

1 **Calorie restriction activates new adult born olfactory-bulb neurones in**
2 **a ghrelin-dependent manner but acyl-ghrelin does not enhance sub-**
3 **ventricular zone neurogenesis**

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20 Highlights

- 21 • Acyl-ghrelin receptor, GHSR, is not expressed in the SVZ
- 22 • Acyl-ghrelin does not modulate SVZ cell proliferation
- 23 • Acyl-ghrelin does not increase adult olfactory bulb neurogenesis
- 24 • Genetic ablation of ghrelin does not affect survival of new adult born neurones
- 25 • Acyl-ghrelin receptor, GHSR, is expressed in the olfactory bulb
- 26 • Calorie restriction activates new adult born neurones in a ghrelin-dependent manner

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38 **Abstract**

39 The ageing and degenerating brain show deficits in neural stem/progenitor cell (NSPC) plasticity
40 that are accompanied by impairments in olfactory discrimination. Emerging evidence suggests
41 that the gut-hormone ghrelin plays an important role in protecting neurones, promoting synaptic
42 plasticity and increasing hippocampal neurogenesis in the adult brain. Here, we studied the role
43 of ghrelin in modulating adult sub-ventricular zone (SVZ) NSPCs that give rise to new olfactory
44 bulb (OB) neurones. We characterised the expression of the ghrelin receptor, growth hormone
45 secretagogue receptor (GHSR), using an immuno-histochemical approach in GHSR-eGFP reporter
46 mice to show that GHSR is expressed in several regions, including the OB, but not in the SVZ of
47 the lateral ventricle. These data suggest that acyl-ghrelin does not mediate a direct effect on NSPC
48 in the SVZ. Consistent with these findings, treatment with acyl-ghrelin or genetic silencing of
49 GHSR did not alter NSPC proliferation within the SVZ. Similarly, using a BrdU pulse-chase
50 approach we show that peripheral treatment of adult rats with acyl-ghrelin did not increase the
51 number of new adult-born neurones in the granule cell layer (GCL) of the OB. These data
52 demonstrate that acyl-ghrelin does not increase adult OB neurogenesis. Finally, we studied
53 whether elevating ghrelin indirectly, via calorie restriction (CR), regulated the activity of new
54 adult-born cells in the OB. Overnight CR induced c-Fos expression in new adult-born OB cells, but
55 not in developmentally born cells, whilst neuronal activity was lost following re-feeding. These
56 effects were absent in ghrelin^{-/-} mice, suggesting that adult-born cells are uniquely sensitive to
57 changes in ghrelin mediated by fasting and re-feeding. In summary, ghrelin does not promote
58 neurogenesis in the SVZ and OB, however, new adult-born OB cells are activated by CR in a
59 ghrelin-dependent manner.

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64 **Introduction**

65 The generation of new adult-born neurones in the olfactory bulb (OB) continues throughout life
66 and contributes to olfactory memory. The adult OB receives new neurones that originate from
67 divided neural stem / progenitor cells (NSPCs) residing in the sub-ventricular zone (SVZ)
68 adjacent to the lateral ventricles. Following NSPC division, the cells differentiate into immature
69 neuroblasts and migrate along the rostral migratory stream (RMS) prior to integration with local
70 OB circuitry¹. This process of adult OB neurogenesis (AOBN) is regulated by several intrinsic and
71 extrinsic factors including age, exercise, inflammation and glucocorticoids¹. However, the
72 underlying mechanisms mediating this process are poorly understood.

73

74 Within the OB, new adult-born neurones promote olfactory memory and enhance the ability to
75 discriminate distinct odours^{2,3}. AOBN is also important for OB granule cell replacement and tissue
76 maintenance⁴. Olfactory impairment has been reported as a prodromal indicator of several
77 neurodegenerative diseases⁵. For example, deficits in olfactory discrimination (i.e the ability to
78 distinguish odours) have been described in experimental neurodegenerative animal models and
79 human Parkinson's disease⁶.

80

81 Ghrelin, an orexigenic gut hormone produced in response to calorie restriction, acts on the
82 hypothalamus to stimulate the release of growth hormone (GH), and promote meal initiation and
83 food intake. Emerging evidence suggests that acyl-ghrelin may also have important extra-
84 hypothalamic functions⁷, such as increasing olfactory sensitivity⁸ and regulating activity in brain
85 regions involved in olfaction and appetitive behaviour⁹.

86 In the neurogenic niche of the hippocampus, acyl-ghrelin has been shown to increase cell
87 proliferation¹⁰ and the number of new adult-born neurones in adult rodents¹¹. The ghrelin
88 receptor, GHSR, which is expressed within the dentate gyrus of the hippocampus, mediates the
89 pro-neurogenic effect of calorie restriction (CR)¹², as well as the increase in hippocampal
90 neurogenesis and antidepressant-like effect following P7C3 treatment¹³. Moreover, ghrelin
91 deficient mice are reported to have impaired cell proliferation in the SVZ that is normalised to
92 wild-type levels with exogenous acyl-ghrelin treatment¹⁴. GHSR is the only molecularly identified
93 receptor for ghrelin, mediating the central effects of this hormone on appetite, body weight and
94 energy metabolism¹⁵. However, it is not known whether GHSR is expressed within the neurogenic
95 niche of the SVZ or whether acyl-ghrelin modulates adult olfactory bulb neurogenesis (AOBN).
96 Here, we aimed to determine the expression pattern of GHSR within the SVZ and whether ghrelin
97 modulates AOBN.

