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GLP-1R signaling directly activates arcuate nucleus kisspeptin action in brain slices but does not rescue LH inhibition in OVX mice during negative energy balance

GLP-1R action on reproductive neuroendocrinology

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Conflict of interest: KMH, AFB, CB, AJM and KLG are full-time employees of Novo Nordisk which markets liraglutide for the treatment of type II diabetes mellitus and obesity. SRL, MAK, BB, MAB, OKR, CT, and MSS have nothing to disclose.

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Abstract

Kisspeptin (Kiss1) neurons in the hypothalamic arcuate nucleus (ARC) are key components of the hypothalamic-pituitary-gonadal axis, as they regulate the basal pulsatile release of gonadotropin releasing hormone (GnRH). ARC Kiss1 action is dependent on energy status and unmasking metabolic factors responsible for modulating ARC Kiss1 neurons is of great importance. One possible factor is glucagon-like peptide-1 (GLP-1), an anorexigenic neuropeptide produced by brainstem preproglucagon neurons. As GLP fiber projections and the GLP-1 receptor (GLP-1R) are abundant in the ARC, we hypothesized that GLP-1R signaling could modulate ARC Kiss1 action. Using ovariectomized (OVX) mice, we found that GLP-producing fibers come in close apposition with ARC Kiss1 neurons; these neurons also contain *Glp1r* mRNA. Electrophysiological recordings revealed that liraglutide (a long-acting GLP-1R agonist) increased action potential firing and caused a direct membrane depolarization of ARC Kiss1 cells in brain slices. We determined that brainstem preproglucagon mRNA is decreased following a 48 h fast in mice, a negative energy state in which ARC Kiss1 expression and downstream GnRH/luteinizing hormone (LH) release are potently suppressed. However, activation of GLP-1R signaling in fasted mice with liraglutide was not sufficient to prevent LH inhibition. Furthermore, chronic central infusions of the GLP-1R antagonist, exendin (9-39) in ad libitum fed mice did not alter ARC *Kiss1* mRNA or plasma LH. As a whole, these data identify a novel interaction of the GLP-1 system with ARC Kiss1 neurons but indicate that CNS GLP-1R signaling alone is not critical for the maintenance of LH during fasting or normal feeding.

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74 **Significance Statement**

75 Reproductive dysfunction is associated with metabolic imbalance, and identifying
76 the underlying molecular mechanisms linking metabolic status with reproductive
77 function is of great importance. Kisspeptin neurons (Kiss1) located in the arcuate
78 nucleus of the hypothalamus (ARC) are essential for fertility and are potently inhibited
79 during negative energy balance; this inhibition occurs in the presence or absence of
80 ovarian steroids. Preproglucagon-expressing neurons located in the brainstem send
81 abundant fiber projections to the ARC where they release the anorexigenic
82 neuropeptide, glucagon-like peptide-1 (GLP-1). The aim of these studies was to
83 determine the interaction of the CNS GLP-1 system with ARC Kiss1 activity to
84 potentially provide a link between systems that control energy balance with those that
85 control reproductive neuroendocrine output.

86

87 **Key Words**

88 Kisspeptin, LH, GLP-1, liraglutide, fasting, hypothalamus

89

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Introduction

Adequate nutrient availability is essential to maintain proper reproductive function and states of chronic negative energy balance lead to the suppression of the hypothalamic-pituitary-gonadal (HPG) axis. Kisspeptin (Kiss1) expressing neurons are positive regulators of gonadotropin-releasing hormone (GnRH) release, and Kiss1 signaling through its receptor (Kiss1r) is essential for fertility (de Roux et al., 2003; Seminara et al., 2003). Kiss1 neurons located in the arcuate nucleus (ARC) of the hypothalamus act on GnRH nerve terminals in the median eminence and regulate basal pulsatile GnRH/luteinizing hormone (LH) release (Li et al., 2009; Han et al., 2015). Similar to other ARC neurons, ARC Kiss1 neurons respond to metabolic cues denoting changes in energy status and alter their activity (True et al., 2013; Frazao et al., 2014; Nestor et al., 2014). Therefore, ARC Kiss1 neurons are thought to be key integrators of metabolic status with proper output of GnRH/LH release. However, the identity of nutrient sensing systems that regulate ARC Kiss1 action in response to changes in energy status remains elusive. Previously, our laboratory used rodent models to show that low leptin and insulin levels associated with negative energy balance do not appear to be responsible for the suppression of GnRH/LH release (Xu et al., 2009; True et al., 2011).

We identified glucagon-like peptide-1 (GLP-1) as a potential nutrient sensing system that integrates metabolic status with reproductive neuroendocrine function in part through the regulation of ARC Kiss1. Post-translational processing of the preproglucagon gene (Gcg) gives rise to GLP-1 which is mainly produced in the

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gastrointestinal tract and brain (Sandoval and D'Alessio, 2015). GLP-1 mediates its action through the GLP-1 receptor (GLP-1R), a 7-transmembrane G-protein coupled receptor (Mayo et al., 2003). Peripherally and centrally administered GLP-1R agonists activate the GLP-1R in the central nervous system (CNS) which suppresses feeding and body weight (Heppner and Perez-Tilve, 2015). This effect is mediated in part through activation of anorexigenic neurons that express proopiomelanocortin (POMC) and cocaine-and amphetamine-regulated transcript (CART) neurons, as well as inhibition of orexigenic neurons that express neuropeptide Y (NPY) and agouti-related peptide (AgRP) located in the ARC (Secher et al., 2014). Preproglucagon-expressing neuronal cell bodies produce GLP-1, GLP-2, and oxyntomodulin (Larsen et al., 1997) and are confined to the brainstem, whereas their fibers project to numerous areas of the brain. Preproglucagon-expressing neurons project heavily to areas of the hypothalamus that regulate energy metabolism (Llewellyn-Smith et al., 2011; Vrang and Grove, 2011) which is consistent with a functional role of GLP-1 in the regulation of feeding and body weight. One hypothalamic nucleus with the highest levels of preproglucagon-expressing fibers, as well as the GLP-1R, is the ARC (Merchenthaler et al., 1999; Llewellyn-Smith et al., 2011; Vrang and Grove, 2011; Ronnekleiv et al., 2014; Heppner et al., 2015). GLP-1-producing neurons are activated in response to calorie ingestion (Kreisler et al., 2014). Furthermore, brainstem preproglucagon expression is inhibited during fasting (Huo et al., 2008) and upregulated during high-fat diet feeding (Knauf et al., 2008) suggesting that preproglucagon neurons act as metabolic fuel sensors.

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Reports on the role of GLP-1 in regulating reproductive function, however, are limited. Female *Glp1r-/-* mice have a delayed onset of puberty suggesting a role for GLP-1R signaling in regulating reproductive function (MacLusky et al., 2000). Functional studies indicate that GLP-1 promotes GnRH secretion from isolated hypothalamic tissue and acts centrally to enhance LH levels in male rats (Beak et al., 1998). More recent studies demonstrate that GLP-1 acts centrally to enhance the pre-ovulatory LH surge of intact female rats (Outeirino-Iglesias et al., 2015). Taken together, these data suggest that GLP-1 promotes GnRH/LH release. Based on the neuroanatomical distribution of GLP-1 producing fibers as well as the functional role of stimulating GnRH/LH release, we hypothesized that GLP-1R signaling activates ARC Kiss1 neurons to enhance GnRH/LH release. Thus, decreased brainstem preproglucagon could contribute to the suppression of ARC Kiss1 activity and suppression of downstream GnRH/LH release during negative energy balance. The aims of these studies were to: 1) determine whether the CNS GLP-1 system has neuroanatomical and functional interaction with ARC Kiss1 neurons and 2) assess whether GLP-1R signaling plays a role in the suppression of the reproductive neuroendocrine axis that occurs during fasting, using an ovariectomized (OVX) mouse model.

