Title
Synergistic effects of the serotonin 2c receptor and leptin on glucose homeostasis

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Synergistic effects of the serotonin 2C receptor and leptin on glucose homeostasis

by

Jennifer M. Wade

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biomedical Sciences

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO
Dedication and acknowledgements:

I would like to thank the members of my thesis committee, David Pearce, Bob Farese, Christian Vaisse and Larry Tecott for their helpful input in developing this research project. I would also like to thank members of the Tecott lab who contributed to my learning over the years and also to the development of this thesis, particularly Elaine Storm, Luna Abdallah, and Evan Goulding.

I also would like to give credit to my family, particularly my father, who encouraged me to switch careers and become a scientist like him, and my friends, Iggy Evans, Shannon Kokoska, Jen Larsen, and Monique van den Berg who provided emotional support and helped with the important task of maintaining my mental equilibrium while pursuing a Ph.D.!

Chapters 2-4 of this thesis are adapted from a paper submitted to Diabetes: Wade JM, Juneja P, MacKay AW, Graham J, Havel PJ, Tecott LH, Goulding EH. Synergistic interactions of leptin and serotonin in the regulation of glucose homeostasis.
Abstract:

To investigate how serotonin and leptin interact in the regulation of energy balance and glucose homeostasis, we created a genetic mouse model, the OB2C mouse, that lacks both functional leptin and functional serotonin 2C receptor (5HT2CR). These mice showed increased feeding and drinking behavior compared to mice with mutations of only leptin (OB) or the 5HT2CR (2C). They also had an enhanced diabetes phenotype, with elevated fasting glucose and impaired glucose tolerance. This enhanced diabetes develops at an age at which OB2C mice do not differ in body weight from OB mice, suggesting that the enhanced diabetes is not a consequence of increased adiposity. We further demonstrated that a serotonin agonist, fenfluramine, and a 5HT2CR antagonist, SB242084, had acute effects on glucose tolerance in wild-type mice which were blunted in 2C mutant mice, suggesting further body-weight independent effects of the 5HT2CR on glucose homeostasis. These findings may provide important insights into the obesogenic and diabetogenic side effects of serotonergic atypical antipsychotic drugs.
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Chapter 1: Background

Obesity and Diabetes

One need only open practically any newspaper to see that public health experts today are alarmed by the rising rate of obesity both in the United States and worldwide. Obesity is defined on the basis of body mass index (BMI), a ratio which compares a person's height and weight. Persons with a BMI over 30 kg/m\(^2\) are considered to be obese. From 1991 to 2001, the prevalence of obesity among U.S. adults rose a remarkable 74%, from 12% to 20.9% of the population (Mokdad et al.) No one factor has been identified as being primarily responsible for this trend, but rather it is generally attributed to a number of lifestyle changes throughout the latter part of the 20th and early 21st centuries, including smoking cessation (Flegal et al.), decreased physical activity (Flegal et al.), and increased caloric intake.

In parallel with the rise in obesity, rates of type II diabetes mellitus have also increased dramatically, by 61% from 1991 to 2001 (Mokdad et al.). It is now estimated that 14.7 million Americans are living with diagnosed type II diabetes (Centers for Disease Control) and that many more have undiagnosed diabetes. While type II diabetes is just one of many health problems associated with obesity, it is of particular public health interest because obesity and diabetes are so tightly linked, both epidemiologically and physiologically. While not all type II diabetes patients are obese or even overweight, countless studies have linked the two conditions (Bray, Astrup and Finer). Women with a BMI of 35 kg/m\(^2\) have a 40-fold greater risk of developing type II diabetes than women with a BMI of 22 kg/m\(^2\) or below (Colditz et al.) For men, the association of obesity and diabetes is only slightly less strong (Chan et al.). Indeed, obesity and type II diabetes are so tightly linked in the current
biomedical zeitgeist that a new term has been created to describe the rise in rates of obesity and type II diabetes: the "Diabesity" epidemic.

**Causes of Type II diabetes: beyond "Diabesity"**

While the mechanistic link between diabetes and obesity is not yet fully understood, several associations between the two have been identified. While adipocytes were once thought of as inert sacs of lipids, we now know that adipose tissue secretes a number of hormones that influence insulin sensitivity throughout the body, including leptin, adiponectin, resistin, tumor necrosis factor α, and interleukin-6. Obesity is associated with changes in the levels of these hormones in the bloodstream (Fasshauer and Paschke). Another indicator of the significance of endocrine actions of adipocytes in regulation of glucose homeostasis is the finding that patients and animal models with an impaired ability to store fat typically display severe insulin resistance and diabetes (Heilbronn et al.). Obesity and diabetes are also both associated with increased adipocyte size. Lifestyle risk factors, including low physical activity and high caloric intake, are common to both obesity and type II diabetes, as well.

The multiple common risk factors of obesity and type II diabetes complicate the study of impaired glucose homeostasis in obese subjects and animals. To what extent is type II diabetes caused by lifestyle factors that may also cause obesity, and to what extent is it a secondary consequence of obesity itself? Genetic and pharmacological animal models are being used to address this question.
Leptin and OB mice

Leptin, one of the hormones secreted by adipose tissue, is a member of the cytokine superfamily. Leptin acts on leptin receptors in the brain and in the periphery as an indicator of energy status to the central nervous system. Mice which lack functional leptin (OB mice) or functional leptin receptor b (DB mice) develop dramatic obesity early in life, along with serious endocrine and metabolic abnormalities, including diabetes, and profound behavioral changes, including marked hypoactivity. OB mice have been known to obesity researchers since 1950, when this naturally occurring single point mutation was first noted due to its dramatic phenotype (Ingalls et al.). However, the leptin gene was not cloned and identified as the culprit until 1995 (Halaas et al.).