98 In addition, as fasting and feeding increase¹⁶ and decrease olfactory sensitivity¹⁷, respectively, we
99 sought to determine whether ghrelin modulates the fasting-induced activation of both new adult-
100 born and developmentally-born OB neurones.

101 **Materials and Methods**

102

103 **Animals and procedures**

104 All experiments involving animals were performed with appropriate ethical approval. Mouse
105 studies were performed at Cardiff University (GHSR-null, Ghrelin^{-/-}) and Monash University
106 (GHSR-eGFP). Studies involving rats were performed at the University of Cambridge.

107

108 **Mice**

109 ***GHSR-eGFP mice***: Adult male GHSR-eGFP reporter mice were housed at room
110 temperature on a 12h light, 12h dark cycle (0700-1900h) with free access (*ad libitum*) to
111 food and water. GHSR-eGFP reporter mice¹⁸ (n=6) were obtained from the Mouse Mutant
112 Regional Resource Center at the University of California Davis, and the hemizygous mice
113 back-crossed to C57BL/6J mice. GHSR-eGFP reporter mice were terminally anaesthetised
114 and trans-cardially perfused with 4% paraformaldehyde (PFA) in 0.1M PBS. Whole brains
115 were rapidly removed and post-fixed in ice cold 4% PFA for 24h at 4°C before being sunk
116 in 30% sucrose. Finally, brains were transferred to PBS + 0.1% sodium azide (Sigma
117 Aldrich, St Louis, USA) and stored at 4°C prior to analysis. Brains were frozen using a fine
118 powder of ground-up dry ice and mounted on a sliding sledge freezing microtome (Zeiss,
119 Microm HM 450) using Jung's freezing medium. The thermostat was set to -30°C to
120 ensure brains remained frozen. 30µm thick coronal sections cut along the entire rostral-
121 caudal axis (bregma +5.345mm to -4.08) were collected in a 96-well plate (Nunc, nunclon
122 surface) filled with PBS + 0.1% sodium azide and stored at 4°C until required. Ghsr-eGFP
123 mouse brains were also collected in a sagittal orientation (bregma +3.925 to -0.20).

124 ***Immunofluorescence for GHSR-GFP***: All experiments were performed on free-floating tissue
125 sections at room temperature, unless stated otherwise. A 1 in 6 series of coronal or sagittal brain
126 sections were selected (minimum of 10 sections per mouse), transferred into a 24-well culture
127 plate (Nunc, nunclon surface) and washed in PBS (Sigma Aldrich, St Louis, USA) three times for 5
128 minutes each. Tissue sections were then permeabilised in methanol (Fisher Scientific,
129 Loughborough, UK) at -20°C for 2 minutes and washed (as before) in PBS. Non-specific binding
130 sites were blocked with 5% normal goat serum (NGS) (Sigma Aldrich, St Louis, USA) in PBS +
131 0.1% triton x-100 (Sigma Aldrich, Gillingham, UK) (PBS-T) for 1h. Excess block was removed and
132 tissue sections incubated with chicken anti-GFP (Chicken polyclonal, Abcam, Cambridge, UK,
133 Ab13970), diluted 1:1000 in PBS-T, for 24h at 4°C. Primary antibody was omitted from the

134 negative control. Sections were washed and incubated in goat anti-chicken Alexa-fluor 488 (Goat
135 polyclonal, Life technologies, USA, A11039), diluted 1:500 in PBS-T, for 30 minutes in the dark.
136 Finally, sections were washed, mounted onto Superfrost⁺ slides (Fisherbrand, Superfrost⁺ slides)
137 and cover-slipped with vectashield (containing DAPI) (Vector Labs, Burlingame, USA) before
138 being stored at 4°C. The slides were analysed by laser scanning confocal microscopy (Zeiss,
139 LSM710) and Zen software (Zeiss, Zen 2010 edition) after 24h. Freely available GIMP v2.8
140 software was used to prepare tiled images of coronal and sagittal sections (www.gimp.org).

141 ***GHSR-null mice:*** For assessing exogenous acyl-ghrelin regulation of SVZ cell proliferation,
142 homozygous male loxTB-GHSR mice (GHSR-null) (a gift from Prof Jeffrey Zigman, University of
143 Texas Southwestern Medical Center, Dallas, TX) and W-T (C57BL/6J; W-T) controls (Harlan UK
144 Ltd.) (14 weeks-old, n = 3 / group) were used¹⁹. The methodological and metabolic aspects of this
145 study have previously been described²⁰. Briefly, mice were prepared with jugular vein cannulae
146 attached to osmotic mini-pumps (Alzet model 2001) under isoflurane anesthesia. The mini-
147 pumps delivered either vehicle or acyl-ghrelin (48µg/day; Phoenix Pharmaceuticals, USA) for 7
148 days. This treatment protocol was shown to increase abdominal adiposity via GHSR, but had no
149 effect on body weight²⁰. Mice were euthanised by cervical dislocation and whole trunk blood was
150 collected into heparinized tubes for plasma separation by centrifugation at 4,000g for 10 minutes
151 at 4°C. Whole brain was removed and immediately snap frozen on dry ice and stored at -80°C
152 prior to analysis.

