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**Complexes of ghrelin GHS-R1a, GHS-R1b and dopamine D₁ receptors
localized in the ventral tegmental area as main mediators of the dopaminergic
effects of ghrelin**

Abbreviated title: Ghrelin-dopamine receptor oligomers in the VTA

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36

37

38 **Abstract**

39 Ghrelin receptor, also known as growth hormone secretagogue receptor or GHS-R1a, is co-
40 expressed with its truncated isoform GHS-R1b, which does not bind ghrelin or signal, but
41 oligomerizes with GHS-R1a, exerting a complex modulatory role that depends on its relative
42 expression. D₁ and D₅ dopamine receptors (D1R and D5R) constitute the two D₁-like receptor
43 subtypes. Previous studies showed GHS-R1b also facilitates oligomerization of GHS-R1a with
44 D1R, conferring GHS-R1a distinctive pharmacological properties. Those include a switch in the
45 preferred coupling of GHS-R1a from G_q to G_s and the ability of D1R/D5R agonists and
46 antagonists to counteract GHS-R1a signaling. Activation of ghrelin receptors localized in the
47 ventral tegmental area (VTA) seems to play a significant role in ghrelin's contribution to
48 motivated behavior. In view of the evidence indicating that dopaminergic cells of the VTA
49 express ghrelin receptors and D5R but not D1R, we investigated the possible existence of
50 functional GHS-R1a:GHS-R1b:D5R oligomeric complexes in the VTA. GHS-R1a:GHS-
51 R1b:D5R oligomers were first demonstrated in mammalian transfected cells and their
52 pharmacological properties were found to be different from those of GHS-R1a:GHS-R1b:D1R
53 oligomers, including weak G_s coupling and the ability of D1R/D5R antagonists, but not agonists,
54 to counteract the effects of ghrelin. However, analyzing the effect of ghrelin in the rodent VTA
55 on MAPK activation with *ex vivo* experiments, on somato-dendritic dopamine release with *in*
56 *vivo* microdialysis and on activation of dopaminergic cells with patch-clamp electrophysiology,
57 provided evidence for a predominant role of GHS-R1a:GHS-R1b:D1R oligomers in the rodent
58 VTA as main mediators of the dopaminergic effects of ghrelin.

59

60

61 **Significance statement**

62 The activation of ghrelin receptors localized in the ventral tegmental area (VTA) plays a
63 significant role in ghrelin's contribution to motivated behavior. We present evidence that
64 indicates these receptors form part of oligomeric complexes that include the functional ghrelin
65 receptor GHS-R1a, its truncated non-signaling isoform GHS-R1b and the dopamine D₁ receptor
66 (D1R). Binding of ghrelin to these complexes promotes activation of the dopaminergic neurons
67 of the VTA by activation of AC-PKA signaling, which can be counteracted by both GHS-R1a
68 and D1R antagonists. Our study provides evidence for a predominant role of GHS-R1a:GHS-
69 R1b:D1R oligomers in rodent VTA as main mediators of the dopaminergic effects of ghrelin.

71 **Introduction**

72 The orexigenic hormone ghrelin acts as an internal signal for animals to engage in food-directed
73 behaviors (Silver and Balsam, 2010; Mason et al., 2014; Al Massadi et al., 2019). It is mainly
74 produced by the stomach oxyntic cells during anticipated mealtimes and its blood levels decrease
75 after meals (Silver and Balsam, 2010). The hypothalamic arcuate nucleus is a major site of
76 expression for ghrelin receptors in the brain, specifically in the cells that synthesize the
77 neuropeptide agouti-related-peptide. Ghrelin reaches the arcuate nucleus by its proximity to the
78 median eminence, where the blood brain barrier is more permissive and allows the passage of
79 peptides (reviewed in Ferré, 2017). Agouti-related-peptide neurons project to several
80 hypothalamic nuclei, as well as to the ventral tegmental area (VTA), and their activation
81 promotes feeding (Aponte et al., 2011; Krashes et al., 2011; Betley et al., 2013). Ghrelin
82 receptors are also expressed in other brain regions, including the hippocampus, substantia nigra,
83 and VTA (Zigman et al., 2006). Several studies suggest that activation of the VTA dopaminergic

84 system plays a significant role in the motivational function of ghrelin (Abizaid et al., 2006;
85 Jerlhag et al., 2007; Skibicka et al., 2011; Al Massadi et al., 2019). The mechanisms proposed to
86 be involved in such activation include direct peripheral transit of ghrelin to the VTA or indirect
87 activation through circuit-level effects of ghrelin from either peripheral or central origin (see
88 Discussion).

89 The ghrelin receptor is a G protein-coupled receptor (GPCR) also known as growth
90 hormone secretagogue receptor (GHSR) or GHS-R1a. Cells expressing GHS-R1a also express
91 GHS-R1b, a truncated isoform lacking transmembrane domains 6 and 7. Although ghrelin is the
92 canonical ligand for GHS-R1a, ghrelin does not bind and does not signal through GHS-R1b.
93 However, GHS-R1b oligomerizes with GHS-R1a exerting an expression-level dependent
94 modulatory role (Navarro et al., 2016). Low relative GHS-R1b expression facilitates GHS-R1a
95 function by promoting its trafficking to the plasma membrane, while a high relative GHS-R1b
96 expression inhibits GHS-R1a function by exerting a negative allosteric effect on GHS-R1a
97 signaling (Mary et al., 2013; Navarro et al., 2016).

98 Another functional role of GHS-R1b is facilitating the oligomerization of GHS-R1a with
99 other GPCRs, specifically with the dopamine D₁ receptor (D1R), which was shown both in
100 mammalian transfected cells and in striatal cells in culture (Navarro et al., 2016). Among the
101 pharmacological properties of the GHS-R1a:GHS-R1b:D1R oligomer, ghrelin promoted
102 adenylyl cyclase (AC) activation, indicating a switch in the usually preferred Gq coupling of
103 GHS-R1a to the preferred Gs coupling of D1R (Navarro et al., 2016). Other studies in
104 mammalian transfected cells and hippocampal neurons have also provided evidence for the
105 ability of GHS-R1a to heteromerize with D1R without the involvement of GHS-R1b, in which
106 case ghrelin did not produce AC activation, while D1R could signal by coupling to Gq (Jiang et

107 al., 2006; Kern et al., 2015; Casanovas et al., 2021). Immunohistochemical experiments in
 108 transgenic mice that express GFP in cells also expressing GHS-R1a indicated a prominent co-
 109 localization of GHS-R1a and D1R in the hippocampus, cortex and in the midbrain, in the
 110 substantia nigra and VTA (Jiang et al., 2006; Kern et al., 2015). Therefore, we investigated if
 111 GHS-R1a:D1R complexes localized in the VTA constitute a significant population of GHS-R1a
 112 receptors mediating the dopaminergic effects of ghrelin.

113 Since some studies indicate that dopamine D₅ receptor (D5R) is the predominant D₁-like
 114 receptor localized in mesencephalic dopaminergic cells (Ciliax et al., 2000; Khan et al., 2000),
 115 we aimed first at analyzing the possible existence of oligomers of GHS-R1a, GHS-R1b and D5R
 116 in mammalian transfected cells and comparing their pharmacological properties to those of GHS-
 117 R1a:GHS-R1b:D1R oligomers. We then evaluated the possible existence of these oligomeric
 118 complexes in the rodent VTA as well as their role in the dopaminergic effects of ghrelin. Using
 119 different *in vitro* and *in vivo* approaches, we obtained evidence for a predominant role of GHS-
 120 R1a:GHS-R1b:D1R, rather than R1a:GHS-R1b:D5R, oligomers in rodent VTA as main
 121 mediators of the dopaminergic effects of ghrelin.

