Compare

Animal Research Protocol

Protocol Number: AN-2770
Status: Closed
Initial Submit Date: 5/22/2002
Approval Period: 8/7/2014 - 8/6/2017

Category of Experiment: E - Vertebrate, unrelieved pain or distress

Section Aa: Title & PI

A1. Protocol Title(s):

METABOLIC ACTIONS OF ORPHAN GROWTH HORMONE SECRETAGOGUE RECEPTOR AND GHRELIN AND THEIR INFLUENCE ON AGING.

HFCS INDUCES ADIPOSE INFLAMMATION AND IS A DIETARY RISK FACTOR FOR CARDIOVASCULAR DISEASE AND STROKE

A2. Principal Investigator

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Does PI have direct contact with animals?: Yes

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A4. Sponsor/Mentor

None

Section Ab: General Information

A5. Additional Research Personnel

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A6. Funding Source:

ARS Project No. 6250-51000-055, expiration data: 09/31/2014

A7. Institutions where work will be performed:

CNRC: Children's Nutrition Research Center

Section B: Personnel & Training

B1. Who will perform the experimental manipulations on animals?

The Principal Investigator is responsible for ensuring that personnel are adequately trained to care for research animals and to conduct animal experiments.

Experimenter: YUXIANG SUN

Humane handling and care of species used in this protocol?	Yes
Non-invasive procedures described in this protocol?	Yes
Invasive procedures described in this protocol?	Yes
Proper use of anesthesia as described in this protocol?	Yes
Recognizing sick animals?	Yes
Pre-operative and Post-operative care?	Yes
Proper euthanasia method as described in this protocol?	Yes

Experimenter: SHAJI K CHACKO

Humane handling and care of species used in this protocol?	Yes
Non-invasive procedures described in this protocol?	Yes
Invasive procedures described in this protocol?	N/A
Proper use of anesthesia as described in this protocol?	Yes
Recognizing sick animals?	Yes
Pre-operative and Post-operative care?	N/A
Proper euthanasia method as described in this protocol?	Yes

Experimenter: ALLI M. ANTAR

Humane handling and care of species used in this protocol?	Yes
Non-invasive procedures described in this protocol?	Yes
Invasive procedures described in this protocol?	N/A
Proper use of anesthesia as described in this protocol?	Yes
Recognizing sick animals?	Yes
Pre-operative and Post-operative care?	N/A
Proper euthanasia method as described in this protocol?	Yes

Experimenter: CHIA-SHAN WU

Humane handling and care of species used in this protocol?	Yes
Non-invasive procedures described in this protocol?	Yes
Invasive procedures described in this protocol?	Yes
Proper use of anesthesia as described in this protocol?	Yes
Recognizing sick animals?	Yes
Pre-operative and Post-operative care?	Yes
Proper euthanasia method as described in this protocol?	Yes

Experimenter: MIAO-HSUEH CHEN

Humane handling and care of species used in this protocol?	Yes
Non-invasive procedures described in this protocol?	Yes
Invasive procedures described in this protocol?	N/A
Proper use of anesthesia as described in this protocol?	Yes
Recognizing sick animals?	Yes
Pre-operative and Post-operative care?	N/A
Proper euthanasia method as described in this protocol?	Yes

Experimenter: GEETALI PRADHAN

Humane handling and care of species used in this protocol?	Yes
Non-invasive procedures described in this protocol?	Yes
Invasive procedures described in this protocol?	Yes
Proper use of anesthesia as described in this protocol?	Yes
Recognizing sick animals?	Yes
Pre-operative and Post-operative care?	Yes
Proper euthanasia method as described in this protocol?	Yes

Experimenter: JONG HAN LEE

Humane handling and care of species used in this protocol?	Yes
Non-invasive procedures described in this protocol?	Yes
Invasive procedures described in this protocol?	Yes
Proper use of anesthesia as described in this protocol?	Yes
Recognizing sick animals?	Yes
Pre-operative and Post-operative care?	Yes
Proper euthanasia method as described in this protocol?	Yes

Experimenter: PREETI KANIKARLA MARIE

Humane handling and care of species used in this protocol?	Yes
Non-invasive procedures described in this protocol?	Yes
Invasive procedures described in this protocol?	No
Proper use of anesthesia as described in this protocol?	Yes
Recognizing sick animals?	Yes
Pre-operative and Post-operative care?	No
Proper euthanasia method as described in this protocol?	Yes

Experimenter: ALVARO MUNOZ

Humane handling and care of species used in this protocol?	Yes
Non-invasive procedures described in this protocol?	Yes

Invasive procedures described in this protocol?	Yes
Proper use of anesthesia as described in this protocol?	Yes
Recognizing sick animals?	Yes
Pre-operative and Post-operative care?	N/A
Proper euthanasia method as described in this protocol?	Yes

B2. If experimenters are not experienced with the specific procedures involved and the humane handling and care of the species listed on this protocol - who will be responsible for training?

They will receive training before the experiments begin by Jong Han Lee

Section C: Animal Housing

MICE

Strain: C57BL6, S129
Age: 4 - 52 week
Sex: Both
Weight: 10-60g
Nbr Requested: 852

C1. Preferred location of animal housing?

Children's Nutrition Research Center

C1a. If you requested Taub for mice, explain why you must house the mice in Taub instead of TMF.

Sometimes we need to send the mice to BCM Mouse Phenotyping Center for testing, which is located in Taub facility.

C2. Will you need special housing conditions? If yes, please describe.

Yes. Occasionally we need to fast animals for 4 - 24 hours, or fed the mice with special diets or drinks, for which we will request “Special Care Instruction Form (SCIF)”. We will use following commercially available special diets: high fat diet (“Western”) diet, high protein diet, high carbohydrate diet; high glucose or high fructose corn syrup (HFCS) drink. The HFCS is being added into the water at 8% concentration to mimic soft drink, which will be used for 2-10 months to study short term and long term effects.

We would appreciate the committee continue to grant us the exception to the policy of maximum 4 mice/cage, when the mouse is over 25g. These mice are used to investigate metabolic changes during aging. Our historic data has been generated with 5 mice /cage housing conditions. Because these mice are males we cannot house them by mixing them with aging male from another age (they will fight). Therefore, unless we qualify for an exception, we will be forced to euthanize one mouse in each cage, this will not only be a huge waste of the valuable aging mice, most importantly this changing of housing condition will affect the aging mice, which will make it impossible for us to compare our current data with our previous data. So we would appreciate the committee continue to grant us 5/cage through out the life span of the mice. We will request appropriate SCIF and our staff will do additional cage and food/and water changes in addition to regular CCM care. No new aging study which requires 5 mice per cage will be conducted without prior IAUC approval. The current study is anticipated to be end in 2 years (by June 2016).

Occasionally we also need to feed mice tamoxifen diet to induce Cre activation. Customized tamoxifin-containing diet containing 400mg/kg (Harlan, TD.120681) for 7-10 days. In order to increase the tolerance of the diet, we may mix 2/3 of the customized tamoxifin-containing diet with 1/3 of the chow in powered form to feed the mice. During the tamoxifen treatment and 1 week after, body weight of the mice will be monitored daily. If the body weight of the mice reduces more than 20%, we will stop the tamoxifin treatment or euthanize the mice immediately.