Materials and Methods

Animals

All animals were fed a standard chow diet (Purina lab chow; catalog # 5001) and maintained on a 12:12-h light-dark cycle at 22°C with free access to food and water unless noted otherwise. For histology experiments and electrophysiological recordings,

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158 *Kiss1*-CreGFP mice on a C57BL/6 background were produced by Elias and colleagues
159 at the University of Michigan (Cravo et al., 2011) and bred at the facilities at OHSU. For
160 single cell RT-PCR experiments, female *Kiss1*-CreGFP mice (C57BL/6J and S129
161 background) were originally produced by Steiner and colleagues at the University of
162 Washington (Gottsch et al., 2011) and then bred at the facilities at OHSU. For studies
163 involving fasting or intracerebroventricular (icv) infusion, adult female C57BL/6J mice
164 (12-14 weeks old) were purchased from the Jackson Laboratory. For studies involving
165 dual in situ hybridization, adult female mice were purchased and ovariectomized at
166 Jackson Laboratory. All animal procedures were approved by the Oregon Health and
167 Science University and the Novo Nordisk Research Center Institutional Animal Care
168 and Use Committees.
169
170 *Ovariectomy (OVX) and estradiol (E_2) replacement*
171 Adult female mice were anesthetized using 2.5 % isoflurane in oxygen delivered
172 by a nose cone. After receiving a pre-operative dose of carprofen (5 mg/kg), the
173 ovaries were removed through bilateral lumbar incisions. The vasculature to the ovary
174 and body wall were sutured, and wound clips were used to close the incision. For
175 surgeries involving E_2 replacement, an E_2-filled capsule was implanted in the
176 interscapular region immediately after OVX surgery. The E_2 implants were made of
177 Silastic tubing (0.59 in long, 0.078 in inner diameter, 0.125 in outer diameter; Dow
178 Corning) and filled with a low dose of crystalline E_2 (20 µg/mL, in sesame oil) as
179 previously described (Navarro et al., 2015).
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Immunohistochemistry

Female *Kiss1*-CreGFP mice (Cravo et al., 2011) underwent OVX surgery and a week later were sedated with ketamine (80 mg/kg) and xylazine (10 mg/kg) and were then perfused with 4% PFA in 0.1 m phosphate (PB) buffer, pH 7.4. Brains were removed and post-fixed in 4% PFA overnight at 4°C and then cryoprotected with 25% sucrose in 0.05 M potassium phosphate buffered saline (KPBS) and stored at -80°C until sectioning. Sections were cut at 25 μm on a freezing microtome in a one-in-six series. For analysis of preproglucagon-expressing fiber contacts onto Kiss1 neurons, the tissue sections were washed in KPBS several times and preincubated in blocking buffer (KPBS plus 0.4% Triton X-100 plus 2% normal donkey serum) for 30 min before incubating in chicken anti-GFP (1:5K; Aves Labs cat # GFP-1020) and mouse anti-GLP-2 (1:2K; Novo Nordisk A/S) in blocking buffer for 24 h at 4°C. The monoclonal GLP-2 antibody was raised against full-length human GLP-2 (GLP-$2_{1–33}$) and has shown to have complete overlap with GLP-1 immunostaining ((Tang-Christensen et al., 2000; Vrang et al., 2007). Because of this overlap, no distinction is made whether fibers are GLP-1 or GLP-2, and are referred to as GLP fibers. Following washes in KPBS, tissue sections were incubated for 1 h in a cocktail of Alexa Fluor 568 donkey anti-mouse antibody (1:1K; Life Technologies; catalog #A10037) and Alexa Fluor 488 goat anti-chicken (1:1K; Life Technologies cat # A11039) at room temperature, then subsequently washed and mounted on gelatin-coated glass slides and coverslipped with SlowFade Gold antifade reagent (Invitrogen; catalog #S36936). For analysis of GLP contacts onto GnRH neurons the same protocol as above was implemented using the primary antibodies mouse anti-GLP-2 (1:2K; Novo Nordisk A/S) and rabbit anti-GnRH (1:32K;

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EL-14;(Ellinwood et al., 1985)) and the secondary antibodies Alexa Fluor 647 donkey anti-mouse (1:1K; Life Technologies, catalog #A31571) and Alexa Fluor donkey anti-rabbit 568 (1:1K; Life Technologies, catalog #A10042).

Confocal analysis

Immunofluorescence images were taken with a Leica SP5 confocal microscope with Acousto-Optical Beam Splitter (Buffalo Grove, Illinois). Analyses of GLP-immunoreactive (GLP-ir) fibers making close appositions to ARC Kiss1 fibers and GnRH cell bodies were performed as previously described (True et al., 2013). Photomicrographs were taken at a 40X magnification at 1024 × 1024 pixel resolution and at a speed of 700 Hz. Focal planes were 1 µm apart for analysis, and 4 ARC sections and 5-7 preoptic area (POA) were analyzed per animal. For more abundant ARC Kiss1 cells, all visible cells in confocal photomicrographs of 4 ARC sections (unilateral) were analyzed for contact analysis. Stacks were analyzed using ImageJ software (NIH).

Single Cell RT-PCR

Kiss1-CreGFP mice (Gottsch et al., 2011) were OVX bilaterally and euthanized a week later for tissue collection. Single cell transcriptomes were isolated from *Kiss1*-CreGFP cells as previously described (Bosch et al., 2013; Navarro et al., 2015). Primers were designed to span at least one intron-exon boundary using the Clone Manager software program (Scientific and Educational software). Stringent PCR conditions were tested to determine the optimal primer concentration, magnesium

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concentration and annealing temperature to produce a single clear band. The primer sequences for *Glp1r* and *Kiss1* were *Glp1r* (149 bp product, accession number NM_021332, forward primer 474-494 nt, reverse primer 602-622 nt); *Kiss1* primers were described previously (Zhang et al., 2013). PCR was performed on 3 µl of cDNA in a 30 µl final volume containing 1X Go *Taq* Flexi buffer (Promega), 2mM $MgCl_2$, 0.33mM deoxynucleoside triphosphate, 0.33 µM forward and reverse primers, 2-U Go *Taq* and 0.22 µg TaqStart antibody (Clontech) for 50 cycles of amplification with specific annealing temperatures (*Glp1r*, 60°C; *Kiss1*, 57°C). PCR products were visualized with ethidium bromide on a 2% agarose gel and confirmed by sequencing. As a negative control, aCSF samples were collected in the vicinity of the dispersed cells and processed in the RT-PCR assays. Water blanks were also included in each RT-PCR assay. In addition, several single cells were processed in the RT-PCR but without reverse transcriptase (RT) in order to assure that genomic DNA was not being amplified. Basal hypothalamic tissue RNA was also included as a positive control (with RT) and negative control (without RT).

For determination of neuronal expression of a particular transcript, 127 neurons (16-33 cells/animal) were harvested from 5 animals. The number of arcuate Kiss1-GFP neurons expressing *Glp1r* was counted for each animal and the mean number of neurons/animal was determined and used for further analysis of mean, SEM, and percentage expression.