153 For analysis of Ki67, snap-frozen brains were sectioned at 10µm thickness using a cryostat
154 (Leica) and mounted directly onto superfrost⁺ coated slides (VWR). A one-in-fifteen series of
155 10µm sections (150µm apart) from each animal, a minimum of 8 sections per mouse, was
156 immunostained using rabbit anti-Ki67 (1:500, ab16667, Abcam) along with a biotinylated goat
157 anti-rabbit for Ni-DAB based detection, as previously described¹¹. Cells were imaged by light
158 microscopy (Nikon 50i) prior to quantification using Image J software.

159 A separate cohort of 19-week old male GHSR-null mice, derived from crosses between animals
160 that were heterozygous for the GHSR-null allele and that had been backcrossed >10 generations
161 onto a C57BL/6J genetic background, and WT littermate mice were housed under normal
162 laboratory conditions (12 h light: 12 h dark, lights on at 06.00 h) (n=3/genotype). Mice were
163 killed by cervical dislocation under terminal anaesthesia, whole brain was removed, immersed in
164 4% PFA for 24h at 4°C and cryoprotected in 30% sucrose prior to preparation of coronal sections
165 (30µm) cut into a 1:12 series along the entire rostro-caudal extent of the brain using a freezing-
166 stage microtome (MicroM, ThermoScientific) and collected for IHC. For DAB-

167 immunohistochemical analysis of GHSR labelling, a minimum of 6 sections per mouse were
168 washed in 0.1M PBS (2× 10min) and 0.1M PBS-T (1× 10min). Subsequently, endogenous
169 peroxidases were quenched by washing in a PBS plus 1.5% H₂O₂ solution for 20min. Sections
170 were washed again (as above) and incubated in 5% NDS in PBS-T for 1h. Sections were incubated
171 overnight at 4°C with rabbit anti-GHSR1a (Phoenix Pharmaceuticals, H-001-62), diluted 1:2000
172 in PBS-T and 2% NGS solution. Another wash step followed prior to incubation with biotinylated
173 goat anti-rabbit (1:400; Vectorlabs, USA) in PBS-T for 70min. The sections were washed and
174 incubated in ABC (Vector- labs, USA) solution for 90min in the dark prior to another two washes
175 in PBS, and incubation with 0.1 M sodium acetate pH6 for 10min. Immunoreactivity was
176 developed in Nickel enhanced DAB solution followed by two washes in PBS. Sections were
177 mounted onto superfrost⁺ slides (VWR, France) and allowed to dry overnight before being de-
178 hydrated and de-lipified in increasing concentrations of ethanol. Finally, sections were incubated
179 in histoclear (2× 3min; National Diagnostics, USA) and coverslipped using entellan mounting
180 medium (Merck, USA). Slides were allowed to dry overnight prior to imaging.

181 **Calorie restriction in Ghrelin^{-/-} mice:** Adult female homozygous ghrelin knockout (ghrelin^{-/-})
182 mice²¹ and their wild type (WT) littermates were derived from crosses between animals that
183 were heterozygous for the ghrelin-null allele. These mice were backcrossed >10 generations on
184 a C57BL/6J genetic background and acclimatized to being individually housed for 7 days under
185 normal laboratory conditions (12h light, 12h dark cycle; 0700-1900h) prior to the onset of the
186 study. Mice were divided into six groups (n=5-8/group) that included *ad-libitum* fed WT, calorie
187 restricted (CR) WT, calorie restricted/re-fed (CR/RF) WT, *ad-libitum* fed ghrelin^{-/-}, CR ghrelin^{-/-}
188 and CR/RF ghrelin^{-/-}. For the first 28 days of the study, mice were fed on an *ad-libitum* diet with
189 daily injections (from days 1-4) of the thymidine analogue, BrdU (50mg/kg; i.p), to label actively
190 dividing cells. On day 28, food was withdrawn at 17.30h from the CR and CR/RF mice. On the
191 subsequent day, CR/RF mice were allowed to feed *ad-libitum* for 1h prior to all animals
192 undergoing cervical dislocation whilst under terminal anaesthesia (~18h CR). Ghrelin^{-/-} mice
193 have growth rates and appetite similar to WT littermates, with no impairment in hyperphagia
194 after fasting^{21,22}. Similarly, adult ablation of ghrelin in mice does not impair growth nor appetite²³.
195 Whole brains were removed, immersed in ice cold 4% PFA for 24h and cryoprotected in 30%
196 sucrose. Coronal sections (30µm) were cut in a 1:12 series along the entire rostral-caudal axis of
197 the olfactory bulb (bregma +5.345mm to +2.445mm) using a freezing stage microtome (MicroM,
198 Thermo Scientific) and collected for IHC.

199

200 **Quantification of BrdU⁺/c-Fos⁺:** All IHC was performed on free-floating sections at room
201 temperature, unless otherwise stated. A 1:6 series of 30µm sections (180µm apart) were washed

202 three times in PBS for 5 min, permeabilised in methanol at -20°C for 2 min, and washed as before.
203 DNA was denatured with 2M HCL for 30 min at 37°C prior to washing sections in 0.1M borate
204 buffer (pH 8.5) for 10 minutes. Sections were washed, blocked with 5% normal goat serum (NGS)
205 plus 5% bovine serum albumin (BSA) in PBS plus 0.1% Triton (PBS-T) for 60 min and incubated
206 in a cocktail of primary antibodies that included rat anti-BrdU (1:400; MCA2060, ABD Serotec)
207 and rabbit anti-c-Fos (1:1000; SC-52, Santa Cruz) in PBS-T overnight at 4°C. The primary antibody
208 was omitted from the negative control.