Calorimetry: Energy expenditure will be measured in the mice using the CLAMS Instrument located in the CNRF. They will be housed for at least 48-72 hours in specialized CLAMS feeder cages to familiarize themselves with the feeding system and to estimate food intake. Maximal CLAMS acclimatization will be 5 days for old mice. They are then transferred to the calorimetry chambers for 72 hours for the simultaneous monitoring of food intake, activity, and energy expenditure. To determine energy expenditure, the system monitors O2 and CO2 gas fractions at both the inlet and output ports of up to 16 chambers through which a known flow of air is passing. The gas fraction and flow measurements are used to compute VO2, VCO2, RQ (respiratory exchange ratio) and heat. Food intake and feeding pattern are measured automatically from the weight of feed removed from specialized feed hoppers installed with the chamber. Activity in the x and z axes is estimated by monitoring the number of infra-red beams broken by the mouse movements. Precise details of the measurements can be found in AN5020. At the end of the study, the mice will be returned to regular animal housing facility in CNRC.

Cold Exposure test - The animals will be transferred to the 4 C cold room on the 5th floor of CNRC room 5015. The mice will be exposed in 4 C for up to 6 hours (with food and water) and core body temperatures will be monitored hourly using an anal probe. Mice showing sign of hypothermia (rectal temperature lower than 25C) will be pulled from study and sacrificed immediately. At the end of cold exposure experiments, all mice will be sacrificed for tissue collections.

C3. Will animals be taken outside of the vivarium?

Yes.

Hours Outside: 0-12 hours
Laboratory: CNRC 5004 & cold room 5015

Justification:
Live mice will be taken to the lab (CNRC 5004) to sacrifice. No live mice will not be returned to animal facility.

Cold exposure test will be conducted in CNRC 4C cold room 5015. The maximal length of test is 6 hours, food and water will be provided during the test. At the end of cold exposure experiments, mice will be brought back to the lab CNRC 5004 and sacrificed for tissue collections immediately.

Section D: Subcommittee Approvals

Biohazardous Agents in animals

rDNA protocol: D-1561.

Section E: Hazardous Agents

None

Section F: Tissue Sharing

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Section G: Lay Summary

G1. How might a patient population (human or animal) ultimately benefit from your experiments?

The quality of life of the elderly would be improved because the potential of this research is to target receptors in the brain that inhibit/reverse certain functional deficits that occur in the brain and peripheral organism during aging.

Aging is associated with reduced ability of heat generation (reduced thermogeneis), which in turn reduces energy expenditure and promotes obesity. Also aging is also associated with adipose tissue inflammation, which direly contributes to insulin unresponsiveness (insulin resistance). Our preliminary studies have suggested that suppression of ghrelin signaling pathway may reverse these age-associated defects. We hope to further investigate whether ghrelin blockers could potentially be used as therapeutic options to improve thermogenesis and reduce inflammation during aging.

G2. What experimental manipulations will be done with the animals (in general terms)?

Animals will be treated orally, intraperitonealy, intravenously with agents (glucose, insulin, ghrelin, leptin, mercaptoacetate, or 2-DG). Blood will be collected and assayed for glucose, insulin, epinephrine, growth hormone and insulin-like growth factor (IGF-1).

Hypothalamic obesity can classically be induced in mice by neonatal administration of monosodium glutamate (MSG) or treatment of adult mice with gold thioglucose (GTG). Our studies of ghrelin and GHS-R physiology have indicated that ghrelin and its receptor may be involved in central glucose sensing and response. Using the above classical methods to induce lesions in glucose-sensing regions of the brain of wild-type, ghrelin KO, and GHS-R KO mice will enable us to determine if ghrelin action is centrally or peripherally mediated.

Streptozocin (STZ) is the most commonly used agent to induced pancreatic b-cell damage (Physiol Res. 50:537-46; Acta histochemica et cytochemica. 33: 201-208). STZ liberates toxic nitric oxide that induces DNA damage. We will use STZ to induce b-cell damage in wild-type (WT) and ghrelin- and Ghst- null mice to study the protective effects of ghrelin signaling on b-cell damage.

Norepinephrine is a catecholamine with multiple roles functioning as a hormone and a neurotransmitter. The actions of norepinephrine are carried out via the binding to adrenergic receptors, which increase O2 consumption and increase energy expenditure (Cell Metabolism 9:203-209). We will use norepinephrine to induce acute increase in energy expenditure in WT and ghrelin- and Ghsr- null mice to characterize the thermogenic effects of ghrelin signaling.

Thioglycollate , LPS and IFNgamma are commonly used reagents to induce peritoneal macrophages (Ray & Dittel. “Isolation of mouse peritoneal cavity cells” 2010 Journal of Visualized Experiments; Journal of Biological Chemistry 258:9631; BMC Complimentary and alternative medicine 8:1). We will use these agents to activate peritoneal macrophages and induce inflammation in WT and ghrelin- and Ghsr-/- null mice to assess the effects of ghrelin signaling on inflammation.

G3. Why must animals be used in these experiments and why is this particular species selected?

These studies must be done in animal species to study the physiology as it relates to humans. There is no evidence that lower organisms such as worms or flies have the same physiological pathways that involve this receptor. After searching for over last 10 years I have found no cell line that expresses a functional endogenous GHS-R. Nevertheless, we have generated and routinely use engineered cell lines expressing the GHS-R for in vitro experiments to establish mechanisms of signaling whenever possible, before doing animal experiments.

Section H: Experimental Summary

H1. What are the objectives or underlying hypotheses of the experiments?

The underlying hypothesis is that the physiology associated with aging can be reversed by stimulation of the growth hormone secretagogue receptor (GHS-R) with highly specific small molecule ligands already proven to be safe in mice and humans. We are investigating the mechanism by which these ligands rejuvenate the growth hormone (GH) axis by amplifying pulsatile GH release from the pituitary, which it has the potential to prevent the functional deficits associated with aging. The experiments are designed to determine how neuropeptide Y (NPY), somatostatin, GHRH and GH regulate the amplitude of GH pulsatility and how these hormones and neurotransmitters affect aging of the brain and peripheral tissues. In rats and humans ghrelin stimulates feeding behavior, fat deposition, glucocorticoid release and GH release. Ghrelin is a functional antagonist of leptin. Therefore, both hormones may play an important role in controlling body composition by