Dual in situ hybridization

For dual *in situ* hybridization (ISH), formalin-fixed paraffin embedded (FFPE) brain tissue was cut into 5 µm sections using a rotary microtome (RM2255, Leica

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Biosystems) and mounted onto Superfrost Plus glass (Fisher Scientific). We sectioned brains from OVX females (n=4) and sampled the ARC at two distinct anatomical locations within the ARC (-1.3 from bregma, and -1.8 from bregma, respectively). Brain tissues were prepared for RNAscope ISH (ACD Bio, Hayward, CA) following the manufacturer's recommendations (ACD Bio #322452-USM), and duplex chromogenic ISH was executed in a HybEZ System following the protocol from ACD Bio (#322500). Our experiments utilized probes to Mm-*Glp1r* (#418851-C2) and Mm-*KISS1* (#408001), which were labeled with red and green chromogens after signal amplification steps, respectively. After staining, slides were counterstained in twenty dips of Mayer's hematoxylin (Sigma Aldrich), dried in an oven a 60°C for 2 hours, and were cover slipped with Ecomount mounting medium (BioCare). Finished slides were scanned at 40x on a Zeiss AxioScan.Z1 for post-hoc analysis. All representative images were matched for zoom level and brightness/contrast.

Electrophysiology

All recordings were performed in ARC *Kiss1*-CreGFP neurons (Cravo et al., 2011) between 60-90 days of age. OVX and OVX + E_2 surgeries were performed 8-10 days prior to recordings using the methods described above. Coronal slices containing the ARC were prepared as previously described (Qiu et al., 2010). Briefly, brain slices (200 µm) containing ARC were maintained with constant flow (1–2 ml/min) of aCSF containing the following (in mM): 124 NaCl, 5 KCl, 2.6 NaH_2PO_4, 2 $MgSO_4$, 1 $CaCl_2$, 10 HEPES, 10 glucose; oxygenated (95% O_2, 5% CO_2) osmolarity ~300 at 32°C–33°C. For current-clamp experiments, microelectrodes had resistances of 3–6 mΩ and were filled

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with an internal solution containing the following (in mM): 125 K-gluconate, 2 KCl, 10 EGTA 5 HEPES, 1 ATP, 0.3 GTP; pH 7.25 with KOH, osmolarity ~295 mosmM. Data acquisition was performed using a multiclamp 700B amplifier (Molecular Devices). Data were filtered at 3 KHz and sampled at 5–10 KHz using a computer interface Digidata 1322 and pClamp 9.2 software (Molecular Devices). The liquid junction potential of 5 mV was corrected in the analysis. All solutions were made fresh the day of the experiment. Liraglutide was obtained from Novo Nordisk Inc. 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and DL-2-amino-5-phosphonovaleric acid (APV) were obtained from Tocris, (Ellisville, MO) and tetrodotoxin (TTX) from Alomone Labs (Jerusalem, Israel).

48 h fasting and brainstem dissection

Adult C57BL/6J female mice from the Jackson Laboratory (12-14 weeks) underwent OVX surgery as described above. One week after surgery animals were maintained on an ad libitum chow-fed diet or fasted for 48 h. We chose a 48 h fast because both brainstem preproglucagon (Huo et al., 2008) and LH levels (Huang et al., 2008) have previously been reported to be inhibited in mice using this paradigm. After the 48 h fast, all animals were anesthetized with isoflurane and decapitated. The brain was placed into a 1 mm coronal brain matrix and a 3 mm section containing the brainstem was collected (-6 mm to -9 mm posterior to bregma). The tissue was then frozen immediately on liquid nitrogen and stored at -80°C until RNA extraction was performed.

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297

298 *48 h fasting and liraglutide treatment*

299 Adult C57BL/6J female mice from the Jackson Laboratory (12-14 weeks old)
300 underwent OVX surgery as described above. One week after surgery animals were fed
301 ad libitum or had their food removed at 0900h. Also at this time, ad libitum fed animals
302 received twice-daily subcutaneous saline injections and fasted animals received twice-
303 daily subcutaneous injections of saline or the GLP-1R agonist, liraglutide (30nmol/kg
304 per injection; Novo Nordisk). Peripheral injection of liraglutide has been reported to
305 penetrate into the ARC (Secher et al., 2014). After 48h, all animals were anesthetized
306 with isoflurane and decapitated to collect trunk blood.

307

308 *Intracerebroventricular Exendin (9-39) infusion and arcuate dissection*

309 Adult C57BL/6J female mice from Jackson laboratory (12-14 weeks old)
310 underwent OVX surgery as described above. One week later, animals were
311 stereotaxically implanted (David Kopf Instruments, Tujunga, CA) with a cannula (brain
312 infusion kit #3, Alzet, Cupertino, CA) placed in the lateral cerebral ventricle as
313 previously described (Heppner et al., 2012). A polyethylene catheter attached the
314 cannula to an osmotic mini-pump (1007D Alzet, Cupertino, CA) that was
315 subcutaneously implanted. The osmotic mini-pump infused either saline or Exendin 9-
316 39 (Ex-9; 7.5nmol/day; American Peptide, catalog # 46-3-10). After 6 days of icv
317 infusion animals were anesthetized with isoflurane and decapitated. Trunk blood was
318 collected and then the brain was dissected out from the skull and placed in a 1mm
319 coronal brain matrix. The first blade was placed at the caudal extent of the

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hypothalamus and the second blade was placed 2 mm rostral. The 2 mm thick coronal slice was placed on chilled petri dish and a dissection razor (Harris Uni-Core; catalog # 7093508) was used to collect the ventral aspect of the brain containing the ARC. The tissue was then immediately frozen on liquid nitrogen and stored at -80°C until RNA extraction was performed. Cannula placement was confirmed by increased expression of *Agrp* mRNA in ARC tissue of Ex-9 treated mice as compared to saline treated controls.

RNA extraction and qPCR for brainstem and arcuate tissue

RNA was isolated using Trizol and the RNeasy micro kit with on-column deoxyribonuclease I treatment (Qiagen). Quality and integrity of RNA was determined using nanodrop spectrophotometer ND-1000. Reverse transcriptase reactions were prepared using 1 µg of RNA and iScript cDNA Synthesis Kit (Bio-Rad). Quantitative real-time PCR was completed using TaqMan probes (Applied Biosystems) for Gcg (Mm01269055_m1), Agrp (Mm00475829_g1), Npy (Mm00445771_m1), Cart (Mm04210469_m1), Kiss1 (Mm03058560_m1), Pomc (Mm00435874_m1), and housekeeping gene 18s (Hs03003631_g1) was used as an endogenous control to normalize each sample and gene. PCRs were in a 10 µl volume using 0.5 µl TaqMan probe, 10 ng cDNA template, 5 µl TaqMan Gene Expression Master Mix II with UNG (Applied Biosystems), and 2.5 µl DNase/RNase free molecular grade water (Qiagen). Real-time PCR was run using an Applied Biosystems 7900HT Fast Real-Time PCR system with an initial denaturing at 50°C for 2 min, 95°C for 10 min, followed by 40

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cycles at 95°C for 15 s, and annealing at 60°C for 1 min. Results were calculated using the Pfaffl method (Pfaffl, 2001).

LH measurements

For experiments involving LH measurements, trunk blood was collected into a tube containing a cocktail of heparin (10 µl of 1000 USP/mL) and protease inhibitor (10 µl of aprotinin 10,000KIU/ml; Fisher Scientific, Catalog # BP2503-10). Plasma was sent to the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core (Charlottesville, VA) to be measured for LH by radioimmunoassay.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 6.0 (GraphPad Software, San Diego, California, USA). Statistical significance was determined either by unpaired Student's t-test, one-way ANOVA followed by Tukey's multiple comparison or Bonferroni's correction post hoc test or two-way ANOVA followed by Bonferroni's multiple comparison post hoc test. The statistical analysis for each experiment is stated in the figure legend. All results are given as means ± SEM. Results were considered statistically significant when $p < 0.05$.

