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1 **Ghrelin action in the PVH of male mice: accessibility, neuronal targets and CRH neurons**
2 **activation**

3
4 **Gimena Fernandez¹, Pablo N. De Francesco¹, María P. Cornejo¹, Agustina Cabral¹, Julieta**
5 **P. Aguggia¹, Victor J. Duque², Nilufer Sayar³, Sonia Cantel⁴, Juan I. Burgos^{5,#}, Jean-Alain**
6 **Fehrentz⁴, Rodrigo Rorato², Deniz Atasoy³, André S. Mecawi², Mario Perello^{1,6}**

7
8 ¹Laboratory of Neurophysiology of the Multidisciplinary Institute of Cell Biology [IMBICE, Argentine
9 Research Council (CONICET) and Scientific Research Commission, Province of Buenos Aires
10 (CIC-PBA), National University of La Plata], La Plata, Buenos Aires, Argentina.

11 ²Department of Biophysics, Escola Paulista de Medicina, Universidade Federal de São Paulo
12 (UNIFESP), São Paulo, São Paulo, Brazil.

13 ³Department of Neuroscience and Pharmacology, Carver College of Medicine, Iowa Neuroscience
14 Institute and Fraternal Order of Eagles Diabetes Research Center (FOEDRC), University of Iowa,
15 Iowa City, Iowa, USA.

16 ⁴Institut des Biomolécules Max Mousseron, Univ Montpellier, CNRS, ENSCM, Montpellier, France.

17 ⁵Centro de Investigaciones Cardiovasculares "Dr. Horacio Eugenio Cingolani" (CONICET and
18 National University of La Plata), La Plata, Buenos Aires, Argentina.

19 ⁶Department of Surgical Sciences, Functional Pharmacology and Neuroscience, University of
20 Uppsala, Uppsala, Sweden.

21
22 Corresponding Author:

23 Dr. Mario Perelló

24 ORCID ID: 0000-0003-2114-6765

25 Laboratory of Neurophysiology, Multidisciplinary Institute of Cell Biology

26 Calle 526 S/N entre 10 y 11-PO Box 403. La Plata, Buenos Aires, Argentina 1900

27 Phone +54 221 4210112

28 Email: mperello@imbice.gov.ar

29
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31
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33
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36 #Current address : Multidisciplinary Institute for Investigation in Pediatric Pathologies (IMIPP),
37 Immunology division, CONICET- Government of the City of Buenos Aires (GCBA), Ricardo
38 Gutierrez Children's Hospital, Buenos Aires, Argentina.

39 **ABSTRACT**

40

41 The hormone ghrelin displays several well-characterized functions, including some with
42 pharmaceutical interest. The receptor for ghrelin, the growth hormone secretagogue receptor
43 (GHSR), is expressed in the hypothalamic paraventricular nucleus (PVH), a critical hub for the
44 integration of metabolic, neuroendocrine, autonomic and behavioral functions. Here, we performed
45 a neuroanatomical and functional characterization of the neuronal types mediating ghrelin actions
46 in the PVH of male mice. We found that fluorescent-ghrelin mainly labels PVH neurons
47 immunoreactive for nitric oxide synthase 1 (NOS1, which catalyze the production of nitric oxide -
48 NO). Centrally-injected ghrelin increases c-Fos in NOS1 PVH neurons and NOS1 phosphorylation
49 in the PVH. We also found that a high dose of systemically-injected ghrelin increases ghrelin level
50 in the cerebrospinal fluid and in the periventricular PVH, and induces c-Fos in NOS1 PVH neurons.
51 Such high dose of systemically-injected ghrelin activates a subset of NOS1 PVH neurons, which
52 does not express oxytocin, via an arcuate nucleus-independent mechanism. Finally, we found that
53 pharmacological inhibition of NO production fully abrogates ghrelin-induced increase of calcium
54 concentration in corticotropin-releasing hormone neurons of the PVH whereas it partially impairs
55 ghrelin-induced increase of plasma glucocorticoid levels. Thus, plasma ghrelin can directly target
56 a subset of NO-producing neurons of the PVH that is involved in ghrelin-induced activation of the
57 hypothalamic-pituitary-adrenal neuroendocrine axis.

58 INTRODUCTION

59

60 Ghrelin is a stomach-derived peptide hormone that acts via the growth-hormone
61 secretagogue receptor (GHSR) ^{1,2}. Due to its ability to release growth hormone (GH), ghrelin (or
62 ghrelin mimetics) rapidly emerged as promising candidates to enhance GH secretion and
63 consequently treat a variety of conditions, such as preventing the deleterious effects of aging on
64 the GH axis ³. Moreover, the finding that ghrelin is the most potent known orexigenic hormone has
65 expanded the potential pharmacological implications for ghrelin mimetics as viable therapeutic
66 options in the management of anorexia and cancer-induced cachexia ⁴. However, ghrelin treatment
67 also displays a variety of undesirable effects, including increments of glycemia, glucocorticoid
68 levels and fat mass as well as a reduction in insulin sensitivity ^{3,5}. The neurobiological substrates
69 that mediate some effects of ghrelin are only partially known, and consequently, it is uncertain if
70 such actions can be independently manipulated. For instance, GHSR is expressed in the
71 hypothalamic paraventricular nucleus (PVH), a critical brain center that integrates diverse
72 physiological functions. Surprisingly, however, little is known about the neurobiological basis
73 mediating ghrelin's actions in the PVH.

74

75 The PVH contains a variety of molecularly defined neuronal populations that display
76 different metabolic, neuroendocrine, autonomic and behavioral functions ⁶. PVH neurons are
77 classified into magnocellular, which mainly project to the posterior pituitary, and parvocellular that
78 project to the median eminence or autonomic control centers ⁷. Furthermore, PVH neurons can
79 synthesize different neuropeptides, including vasopressin (AVP), oxytocin (OXT), corticotropin-
80 releasing hormone (CRH), thyrotropin-releasing hormone (TRH) or neurotensin (NT), hereafter
81 named PVH^{AVP}, PVH^{OXT}, PVH^{CRH}, PVH^{TRH} or PVH^{NT} neurons, respectively. PVH neurons also
82 synthesize neurotransmitters such as nitric oxide (NO), norepinephrine or gamma-aminobutyric
83 acid (GABA); these neurons can be recognized by the expression of specific enzymes such as
84 nitric oxide synthase 1 (NOS1), tyrosine hydroxylase (TH) or glutamate decarboxylase 2 (GAD2),
85 hereafter named PVH^{NOS1}, PVHTH or PVH^{GAD2} neurons, respectively. Some PVH neurons show a
86 dual phenotype. For instance, PVH^{OXT} neurons may also be PVH^{NOS1} neurons and, consequently,
87 are referred to as PVH^{OXT/NOS1} neurons ⁸⁻¹¹. The PVH neurons are targeted by ghrelin in mice,
88 which are an instrumental animal model to delineate the neurobiological basis mediating ghrelin's
89 actions in mammals ¹². Studies using *in situ* hybridization ¹³⁻¹⁵, binding of fluorescently-labeled
90 ghrelin ¹⁶ or a reporter mouse ¹⁷ have shown that the mouse PVH contains GHSR-expressing
91 neurons (hereafter named PVH^{GHSR} neurons). In mice, systemically-injected ghrelin activates PVH
92 neurons, as indicated by the assessment of the expression of the marker for transcriptional activity

93 c-Fos, magnetic resonance or positron emission tomography ^{18–21}. To the best of our knowledge,
94 however, the neurochemical identity of the PVH^{GHSR} neurons is unknown.

95
96 The local action of ghrelin in the mouse PVH displays selective effects ¹². For instance,
97 intra-PVH-injected ghrelin does not induce food intake in mice, but activates the hypothalamic-
98 pituitary-adrenal axis (HPA) in a similar extent as seen in response to systemically or centrally-
99 injected ghrelin ²². Ghrelin does not directly act on PVH^{CRH} neurons, as they do not express GHSR
100 ²², but rather reduces the inhibitory post-synaptic currents in PVH^{CRH} neurons by acting at
101 presynaptic GABA terminals ^{23,24}. Conversely, ghrelin was shown to inhibit unidentified mouse PVH
102 neurons by decreasing the excitatory inputs in an endocannabinoid-dependent manner ²⁵. Thus,
103 ghrelin's action in the mouse PVH seems to involve complex local neuronal circuits and molecular
104 mechanisms, whose intricacies have been poorly elucidated.

105
106 Ghrelin's actions in the mouse hypothalamus are restricted due to the limited accessibility
107 of this hormone into the brain ^{26,27}. In particular, plasma ghrelin does not cross the blood-brain
108 barrier ²⁸, and rather only diffuses through fenestrated capillaries of the median eminence in order
109 to reach the neighboring hypothalamic arcuate nucleus (ARH) ^{24,29}. In the ARH, ghrelin activates
110 the agouti-related peptide (AgRP) neurons (hereafter named ARH^{AgRP} neurons) that, in turn,
111 strongly innervate the PVH ^{30–33}. Ghrelin can also act in the PVH via ARH-independent
112 mechanisms since systemically-injected ghrelin induces c-Fos expression in the PVH of ARH-
113 ablated mice ²⁴. It is currently unknown if plasma ghrelin can directly target the PVH. The PVH
114 contains the highest density of capillaries in the hypothalamus ³⁴; thus, plasma ghrelin may locally
115 diffuse into the PVH in a physiologically significant extent. Since ghrelin crosses the blood-
116 cerebrospinal fluid (CSF)-barrier ³⁵, plasma ghrelin could also target the PVH from the CSF. Here,
117 we first mapped the localization of PVH^{GHSR} neurons in the mouse brain and established its identity.
118 Since we found that a significant fraction of mouse PVH^{GHSR} neurons are PVH^{OXT/NOS1} or PVH^{NOS1}
119 neurons, we investigated if plasma ghrelin can reach and activate them in an ARH-independent
120 manner. Finally, we tested the hypothesis that ghrelin-induced indirect activation of the PVH^{CRH}
121 neurons involves NO signaling.