209 Following primary antibody treatment, sections were washed, incubated with biotinylated goat
210 anti-rat (1:400; BA-9400, Vector Labs) in PBS-T for 60 min in the dark and then washed as before.
211 Similarly, secondary antibodies were also applied as a cocktail that included goat anti-rabbit
212 (1:400; BA-1000, Vector Labs) and streptavidin AF-594 (1:500; S11227, Life Technologies) in
213 PBS-T for 30 min. Following another wash, including one containing Hoechst nuclear stain,
214 sections were mounted onto superfrost+ slides (VWR, France) and cover-slipped with prolong
215 gold anti-fade solution (Life Technologies, USA).

216

217 **Quantification of immunolabelled cells:** A 1:6 series of 30µm sections (180µm apart) from each
218 animal was analysed for immunoreactivity using an epi-fluorescent microscope system (Zeiss,
219 Imager M1 with Axiocam MRm). Immunolabelled cells were manually counted bilaterally using a
220 ×40 objective through the z-axis of the entire rostral-caudal extent of the dorsal granule cell layer
221 (GCL), glomerular layer (GL), subependymal zone (SEZ) and the lateral olfactory tract body (LOT).
222 Resulting numbers were divided by the total area measurement to give a count per pixel, which
223 was converted into mm² and averaged for each brain. All analyses were performed blind to both
224 genotype and treatment.

225

226 **Rats**

227 Adult male lister hooded rats (n=10/11 per group, weighing 250-300g; Harlan, Bicester, UK)
228 were housed in groups of four and maintained at room temperature on a 12h light, 12h dark cycle
229 (0700-1900h). These experimental procedures have previously been described¹¹. Briefly, from
230 days 0-14, rats received daily intra-peritoneal injections of acyl-ghrelin (Phoenix
231 Pharmaceuticals, 031-31) or saline (10µg/kg body weight) with BrdU injections (50mg/kg) on
232 days 5-8. On day 29, rats were terminally anaesthetised, trans-cardially perfused with 4% PFA
233 and brains were removed for immersion fixation and cryoprotection (as before). Analysis of adult
234 hippocampal neurogenesis (AHN) in these rats demonstrated that acyl-ghrelin significantly
235 increased the number of new adult born neurones¹¹.

236

237 **Double immunofluorescence for BrdU/NeuN:** A 1 in 6 series of coronal OB brain sections
238 (bregma +5.345mm to +2.445) were transferred into a 24-well culture plate and washed in PBS,
239 permeabilised in methanol at -20°C for 2 minutes and washed in PBS as before. DNA was
240 denatured using 2M hydrochloric acid (HCL) (Fisher Scientific, Loughborough, UK) and incubated
241 at 37°C for 30 minutes. Excess HCL was removed and the sections washed in 0.1M borate buffer,
242 pH 8.5, for 10 minutes to neutralise the remaining HCL. Tissue sections were then washed,
243 blocked with 5% NGS diluted in PBS-T for 1h and incubated with rat anti-BrdU (Rat monoclonal,
244 ABD Serotec, Oxfordshire, UK, MCA2060), diluted 1:3000 in PBS-T for 24h at 4°C. The primary
245 antibody was omitted from the negative control. Sections were washed and incubated with
246 biotinylated goat anti-rat (Goat polyclonal, Vector labs, Burlingham, USA, BA-9400), diluted 1:400
247 in PBS-T for 1h in the dark. Tissue sections were subsequently washed, incubated in streptavidin
248 AF594 (Life technologies, Eugene, USA, S11227), diluted 1:500 in PBS-T for 30 minutes and
249 washed as before. Sections were then incubated in mouse anti-NeuN (Mouse monoclonal, EMD
250 Millipore, Massachusetts, USA, MAB377), diluted 1:1000 in PBS-T for 1h. The negative control
251 contained PBS-T. Tissue sections were then washed and incubated in goat anti-mouse AF 488
252 diluted 1:500 in PBS-T for 30 minutes, prior to being washed with Hoescht, diluted 1:10000 in
253 PBS, for 5 minutes. Finally, sections were washed, mounted onto Superfrost+ slides and cover-
254 slipped with prolong gold anti-fade reagent, prior to storage at 4°C.

255

256 **Quantification of BrdU⁺:** Image J software (version 1.47) was used to quantify the number of new
257 adult-born cells in the dorsal and ventral granular cell layer (GCL) of the OB. Images taken by the
258 fluorescent microscope were inverted and unsharp-masked, using a radius of 10.0 pixels and a
259 mask weight of 0.60. The polygon tool was then used to draw around the granular cell layer and
260 the total area measured. Each image's threshold was individually optimised, typically ranging
261 from 0.100 – 0.180. The particle size was set to 20-300 pixel² and circularity at 0.0-1.0. Resulting
262 numbers were divided by the total area measurement to give a count per pixel, which was then
263 averaged for each brain.

