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Combined loss of ghrelin receptor and cannabinoid CB1 receptor in mice decreases survival but does not additively reduce body weight or eating

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Abstract

Ghrelin administration increases food intake, body weight (BW), adiposity, and blood glucose. In contrast, although mouse models lacking ghrelin or its receptor (GHSR) exhibit life-threatening hypoglycemia in starvation-like states, they do not exhibit appreciable reductions in food intake, BW, adiposity, blood glucose, or survival when food availability is unrestricted. This suggests the existence of a parallel neuromodulatory system that can compensate for disruptions in the ghrelin system in certain settings. Here, we hypothesized that the cannabinoid CB1 receptor (CB1R) may encode this putative redundancy, and as such, that genetic deletion of both GHSR and CB1R would exaggerate the metabolic deficits associated with deletion of GHSR alone. To test this hypothesis, we assessed food intake, BW, blood glucose, survival, and plasma acyl-ghrelin in *ad libitum*-fed male wild-type mice and those that genetically lack GHSR (GHSR-nulls), CB1R (CB1R-nulls), or both GHSR and CB1R (double-nulls). BW, fat mass, and lean mass were similar in GHSR-nulls and wild-types, lower in CB1R-nulls, but not further reduced in double-nulls. Food intake, plasma acyl-ghrelin, and blood glucose were similar among genotypes. Deletion of either GHSR or CB1R alone did not have a statistically-significant effect on survival, but double-nulls demonstrated a statistical trend towards decreased survival ($p=0.07$). We conclude that CB1R is not responsible for the normal BW, adiposity, food intake, and blood glucose observed in GHSR-null mice in the setting of unrestricted food availability. Nor is CB1R required for plasma acyl-

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ghrelin secretion in that setting. However, GHSR may be protective against exaggerated mortality associated with CB1R deletion.

Keywords

Ghrelin; endocannabinoids; food intake; body weight; survival

Introduction:

The neuronal circuitry that regulates ingestion of sufficient food to sustain life is complex and is modulated by multiple hormones and neurotransmitters. One such hormone, ghrelin, secreted predominantly from the stomach and acting through the growth hormone secretagogue receptor (GHSR), is a key hormonal signal of negative energy balance that interacts with the brain and other organs to stimulate food intake and promote survival (Mani, et al. 2017a, Muller, et al. 2015). Supporting this notion, plasma ghrelin increases upon food deprivation and body weight loss and decreases post-prandially and in obese states, and ghrelin administration robustly stimulates food intake in rodent models and humans (Toshinai, et al. 2001, Mani, et al. 2016, Zhao, et al. 2010, Ariyasu, et al. 2001, Tschop, et al. 2000, Liu, et al. 2008, Cummings, et al. 2001, Frecka, et al. 2008, Cummings, et al. 2002). Also, chronic ghrelin administration stimulates body weight gain and adiposity *via* stimulatory effects on food intake and inhibitory effects on energy expenditure (Tschop, et al. 2000, Wren, et al. 2001, Nakazato, et al. 2001, Kamegai, et al. 2000). Apart from contributing to these homeostatic functions, and its initially characterized growth hormone (GH) secretagogue actions, the ghrelin system also is implicated in stimulating hedonic aspects of feeding, as assessed, for instance by observing ghrelin-induced activation of CNS reward circuits using imaging modalities in human subjects and with operant responding, conditioned place preference, cue-potentiated feeding, and food preference paradigms in rodent models (Muller, et al. 2015, Lockie, et al. 2013, Perello, et al. 2015, Goldstone, et al. 2014, Perello, et al. 2010, Walker, et al. 2012, St-Onge, et al. 2016, Skibicka, et al. 2011, Egicioglu, et al. 2010). Many of these food reward behaviors are blunted in genetic mouse models lacking components of the ghrelin system, such as ghrelin, GHSR, or GOAT (ghrelin *O*-acyltransferase, the enzyme that acylates ghrelin, thus allowing it to bind GHSR) (Perello, et al. 2010, Egicioglu, et al. 2010, Abizaid, et al. 2006, Chuang, et al. 2011a, Davis, et al. 2012). These studies suggest a required role of ghrelin in modulating many hedonic feeding behaviors.

Ghrelin system-deficient mouse models also demonstrate life-threatening hypoglycemia during severely calorically restricted states, indicating a required metabolic role of ghrelin during starvation-like states (Mani, et al. 2016, Zhao, et al. 2010, McFarlane, et al. 2014, Li, et al. 2012, Wang, et al. 2014, Zhang, et al. 2015). However, when food availability is unrestricted, the loss-of-ghrelin function mouse models do not exhibit marked reductions in food intake, body weight, or adiposity. Indeed, genetic mouse models with loss-of-ghrelin function [GOAT-knockout (KO), ghrelin-KO, GHSR-KO, and GHSR-null] demonstrate similar food intake and body weight when fed standard chow, and either no or only partial resistance to diet-induced obesity (Zhao, et al. 2010, Sun, et al. 2004, Zigman, et al. 2005,

Wortley, et al. 2004, Sun, et al. 2003, De Smet, et al. 2006, Kirchner, et al. 2009, Pfluger, et al. 2008). Also, diphtheria toxin-induced ablation of ghrelin cells in adult mice does not reduce food intake or reduce body weight, thus ruling out the potential for early developmental compensatory changes (that would not necessarily occur in adults upon the acute ablation of ghrelin cells) as the reason for the lack of a food intake or body weight phenotype in ghrelin-KO mice (McFarlane, et al. 2014).