balancing fat and lean mass as well as controlling appetite. GHS-R -/- and ghrelin -/- mice have been generated and will be used to understand how the GHS-R and its ligands regulate feeding and metabolism. Part of these investigations will involve studying tolerance to glucose or insulin challenge. A previous study indicates that ghrelin levels are significantly elevated in rats maintained on a high carbohydrate diet and that ghrelin levels drop when serum glucose levels are high. We wish to investigate whether the absence of ghrelin or GHS-R causes an altered tolerance to a glucose or insulin challenge. Two standard tests of carbohydrate metabolism, glucose tolerance (GTT) and insulin tolerance test (ITT) will be used. The glucose bolus for the glucose tolerance testing will be administered either orally or i.p. To determine if ghrelin or the GHS-R is required for normal sensitivity and/or response to negative energy balance, we will induce a negative energy balance in the ghrelin and GHS-R deficient animals by either inducing acute glucoprivation by administering 2-deoxyglucose, acute lipoprivation by administering mercaptoethanol or by fasting. The hypothalamic-pituitary-adrenal (HPA) axis coordinates the integrative response to a variety of stressful stimuli. The three main components of this axis are corticotrophin-releasing hormone (CRH), adrenocorticotrophic hormone (ACTH), and glucocorticoids. Peripheral administration of ghrelin in rodents causes increased expression of CRH in the hypothalamus and an increase in corticosterone in the bloodstream. I.C.V. administration of ghrelin stimulates the HPA axis at the hypothalamic level, at least in part via increased expression of CRH and AVP. To investigate the physiologic role of ghrelin and the GHS-R in the proper functioning of the HPA axis we will cause acute cold or restraint stress in our knockout models and assessing their responses to these stressors. It has also been reported that central administration of ghrelin suppresses energy expenditure and thermogenesis in brown fat via its inhibitory effect on sympathetic nerve activity (Neurosci Lett. 2003, 349:75). We plan to study the thermogenesis characteristics of ghrelin KO in order to understand the ghrelin's role in thermogenesis. Many of our experiments have suggested that ghrelin's functions only become obvious when animals are placed in stressful conditions. We feel that by putting animals in lower temperature, we might be able to detect ghrelin's contribution in thermogenesis. Our studies to date show that compared to WT mice, our ghrelin- or GHS-R-deficient mice have an augmented glucose disposal rate. Alternatively, they lack sufficient glycogenolysis or gluconeogenesis to maintain the same glucose levels as WT mice during situations of energy deficit; however, the mice do not become hypoglycemic or develop behavioral differences or exhibit suffering in any way. The most widely acceptable methods of determining glucose turnover rate are the glucose clamps. We intend to establish venous and arterial catheters in the test mice so that these clamps may be performed on healthy, conscious, and unrestrained mice. Once catheters are established, hyperinsulinemic, hyperglycemic, and hypoglycemic clamp studies will be performed to study the basal glucose flux in the presence of different stimuli. Paired with the use of a radiolabeled glucose analog (labeled 2-deoxyglucose), we will also determine tissue-specific glucose uptake (Dr. Sun has completed an official training course at Vanderbilt University so that she is adept at using the necessary surgery techniques). Our studies of ghrelin and GHS-R physiology have indicated that ghrelin and its receptor may be involved in central glucose sensing and response. Central or hypothalamic obesity can classically be induced in mice by neonatal administration of monosodium glutamate (MSG) or treatment of adult mice with gold thioglucose (GTG). Daily subcutaneous injections of MSG to pups from neonatal days 1 to 5 causes cerebral lesions in mice that extended into the neocortex, hypocampus, and habenular nucleus and hypothalamic lesions that extend to the preoptic area, the mamillary nucleus, and the ventral two-thirds of the ventromedial nucleus. Most animals treated in this way subsequently developed overt obesity by the 10th week of age as evaluated by the ratio of mean weight and length. In contrast, a single ip injection of GTG to adult mice produces a lesion in the ventromedial hypothalamus whose localization is reproducible and which induces a severe obese phenotype. This hypothalamic lesion is dependent on insulin and the glucose moiety of the GTG molecule and is blocked by glucose uptake inhibitors, so GTG has been thought to primarily target the glucose-sensitive or glucose-responsive neurons of the hypothalamus. Using the above classical methods to induce lesions in glucose-sensing regions of the brain of wild-type, ghrelin KO, and GHS-R KO mice will enable us to determine if ghrelin action is centrally or peripherally mediated.

Amendment on Special diets: Ghrelin increases growth hormone release, stimulates appetite and promotes obesity. The regulation of postprandial ghrelin release in man depending on the ingested macronutrients. Ghrelin levels decrease after ingestion of carbohydrate- and fat -rich meals, but with a different time pattern. In contrast, ghrelin levels increase after the ingestion of protein-rich meals. It is known that fructose produces lower levels of leptin and insulin than glucose. Raising leptin and insulin levels trigger the feeling of "fullness" while eating. Intestinally, it has been shown that ghrelin is higher with consumption of fructose than it does with glucose. Hence, we hypothesize that ghrelin is orexigenic hormone control "hunger" in mice and responses differentially to different macronutrients, and we would like to test the ghrelin regulation of different special diets.

H2. What will happen to the animals, from start to finish?

All genotyping required tail biopsy will be conducted before mice reach 4 weeks of age. 2-3mm mouse tail will be clipped without general anesthesia. Hemostasis will be achieved by direct pressure on the end of the tail. If the direct pressure doesn't work, styptic power will be allowed to stop the bleeding. In rare situation, we need to reconfirm genotyping of mice older than 4 weeks of age. In that case, we will use general anesthesia (such as isolurance) on the mice, before tail biopsy is conducted.

Bleeding methods: Bleeding methods: Generally for collection of small amount of blood (under 50ul), lancet piercing in the tail vein near the tail tip will be used. For collection of big volume of blood (more than 100ul), facial bleeding or retro-orbital bleeding under anesthetized condition will be used.

Glucose tolerance test - Groups of mice will be fasted overnight (18 h) prior to testing. After the mice are weighed, a specimen of blood is obtained for a fasting glucose and insulin level. A bolus of 50 % dextrose (2.5 g/kg) is given to each mouse by oral gavage or injected i.p. Immediately following gavage or injection, ghrelin or synthetic GH secretagogues (MK-0677). Blood specimens for glucose and insulin levels are then obtained 30, 60, and 120 min after dextrose administration. Only a single drop of blood is enough for each glucose measurement and 25 uL of blood will be collected for insulin measurement. Mice are allowed to recover for 2 weeks before additional testing is performed.

Insulin tolerance test - Groups of mice will be fasted for 2-4 hrs. After the mice are weighed, a specimen of blood is obtained by lancet piercing in the tail vein near the tail tip for a fasting glucose level. Insulin (0.75 U/kg of Humulin in saline) will be administered i.p. to each mouse. Immediately following insulin administration, ghrelin or other GH secretagogues may be administered to some mice. Blood specimens for glucose levels will be obtained 15, 30, 60, and 90 minutes after insulin administration. Only a single drop of blood is enough for each glucose measurement. Mice are allowed to recover for 2 weeks before additional testing is performed.

2 - deoxyglucose (2-DG) challenge - Groups of mice will be fasted overnight (18 h) prior to testing. After the mice are weighed, a specimen of blood is obtained for fasting glucose and glucagon levels. 2-deoxyglucose (500 mg/kg mouse) is administered to each mouse by i.p. injection. Immediately following injection, ghrelin or other GH secretagogues may be administered to some mice. Blood specimens for glucose and glucagon levels are then obtained 30, 60, and 120 min after 2-DG administration. Only a single drop of blood is enough for each glucose measurement and 25 uL of blood will be collected for glucagon measurement. Mice are allowed to recover for 2 weeks before additional testing is performed.