264

265 **Quantification of BrdU⁺/NeuN⁺:** To quantify the number of new adult-born neurons in the dorsal
266 and ventral granular cell layer of the OB, BrdU⁺/NeuN⁺ immunoreactive cells were manually
267 counted through the z-axis of the entire rostral-caudal extent of the OB. Resulting numbers were
268 divided by the area of the z-stack to give a count per pixel, which was then averaged for each
269 brain.

270

271 **Microscopy**

272 Tissue sections were analysed using a fluorescent microscope (Zeiss, Imager M1 with AxioCam
273 MRm) with Axiovision software (version 4.6) and a laser scanning confocal microscope (Zeiss,
274 LSM 710) with Zen software (Zen 2010 edition). Images were collected using $\times 4$, $\times 10$ and $\times 40$
275 objectives. BrdU⁺/NeuN⁺ immunoreactive newborn adult neurones, in the dorsal and ventral GCL,
276 were imaged using a $\times 40$ oil immersion objective. A z-stack consisting of 21-25 tissue slices at
277 $0.7\mu\text{m}$ intervals ($14.0\text{-}16.8\mu\text{m}$ range) were taken throughout the rostral-caudal extent of the OB.
278 All experiments and analyses were performed blind to genotype and treatment.

279

280 **Statistical Analysis**

281 Statistical analyses were performed using GraphPad Prism 6.0 for Mac (GraphPad Software, San
282 Diego, CA). Statistical significance was assessed by an unpaired two-tailed Student's *t*-test or one-
283 way ANOVA with Bonferroni's *post-hoc* test. Where there was more than one variable a two-way
284 ANOVA with Tukey's multiple comparisons test was used or a Kruskal-Wallis test followed by a
285 Dunn's multiple comparisons test was used when a normal distribution of data could not be
286 assumed. Data are presented as a mean \pm SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ and ****,
287 $P < 0.0001$ were considered statistically significant.

288

289

290 **Results**

291 **GHSR is expressed in the adult OB but not in the SVZ**

292 The expression of GHSR was assessed to determine whether ghrelin could directly influence the
293 proliferation of NSPCs in the SVZ. To achieve this aim we used GHSR-eGFP reporter mice to show
294 that eGFP immunoreactivity was present within the anterior olfactory nucleus (AON) and orbital
295 and motor orbital cortex in the caudal OB (figure 1i). Immunoreactivity was observed within the
296 anterior cingulate cortex, motor cortex and lateral septal nucleus (figure 1ii). Sagittal sections
297 revealed strong immunoreactivity in the anterior amygdala area, granule cell layer of the
298 hippocampal dentate gyrus (DG) and the medial amygdala nucleus (figure 1iii). Notably, staining
299 was absent within the lateral lining of the SVZ in tissue sectioned in both a coronal and sagittal
300 orientation (figure 1iiD and iiiC). To determine whether the GHSR-eGFP immunoreactivity was
301 similar to that observed with GHSR1a antisera, we performed IHC using a rabbit anti-GHSR1a
302 antibody on adult WT and GHSR-null mouse brain tissue. These analyses revealed a similar
303 pattern of immunoreactivity on both WT and GHSR-null tissues, including the SVZ (figure S1),
304 suggesting a lack of binding specificity for the GHSR1a antigen. These data suggest that ghrelin
305 may be involved in olfactory function but not through direct modulation of NSC's lining the lateral
306 wall of the SVZ.

307

308 **Acyl-ghrelin does not increase cell proliferation in the adult SVZ**

309 The proliferative effect of ghrelin and GHSR-agonists have been widely reported within CNS and
310 peripheral tissues²⁴⁻²⁶. A recent study reported that ghrelin promoted proliferation of cells within
311 the SVZ¹⁴. Here, we took advantage of genetically modified mice to analyse the effect of acyl-
312 ghrelin treatment on SVZ cell proliferation in adult WT and GHSR-null mice, where GHSR is
313 transcriptionally silenced. Using the mitotic marker, Ki67, we report that acyl-ghrelin treatment
314 had no effect on the number of proliferating cells within the SVZ niche in WT mice (figure 2, $P =$
315 >0.99). Similarly, transcriptional silencing of GHSR did not affect the rate of SVZ cell division in
316 vehicle (WT veh vs GHSR-null veh, $P = 0.6388$) or acyl-ghrelin treated mice (GHSR-null veh vs
317 GHSR-null acyl-ghrelin, $P = 0.0944$). The low number of replicates means that the statistical
318 analysis is of low power, however, the data suggest that acyl-ghrelin does not regulate cell
319 proliferation in the adult mouse SVZ and that genetic silencing of GHSR does not decrease cell
320 division in this niche.

321

322 **Acyl-ghrelin does not increase the number of new adult-born olfactory bulb neurones**

323 We recently showed that treatment with acyl-ghrelin increased adult hippocampal neurogenesis
324 (AHN) in adult rats¹¹. To determine whether acyl-ghrelin treatment modulates AOBN in a similar
325 way to AHN, we quantified the number of new adult-born neurones in the OB of adult rats from
326 the same study. Using OB tissue from the same rats whereby acyl-ghrelin increased AHN provides
327 us with valuable experimental controls. We show there was no significant difference in the
328 number of new adult-born cells (BrdU⁺/NeuN⁻) in the GCL of the OB following acyl-ghrelin
329 treatment compared to saline treatment (figure 3G, $P = 0.8482$). Similarly, no differences were
330 observed in the number of new adult-born neurones (BrdU⁺/NeuN⁺) (figure 3H, $P = 0.7388$) or in
331 the rate of neurone differentiation (figure 3I, $P = 0.6870$).