An alternative hypothesis to explain the lack of marked food intake, body weight, and blood glucose effects in *ad libitum*-fed conditions upon deletion of components of the ghrelin system might be the existence of a parallel neuromodulatory system that can compensate for disruptions in the ghrelin system. Such redundancy seems plausible, especially given the fundamental importance of eating for survival. One possible candidate is the endogenous cannabinoid (endocannabinoid) system which works by engaging both CB1R and CB2R. CB1R is a G-protein coupled receptor (GPCR) that is abundantly-expressed in the central nervous system (Piazza, et al. 2017). Similar to the ghrelin system, activation of CB1R induces food intake, body weight gain, and food reward behaviors, and endocannabinoid release increases in settings of calorie restriction (Piazza, et al. 2017, Edwards, et al. 2016, Kirkham, et al. 2002). Of particular interest, the expression pattern of CB1Rs and GHSRs overlap in several brain nuclei known to regulate food intake and body weight, including the hippocampus, anterior hypothalamic area, medial preoptic area, dorsomedial hypothalamus, ventromedial hypothalamus, lateral hypothalamic area and ventral tegmental area (Edwards, et al. 2016, Mani, et al. 2017b, Mani, et al. 2014, Zigman, et al. 2006, Wittmann, et al. 2007, Herkenham, et al. 1991). Overlapping CB1R and GHSR expression also occurs in the arcuate hypothalamic nucleus. However, while ghrelin's orexigenic actions are partially mediated by GHSRs highly expressed on arcuate NPY/AgRP neurons, CB1R's food intake stimulatory actions in the arcuate nucleus instead have been proposed to be mediated via CB1R-expressing axons that form synapses with the NPY/AgRP neurons, thus disinhibiting them (Nakazato, et al. 2001, Wang, et al. 2014, Mani, et al. 2017b, Mani, et al. 2014, Zigman, et al. 2006, Cowley, et al. 2003, Cota, et al. 2003, Morozov, et al. 2017, Willeesen, et al. 1999). A search for co-localization of GHSRs and CB1Rs within anorexigenic arcuate POMC neurons has not explicitly been reported, although expression of GHSRs is poor in this population, while CB1Rs within the mitochondria of POMC neurons have been suggested to contribute to endocannabinoid-stimulated feeding (Willeesen, et al. 1999, Koch, et al. 2015). Endocannabinoids also bind CB2R and other targets, but pharmacological and genetic studies suggest that their metabolic actions mainly are mediated *via* CB1R (Piazza, et al. 2017).

Moreover, other studies have revealed functional interactions between the ghrelin and endocannabinoid systems, which may suggest an interdependency between the two systems. For instance, peripheral administration of CB1R inverse agonist SR141716A (rimonabant) or CB1R antagonists AM-251 or LH-21 completely prevents the orexigenic action of central or s.c. administered ghrelin or the ghrelin mimetic hexarelin in rodents, suggesting that ghrelin's orexigenic efficacy depends on endocannabinoid release and activation of CB1R (Alen, et al. 2013, Ting, et al. 2015, Tucci, et al. 2004, Rigamonti, et al. 2006, Senin, et al. 2013). Ghrelin-induced food intake is absent in CB1R-KO mice and in wild-type mice administered SR141716A i.c.v. (Kola, et al. 2008). Direct microinjection of SR141716A

into the ventral tegmental area (VTA) of mice prevents locally injected ghrelin's action to increase locomotor activity, but not food intake (Kalafateli, et al. 2018). In contrast, direct microinjection of AM-251 into the paraventricular nucleus of the hypothalamus (PVN) potentiates PVN injected ghrelin's action to stimulate food intake (Soria-Gomez, et al. 2014). Local administration of either ghrelin or the CB1R agonist anandamide to the PVN of rats stimulates food intake and increases substrate utilization, as reflected by an increased respiratory exchange ratio (RER) (Wren, et al. 2001, Abtahi, et al. 2019, Chapman, et al. 2012, Melis, et al. 2002, Olszewski, et al. 2003). In another study, i.p.-administered but not i.c.v.-administered SR141716A prevented ghrelin-induced GH secretion (Al-Massadi, et al. 2010). Conversely, the effect of a CB1R agonist on hypothalamic, visceral fat, and liver AMPK (AMP-activated protein kinase) activity was absent in GHSR-KO mice, suggesting requirement of intact ghrelin signaling for cannabinoid-induced AMPK activity in those tissues (Lim, et al. 2013). In summary, these studies suggest that the endocannabinoid and ghrelin systems interact in multiple brain nuclei and in the periphery to regulate food intake and behavior.