Synergy of leptin and ghrelin in stimulation of growth hormone secretion - Groups of mice (at least 6 each of WT, ghrelin(-/-), leptin(ob/ob), or ghrelin(-/-)+ leptin(ob/ob)) will be anesthetized and then given doses (400 ug/kg body weight of each agent) of ghrelin, leptin, or ghrelin+leptin. This will be done on each of 3 consectutive weeks with a different agent used on each mouse every week. Blood will be drawn for the determination of growth hormone levels prior to and 5 minutes after each dose.

Acute cold stress - Blood specimens for glucose, glucagon, and corticosterone levels will be obtained. Styptic powder will be used to stop bleeding if necessary to ensure no additional blood loss in the subsequent period. Anesthesia cannot be used because administration of anesthetics will reduce one of the readout parameters of the experiment: core body temperature. The mice will then be placed in a tub containing enough room temperature water to create body buoyancy but not enough so that the mice are forced to swim. Experience has shown us that a 20-minute period in RT water will reduce the core body temperatures of the mice to approximately room temperature. If the mouse becomes unable to stand and keep its head above water during the 20-min immersion, it will be promptly removed from the water. After this cold immersion, the mice will be toweled dry, the body temperatures of the mice will be determined by rectal probe, and 100ul blood will be collected. The mice will be placed in warmed holding cages (warmed by a 100W light bulb) until dry and another specimen of blood and core body temperatures will be obtained in 60 minutes.

Restraint stress- (maximal 3 times every 12months). Blood (50ul) will be obtained from the mice before the start of stress test. Each mouse will be placed tail-first into a ventilated 50-mL conical centrifuge tube and a ventilated cap will be placed on the top of the tube in a vertical position. During the 2-hour restraint, the mice will be monitored every 15 min for signs of too much struggle against the restraint and to ensure that the mouse has not assumed an uncomfortable position. Blood specimen will be obtained right after the test. The test will be conducted outside of home cages. After the test, the mice will be returned to home cages. This test has been used by others in publications. Eg . Neuropsychopharmacology 39, 1262(2014); BLOOD, 99: 2455 (2002).

Cold Exposure Protocol- Our animals currently are held in CNRF (1064), for the pilot study we are planning to use only male mice. The animals will be transferred to the 4 C cold room on the 5th floor of CNRC 5015. The mice will be exposed in 4 C for up to 6 hours (with food and water) and core body temperatures will be monitored hourly using an anal probe. For mice show sign of hypothermia (rectal temperature lower than 25C) will be pulled from study and sacrificed immediately. At the end of cold stress experiments, all mice will be sacrificed for tissue collections and will not be put back to home cages for recovery. This cold exposure approach has been widely used by many laboratories (PNAS 2003,100:10085).

Monosodium glutamate-induced obesity – Mice will be injected s.c. with 2 mg/g body weight MSG once a day for 5 days. Body weights of these mice will be monitored weekly to detect obesity. At approximately 2 months of age, IPGTTs or 2DG challenges will be performed with or without the administration of exogenous ghrelin. Blood will be obtained for glucose, insulin, glucagon, and catecholamine levels.

Gold-thioglucose-induced obesity - Mice will be given a single i.p. injection of gold-thioglucose (500 mg/kg body weight). Body weights of these mice will be monitored weekly to detect obesity. At approximately 10 weeks post GTG dose, IPGTTs or 2DG challenges will be performed with or without the administration of exogenous ghrelin. Blood will be obtained for glucose, insulin, glucagon, and catecholamine levels.

Thioglycollate challenge- Thioglycollate is commonly used reagents to induce peritoneal macrophages (Journal of Biological Chemistry 258:9631; BMC Complimentary and alternative medicine 8:1). We will ip inject 1ml 3% thioglycollate into the mice to activate peritoneal macrophages and induce inflammation. Mice will be scarified 48 hours later for analysis.

Calorimetry: Energy expenditure will be measured in the mice using the CLAMS Instrument located in the CNRF. They will be housed for at least 48-72 hours in specialized CLAMS feeder cages to familiarize themselves with the feeding system and to estimate food intake. Maximal CLAMS acclimatization will be 5 days for old mice. They are then transferred to the calorimetry chambers for 72 hours for the simultaneous monitoring of food intake, activity, and energy expenditure. To determine energy expenditure, the system monitors O2 and CO2 gas fractions at both the inlet and output ports of up to 16 chambers through which a known flow of air is passing. The gas fraction and flow measurements are used to compute VO2, VCO2, RQ (respiratory exchange ratio) and heat. Food intake and feeding pattern are measured automatically from the weight of feed removed from specialized feed hoppers installed with the chamber. Activity in the x and z axes is estimated by monitoring the number of infra-red beams broken by the mouse movements. Details of the measurements can be found in AN5020.

Body Composition: Measurement of body composition (fat and lean ratio) is achieved in a non-invasive manner through use of the EchoMRI-100 instrument on conscious mice.

Reply for Amendment on Special diets: 1: Study the obese and diabetic phenotypes of the mice under different macronutrients: high fat diet (“Western”) diet, high carbohydrate diet - high glucose and high fructose, and high protein diet. Mouse body weight, food intake and blood glucose will be monitored. Plasma insulin, leptin, adiponectin, glucagon, and plasma lipid and free fatty acids will be analyzed. 2. Characterize the metabolic profiles of the ll mice under different diets. Indirect calorimetry will be analyzed by CLAMS system, total body composition will be analyzed by Piximus scan or MRI. 3. Functional analysis of the impacts of different macronutrients on the mice. Glucose and insulin tolerance tests, and insulin clamps will performed. Fuel efficiency of different diets will be calculated, and pair-feeding study will be carried out to address whether the phenotypes observed is depend on food intake.

Animals will be put on different special diets right after weaning. Body weight, food intake, blood glucose will be recorded every 2 weeks and 50ul blood will be collected every 4 weeks for insulin and glucagon analysis. After 4-6 months of feeding, body composition and calorimetry analysis will be carried out. Subsequently, glucose tolerance test and insulin tolerance test will be performed.

We will conduct pair-feeding experiments using metabolic cages in CNRF. Our preliminary experiments show that our null mice consume less food than that of wild-type mice. For the paired-feeding study, the feeders for KO mice are open all the time, but the feeders for wild-type mice will only open when the food consumption is lower than their null counterparts (once over it, the feeders will be shut down automatically). The difference in food intake between WT and KO is less than 10%, thus the amount of automatically controlled food restricted in WT mice is less than 10% of their ad lid fed amount. With this very modest reduction of food intake, the

health of the mice will not be affected. Should any mice lose weight significantly (more than 20%), they will be sacrificed right the way (see S1).

Tamoxifen treatment: To induce Cre activation in our Cre containig mouse models, mice will be administrated with tamoxifen via one of the following routes:a single dose of 8mg (200ul) i.p. injection, 4mg gavage 5 times every other day, or customized tamoxifin-containing diet containing 400mg/kg for 7-10 days.

Norepinephrine-induced O2 consumption: In order to study norepinephrine-induced O2 consumption, we have to keep the mice at the lowest metabolic rate for 60-90 min, so we can record the effect of norepinephrine-induced O2 consumption in metabolic chambers. Pentobarbital 50 mg/kg BW IP will be used to anesthetize the mice for long period of time. We will then subcutaneously inject norepinephrine 1-1.5mg/kg (in 0.1-0.2ml saline solution) to induce acute increase of O2 consumption in the mice to characterize the thermogenic effects of ghrelin signaling.