123 **Materials and methods**

124
 125 *Vectors, fusion proteins, cell culture and transfection.* Human cDNAs for GHS-R1a, GHSR1b
 126 and D5R, cloned into pcDNA3.1, were amplified without their stop codons using sense and
 127 antisense primers harboring EcoRI and KpnI sites to clone D5R in the pRLuc-N1 vector (pRLuc-
 128 N1, PerkinElmer Life Sciences) and GHS-R1a or GHS-R1b to the pEYFP-N1 vector (enhanced
 129 yellow variant of GFP, Clontech) or EcoRI and KpnI sites to clone GHS-R1a in a GFP²-

130 containing vector (p-GFP², Packard BioScience, Meriden, CT). Amplified fragments were
131 subcloned to be in-frame with restriction sites of pRLuc-N1, pEYFP-N1, or p-GFP² vectors to
132 provide plasmids that express proteins fused to RLuc, YFP, or GFP² on the C-terminal end
133 (D5R-RLuc, GHS-R1a-YFP, GHS-R1b-YFP and GHS-R1a-GFP²). HEK-293T cells were
134 maintained at 37 °C in an atmosphere of 5% CO₂. They were grown in DMEM (Gibco)
135 supplemented with 2 mM L-glutamine, 100 µg/ml sodium pyruvate, 100 units/ml penicillin/
136 streptomycin, minimum Eagle's medium non-essential amino acid solution (1/100) and 5% (v/v)
137 heat inactivated FBS (all supplements were from Invitrogen). HEK-293T cells were transiently
138 transfected with the corresponding protein cDNA by the PEI (Sigma-Aldrich, St. Louis, MO)
139 method. Cells were incubated for 4 h with the corresponding cDNA together with PEI (5.47 mM
140 in nitrogen residues) and 150 mM NaCl in a serum-starved medium. After 4 h, the medium was
141 changed to a fresh complete culture medium. Forty-eight hours after transfection, cells were
142 washed twice in quick succession in Hanks' balanced salt solution with 10 mM glucose,
143 detached, and resuspended in the same buffer and used for BRET, SRET, cAMP accumulation or
144 ERK1/2 phosphorylation experiments.

145
146 *Resonance energy transfer-based assays.* For bioluminescence resonance energy transfer
147 (BRET) experiments, HEK-293T cells were transiently co-transfected with a constant cDNA
148 encoding for receptor-RLuc and with increasing amounts of cDNA corresponding to receptor-
149 YFP (BRET¹) or receptor-GFP² (BRET²). To control the cell number, the sample protein
150 concentration was determined using a Bradford assay kit (Bio-Rad) using bovine serum albumin
151 dilutions as standards. To quantify fluorescence proteins, cells (20 µg protein) were distributed in
152 96-well microplates (black plates with a transparent bottom) and the fluorescence was read in a

153 Fluostar Optima fluorimeter (BMG Labtech, Offenburg, Germany) equipped with a high energy
 154 xenon flash lamp using a 10-nm bandwidth excitation filter at 410 nm for receptor-GFP² reading
 155 or 485 nm for receptor-YFP reading. Receptor fluorescence expression was determined as
 156 fluorescence of the sample minus the fluorescence of cells expressing receptor-RLuc alone. For
 157 BRET measurements, the equivalent of 20 µg of cell suspension was distributed in 96-well white
 158 microplates with white bottoms (Corning 3600, Corning, NY) and 5 µM of coelenterazine H (for
 159 the YFP acceptor) or DeepBlueC (for the GFP² acceptor) (Molecular Probes, Eugene, OR) were
 160 added. Using DeepBlueC or coelenterazine H as substrates results in respective 410- and 485-nm
 161 emissions from RLuc, which allows the respective selective energy transfer to GFP² and YFP
 162 (Carriba et al., 2008). One minute after adding coelenterazine H or immediately after addition of
 163 DeepBlueC, BRET was determined using a Mithras LB 940 reader (Berthold Technologies,
 164 DLReady), which allows the integration of the signals detected in the short-wavelength filter at
 165 485 nm and the long-wavelength filter at 530 nm when YFP is the acceptor or the short-
 166 wavelength filter at 410 nm and the long-wavelength filter at 510 nm when GFP² is the acceptor.
 167 To quantify receptor-RLuc expression, luminescence readings were performed after 10 min of
 168 adding 5 µM of coelenterazine H irrespective of the acceptor used. Net BRET is defined as
 169 $[(\text{long wavelength emission})/(\text{short wavelength emission})] - C_f$, where C_f corresponds to $[(\text{long}$
 170 $\text{wavelength emission})/(\text{short wavelength emission})]$ for the RLuc construct expressed alone in
 171 the same experiment. For sequence resonance energy transfer (SRET) experiments (Carriba et
 172 al., 2008), HEK-293T cells were transiently co-transfected with constant amounts of cDNAs
 173 encoding both the receptor fused to RLuc or GFP² and with increasingly amounts of cDNA
 174 corresponding to the receptor fused to YFP. Using aliquots of transfected cells (20 µg of
 175 protein), different determinations were performed in parallel: quantification of protein-YFP

176 expression and quantification of protein-RLuc expression as described above. For SRET, cells
177 were distributed in 96-well microplates, and 5 μ M DeepBlueC was added. The SRET signal was
178 collected using a Mithras LB 940 reader with detection filters for short wavelength (410 nm) and
179 long wavelength (530 nm). By analogy with BRET, net SRET is defined as ((long wavelength
180 emission)/(short wavelength emission)) – Cf, where Cf corresponds to long wavelength emission
181 / short wavelength emission for cells expressing protein-RLuc and protein-GFP². Linear
182 unmixing was done for SRET quantification, taking into account the spectral signature to
183 separate the two fluorescence emission spectra (Zimmermann et al., 2002). BRET and SRET are
184 expressed as milliBRET and milliSRET units (mBU and mSU; net BRET and net SRET X
185 1000).

186
187 *cAMP Accumulation assay.* Homogeneous time-resolved fluorescence energy transfer assays
188 were performed using the Lance Ultra cAMP kit (PerkinElmer Life Sciences). The optimal cell
189 density was first established for an appropriate fluorescent signal by measuring the time-resolved
190 FRET signal as a function of forskolin concentration using different cell densities. Forskolin
191 dose-response curves were related to the cAMP standard curve to establish which cell density
192 provides a response that covers most of the dynamic range of the cAMP standard curve. HEK-
193 293T cells growing in medium containing 50 μ M of the phosphodiesterase inhibitor zardaverine
194 were incubated for 15 min before either ghrelin or vehicle was added. Fluorescence at 665 nm
195 was analyzed on a PHERAstar Flagship microplate reader equipped with a homogeneous time
196 resolved fluorescence energy transfer optical module (BMG Labtech).

197

198 *Determination of phosphorylated ERK1/2 in HEK-293T cells or in VTA slices.* Transfected
 199 HEK-293T cells were cultured in serum-free medium for 16 h before the addition of any ligand.
 200 Cells or rat VTA slices were treated or not with the indicated ligands for the indicated time (see
 201 corresponding Figs.) and were lysed by the addition of 300 µl of ice-cold lysis buffer (50 mM
 202 Tris-HCl, pH 7.4, 50 mM NaF, 150 mM NaCl, 45 mM β-glycerophosphate, 1% Triton X-100, 20
 203 µM phenyl-arsine oxide, 0.4 mM NaVO₄, and protease inhibitor mixture). Cellular debris was
 204 removed by centrifugation at 13,000 g for 5 min at 4°C, and the protein was quantified by the
 205 bicinchoninic acid method using BSA dilutions as standard. Phosphorylated proteins were then
 206 determined by Western blot, using a mouse anti-phospho-ERK1/2 antibody (1:2500; Sigma) and
 207 rabbit anti-total-ERK1/2 antibody (1:40,000; Sigma) to quantify phospho-ERK1/2. Bands were
 208 visualized by the addition of a mixture of IRDye 800 (anti-mouse) antibody (1:10,000; Sigma)
 209 and IRDye 680 (anti-rabbit) antibody (1:10,000; Sigma) and scanned by an Odyssey infrared
 210 scanner (LI-COR Biosciences). Band densities were quantified using the scanner software
 211 exported to Excel (Microsoft). The level of phosphorylated proteins was normalized for
 212 differences in loading using the total (phosphorylated plus non-phosphorylated) protein band
 213 intensities.

214
 215 *Animals.* All animals used in the study were maintained in accordance with the National
 216 Institutes of Health Guide for the Care and Use of Laboratory Animals, and the animal research
 217 conducted to perform this study was reviewed and approved by the NIDA IRP Animal Care and
 218 Use Committee (protocol number 18-MTMD-13), the Catalan Ethical Committee for Animal
 219 Use (protocol numbers CEEA/DMAH 4049 and 5664) and the Institutional Animal Care and
 220 Use Committee at Marquette University (protocols AR-290 and -291). Male Sprague Dawley

221 rats (from the in-house colony of the Faculty of Biology, University of Barcelona or Charles
 222 River Laboratories) were used to obtain VTA slices and for microdialysis experiments. Male
 223 GHS-R CRISPR/Cas9 KO Wistar rats and wild-type (WT) littermates (Zallar et al., 2019; in-
 224 house colony at NIDA IRP) were also used in microdialysis experiments. Pitx3-eGFP(+) mice
 225 (Zhao et al., 2004), generously provided by Dr. Kevin Wickman (University of Minnesota) with
 226 permission from Dr. Meng Li (Cardiff University) and C57BL6/J mice from the in-house colony
 227 at Marquette University were used for electrophysiology experiments.