332

333 **Calorie restriction induces activation of new adult-born OB cells in a ghrelin-dependent**
334 **manner**

335 To determine whether a CR-mediated increase in endogenous acyl-ghrelin was able to increase
336 the expression of the proto-oncogene, c-Fos, in new adult-born OB cells, we analysed the number
337 of active c-Fos⁺ cells within the GCL, GL, SEZ and LOT in WT and ghrelin^{-/-} mice. A two-way ANOVA
338 revealed a statistically significant main effect of treatment on BrdU⁺ ($p=0.0031$) and BrdU⁻/c-Fos⁺
339 ($p=0.0487$) cells within the GCL. Comparatively, genotype and the interaction (treatment and
340 genotype) showed a significant effect on c-Fos⁺ ($p=0.0001$ and 0.017 , respectively) and BrdU⁺/c-
341 Fos⁺ ($p=0.0002$ and 0.00021 , respectively) cells within the GCL. Outside of the GCL, a significant
342 main effect of treatment was reported in BrdU⁺/c-Fos⁺ cells of the SEZ ($p=0.0118$) and BrdU⁺ cells
343 of the LOT ($p=0.0075$). No increase was observed within the GL (Table S1).

344 A Tukey post hoc test revealed a reduction in the number of new adult-born cells (BrdU⁺) in
345 CR/re-fed (CR/RF) ghrelin^{-/-} mice, compared with CR ghrelin^{-/-} mice (figure 4C, $P = 0.0086$) within
346 the GCL. Furthermore, there was an increased number of activated cells (c-Fos⁺) in CR WT mice,
347 compared with *ad libitum* WT ($P = 0.0258$) and CR/RF WT ($P = 0.0043$) mice. Notably, CR also
348 increased activated cells in WT relative to CR ghrelin^{-/-} mice ($P < 0.0001$) within the GCL (figure
349 4D). Further analysis revealed that the number of active new adult-born cells (BrdU⁺/c-Fos⁺) was
350 increased in CR WT mice compared with CR/RF WT ($P = 0.0169$) and CR ghrelin^{-/-} mice
351 ($p < 0.0001$) within the GCL (figure 4E). Whereas, there were very few active developmentally
352 born cells (BrdU⁻/c-Fos⁺) and these cells were not significantly affected by treatment or genotype
353 (figure 4F). Outside of the GCL, the number of active new adult-born cells (BrdU⁺/c-Fos⁺) was
354 reduced in CR ghrelin^{-/-} mice, compared with *ad-libitum* fed ghrelin^{-/-} mice ($P = 0.0169$) within
355 the SEZ. No significant differences were reported in the other regions tested (Table S1). There
356 was no significant difference in body weight change in ghrelin^{-/-} mice relative to WT mice in either
357 of the groups (Two-way ANOVA; main effect of genotype, $P = 0.9335$; main effect of feeding

358 pattern, $P = 0.0049$; main effect of interaction (feeding pattern vs genotype), $P = 0.3469$).
359 Collectively, these data suggest that CR increases the activation of new adult-born cells in a
360 ghrelin-dependent manner.

361

362 **Discussion**

363 The generation of new OB neurones in the adult brain is important for olfactory discrimination, a
364 process that is impaired in ageing and several neurodegenerative disorders. Here, we tested the
365 hypothesis that ghrelin is an important regulator of AOBN. First, we characterised expression of
366 GHSR in the adult mouse brain. Numerous studies have attempted to characterise the expression
367 pattern of GHSR in several species, including mouse, rat and lemur²⁷⁻³⁰, though the lack of reliable
368 anti-GHSR antibodies have limited progress. More recently, a report using the GHSR-eGFP mouse
369 and in situ hybridisation histochemistry demonstrated GHSR expression within the OB,
370 hippocampus and hypothalamic nuclei¹⁵. Furthermore, Cre-activity in *Ghsr-IRES-Cre/ROSA26-*
371 *ZsGreen* reporter mice was also reported in the main and accessory OB³¹.

372 Here, using adult GHSR-eGFP mice, we report GHSR immunoreactivity in the MCL, AON and
373 orbital and motor orbital cortices of the OB, as well as within the anterior cingulate cortex, motor
374 cortex, lateral septal nucleus, entopeduncular nucleus, hippocampus and the medial amygdaloid
375 nucleus. However, GHSR was not expressed within the neurogenic niche of the SVZ in GHSR-eGFP
376 reporter mice. Indeed, this finding is consistent with previous studies that do not report GHSR
377 immunoreactivity within the SVZ niche. Together, these findings suggest that ghrelin does not
378 mediate direct effects on NSPC proliferation.