H3. How many animals will be used and how did you determine that this will be sufficient?

The following questions will be addressed (number of animals needed): To avoid dealing with the effects of cyclic female hormones, most of the testing will be perfomed in male mice. Female mice not used for testing or for breeding under protocol AN-1804 will either be used during the training period for arterial and venous catheter placement or they will be euthanized.

In general, groups of mice for testing under this protocol will be comprised of 10 male mice per group. 10 M ghrelin -/-, 10 M ghrelin +/+, 10 M GHS-R -/-, and 10 M GHS-R +/+ (40 mice total per test).

Glucose, insulin tolerance, mecatoacetate challenge and 2-DG challenge function tests: all four tests may be performed on the same animal, only one study group will be required at a time. However, these tests will need to be repeated in 3 independent groups at different ages to make sure the data are reproducible. (120 mice total).

Two groups are needed for fasting and inflammation studies. Another 2 groups are needed for the cold stress and restraint stress studies. (160 mice total)

One-hour cold exposure experiments will be performed on 2 groups of mice.(80 mice total)

Clamp studies: 3 types of clamps (Hyperinsulinemic-euglycemic, hyperglycemic, and hypoglycemic) will be performed, which add to 120 mice. Also included in this number are approximately 42 mice used for donor mice and practice catheter placements. (162 mice total, the procedures are described in Dr. Chan's AN-625).

For central-induced obesity and calorie restriction studies each will require 3 groups of mice. (240 mice total)

Besides experimental mice above, we budget 45 male and 45 female mice for breeding of various mouse lines.(90 mice total)

Section I: Exogenous Substances

MICE

I1. Exogenous substances to be administered:

Drug/Substance:	2-deoxyglucose
Dosage:	Up to 500 mg/kg
Route:	i.p. or i.v. (through jugular catheter)
Volume:	0.1-0.3 mL
Frequency:	Once per age point - up to 3 age points

I2. Will the animals(s) be anesthetized or sedated during administration?

No

I3. Will any physical or physiological impairment (i.e. pain, distress, change in activity) of the animals(s) result from this drug?

2-DG causes central glucoprivation. The mice will go into acute energy deprivation and will exhibit signs of hunger (hunting for any available food source) and lethargy. Refeeding after the testing will aid in the recovery from the central glucoprivation. We will use pharmaceutical grade compounds and dilute in sterile medical-grade saline, then use filter with pore size 0.2 μm for sterilization

I1. Exogenous substances to be administered:

Drug/Substance:	8% High fructose corn syrup
Dosage:	Ad lib
Route:	Oral
Volume:	As much as the mice desire to drink
Frequency:	Continuously

I2. Will the animals(s) be anesthetized or sedated during administration?

No

I3. Will any physical or physiological impairment (i.e. pain, distress, change in activity) of the animals(s) result from this drug?

No

I1. Exogenous substances to be administered:

Drug/Substance:	Ghrelin
Dosage:	1 - 100 mcg
Route:	i.p. or oral
Volume:	0.1 - 0.2 ml
Frequency:	Once

I2. Will the animals(s) be anesthetized or sedated during administration?

No

I3. Will any physical or physiological impairment (i.e. pain, distress, change in activity) of the animals(s) result from this drug?

We will use pharmaceutical grade compounds and dilute in sterile medical-grade saline, then use filter with pore size 0.2 μm for sterilization

I1. Exogenous substances to be administered:

Drug/Substance:	Ghrelin angonist MK-0677
Dosage:	1-10ug
Route:	ip
Volume:	100ul
Frequency:	one time

I2. Will the animals(s) be anesthetized or sedated during administration?

No

I3. Will any physical or physiological impairment (i.e. pain, distress, change in activity) of the

animals(s) result from this drug?

We will use pharmaceutical grade compounds and dilute in sterile medical-grade saline, then use filter with pore size 0.2 µm for sterilization

I1. Exogenous substances to be administered:

Drug/Substance:	Glucose (dextrose)
Dosage:	Up to 2.5 g/kg body weight
Route:	oral, i.p., i.v. (through jugular catheter)
Volume:	0.1 - 0.3 mL
Frequency:	Up to 4 challenges - 2, 6, 10 and 14 months of age

I2. Will the animals(s) be anesthetized or sedated during administration?

No

I3. Will any physical or physiological impairment (i.e. pain, distress, change in activity) of the animals(s) result from this drug?

No physical or physiological impairment result from doses of glucose at this level. Pharmaceutical grade medical preparation of dextrose in sterile liquid will be used.

I1. Exogenous substances to be administered:

Drug/Substance:	Gold-thioglucose
Dosage:	500 mg/kg
Route:	i.p.
Volume:	volume = to 1/100th body weight
Frequency:	once

I2. Will the animals(s) be anesthetized or sedated during administration?

No

I3. Will any physical or physiological impairment (i.e. pain, distress, change in activity) of the animals(s) result from this drug?

GTG injections will cause lesions in feeding and glucose-sensing areas of the brain. Mice treated in this way will become hyperphagic and obese. We will use pharmaceutical grade compounds and dilute in sterile medical-grade saline, then use filter with pore size 0.2 µm for sterilization

I1. Exogenous substances to be administered:

Drug/Substance:	Humulin (insulin)
Dosage:	Up to 0.75 U/kg
Route:	i.p. or i.v. (through jugular catheter)
Volume:	0.1 - 0.3 ml
Frequency:	Once per age point - up to 3 age points

I2. Will the animals(s) be anesthetized or sedated during administration?

No

I3. Will any physical or physiological impairment (i.e. pain, distress, change in activity) of the animals(s) result from this drug?

Hypoglycemia due to hyperinsulinemia may occur. Mice may show lethargy or seizures. A bolus of 50 % dextrose by oral gavage will be given to mice exhibiting such behavior. Pharmaceutical grade medical preparation of insulin in sterile liquid will be used.

I1. Exogenous substances to be administered:

Drug/Substance: IFNgama
Dosage: 100ng
Route: i.p.
Volume: 0.2ml
Frequency: single dose

I2. Will the animals(s) be anesthetized or sedated during administration?

No

I3. Will any physical or physiological impairment (i.e. pain, distress, change in activity) of the animals(s) result from this drug?

At the dose of dug was given, the mice should not show detrimental signs of physiological impairment. However the mice will likely to reduce food intake, lose body weight, and/or induce fever. We will monitor the body weight and rectal core body temperature daily. We will make timely decision to euthanize the animals should the condition of the animals become severe (eg. lose more than 20% of body weight, or body temporature is too high). We will use pharmaceutical grade compounds and dilute in sterile medical-grade saline, then use filter with pore size 0.2 μm for sterilization.

I1. Exogenous substances to be administered:

Drug/Substance: Leptin
Dosage: 400 mcg/kg body weight
Route: i.p.
Volume: 0.1 - 0.3 ml
Frequency: twice over a 3 - week period

I2. Will the animals(s) be anesthetized or sedated during administration?