Results

Neuroanatomical interaction of the CNS GLP system with ARC Kiss1 cells

We used immunohistochemistry to assess GLP fiber contacts onto ARC *Kiss1*-CreGFP neurons in OXV mice (Figure 1A-C). The GLP-2 primary antibody has

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previously been described and has complete overlap with GLP-1 distribution (Tang-Christensen et al., 2000; Vrang et al., 2007; Vrang and Grove, 2011). We determined that GLP-ir fibers come in close apposition with an average of 22% of ARC Kiss1 cells (range of 10.1%-28.6%; n=5 animals, 4 sections per animal) (Figure 1A-C). We also determined that the *Glp1r* mRNA is expressed within a subpopulation (20%) of *Kiss1* cells using single cell RT-PCR (range of 18-25%; 16-33 cells/animal; n=5 animals; Figure 1D). Furthermore, GLP-ir fibers come in close apposition to an average of 10.9% of GnRH cell bodies (range of 8.1%-14.7; n=4 animals, 5-7 sections per animal; data not shown). Our data are consistent with studies in male mice showing GLP-1-ir fiber contacts onto GnRH cells (Farkas et al., 2016).

To gain a better understanding of the neuroanatomical location of Kiss1 cells that co-express *Glp1r* mRNA we performed dual *in situ* hybridization (ISH) on brain sections from OVX mice. We detected an average of 21.3% of ARC Kiss1 neurons co-express *Glp1r* mRNA (Figure 1E,F), which is consistent with our single-cell RT-PCR co-expression analysis (Figure 1D). In this mixed population of *Glp1*+ and *Kiss1*+ neurons, we observed a higher number of *Kiss1/Glp-1r* co-expressing cells in the ventrolateral portion of the ARC (Figure 1F). Taken together, these data provide neuroanatomical and molecular evidence that the CNS GLP system interacts with the reproductive neuroendocrine axis in an OVX mouse model.

Electrophysiological recordings in ARC Kiss1 neurons treated with the GLP-1R agonist liraglutide

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We determined that GLP-producing neurons come in close contact with ARC Kiss1 cells and that the GLP-1R is expressed within a subset of ARC Kiss1 neurons in OVX mice (Figure 1). To assess the function of GLP-1R signaling within ARC Kiss1 cells we performed current clamp recordings with application of the long acting GLP-1R agonist, liraglutide. We found that liraglutide at 100 nM and 300 nM caused a membrane depolarization in ARC Kiss1 neurons (Figure 2A) and an increase in action potential firing with the 300 nM concentration of liraglutide (Figure 2A). To determine if GLP-1R signaling is directly activating ARC Kiss1 cells, we performed similar experiments in the presence of presynaptic blockers. Liraglutide at 300 nM concentration caused a membrane depolarization even in the presence of presynaptic blockers (Figure 2B), which occurred in 60% of the ARC Kiss1 neurons that were tested. It should be noted that a greater percentage of ARC Kiss1 cells responded to GLP-1R agonism (~60%) than expressed *Glp1r* mRNA (~20%), which may reflect a greater sensitivity of electrophysiology methods compared to single-cell RT-PCR/dual ISH. Alternatively, there may be a higher level of functional GLP-1R protein at the cell surface as compared to *Glp1r* mRNA expression. Nevertheless, these electrophysiological data indicate that GLP-1R signaling directly activates ARC Kiss1 cell action suggesting that GLP-1R signaling may have a stimulatory effect on downstream GnRH/LH release.

The stimulatory effect of GLP-1R signaling on ARC Kiss1 cells in OVX mice led us to determine whether this effect is sex specific or if estradiol modifies the action of GLP-1R signaling in ARC Kiss1 neurons. We then performed current clamp recordings

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in male and OVX+E_2 *Kiss1*-CreGFP mice. ARC Kiss1 neurons from OVX+E_2 responded to liraglutide with membrane depolarization (Figure 3A) although there was a reduction in the magnitude of liraglutide-mediated responses in OVX+E_2 as compared to OVX, these differences were not significant. Male ARC Kiss1 neurons showed similar depolarization in the presence of liraglutide treatment. Overall, we observed that liraglutide caused a membrane depolarization in 52% of ARC Kiss1 neurons from OVX+E_2 mice and 60% of ARC Kiss1 neurons from males. From these data we also determined that the initiation of spontaneous action potentials depends upon the magnitude of liraglutide-mediated depolarization (Figure 3C). We observed that the magnitude of liraglutide-mediated depolarization was greater in males. However, this was only statistically significant between OVX+E_2 and male mice. As a whole, these data suggest that GLP-1R signaling activates ARC Kiss1 neurons and this activation is not sex specific or modified by the presence of estradiol.

Brainstem preproglucagon (Gcg) expression during calorie restriction and effect on LH levels in response to GLP-1R agonism during 48 h fasting

It is well established that hypothalamic Kiss1 is inhibited during fasting and calorie restriction (Luque et al., 2007; True et al., 2011). Data in male mice also indicate that brainstem preproglucagon expression is suppressed in response to prolonged fasting (Huo et al., 2008). To determine if brainstem preproglucagon is decreased in response to fasting in OVX mice, we exposed OVX mice to a 48 h fast. Consistent with what has been observed in males, female OVX mice also have a decrease in brainstem preproglucagon expression following a 48 h fast (Figure 4A;

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433 p=0.0006, unpaired t-test). As we determined that GLP-1R signaling stimulates ARC
434 Kiss1 cell action in brain slices from OVX mice we hypothesized that lack of this
435 stimulatory signal coming from brainstem preproglucagon neurons is contributing to the
436 downstream suppression GnRH/LH. To determine whether restoring GLP-1R signaling
437 will relieve the inhibition of LH during fasting, we treated OVX mice with liraglutide
438 during a 48 h fast. Liraglutide is a long acting GLP-1R agonist that has been
439 demonstrated to enter into the ARC upon peripheral administration (Secher et al.,
440 2014). We took advantage of this property of liraglutide to penetrate into ARC tissue
441 and gave twice-daily subcutaneous injections of liraglutide during a 48 h fast in OVX
442 mice. After the 48 h fast, body weight was significantly reduced in fasted mice treated
443 with either saline or liraglutide as compared saline-treated fed controls (Figure 4B;
444 p=0.0007, two-way ANOVA with Bonferroni's post-hoc test). As expected, fasted
445 animals treated with saline experience an inhibition of LH (Figure 4C; p<0.0001, one-
446 way ANOVA with Tukey's post-hoc test). In contrast to what we had predicted, animals
447 that were fasted and treated with liraglutide also experienced a similar inhibition of LH
448 (Figure 4C; p<0.0001, one-way ANOVA with Tukey's post-hoc test) indicating that
449 enhancing GLP-1R signaling with peripheral injections of liraglutide is not sufficient to
450 prevent LH inhibition during fasting in OVX mice.
451
452 *Chronic ICV infusion of Ex 9-39 to OVX mice*
453 The data from Figure 3 and 4 suggest that although GLP-1R signaling can
454 stimulate ARC Kiss1 action, it may not be a potent enough signal to override the
455 inhibition on the reproductive neuroendocrine axis during extreme cases of nutrient

