 hormone (MCH) on luteinising hormone release involving MCH1 and  
755 melanocortin-5 (MC-5) receptors. *J Neuroendocrinol* 2006; 18: 157-167.
- 756 56. Brown RSE, Khant Aung Z, Phillipps HR, Barad Z, Lein HJ, Boehm U, Szawka RE,  
757 Grattan DR. Acute suppression of LH secretion by prolactin in female mice is  
758 mediated by kisspeptin neurons in the arcuate nucleus. *Endocrinology* 2019;  
759 160: 1323–1332.
- 760 57. Silva JP, von Meyenn F, Howell J, Thorens B, Wolfrum C, Stoffel M. Regulation of  
761 adaptive behaviour during fasting by hypothalamic FOXa2. *Nature* 2009; 462:  
762 646-650.

763 58. Cripps AW, Williams VJ. The effect of pregnancy and lactation on food intake,  
764 gastrointestinal anatomy and the absorptive capacity of the small intestine in  
765 the albino rat. *Br J Nutr* 1975; 33: 17-32

766 59. Woodside B. Prolactin and the hyperphagia of lactation. *Physiol Behav* 2007; 91:  
767 375-382.

768 60. Nobel EE, Hahn JD, Konanur VR, Hsu TM, Page SJ, Cortella AM, Liu CM, Song MY,  
769 Suarez AN, Szujewski CC, Rider D, Clarke JE, Darvas M, Appleyard SM, Kanoski  
770 SE. Control of feeding behavior by cerebral ventricular volume transmission of  
771 melanin-concentrating hormone. *Cell Metabolism* 2018; 28: 55-68.