No

I3. Will any physical or physiological impairment (i.e. pain, distress, change in activity) of the animals(s) result from this drug?

At a single dose of 400 mcg/kg body weight the mice should not show signs of significant physiological impairment. Exogenous leptin may cause a transient loss of appetite and increased energy expenditure in these mice. We will use pharmaceutical grade compounds and dilute in sterile medical-grade saline, then use filter with pore size 0.2 μm for sterilization

I1. Exogenous substances to be administered:

Drug/Substance: Lipopolysaccharide
Dosage: 30-300ug
Route: intraperitoneal
Volume: 0.2 ml
Frequency: single dose

I2. Will the animals(s) be anesthetized or sedated during administration?

No

I3. Will any physical or physiological impairment (i.e. pain, distress, change in activity) of the animals(s) result from this drug?

At the dose of dug was given, the mice should not show detrimental signs of physiological impairment. However the mice will likely to reduce food intake, lose body weight, and/or have fever. We will monitor the body weight and rectal core body temperature daily. We will make timely decision to euthanize the animals should the condition of the animals become severe (eg. lose more than 20% of body weight, or body

temporature is too high). We will use pharmaceutical grade compounds and dilute in sterile medical-grade saline, then use filter with pore size 0.2 μm for sterilization

I1. Exogenous substances to be administered:

Drug/Substance: Monosodium glutamate
Dosage: 2 mg/g BW
Route: subcutaneously
Volume: 20 uL
Frequency: once per day for 5 days

I2. Will the animals(s) be anesthetized or sedated during administration?

No

I3. Will any physical or physiological impairment (i.e. pain, distress, change in activity) of the animals(s) result from this drug?

MSG injections will cause lesions in feeding and glucose-sensing areas of the brain. Mice treated in this way will become hyperphagic and obese. We will use pharmaceutical grade compounds and dilute in sterile medical-grade saline, then use filter with pore size 0.2 μm for sterilization

I1. Exogenous substances to be administered:

Drug/Substance: Streptozocin
Dosage: 40-200mg/kg
Route: i.p.
Volume: 0.2 -0.5ml
Frequency: sigle dose for high or multiple dose for low

I2. Will the animals(s) be anesthetized or sedated during administration?

No

I3. Will any physical or physiological impairment (i.e. pain, distress, change in activity) of the animals(s) result from this drug?

At the dose of dug was given, the mice should not show detrimental signs of physiological impairment. However the mice will likely to reduce food intake, lose body weight, and/or have fever. We will monitor the body weight and rectal core body temperature daily. We will make timely decision to euthanize the animals should the condition of the animals become severe (eg. lose more than 20% of body weight, or body temporature is too high). We will use pharmaceutical grade compounds and dilute in sterile medical-grade saline, then use filter with pore size 0.2 μm for sterilization

I1. Exogenous substances to be administered:

Drug/Substance: Tamoxifen
Dosage: 8mg
Route: i.p.
Volume: 200ul
Frequency: 1 time

I2. Will the animals(s) be anesthetized or sedated during administration?

No

I3. Will any physical or physiological impairment (i.e. pain, distress, change in activity) of the animals(s) result from this drug?

Body weight of the mice will be monitored daily 1 week after the treatment. If the body weight of the mice

reduces significantly, we will euthanize the mice immediately. We will perform validation studies at least 2 weeks after the end of tamoxifen treatment. We will use pharmaceutical grade compounds and dilute in sterile medical-grade saline, then use filter with pore size 0.2 μm for sterilization.

I1. Exogenous substances to be administered:

Drug/Substance: Tamoxifen
Dosage: 4mg
Route: gavage
Volume: 200ul
Frequency: 5 times every other day

I2. Will the animals(s) be anesthetized or sedated during administration?

No

I3. Will any physical or physiological impairment (i.e. pain, distress, change in activity) of the animals(s) result from this drug?

During the tamoxifen treatment, body weight of the mice will be monitored daily. If the body weight of the mice reduces significantly, we will stop the tamoxifin treatment or euthanize the mice immediately. We will use pharmaceutical grade compounds and dilute in sterile medical-grade saline, then use filter with pore size 0.2 μm for sterilization.

I1. Exogenous substances to be administered:

Drug/Substance: Tamoxifen
Dosage: 400mg/kg tamoxifin-containing diet
Route: Feeding
Volume: ad. lib.
Frequency: 7-10 days.

I2. Will the animals(s) be anesthetized or sedated during administration?

No

I3. Will any physical or physiological impairment (i.e. pain, distress, change in activity) of the animals(s) result from this drug?

During the tamoxifen treatment, body weight of the mice will be monitored daily. If the body weight of the mice reduces significantly, we will stop the tamoxifin treatment or euthanize the mice immediately. In order to increase the tolerance of the diet, we may mix 2/3 of the customized tamoxifin-containing diet with 1/3 of the regular chow in powered form to feed the mice.

I1. Exogenous substances to be administered:

Drug/Substance: Thiogycollate
Dosage: 3%
Route: i.p.
Volume: 1ml
Frequency: single dose

I2. Will the animals(s) be anesthetized or sedated during administration?

No

I3. Will any physical or physiological impairment (i.e. pain, distress, change in activity) of the animals(s) result from this drug?

At the dose of dug was given, the mice should not show detrimental signs of physiological impairment. However the mice will likely to reduce food intake, lose body weight, and/or have fever. We will monitor the body weight and rectal core body temperature daily. We will make timely decision to euthanize the animals should the condition of the animals become severe (eg. lose more than 20% of body weight, or body temporature is too high). We will use pharmaceutical grade compounds and dilute in sterile medical-grade saline, then use filter with pore size 0.2 μm for sterilization

I1. Exogenous substances to be administered:

Drug/Substance: norepinephrine
Dosage: 1-1.5mg/kg
Route: Subcutaneously
Volume: 0.1-0.2ml
Frequency: single dose

I2. Will the animals(s) be anesthetized or sedated during administration?

Yes

I3. Will any physical or physiological impairment (i.e. pain, distress, change in activity) of the animals(s) result from this drug?

Norepinephrine will be given to anesthetized animals. Its stimulatory effect on sympathetic nervous activity will only last will last 20min - 60min depend on the animals. At the dose of the dug was given, the mice should not show long detrimental long term effect. The animals will be monitored in metabolic chambers (30o C) during the entire process until they wake up. This is a frequently used approach, reference can be found in publications such as Cell Metabolism 9:203-209. We will use pharmaceutical grade compounds and dilute in sterile medical-grade saline, then use filter with pore size 0.2 μm for sterilization.

I1. Exogenous substances to be administered:

Drug/Substance:
Dosage:
Route:
Volume:
Frequency:

I2. Will the animals(s) be anesthetized or sedated during administration?

No

I3. Will any physical or physiological impairment (i.e. pain, distress, change in activity) of the animals(s) result from this drug?

No

I1. Exogenous substances to be administered:

Drug/Substance:
Dosage:
Route:
Volume:
Frequency:

I2. Will the animals(s) be anesthetized or sedated during administration?

No

I3. Will any physical or physiological impairment (i.e. pain, distress, change in activity) of the

animals(s) result from this drug?