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deprivation such as a 48 h fast in mice. It could also suggest that other stimulatory signals may be more important to maintaining ARC Kiss1 action and downstream GnRH/LH release. Therefore, we next aimed to determine whether GLP-1R signaling is critical for maintaining ARC Kiss1 expression and circulating LH levels. To do this, we gave chronic ICV infusion of the GLP-1R antagonist Exendin 9-39 (Ex-9) to OVX mice. We chose a dose of 7.5 nmol/day of Ex-9 as this dose has been previously used in adult male mice (Nogueiras et al., 2009). After 6 days of ICV infusion of Ex-9 in OVX mice, no differences in cumulative food intake (24.62 ± 0.57 g vs 24.99 ± 0.46 g; Saline vs Ex-9; p=0.62, unpaired t-test, n=9 animals per group) were observed between saline and Ex-9 treated animals which is consistent with previous reports in male mice (Nogueiras et al., 2009). We did not detect a difference in body weight in Ex-9 treated animals as compared to saline-treated controls, however we did note a sizable body weight gain in both groups at the end of the infusion period (percent increase in body weight, Saline 12.79 ± 1.02% and Ex-9 13.01 ± 1.39%). The ICV implantation was started 1-week post-OVX surgery which is about the time that mice tend to increase their body weight in response to removal of ovarian hormones (Witte et al., 2010). Therefore, the rise in body weight in response to removal of ovarian hormones may be masking the body weight effects of Ex-9 at this dose. Despite seeing no differences in body weight between saline and Ex-9 treated animals, ARC expression of *Agrp* was increased in Ex-9 treated mice (Figure 5A; p=0.0023, unpaired t-test) confirming proper cannula placement. We did not detect differences in ARC *Kiss1 (*p=0.26*,* unpaired t-test*)* expression or in plasma LH levels (p=0.91, unpaired t-test) in Ex-9 treated animals

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as compared to saline controls (Figure 5B,C), suggesting that GLP-1R signaling is not essential to maintaining ARC Kiss1 and circulating LH.

Discussion

These data are the first to provide direct neuroanatomical, molecular and electrophysiological evidence of the interaction of the CNS GLP-1 system with ARC Kiss1 neurons to stimulate their activity. Although our studies focus on CNS-preproglucagon interactions with ARC Kiss1, we cannot discount that GLP-1 produced by the gastrointestinal tract may also activate GLP-1Rs on ARC Kiss1 neurons. Nevertheless, our reports are consistent with others demonstrating that GLP-1R signaling stimulates the reproductive neuroendocrine axis as GLP-1 increases GnRH/LH levels in animals under normal feeding conditions (Beak et al., 1998; Outeirino-Iglesias et al., 2015). Our data suggest that GLP-1 stimulatory action on GnRH/LH may be due, in part to upstream activation of ARC Kiss1 neurons. We show that the GLP-1R agonist liraglutide causes a membrane depolarization in approximately 60% of ARC Kiss1 neurons from OVX mice. Furthermore, we find that liraglutide depolarizes ARC Kiss1 cells from intact male and OVX+E_2 mice suggesting that this effect is not sex or estrogen dependent. Follow-up studies will be necessary to further characterize the pharmacological properties of GLP-1R signaling in both female and male ARC Kiss1 neurons.

In addition to acting indirectly through ARC Kiss1 neurons to modulate LH release we also find that GLP-producing fibers come in close contact with GnRH neurons which is consistent with the findings of other groups (Farkas et al., 2016).

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Furthermore, recent electrophysiological studies demonstrated that the GLP-1R agonist, exendin-4 activates GnRH neurons (Farkas et al., 2016). Although our studies focused on GLP-1R activation of ARC Kiss1 neurons, it appears that GLP-1R signaling may modify GnRH/LH release through activation of both ARC Kiss1 and GnRH neurons.

Our current electrophysiological data, as well as data in the literature (Beak et al., 1998; Outeirino-Iglesias et al., 2015), describe an interaction of GLP-1R signaling with CNS Kiss1 action and downstream GnRH/LH in animals under normal energy balance, but no reports have investigated this interaction in animals under negative energy balance. Decreased circulating leptin and insulin during negative energy balance were believed to be key metabolic signals that reduced the activation of CNS kisspeptin neurons resulting in suppressed downstream GnRH/LH release. However, previous work from our group indicates that restoration of leptin and/or insulin infused at physiological levels was not sufficient to prevent this inhibition (Xu et al., 2009; True et al., 2011). Therefore, the factors that contribute to the inhibition of the reproductive axis during negative energy balance remain elusive. Our current data reveal that GLP-1R activation stimulates ARC Kiss1 neuronal activity leading us to hypothesize that a reduction in CNS preproglucagon may be one of these key metabolic factors. Although we do find that brainstem preproglucagon expression is reduced following a 48h fast in OVX mice, restoring GLP-1R signaling with peripheral injections of liraglutide was not sufficient to prevent LH inhibition. Assessing GLP-1R action in electrophysiological recordings of ARC Kiss1 neurons from 48h fasted mice would clarify whether GLP-1R signaling has full potency during fasting. Previous data demonstrate that the anorectic

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524 action of central GLP-1R signaling is blunted in fasted rats (Sandoval et al., 2012).
525 Furthermore, activation of GLP-1 producing neurons by cholecystokinin as measured by
526 cfos is reduced in food deprived rats (Maniscalco and Rinaman, 2013). The decreased
527 function of CNS GLP-1R signaling or brainstem preproglucagon activity during negative
528 energy balance could be due to a lack of other metabolic signals necessary for full
529 potency of action. For example, leptin is significantly reduced during nutrient deprivation
530 (Ahren et al., 1997), and leptin relieves the blunted anorexigenic action of CNS GLP-1R
531 in fasted rats (Sandoval et al., 2012). Moreover, leptin prevents the suppression of
532 brainstem preproglucagon expression in fasted mice (Huo et al., 2008) which may be
533 due to direct action on brainstem preproglucagon neurons (Hisadome et al., 2010). In
534 the future, studies that aim to restore multiple metabolic factors (ie leptin, GLP-1,
535 insulin) may more effectively restore GnRH/LH release during negative energy balance.
536 This multi-agonist approach is currently being explored as a potential therapeutic for
537 obesity, a disease that encompasses the dysfunction of multiple metabolic pathways
538 (Finan et al., 2015a). Promising preclinical studies demonstrate that treating obese
539 animal models with dual (Finan et al., 2013) and triagonists (Finan et al., 2015b) could
540 have more potent effects on weight loss as compared to single molecule therapies.
541 Further investigation is necessary to determine whether a similar multiagonist
542 therapeutic approach will ameliorate reproductive dysfunction associated with negative
543 energy balance.
544
545 It is possible that the lack of effectiveness of GLP-1R agonism on LH levels
546 during fasting reflects the presence of multiple inhibitory pathways that block GnRH/LH

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release. For example, ghrelin (Tschop et al., 2000), corticosterone (Dallman et al., 1999), and FGF21 (Zhang et al., 2015) are all significantly elevated during food deprivation. Interestingly, all of these hormones inhibit the reproductive neuroendocrine axis (Barreiro and Tena-Sempere, 2004; Kinsey-Jones et al., 2009; Owen et al., 2013). Similarly, upregulation of brainstem glucose-sensing neurons during fasting may be overriding excitatory signals on the reproductive neuroendocrine axis. Noradrenergic glucose-sensing neurons in the A1 region of the ventral lateral medulla (VLM) are potent regulators of LH (Ritter et al., 2006) and ablation of these neurons prevents LH inhibition in response to glucoprivation (I'Anson et al., 2003). Recent studies reveal that preproglucagon-expressing neurons make close appositional contacts onto catecholaminergic neurons of the A1/C1 region of the VLM (Llewellyn-Smith et al., 2013). The physiological significance of these contacts has not been studied. It is interesting to hypothesize that under normal feeding conditions, preproglucagon-expressing neurons inhibit A1 glucose-sensing neurons. Therefore, decreased brainstem preproglucagon expression during fasting allows for the disinhibition of A1 glucose-sensing neurons in the VLM contributing to the shutdown of the reproductive neuroendocrine axis. This interpretation is in accordance with our studies where liraglutide did not prevent LH inhibition as preproglucagon neurons lack GLP-1R expression and are not activated by exogenous GLP-1R agonism (Hisadome et al., 2010). Determining whether preproglucagon-expressing neurons aid in the regulation of glucose-sensing neurons in the brainstem to control proper neuroendocrine output according to metabolic status would be of interest to explore in the future.