228

229 *Rat VTA slice preparation.* Male Sprague Dawley rats (2 months old) were killed by decapitation
 230 under 4% isoflurane anesthesia, and brains were rapidly removed, placed in ice-cold oxygenated
 231 (O_2/CO_2 , 95%/5%) Krebs- HCO_3^- buffer (in mM: 124 NaCl, 4 KCl, 1.25 KH_2PO_4 , 1.5 $MgCl_2$,
 232 1.5 $CaCl_2$, 10 glucose, and 26 $NaHCO_3$, pH 7.4), and sliced at 4 °C using a brain matrix (Zivic
 233 Instruments). VTA slices (500 μm thick) were dissected at 4°C in Krebs- HCO_3^- buffer; each
 234 slice was transferred into a 12-well plate with Corning Netwell inserts containing 2 ml of ice-
 235 cold Krebs- HCO_3^- buffer. The temperature was raised to 23°C, and after 30 min, the medium
 236 was replaced by 2 ml of fresh buffer (23°C). Slices were incubated under constant oxygenation
 237 (O_2/CO_2 , 95%/5%) at 30°C for 4 h in an Eppendorf Thermomixer (5 PRIME), and the medium
 238 was replaced by fresh buffer and incubated for 30 min before the addition of any ligand. After
 239 incubation, the solution was discarded, and slices were frozen on dry ice and stored at 80°C until
 240 ERK1/2 phosphorylation was determined.

241

242 *In vivo microdialysis.* Male Sprague Dawley rats or GHS-R CRISPR/Cas9 KO Wistar rats or
 243 their WT littermates (3 months old) were used. Experiments were performed during the light

244 cycle. Rats were deeply anesthetized with 3 ml/kg Equithesin (4.44 g of chloral hydrate, 0.972 g
245 of Na pentobarbital, 2.124 g of MgSO₄, 44.4 ml of propylene glycol, 12 ml of ethanol, and
246 distilled H₂O up to 100 ml of the final solution; NIDA Pharmacy) and implanted unilaterally in
247 the VTA (coordinates in millimeters from bregma with a 10° angle in the coronal plane: anterior,
248 -5.6; lateral, 2.4; vertical, -9) with a specially designed microdialysis probe that allows the direct
249 infusion of large peptides within the sampling area (Navarro et al., 2015). Some animals were
250 implanted simultaneously with two regular probes, one in the VTA and the second one in the
251 ipsilateral shell of the nucleus accumbens (NAc; coordinates in millimeters from bregma:
252 anterior, 1.6; lateral, 0.5; vertical, -5.1) with a regular microdialysis probe. After surgery, rats
253 were allowed to recover in hemispherical CMA-120 cages (CMA Microdialysis) equipped with
254 two-channel overhead fluid swivels (Instech) connected to a sample collector (CMA 470, CMA).
255 Twenty-four hours after implanting the probes, experiments were performed on freely moving
256 rats in the same hemispherical home cages in which they recovered overnight from surgery. An
257 artificial cerebrospinal solution containing (in mM) 144 NaCl, 4.8 KCl, 1.7 CaCl₂, and 1.2
258 MgCl₂ was pumped through the probe at a constant rate of 1 µl/min. After a washout period of
259 90 min, dialysate samples were collected at 20 min intervals. For peptide infusion, ghrelin and
260 the liver expressed antimicrobial peptide 2 (LEAP2), and endogenous antagonist of GHS-R1a
261 (Ge et al., 2018), were dissolved in ACSF to a final concentration of 10 µM. The peptides were
262 injected with a 1 µl syringe (Hamilton) driven by an infusion pump and coupled with silica
263 tubing (73 µm inner diameter; Polymicro) to the microdialysis probe infusion cannula (dead
264 volume, 40 nl), which was primed with ACSF and plugged during implantation and delivered at
265 a rate of 16.6 nl/min. The GHS-R1a antagonist YL781, the D1R/D5R antagonist SCH23390 and
266 the PKA inhibitor H-89 were administered within the VTA by constant perfusion by reverse

267 dialysis within the VTA (all at 10 μ M). At the end of the experiment, rats were given an
 268 overdose of Equithesin, the brains were extracted and fixed in formaldehyde, and probe
 269 placement was verified using cresyl violet staining. Dopamine content was measured by HPLC
 270 coupled with a coulometric detector (5200a Coulochem III; ESA).

271

272 *Patch-clamp electrophysiology.* Horizontal slices containing the VTA (225 μ m) were prepared as
 273 described in detail elsewhere (Kotecki et al., 2015) from 6-8 week-old Pitx3-eGFP(+) or
 274 C57BL6/J mice using previously identified putative dopaminergic neuron characteristics (Cruz et
 275 al., 2004; Arora et al., 2011). Results did not differ from those in eGFP+ cells and thus were
 276 combined. Current clamp recordings (were performed at 32-34°C using borosilicate electrodes
 277 (2.5-4.5 M Ω) filled with a K-Gluconate solution (Hearing et al., 2013; Anderson et al., 2019) to
 278 assess spontaneous activity ($I=0$). Upon achieving whole-cell, cells were permitted to stabilize
 279 over a 2-5 min period prior to drug application. Drugs were bath applied using a gravity
 280 perfusion system at a rate of 2-2.5 ml/min. Each drug was applied for at least 5 min, with
 281 measures of frequency taken only after at least 3 consecutive sweeps of stable firing was
 282 obtained. For studies involving receptor antagonists (eticlopride, SCH39166, JMV2959), drugs
 283 were applied prior to and during subsequent application of ghrelin. Drug concentrations were as
 284 follows: ghrelin (500 nM), SCH39166 (10 μ M), JMV2959 (10 μ M), eticlopride (2 μ M).

285

286 *Statistics.* Parametric statistics (paired or non-paired t test, one-way, bifactorial or repeated-
 287 measures ANOVA followed by Dunnett's or Tukey's multiple comparison tests) were used,
 288 since the different groups analyzed showed normality and homogeneity of variance. For patch-
 289 clamp electrophysiological experiments paired t test or repeated-measures ANOVA were used

290 because measures (neural spike firing) were taken two or three times (baseline *versus* post-drug
291 *versus* post-drug combinations) within the same cell to determine whether the mean difference
292 between the repeated observations is zero. Details of the statistical analysis for each experiment,
293 statistical test, sample size and degree of significance, are described in the corresponding figure
294 legend. GraphPad Prism software version 7 was used for the statistical analysis.

295

296 **Results**

297

298 **GHS-R1b-dependent oligomerization of GHS-R1a with D5R**

299 The biophysical techniques Bioluminescence Resonance Energy Transfer BRET¹ and BRET²
300 and Sequential Resonance Energy Transfer (SRET) were performed in HEK-293T cells
301 transfected with different combinations of D5R fused to RLuc (D5R-RLuc), GHS-R1a fused to
302 YFP (GHS-R1a-YFP) or GFP² (GHS-R1a-GFP²) and either non-fused GHS-R1b or GHS-R1b
303 fused to YFP (GHS-R1b-YFP). In BRET¹ and BRET², RLuc was used as the donor
304 chromophore, but with two different RLuc substrates, coelenterazine H and DeepBlueC, which
305 induce two different bioluminescent light emissions (485 nm and 410 nm, respectively), allowing
306 energy transfer to a close YFP or GFP² acceptor chromophore, respectively. These acceptors
307 emit 530-nm (YFP) or 510-nm (GFP²) light, which can either be detected (for the respective
308 BRET¹ or BRET² measurements) or result in a Fluorescence Resonance Energy Transfer (FRET)
309 to a second acceptor chromophore (for SRET measurements). In SRET, YFP was used as the
310 second acceptor upon transfer of energy from GFP², which was the first acceptor from
311 DeepBlueC-induced RLuc bioluminescence (techniques described in detail in Carriba et al.,
312 2008).

313 Saturation BRET¹, BRET² and SRET experiments were performed to support a specific
 314 interaction between the receptors fused to the corresponding chromophores from a non-specific
 315 random collision. Energy transfer was then determined using the same transfected quantities of
 316 the receptor fused to the donor (and first acceptor in SRET experiments) *versus* increasing
 317 quantities of the receptor fused to the acceptor chromophore (or second acceptor in SRET
 318 experiments; Carriba et al., 2008). Under the present experimental conditions, a BRET saturation
 319 curve, indicative of oligomerization, could be obtained with BRET¹ experiments in cells
 320 transfected with D5R-RLuc and GHS-R1b-YFP (Fig. 1C), but not with GHS-R1a-YFP, which
 321 showed a straight line, indicative of non-specific random collision (Fig. 1A). The same lack of
 322 saturation was also observed with BRET² experiments in cells transfected with D5R-RLuc and
 323 GHS-R1a-GFP² (Fig. 1B). Nevertheless, a clear saturation curve was obtained when the cells
 324 were co-transfected with non-fused GHS-R1b (Fig. 1B). In the same way, a clear saturation
 325 curve was obtained in SRET experiments in cells transfected with D5R-RLuc, GHS-R1a-GFP²
 326 and GHS-R1b-YFP (Fig. 1D). These results indicate that, the same as with D1R (Navarro et al.,
 327 2016), GHS-R1b facilitates oligomerization of D5R with GHS-R1a. Since GHS-R1b, but not
 328 GHS-R1a, oligomerizes with D5R, these results strongly suggest that GHS-R1b establishes the
 329 link between D5R and GHS-R1a in the GHS-R1a:GHS-R1b:D5R oligomer.