While many factors have been implicated in the regulation of body weight, leptin's effects are by far the most profound. While null mutations in leptin or the leptin receptor only account for a handful of obesity cases worldwide, most obese patients suffer from hyperleptinemia and leptin resistance, and it is believed that a fuller understanding of how leptin regulates energy balance may lead to more effective treatments for obesity.

Leptin and glucose homeostasis

As noted above, both OB mice and DB mice develop diabetes. While initially, researchers wondered if this might be a consequence of these animals' obesity, subsequent research has revealed direct effects of leptin on glucose homeostasis. Pelleymounter et al. found that low-
dose daily leptin administration at a dose not sufficient to cause weight loss in OB mice lowered blood glucose without affecting body weight. Similarly, Pocai et al. (2005) found that IC3V administration of leptin for 3 days inhibited glycogenolysis in diet-induced obese rats, normalizing their blood glucose levels. Schwartz et al. (1996b) also showed weight-independent effects of leptin on glucose homeostasis by pair feeding leptin-treated and untreated OB mice and found that the leptin treated mice showed a 40% greater reduction in blood glucose than the non-treated mice.

In addition, studies of the effect of mouse background strain on the OB and DB phenotypes have given further insight into the direct effects of leptin on glucose homeostasis. Haluzik et al. showed that OB mice on the FVB/N background were more severely diabetic than those on the C57BL/6J background, despite similar levels of obesity. Similarly, Chua et al. compared DB mice on the FVB strain background (FVB-Lepr\textsuperscript{db}) and on the DBA background (DBA-Lep\textsuperscript{db}). They found that while both lines of mice had similar body weights, the FVB-Lepr\textsuperscript{db} mice had far higher levels of blood glucose and serum insulin, and had worsened sensitivity to insulin. Both lines showed reduced blood glucose and increased insulin levels with age, despite the fact that their obesity continued to worsen with age. Interestingly, Leiter et al. also saw that leptin receptor mutant mice on a 129/J background also improved their diabetes over time, but these mice decreased both their blood glucose and insulin with age, suggesting that insulin sensitivity improves with age in some leptin-deficient animal models.
**Downstream targets of leptin**

Leptin receptors are expressed throughout the body, including muscle, liver and adipose tissue. However, leptin's effects on feeding, metabolism and glucose homeostasis are believed to be primarily regulated by leptin receptors in the brain. Leptin receptor b, one of several splice variants of the leptin receptor gene and the only one yet shown to be biologically active, is expressed in all the major hypothalamic sites implicated in the regulation of energy balance. It is expressed robustly in the arcuate nucleus of the hypothalamus (Arc), the ventromedial nucleus of the hypothalamus (VMH), and the dorsomedial hypothalamus (DMH), and to a lesser extent in the paraventricular nucleus of the hypothalamus (PVN) and the lateral hypothalamus (LH) (Schwartz et al. 1996a, Elmquist et al. 1998). The Arc is believed to be one of the most important sites of integration of energy balance signals, and is of additional interest because of its location at the base of the brain where the blood-brain barrier is permeable (Banks et al.). Leptin is transported across the blood-brain barrier, however, it is believed that leptin transporters can become saturated, contributing to obesity (Banks et al.). Perhaps the most dramatic illustration of the effects of leptin in the Arc on glucose homeostasis comes from the work of Coppari et al., who showed that adenoviral re-expression of leptin receptors only in the Arc of DB mice markedly improved glucose homeostasis and modestly reduced body weight.

Two distinct populations of leptin-sensitive neurons in the Arc have been identified: POMC/CART neurons, which produce anorexigenic effects and AgRP/NPY neurons, which produce orexigenic effects. POMC/CART neurons are defined on the basis of their expression of the
Proopiomelanocortin (POMC) and Cocaine and Amphetamine Related Transcript (CART) genes. AgRP/NPY neurons, similarly, are named for their expression of the Agouti-related Peptide (AgRP) and Neuropeptide Y (NPY) genes. Leptin depolarizes POMC/CART neurons and hyperpolarizes AgRP/NPY neurons (Coppari et al.). The POMC gene product α-MSH and AgRP act as natural antagonists of one another at melanocortin 4 receptors (MC4R), which are expressed at high levels in the PVN, the DMH, and the LH (Elmquist et al., 1999). α-MSH activates MC4 receptors, and AgRP inhibits them. MC4 receptors are believed to be a major mediator of the effects of leptin on feeding, body weight, and glucose homeostasis. OB mice have dramatically lowered levels of POMC mRNA, and a melanocortin receptor antagonist has been shown to block leptin's anorexic effects and effects on sympathetic nervous system activation (Elmquist et al., 1999). Several animal models of melanocortin disruption, including MC4R null mice, mice that ectopically express the MC4R antagonist Agouti, and mice that have targeted disruption of the leptin receptor only in POMC neurons, are hyperphagic and obese, and develop diabetes and hyperleptinemia (Huszar et al., Balthasar et al., 2004). However, when Boston et al. crossed Agouti mice with OB mice, they found that the resulting animals were significantly more obese and hyperinsulinemic than OB mice, suggesting an additional leptin-independent role of the MC4R in regulation of body weight and glucose homeostasis. Additional studies have shown that the MC4R has direct effects on glucose homeostasis that are independent of changes in body weight. Obici et al. found that ICV infusion of α-MSH enhanced insulin action in rats, whereas infusion of the melanocortin antagonist SHU9119 decreased insulin action.
NPY is another important mediator of leptin's effects in the Arc. OB mice have elevated expression of Arc NPY (Elmquist et al., 1999). When OB mice were crossed with mice with targeted disruption of NPY, the resulting mice had less severe hyperphagia and obesity than that seen in OB mice, and also had improved (though still abnormally high) serum glucose and insulin (Erickson et al.).