There is also evidence that endocannabinoids working *via* CB1R stimulate ghrelin secretion. For instance, a single i.p. injection of the cannabinoid agonists R-(+)-methanandamide or CP55,940 attenuates ghrelin-immunoreactivity within the gastric mucosa and increases plasma ghrelin in rats (Zbucki, et al. 2008). Also, plasma ghrelin is lower in fasted rats given SR141716A or another CB1R blocker, oleylethanolamine (Senin, et al. 2013, Cani, et al. 2004). Blockade of peripheral CB1R also reduces ethanol ingestion-induced increases of plasma acyl-ghrelin in mice (Godlewski, et al. 2019). Altogether, these results suggest that endocannabinoids acting through CB1R may increase food intake in part by stimulating ghrelin secretion.

The aforementioned pharmacological studies demonstrating interaction between the ghrelin and the endocannabinoid systems, along with their overlapping functions, receptor distributions, and activation of similar signal transduction cascades suggest the possibility that the two systems function in a complementary fashion to regulate food intake and body weight. Furthermore, this evidence leads us to hypothesize that the endocannabinoid-CB1R system can compensate for disruptions in the ghrelin system. If so, we would predict that in the setting of unrestricted access to food, genetic deletion of both GHSR and CB1R would exaggerate the metabolic deficits associated with deletion of GHSR or CB1R alone, possibly leading to marked reduction in food intake. In the present study, we tested this hypothesis by comparing food intake, body weight, blood glucose, and survival of wild-type mice, GHSR-null mice, CB1R-null mice, and mice that lack both GHSRs and CB1Rs (double-nulls) during *ad libitum* exposure to regular chow. We also tested if CB1Rs are required for normal ghrelin secretion by comparing plasma ghrelin in CB1R-null mice to that in controls and by determining the effects of CB1R agonists on ghrelin secretion from primary cultures of gastric mucosal cells from wild-type mice.

Materials and Methods:

Animal studies:

All animal experiments were approved by the UT Southwestern Medical Center Institutional Animal Care and Use Committee, and were carried out in accordance with the National Institute of Health *Guide for the Care and Use of Laboratory Animals*. Mice were housed under a 12 h dark-light cycle in standard environmentally-controlled conditions, at room temperature. The GHSR-null mouse line was described previously (Zigman, et al. 2005) and has been used in numerous studies by our group and others (Perello, et al. 2010, Wang, et al. 2014, Zigman, et al. 2005, Kurashina, et al. 2015, Walker, et al. 2015, Scott, et al. 2012).

Generation of a novel CB1R-null mouse line:

A novel CB1R-null mouse line was generated by inserting a loxP-flanked transcriptional blocking cassette (TBC) into the endogenous murine CB1R gene (*cnr1*) locus using previously-described BAC (bacterial artificial chromosome) recombineering techniques (Zigman, et al. 2005). The TBC also was as described previously (Zigman, et al. 2005) and included the following elements (in the order listed): a splice acceptor site from the mouse *engrailed 2* gene (*En-2*), an *SV40* poly(A) signal, an *SV40* enhancer, a neomycin resistance (*neo^R*) gene, two *HSV-TK* poly(A) signals, a synthetic poly(A) signal/transcriptional pause signal, another synthetic poly(A) signal, and a Myc-associated zinc finger protein-binding site, and was flanked by loxP sites. This construct was inserted into a mouse *cnr1*-containing BAC (BMQ-337H13; Source Bioscience, Nottingham, UK), at a site 11 base pairs upstream of the translational start site of the *cnr1* gene, in exon 2. The final targeting construct, spanning approximately 6 kb upstream and 7 kb downstream of the TBC insertion site, was cloned into a commercially available pGEM vector (Promega, Madison, WI), which subsequently was electroporated into 129J6 ES cells by the UT Southwestern Medical Center Transgenic Technology Core Facility. Correctly-targeted ES cell clones were identified by Southern blot and PCR analyses, and injected into mouse blastocysts. Confirmation of targeted insertion and germline transmission of the *loxP*-TBC-*loxP*-containing *cnr1* allele was determined with the help of PCR amplification of genomic DNA of resulting pups and sequencing the PCR products. CB1R-null mice (harboring 2 copies of the recombinant *cnr1* allele) and their littermates, which included heterozygotes (harboring 1 copy of the recombinant *cnr1* allele and 1 copy of the wild-type *cnr1* allele) and wild-type mice (harboring 2 copies of the wild-type *cnr1* allele), were viable and were born with the expected Mendelian distribution upon mating of heterozygotes. The CB1R-null line was backcrossed to C57BL/6J mice for more than 10 generations prior to their use here.