330

331 **Differences between the signaling of GHS-R1a:GHS-R1b:D1R and GHS-R1a:GHS-** 332 **R1b:D5R oligomers in mammalian transfected cells**

333 The inability of GHS-R1b to bind ghrelin and signal indicates that any differential interaction
 334 between GHS-R1a and D1R/D5R ligands that depends on the presence of GHS-R1b is
 335 dependent on the formation of GHS-R1a:GHS-R1b:D1R or GHS-R1a:GHS-R1b:D5R oligomers.

336 In our previous study, both D1R/D5R agonists and ghrelin were able to produce a Gs-dependent
337 cAMP formation upon GHS-R1a:GHS-R1b:D1R oligomerization (Navarro et al., 2016). The
338 analysis of cAMP formation showed that co-administration of ghrelin and the D1R/D5R agonist
339 SKF81297 did not produce an additive effect, indicative of a negative crosstalk (Navarro et al.,
340 2016). Also dependent on GHS-R1a:GHS-R1b:D1R oligomerization, the D1R/D5R antagonist
341 SCH23390 counteracted the effects of both SKF81297 and ghrelin on cAMP formation. In
342 addition, the GHS-R1a antagonist YIL781 also counteracted the effects of both ghrelin and
343 SKF81297. In the present study, we also analyzed the effect of ghrelin on cAMP formation in
344 HEK-293T cells co-transfected with GHS-R1a and D1R cDNA and increasing amounts of GHS-
345 R1b cDNA (Fig. 2A), with parallel experiments in cells co-transfected with GHS-R1a, D5R and
346 increasing amounts of GHS-R1b (Fig. 2B). The results from cells transfected with the D1R
347 reproduced our previous finding of a ghrelin-induced cAMP accumulation facilitated by co-
348 transfection with GHS-R1b (Navarro et al., 2016). Also, as previously shown, the effect of
349 ghrelin (100 nM) depended on the GHS-R1b/GHS-R1a cDNA ratio, following an inverted u-
350 shaped curve, with an increase of 26%, 72%, 46% and 15% *versus* control values at cDNA ratios
351 of 0, 0.3, 1 and 2, respectively (Fig. 2A). Different from our previous studies (Navarro et al.,
352 2016; Casanovas et al., 2021), the small effect of ghrelin observed in the absence of GHS-R1b
353 reached statistical significance (Fig. 2A). This would agree with GHS-R1a being still able to
354 induce a certain degree of activation of Gs when co-transfected with D1R and in the absence of
355 GHS-R1b. On the other hand, ghrelin (100 nM) was practically ineffective at promoting cAMP
356 accumulation when co-transfected with GHS-R1b and D5R and only showed a small but
357 significant effect (26% increase *versus* control) at a GHS-R1b/GHS-R1a ratio of 0.3 (Fig. 2B).

358 If comparable, the results with cAMP accumulation would indicate a clear difference in
359 the functional properties of the D₁-like receptors upon oligomerization with GHS-R1a and GHS-
360 R1b, with D1R, but not D5R, increasing the ability of ghrelin to signal through Gs and activate
361 AC. Therefore, the same cells (HEK-293T) and transfection methodology (amount of D1R or
362 D5R cDNAs as well as GHS-R1a and GHS-R1b cDNAs, at a GHS-R1b/GHS-R1a ratio of 0 or
363 0.3) was applied to compare differences between GHS-R1a:GHS-R1b:D1R and GHS-R1a:GHS-
364 R1b:D5R oligomers on MAPK signaling, which does not necessarily depend on Gs activation.
365 We first analyzed the effect of different concentrations and incubation times of ghrelin and
366 SKF81297 in HEK-293T cells transfected with either D5R (Fig. 3A) GHS-R1a (Fig. 3B) or
367 GHS-R1b (Fig. 3C) on ERK1/2 phosphorylation. Fused receptors were used to demonstrate their
368 functionality. As expected, ghrelin was effective in cells transfected with GHS-R1a (Fig. 3B),
369 but not with GHS-R1b (Fig. 3C). From the results of the different agonist concentrations and
370 time exposures (Fig. 3), 100 nM of SKF81297 and 50 nM of ghrelin and a time of incubation of
371 7 min were selected as most appropriate to analyze the effect of different ligand interactions on
372 MAPK signaling in cells co-transfected with GHS-R1a with or without GHS-R1b and D5R (Fig.
373 4) or D1R (Fig. 5). In both cases, SKF81297 and ghrelin were effective and produced the same
374 effect in the absence (Figs. 4A and 5A) or presence of GHS-R1b (Figs. 4B and 5B). Remarkably,
375 ghrelin was very effective in cell transfected with D5R, indicating that the scarce activating
376 effect of AC in cells co-transfected with GHS-R1a, GHS-R1b and D5R, *versus* those co-
377 transfected with GHS-R1a, GHS-R1b and D1R, was not dependent on a general reduction of
378 D5R function, but on the selective ability of the D1R to promote a ghrelin-mediated Gs
379 activation in the GHS-R1a:GHS-R1b:D1R oligomer.

380 The comparison of the compound effect of ghrelin and SKF81297 uncovered another
381 pharmacodynamic difference that depends on the D₁-like receptor subtype. Only in cells co-
382 transfected with GHS-R1a, GHS-R1b and D1R, there was evidence for a negative crosstalk upon
383 co-administration of ghrelin and SKF81297 (Figs. 5B). On the other hand, negative crosstalk was
384 not observed in cells co-transfected with GHS-R1a, GHS-R1b and D5R, where co-administration
385 of both agonists produced an additive response (with the effect of the compound administration
386 being significantly higher than SKF81297 alone; Fig. 4B). These results would also complement
387 the previously reported selective negative crosstalk on AC signaling in cells co-transfected with
388 GHS-R1a, GHS-R1b and D1R, but not in cells not co-transfected with GHS-R1b, indicating its
389 dependence on GHS-R1a:GHS-R1b:D1R oligomerization (Navarro et al., 2016).

390 As an orthogonal measure from the negative crosstalk between agonists, a strong cross-
391 antagonism of the D1R/D5R antagonist SCH23390 and the GHS-R1a antagonist YL781 (Esler et
392 al. 2007) could be demonstrated in cells co-transfected with either D5R or D1R and GHS-R1a,
393 but only when also co-transfected with GHS-R1b (Figs. 4D and 5D *versus* Figs. 4C and 5C).
394 Thus, in cells expressing D5R and GHS-R1a, but in the absence of GHS-R1b, the ability of
395 ghrelin to activate GHS-R1a and produce ERK1/2 phosphorylation could only be counteracted
396 by the GHS-R1a antagonist YL781 (1 μ M) (Fig. 4C), while it could also be counteracted by the
397 D1R/D5R antagonist SCH23390 (1 μ M) in the presence of GHS-R1b (Fig. 4D). Likewise, the
398 ability of the D1R/D5R agonist SKF81297 to activate MAPK signaling could only be
399 counteracted by SCH23390 in the absence of GHS-R1b (Fig. 4C) and was also counteracted by
400 YIL781 in the presence of GHS-R1b (Fig. 4D). This same pattern was observed when D1R was
401 present in the oligomeric complex, instead of D5R (Figs. 5C and 5D). Thus, the presence of
402 GHS-R1b induces the ability of GHS-R1a and D1R/D5R antagonists to respectively cross-

403 antagonize D1R/D5R and GHS-R1a agonists, which is consistent with its proposed role in
404 supporting oligomerization between GHS-R1a and D1R or D5R.

405

406 **MAPK signaling of GHS-R1a:GHS-R1b:D1R oligomers in the VTA**

407 Ghrelin and SKF81297-induced ERK1/2 phosphorylation were then evaluated in VTA slices of
408 Sprague-Dawley rats using a previously reported method that allowed the study of MAPK
409 signaling of other GPCR complexes (Navarro et al., 2015; Moreno et al., 2017; see also
410 Materials and Methods). As in previous studies, higher ligand concentrations were used
411 compared with transfected cell preparations to allow sufficient penetration into the tissue. At 500
412 nM, both ghrelin and the D1R agonist SKF81297 produced a significant increase in ERK1/2
413 phosphorylation, but not when co-administered, indicative of a negative crosstalk (Fig. 6). The
414 effects of both agonists were counteracted by either antagonist, SCH23390 or YL781 (at 5 μ M),
415 indicative of cross-antagonism (Fig. 6). That this cross-antagonism represents a biochemical
416 characteristic of GHS-R1a:GHS-R1b:D1R and GHS-R1a:GHS-R1b:D5R oligomers implies that
417 either of them could represent a major population of functional GHS-R1a receptors in the VTA.
418 In addition, to our knowledge, there are not available ligands that can discriminate between D1R
419 and D5R, including SKF81297 and SCH23390. While not conclusive, the evidence of negative
420 crosstalk between ghrelin and SKF81297, which was specific for D1R in HEK-293T cells, is
421 more consistent with D1R as the predominant D₁-like receptor forming functional complexes
422 with GHS-R1a and GHS-R1b in the VTA.