122 METHODS

123
124 Mice. Studies were performed using wild-type (WT) C57BL/6 mice as well as ARH-intact or
125 ARH-ablated mice, which were generated using subcutaneous (SC) injections of saline alone or
126 containing monosodium glutamate (Sigma-Aldrich, cat# G1626, 2.5 mg/g body weight (BW)),
127 respectively, in 4-day-old WT mice. The ARH was analyzed at the end of the experiments using
128 thionin staining and AgRP immunostaining, and was confirmed to be similarly lesioned in all
129 monosodium glutamate-treated mice, as previously described^{20,32,36}. Also, we used $Gad2^{tdTomato}$
130 mice, which express tdTomato in $Gad2$ -expressing GABA cells, and were generated by crossing
131 Ai14 reporter mice (Allen Institute, 129S6-Gt(ROSA)26Sor^{tm14(CAG-tdTomato)Hze/J}; Stock# 007908,³⁷)
132 and $Gad2$ -CreER mice (Jackson Laboratory, $Gad2^{tm1(cre/ERT2)Zjh/J}$; Stock# 010702,³⁸) that express
133 a tamoxifen-inducible Cre recombinase under the endogenous promoter of $Gad2$ gene. Adult
134 $Gad2^{tdTomato}$ mice received daily injections of tamoxifen (Sigma-Aldrich, cat# T5648; 70 mg/kg BW,
135 intraperitoneal, IP) for 4 consecutive days to induce Cre recombination, and used 3 weeks later.
136 Also, we used 4 Crh -IRES-Cre mice, which express Cre recombinase under the promoter of Crh
137 gene (Jackson Laboratories, B6(Cg)- $Crh^{tm1(cre)Zjh/J}$, Stock# 012704,³⁸). All mice were backcrossed
138 for more than 10 generations onto a C57BL/6 genetic background. Experimental mice were 10-14-
139 week-old males that were maintained under controlled temperature ($22\pm 1^\circ\text{C}$) and photoperiod (12-
140 h light cycle from 7:00 am to 7:00 pm). Five days before the experimental day, mice were
141 individually housed and *ad libitum* fed with regular chow diet, which was available until the time of
142 anesthesia/treatment and then taken away. The protocols received approval from the Institutional
143 Animal Care and Use Committee of the IMBICE, UNIFESP and the FOEDRC, where the mouse
144 studies were performed.

145
146 Labeling with fluorescent ghrelin. PVH cells directly responsive to ghrelin were labeled with
147 far red-ghrelin (Fr-ghrelin), which is a variant of ghrelin conjugated to DY-647P fluorophore at its
148 C-terminal end³⁹. We have shown that centrally-administered fluorescent ghrelin in mice
149 selectively labels GHSR-expressing neurons present in periventricular hypothalamic nuclei,
150 including not only the PVH but also the ARH, the ventromedial nucleus and the dorsomedial
151 nucleus^{16, 39}. Anesthetized mice were stereotaxically implanted with a single indwelling guide
152 cannula (4-mm long, 22-gauge, PlasticsOne) into the lateral ventricle (coordinates: 0.34 mm
153 anterior; ± 1 mm lateral and 2.3 mm ventral) and intracerebroventricularly (ICV) injected with 2 μL
154 of Fr-ghrelin (60 pmol/mouse, which is minimum amount of tracer that can be directly visualized).
155 Central injections were made over 2 min through a 30-gauge needle. Mice were perfused with
156 formalin after 30 min. Brains were removed, post-fixed, immersed in 20% sucrose, and coronally

157 cut into four equal 40- μ m series on a cryostat. Correct location of cannulas was confirmed by
158 histological observation at the end of experiments. Here, we used (a) naïve WT mice (n=12), (b)
159 WT mice that were ICV-injected with colchicine (16 μ g/mouse) 2-day before the experiment (n=3),
160 and (c) Gad2^{tdTomato} mice (n=3).

161
162 Immunolabeling of the subsets of PVH neurons. In order to reveal the identity of PVH cells
163 labeled by Fr-ghrelin, one series of brain sections containing the PVH of mice ICV-injected with Fr-
164 ghrelin were treated with blocking solution (2% normal donkey serum and 0.25% Triton X-100 in
165 PBS). Brain sections of WT mice were incubated with the following antibodies: rabbit anti-TH
166 (Santa Cruz Biotechnology, Cat# sc-14007, 1:5000, RRID: [AB_671397](#)), rabbit anti-NOS1 (Thermo
167 Fisher Scientific, Cat# 61-7000, 1:3000, RRID: [AB_2313734](#)), mouse anti-neurophysin I (marker
168 of PVH^{OXT} neurons, National Institute of Neurological Disorders and Stroke (NINDS), Cat# PS38,
169 1:1000, RRID: [AB_2315026](#)), or mouse anti-neurophysin II (marker of PVH^{AVP} neurons, NINDS,
170 Cat# PS41, 1:1000, RRID: [AB_2313960](#)). Brain sections of colchicine-treated mice were incubated
171 with the following antibodies: goat anti-CRH (recognizes CRH ⁴⁰, Santa Cruz Biotechnology, Cat#
172 sc-1759, 1:500, RRID: [AB_631300](#)), rabbit anti-TRH (recognizes proTRH ⁴¹, 1:500, RRID:
173 [AB_2315485](#)) or rabbit anti-NT (ImmunoStar, Cat# 20072, 1:1000, RRID: [AB_572254](#)). In order to
174 label PVH^{OXT/NOS1} neurons, subsets of sections were simultaneously incubated with antibodies
175 against neurophysin I and NOS1. After 48 h at 4°C, sections were incubated with donkey anti-
176 rabbit AlexaFluor594 (Thermo Fisher Scientific, Cat# A-21207, 1:1000, RRID: [AB_141637](#)),
177 donkey anti-mouse AlexaFluor594 (Thermo Fisher Scientific, Cat# A-21203, 1:1000, RRID:
178 [AB_141633](#)) or donkey anti-goat AlexaFluor594 (Thermo Fisher Scientific, Cat# A-11058, 1:1000,
179 RRID: [AB_2534105](#)) secondary antibodies, depending on the primary antibody used in each case,
180 for 2 h at room temperature. Finally, slices were mounted on glass slides and coverslipped with
181 mounting media.

182
183 scRNA-seq data analysis. Raw and processed scRNA-Seq datasets of fluorescent GFP+
184 PVH neurons isolated from mice generated by crossing an Oxt-Flp recombinase driver line to a
185 Flp-dependent GFP reporter line ⁴² were analyzed to identify Ghsr expressing neurons. Quality
186 control (QC) assessment and cell clustering were preserved from the original analysis. Starting
187 with 146 QC-accepted neurons, cells were classified as *Ghsr+*, *Oxt+* and/or *Nos1+* based on their
188 respective transcript counts being greater than zero. Subsequently, they were grouped and
189 counted according to the resulting joint positivity, along with their original magnocellular or
190 parvocellular classification. Analysis was performed in RStudio 2023.06.0, R version 4.3.0. Original
191 datasets are publicly available at https://github.com/gofflab/OT_neuron_study_2020.

192
193 Central injection of ghrelin. WT mice were implanted with a single indwelling ICV guide
194 cannula into the lateral ventricle using stereotaxic surgeries, as described above. Here, the guide
195 cannulas were permanently attached to the skull with dental cement and plugged with a 28-gauge
196 dummy cannula. After surgery, mice were allowed to recover for at least 5 days. During these days,
197 mice were accustomed to handling and removal of the dummy cannula. At around 9:30–10:00 am
198 of the experimental day, mice were ICV-injected with 2 μ L of artificial CSF alone or containing
199 ghrelin (Global Peptide, cat# PI-G-03, 60 pmol/mouse). Mice were anesthetized and perfused at
200 120 min after treatment for c-Fos immunostaining (n=4 and 4 for vehicle or ghrelin, respectively)
201 or at 15, 30 and 120 min after treatment for phospho-NOS1 immunostaining (n=3 for vehicle, and
202 n=4, 3 and 3 for ghrelin, respectively). Brains of perfused mice were obtained and used to generate
203 coronal sections for immunostainings, as described above. An independent set of cannulated WT
204 mice were ICV-injected with 2 μ L of artificial CSF alone (n=6) or containing 60 pmol/mouse of
205 ghrelin (n=6) and euthanized 30-min post-treatment. Here, brains were obtained and cut in coronal
206 sections in order to obtain PVH punches, which were, in turn, used to isolate total RNA (see below).
207 In all cases, the correct location of ICV injections was verified at the end of the experiment by
208 histological analysis.

209
210 Systemic injection of ghrelin. For dose-response studies, WT mice were SC-injected with
211 saline alone or containing ghrelin (60 or 600 pmol/g BW). Mice were perfused, as described above,
212 at 120-min post-treatment for c-Fos immunostaining (n= 5, 8 and 3 per group, respectively) or
213 anesthetized at 10-min post-treatment for CSF extraction. In the latter case, anesthetized mice
214 were placed in a stereotaxic frame and used to collect CSF from the cisterna magna at 30-min
215 post-treatment, as recently reported³⁹. CSF samples were collected on EDTA (1 mg/mL final) and
216 p-hydroxy-mercuribenzoic acid (0.4 mM final) and acidified with HCl (0.1 M final). Ghrelin was
217 assessed in pools of 30 μ L of CSF (n=4 per group). An independent set of WT mice were SC-
218 injected with vehicle alone (n=4) or containing 600 pmol/g BW of fluorescein-ghrelin (F-ghrelin,
219 n=4). F-ghrelin is a 19-residue analog of ghrelin conjugated to fluorescein isothiocyanate at its C-
220 terminal end^{16,43}. These mice were anesthetized and perfused at 20-min post-treatment, and their
221 brains were processed for immunostainings against fluorescein (see below).

222 Two additional experiments were performed to further study the action of the high dose of
223 ghrelin in the PVH. A set of ARH-intact and ARH-ablated mice were SC-injected with vehicle (n=4
224 per group) or ghrelin (600 pmol/g BW, n=4 per group), anesthetized and perfused 120-min post-
225 treatment. These brains were processed for c-Fos, NOS1 and neurophysin I immunostainings (see
226 below). Another cohort of WT mice was SC-treated with vehicle alone or containing either ghrelin

227 (600 pmol/g BW), N-nitro-L-Arginine methyl ester (L-NAME, 10 µg/g BW), or a combination of both.
228 Mice were euthanized at 120-min post-treatment for c-Fos immunostainings (n=3, 4, 3 and 8 per
229 group, respectively) or at 30-min post-treatment to obtain both PVH punches for mRNA
230 quantification and blood samples for corticosterone assessment (n= 9, 8, 9 and 7 per group,
231 respectively).

232
233 Chromogenic immunostaining. For c-Fos, phospho-NOS1 or fluorescein immunostaining,
234 one series of sections were pretreated with 0.5% H₂O₂, next treated with blocking solution and then
235 incubated with the following antibodies: rabbit anti-c-Fos antibody (Millipore, Cat# PC38, 1:20.000,
236 RRID: [AB_2106755](#)), rabbit anti-phospho-NOS1 (Abcam, Cat# ab5583, 1:1500, RRID:
237 [AB_304964](#)) or goat anti-fluorescein (Molecular Probes, Cat# A-11096, 1:1500, RRID:
238 [AB_221558](#)) for 48 h at 4°C. Then, sections were incubated with biotinylated donkey anti-rabbit
239 (Jackson ImmunoResearch Labs, Cat# 711-065-152, 1:3000, RRID: [AB_2340593](#)) or biotinylated
240 rabbit anti-goat (Vector Laboratories, Cat# BA-5000, 1:1500, RRID: [AB_2336126](#)) secondary
241 antibodies depending on the primary antibody used in each case, for 1 h at room temperature and
242 then treated with avidin-peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories, Cat#
243 PK-6200) for 1 h, according to manufacturer's protocols. Finally, sections were incubated with 3-
244 3'-diaminobenzidine (Sigma-Aldrich, Cat# D8001)/Nickel solution in order to generate a
245 black/purple precipitate in immunoreactive cells. Afterward, sections were sequentially mounted
246 on glass slides and coverslipped with mounting media. For triple immunostaining against c-Fos,
247 neurophysin I and NOS1, brain sections were first subjected to chromogenic immunostaining
248 against c-Fos and then to fluorescent immunostaining against neurophysin I and NOS1, as
249 described above.