772

773

774

775

776

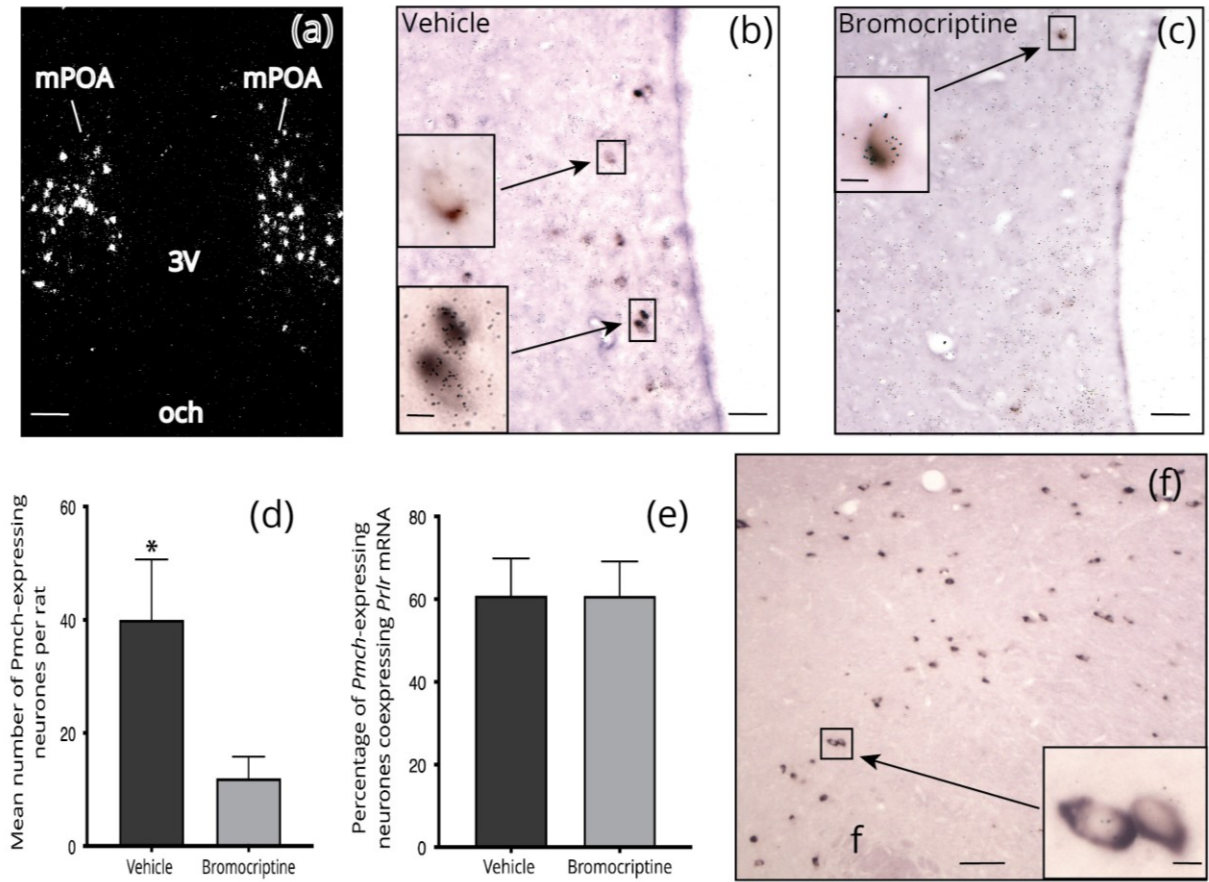
777

778

779

780

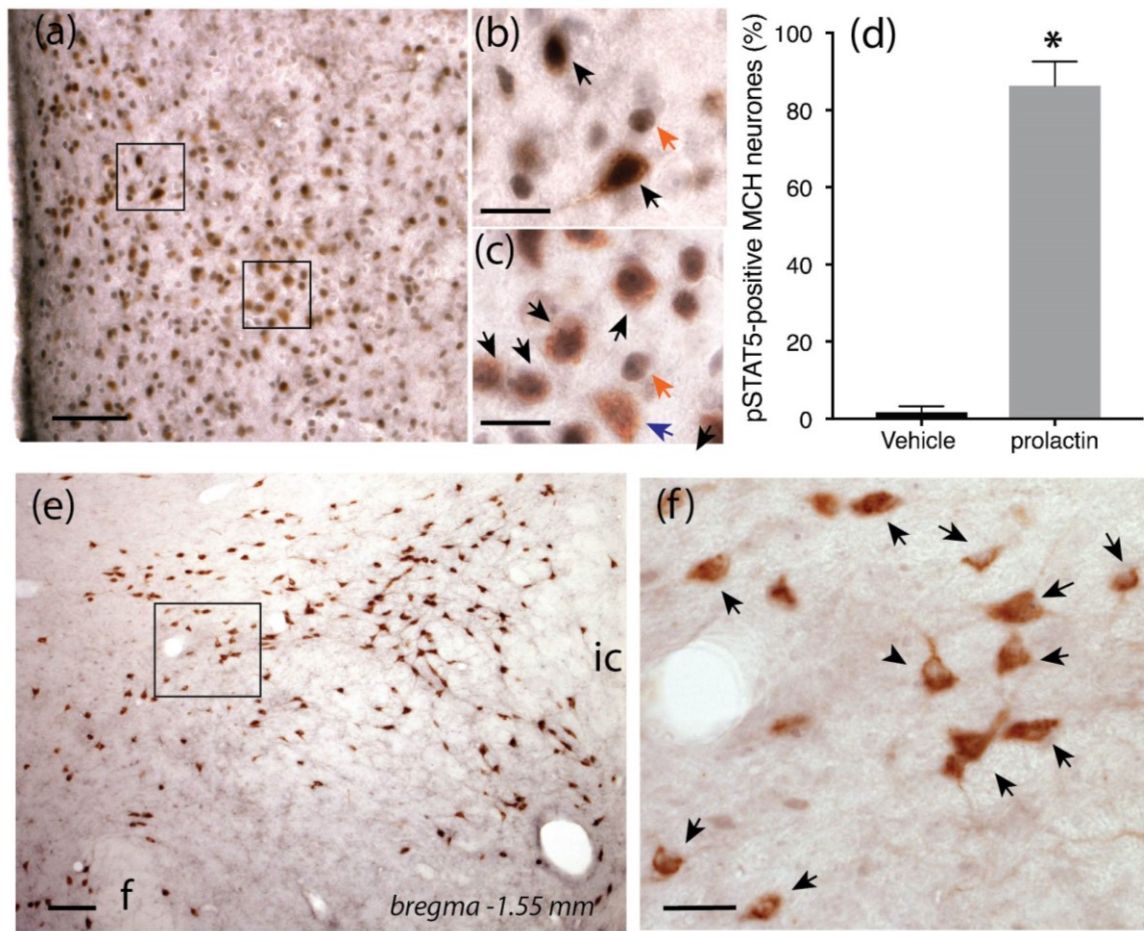
781 Figure 1



782

783

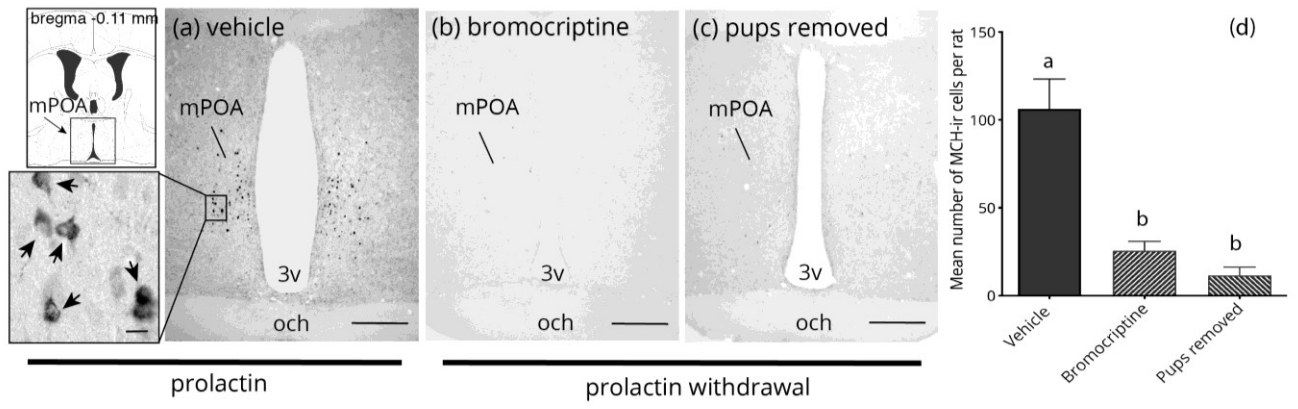
784 Figure 2



785



786 Figure 3



787