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569 Our present data demonstrate that pharmacological inhibition with the GLP-1R
570 antagonist, Ex-9 does not alter ARC Kiss1 gene expression or circulating LH levels.
571 This is consistent with transgenic mouse data demonstrating that global deletion of the
572 GLP-1R does not alter the number or distribution of gonadotrophs and adult *Glp1r-/-*
573 mice are fertile (MacLusky et al., 2000). Together, these data suggest that GLP-1R
574 signaling may not be essential for maintaining ARC Kiss1 and LH in animals that are in
575 normal energy balance. In contrast, a recent publication examined CNS GLP-1R action
576 in prepubertal female rats and demonstrated that low doses of icv GLP-1 synchronized
577 vaginal opening and increased LH whereas the GLP-1R agonist exendin-4 inhibited
578 vaginal opening and decreased LH independently of reduced feeding (Outeirino-Iglesias
579 et al., 2015). These data are inconsistent with our current data where pharmacological
580 doses of a GLP-1R agonist failed to alter LH release in adult animals during fasting.
581 The reason for this discrepancy is unclear.
582 Our in vivo pharmacological studies were all performed in OVX mice, so as to be
583 able to measure the inhibitory effect of fasting on basal LH levels. Intact and OVX + E_2
584 mice have very low levels of basal LH, making it technically difficult to measure the
585 inhibition of LH in these models. Therefore, if GLP-1 is playing a role in the inhibition of
586 LH due to negative energy balance, its effects should be manifested in the OVX model.
587 Although our results show that estradiol appears to have little effects on the ability of
588 Kiss1 cells to be activated by GLP-1, it is possible that there may be estradiol
589 dependent effects of GLP-1 during other reproductive states such as puberty, and
590 follow-up studies in OVX + E_2 models may be warranted.
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592 In addition to regulating reproductive neuroendocrine function, Kiss1r signaling
593 may regulate energy homeostasis as loss of Kiss1r signaling leads to body weight gain
594 (Tolson et al., 2014). The kisspeptin population responsible for this effect is unknown
595 but ARC Kiss1 neurons may be prime candidates as they are in a primary brain area
596 that regulates energy homeostasis and send fiber projections to numerous
597 hypothalamic nuclei that regulate feeding and energy expenditure (Yeo and Herbison,
598 2011; Yeo, 2013). Furthermore, kisspeptin-ir neurons are in close apposition with ARC
599 proopiomelanocortin (POMC) neurons and electrophysiological recordings demonstrate
600 that kisspeptin directly excites ARC POMC and indirectly inhibits ARC neuropeptide Y
601 (NPY) neurons (Fu and van den Pol, 2010). Although GLP-1R signaling was reported
602 to regulate GnRH/LH release (Beak et al., 1998; Outeirino-Iglesias et al., 2015), the
603 most consistent physiological output of GLP-1 mimetics is reduced body weight, which
604 requires CNS GLP-1R signaling (Sisley et al., 2014). Activation of GLP-1R signaling
605 directly stimulates ARC POMC neurons and indirectly inhibits ARC NPY neurons, which
606 are thought to be important mechanisms whereby GLP-1R agonists mediate a reduction
607 in body weight (Secher et al., 2014). In addition to regulating ARC POMC and NPY, our
608 electrophysiological data may suggest that GLP-1R signaling regulates energy
609 homeostasis through activation of ARC Kiss1 neurons. Determining the effectiveness of
610 GLP-1R agonists on weight loss in transgenic animals with a specific inhibition of ARC
611 Kiss1 neurons may help to clarify this role of ARC Kiss1.
612
613 In summary, we find that GLP-producing fibers interact with ARC Kiss1 cells
614 which express the GLP-1R. Furthermore, GLP-1R signaling directly activates ARC

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615 Kiss1 function in an estradiol independent manner. Despite a clear stimulatory effect on
616 ARC Kiss1 action we find that pharmacological activation of GLP-1R signaling during
617 fasting or pharmacological inhibition of CNS GLP-1R signaling during normal feeding
618 does not alter circulating LH levels suggesting that GLP-1R activation is not critical for
619 the maintenance of LH in adult animals. Alternatively, GLP-1R signaling within ARC
620 Kiss1 cells may regulate an unidentified physiological output of ARC Kiss1 activation.
621 Further studies are necessary to fully understand the significance of GLP-1R activation
622 of ARC Kiss1. Collectively, these data not only identify a novel signal that stimulates
623 ARC Kiss1 cell activity, but also highlights the complexity of metabolic signals that
624 regulate the reproductive neuroendocrine axis.
625
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627 **Figure Legend**

628 **Figure 1: Interaction of the CNS GLP system with ARC Kiss1 in OVX mice.** GLP-ir
629 fibers (red) come in close apposition with an average of 22% of ARC kisspeptin-ir cells
630 (green). A) Maximal projection at 40x zoom B) Maximal projection at 63x zoom C) 1µm
631 plane at 63x zoom. Scale bars= 10 µm. n= 5 animals, 4 sections per animal. D)
632 Representative gel of single cell RT-PCR demonstrating that a subpopulation (20%) of
633 ARC Kiss1 cells from OVX mice express *Glp1r* mRNA. n=5 animals, 16-33 cells per
634 animal. The expected sizes for the PCR products are 120 bp for Kiss1 and 148 bp for
635 *Glp1r*. MM, molecular marker; -RT, Kiss1-GFP cell reacted without reverse transcriptase
636 (RT); Tissue controls (+, -), basal hypothalamic RNA reacted with (+) or without (-) RT. E)
637 Dual *in situ* hybridization demonstrating co-expression of *Glp1r* (red) and *Kiss1* (green)
638 mRNA in the ARC (51 out of 240 cells, 21.3% co-expression). In this example, an OVX
639 animal showed robust *Kiss1* mRNA expression in neurons intermingled with a larger
640 population of *Glp1r*+ neurons in the ARC. F) Inset from panel E. At higher magnification,
641 a subpopulation of ARC Kiss1 neurons express robust and detectable mRNA signal for
642 *Glp1r*. Filled black arrows indicate high *Glp1r* expression, open arrows indicate low
643 *Glp1r* expression. n=4 animals; Scale bars = 100 µm. 3V = third ventricle.
644
645 **Figure 2: Electrophysiological recordings in brain slices demonstrating effects of**
646 **GLP-1R signaling on ARC Kiss1 cells of OVX mice.** Current clamp recordings in
647 brain slices from OVX mice demonstrate that ARC Kiss1 cells treated with the long-
648 acting GLP-1R agonist, liraglutide, showed a membrane depolarization and increased
649 action potential firing (A). *p<0.05, **p<0.01 vs RMP, one-way RM-ANOVA with
650 Bonferroni's post-hoc test; ††p0.01, 100nM vs 300nM liraglutide, one-way RM-ANOVA