423

424 **GHS-R1a:GHS-R1b:D1R oligomers-mediated modulation of dopaminergic neuronal** 425 **function in the VTA**

426 The local application of ghrelin in the rodent VTA has been previously shown to promote an
 427 increase in the activity of the dopaminergic neurons with an increase in their firing rate (Abizaid
 428 et al., 2006) and in dopamine release in the ipsilateral nucleus accumbens (NAc) (Jerlhag et al.,
 429 2007), using patch-clamp electrophysiology in brain slices and *in vivo* microdialysis,
 430 respectively. The lack of availability of specific antagonists hampered the demonstration of the
 431 specific involvement of GHS-R1a in these effects of ghrelin. Nevertheless, brain slices from
 432 GHSR knockout (KO) mice were used in the electrophysiological experiments and shown to be
 433 insensitive to the effect of ghrelin (Abizaid et al., 2006). Selective and potent small-molecule
 434 GHS-R1a competitive antagonists have now been developed, such as YIL781 (Esler et al. 2007)
 435 and JMV2959 (Moulin et al., 2007, 2013). In addition, an endogenous antagonist of GHS-R1a,
 436 LEAP2, has recently been discovered (Ge et al., 2018). LEAP2 is mainly produced in the liver
 437 and small intestine. Its secretion is suppressed by fasting and has been shown to block the effects
 438 of ghrelin *in vivo*, including food intake, GH release and maintenance of viable glucose levels
 439 during chronic caloric restriction (Ge et al., 2018). Although it was initially characterized as a
 440 negative allosteric modulator (Ge et al., 2018), subsequent studies indicate it is an inverse
 441 agonist (M'Kadmi et al., 2019).

442 By using a previously described *in vivo* microdialysis technique that allows the slow
 443 infusion of large peptides (Navarro et al., 2015; Moreno et al., 2017; Cai et al., 2019), we first
 444 investigated the ability of the local application of ghrelin and LEAP2, alone and in combination,
 445 to modulate the respective somato-dendritic and terminal release of dopamine in the VTA and
 446 ipsilateral NAc. The recently developed and characterized GHS-R1a CRISPR/Cas9 KO Wistar
 447 rat and wild-type (WT) littermates (Zallar et al., 2019) were used to further demonstrate the
 448 dependence on GHS-R1a of the pharmacological effects of ghrelin and LEAP2. As previously

449 reported by Jerlhag et al. (2006) with bolus infusion (1 μ g in 1 μ l in 1 min), the slow local
 450 infusion of ghrelin (10 μ M at 16 nl/ml) in the VTA of WT rats produced a significant increase in
 451 the extracellular levels of dopamine in the ipsilateral NAc (Fig. 7A). Furthermore, we
 452 demonstrate that ghrelin induces a significant somato-dendritic dopamine release (Fig. 7A). This
 453 is expected since local dopamine release in the VTA is a correlate of dopaminergic cell firing,
 454 induced by the antidromic propagation of action potentials (Legault and Wise, 1999). On the
 455 other hand, somato-dendritic dopamine release promotes activation of inhibitory autoreceptors of
 456 the dopamine D₂ receptor subtype (D2-autoreceptors), with the consequent inhibition of cell
 457 excitability, limiting excessive neuronal activation (Adell and Artigas, 2004). As expected,
 458 ghrelin was ineffective in GHS-R KO rats and no significant changes were observed with the
 459 extracellular levels of dopamine in the VTA or in the ipsilateral NAc (Fig. 7B). Less expected
 460 was the lack of effect of the VTA infusion of LEAP2 (10 μ M) in the VTA or ipsilateral NAc of
 461 WT rats (Fig. 7C), since there *is in vitro* and *in vivo* evidence for a significant constitutive
 462 activity of GHS-R1a (Petersen et al., 2009; Damian et al., 2012; M'Kadmi et al., 2019).
 463 Nevertheless, co-infusion of LEAP2 completely counteracted the effect of ghrelin (both at 10
 464 μ M) (Fig. 7D).

465 The effect of the slow local infusion of ghrelin on the somato-dendritic dopamine release
 466 in the VTA was then evaluated in Sprague-Dawley rats to compare with the efficacy of other
 467 previously reported neuropeptides using the same rat strain and methodology. At the same
 468 concentration (10 μ M), ghrelin produced a much larger dopamine release (over 200%; Fig. 6E)
 469 as compared to orexin-A or endomorphin-1 (below 100% in both cases; Navarro et al., 2015;
 470 Moreno et al., 2017). Constant perfusion by reverse dialysis within the VTA of the GHS-R1a
 471 antagonist YL781 (10 μ M) or the D1R/D5R antagonist SCH23390 (10 μ M) counteracted the

472 dopamine releasing effect of ghrelin (Fig. 7E). Finally, the perfusion of the protein kinase A
473 (PKA) inhibitor H-89 (10 μ M) also completely counteracted the effect of ghrelin (Fig. 7E). The
474 cross-antagonism by SCH23390 supports that GHS-R1a:GHS-R1b:D₁-like receptor oligomers
475 localized in the VTA play a significant role in the modulation of the dopaminergic cell function.
476 The inhibitory effect of the PKA inhibitor indicates a significant role of Gs-AC-cAMP signaling
477 in the dopamine releasing effect of ghrelin, which is again consistent with the interpretation that
478 GHS-R1a:GHS-R1b:D₁R, rather than GHS-R1a:GHS-R1b:D₅R, oligomers represent the
479 functionally dominant population of GHS-R1a receptors in the VTA.

480 However, the counteracting effect of the SCH23390 on ghrelin-induced somato-dendritic
481 dopamine release could just be an apparent cross-antagonism, since it has been previously shown
482 that D₁-like receptors localized in the VTA dopaminergic cells facilitate the desensitization of
483 D₂-autoreceptors upon prolonged exposure to dopamine or D₂R agonists (Nimitvilai and Brodie,
484 2010; Nimitvilai et al., 2012a,b), and the application of D₁R/D₅R antagonists can prevent this
485 desensitization (Nimitvilai and Brodie, 2010). Another possible mechanism responsible for the
486 counteracting effect of the SCH23390 could be its also demonstrated agonism on serotonin 5-
487 HT_{2C} receptors (5-HT₂CR) (Millan et al., 2001), which are localized in GABAergic and
488 dopaminergic cells in the VTA (Bubar and Cunningham, 2007), where they appear to mediate an
489 inhibitory influence of serotonin upon dopamine neurotransmission (Bubar and Cunningham,
490 2006). Therefore, we also performed electrophysiologic measurements in identified VTA
491 dopaminergic neurons, controlling the inhibitory role of D₂-autoreceptors (with the addition of
492 the D₂R antagonist eticlopride) and using the selective D₁R antagonist SCH39166, which does
493 not bind to 5-HT₂CR (Wamsley et al., 1991).

494 We initially examined whether ghrelin influences the firing properties of VTA
 495 dopaminergic neurons with whole-cell patch clamp recordings in C57BL6/J (WT) mice using
 496 classical electrophysiological properties to identify putative dopaminergic neurons. Due to the
 497 unexpected variability of the effect of ghrelin (increased or decreased firing), subsequent studies
 498 were performed in mice with C57BL6/J background expressing eGFP under the control of the
 499 promoter for the dopaminergic neuron-specific transcription factor Pitx3 (Pitx3-eGFP; Zhao et
 500 al., 2004; Fig. 8A). This genetic approach represents a more reliable method than classical
 501 electrophysiological measures for the identification of dopaminergic neurons and there are
 502 numerous publications that have validated the use of Pitx3 mice to identify dopaminergic
 503 neurons (i.e., Labouebe et al., 2007; Kotecki et al, 2015; McCall et al., 2017). In fact, VTA
 504 dopaminergic neurons are now known to be comprised of a phenotypical heterogenous
 505 population of neurons that differ in their previously thought unique electrophysiological profile,
 506 which included the presence of a large hyperpolarization-activated cation current (I_h), long
 507 action potential durations, narrow range of basal firing frequency and a capacity for inhibition by
 508 D2R agonists, related D2-autoreceptor-mediated feedback inhibition via somato-dendritic
 509 dopamine release (Ford et al., 2006; Lammel et al., 2008; Margolis et al., 2008; Morales and
 510 Margolis, 2017). Only one of those measures, I_h , was used to identify dopaminergic neurons in
 511 the study by Abizaid et al. (2006), which showed a significant ghrelin-induced increase in
 512 action potential frequency in putative dopaminergic cells from rat and mouse VTA.