250
251 Imaging and quantitative analysis. Images were acquired with 20x/0.80 and 40x/0.95
252 objectives using a Zeiss AxioObserver D1 equipped with an Apotome.2 structured illumination
253 module and an AxioCam 506 monochrome camera. For samples subjected to chromogenic
254 immunostaining alone, bright-field images were acquired using a Nikon Eclipse 50i and a DS-Ri1
255 Nikon digital camera with a 0.45X adapter using 10X/0.3 and 60X/0.80 objectives. Images were
256 taken in comparable areas and under the same optical and light conditions. Image processing and
257 analysis were performed in the ImageJ-based open-source image-processing package Fiji ⁴⁴.
258 Quantitative analyses were performed in sections containing the PVH (between bregma -0.58 and
259 -1.22 mm) or ARH (between bregma -1.58 and -2.06 mm) that were identified using the mouse
260 brain atlas ⁴⁵. Quantifications were bilaterally performed and corrected by double counting. The
261 total number of labeled cells in each area was determined by multiplying the number of cells

262 counted by four to correct for uncounted sections of the nucleus. Additionally, data were corrected
263 using the Abercrombie method ⁴⁶, which takes into account the thickness of the sections and the
264 mean diameter of the neuron along the z-axis. The mean diameter of the neurons was determined
265 using Fiji.

266 The number of cells labeled with Fr-ghrelin (hereafter Fr-ghrelin+ cells) within the PVH was
267 expressed as total Fr-ghrelin+ cells per PVH. For the phenotypical characterization of Fr-ghrelin+
268 PVH cells, the fraction of Fr-ghrelin+ cells positive for each marker or set of markers were
269 expressed as a percentage, which represents the fraction of Fr-ghrelin+ cells and a given marker
270 (or markers) as compared to the total number of either Fr-ghrelin+ cells or cells positive for a given
271 marker (or markers). The number of phospho-NOS1+ cells was expressed per PVH section per
272 side. The total number of c-Fos+ in the ARH or the PVH was expressed per PVH section per side,
273 whereas the fraction of c-Fos+ cells of a given cell type are shown as a percentage versus the total
274 number of the given cell type in the PVH, or in a subregion of the PVH (e.g., periventricular vs.
275 lateral regions of the PVH). The diffusion of F-ghrelin within the PVH was measured as described
276 in detail in the past ³⁵. Briefly, bright-field images were converted into 16-bit images. Images
277 underwent flat-field correction, and the pixel intensity values were transformed into optical density
278 data by taking the negative decimal logarithm of the original value divided by the flat-field value.
279 Optical density profiles were acquired perpendicular to the 3V along the length of a 300- μ m box at
280 the PVH level. The average optical density of a 25- μ m region farthest from the 3V was subtracted
281 from each profile, and the resulting curve was aligned using the first zero-crossing point nearest to
282 the 3V. The software Fiji was employed for all image processing and analysis steps.

283
284 *qPCR for RNA quantification in PVH punches.* After euthanasia, brains were rapidly
285 removed, frozen on dry ice and stored at -80°C . Brains were sliced into 60- μ m coronal sections
286 using a cryostat. The PVH was identified, according to the mouse brain atlas ⁴⁵, and collected using
287 a 500- μ m micropunch needle. The punch location was confirmed by toluidine blue (0.1%) staining
288 of the sections. PVH punches were collected in TRIzol[®] reagent, and total RNA was extracted
289 following the manufacturer's instructions. RNA concentration was measured using a NanoDrop[™]
290 One spectrophotometer. Samples were diluted to the same concentration and treated with the
291 DNase I Amp Grade kit (Life Technologies) to eliminate genomic DNA. RNA integrity was
292 confirmed by the detection of 18S and 28S bands after agarose-formaldehyde gel electrophoresis
293 (not shown), and RNA quality was verified by optical density absorption ratio 260 nm/280 nm. The
294 cDNA was synthesized using the QuantiTect Reverse Transcription Kit (Qiagen) using 300 ng of
295 total RNA. The optimization and validation of primers were performed using a qPCR standard
296 curve. Primers used were: sense: 5'-AGCATGGGCTCTCCTGTCA-3', antisense: 5'-

297 GAGACCAGAGTGGGCTGCA-3' for *c-Fos*; sense:5'-TCAAAGCCATCCAGCGCATA-3',
298 antisense: 5'-GGTACCGGTTGTCATCCCTC-3' for *Nos1*; sense: 5'-TGCCCCAGTCTTGCTTGCT-
299 3', antisense: 5'-TCCAGGTCTAGCGCAGCCC-3' for *Oxt*; and sense: 5'-
300 GAAGGTCAAAGGGAATGTGTTCA-3', antisense: 5'-CCTTGTCTGCCTTCAGCTTGT-3' for
301 *Rpl19*. Samples were 1:5 diluted and qPCRs were performed in triplicate using SYBR green
302 (Applied Biosystems) in a QuantStudio™ 5 Real-Time PCR System, with 384-well following the
303 manufacturer's instructions. The ribosomal protein L19 mRNA (*Rpl19*) did not show statistical
304 differences among groups and was used as an internal control gene for the assays. For relative
305 quantification of gene expression, the $2^{-\Delta\Delta CT}$ method was employed.

306
307 ELISA: CSF ghrelin concentration was assayed using a specific ghrelin ELISA kit (Bertin
308 Pharma, cat# A05117, RRID: [AB_2619624](#), intra-assay variation 8.1%), whose detection limit was
309 6.7 pg/mL. For corticosterone assessment, blood samples were collected in EDTA-coated tubes,
310 and plasma was obtained after centrifugation and stored at -20°C . Plasma corticosterone
311 concentration was determined using a competitive ELISA (Arbor Assays, cat# K014-H1, RRID:
312 [AB_2877626](#), intra-assay variation 5.2%).

313
314 In vivo calcium imaging. Here, anesthetized Crh-IRES-cre mice were stereotaxically-
315 injected with 300 to 500 nL of AAV-CAG-FLEX-GCaMP8m (Addgene, #162381) virus in both PVH
316 (coordinates: 0.8 mm anterior, ± 0.2 mm bilateral, 5.6 mm ventral) using a pulled glass pipette
317 controlled by a micromanipulator. Next, a metal ferrule-capped optical fiber (400 μm core diameter,
318 NA=0.48, Thorlabs) was implanted ~ 100 μm above the viral injection site in the midline and ferrules
319 were fixed with dental cement. After 5-6-week recovery, Crh-IRES-cre mice were single-housed in
320 custom plexiglass cages. After 1-2 days of habituation, mice were tethered to the optical fiber (400
321 μm core, 0.48 NA, bundled fibers, Doric Lenses) and allowed to acclimatize for a few hours before
322 the starting experiments. Signals from 405 nm and 465 nm excitation (at 30-50 μW) were acquired
323 at a 3 Hz sampling rate with Doric FP Bundle Imager (Doric Lenses). Recordings were performed
324 in the absence of food in otherwise *ad libitum* fed animals. After 30 min baseline recording, mice
325 were systemically injected with vehicle, different doses of ghrelin (60 or 600 pmol/g BW) and a high
326 dose of ghrelin (600 pmol/g BW) plus L-NAME (10 $\mu\text{g/g}$ BW) under brief (5 sec) anesthesia with
327 isoflurane. For the analysis of the fiber photometry data, the isosbestic signal (405 nm) was fit to
328 the calcium-dependent (465 nm) signal using the linear least squares fit in a custom MATLAB
329 script. $\Delta F/F$ was calculated as $(465\text{ nm-fitted } 405\text{ nm})/(\text{fitted } 405\text{ nm})$, and from these values, z-
330 scores were calculated for the baseline period to account for the inter-animal differences in signal
331 intensities $(F-\mu)/\delta$ where F is the calculated $\Delta F/F$ value, μ and δ are the mean and standard

332 deviation for the baseline period, respectively. After the *post hoc* analysis, mice were eliminated
333 from the analyses if the fiber tip location and/or sensor expression were off-target.

334
335 Statistical analyses. Normality and homogeneity of variances were tested using the
336 D'Agostino & Pearson omnibus test or Bartlett's test, respectively. Data were expressed as the
337 mean \pm standard error of the mean (SEM), and the experimental groups were compared with
338 Student's unpaired t-test, One-sample t-test or One-way ANOVA, depending on the number of
339 groups or experimental design. Tests used for each comparison and statistical outputs are
340 indicated in the figure legends or the text depending if data summaries are shown as text or graphs,
341 respectively. Differences were considered significant when $p < 0.05$. All statistical analyses were
342 conducted with the software GraphPad Prism, version 9.0.

343 RESULTS

344

345 1) Ghrelin directly targets OXT/NOS1+ cells of the PVH. We first performed a systematic
346 analysis of the number and the localization of PVH cells that were labeled in mice ICV-injected with
347 Fr-ghrelin. Fr-ghrelin labels 789 ± 20 cell bodies in the mouse PVH that were located between
348 bregma -0.58 and -1.22 mm of the Paxinos brain atlas (**Figure 1A-B**). Fr-ghrelin+ cells were
349 present in all sub-regions of the PVH, and the majority of them (718 ± 19) were enriched in the
350 anteromedial part of the nucleus, between bregma -0.58 and -0.94 mm (**Figure 1B**). Fr-ghrelin+
351 cells were relatively homogeneous in shape, with $15.8 \pm 0.3 \mu\text{m}$ of diameter (**Figure 1C**). Strikingly,
352 Fr-ghrelin+ signal was also observed in fiber-like structures that were enriched in the periventricular
353 region of the PVH, and laterally-oriented (**Figure 1D-E**). In terms of the neurochemical phenotype
354 of the Fr-ghrelin+ cells (**Figure 1F**), Fr-ghrelin did not label PVH^{CRH}, PVH^{TRH} or PVH^{NT} neurons in
355 brain sections of colchicine-treated mice. In *Gad2^{tdTomato}* mice, PVH^{GAD2} cells represented $1.0 \pm 0.6\%$
356 of all Fr-ghrelin+ cells. In naïve mice, PVH^{NOS1}, PVH^{OXT}, PVH^{AVP} and PVHTH neurons represented
357 42.2 ± 4.5 , 37.1 ± 4.0 , 7.0 ± 2.7 and $1.4 \pm 0.8 \%$ of all Fr-ghrelin+ cells.