It is important to note that while the Arc is certainly a very important target of leptin, it is not the only one. As stated earlier, Coppari et al. found that expression of the leptin receptor only in Arc neurons partially rescued the leptin receptor null phenotype, but these animals still showed increased fat mass and body weight, hyperinsulinemia, and a trend towards decreased movement. Leptin receptors are also expressed widely in the CNS outside the Arc, as are NPY and AgRP.

**Central regulation of glucose homeostasis**

It has been well established that the Arc is an important brain region for regulation of glucose homeostasis in the periphery. Arc neurons mediate their effects on the peripheral tissues involved in glucose homeostasis via projections to sympathetic and parasympathetic preganglionic spinal cord neurons which project to peripheral tissues involved in glucose homeostasis via the vagus nerve. Both NPY/AgRP neurons and POMC/CART neurons in the Arc send projections to the PVN and the LH, which in turn project to autonomic preganglionic neurons (Elmquist et al., 1998). In addition, POMC/CART neurons have been
shown to have direct projections from the Arc to preganglionic spinal cord neurons (Elías et al.).

Determination of the peripheral neuronal pathways regulating glucose homeostasis have been a more recent undertaking. Earlier this year, Kreier et al. used the neuronal tracer cholera toxin b (CTB) to find neural inputs to visceral fat, liver, and pancreas (each tissue's CTB was labeled with a different fluorescent label) in order to further characterize the pathways by which the brain regulates glucose homeostasis. They found that liver, pancreas, and intraabdominal fat shared a set of vagal motor neurons. In addition, they used injections of the retrograde tracer pseudorabies virus to determine projections from hypothalamus and brainstem to the intra-abdominal compartment, and found that the intermediolateral cell column of the spinal cord (where vagal sympathetic neurons originate) receives inputs from the PVN.

**Serotonin and the serotonin 2C receptor**

Serotonin (5-HT) is a neurotransmitter synthesized in serotonergic neurons in the central nervous system and in peripheral cells, primarily in the gut. Serotonin exerts effects on many diverse facets of mood and behavior, including depression, anxiety, feeding behavior, sexual behavior, and physical activity. Many pharmacologic therapies for mood disorders work at least in part through their effects on the serotonin system. Serotonin acts through its 14 known receptor subtypes, which are expressed in discrete patterns.
The serotonin 2C receptor (5-HT$_{2c}$R) is expressed widely but exclusively in the central nervous system, including all the hypothalamic areas implicated in regulation of body weight and glucose homeostasis. The 5-HT$_{2c}$R is the serotonin receptor subtype most strongly implicated in serotonin's regulation of feeding behavior and body weight. Much of our understanding of the importance of this receptor has come from the generation of the 5-HT$_{2c}$R null mutant mouse. These 5-HT$_{2c}$R null mice exhibit hyperphagia, hyperactivity and a tendency towards increased body weight late in life (Tecott et al.). These mice also have endocrine abnormalities late in life: they develop hyperinsulinemia and hyperleptinemia compared to their wild-type littermates and they have impaired glucose tolerance (Nonogaki et al.). Exposure to a high-fat diet has been shown to enhance and accelerate obesity and diabetes in these mice (Nonogaki et al.). 5-HT$_{2c}$R mutant mice are also resistant to the anorectic effects of the 5-HT$_{2c}$R agonist 1-(3-chlorophenyl)piperazine (mCPP), suggesting that mCPP-induced anorexia is mediated by the 5-HT$_{2c}$R (Tecott et al.).

**Serotonin and glucose homeostasis**

In addition to the phenotype of the 5-HT$_{2c}$R null mouse, there have been several other indications that serotonin is involved in both the regulation of feeding and body weight and of glucose homeostasis. Serotonin has been implicated in the onset of weight gain and the development of type II diabetes mellitus in patients taking atypical antipsychotic drugs, which decrease serotonergic tone. A polymorphism in the 5-HT$_{2c}$R gene has been found to be associated with resistance to atypical antipsychotic drug-induced weight gain (Templeman et al.), and the morbidity rate from type II diabetes of six atypical antipsychotic agents was
found to be positively correlated with the drugs' 5-HT$_{2C}$R occupancy rate (Matsui-Sakata et al.). Human polymorphisms in the 5-HT$_{2C}$R promoter have also been associated with resistance to obesity and diabetes (Yuan et al.).

Conversely, the nonspecific serotonin agonist fenfluramine has been demonstrated to reduce food intake and to improve glucose tolerance and insulin action (Pinder et al.). The effects of fenfluramine on insulin action have been shown to be independent of the drug's effects on food intake and body weight (Scheen et al., Verdy et al.) and have been observed in normal rats (Jorgensen), streptozotocin diabetic rats (Jorgensen), diet-induced obese rats (Storlien et al.), and type II diabetes patients (Verdy et al., Scheen et al.). The effects of fenfluramine on insulin action are believed to be due to an increase in insulin sensitivity, as fenfluramine treatment decreases serum levels of both glucose and insulin (Verdy et al., Scheen et al.). A hyperinsulinemic-euglycemic clamp study revealed that in humans, 3 days of fenfluramine treatment increased insulin action in diabetic patients (Pestell et al.). A clamp study in diet-induced obese rats also showed improved insulin action with fenfluramine treatment, however, it also showed improved glucose uptake in muscle, and the most dramatic effects were seen in the liver, where fenfluramine treatment completely reversed diet-induced impaired suppression of liver glucose production (Storlien et al.).

The 5-HT$_{2C}$R has been implicated as a primary mediator of fenfluramine's effects on feeding behavior. Vickers et al. (2001) showed that the 5-HT$_{2C}$R antagonist SB242084 blocks the anorectic effects of fenfluramine and Vickers et al. (1999) showed that fenfluramine-induced anorexia was blunted in 5-HT$_{2C}$R mutant mice. To date, no studies have been published
demonstrating that the 5-HT$_2C$R is responsible for fenfluramine's effects on glucose homeostasis, however, given the evidence presented above, it seems a likely candidate.