513 Spontaneous action potentials were recorded in current clamp ($I=0$). For the initial
 514 assessment of the effect of ghrelin, the baseline firing frequency in cells collected from
 515 C57BL6/J (WT) (n=6) and Pitx3 (n=5) mice (Fig. 8B) did not differ significantly (WT: 3.47 ± 1.3
 516 Hz; Pitx3: 5.40 ± 2.1 Hz; non-paired t test: $p=0.412$). These data were therefore combined to

illustrate the variable effect of ghrelin. Following at least 3-5 min of stable recording of action potentials, 0.5 μ M ghrelin was applied via bath perfusion. Within cell comparisons initially showed that while 10 of 11 cells exhibited a significant change in firing frequency ($>20\%$ change), but there was no significant effect of ghrelin on action potential frequency (Fig. 8B) (paired t test, $p=0.49$). Subsequent examination of within-cell effects of ghrelin compared to baseline revealed that 5 cells exhibited an increase in firing frequency, while 5 showed a reduction in firing rate. Post hoc analysis of these two populations showed a mean 65% increase in firing frequency compared to baseline (baseline: 2.45 ± 0.55 Hz, ghrelin: 4.28 ± 1.2 Hz; paired t test, $p=0.052$), whereas 5 showed a mean 47% reduction in firing (baseline: 6.75 ± 2.13 , ghrelin: 3.64 ± 1.60 ; paired t test, $p=0.034$). This differential effect of ghrelin could depend on the already established existence of two subsets of VTA dopaminergic neurons, with different sensitivity to D2-autoreceptor-mediated inhibition. The subset less sensitive to D2-autoreceptor inhibition is species-dependent and seems to be mostly represented by dopaminergic cells projecting to the amygdala and to the frontal cortex in rats and mice, respectively (reviewed in Morales and Margolis, 2017). As reductions in firing were often preceded by acute elevations in activity (data not shown), it was possible that ghrelin-induced increases in activity and associated somato-dendritic release could drive a subsequent inhibition of activity in the subset of dopaminergic neurons more sensitive to D2-autoreceptor-mediated inhibition. To determine if ghrelin-induced reductions in firing observed in a subset of cells reflects a D2R-dependent pause in firing (Beckstead et al., 2004, Beckstead and Williams, 2007, Ford et al., 2010, Bello et al., 2011, Courtney et al., 2012), we applied the D2R antagonist eticlopride prior to and in combination with ghrelin (Figs 8C and 8D). Bath application of eticlopride alone slightly elevated firing rates compared to ACSF baseline (Fig. 8C). However, co-application of eticlopride and ghrelin led to

540 a significant increase in firing in 5 out of 5 cells (repeated-measures ANOVA: $p < 0.001$; Fig. 8C)
541 with a mean increase of $81 \pm 0.8\%$ compared to eticlopride alone ($p < 0.001$) and $115 \pm 0.8\%$
542 compared to ACSF ($p = 0.002$; Figs 8C and 8D). Firing frequency did not differ between ACSF
543 and eticlopride ($p = 0.495$).

544 Finally, we investigated whether GHS-R1a formation of oligomers with GHS-R1b and
545 D₁-like receptors mediated the observed ghrelin-induced increases in dopaminergic neuron
546 firing. Baseline recordings were taken, then the GHS-R1a antagonist JMV2959 or the D1R/D5R
547 antagonist SCH39166, plus eticlopride, were applied either alone or followed by the application
548 of ghrelin. Repeated-measures ANOVA revealed no differences in firing frequency following
549 application of JMV2959 alone or JMV2959 plus ghrelin compared to ACSF (plus eticlopride;
550 $p = 0.83$; Fig. 8E). Thus, similar to LEAP2 in the microdialysis experiments, JMV2959 did not
551 behave as an inverse agonist on dopaminergic cell activity, even though it has been described as
552 an inverse agonist in some in vitro assays (Ramirez et al., 2019). This observation seems to
553 disclose another property of GHS-R1a-GHS-R1b-D1R oligomers, a decrease in the constitutive
554 activity of GHS-R1a. The same as JMV2959, SCH39166 alone or SCH39166 plus ghrelin did
555 not differ from ACSF baseline (plus eticlopride; $p = 0.07$; Fig. 8F). Taken together, these findings
556 indicate that ghrelin augments firing frequency in dopaminergic neurons that are both sensitive
557 and insensitive to D2R-inhibition and that these actions are probably predominantly mediated by
558 GHS-R1a-GHS-R1b-D1R oligomers.

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563 Discussion

564 The present study supports that GHS-R1a:GHS-R1b:D1R oligomers are the main mediators of
 565 the excitatory effects of ghrelin on VTA dopaminergic cells. We previously showed in
 566 mammalian transfected cells and in striatal neurons in culture that GHS-R1b facilitates
 567 oligomerization of GHS-R1a with D1R, switching GHS-R1a from its canonical Gq-mediated
 568 signaling to a Gs-AC-cAMP signaling (Navarro et al., 2016). As a biochemical signature of the
 569 GHS-R1a:GHS-R1b:D1R oligomer, a D1R/D5R antagonist could block ghrelin-induced cAMP
 570 formation in both cell preparations (Navarro et al., 2016). In the present study, the ghrelin-
 571 induced increase in the activity of VTA dopaminergic cells was found both to be dependent on
 572 Gs-AC-cAMP-PKA signaling and counteracted by D1R/D5R antagonists.

573 Our initial hypothesis was that D5R should be the principal D₁-like receptor involved, in
 574 view of its established predominant expression in the mesencephalic dopaminergic cells in the
 575 rodent and human and non-human primates (Ciliax et al., 2000; Khan et al., 2000). In fact, the
 576 immunohistochemical results by Jiang et al. (2006), showing a significant co-localization of
 577 GHS-R1a and D1R in the mouse substantia nigra and VTA contradicted the consistently reported
 578 lack of D1R mRNA expression in the mesencephalic cells of the mammalian brain (Mansour et
 579 al., 1991; Mengod et al., 1991; Hurd et al., 2001). We first demonstrated that, similarly to D1R,
 580 D5R can form oligomers with GHS-R1a and GHS-R1b. With RET techniques in mammalian
 581 transfected cells, it was possible to show that D5R can form a complex with GHS-R1a, but only
 582 in the presence of GHS-R1b, which alone could also oligomerize with D5R. This strongly
 583 suggests that GHS-R1b acts as a link between D5R and GHS-R1a in the GHS-R1a:GHS-
 584 R1b:D5R complex, in view of the well-established ability of GHS-R1b to oligomerize with
 585 GHS-R1a (see Introduction).

586 Significantly, we could find specific differences in the pharmacological properties of
587 GHS-R1a:GHS-R1b:D1R and GHS-R1a:GHS-R1b:D5R oligomers. The pharmacological
588 properties were dependent on the existence of the respective oligomeric complex and could be
589 demonstrated by their dependence on the presence of GHS-R1b. In comparison to the GHS-
590 R1a:GHS-R1b:D1R oligomer, ghrelin promoted a weaker Gs-AC-cAMP signaling by the GHS-
591 R1a:GHS-R1b:D5R oligomer. Nevertheless, MAPK signaling was pronounced with both
592 oligomers and allowed us to disclose a second pharmacological difference. This is a negative
593 crosstalk between ghrelin and a D1R/D5R agonist within the GHS-R1a:GHS-R1b:D1R, but not
594 the GHS-R1a:GHS-R1b:D5R oligomer. On the other hand, both oligomers showed cross-
595 antagonism with MAPK signaling, with the D1R/D5R antagonist SCH23390 counteracting
596 ghrelin and the GHS-R1a antagonist YL781 completely counteracting the effect of the D1R/D5R
597 agonist SKF81297. Cross-antagonism has been repeatedly shown to be a specific property of
598 GPCR heteromers (Ferré et al., 2014). That the presence of GHS-R1b induces the ability of
599 GHS-R1a and D1R/D5R antagonists to respectively cross-antagonize D1R/D5R and GHS-R1a
600 agonists, strongly argues for cross-antagonism between ghrelin and D1R/D5R ligands as a
601 biochemical property of GHS-R1a:GHS-R1b:D1R or GHS-R1a:GHS-R1b:D5R oligomers. Even
602 though there are no available ligands that can differentiate pharmacologically from D1R and
603 D5R, the present results disclose significant pharmacological differences between both receptors
604 that depend on their oligomerization with GHS-R1a and GHS-R1b. These differences could then
605 be used to identify the predominant D₁-like receptor subtype forming oligomeric complexes with
606 GHS-R1a and GHS-R1b which modulate dopamine function within de VTA. However, it is
607 important to point out that the evidence for the existence of GHS-R1b in the VTA is still
608 correlational and that it needs still to be demonstrated.