358

359 Since PVH^{OXT/NOS1} neurons exist in the mouse hypothalamus⁸⁻¹¹, we assessed if they were
360 labeled by Fr-ghrelin (**Figure 2A**). We found that PVH^{OXT/NOS1} neurons were enriched in the
361 anteromedial part of the nucleus and represented $74.2 \pm 4.8\%$ of all PVH^{NOS1} neurons and
362 $83.4 \pm 7.3\%$ of all PVH^{OXT} neurons, in agreement with previous reports⁸⁻¹¹. Fr-ghrelin labeled
363 258 ± 17 , 107 ± 17 and 70 ± 7 PVH^{OXT/NOS1}, PVH^{NOS1} and PVH^{OXT} neurons, respectively. Thus, Fr-
364 ghrelin labeled 22.2 ± 7.0 , 11.9 ± 6.1 and $29.3 \pm 10.4 \%$ of all PVH^{OXT/NOS1}, PVH^{NOS1} and PVH^{OXT}
365 neurons, respectively, which represented 32.6 ± 6.1 , 9.8 ± 5.2 and $7.6 \pm 2.8 \%$ of all Fr-ghrelin+ cells
366 (~50% all Fr-ghrelin+ cells, **Figure 2B**). In order to clarify the nature of the PVH^{OXT/NOS1} and PVH^{OXT}
367 neurons targeted by ghrelin, we analyzed a dataset of the transcriptional profile of OXT-producing
368 neurons that was obtained by scRNA-seq analysis of the cells expressing green fluorescent protein
369 (hereafter named GFP+ cells) collected from PVH samples of OXT-reporter mice systemically
370 injected with fluorogold to label magnocellular neurons⁴². The analysis of the transcriptional profile
371 of 146 GFP+ cells reported in the mentioned study revealed that *Ghsr* was expressed in 9 GFP+
372 cells, all of which were magnocellular PVH cells (**Figure 2C-D**), as indicated by the transcriptional
373 profile and the presence of fluorogold. Six of the 9 GFP+ cells expressing *Ghsr* also express *Nos1*
374 (**Figure 2E**). Thus, *Ghsr* was expressed in ~7.1 and ~9.4 % of all PVH^{OXT/NOS1} and PVH^{OXT} neurons,
375 respectively (**Figure 2F**).

376

377 2) Centrally-injected ghrelin acts on PVH^{OXT/NOS1}, PVH^{NOS1} and PVH^{OXT} neurons. Since
378 centrally injected ghrelin induces c-Fos in the PVH ²², we tested if centrally-injected ghrelin
379 activates PVH^{OXT/NOS1}, PVH^{NOS1} and PVH^{OXT} neurons. We found that ICV-injected ghrelin increased
380 the fraction of PVH^{OXT/NOS1} (35.5±1.4 vs. 0.2±0.1 % of all PVH^{OXT/NOS1}), PVH^{NOS1} (24.4±7.0 vs.
381 0.0±0.0 % of all PVH^{NOS1}) and PVH^{OXT} (13.6±4.9 vs. 0.0±0.0 % of all PVH^{OXT}) neurons positive for
382 c-Fos, as compared to vehicle treatment (p<0.05 for all cases, Student's t-test, **Figure 3A**). Since
383 the phosphorylation of NOS1 at Ser1417 increases its activity and, consequently, the production
384 of NO ^{47,48}, we performed immunostaining against phospho-NOS1 in brain sections of mice ICV-
385 injected with ghrelin (**Figure 3B**). We found that ICV-injected ghrelin transiently increased the
386 number of phospho-NOS1 positive cells in the PVH, reaching the peak 15 min after treatment
387 (**Figure 3C**). Conversely, ICV-injected ghrelin increased the mRNA levels of c-Fos in the PVH at
388 30-min post-treatment (1.00±0.16- vs. 1.99±0.22-fold, p<0.05, Student's t-test) but it did not affect
389 mRNA levels of *Nos1* and *Oxt* (1.00±0.18- vs. 1.05±0.21-fold and 1.00±0.15- vs. 0.86±0.22-fold,
390 respectively), as compared to vehicle treatment.

391
392 3) A high dose of systemically-injected ghrelin is required to activate the PVH. In order to
393 test the extent to which plasma ghrelin can act in the PVH, we systemically-injected WT mice with
394 60 and 600 pmol/g BW of ghrelin, which induce a ~2.3- and ~16.8-fold transient increase of plasma
395 ghrelin levels at 30-min post-treatment, respectively ²⁰. At 2-h post-treatment, we confirmed that
396 both doses of ghrelin increase the number of c-Fos+ cells in the ARH (9±2, 44±10 and 129±11 for
397 vehicle, 60 pmol/g of ghrelin and 600 pmol/g of ghrelin, respectively. One way ANOVA, [$F_{treat}(2,13)$
398 = 26.11, p<0.0001]), as previously shown ²⁰. Conversely, only the high dose of ghrelin increased
399 the number of c-Fos+ cells in the PVH (7±2, 8±1 and 153±23 cells/side for vehicle, 60 pmol/g of
400 ghrelin and 600 pmol/g of ghrelin, respectively. One way ANOVA, [$F_{treat}(2,13)$ = 102.2, p<0.0001],
401 **Figure 4A**). Next, we tested if systemically-injected ghrelin reaches the CSF in our experimental
402 conditions. At 30-min post-treatment, ghrelin was undetectable in the CSF of vehicle-injected mice,
403 as we reported in the past ³⁵, as well as in the CSF of mice injected with 60 pmol/g BW of ghrelin,
404 whereas we detected 29.9±12.3 pg/mL of ghrelin in the CSF of mice injected with 600 pmol/g BW
405 of ghrelin. To assess if the elevation of ghrelin in the CSF could impact the PVH, we systemically-
406 injected 600 pmol/g BW of F-ghrelin in mice and estimated the presence of tracer in this
407 hypothalamic area using immunostaining (**Figure 4B**). As previously reported, fluorescein signal
408 was mainly detected at the ARH of F-ghrelin injected mice (not shown, ²⁰). In the PVH, quantitative
409 analysis of optical intensity in the medial-lateral axis at the level of the PVH showed that the F-
410 ghrelin signal was higher at all distances from 0 to ~70 µm from the ventricle wall as well as at
411 some, but not all, distances comprised between the ~70 and 130 µm range, as compared to

412 vehicle-treated mice (**Figure 4C**). F-ghrelin signal beyond ~130 μm away from the ventricle wall
413 showed no significant difference between mice injected with vehicle or F-ghrelin. Thus, a high dose
414 of systemically-injected ghrelin is required to investigate the direct effects of the hormone on the
415 PVH.

416
417 4) Systemically-injected ghrelin acts on PVH^{OXT/NOS1} and PVH^{NOS1} neurons. Next, we tested
418 if the high dose of systemically-injected ghrelin activates PVH^{OXT/NOS1}, PVH^{NOS1} and PVH^{OXT}
419 neurons. We found that ghrelin induced a significant increase in the fraction of PVH^{OXT/NOS1}
420 (26.7 ± 5.2 vs. $1.0\pm 0.6\%$ of all PVH^{OXT/NOS1}) and PVH^{NOS1} (25.1 ± 8.3 vs. $0.0\pm 0.0\%$ of all PVH^{NOS1})
421 neurons positive for c-Fos, as compared to vehicle treatment ($p < 0.05$ for all cases, Student's t-test)
422 but did not affect the fraction in PVH^{OXT} neurons positive for c-Fos (6.2 ± 2.1 vs. $0.2\pm 0.2\%$ of all
423 PVH^{OXT}). The high dose of ghrelin increased the mRNA levels of c-Fos in the PVH at 30-min post-
424 treatment (1.00 ± 0.26 vs. 2.71 ± 0.86 , $p < 0.05$, Student's t-test), but it did not affect mRNA levels of
425 *Nos1* and *Oxt* (1.00 ± 0.19 - vs. 1.11 ± 0.17 -fold and 1.00 ± 0.10 - vs. 0.93 ± 0.09 -fold, respectively) as
426 compared to vehicle treatment.

427
428 In order to test if the ARH is required for the ghrelin-induced increase of c-Fos in
429 PVH^{OXT/NOS1} and PVH^{NOS1} neurons, we assessed c-Fos induction in the hypothalamus of ARH-
430 intact and ARH-ablated mice systemically-injected with 600 pmol/g BW of ghrelin (**Figure 5A**).
431 Here, we separately estimated the fraction of PVH^{OXT}, PVH^{OXT/NOS1} and PVH^{NOS1} neurons positive
432 for c-Fos in the periventricular and lateral regions of the PVH, since these regions were differentially
433 reached by systemically-injected F-ghrelin. Here, quantitative analysis indicated that all quantified
434 PVH^{OXT/NOS1}, PVH^{NOS1} and PVH^{OXT} neurons of vehicle-treated ARH-intact and ARH-ablated mice
435 lacked c-Fos. We found that ghrelin treatment increased the fraction of c-Fos+ PVH^{OXT/NOS1}
436 neurons in both regions of PVH of ARH-intact mice, as compared to basal levels, but not in ARH-
437 ablated mice, in which the fractions of c-Fos+ PVH^{OXT/NOS1} neurons were not different than zero
438 (**Figure 5B-C**). Conversely, the high dose of ghrelin induced a similar increase in the fraction of c-
439 Fos+ PVH^{NOS1} neurons in each region of PVH of ARH-intact and ARH-ablated mice (**Figure 5D-
440 E**). Ghrelin did not affect the fraction of PVH^{OXT} neurons positive for c-Fos in any region of the PVH
441 of ARH-intact and ARH-ablated mice (**Figure 5F-G**). Thus, a high dose of systemically-injected
442 ghrelin activates PVH^{NOS1} via ARH-independent mechanisms and the PVH^{OXT/NOS1} neurons via
443 ARH-dependent mechanisms.