No

Section J. Antibody Production

None

Section K. Tumor Studies

None

Section L. Antemortem Fluid

MICE

L1. Fluid to be collected:

Blood

L2. Volume to be collected:

No more than 0.25 ml per mouse every two weeks.

L3. Frequency of collection:

2 or 3 time points.

L4. Method (or Route) of collection:

Tail bleed or lancet piercing in the tail vein.

L5. Is anesthesia or sedation used?

No

Section M. Transgenics

MICE

M1. Who will develop the transgenic line?

Transgenic is already available.

M2. Are there any phenotypes that will affect the animal's health and well being associated with this transgenic line(s)?

ob/ob mice and the double knockouts developed from them (ob/ob-ghrelin and ob/ob-GHSR) are obese. We will take care to ensure that the ventral surfaces of these obese mice remain as dry and as clean as possible (cage bottoms will be changed more frequently).

Section N. Restraint

MICE

N1. Method(s) of restraint:

Restraint device or cage: Each mouse will be pulled tail-first into a ventilated 50-mL conical centrifuge tube and a ventilated cap will be placed on the top of the tube. The restrained mouse will then be placed back in its home cage for 2 hours. During this 2-hour restraint, the mice will be monitored every 15 min for signs of too much struggle against the restraint and to ensure that the mouse has not assumed an uncomfortable position (doubled up in the tube).

N2. Maximum length of time any one animal would be restrained within a 24 hour period:

2 hours

Section O. Conditioning

MICE

O1. Purpose of conditioning

To standardize metabolic function

O2. Type(s) of conditioning:

O3. Criteria for monitoring the condition of the animals during food and water deprivation:

Mice will be euthanized if they lose more than 20% of body weight, or have severe skin infections.

Section P. Nutrition

MICE

P1. Method of dietary manipulations:

Fasting - food removed from cage wiretop and cage-bottom replaced with a clean cage bottom.

Special diets (high fat, carbohydrate or high protein): We will need to feed our mice special diets such as high fat, high carbohydrate, or high protein from 2 months to 5 months of age. All of the diets will be purchased from commercially available and BCM proven vendors such as Research Diets, Inc. and Harland Teklad. During the special diet feeding period, we will provide complete care (change food, water and cages), no CCM care is needed.

Occasionally customized tamoxifin-containing diet containing 400mg/kg (Harlan, TD.120681) will be used for 7-10 days. In order to increase the tolerance of the diet, we may mix 2/3 of the customized tamoxifin-containing diet with 1/3 of the chow in powered form to feed the mice.

Automatically controlled pair-feeding in metabolic cages.

P2. Duration of dietary manipulations:

Fasting for 6 - 24 hrs.

Special diets for 2-12 months.

Tamoxifin-containing diet containing 400mg/kg (Harlan, TD.120681) will be used for 7-10 days.

Pair-feeding in metabolic cages may last 2-4 weeks.

Overnight fasting, GTT, ITT may be performed once every 6 months on a given mouse. Special diet will be fed from 2 months to 5 months of age

P3. Describe any physical or psysiological impairment that may result and how it will be monitored and/or minimized:

None.

Section Q. Sedation

MICE

Q1. When is this sedation used?

In rare situation, we need to reconfirm genotyping of mice older than 4 weeks of age. In that case, we will use general anesthesia (such as isolurance) on the mice, before tail biopsy is conducted.

Norepinephrine-induced O2 consumption: In order to study norepinephrine-induced O2 consumption, we have to keep the mice at the lowest metabolic rate for 60-90 min, so we can record the effect of norepinephrine-induced O2 consumption in metabolic chambers. Pentobarbital 50 mg/kg BW IP will be used to anesthetize the mice for long period of time. We will then subcutaneously inject norepinephrine 1-1.5mg/kg (in 0.1-0.2ml saline solution) to induce acute increase of O2 consumption in the mice to characterize the thermogenic effects of ghrelin signaling.

Q2. Drug Name, Dose and Route:

Drug Name:
Other

Dose and Route:
Inhalable isoflurane as general anesthesia: A vapor chamber will be used, so the isolflurane will not be in direct contact with the mice and also we can observe the braking pattern of the mice. The procedure will be carried out in fume hood.

For norepinephrine-induced O2 consumption experiments, we will use: 1) For repeated experiment only: Pentobarbital 50 mg/kg BW, IP will be used to anesthetize the mice for norepinephrine-induced O2 consumption experiments; 2) For new experiments: 30 - 45 minutes anesthesia: 80 mg/kg BW ketamine + 16 mg/kg BW xylazine IM. Mix equal volume of ketamine (100 mg/ml) sylazine (20 mg/ml) For a 30 g mouse this is 0.024 ml ketamine + 0.024 ml xylazine.

Section R. Surgical Procedures

MICE

PRE-OPERATIVE CARE

R1. What special care will be given to prepare animal for surgery?

R2. Who will administer the anesthesia?

R3. What drugs are to be used? (Include dose and route)

Ketamine / Xylazine DEA Schedule III: 30 - 45 minutes anesthesia: 80 mg/kg BW ketamine + 16 mg/kg BW xylazine IM. Mix equal volume of ketamine (100 mg/ml) sylazine (20 mg/ml) For a 30 g mouse this is 0.024 ml ketamine + 0.024 ml xylazine.

R4. What is the estimated duration of anesthesia?

R5. How will the depth of anesthesia be monitored?

Absence of withdrawal reflex to toe pinch

If a paralytic agent is used, explain its purpose and how the level of anesthesia will be monitored in the paralyzed animal:

SURGICAL PROCEDURE

R6. Survival Procedure?

Yes

R7. Major Procedure?

No

R8. Does the study involve multiple surgeries?

No

R9. Describe the surgical procedure(s):

Other:
Insulin clamps will be conducted under Dr. Chan's protocol AN-625.

R10. How will skin be closed?

Clips or non-absorbable suture

R11. Who will perform the surgical procedure?

R12. Where will the surgery take place? (Room / Building)

R13. Will surgery take place under sterile conditions or aspetic conditions?

Sterile

R14. Who will perform post-surgical care?

R15. What precautions will be taken during and after surgery to prevent hypothermia and/or dehydration?

Warming Blanket

R16. What criteria will be used to determine that animals are ready to return to their housing?

Sternal Recumbancy

R17. Describe post-operative monitoring:

R18. What post-operative analgesics will be used?

R19. Provide a justification if no post-op analgesics will be used:

R20. What post-operative antibiotics will be used?

Antibiotic:

Dosage:
Route: ;
Frequency:

Indications for antibiotics:

R21. Describe any special diet or other supportive care required post-op:

Section S. Minimization of Pain

S1. What criteria will be used for early euthanasia?

Immobility, huddled posture, inability to eat, ruffled fur, self mutilation, vocalization, dehiscent wound, hypothermia, >20% weight loss. Additionally, obese mice may not be able to effectively groom themselves which may result in skin abrasions or damage from excessive urine accumulation in the bedding. Obese mice with lesions on the ventral surface will be treated immediately with antibiotics or euthanized.

For thioglycollate-induced peritonitis, body weights of the mice will be monitored daily. If the body weight reduces more than 20%, or the mice show seizures, signs of abdominal pain, distension or other neurologic signs, the mice will be euthanized immediately.