609 Three different models were used to examine the activating effects of ghrelin in the VTA:

610 (1) MAPK activation in VTA slices, (2) somato-dendritic dopamine release in microdialysis
 611 experiments, and (3) increased activity of dopaminergic cells in patch-clamp experiments. The
 612 most pronounced effect was the increase of somato-dendritic dopamine release in the VTA and
 613 its corresponding projecting area, the NAc, after a slow and sustained infusion of ghrelin (60
 614 min) into the VTA. On the other hand, the electrophysiological experiments required D2-
 615 autoreceptors blockade to disclose the stimulatory effects of ghrelin. The most likely explanation
 616 for this difference is the reported desensitization of D2-autoreceptors after prolonged exposure to
 617 dopamine, which depends on co-stimulation of D₁-like receptors, probably D5R localized in the
 618 VTA dopaminergic cells (Nimitvilai and Brodie, 2010; Nimitvilai et al., 2012a,b). The
 619 dependence on D1R/D5R activation, as well as on activation of a Gq/PLC/PKC pathway and
 620 calcium signaling, suggests that a significant population of D2-autoreceptors could represent
 621 D2R-D5R heteromers (Rashid et al., 2007; So et al., 2009; Nimitvilai et al., 2012a,b). Inhibition
 622 of ghrelin-induced somato-dendritic dopamine release by a D1R/D5R antagonist should
 623 therefore involve a reversal of autoreceptor sensitization. Nevertheless, the electrophysiological
 624 experiments demonstrated a clear cross-antagonism with a complete D1R/D5R antagonist-
 625 mediated blockade of ghrelin-induced activation of VTA dopaminergic cells upon D2-
 626 autoreceptor blockade. These results implied that oligomers of GHS-R1a, GHS-R1b and D₁-like
 627 receptors could represent a main population of GHS-R1a within the VTA that modulate
 628 dopaminergic cell function.

629 Unexpectedly, negative crosstalk was observed on MAPK activation in VTA slices
 630 induced by ghrelin and the D1R/D5R agonist SKF81297. In addition, the local perfusion in the
 631 VTA of a PKA inhibitor counteracted ghrelin-induced somato-dendritic dopamine release,

disclosing the pharmacological profile of the GHS-R1a:GHS-R1b:D1R oligomer. The lack of expression of D1R in the VTA dopamine neurons would therefore favor a presynaptic localization of the GHS-R1a:GHS-R1b:D1R oligomer in VTA glutamatergic or cholinergic excitatory inputs. Indeed, past work has shown that ghrelin-mediated increases in the firing frequency of putative VTA dopaminergic neurons are inhibited by blockade of AMPA- and NMDA-type glutamate receptors and aligns with elevations in the frequency of miniature excitatory postsynaptic currents – a phenomenon often equated with increased presynaptic glutamate release (Abizaid et al., 2006). Our results using patch-clamp electrophysiology are also compatible with a presynaptic effect of ghrelin, which may also explain its non-selective effect on those subsets of dopaminergic neurons that either do or do not express somato-dendritic D2-autoreceptors.

There is evidence for a functional role of presynaptic D₁-like receptors localized in glutamatergic terminals in the VTA, which when activated upon somato-dendritic dopamine release promote glutamate release, and which seem to play an important role in the pharmacological effects of alcohol and psychostimulants (Kalivas and Duffi, 1995; Deng et al., 2009; Xiao et al., 2009). Cholinergic or glutamatergic neurons from the laterodorsal tegmental area (LDTg) constitute a major excitatory input to the VTA (Geisler et al., 2007; Watabe-Uchida et al., 2012) and the LDTg has been shown to express GHS-R1a mRNA (Guan et al., 1997; Zigman et al., 2006). To our knowledge, there is no evidence for D₁-like receptor expression in the LDTg, but there are other brain areas that also provide significant glutamatergic inputs to the VTA and express both GHS-R1a and D1R, which could therefore be expressed in their VTA nerve terminals. Those include the paraventricular nucleus of the hypothalamus, the retrorubral field and the lateral parabrachial nucleus (Thibaut et al., 1990; Guan et al., 1997; Rivkees and

655 Lachowicz, 1997; Czyrak et al., 2000; Maltais et al., 2000; Zigman et al., 2006; Geisler et al.,
656 2007; Coizet et al., 2010; Watabe-Uchida et al., 2012; Mani et al., 2014). Experiments are in
657 progress to determine the possible existence of GHS-R1a:GHS-R1b:D1R oligomers in these
658 VTA excitatory inputs, which should provide a better understanding on the mechanisms by
659 which GHS-R1a signaling is able to influence the function of VTA dopaminergic neurons.

660 Apart from the localization of GHS-R1a:GHS-R1b:D1R oligomers within the VTA, the
661 origin of the endogenous ghrelin involved in the functional activation of these receptor
662 complexes needs still to be determined. Some of the proposed mechanisms by which endogenous
663 ghrelin could activate GHS-R1 in the VTA include its local synthesis, its peripheral transit to the
664 VTA, and an indirect activation through circuit-level effects (Cabral et al., 2017; Edwards and
665 Abizaid, 2017; Perello et al., 2019; Wenthur et al., 2019). The evidence for central synthesis of
666 ghrelin is modest and hotly debated (Cabral et al., 2017; Edwards and Abizaid, 2017).
667 Nevertheless, we have recently demonstrated that cocaine-mediated reward is sensitive to
668 systemically administered GHS-R1a antagonists, but not to sequestration of peripheral ghrelin
669 with peripherally acting specific antibodies (Wenthur et al., 2019), which would argue against
670 the transport of peripheral ghrelin to the CNS as the primary source of its facilitatory effects on
671 reward-associated behaviors.

672 Irrespective of the specific origin of endogenous ghrelin, the present study strongly
673 suggests that the GHS-R1a:GHS-R1b:D1R oligomer localized in the VTA can provide an
674 important pharmacological target for alcohol and psychostimulant use disorders, in view of the
675 preclinical and clinical evidence suggesting that GHS-R1a antagonists can be useful
676 pharmacological approaches to treat in these disorders (Jerlhag et al., 2009; Jerlhag et al., 2010;
677 Zallar et al., 2017; Farokhnia et al., 2019; Wenthur et al., 2019; Edvardson et al., 2021).

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938 **Figure 1.** GHS-R1a:GHS-R1b:D15R oligomerization in HEK-293T cells. **A**, BRET¹ saturation
 939 experiment, showing a straight line, in cells co-transfected with D5R-RLuc cDNA (0.8 µg) and
 940 increasing amounts of GHS-R1a-YFP (0.25-3 µg). **B**, BRET² saturation experiments in cells co-
 941 transfected with D5R-RLuc cDNA (0.8 µg) and increasing amounts of GHS-R1a-GFP² cDNA
 942 (0.25-2 µg) in the presence (red saturation curve) and absence (black straight line) of co-
 943 transfected non-fused GHS-R1b (0.3 µg). **C**, BRET¹ saturation experiment showing a saturation

944 curve in cells co-transfected with D5R-RLuc cDNA (0.8 μ g) and increasing amounts of GHS-
 945 R1b-YFP (0.1-1.5 μ g). **D**, SRET saturation experiment showing a SRET saturation curve in cells
 946 co-transfected with a constant amount of GHS-D5R-RLuc cDNA (1 μ g) and GHS-R1a-GFP²
 947 cDNA (1.5 μ g) and increasing amounts of GHS-R1b-YFPcDNA (0.1-1.5 μ g). The relative
 948 amounts of BRET or SRET are given as a function of 100 X the ratio between the fluorescence
 949 of the acceptor and the luciferase activity of the donor. BRET and SRET are expressed as
 950 milliBRET units (mBU) or milliSRET units (mSU) and given as the means $\pm$ S.D. of six to eight
 951 experiments grouped as a function of the amount of BRET or SRET acceptor.