444
445 5) Ghrelin-induced activation of hypophysiotropic PVH^{CRH} neurons involves NO signaling.
446 Centrally and systemically-injected ghrelin activates PVH^{CRH} neurons in mice, in an ARH-

447 independent manner^{22,24}. Since NO activates PVH^{CRH} neurons^{49,50}, we hypothesized that ghrelin-
448 induced activation of PVH^{NOS1} and PVH^{OXT/NOS1} neurons could contribute to mediate the stimulatory
449 action of systemically-injected ghrelin on the PVH^{CRH} neurons. Thus, we tested if ghrelin affects
450 calcium levels in PVH^{CRH} neurons using fiber photometry-based calcium assessment in a reporter
451 mouse model that expresses GCaMP8m selectively in such neuron type. We found that 60 pmol/g
452 BW of ghrelin does not affect the calcium signal in PVH^{CRH} neurons, whereas 600 pmol/g BW of
453 ghrelin increased it at the 10- to 20 min post-treatment time period (**Figure 6A-C**). Notably,
454 simultaneous injection of ghrelin and the NOS inhibitor L-NAME fully abrogated the ghrelin-induced
455 increase of calcium signal in PVH^{CRH} neurons. Additionally, systemically-injected ghrelin did not
456 affect *Crh* mRNA levels in the PVH at 30 min post-treatment (not shown), and we found no
457 evidence that L-NAME affects such ghrelin-induced increase of c-Fos in the PVH (**Figure 6D**).
458 However, the high dose of ghrelin increased plasma corticosterone concentration in mice treated
459 or not with L-NAME, as compared with their control groups; but the increment was smaller in mice
460 treated with ghrelin and L-NAME (**Figure 6E**) suggesting that L-NAME-mediated impairment of the
461 ghrelin-induced activation of PVH^{CRH} neurons affects the overall activation of the HPA axis.

462 DISCUSSION

463

464 Here, we provide a neuroanatomical and functional characterization of PVH^{GHSR} neurons in
465 male mice. First, we describe that mouse PVH^{GHSR} neurons include PVH^{OXT/NOS1} and PVH^{NOS1}
466 neurons. Then, we show that centrally-injected ghrelin activates PVH^{OXT/NOS1} and PVH^{NOS1}
467 neurons. Conversely, a high, but not a low, dose of systemically-injected ghrelin is required to
468 reach the CSF and further act into the PVH. Such high dose of systemically-injected ghrelin
469 activates PVH^{OXT/NOS1} and PVH^{NOS1} neurons, and ghrelin-induced activation of PVH^{NOS1} neurons
470 occurs in an ARH-independent manner. Thus, only PVH^{NOS1} neurons may be able to directly
471 respond to plasma ghrelin. Finally, we found that pharmacological inhibition of NO production
472 impairs ghrelin-induced activation of PVH^{CRH} neurons suggesting that PVH^{NOS1} neurons play a role
473 mediating the central stimulatory effects of ghrelin on the HPA axis.

474

475 We found that PVH^{GHSR} neurons are mainly PVH^{OXT/NOS1} neurons (~33%) or PVH^{NOS1}
476 neurons (~10%) whereas smaller subsets of them are PVH^{OXT} neurons (~8%), PVH^{AVP} neurons
477 (~7%), PVH^{GAD2} neurons (~1%) or PVHTH neurons (~1%). Thus, we revealed the phenotype of
478 ~60% of PVH^{GHSR} neurons. The phenotype of the remaining PVH^{GHSR} neurons remains unknown
479 but may include glutamatergic neurons, which are enriched in the PVH⁵¹⁻⁵⁴. We estimated that the
480 mouse PVH contains ~2000 PVH^{NOS1} neurons and ~1500 PVH^{OXT} neurons, slightly above what
481 was reported in previous reports^{6,11,55}. Among these neurons, we found that PVH^{OXT/NOS1} neurons
482 represent ~84% of all PVH^{OXT} neurons and ~63% of all PVH^{NOS1}, estimations that agree with
483 previous reports in mice¹¹ and in rats, in which the majority of the magnocellular PVH^{OXT} are
484 PVH^{OXT/NOS1} neurons that, in turn, constitute the main PVH^{NOS1} cell type^{8,56-58}. Here, we found Fr-
485 ghrelin labels ~370 NOS1+ neurons (including PVH^{OXT/NOS1} and PVH^{NOS1} neurons) and presumably
486 activates them since centrally-injected ghrelin induces c-Fos expression in both neuron types as
487 well as NOS1 phosphorylation that enhances its enzymatic activity^{47,48}. Thus, it seems reasonable
488 to hypothesize that ghrelin induces local PVH NO production, although this parameter was not
489 assessed. We could not identify if Fr-ghrelin labels magnocellular or parvocellular PVH neurons,
490 as these cell types are intermingled in the mouse PVH and are hard to discriminate without
491 additional assessments⁶. However, the analysis of a scRNAseq dataset revealed that *Ghsr*
492 expression is enriched in magnocellular PVH^{OXT} neurons that, in turn, are mainly PVH^{OXT/NOS1}
493 neurons⁴². Thus, current neuroanatomical and transcriptomic analyses indicate that ghrelin can
494 directly target subsets of PVH^{OXT/NOS1} and PVH^{NOS1} neurons. Of note, neuroanatomical analyses
495 indicated that ghrelin targets larger fractions of each neuronal subset as compared to the
496 estimations based on the transcriptomic analyses. Neuroanatomical analyses are presumably

497 more reliable as they are based on a larger sample size (~1800 cells/mouse in 5 mice vs. 146 total
498 cells). Transcriptomic analyses, in turn, have likely underestimated the number of *Ghsr*-expressing
499 cells due to low gene expression/copy number of *Ghsr* compared to a typical single-cell sequencing
500 depth. Indeed, low recovery rates of *Ghsr*-expressing cells in other available scRNAseq datasets
501 that included hypothalamic cells^{53,59} prevented us from performing additional analysis of the
502 subsets of PVH neurons directly responsive to ghrelin.

503

504 Our results indicate that high plasma ghrelin concentrations, as those detected under
505 calorie restricted mice^{20,36}, are required to activate NO-producing neurons of the PVH. In particular,
506 we found that only high (~16.8-fold at 30-min post-treatment), but not low transient elevations in
507 plasma ghrelin concentration increase c-Fos expression in the entire PVH and specifically in
508 PVH^{OXT/NOS1} neurons and PVH^{NOS1} neurons. The observations that only the administration of the
509 higher dose of systemically-injected ghrelin (or F-ghrelin) induces detectable levels of ghrelin in
510 the CSF, and leads to the presence of the tracer in the periventricular region of the PVH, with a
511 medial-to-lateral diffusion pattern, suggest that ghrelin needs to cross the blood-CSF-barrier and
512 reach the CSF in order to act in the PVH, as we have shown for other brain targets²⁷. Notably, we
513 also found here that centrally-injected fluorescent ghrelin labels fibers in the periventricular region
514 of the PVH. Although the identity of these fibers was not revealed, a previous study showed that
515 magnocellular PVH^{OXT/NOS1} neurons of rats send processes that break through the ependymal layer
516 of the third ventricle and contact the CSF⁶⁰. Thus, ghrelin-induced activation of the PVH may also
517 involve some direct sensing of ghrelin present in the CSF. At the functional level, we found that
518 systemically-injected ghrelin increases c-Fos in PVH^{NOS1} neurons, which are not OXT+ cells, via
519 ARH-independent mechanisms, whereas it increases c-Fos in PVH^{OXT/NOS1} neurons via ARH-
520 dependent mechanisms, despite centrally-injected Fr-ghrelin labels a subset of both types of NO-
521 producing neurons. The ARH-dependent ghrelin-induced activation of PVH^{OXT/NOS1} neurons may
522 involve ARH^{AgRP/NPY} neurons, which are enriched in GHSR, sense plasma ghrelin and densely
523 innervate the PVH³⁰⁻³³. ARH^{AgRP/NPY} neurons target a subset of NOS1-expressing PVH neurons
524 that expresses receptors for AgRP and NPY^{61,62}. Also, ARH^{AgRP/NPY} neurons innervate and inhibit
525 a subset of PVH^{OXT} neurons, which mediate the orexigenic effects of ARH^{AgRP/NPY} neuronal
526 activation³⁰. The low dose of systemically-injected ghrelin also induces c-Fos in the ARH^{AgRP/NPY}
527 neurons and feeding³⁶ but such effect does not seem to be sufficient to induce c-Fos in the PVH,
528 an observation that is in line with the concept that selective activation of ARH^{AgRP/NPY} neurons
529 results in inhibition of food intake regulating PVH neurons³⁰. These observations, together with
530 previous studies²⁴, support the notion that high doses of systemically-injected ghrelin targets a
531 subset of PVH neurons to activate HPA axis that is different than the subset of PVH neurons

532 recruited by ARH^{AgRP/NPY} neurons to induce food intake. Thus, ghrelin-mediated recruitment of
533 PVH^{OXT/NOS1} and PVH^{NOS1} neurons seems to involve not only the local action of the hormone but
534 also additional indirect mechanisms that remain to be studied.

535
536 It is well documented that ghrelin infusion increases plasma cortisol concentration in
537 humans ^{63–65}. In mice, centrally- and systemically-injected ghrelin increases c-Fos in PVH^{CRH}
538 neurons and plasma corticosterone levels ²². Such ghrelin-induced activation of the PVH^{CRH}
539 neurons in mice is indirect, since PVH^{CRH} neurons lack GHSR ^{17,22} (as confirmed here), and does
540 not require the ARH integrity, as it is observed in ARH-ablated mice ²⁴. Even so, the molecular
541 bases mediating ghrelin-induced activation of mouse PVH^{CRH} neurons remain uncertain. Previous
542 studies in mice showed that ICV infusion of ghrelin increases NOS activity in the hypothalamus
543 and that L-NAME reduces ghrelin-induced food intake ^{66,67}. Also, NOS1 knockout (KO) mice do not
544 increase food intake in response to ghrelin ⁶⁸, and NOS1 inhibition blocks ghrelin's ability to induce
545 reward-related behaviors in rats ⁶⁹. Besides, several evidences indicate that NO activates PVH^{CRH}
546 neurons. For instance, NO depolarizes rat type II PVH neurons that include the hypophysiotropic
547 PVH^{CRH} neurons ^{70,71}. In rat PVH cultures, NO induces CRH release and NOS inhibitors reduce
548 CRH release induced by noradrenaline, IL-1beta, IL-2 and cholinergic agonists ^{50,72,73}. HPA axis
549 activation in rats induced by adrenergic receptor agonists is reduced by L-NAME or 7-nitroindazole,
550 a specific NOS1 inhibitor ⁷⁴. The observations that systemically-injected ghrelin can reach the PVH
551 and activate NOS1+ neurons led us to hypothesize that NO could mediate ghrelin-induced
552 activation of the PVH^{CRH} neurons. Here, we found that L-NAME fully abrogates ghrelin-induced
553 increase of calcium levels in PVH^{CRH} neurons and partially reduces ghrelin-induced increase of
554 plasma corticosterone levels suggesting that indeed ghrelin-induced activation of the HPA axis
555 involves NO signaling. Ghrelin may increase corticosterone levels, in part, via the PVH^{AVP} neurons,
556 a fraction of which is labeled by Fr-ghrelin and control the adrenocorticotrophic hormone release
557 from the pituitary ⁷⁵. Also, it is possible that different experimental conditions could have made
558 more evident the role of NO signaling on the HPA axis activation at systemic level (e.g., use of
559 selective NOS1 inhibitors, higher doses, chronic treatment, etc.). Strikingly, the lack of endogenous
560 ghrelin signaling in genetically-modified mice was found to either impair or enhance the activation
561 of the PVH^{CRH} neurons ^{17,76,77}. Thus, future studies are required to further investigate the extent to
562 which current observations in non-stressed mice treated with ghrelin also take place when
563 endogenous ghrelin levels fluctuate under physiological or stress conditions.