**Serotonin and leptin**

Several studies have indicated that the serotonin and leptin systems affect feeding, body weight, and glucose homeostasis independent of one another. Young 5-HT$_2C$R mutant mice display marked hyperphagia despite normal levels of serum leptin (Nonogaki et al.). Halford and Blundell have proposed that since serotonin's regulation of feeding is primarily short-acting whereas leptin has more chronic effects on food intake that the two systems work through separate pathways. However, there is evidence that serotonin and leptin may interact in their regulation of energy balance. Heisler et al. found that administration of fenfluramine doubled the firing rate of leptin-sensitive POMC neurons in the Arc, and that the 5-HT$_2C$R is co-expressed with POMC in these cells. Finn et al. found in monkeys that some cells in the dorsal raphé, the area of the brain where most serotonin in the brain is produced, co-express leptin receptor and serotonin transporter mRNA. This finding was bolstered by another report from Fernandez-Galaz et al., who found in rats that digoxigenin-labeled leptin was taken up by serotonergic cells in the dorsal raphé. Most recently, Wang and Chehab found that deletion of the 5-HT$_2C$R from transgenic mice overexpressing leptin exacerbates diet induced obesity, hyperleptinemia, and hyperinsulinemia in these mice. They also found using quantitative PCR that 5-HT$_2C$R mutant mice had decreased hypothalamic mRNA levels of NPY, AgRP,
and POMC compared to wild-type mice, however, this finding has not been replicated by our own lab.

In addition, there is some evidence that manipulation of the serotonin and leptin systems have a mutual influence on one another, however, the evidence is somewhat paradoxical. The serotonin precursor 5-HTP has been shown to increase leptin levels in mice, however, acute and chronic administration of the serotonin releaser and reuptake inhibitor fluoxetine has been shown to decrease serum leptin (Halford and Blundell). Conversely, Clark et al. found that IP and ICV leptin treatment of rats decreased serotonin concentrations in the PVN, and that ICV leptin also decreased serotonin levels in the VMH. And Collin et al. found that OB mice showed decreased serotonin transporter mRNA in their dorsal raphés.

Despite these suggestions of an interaction between these systems, to this date it is not well understood how serotonin and leptin might interact in their shared regulation of food intake, body weight, and glucose homeostasis. In the studies described in the following chapters, we use a genetic mouse model to better understand the relationships between these systems, and also to determine whether the serotonin 2C receptor has direct effects on glucose homeostasis that are independent of body weight.
Chapter 2: Genetic interactions of serotonin and leptin in the regulation of glucose homeostasis

Introduction:

Susceptibility to obesity and type II diabetes is characterized by polygenic modes of inheritance (Sladek et al.). This is in accord with a complex set of interactions among the multiple pathways through which energy balance and glucose homeostasis are regulated. Insights into the pathophysiology of these disorders would therefore be facilitated by an understanding of how these pathways interact. For example, serotonin- and leptin-responsive pathways have both been implicated in the regulation of energy balance and glucose homeostasis, yet the extent to which they interact has been unclear.

Several lines of evidence support this possibility that serotonin and leptin may regulate energy balance and glucose homeostasis by influencing common neural pathways. Both leptin receptors and 5HT2CRs are found within multiple hypothalamic structures implicated in energy balance. These include the ventromedial, dorsomedial, paraventricular, lateral and arcuate hypothalamic nuclei (Hoffman and Mezey, Wright et al., Elmquist et al. 1998, Schwartz et al. 1996a).

In order to investigate potential interactions between 5HT2CR- and leptin-responsive pathways, we crossed mice with null mutations in the htr2c gene with mice with null mutations in the lep gene to create mice deficient for both genes, the OB2C double mutant.
mouse. Here we report that 5HT2CRs mediate central serotonergic influences on peripheral glucose homeostasis in a manner that is sensitive to leptin signaling.

**Materials and Methods:**

**Animals:** Male mice heterozygous for the obese spontaneous mutation (Lep\textsuperscript{ob}/Lep\textsuperscript{+}, B6.V-Lep\textsuperscript{ob}/J) were obtained from The Jackson Laboratory (Bar Harbor, ME) and bred with female mice heterozygous for a null mutation of the X-linked htr2c gene (Tecott et al., 1995) congenic on a C57BL/6J background. From this cross, female mice heterozygous for both the lep and htr2c mutations (htr2c\textsuperscript{c}/htr2c\textsuperscript{c}, Lep\textsuperscript{ob}/Lep\textsuperscript{+}) and male mice heterozygous for the lep mutation (Lep\textsuperscript{ob}/Lep\textsuperscript{+}) were then bred to produce the male experimental mice (WT: htr2c\textsuperscript{c}/Y, Lep\textsuperscript{+}/Lep\textsuperscript{+}; 2C: htr2c\textsuperscript{c}/Y, Lep\textsuperscript{+}/Lep\textsuperscript{+}; OB: htr2c\textsuperscript{c}/Y, Lep\textsuperscript{ob}/Lep\textsuperscript{ob}; OB2C: htr2c\textsuperscript{c}/Y, Lep\textsuperscript{ob}/Lep\textsuperscript{ob}) and heterozygous mice for additional breeding. Genotyping for the htr2c gene mutation was performed by PCR analysis using a primer in the neomycin resistance gene (NeoD: 5’-CACCTTGCTCCTGCGCGAGAAA-3’) and flanking primers in the htr2c gene (2Cfor: 5’-GCTCAGAATTCTGGAAATGTGT-3’; 2Crev: 5’-CGGACTGCTAAATTGGGTC-3’) to produce a 114 bp band for WT mice and a 600bp band for 2C mutant mice. Genotyping for the obese mutation was performed as described (Erickson et al., 1996). All animals were housed at room temperature (20-24°C) on a 12-hr light/dark cycle (lights on at 7 am) with free access to water and a standard chow diet (PicoLab Mouse Diet 20 5150, Purina Mills, Richmond, IN) except were indicated below. Experiments were performed in accordance with the guidelines of the National Institutes of
Food and Water Intake Measurements: Mice were individually housed for 16 days in cages with feeders and water bottles mounted at one end. Food and water were weighed in and out daily to determine total intake. Animals were weighed prior to placement in the monitoring apparatuses and at the end of data collection but were otherwise unhandled. For each mouse, the food and water intake values used in the comparison of groups was the mean of the intake values for the last 12 days of data collection thus allowing for 4 days of acclimation to the novel housing. The mice were run in 12 cohorts of 3-21 mice determined by the generation of mice from the breeding colony. The mice ranged in age from 2-8 months (2 mos: WT 10, 2C 10, OB 15, OB2C 10; 3 mos: WT 4, 2C 2, OB 6, OB2C 4; 4 mos: WT 7, 2C 10, OB 10, OB2C 7; 5 mos: WT 3, 2C 5, OB 6, OB2C 3; 6 mos: WT 3, 2C 3, OB 1, OB2C 2; 7 mos: WT 2, 2C 5, OB 4; OB2C 4; 8 mos: WT 4, 2C 6, OB 7; OB2C 4) and where possible an individual mouse was tested at multiple ages (1 run: 47 mice; 2 runs: 27; 3 runs: 12; 4 runs: 4) to assess the change in intake with age.