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 953 **Figure 2.** Analysis of cAMP accumulation in HEK-293T cells expressing GHS-R1a, GHS-R1b
 954 and D1R or GHS-R1a, GHS-R1b and D1R. **A**, Cells were transfected with GHS-R1a cDNA (1
 955 μ g) and D1R cDNA (1 μ g) in the presence of increasing amounts of GHS-R1b cDNA (0, 0.3, 1
 956 or 2 μ g). **B**, Cells were transfected with GHS-R1a cDNA (1 μ g) and D5R cDNA (1 μ g) in the
 957 presence of increasing amounts of GHS-R1b cDNA (0, 0.3, 1 or 2 μ g); cAMP levels were
 958 analyzed after the addition of ghrelin (50 nM) or vehicle and are expressed as percentage of cells
 959 treated with vehicle and not transfected with GHS-R1b (broken line) and represented as means +
 960 S.E.M. of six experiments performed in triplicate. In **A**, a bifactorial ANOVA demonstrates a
 961 significant effect of ghrelin ($p < 0.0001$) and of GHS-R1b ($p < 0.0001$); *, ***, and ****:
 962 significantly different *versus* the respective vehicle-treated control group ($p < 0.05$, $p < 0.001$ and
 963 $p < 0.0001$, respectively; Tukey's multiple comparisons test). In **B**, a bifactorial ANOVA
 964 demonstrates a significant effect of ghrelin ($p < 0.05$) without a GHS-R1b effect; *: significantly
 965 different *versus* the respective vehicle-treated control group ($p < 0.05$; Tukey's multiple
 966 comparisons test).

967

968 **Figure 3.** Analysis of ligand-induced ERK1/2 phosphorylation in HEK-293T cells expressing
 969 D5R, GHS-R1a or GHS-R1b. **A**, Cells were transfected with D5R cDNA (1 μ g) and treated with
 970 increasing concentrations of SKF81297 (50-500 nM) for 5 min (upper graph) or for different
 971 times (2.5-10 min) with 100 nM of SKF81297 (lower graph). **B** and **C**, Cells were transfected
 972 with GHS-R1a or GHS-R1b cDNA (1 μ g in both cases) and treated with increasing
 973 concentrations of ghrelin (10-1000 nM) for 7.5 min (upper graphs) or for different times (2.5-15
 974 min) with 50 nM of ghrelin (lower graphs). Phosphorylated ERK1/2 levels are expressed as
 975 percentage of non-treated cells (broken line) and represented as means + S.E.M of five different
 976 experiments performed in duplicate); *, ** and ***: significantly different *versus* non-treated
 977 cells ($p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively; one-way ANOVA followed by Tukey's
 978 multiple comparisons test).

979

980 **Figure 4.** Comparison of ligand-induced ERK1/2 phosphorylation in HEK-293T cells expressing
 981 D5R and GHS-R1a with or without GHS-R1b. **A** and **C**, Cells were transfected with D5R and
 982 GHS-R1a cDNA (1 μ g in both cases). **B** and **D**, Cells were transfected with D5R cDNA (1 μ g),
 983 GHS-R1a cDNA (1 μ g) and GHS-R1b cDNA (0.3 μ g). In **A** and **B**, cells were treated for 7 min
 984 with ghrelin (50 nM), the D1R/D5R agonist SKF81297 (100 nM) or both compounds. In **C** and
 985 **D**, cells were also treated for 7 min with either ghrelin (50 nM) or SKF81297 (100 nM), after
 986 previous treatment for 10 min with the GHS-R1a antagonist YL781 (1 μ M) or the D1R/D5R
 987 antagonist SCH23390 (1 μ M). Phosphorylated ERK1/2 levels are expressed as percentage of
 988 non-treated cells (broken line) and represented as means + S.E.M of six to eight different
 989 experiments performed in duplicate); # and ##: significantly different *versus* cells only treated

990 with SKF81297 ($p<0.05$ and $p<0.01$, respectively; one-way ANOVA followed by Tukey's
 991 multiple comparisons test); ** and ***: significantly different *versus* non-treated cells ($p<0.01$
 992 and $p<0.001$, respectively; one-way ANOVA followed by Tukey's multiple comparisons test).

993

994 **Figure 5.** Comparison of ligand-induced ERK1/2 phosphorylation in HEK-293T cells expressing
 995 D1R and GHS-R1a with or without GHS-R1b. **A** and **C**, Cells were transfected with D1R and
 996 GHS-R1a cDNA (1 μ g in both cases). **B** and **D**, Cells were transfected with D1R cDNA (1 μ g),
 997 GHS-R1a cDNA (1 μ g) and GHS-R1b cDNA (0.3 μ g). In **A** and **B**, cells were treated for 7 min
 998 with ghrelin (50 nM), the D1R/D5R agonist SKF81297 (100 nM) or both compounds. In **C** and
 999 **D**, cells were also treated for 7 min with either ghrelin (50 nM) or SKF81297 (100 nM), after
 1000 previous treatment for 10 min with the GHS-R1a antagonist YL781 (1 μ M) or the D1R/D5R
 1001 antagonist SCH23390 (1 μ M). Phosphorylated ERK1/2 levels are expressed as percentage of
 1002 non-treated cells (broken line) and represented as means + S.E.M of six to eight different
 1003 experiments performed in duplicate); #: significantly different *versus* cells only treated with
 1004 SKF81297 ($p<0.05$; one-way ANOVA followed by Tukey's multiple comparisons test); *, **
 1005 and ***: significantly different *versus* non-treated cells ($p<0.05$, $p<0.01$ and $p<0.001$,
 1006 respectively; one-way ANOVA followed by Tukey's multiple comparisons test).

1007

1008 **Figure 6.** Analysis of ligand-induced ERK1/2 phosphorylation in rat VTA slices. ERK1/2
 1009 phosphorylation was determined in rat VTA slices pretreated for 3 h with medium. Slices were
 1010 then incubated for 20 min with medium, the GHS-R1a antagonist YIL781 (5 μ M), or the
 1011 D1R/D5R antagonist SCH23390 (5 μ M) and treated for 12 min with medium, ghrelin (500 nM),
 1012 the D1R/D5R agonist SKF81297 (500 nM), or both. Phosphorylated ERK1/2 levels are

expressed as percentage of non-treated slices (broken line) and represented as means + S.E.M. of 4 to 5 slices. * and **: significantly different *versus* non-treated slices ($p < 0.05$ and $p < 0.01$, respectively; one-way ANOVA followed by Tukey's multiple comparisons test).

Figure 7. Activation of VTA dopaminergic cells by ghrelin: Microdialysis experiments. **A-D**, Somato-dendritic and NAc dopamine release in GHS-R1 CRISPR/Cas9 KO Wistar rats (GHSR-KO) and wild-type (WT); values represent dopamine concentrations from the VTA and the ipsilateral NAc, in means + SEM (n=7-14 per group) of percentage of baseline (average of three samples before infusion of ghrelin or LEAP2); white or gray rectangles in x-axis: periods of infusion of ghrelin or LEAP2 (10 μ M for both peptides). **E**, Somato-dendritic dopamine release in Sprague-Dawley rats; values represent dopamine concentrations from the VTA, in means + SEM (n=7-14 per group) of percentage of baseline (average of three samples before infusion of ghrelin); white or lined rectangles in x-axis: periods of infusion of ghrelin or perfusion (reverse dialysis) of the GHS-R1a antagonist YIL781, the D1R/D5R antagonist SCH23390 or the PKA inhibitor H-89 (10 μ M for all compounds). Filled symbols represent significantly different means as compared to the mean of the first three basal values ($p < 0.05$; repeated-measures ANOVA followed by Dunnett's multiple comparisons test).

Figure 8. Activation of VTA dopaminergic cells by ghrelin: Electrophysiological experiments. **A**, eGFP positive cell in the VTA of an acutely isolated brain slice from a Pitx3 mouse, as visualized via infrared microscopy (top) and epifluorescence (bottom); the dashed circle highlights the cell targeted for evaluation (pipette visible in the top). **B**, Mean firing frequency of dopaminergic neurons in ACSF (baseline) and following 5-minute bath application of ghrelin

1036 using a within-cell approach. **C**, Mean firing frequency in ACSF and following bath application
1037 of the D2R antagonist eticlopride (5 minutes) and subsequent application of ghrelin and
1038 eticlopride. **D**, Representative trace showing impact of eticlopride and ghrelin in the presence of
1039 eticlopride on firing frequency followed by washout in ACSF; lines denote duration of drug
1040 on/off period and highlighted boxes denote the time data was collected and averaged for firing
1041 frequency in ACSF, eticlopride, and ghrelin plus eticlopride. **E**, Mean firing frequency of
1042 dopaminergic neurons in the presence of ACSF, the GHS-R1a antagonist JMV2959 and ghrelin
1043 plus JMV2959. **F**, Mean firing frequency in ACSF and following bath application of the
1044 D1R/D5R antagonist SCH39166 and subsequent application of ghrelin in the presence of
1045 SCH39166. *** and ##: $p < 0.001$ versus ACSF and $p < 0.01$ versus eticlopride alone, respectively
1046 (repeated-measures ANOVA followed by Tukey's multiple comparisons test).

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