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651 with Bonferroni's post-hoc test. Current clamp recordings performed in the presence of
652 presynaptic blockers demonstrate that liraglutide caused a membrane depolarization in
653 ARC Kiss1 cells of OVX mice (B). **p<0.01 RMP vs TTX+CNXQ+AP5+Liraglutide
654 300nM, one-way RM-ANOVA with Bonferroni's post-hoc test; ††p0.01, 100nM vs
655 300nM liraglutide, one-way RM-ANOVA with Bonferroni's post-hoc test. ##p<0.01
656 TTX+CNXQ+AP5 vs TTX+CNXQ+AP5+Liraglutide 300nM, one-way RM-ANOVA with
657 Bonferroni's post-hoc test; n=23 cells from 16 animals. ~60% of ARC Kiss1 cells
658 respond to liraglutide.
659
660 **Figure 3: Electrophysiological recordings in brain slices demonstrating effects of**
661 **GLP-1R signaling on ARC Kiss1 cells of OVX+E_2 and male mice.** Current clamp
662 recordings in ARC Kiss1 cells from brain slices treated with the long-acting GLP-1R
663 agonist, liraglutide, showed a membrane depolarization in both OVX+E_2 (A; 52% of cells
664 responded) and male (B; 60% of cells responded). ***p<0.001, ****p<0.0001 vs RMP,
665 one-way RM-ANOVA with Bonferroni's post-hoc test; †††p<0.001, 100nM vs 300nM
666 liraglutide; one-way RM-ANOVA with Bonferroni's post-hoc test. The magnitude of
667 depolarization was greater in males as compared to OVX+E_2 females at 100nM and
668 300nM concentrations (C). *p<0.05 OVX+E_2 vs males, one-way ANOVA with
669 Bonferroni's post-hoc test. n=13 male and 27 OVX +E_2 mice.
670
671 **Figure 4: Effects of fasting in OVX mice on brainstem preproglucagon**
672 **expression and on GLP-1R agonism to restore fasting-suppressed LH levels.**
673 Brainstem preproglucagon (Gcg) expression was assessed using qPCR and is

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decreased following a 48h fast (A;***p<0.001, unpaired t-test). To determine whether GLP-1R agonism prevents LH inhibition during calorie restriction, liraglutide (30nmol/kg) was administered subcutaneously twice-daily at the start of a 48h fast (B,C). Saline-fasted and liraglutide-fasted animals display decreased body weight compared to saline-fed controls (B; **p<0.01, two-way ANOVA with Bonferroni's post-hoc test). Saline-fasted and liraglutide-fasted animals display significantly lower levels of LH as compared to saline-fed controls (C; ****p<0.0001, one-way ANOVA with Tukey's post-hoc test). n=7-8 animals per group.

Figure 5: Effect of chronic icv Ex-9 on food intake, body weight, ARC gene expression and plasma LH in OVX mice. C57BL/6 mice were OVX and one week later received an icv infusion of saline or the long-acting GLP-1 antagonist, Ex-9, for 6 days (7.5 nmol/ day). Ex-9 caused a significant increase in ARC expression of *Agrp* (A; **p<0.01, unpaired t-test), but did not alter ARC expression of *Kiss1* (B). Plasma LH levels were similar in saline and Ex-9 treated mice (C). n= 9 animals per group.

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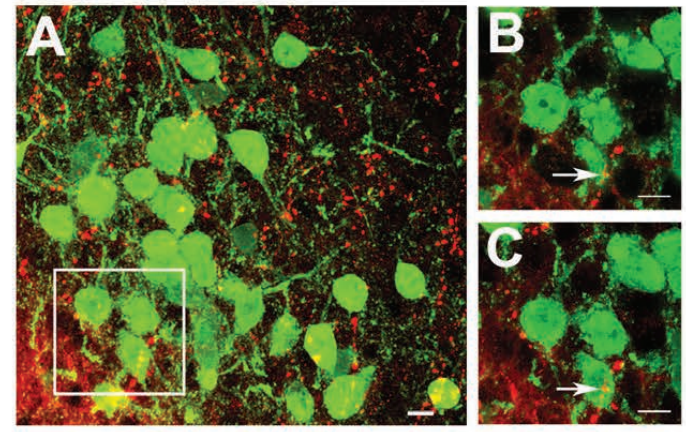

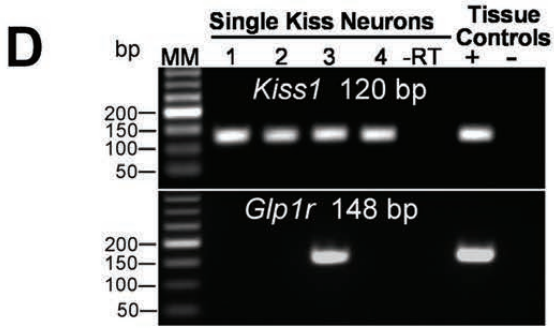

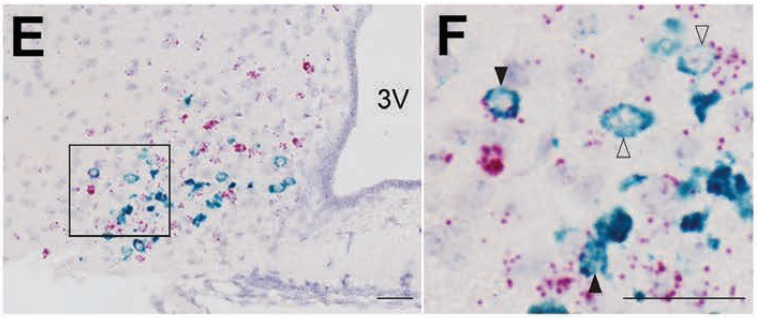

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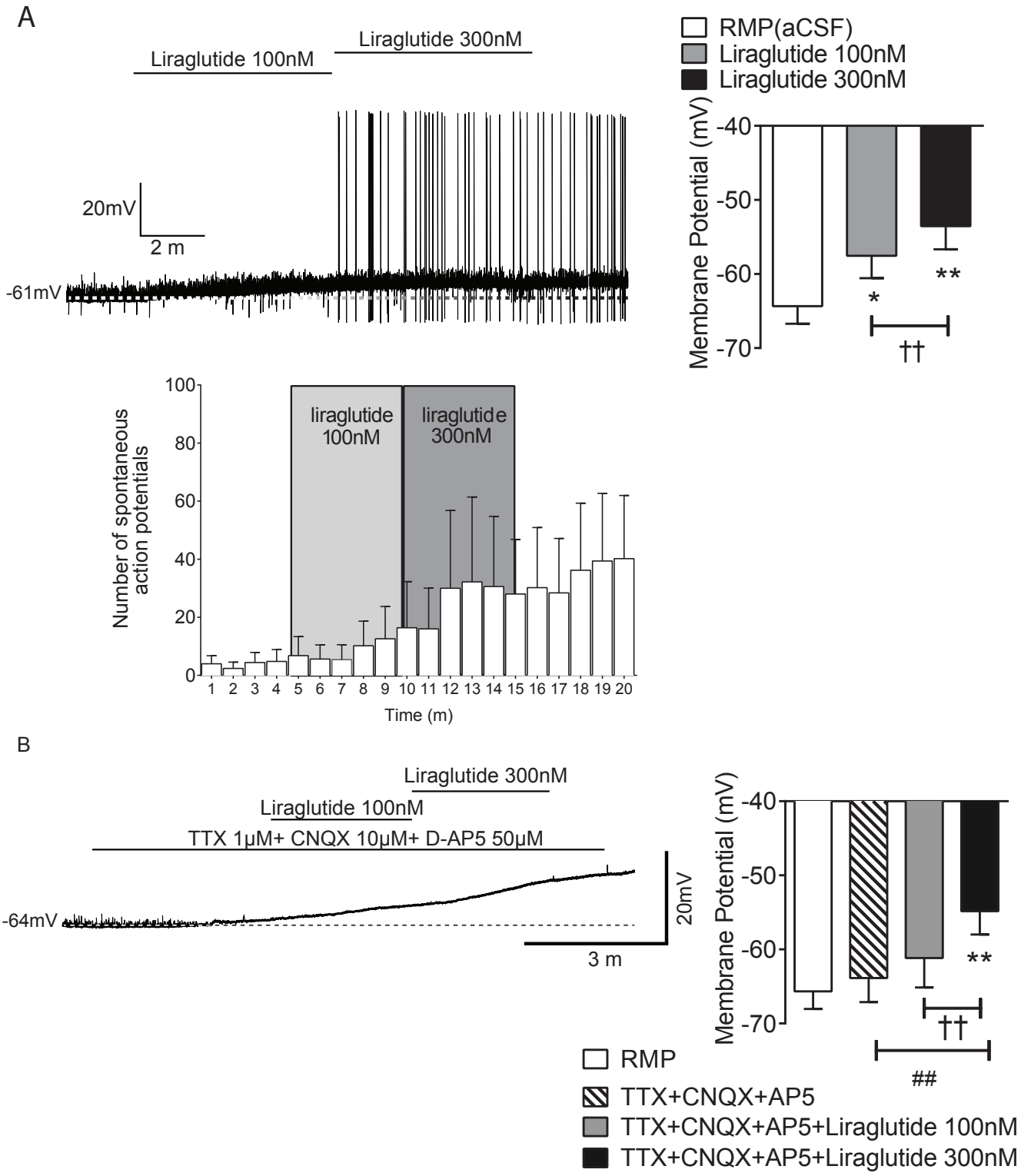