Fasting serum physiology: Mice were weighed and individually housed for 16 hours prior to an 8 hour fast beginning at 7 a.m. Mice were then rapidly decapitated for collection of blood from the neck cavity. Samples were centrifuged to collect serum for measurement of glucose, insulin, glucagon, and corticosterone. Mice were tested from 2-10 months of age (2 mos: WT 7, 2C 8, OB 10, OB2C 11; 4 mos: WT 6, 2C 8, OB 11, OB2C 8; 6 mos: WT 6, 2C 10, OB 12, OB2C 9; 10 mos: WT 11, 2C 10, OB 15, OB2C 7). Glucose was measured using
a GM7 Analox instrument (Analox Instruments Ltd., Lunenberg, MA) using the glucose oxidase method. Insulin, glucagon, and corticosterone were measured by radioimmunoassay, using the Linco Rat Insulin RIA Kit #RI13K (Linco Research, St. Charles, MO), the Linco Glucagon RIA Kit #GL-32K (Linco Research, St. Charles, MO), and the MP Biomedicals rat and mouse Corticosterone RIA kit #07-120103 (MP Biomedicals, Solon, OH).

**Glucose tolerance tests:** Beginning at 7 a.m., mice were fasted for 8 hours prior to initial blood collection from the tail vein for determination of baseline glucose levels. Mice were then weighed and given an intraperitoneal injection of 2 g/kg d-glucose (Sigma, St. Louis, MO) in distilled water. Subsequently, blood was collected from the tail vein and centrifuged for serum collection at multiple time points for up to 2 hours.

**Pancreatic histology:** Mice were anesthetized with avertin (2-2-2-tribromoethanol) and perfused with 0.9% NaCl followed by 4% paraformaldehyde. Histological analysis, quantification of the tissue area, and counting of cells were performed as described previously (Hebrok et al.). Immunohistochemical and immunofluorescence analyses were performed on paraffin sections as described previously (Kim et al.). The following primary antibodies were used: guinea pig anti-insulin diluted 1:500 (Linco Research, St. Charles, MO), rabbit anti-glucagon diluted 1:500 (Linco Research, St. Charles, MO). The following secondary antibodies were used for immunofluorescence: fluorescein isothiocyanate (FITC)-conjugated anti–guinea pig (Invitrogen, Carlsbad, CA), Cy3-conjugated anti-rabbit diluted 1:500 (Invitrogen, Carlsbad, CA).
**Glucose-stimulated insulin release:** Eleven week old mice were euthanized by cervical dislocation, and their pancreases were immediately inflated by injection of 3 ml of collagenase into the bile duct. The pancreases were removed and digested in collagenase at 37°C for 13-17 minutes followed by straining, centrifugation and washing. Islets were then isolated by ficoll gradient centrifugation, and 6 medium-sized islets were picked into 5 ml round-bottom tubes containing RPMI 1640 media with 0.1% BSA. Stock glucose was added to each tube to achieve concentrations of 75, 150, 300, and 600 mg/dl. Samples were then incubated with shaking for one hour at 37°C followed by brief centrifugation and removal of supernatant for measurement of insulin by radioimmunoassay as described above.

**Statistics:** The analysis of the behavioral monitoring variables (chow and water intake, body weight) used a mixed linear model (mixed procedure, SAS Institute Inc., Cary NC) with mouse, htr2c and lep genotypes, and age in months defined as class variables and with age as a repeated measure. The analysis of urine glucose levels was also carried out using a mixed linear model with mouse, htr2c and lep genotypes, age in months, and urine glucose defined as class variables and with age as a repeated measure. Analysis of variance (ANOVA, SPSS, Chicago IL) was used in comparing fasting serum values. The analysis of the glucose tolerance tests and glucose stimulated insulin release used repeated measures ANOVA (SPSS, Chicago IL).

**Results:**

**Regulation of intake:** To determine how mutations in the htr2c and lep genes interact in the regulation of ingestion, daily food and water intake were measured in WT (htr2c+/Y, Lep+/Lep+), 2C mutant (htr2c+/Y, Lep+/Lep+), OB mutant (htr2c+/Y, Lep+/Lep+), and OB2C
double mutant \((htr2c/Y, Lep^{ob}/Lep^{ob})\) mice over an age range of 2 to 8 months. These measurements revealed significant effects of age and of the \(htr2c\) and \(lep\) gene mutations on both food and water intake (Fig 1A and B). In addition, a significant synergistic interaction of the two mutations with age was observed for both food and water with the OB2C double mutant mice exhibiting a large non-additive increase in intake that declined slowly over age compared with the WT and single mutant mice (Fig 1A and B). This synergistic interaction was particularly striking for water intake yielding a significant interaction of the two mutations across all ages despite the decline in water intake to similar levels by 8 months of age. There was also a significant effect of age and of the \(htr2c\) and \(lep\) gene mutations on body weight, however in contrast to the effect of the combined mutations on intake, a significant interaction of the two mutations was not observed in the regulation of body weight (Fig. 1C).