Validation of the CB1R-null line:

The CB1R-null mouse line was validated by demonstrating the absence of CB1R mRNA expression in brain sections using *in situ* hybridization histochemistry (ISHH), as previously described (Vianna, et al. 2012). The [³⁵S]UTP antisense cRNA riboprobe used in ISHH was transcribed from a template generated by PCR amplification of genomic DNA with the following CB1R-specific primers: forward, 5'-CTGCAAGAAGCTGCAATCTG-3' and reverse, 5'-TGGCGATCTTAACAGTGCTC-3'. ISHH was performed in 3 CB1R-null mice and 3 wild-type controls. The line was functionally validated by demonstrating the absence

of hyperphagia in response to administration of a potent CB1R-selective agonist WIN-55,212-2 (Tocris Bioscience, Minneapolis, MN). WIN 55,212-2 was prepared in saline solution containing 5% DMSO and Tween 80 (1drop/5mL). Adult CB1R-null mice and wild-type littermates were administered vehicle and then with WIN 55,212-2 (0.6 mg/kg BW i.p.) 24h later. The experiments were performed during the first hour after the lights are switched on. One-hour and 2h food intake was measured after both vehicle and WIN 55,212-2 administration.

Experimental groups and measurement of metabolic parameters:

GHSR-null mice (which lack GHSRs) (Zigman, et al. 2005) maintained on a C57BL/6N background were crossed with CB1R-null mice to generate mice heterozygous for both the GHSR-null and CB1R-null alleles. Male and female double heterozygotes were crossed to generate the following four experimental mouse groups: wild-type mice, GHSR-nulls, CB1R-nulls, and double-nulls (that lack both GHSRs and CB1Rs). The mice were weaned and individually housed beginning at 4 wks-of-age and were allowed free access to regular chow diet [2016 Teklad Global 16% protein diet (Envigo, Indianapolis, IN)]. Only male mice were used for the study. Weekly food intake, weekly body weight, and overall survival were assessed until 20 wks-of-age, at which time body composition was determined with an EchoMRI-100 analyzer (EchoMRI LLC, Houston, TX). Thereafter, blood samples for acyl-ghrelin estimation were collected by a quick superficial temporal vein bleed into EDTA-coated microcentrifuge tubes containing p-hydroxymercuribenzoic acid (final concentration 1 mM) placed on ice. The blood samples were immediately centrifuged at 4°C at 1,500 g x 15 min. Hydrochloric acid was added to the plasma aliquot for ghrelin estimation to achieve a final concentration of 0.1 N for stabilization of acyl-ghrelin. The processed samples were stored at -80°C until analysis. Blood glucose was measured in untreated blood at the time of the bleeds using a Bayer Contour blood glucose monitoring system (Mishawaka, IN).

Mouse gastric mucosal cell primary culture:

Gastric mucosal cells were isolated from 8–12 wk-old male C57BL/6N mice as reported previously (Mani, et al. 2016, Sakata, et al. 2012). The mice were anesthetized with chloral hydrate (700 mg/kg i.p.), followed by laparotomy to dissect out the stomachs. The stomachs were tied off at both ends with surgical sutures, excised and placed in ice-cold PBS, and then incised at the non-glandular portion to clear the digesta. The stomachs were then inverted inside-out by passing the distal part of the stomach through the incision and inflated with and briefly placed in ice-cold DMEM medium with no glucose (Life Technologies, Grand Island, NY). The residual digesta adhering to the stomach mucosa was cleared off gently by using soft paper pads. The inverted stomachs were then incubated for 1.5 hrs at 37°C in 35 U dispase II/15 mL PBS (Roche Diagnostics, Indianapolis, IN) in a 50 mL centrifuge tube, and then the mucosa was scraped off using a transfer pipette into sterile DMEM/F-12 (1:1) medium (Mediatech Inc. Manassas, VA) containing 10% (vol/vol) FBS (Atlanta Biologicals, Lawrenceville, GA), supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin sulfate. The cells were centrifuged at 310 g for 3 min, and the media was removed and subjected to digestion with 0.25% trypsin EDTA (Mediatech Inc. Manassas, VA). After 5 min, the trypsin was inactivated by adding FBS containing DMEM/F-12 medium. The mucosal cells were triturated to disperse them, filtered through a 100 µM Falcon nylon

mesh filter, and then centrifuged at 310 g for 3 min to remove the supernatant medium. The cells were resuspended in FBS containing DMEM/F-12 medium supplemented with sodium octanoate-bovine serum albumin (BSA) to achieve a final concentration of 50 μ M sodium octanoate and plated at a density of 1×10^5 cells/mL/well in poly-D-lysine pre-coated 24-well plates. The cells were placed overnight in a 37°C incubator with 5% CO₂. The next day, the cells were treated with vehicle or different concentrations of anandamide or R-(+)-methanandamide (Millipore Sigma, Burlington, MA) for 6 h in serum free DMEM (Life Technologies, Grand Island, NY) containing 5 mM glucose and 50 μ M sodium octanoate-BSA. Both anandamide and R-(+)-methanandamide stock solutions were prepared using ethanol, which was added to the vehicle control wells. At the end of 6 h incubation period, the medium was collected, placed on ice and immediately centrifuged at 4°C at 800 g x 5 min. Hydrochloric acid was added to the supernatant to achieve a final concentration of 0.1 N (for stabilization of acyl-ghrelin) and stored at -80°C until analysis.