564
565 Ghrelin targets different PVH neurons and affects a variety of neurobiological mechanisms
566 within this hypothalamic area ^{12,78}. Here, we propose that ghrelin action in the mouse PVH results

567 in production of NO that has important functional implications at a local level. The NO rapidly
568 diffuses through the brain tissue and affects neighboring cells in a radius of ~150 μm ^{79,80}. Indeed,
569 selective activation of NOS1-expressing neurons of the PVH using pharmacogenetics results in c-
570 Fos induction not only in NOS-expressing cells but also in neighboring PVH neurons ¹¹. Thus, it is
571 likely that the release of NO by ~800 NOS1+ cells within the PVH in response to systemically-
572 injected ghrelin (as estimated based on c-Fos induction) is sufficient to impact on a significant
573 number of neurons and terminals within the nucleus. Here, we systemically-injected L-NAME, but
574 the short half-life of NO (~1-seg ⁷⁹) makes very unlikely that extra-PVH NO could impact on our
575 observations. It remains to be established if the putative release of NO affects the PVH^{CRH} neurons
576 directly or indirectly, via regulation of GABA inputs innervating them that are known to integrate
577 multiple signals and shape PVH^{CRH} neurons activity ^{81,82}. PVH GABA inputs are susceptible to NO
578 since the intra-PVH injections of a GABAA receptor antagonist prevent the sympathetic effects of
579 intra-PVH injected NO donors ⁸³. Of note, we found that systemically-injected ghrelin does not
580 affect *Nos1* mRNA levels in the PVH, but rather increases NOS1 protein phosphorylation. Also,
581 systemically-injected ghrelin increased intracellular calcium in PVH^{CRH} neurons but not affect *Crh*
582 mRNA levels in the PVH, unlike our previous study showing that ICV-injected ghrelin increases
583 *Crh* mRNA levels in the PVH ²². Conversely, systemically-injected ghrelin increases *c-Fos* mRNA
584 levels in the PVH. Thus, it seems that systemic ghrelin treatment primarily induces rapid regulatory
585 effects in the signaling pathways of the PVH but not gene expression, which was restricted to c-
586 Fos, a transcription factor that likely modulates later transcriptional effects in PVH neurons.

587
588 In summary, current studies help to gain insights regarding the neurobiological basis by
589 which ghrelin acts in the PVH and activates the HPA axis in male mice. It is important to highlight
590 that the current study was performed on exclusively males because ghrelin is known to display
591 stronger orexigenic effects in males than in females, in both rats and mice ^{84,85}. However, the HPA
592 axis usually mediates more rapid and greater responses in females ⁸⁶. Thus, future studies should
593 be performed to investigate if the reported observations regarding to the action of ghrelin in the
594 PVH involve any type of sex dependent differences.

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605 **Disclosure Statement:** The authors have nothing to disclose.

606 **Data Availability**

607

608 Some or all datasets generated during and/or analyzed during the current study are not publicly
609 available but are available from the corresponding author on reasonable request.

610

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- 849

850 **LEGENDS**

851 **Figure 1. A.** Schematic diagrams from the Mouse Brain Atlas ⁴⁵ showing coronal brain sections
852 containing different rostro-caudal levels of the PVH (between bregma -0.58 mm and -1.22 mm). **B.**
853 Representative images of coronal brain sections containing the PVH of WT mice ICV-injected with
854 Fr-ghrelin (pseudo-colored to cyan). Subdivisions of the PVH are overlaid following the
855 delineation described in the Mouse Brain Atlas ⁴⁵. fx: fornix, PaAP: PVH anterior parvicellular part,
856 PaDC: PVH dorsal cap, PaLM: PVH lateral magnocellular part, PaMM: PVH medial magnocellular
857 part, PaMP: PVH medial parvicellular part, PaPo: PVH posterior part, PaV: PVH ventral part, pe:
858 periventricular hypothalamic nucleus, V: third ventricle. **C-E.** Representative high magnification
859 images of Fr-ghrelin+ cells in the PVH (**C**) and fiber-like structures in the periventricular region of
860 the PVH of WT mice ICV-injected with Fr-ghrelin (**D-E**). Arrows point to Fr-ghrelin+ cells and arrow-
861 heads point to Fr-ghrelin+ fiber-like structures. **F.** Representative images of brain coronal sections
862 containing the PVH of *Gad2^{tdTomato}* (red) or WT mice ICV-injected with Fr-ghrelin subjected to a
863 fluorescent immunostaining against diverse neuropeptides including CRH, TRH, AVP, NT, TH,
864 OXT or NOS1 (red), respectively. Insets depict high magnification of areas marked in low
865 magnification images. Arrows point dual-labelled cells (Fr-ghrelin/OXT+ or Fr-ghrelin/NOS1+) and
866 arrow-heads point single-labelled cells (OXT+ or NOS1+). Scale bars: 100 μ m (low magnification)
867 and 20 μ m (high magnification). Cell nuclei labelled with Hoechst (blue).

868
869 **Figure 2. A.** Representative images of coronal brain sections containing the PVH of WT
870 mice ICV-injected with Fr-ghrelin (pseudo-colored to cyan) and subjected to a double-fluorescent
871 immunostaining against OXT (red) and NOS1 (green). Inset depict a high magnification of the area
872 marked in low magnification image. Arrows point to triple-labelled cells (Fr-ghrelin/OXT/NOS1+).
873 Scale bars: 50 μ m (low magnification) and 20 μ m (high magnification). Cell nuclei labelled with
874 Hoechst (blue). **B.** Venn diagram of Fr-ghrelin+, OXT+ and NOS1+ cells in the PVH of mice. **C-E.**
875 Plots of relative gene expression of *Ghsr* (**C**), *Oxt* (**D**) and *Nos1* (**E**) in neurons classified as
876 magnocellular or parvocellular, taken from the dataset of Lewis *et al.* ⁴². Cyan circles represent
877 neurons with non-zero relative expression (at least one read) for *Ghsr*. **F.** Venn diagram of
878 magnocellular neurons from the same dataset, classified as *Ghsr+*, *Oxt+* and/or *Nos1+*
879 presenting a relative expression greater than zero for each respective gene. Venn diagrams were
880 generated using eulerr (<https://cran.r-project.org/package=eulerr>) ⁸⁷ in order to have the areas of
881 circles and their intersections proportional to the number of neurons in each subgroup.

882
883 **Figure 3. A.** Representative images of brain coronal sections containing the PVH of WT
884 mice ICV-injected with vehicle or ghrelin, respectively, subjected to a triple-immunostaining against

885 c-Fos (white), OXT (red) or NOS1 (green). Insets depict a high magnification image of the areas
886 marked in low magnification images. Arrows point to triple-labelled cells (c-Fos/OXT/NOS1+ cells)
887 and arrow-heads point c-Fos+ cells. Scale bars: 50 μm (low magnification) and 20 μm (high
888 magnification). Cell nuclei labelled with Hoechst (blue). **B.** Representative greyscale images of
889 coronal brain sections containing the PVH of WT mice ICV-injected with vehicle or ghrelin,
890 respectively, perfused 15 min later and subjected to a chromogenic immunostaining against
891 phospho-NOS1. Insets depict a high magnification image of the areas marked in low magnification
892 images. Arrows point to phospho-NOS1+ cells. Scale bars: 50 μm (low magnification) and 20 μm
893 (high magnification). **C.** Bar graph displaying the quantitative analysis of the number of phospho-
894 NOS1+ cells in the PVH of WT mice ICV-injected with vehicle or ghrelin, respectively, and perfused
895 15, 30 or 120 min later. Data represent the mean \pm SEM and were compared by One-way ANOVA
896 [$F_{\text{treat}}(3,9) = 59.06, p < 0.0001$] followed by Tukey's multiple comparisons tests. ***, $p < 0.001$ vs
897 vehicle-treated group.

898
899 **Figure 4. A.** Representative images of coronal brain sections containing the PVH of WT
900 mice SC-injected with vehicle, 60 pmol/g BW or 600 pmol/g BW of ghrelin, respectively, and
901 subjected to a chromogenic immunostaining against c-Fos (black/purple). Scale bars: 100 μm . **B.**
902 Pseudo-colored images of coronal brain sections containing the PVH of WT mice SC-injected with
903 vehicle or F-ghrelin 600 pmol/g BW, respectively, and subjected to a chromogenic immunostaining
904 against fluorescein. Scale bars: 100 μm . **C.** Line graph displaying the quantitative analysis of the
905 optical density signal in a 300 μm box length placed over the PVH as shown in panel B. Data
906 represent the mean \pm SEM and were compared by unpaired Student's t-test with Welch correction.
907 *, $p < 0.05$ vs vehicle-treated group.

908
909 **Figure 5. A.** Representative images of coronal brain sections containing the PVH of ARH-
910 intact or ARH-ablated mice SC-injected with vehicle or 600 pmol/g BW of ghrelin, respectively,
911 subjected to a triple-immunostaining against c-Fos (white), OXT (red) or NOS1 (green). Insets
912 depict a high magnification image of the areas marked in low magnification images. Arrows point
913 to triple-labelled cells (c-Fos/OXT/NOS1+ cells) and arrow-heads point to c-Fos+ cells. Scale bars:
914 50 μm (low magnification) and 20 μm (high magnification). Cell nuclei labelled with Hoechst (blue).
915 **B-G.** Bar graphs displaying the quantitative analysis of the percentage of PVH^{OXT/NOS1}, PVH^{NOS1}
916 and PVH^{OXT} neurons positive for c-Fos in the periventricular (**B, D and F**) or lateral region (**C, E**
917 and **G**) of the PVH, respectively, of ARH-intact or ARH-ablated mice SC-treated with 600 pmol/g
918 BW of ghrelin. Data represent as mean \pm SEM. Data of each neuronal set and in each PVH region
919 were compared using both one-sample t-test vs. 0 %. to test if groups responded to ghrelin

920 (comparisons labeled in red **, $p < 0.01$ and *, $p < 0.05$), and unpaired Student's t-test to test if the
921 magnitude of responses were different (comparison labeled in blue #, $p < 0.05$).