**Glucose homeostasis:** To examine the effects of combined \(htr2c\) and \(lep\) mutations on glucose homeostasis, the urine of WT, 2C, OB, and OB2C mice from 2-8 months of age was tested for the presence of glucose. A significant effect of age and the \(lep\) gene mutation as well as an interaction of the \(htr2c\) and \(lep\) gene mutations was observed with the OB2C double mutants exhibiting elevated urine glucose relative to the OB mutants (Fig. 2) consistent with a worsening of the OB diabetes phenotype in the OB2C double mutant mice.

To further investigate the possibility of a synergistic impairment in glucose homeostasis in the OB2C double mutants, the fasting serum physiology of WT, 2C, OB and OB2C mice was investigated. There were significant effects of age, the \(htr2c\), and \(lep\) gene mutations, as well
as a significant interaction of the two mutations on glucose levels (Fig. 2A) with the OB2C mutants exhibiting a non-additive increase in glucose levels relative to the WT and single mutant mice. The elevated fasting glucose levels of the OB and OB2C mutant mice declined with age at a similar rate and by 10 months of age all groups exhibited similar levels of glucose (Fig. 3a). In contrast, insulin levels were only significantly altered by the lep gene mutation without a significant effect of age, the htr2c gene mutation, and without an interaction of the two mutations (Fig. 3b). In addition, examination of corticosterone levels did not reveal a significant interaction of the two mutations although significant effects of age and the lep gene mutation were observed (2X4 ANOVA: 2C p = 0.3, OB p < 0.0001, Age p < 0.0001, 2CxOB p = 0.8 2CxAge p = 0.07, OBxAge p < 0.0001, 2CxOBxAge p = 0.1). Glucagon was also tested in OB and OB2C mutant mice at 2 months of age only and was not significantly different.

Glucose tolerance was tested in WT, 2C, OB, and OB2C mice again revealing a significant effect of the htr2c and the lep gene mutations as well as a significant interaction of the two mutations (Fig. 4). In addition, there was a significant effect of time and interaction of the lep gene mutation with time revealed by the failure of the OB and OB2C mutant mice to return to baseline compared with the WT and 2C mutant mice. In contrast, the interaction of time with the two mutations was not significant with a similar time course of glucose levels in the OB and OB2C mutant mice indicating that the significant interaction of the two mutations results from the altered baseline glucose levels (Fig. 4).
Pancreatic physiology: Because the OB2C double mutants failed to increase insulin levels above the levels exhibited by the OB mutants despite the presence of significantly elevated glucose levels, pancreatic tissue from OB and OB2C double mutants was examined for morphological abnormalities at 11 weeks of age when the mice exhibited the largest difference in glucose levels. No significant difference in islet number or total islet area was observed (number, mean±se: OB 25±4, OB2C 19±3, p = 0.3; normalized area, mean±se: OB 1±0.5, OB2C 0.7±0.2, p = 0.4). To determine if the OB and OB2C mutant mice differed in their ability to produce insulin, a glucose-stimulated insulin release study was also performed. Consistent with the lack of difference in pancreatic morphology, there was no significant difference in glucose-stimulated islet insulin production between the two groups (Fig. 5).
Figure 1a. Average daily chow intake over time in WT (black), 2C (red), OB (blue), and OB2C (purple) mice. There were significant effects of age and of the *htr2c* and *lep* mutations and a synergistic interaction of the two mutations with one another with age. Mixed model: 2C geno p < 0.0001, OB geno p < 0.0001, Age p = 0.0001, 2CxB p = 0.8, 2CxAge p = 0.5, OBxAge p < 0.0001, 2CxBxAge p = 0.004.
Figure 1b. Average daily water intake over time in WT (black), 2C (red), OB (blue), and OB2C (purple) mice. There were significant effects of age and of the htr2c and lep mutations and a synergistic interaction of the two mutations with one another with age. Mixed model: 2C $p < 0.0001$, OB $p < 0.0001$, Age $p < 0.0001$, 2CxOB $p < 0.0001$, 2CxAge $p = 0.0006$, OBxAge $p < 0.0001$, 2CxOBxAge $p < 0.0001$. 

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Figure 1c. Average body weight over time in WT (black), 2C (red), OB (blue), and OB2C (purple) mice. There were significant effects of age and of the *htr2c* and *lep* mutations, but not a significant interaction of the two mutations with each other with age. Mixed model: 2C p = 0.007, OB p < 0.0001, Age p < 0.0001, 2CxOB p = 0.8, 2CxAge p = 0.1, OBxAge p < 0.0001, 2CxCBxAge p = 0.8.
Figure 2. Urine glucose over time in WT (black), 2C (red), OB (blue), and OB2C (purple) mice. There was a significant effect of the \textit{lep} mutation, and an interaction of the \textit{lep} and \textit{htr2c} mutations with one another with age. Mixed model: 2C $p = 0.051$, OB $p < 0.0001$, Age $p < 0.0001$, 2CxOB $p = 0.04$, 2CxAge $p = 0.99$, OBxAge $p < 0.0001$, 2CxBxAge $p = 0.99$. 
Figure 3a. Fasting serum glucose over time in WT (black), 2C (red), OB (blue), and OB2C (purple) mice. There was a significant effect of the *lep* and *htr2c* mutations and of the two with one another. There was also a significant effect of age, but only an interaction of age with *lep* mutation. 2X4 ANOVA: 2C p = 0.001, OB p < 0.0001, Age p < 0.0001, 2CxB p = 0.007, 2CxAge p = 0.5, OBxAge p < 0.0001, 2CxBxAge p = 0.1
3b.