Acyl-ghrelin measurement from plasma samples and cell culture supernatants:

Acyl-ghrelin concentrations in the plasma and cell culture supernatants were determined by ELISA (Catalog # EZRGRA-90K; Millipore-Merck, St. Charles, MO). The endpoint calorimetric measurements for the ELISA were performed using a PowerWave XS Microplate spectrophotometer and KC4 junior software (BioTek Instruments, Inc. Winooski, VT). Acyl-ghrelin secretion in the cell culture experiments was calculated first by determining the mean acyl-ghrelin concentration in the media of vehicle-treated cells. Afterwards, for each condition, the measured acyl-ghrelin concentration in the medium was divided by the mean acyl-ghrelin concentration in the media of vehicle-treated cells, resulting in fold-change in acyl-ghrelin compared to vehicle treatment.

Statistics:

All data are expressed as mean $\pm$ SEM. All statistical analyses and graph preparations were performed using GraphPad Prism 7.04 (Graphpad, San Diego, CA). One-way ANOVA or repeated measures two-way ANOVA followed by the indicated *post hoc* comparison tests were used to test for significant differences among test groups, as indicated in the figure legends. ANOVA results are presented as F statistic and degrees of freedom with the associated p value. Survival curves were calculated by the Kaplan-Meier estimate method, and differences among percent survival were compared using the Mantel-Cox log-rank test. p-values < 0.05 were considered statistically significant. p-values 0.05 and <0.1 were indicative of a statistical trend.

Results:

To validate the novel CB1R-null mouse line, which was generated using similar methodologies and the same TBC as that for the GHSR-null line, we first compared CB1R mRNA expression within the brains of CB1R-null mice and wild-type littermates by ISHH, using riboprobes specific for CB1R mRNA. CB1R mRNA expression was present within brain sections from wild-type littermates and followed a normal CB1R mRNA expression pattern (Marsicano, et al. 1999), including in the ventromedial nucleus of the hypothalamus (VMH), the CA1/CA2 regions of the hippocampus, the amygdala, the caudate putamen, and

the entorhinal and perirhinal cortical areas, as depicted in Fig. 1A. In contrast, CB1R expression as determined using the CB1R-specific riboprobe was nearly undetectable in the brain sections of CB1R-null mice (Fig. 1A).

To validate the absence of CB1R activity in CB1R-null mice, we measured the acute food intake responses to i.p. administration of the CB1R-selective agonist WIN 55,212-2 in CB1R-null mice and wild-type controls. Food intake was measured at 1 h and 2 h post-vehicle administration and again at 1 h and 2 h post-WIN 55,212-2 (0.6 mg/kg b.w., i.p.) administration in the same mice. Food intake measured at both 1 h and 2 h post-WIN 55,212-2 administration was significantly higher when compared to vehicle administration in the wild-type mice, but not in CB1R-nulls (Fig. 1B and C), indicating the absence of CB1R activity in the CB1R-null mice.

Next, we crossed the CB1R-null line to the GHSR-null line and studied four of the resulting genotypes: wild-types, GHSR-nulls, CB1R-nulls, and double-nulls (which lack both GHSRs and CB1Rs) beginning at 4 wks-of-age through 20 wks-of-age. Over that time period, the mean body weight curve of GHSR-null mice did not differ from that of wild-type mice, but CB1R-null mice weighed significantly less than the wild-type and GHSR-null mice (Fig. 2A). The body weight curve of double-null mice also was lower than that of wild-type and GHSR-null mice, but did not differ from the body weight curve of CB1R-null mice (Fig. 2A). These results indicate that the lack of GHSR does not further reduce body weights of CB1R-null mice. Despite the divergent body weight curves, weekly food intake relative to body weight was equivalent among the genotypes (Fig. 2B).

The mice were subjected to body composition analysis at 20 wks-of-age. Similar to the body weight curves, both mean fat mass and mean lean mass of GHSR-null mice were similar to those of wild-type mice (Fig. 2C and D). Fat mass and lean mass were both significantly lower in CB1R-null mice, while the fat mass and lean mass of double-null mice were equivalent to the values in CB1R-nulls (Fig. 2C, 2D). The % lean mass of both CB1R-null mice and double-null mice was significantly higher than that of wild-type and GHSR-null mice ($p < 0.05$; % lean mass of wild-type = 67.51 ± 2.1 , GHSR-nulls = 67.69 ± 1.7 , CB1R-nulls = 74.99 ± 0.9 , double-nulls = 74.23 ± 0.7), suggesting that the loss of CB1R leads to leanness.

We also monitored survival rates of the mice during the 4 to 20-wk-of-age time period. While neither GHSR deletion nor CB1R deletion alone had a statistically-significant effect on survival, double-nulls demonstrated a statistical trend towards decreased survival when compared to wild-type mice (double-nulls: 60% survival vs. wild-types: 100% survival; $p=0.07$; Fig. 3), suggesting that GHSR promotes survival in the absence of CB1Rs or vice versa.