922

923 **Figure 6. A.** Schematic view of the fiber photometry system used for recording GCaMP8m-
924 expressing CRH neurons signal in the mouse PVH. **B.** Line graph displaying the quantitative
925 analysis of the relative changes in fluorescence in the PVH of Crh-cre mice intra-PVH injected with
926 a AAV.DIO-GCaMP8m virus and IP-treated with vehicle, ghrelin 60 pmol/g BW, ghrelin 600 pmol/g
927 BW or ghrelin 600 pmol/g BW + L-NAME. The arrow indicates the time at which IP injections were
928 performed and the dotted line indicates the time period in which fluorescence was analyzed and
929 compared (shown in C). **C.** Bar graph showing the quantitative analysis of the relative changes in
930 fluorescence from 10 to 20 min after IP-treatment in B. Data represent the mean \pm SEM and were
931 compared by One-way ANOVA [$F_{treat}(3,16) = 2.494$, $p = 0.0971$] followed by Fisher's LSD test (*,
932 $p < 0.05$). **D-E.** Bar graphs displaying the quantitative analysis of the total number of c-Fos+ cells in
933 the PVH (**D**) and plasma corticosterone levels (**E**) of WT mice SC-treated with vehicle, L-NAME
934 (10 μ g/g BW), ghrelin (600 pmol/g BW), or ghrelin (600 pmol/g BW) + L-NAME (10 μ g/g BW). Data
935 represent the mean \pm SEM and were compared by One-way ANOVA (in **D** [$F_{treat}(3,14) = 13.42$,
936 $p = 0.0002$]); in **E** [$F_{treat}(3,25) = 64.53$, $p < 0.0001$] followed by Tukey's multiple comparisons tests.
937 ***, $p < 0.001$ and **, $p < 0.01$, vs vehicle- and L-NAME-treated group; #, $p < 0.05$, vs ghrelin-treated
938 group.