379 As eGFP immunoreactivity in this transgenic model may correspond to two structurally different
380 receptors, GHSR1a, which encodes the functional receptor, and the truncated GHSR1b, generated
381 from alternative splicing of GHSR, we sought to identify GHSR1a expressing cells using antisera
382 raised against GHSR1a. The specificity of polyclonal antibodies used to characterise GHSR within
383 the adult brain remains unclear. Li *et al.*¹⁴ reported GHSR expression within the adult mouse
384 neurogenic niche of the SVZ using immuno-fluorescence with the rabbit anti-GHSR1a antibody
385 (Phoenix Pharmaceuticals, H-001-62), diluted 1:500. In our study, using the same antibody, IHC
386 in brain tissue from adult GHSR-null and WT mice revealed detectable immunoreactivity in tissue
387 from both genotypes. Our data suggest that the rabbit anti-GHSR antibody resulted in non-specific
388 staining within the SVZ and cingulate cortex (figure S1), preventing it's use to determine GHSR1a
389 expression in this context. Combined, these studies suggest that the ghrelin receptor is not
390 expressed in the SVZ, and thus does not directly modulate NSPC proliferation.

391 To determine whether ghrelin induces cell proliferation within the SVZ we treated GHSR-null and
392 wild-type mice for 7-days with acyl-ghrelin. Subsequent analysis revealed no effect of genotype

393 or treatment on the number of dividing Ki67⁺ cells in the SVZ. In contrast, a previous study
394 reported that ghrelin^{-/-} mice had a reduced number of proliferating NSPCs, migrating neuroblasts
395 and OB interneurons, that could be restored to WT levels by intraperitoneal administration of
396 acyl-ghrelin¹⁴. Several differences between the two experimental procedures might account for
397 the contrasting results. For example, Li et al. used 8-9 week old WT and ghrelin^{-/-} mice that
398 received acyl-ghrelin (80 µg/kg) via intraperitoneal injection, once daily for 8 consecutive days.
399 Whereas, in our study 14-week old WT and GHSR-null mice were given acyl-ghrelin (48 µg/day)
400 via intravenous mini-pump. Therefore, inconsistencies between studies may be attributable to
401 genetic background, the physiological dose or the route of administration of acyl-ghrelin.

402

403 Next, using a BrdU pulse-chase approach we determined the effect of exogenous acyl-ghrelin
404 treatment on the maturation and survival of new adult-born neurones in the rat OB. Consistent
405 with our previous cell proliferation analysis in mice, acyl-ghrelin did not increase in the number
406 of new adult-born BrdU⁺ cells or BrdU⁺/NeuN⁺ neurones in the GCL of the OB. Furthermore, no
407 differences were observed in the rate of neuronal differentiation. Notably, we have previously
408 reported that adult hippocampal neurogenesis was significantly increased by acyl-ghrelin in
409 these rats¹¹. The high level of GHSR expression within hippocampal neurogenic niche¹² and it's
410 absence in the SVZ niche is likely responsible for this effect. These data provide compelling
411 evidence that acyl-ghrelin does not promote AOBN.

412 Numerous studies have suggested that ghrelin plays an important role in olfactory-related
413 behaviours including odour discrimination and sensitivity^{8,32,33}. Loch *et al.* reported an increased
414 responsiveness of the mouse olfactory epithelium following nasal application of ghrelin. This
415 resulted in a higher reactivity of olfactory sensory neurones within the olfactory epithelium,
416 which in turn, increased the activity of receptor-specific glomeruli. GHSR expression on the
417 surface of olfactory sensory neurones suggest that ghrelin and GHSR may play an important role
418 in enhancing neuronal responsiveness and olfaction. However, the underlying mechanism by
419 which acyl-ghrelin enhances olfaction remains elusive and it is unclear if new adult-born OB
420 neurones are involved in this physiology.

421 As acyl-ghrelin is known to regulate both olfaction and appetite we sought to determine whether
422 new adult-born OB neurones are activated by CR in a ghrelin-dependent manner. Our data
423 demonstrate that overnight CR activated new adult-born cells in the OB. Re-feeding for one hour
424 returned the number of c-Fos positive cells to baseline, suggesting that the new adult-born cells
425 are sensitive to feeding status. Notably, this CR effect was absent in ghrelin^{-/-} mice demonstrating
426 that the activation of new adult-born cells was dependent upon intact ghrelin signalling.
427 Furthermore, there was no CR-mediated activation of developmentally-born cells (BrdU⁻/c-Fos⁺)

428 in the GCL of the OB indicating that adult-born neurones are uniquely responsive to acute changes
429 in food intake. Therefore, we confirm that CR activates new adult-born OB cells in a ghrelin-
430 dependent manner. This finding provides further support for ghrelin acting as a mediator of CR-
431 associated physiology, including, neuroprotection³⁴, anti-anxiety³⁵, hippocampal neurogenesis
432 and cognitive enhancement¹², and glycemic regulation³⁶.

433 Although the relationship between hunger stimulation and olfaction has been long recognised, a
434 molecular mechanism relating the two processes has not been determined³⁷. Soria-Gomez *et al.*
435 observed that cortical feedback projections to the OB crucially regulate food intake, possibly
436 through cannabinoid type-1 receptor (CB1R) signalling. The endocannabinoid system, in
437 particular CB-1Rs, promoted food intake in fasted mice by increasing odour detection. Notably,
438 ghrelin's orexigenic effect is lost in CB-1R knock-out mice³⁸. Although the relationship between
439 ghrelin and the endocannabinoid system in the OB is unknown, both GHSR and CB-1R are GPCRs
440 known to form homo- and heterodimers (or higher-order oligomers) as part of their normal
441 trafficking and function^{39,40}. Therefore, heterodimerisation of CB-1R and GHSR may be important
442 in linking ghrelin to adult-born OB neurones and olfaction.