Next, we assessed the effects of CB1R deletion on plasma ghrelin levels and blood glucose. Plasma acyl-ghrelin and blood glucose were measured in all the surviving wild-type, GHSR-null, CB1R-null, and double-null mice enrolled in the above-described longitudinal study, at 20 wks-of-age, in the *ad libitum*-fed state. Plasma acyl-ghrelin in GHSR-nulls was similar to that in wild-type mice, as noted previously (Fig. 4A) (Chuang, et al. 2011b). Similarly,

plasma acyl-ghrelin in both CB1R-nulls and double-nulls was equivalent to that of the wild-type mice and GHSR-nulls (Fig. 4A). Blood glucose also was similar among the genotypes (Fig. 4B).

Finally, to determine if cannabinoids can directly stimulate acyl-ghrelin secretion from gastric ghrelin cells, we tested the effects on ghrelin secretion of different concentrations of the endocannabinoid anandamide and the synthetic anandamide analog R-(+)-methanandamide using primary gastric mucosal cell cultures. Both anandamide and R-(+)-methanandamide stimulated acyl-ghrelin secretion in the cells, but only at the relatively high concentrations of 10 μ M (Fig. 4C and D), which is 1,000-fold more than the EC₅₀ of the compounds for stimulating CB1R activity (Khajehali, et al. 2015).

Discussion:

The ghrelin and endocannabinoid systems together have been a subject of great interest because of the overlapping distributions of GHSR and CB1R in several brain regions and some peripheral organs, their similar functions in regulating food intake, body weight, energy expenditure, feeding behaviors, and antidepressant-like activity, and their use of common signal transduction intermediates within certain brain nuclei [as reviewed in (Edwards, et al. 2016)]. While such parallelism suggests the possibility that the ghrelin and endocannabinoid systems may be redundant or complementary, there are also several examples in the literature of CB1R ligands modulating the effects of acyl-ghrelin, of abrogated CB1R agonist efficacy in GHSR-KO mice, or of abrogated ghrelin efficacy in CB1R-KO mice (Edwards, et al. 2016). There are also speculations on the possible existence of GHSR-CB1R heterodimers in certain brain regions (Edwards, et al. 2016, Lim, et al. 2013). These latter examples instead suggest that the ghrelin and endocannabinoid systems may be interdependent.

In this study, we tested the effects of dual GHSR and CB1R genetic deletion on several basic metabolic phenotypes observed in mice with *ad libitum* access to regular chow. We also tested the requirement of CB1R actions to maintain plasma ghrelin. Our results show that in *ad libitum*-fed conditions, genetic deletion of GHSR alone has no significant effect on body weight, food intake, or adiposity and CB1R significantly reduces body weight, fat mass, and lean mass. However, dual genetic deletion of both GHSR and CB1R does not exaggerate the reductions in body weight, fat mass, or lean mass otherwise observed in the CB1R-nulls, nor does it affect weekly food intake. Importantly, while neither GHSR deletion nor CB1R deletion alone resulted in a statistically significant reduction in survival over the time course of the study (4 – 20 wks-of-age), their dual deletion did decrease survival ($p=0.07$, as compared to wild-type control mice).

Finally, CB1R deletion did not impact plasma ghrelin in the *ad libitum*-fed condition, nor did CB1R agonists stimulate ghrelin secretion in an *ex vivo* primary gastric mucosal cell culture system except when supraphysiological doses were used. Thus, these data do not support the hypothesis that the metabolic actions of the endocannabinoid-CB1R system mask the development of a starvation-like state – with reduced body weight, reduced fat mass, reduced blood glucose, and anorexia – in GHSR-nulls with unrestricted access of

food. However, our data do suggest that GHSR and CB1R work together to ensure normal survival in settings with *ad libitum* feeding. Also, our data suggest that the endocannabinoid-CB1R system does not contribute substantively to plasma ghrelin regulation in *ad libitum*-fed states.