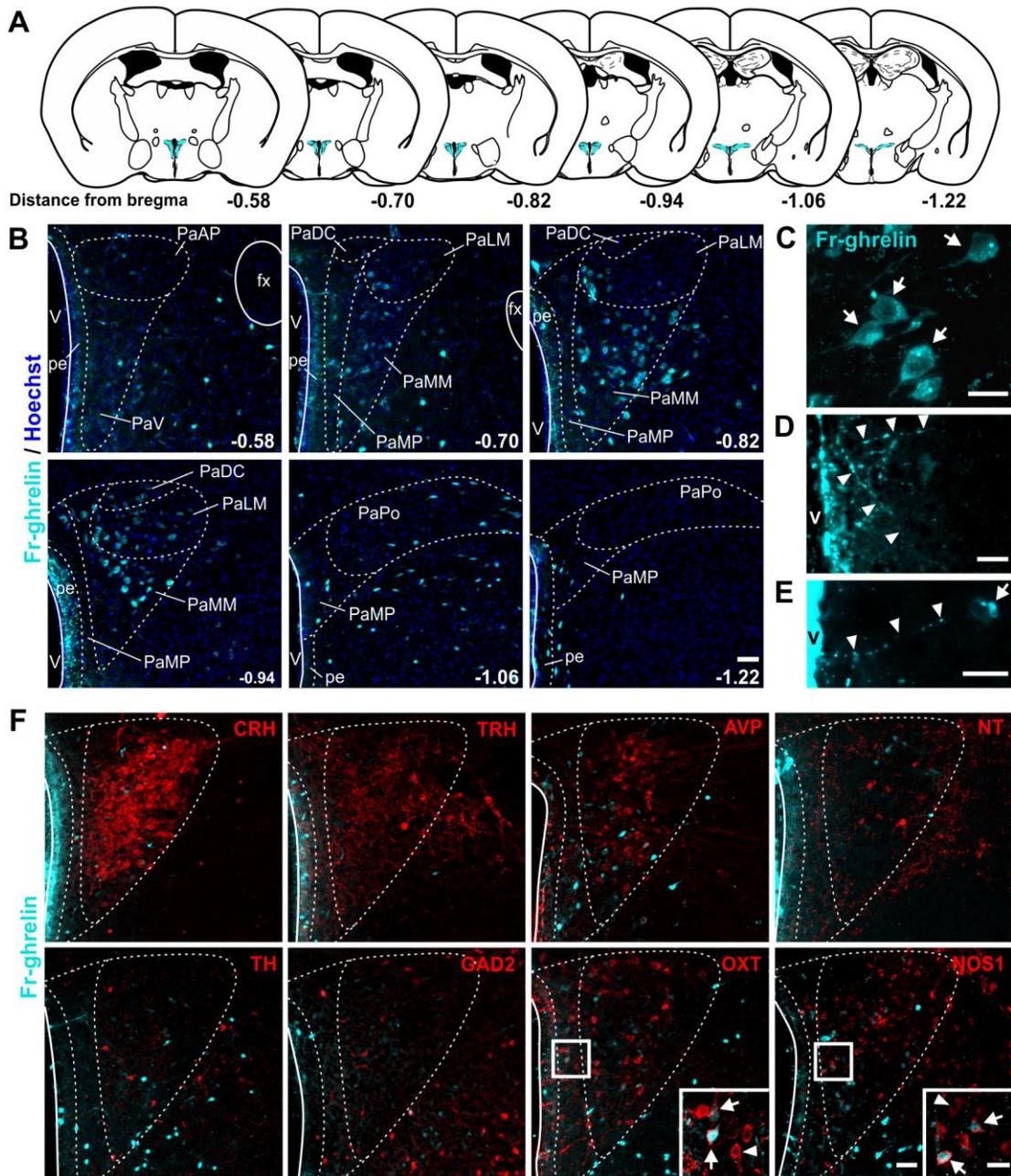


Figure 1

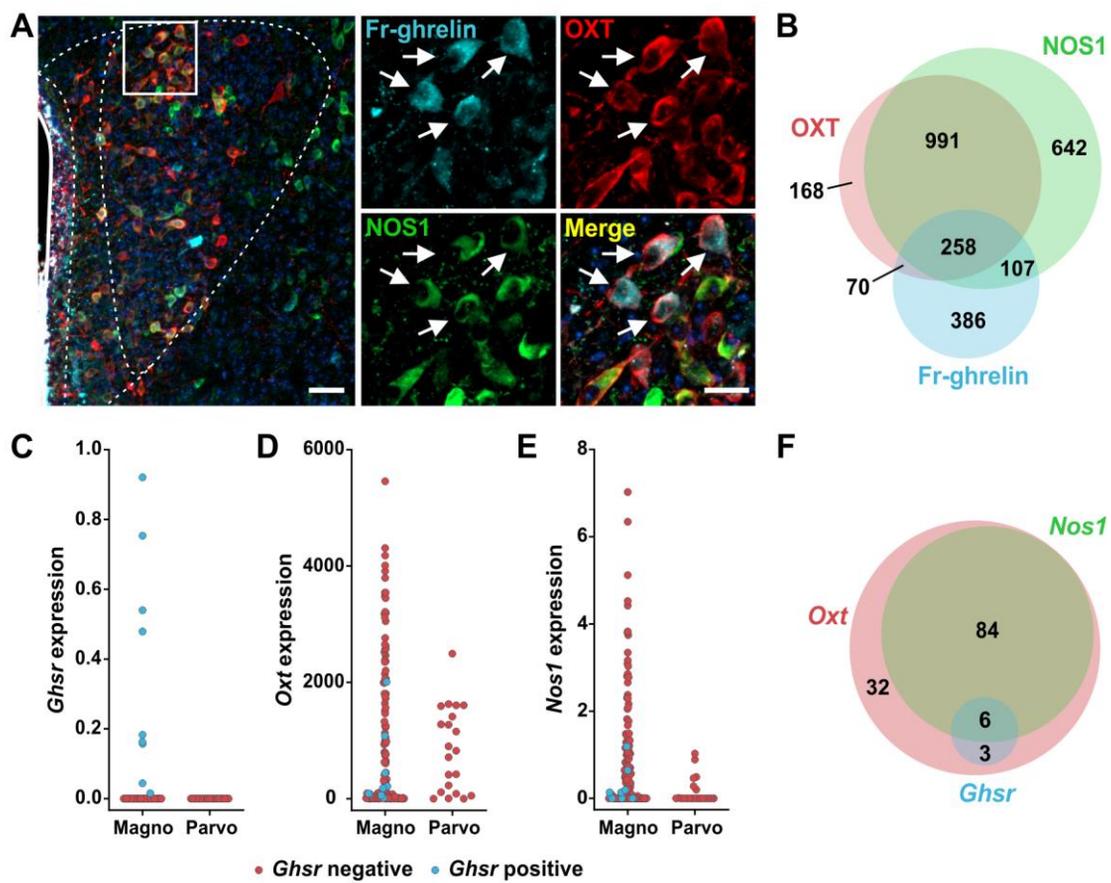


Figure 2

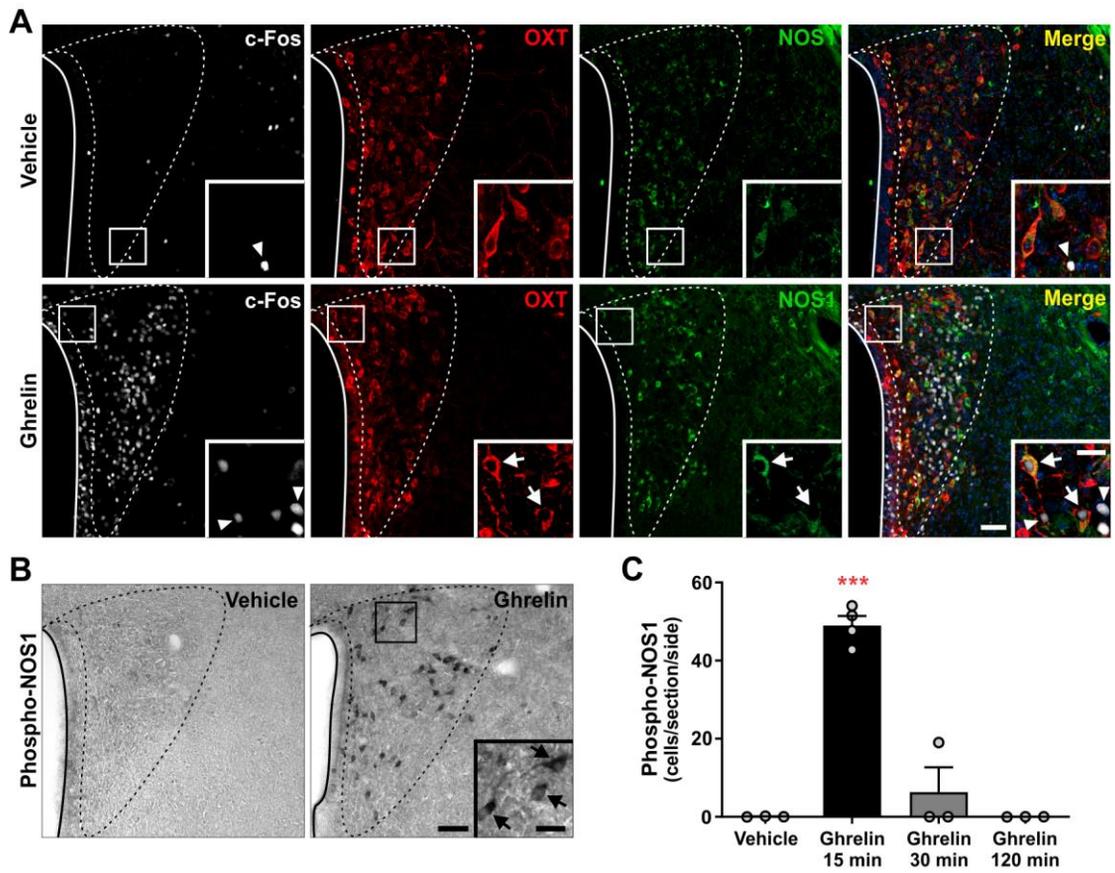


Figure 3

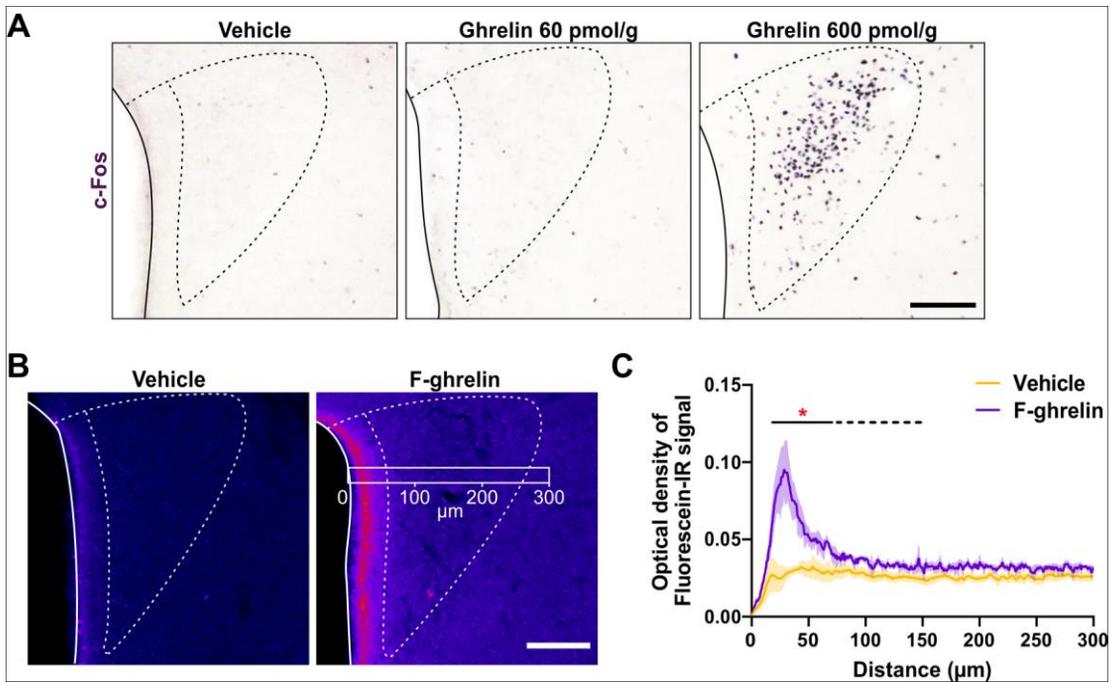


Figure 4

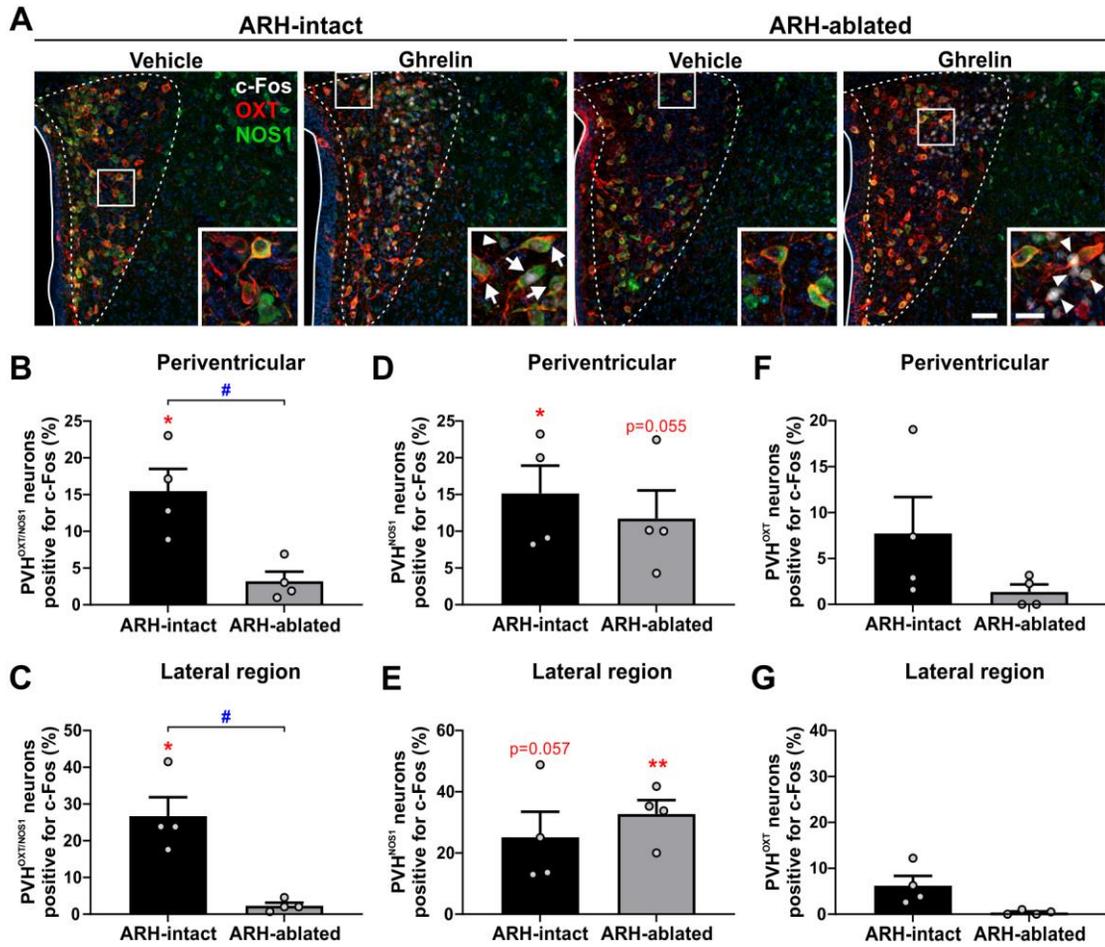


Figure 5

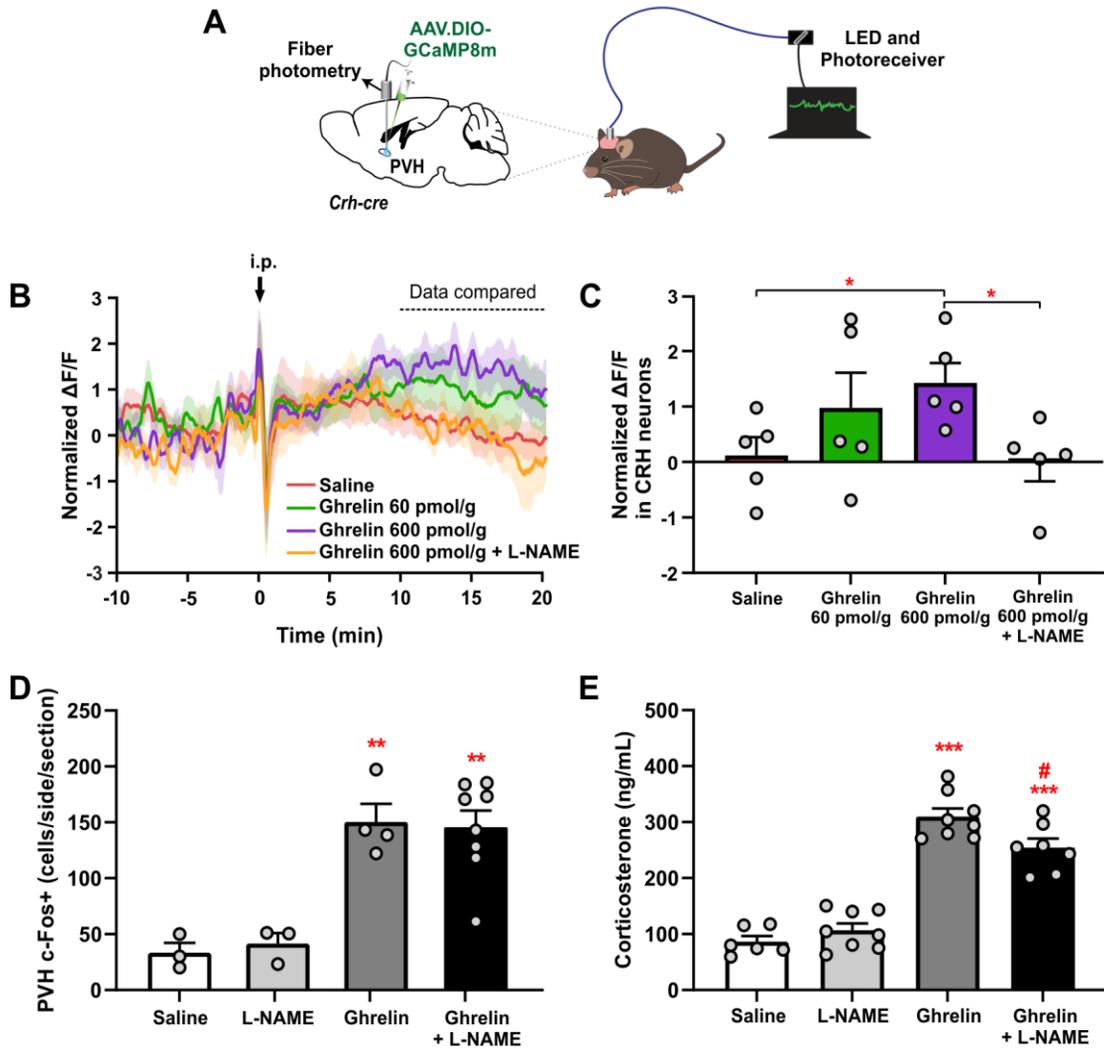


Figure 6