Figure 3b. Fasting serum insulin over time in WT (black), 2C (red), OB (blue), and OB2C (purple) mice. There was a significant effect of *lep* mutation only. 2X4 ANOVA: 2C p = 0.5, OB p < 0.0001, Age p = 0.7, 2CxOB p =0.3, 2CxAge p = 0.99, OBxAge p = 0.6, 2CxOBxAge p = 0.99.
Figure 4. Glucose tolerance test in 2-month old OB (blue), and OB2C (purple) mice. There was a significant interaction of *lep* and *htr2c* mutations. 2X6 ANOVA: 2C p = 0.008, OB p < 0.0001, Time p <0.0001, 2CxB p =0.03, 2CxTime p = 0.7, OBxTime p < 0.0001, 2CxBxTime p = 0.9.
Figure 5. Glucose stimulated insulin release of isolated pancreatic islets from 2-month old OB (blue), and OB2C (purple) mice. There was a significant effect of glucose dose, but not of htr2c mutation. 1X4 ANOVA: 2C p = 0.3, Glucose p < 0.0001, 2CxGlucose p = 0.9.
Chapter 3: Pharmacologic effects of serotonin on glucose homeostasis

Introduction:

Central serotonin (5-hydroxytryptamine; 5-HT) systems have been long implicated in the regulation of feeding and energy balance, as highlighted by the clinical use of the serotonin-releasing agent fenfluramine as an appetite suppressant (Davis and Faulds). More recently, a growing concern regarding the diabetogenic side effects of atypical antipsychotic drugs has raised questions regarding the importance of serotonergic influences on glucose homeostasis (Ramaswamy et al.). These drugs, which are commonly used to treat psychotic disorders such as schizophrenia, have nonspecific 5-HT receptor antagonist properties. Conversely, nonspecific 5-HT receptor agonist compounds have been shown to improve glucose tolerance. In particular, fenfluramine has been shown to improve glucose tolerance and insulin action (Scheen et al.), although the mechanisms underlying this effect have not been fully determined.

Of the at least 14 distinct subtypes of 5-HT receptors, the 5HT2CR has been most strongly implicated in serotonergic effects on energy balance. Expression of the 5HT2CR is restricted to the CNS, where it is found in both hypothalamic and extrahypothalamic regions implicated in energy balance regulation (Hoffman and Mezey, Wright et al.). Mice with targeted null mutations of the htr2c gene are hyperphagic and develop mild obesity, hyperinsulinemia, and impaired glucose tolerance late in life (Nonogaki et al.). These deficits are accelerated and exaggerated in htr2c mutant mice fed a high-fat diet (Nonogaki et al.). However, in the
initial characterization of these mice, it was not clear whether the effects of \textit{htr2c} mutation on glucose homeostasis were secondary to the obesity phenotype seen in 2C mutant mice.

In order to investigate the effects of acute manipulation of the serotonin system on glucose homeostasis in young, lean mice, we looked at the effects of two serotonergic pharmacologic agents on glucose tolerance in WT and 2C mutant mice.

\textbf{Materials and Methods:}

\textbf{Animals:} Experimental mice were generated by breeding female mice heterozygous for the \textit{htr2c} mutation with male C57BL6/J mice obtained from The Jackson Laboratory. Genotyping for the \textit{htr2c} gene mutation was performed by PCR analysis using a primer in the neomycin resistance gene (NeoD: 5’-CACCTTGCTCCTGCGAGAAA-3’) and flanking primers in the \textit{htr2c} gene (2Cfor: 5’-GCTCAGAATTCTGGAAATGTGT-3’; 2Crev: 5’-CGGACTGCTAAATTGGGTTC-3’) to produce a 114 bp band for WT mice and a 600bp band for 2C mutant mice. All animals were housed at room temperature (20-24°C) on a 12-hr light/dark cycle (lights on at 7 am) with free access to water and a standard chow diet (PicoLab Mouse Diet 20 5150, Purina Mills, Richmond, IN) except were indicated below. Experiments were performed in accordance with the guidelines of the National Institutes of Health \textit{Guide for Care and Use of Laboratory Animals} and the University of California Institutional Animal Care and Use Committee.
**Pharmaceutical Agents:** Fenfluramine and SB242084 were obtained from Sigma (St. Louis, MO). Fenfluramine was dissolved in 0.9% NaCl. SB242084 was dissolved in 0.8% beta-cyclodextrin (Sigma, St. Louis, MO) in 0.9% NaCl. All drugs were injected at a volume of 0.1 liters per gram of body weight.

**Glucose tolerance tests:** Beginning at 7 a.m., mice were fasted for 8 hours prior to initial blood collection from the tail vein for determination of baseline glucose levels. Mice were then weighed and given an intraperitoneal injection of drug or vehicle followed by an intraperitoneal injection of 2 g/kg d-glucose (Sigma, St. Louis, MO) in distilled water. Subsequently, blood was collected from the tail vein and centrifuged for serum collection at multiple time points for up to 2 hours. Serum glucose was measured using a Trinder glucose oxidase kit (Mega Diagnostics, Los Angeles, CA). These studies used a cross-over design with each mouse receiving both vehicle and drug on separate test days in a random order, with a one week interval between tests.