The body weight, body composition, food intake, and blood glucose phenotype observed here in the GHSR-null mice was similar to that we had previously reported in males with *ad libitum* access to regular chow diet (Chuang, et al. 2011a, Wang, et al. 2014, Zigman, et al. 2005, Scott, et al. 2012). Similarly, although the CB1R-null mouse model used here is novel, their body weight, body composition, food intake, and blood glucose phenotype was very similar to that reported previously for other CB1R-KO mouse lines (Cota, et al. 2003, Ravinet Trillou, et al. 2004). In particular, the body weights of the C1BR-null mice on average were 25% less and body composition included on average ~50% less fat mass compared to wild-type controls when measured at 20-wks-of age. Weekly body weight-normalized food intake and *ad libitum*-fed blood glucose of the CB1R-null mice were similar to the wild-type mice. However, as mentioned, combined GHSR and CB1R deletion did not unmask a starvation-like phenotype. Instead, the body weight curves, body composition, and blood glucose of the double-null mice in the *ad libitum*-fed state were no different than those in C1BR-null mice. There are many potential explanations for this observation. For instance, if indeed the endocannabinoid-CB1R system is a critical downstream effector of the ghrelin system as some of the aforementioned studies suggest (Alen, et al. 2013, Ting, et al. 2015, Tucci, et al. 2004, Rigamonti, et al. 2006, Senin, et al. 2013, Kola, et al. 2008), an exaggerated phenotype might not necessarily be expected. If the CB1R and GHSR systems instead are parallel and independent, our findings might suggest that the lack of one system's function is not compensated by the other system. Alternatively, it is also evident that maintenance of sufficient stores of nutrients and blood glucose are critical for survival, and thus other hormonal/neuronal systems may be able to compensate for the loss of both GHSR and CB1R. Yet another potential explanation is that in the setting of unrestricted access to regular chow as used here, endogenous acyl-ghrelin actions on food intake, body weight, body composition, and blood glucose might be relatively quiescent due to the persisting actions of and comparatively high levels of liver enriched antimicrobial peptide-2 (LEAP2), the recently discovered endogenous antagonist/inverse agonist of GHSR (Ge, et al. 2018, Mani, et al. 2019). If so, genetic GHSR deletion might not impact the overall metabolic profile in the *ad libitum*-fed condition. Further studies are needed to explore these possibilities. Further studies are also needed to assess whether metabolic derangements resulting from combined loss of GHSR and CB1R might become apparent in settings of calorie restriction, such as that in which ghrelin system loss-of-function models demonstrate life-threatening hypoglycemia (Mani, et al. 2016, Zhao, et al. 2010, Li, et al. 2012, Wang, et al. 2014, Zhang, et al. 2015). Also, the possibility that the lack of overt metabolic changes in the double-null mice could be due to the development of genetic compensation for the lack of both CB1R and GHSR throughout the lives of these animals must be considered, and could be tested by conditionally deleting CB1R and GHSR from adult mice.

The 30% mortality that we observed here for C1BR-null mice between 4 and 20 wks-of-age, although not statistically different from wild-type littermates, was not unexpected. CB1R-

KO mice previously were shown to have a reduced survival rate of 70% between 4 and 24 wks of age (Zimmer, et al. 1999). Lower survival rates also had been described in CB1R-KO neonates and in wild-type neonates administered CB1R antagonists (Fride, et al. 2003). The reason for the higher mortality due to the lack of CB1Rs is unknown, but latent cardiovascular problems and higher incidence of seizures have been postulated as potential causative factors (Zimmer, et al. 1999, Fride, et al. 2003). In our study, mice lacking both CB1R and GHSR exhibited a lower survival rate of 60%, and were the only group in which survival showed a statistical difference from that of wild-type mice ($p=0.07$). Thus, this study adds to a growing body of evidence defining ghrelin as an important survival hormone, for instance during the setting of extreme caloric restriction when it defends against marked hypoglycemia, or in neonatal Snord116del mice modeling Prader-Willi Syndrome, in which GHSR agonist administration significantly reduces mortality (Mani, et al. 2017a, Zhao, et al. 2010, Rodriguez, et al. 2018). Further research is needed to determine the mechanisms by which combined loss of CB1R and GHSR reduce survival.

A final discussion point relates to the effect of CB1R on plasma ghrelin. We did not observe changed plasma acyl-ghrelin in the CB1R-null or double-null groups, as compared to the wild-type mice. These results contrast those from published studies using pharmacological approaches, in which the CB1R antagonists SR141716A and oleylethanolamine reduced plasma ghrelin while the non-selective cannabinoid agonists R-(+)-methanandamide and CP55,940 increased plasma ghrelin in rats (Zbucki, et al. 2008, Cani, et al. 2004). They also contrast a recent study in which raised plasma ghrelin in alcohol-drinking wild-type mice was blocked by administration of a peripheral CB1R inverse agonist JD5037 (Godlewski, et al. 2019). Also, our gastric primary mucosal cultures experiments using the potent partial CB1R agonists anandamide, R-(+)-methanandamide [with EC_{50} of between 5.5 – 6.8 nM for cAMP inhibition and ERK activation in an cell expression systems (Khajehali, et al. 2015)] revealed stimulation of acyl-ghrelin secretion, but only at concentrations of $>1 \mu\text{M}$. This new finding combined with our previous gastric primary mucosal cell culture studies showing no ghrelin secretagogue activity for the potent CB1R/CB2R agonist CP 55,940 [at concentrations up to 100 nM (Engelstoft, et al. 2013)], suggests that while cannabinoids can act directly on ghrelin cells to stimulate ghrelin secretion at extremely high concentrations, they may not have meaningful impact on ghrelin secretion at physiologically relevant concentrations. This assessment is supported by the lack of an effect of CB1R deletion on plasma ghrelin and the previously reported very low CB1R mRNA expression in gastric ghrelin cells (Engelstoft, et al. 2013). In summary, the results presented here suggest that direct endocannabinoid actions mediated through CB1R are not prominent regulators of ghrelin secretion in *ad libitum*-fed states. Further studies are needed to determine the basis of the discrepancies between these data and the evidence noted during the ethanol ingestion studies in mice (Godlewski, et al. 2019) and the pharmacological studies in fasted rats (Senin, et al. 2013, Cani, et al. 2004).