For pair-feeding study, should any mice lose weight significantly (more than 20%), they will be sacrificed right the way.

For cold stress study: If the rectal temperature of the mice is less than 25°C, the mice will be pulled from the study and scarified immediately. All cold tress mice will be scarified at the end of experiment regardless their temperatures, mice will not be put back to home cage for recovery.

For Restraint stress: Each mouse will be placed tail-first into a ventilated 50-mL conical centrifuge tube and a ventilated cap will be placed on the top of the tube in a vertical position. During the 2-hour restraint, the mice will be monitored every 15 min for signs of too much struggle against the restraint and to ensure that the mouse has not assumed an uncomfortable position. If the mice exhibit unusual neurological signs, the mouse will be sacrificed right the way.

S2. Will there be unrelieved pain, discomfort or stress? (defined as lasting for more than a moment, i.e. longer duration or more painful than a needlestick)

Yes:
Many of our experiments have suggested that ghrelin's functions only become obvious when animals are placed in stressful conditions. Mice may be subjected to acute cold stress (20 min in RT water or 4 degrees for 1-6 hours), and restraint stress (2 hours), and 24 hour fast. Ghrelin (the gene we study) is an energy sensor, which is highly regulated by fed and fasting state. We need to study the level of ghrelin under the condition that liver glycogen is depleted, so we need to fast the mice for 24 hours. These are both methods of transient stress that are used to investigate the H/P axis responses in these mice. No more than 100 mice will be stressed by these methods.

In cold stress experiments, core body temperatures will be monitored at 15 min intervals using an anal probe. Severely hypothermic animals (loss of 10 deg of body temperature) will be pulled out and warmed to room temp. For restraint stress, each mouse will be placed tail-first into a ventilated 50-mL conical centrifuge tube and a ventilated cap placed on the top of the tube. During the 2-hour restraint, the mice will be monitored every 15 min for signs of too much struggle against the restraint and to ensure that the mouse has not assumed an uncomfortable position (doubled up in the tube). The restrained mouse will then be placed back in its home cage for 2 hours. After the 2-hour restraint another specimen of blood will be taken for glucose, glucagon, and corticosterone levels.

Rational: It is known that non-shivering thermogenesis is regulated by brown fat which responds to sympathetic nerve impulses. Brown fat in leptin knockout (ob/ob) mice is usually in an inactive state, so leptin

knockout (ob/ob) mice have impaired capacity for non-shivering thermogenesis, and are not able to maintain body temperature under cold conditions. When ob/ob mice are placed at 4oC, core body temperature drops 9oC in an hour compared to 1.4oC in wild-type mice (PNAS 2003, 100:10085). It has also been reported that central administration of ghrelin suppresses energy expenditure and thermogenesis in brown fat via its inhibitory effect on sympathetic nerve activity (Neurosci Lett. 2003, 349:75). We propose to study the thermogenesis characteristics of the ghrelin/leptin KOs in order to understand the ghrelin's role in thermogenesis. A preliminary experiment has shown that the regular body temperature of ghrelin/leptin double KO is comparable to ob/ob, but appears to be lower than wild-type and ghrelin KO. We feel that by putting animals in lower temperature (4oC), we might be able to detect ghrelin's contribution in thermogenesis.

thyoglycollate broth may cause abdominal discomfort and pain.

When use isoflurane, we will place cotton or tissue containing liquid isoflurane in an plastic tube inside the anesthesia jar to prevent the mice from direct contact.

For Restraint stress: Each mouse will be placed tail-first into a ventilated 50-mL conical centrifuge tube and a ventilated cap will be placed on the top of the tube in a vertical position. During the 2-hour restraint, the mice will be monitored every 15 min for signs of too much struggle against the restraint and to ensure that the mouse has not assumed an uncomfortable position. We may correct the position of the mice make them more comfortable. If the mice exhibit unusual neurological signs, the mouse will be sacrificed right the way.

S3. Will protocol involve death as an endpoint?

No

Section T. Euthanasia

MICE

T1. Method:

MICE/BIRDS - Cervical Disarticulation

Cervical Disarticulation while under surgical plane of anesthesia or after CO2 induced unconsciousness.

T2. Provide clear, scientific justification for the use of any method without anesthesia.

T1. Method:

MICE/RATS - Drug Overdose Pentobarbital sodium, DEA Schedule II

120 mg/kg IP, IV

T2. Provide clear, scientific justification for the use of any method without anesthesia.

Section V. Alternative to Animal Models

V1. I have searched the following sources for alternatives to animal models:

Model:
MedLine

Keywords:
growth hormone secretagogues, ghrelin, animal testing alternatives, restraint stress

Date of Search:
5/20/2014

Inclusive Years Of Search:
2000-2014

Model:
Other PubMed

Keywords:
ghrelin, growth hormone secretagogues, animal testing alternatives, restraint stress, cold stress

Date of Search:
5/20/2014

Inclusive Years Of Search:
2000-2014

I have determined that one or more supplemental non-animal model(s) are available for some of these studies and I will be using them in order to reduce the number of animals necessary.

Describe briefly (if applicable):
These studies must be done in animal species to study the physiology as it relates to humans. There is no evidence that lower organisms such as worms or flies have the same physiological pathways that involve this receptor. After searching for over last 10 years I have found no cell line that expresses a functional endogenous GHS-R. Nevertheless, we have generated and routinely use engineered cell lines expressing the GHS-R for in vitro experiments to establish mechanisms of signaling whenever possible, before doing animal experiments.

Certification by Principal Investigator

(1) I certify that my studies do not unnecessarily duplicate previous experiments.

(2) I certify that the use of all animals involved in this project will be carried out according to the provisions of the, PHS Policy on Humane Care and Use of Laboratory Animals, the Animal Welfare Act Regulations, the principals of the Guide for the Care and Use of Laboratory Animals, and the policies and procedures of Baylor College of Medicine. I agree to notify the IACUC of Baylor College of Medicine of any substantive changes in the research use of the animals, including the number of animals, species used, or procedures performed.

(3) I certify that all personnel listed on this protocol will be appropriately trained and will have completed the mandatory species-specific training (AALAS) available through BRAIN Electronic Certification and Training eCAT prior to working with any animals.

(4) I understand that Baylor College of Medicine and its representatives on the IACUC have the authority to suspend any part of my research, should I not be in compliance at any time with any of the above aforementioned policies, procedures, or regulations.

(5) I understand that the Center for Comparative Medicine has been made responsible for administering and assigning animal housing space within the central animal facilities. CCM staff will make space assignments for the efficient utilization of space, which may result in investigators sharing animal housing space. I further realize that I must notify CCM of animal housing needs in order to insure the availability of space before animals are procured.

(6) I am familiar with the Baylor College of Medicine Laboratory Safety Guidelines on Infectious Agents and the Chemical Hygiene Plan. I will abide by the regulations contained in these documents and in all additional policies and procedures that relate to research at Baylor College of Medicine. I will ensure that all laboratory personnel engaged in this project will be informed of potential hazards and adequately trained in procedures of animal experimentation involving hazardous agents.