443 Several questions remain unanswered, including whether ghrelin alters the electrophysiological
444 properties and/or directly activates GCs in the OB to enhance odour discrimination. As new adult-
445 born OB cells enhance the odour-reward association³, further work is needed to determine
446 whether the ghrelin-induced intake of rewarding foods⁴¹ requires signalling via new neurones in
447 the OB. Similarly, it is not known whether ghrelin can increase appetite and improve olfaction in
448 the absence of new adult-born OB cells.

449 In summary, these data demonstrate that while ghrelin does not increase SVZ-OB neurogenesis,
450 it does mediate the CR-induced activation of new adult-born OB cells. We speculate that ghrelin
451 modulates new OB neurone activity to integrate olfactory responses with nutritional status.

452

453 **Figure legends**

454

455 **Figure 1. Characterisation of GHSR1a in the adult GHSR1a-eGFP mouse brain.**

456 (i). GHSR1a-eGFP immunoreactivity is present within the orbital and motor orbital cortex and the
457 anterior olfactory nucleus. CTX, cortex; AON, anterior olfactory nucleus.

458 (ii). Collage of coronal mouse sections (A). Inset images of GHSR1a-eGFP immunoreactivity in
459 anterior cingulate cortex dorsal (B), anterior cingulate cortex (C) and lateral septal nucleus (E).
460 GHSR1a-eGFP immunoreactivity is absent in the lateral lining of the SVZ (D).

461 (iii). Collage of sagittal mouse sections (A). Inset images of GHSR1a-eGFP immunoreactivity in
462 primary motor cortex (B), anterior amygdala area (D), dorsal granule cell layer of the dentate
463 gyrus (E), ventral dentate gyrus (F) and medial amygdalar nucleus (posterodorsal)(G). GHSR1a-
464 eGFP immunoreactivity is absent within the lateral lining of the SVZ (C).

465 Montage image scale bar = 200µm. Inset image scale bar = 50µm.

466

467 **Figure 2. Acyl-ghrelin treatment does not increase cell proliferation in the SVZ of adult**

468 **wild-type or GHSR-null mice.** (A) GHSR-null and WT littermate mice were treated for 7-days
469 with either saline or acyl-ghrelin (48ug/day i.v) via osmotic mini-pump before brains were
470 collected and Ki67 immunoreactivity quantified throughout the rostro-caudal extent of the SVZ.
471 (B) Total number of Ki67+ cells did not change following acyl-ghrelin treatment in either WT or
472 GHSR-null mice. Data are mean +/- SEM, n=3 mice per group. Statistical analysis performed by
473 Kruskal-Wallis test ($P = 0.2087$) followed by a post-hoc Dunn's multiple comparison test.

474

475 **Figure 3. Exogenous acyl-ghrelin does not increase the number of new adult born neurones**

476 **in the granule cell layer of the rat olfactory bulb.** (A) Experimental paradigm. (B) Collage
477 image of the rat olfactory bulb. Representative images of BrdU (red) and NeuN (green) in (C)
478 dorsal granule cell layer (GCL) and (D) ventral GCL of the OB. Scale bar = 200µm. Representative
479 images of new adult-born neurones co-expressing NeuN+ and BrdU+ (yellow) in (E) dorsal GCL
480 and (F) ventral GCL. Scale bar = 50µm. Quantification of new adult-born OB cells (G) ($P = 0.8482$),
481 new adult-born neurones (H) ($P = 0.7388$) and % neuronal differentiation (I) ($P = 0.6870$) after
482 acyl-ghrelin or saline treatment. Data are mean +/- SEM. Statistical analysis was performed by
483 two-tailed unpaired Student's *t*-test. $P < 0.05$ considered significant, ns = not significant. n = 11
484 rats per group.

485

486 **Figure 4. New adult-born OB cells are activated by calorie restriction in a ghrelin-**

487 **dependent manner.** (A) Schematic of experimental paradigm. (B) New adult-born active

488 neurone (yellow; scale bar = 25µm) co expressing BrdU (green) and c-Fos (red) in the GCL of the
489 OB. Scale bar = 50µm. Quantification of (C) new adult-born cells (BrdU⁺), (D) active cells (c-Fos⁺),
490 (E) active new adult-born cells (BrdU⁺/c-Fos⁺) and (F) active developmentally born cells (BrdU⁻
491 /c-Fos⁺) in the GCL of the OB. (G) Representative images of new adult-born cells (BrdU⁺; green),
492 active cells (c-Fos⁺; red) and active new adult-born cells (BrdU⁺/CFos⁺; yellow in merged image).
493 Arrows correspond to new BrdU⁺/c-Fos⁻ cells, whilst arrowheads represent active new adult-
494 born BrdU⁺/c-Fos⁺ cells. Scale bar = 50µm. Statistical analysis was performed by two-way ANOVA
495 with Tukey post hoc test. * P ≤ 0.05, ** P ≤ 0.01, **** P ≤ 0.0001. All data shown are mean +/-SEM;
496 n = 5-8 rats per group. AL (ad-libitum), CR (calorie restriction), CR/RF (calorie restriction / re-
497 fed), WT (wild-type), GKO (ghrelin^{-/-}).
498

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