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Abbreviations:

AgRP	Agouti-related peptide
AMPK	AMP-activated protein kinase
BAC	Bacterial artificial chromosome
BW	Body weight
CB1R	Cannabinoid receptor 1
CB2R	Cannabinoid receptor 2
GHSR	Growth Hormone Secretagogue Receptor
GOAT	Ghrelin O-acyl transferase
GPCR	G-protein coupled receptor
EC₅₀	Effective concentration 50
ISHH	<i>In situ</i> hybridization histochemistry
KO	Knockout
LEAP2	Liver enriched antimicrobial peptide-2
NPY	Neuropeptide Y
POMC	Proopiomelanocortin
PVN	Paraventricular nucleus of the hypothalamus
TBC	Transcriptional blocking cassette
TRP	Transient receptor potential
VMH	Ventromedial nucleus of the hypothalamus
VTa	Ventral tegmental area

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Highlights:

Genetic deletion of GHSR does not exaggerate the reduced body weight, fat mass, or lean mass exhibited by mice lacking CB1R.

Genetic deletion of both GHSR and CB1R in mice reduces survival.

CB1R genetic deletion in *ad libitum*-fed mice does not affect plasma ghrelin.

Application of cannabinoids to cultured gastric mucosal cells does not prominently affect ghrelin secretion.

1. Validation of a novel CB1R-null mouse line.

A. Representative brightfield photomicrographs of coronal brain sections at two different levels demonstrating CB1R mRNA expression (indicated by black silver granules) in wild-type mice (left) and CB1R-null mice (right). Approximate distance of the coronal section from the bregma is indicated in the photomicrographs. B. Food intake measured in wild-type mice (left) and CB1R-null mice (right) at 1 h and 2 h post administration of the CB1R agonist WIN 55,212-2 (open bars) or vehicle (black bars). Data were analyzed by repeated measures two-way ANOVA, $n=9-16$. Administration of WIN 55,212-2 increased food intake in wild-type mice [$F_{(1,8)} = 8.25$, $p=0.02$], but not in CB1R-null mice [$F_{(1,15)} = 0.15$, $p=0.7$]. Post hoc comparisons using Sidak's test indicate the change in food intake with WIN 55,212-2 – n.s.= not significant, ** $p<0.01$, **** $p<0.0001$. Data are expressed as mean $\pm$ standard error of mean (SEM).

2. Effects of GHSR and/or CB1R deletion on body weight, food intake, and body composition.

Body weight (A) and weekly food intake curves (B) of individually-housed wild-type, GHSR-null, CB1R-null, and double-null mice that survived up to 20-wks-of-age. Fat mass (C) and lean mass (D) of the mice when measured at 20-wks-of-age. Data in panels A and B were analyzed by repeated measures two-way ANOVA, $F_{(3,26)} = 9.3$, $p = 0.0002$ for panel A; $F_{(3,26)} = 3.1$, $p = 0.05$ for panel B. Data in panels C and D were analyzed by one-way ANOVA, $F_{(3,26)} = 10.64$, $p < 0.0001$ for panel C; $F_{(3,26)} = 7.59$, $p = 0.0008$ for panel D. $n = 5-12$. Post hoc comparisons using Sidak's test indicate the genotype dependent change in parameters. n.s. - not significant; * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. Data are expressed as mean $\pm$ SEM.

3. Effect of GHSR and/or CB1R deletion on survival.

Survival curves of wild-type, GHSR-null, CB1R-null, and double-null mice between the age of 4 to 20-wks-of age. The survival curves were calculated by Kaplan-Meier method, and the percentage survival among the groups was compared by Mantel-Cox log-rank test.

$p > 0.05$ and < 0.1 . Numbers within the parentheses of the legends indicate the number of mice that survived until 20-wks-of-age over the total number of mice entered in to the study for each genotype.

4. Acyl-ghrelin levels and blood glucose in *ad libitum*-fed mice and as measured in the media of primary cultures of gastric mucosal cells.

Plasma acyl-ghrelin (A) and blood glucose (B) in 20-wk-old wild-type, GHSR-null, CB1R-null, and double-null mice. C & D, indicate the fold change in acyl-ghrelin concentration in cell culture medium (compared to control) in response to 6 h treatment with increasing concentrations of anandamide and R-(+)-methanandamide, respectively. Data were analyzed by one-way ANOVA, $F_{(3,26)} = 0.1$, $p = 0.96$ for panel A; $F_{(3,26)} = 0.98$, $p = 0.41$ for panel B; $F_{(3,25)} = 17.93$, $p < 0.0001$ for panel C; $F_{(3,26)} = 72.05$, $p < 0.0001$ for panel D. Post hoc comparisons using Sidak's test indicate the difference in parameters with genotype or

treatment. . n.s-not significant; **** $p < 0.0001$, significant change when compared to the vehicle treatment (control). Data are expressed in mean $\pm$ SEM.

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