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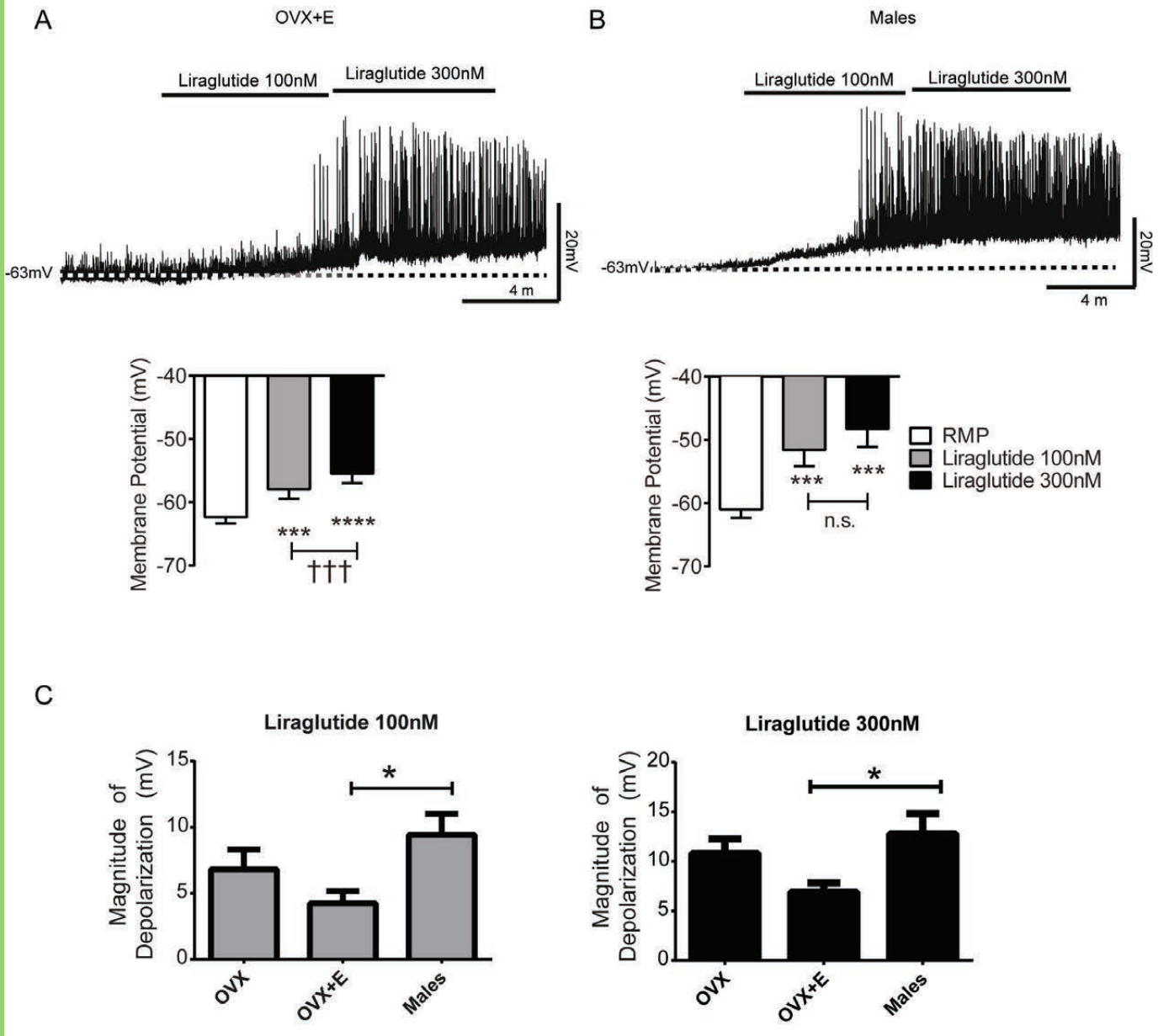

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Figure 4

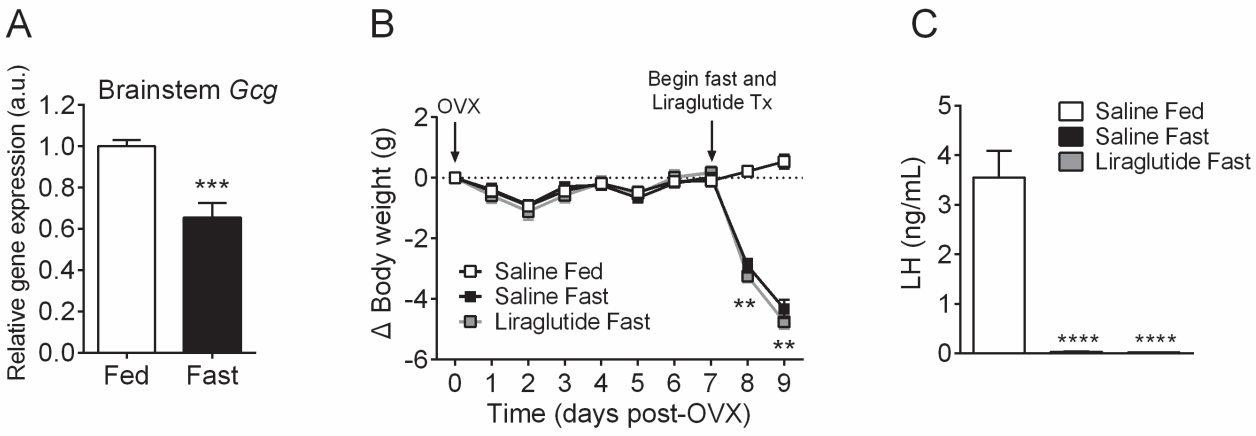

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Figure 5