**Statistics:** The analysis of the glucose tolerance tests used repeated measures ANOVA (SPSS, Chicago IL. Each mouse received vehicle and drug treatment in a cross-over design, and the difference between the glucose levels for drug treatment minus vehicle treatment at each time point was used as the repeated measures variable across time after the glucose load.
Results:

**Fenfluramine:** We tested the ability of the non-specific serotonin releaser and reuptake inhibitor fenfluramine to improve glucose tolerance in young WT and 2C mutant mice prior to a divergence in body weight. Both 2C mutant mice and their WT littermates were injected with either vehicle or 6 mg/kg fenfluramine and then administered a glucose tolerance test. The WT and 2C mutant mice differed in their response to fenfluramine. In comparison with the fenfluramine- and vehicle-treated 2C mutant mice, fenfluramine-treated WT mice exhibited a more rapid decline in glucose levels than when treated with vehicle (Fig. 6A), suggesting that the effects of fenfluramine on glucose homeostasis are at least in part mediated by the 5-HT2CR.

**SB242084:** SB242084, a specific 5HT2CR antagonist, also produced a differential glucose tolerance response in WT and 2C mutant mice. Recovery from glucose challenge was slower in WT mice treated with SB242084 than when treated with vehicle in comparison with 2C mutant mice treated with SB242084 and vehicle (Fig. 6B), suggesting that SB242084's effects on glucose homeostasis are also at least in part mediated by the 5-HT2CR. However, there is a trend for a drug effect in both genotypes, suggesting that there may also be 5-HT2CR-independent mechanisms involved as well.

The differential response of the WT and 2C mutant mice to fenfluramine and SB242084 when the mice are not diverged in body weight (T tests, mean±se: fenfluramine WT 22.0±0.7 2C 23.6±0.6 p = 0.09; SB242084 WT 23.6±0.6 2C 22.1±0.7 p = 0.1) indicates that
the 5HT2CR has a role in regulation of glucose homeostasis that is independent of its role in the regulation of food intake and body weight. This is the first time that the 5-HT2CR has been implicated in the regulation of glucose homeostasis in lean mice.
Figure 6a. Glucose tolerance test with fenfluramine and vehicle in 3-month-old WT and 2C mice. There was a significant interaction of \textit{htr2c} mutation with time. 1X4 ANOVA: 2C p = 0.1, Time p < 0.0001, 2C\times Time p = 0.01
Figure 6b. Glucose tolerance test with SB242084 and vehicle in 3-month-old WT and 2C mice. There was a significant interaction of htr2c mutation with time. 1X6 ANOVA: 2C p = 0.1, Time p = 0.07, 2C\times Time p 0.001.
Chapter 4: Conclusions

In these studies, we have shown that mutation of the *ob* and *htr2c* genes results in a synergistic impairment of glucose homeostasis, accompanied by synergistic increases in food and water intake in OB2C mice, indicating that leptin and the 5HT2CR interact to regulate these processes. The differences in glucose levels and food and water intake seen between OB and OB2C were not attributable to differences in adiposity between the two genotypes, suggesting a body-weight independent role of the 5HT2CR in regulation of food intake and glucose homeostasis. We have also demonstrated for the first time that the 5HT2CR can have effects on glucose homeostasis in lean animals in our pharmacological studies, which showed that 2C mutant mice have a blunted glucose tolerance response to serotonergic drugs.

Several earlier functional and neuroanatomical studies had suggested a link between the leptin and serotonin systems. OB mutant mice have been shown to have decreased expression of serotonin transporter mRNA in the dorsal raphé (Collin et al.), and leptin treatment of OB mice increased the concentration of serotonin in hypothalamic and brainstem neurons (Harris et al.). More recently, the mouse model of Wang and Chehab showed that null mutation of the *htr2c* enhances diet-induced obesity in mice overexpressing *lep*, a model of leptin resistance, although the role of an interaction of the two genes was not investigated in this model. Both the 5HT2CR and leptin receptors are expressed in hypothalamic regions implicated in energy balance and glucose homeostasis, including the Arc, the ventromedial hypothalamus, the dorsomedial hypothalamus, the paraventricular hypothalamus, and the
lateral hypothalamus (Elmquist et al. 1998, Hoffman and Mezey, Schwartz et al., Wright et al.). Co-expression of the 5HT2CR and leptin receptors has also been shown to occur: leptin receptors are expressed on serotonergic neurons in the raphé nuclei (Collin et al., Finn et al., Hay-Schmidt et al.), and these serotonergic neurons have been shown to accumulate leptin (Férrandez-Galaz et al.). The 5HT2CR has also been found to be expressed on leptin-sensitive POMC neurons in the Arc (Heisler et al.), a brain region crucial to leptin's regulation of glucose homeostasis (Coppari et al.).

At two months of age, OB2C double mutant mice have dramatically increased food (70% over OB) and water (197% over OB) intake (fig. 1a-b). The increased water intake seen in the double mutants raised the possibility of a worsening of glucose homeostasis in these mice. OB2C double mutants demonstrate elevated urine glucose levels compared to their OB, WT and 2C littermates (fig. 2), as well as higher fasting glucose (fig. 3a) and impaired glucose tolerance (fig. 4), demonstrating a synergistic interaction of the lep and htr2c genes on glucose homeostasis.

The impaired glucose homeostasis phenotype of both OB and OB2C mice ameliorates as the mice age (fig. 2-3). This effect had previously been demonstrated in OB mice on the C57/Bl6 background (Coleman and Hummel), however, the mechanism is not clear. Interestingly, serum insulin concentrations do not appear to change significantly with age (fig. 3b), suggesting that the euglycemia seen in older OB mice is a result of improved insulin sensitivity, and not a result of increased insulin production. In parallel with the improvement of glucose levels with age, both OB and OB2C mice show a normalization of
food and water intake (fig. 1a-b). Given the well-established effects of elevated blood glucose on osmotic regulation, it is likely that the normalization of water intake is secondary to the animals' improvement in serum glucose levels. Hyperphagia is also linked to diabetes (Plum et al, Sipols et al.), and the normalization of feeding in these mice may also be secondary to the improvement in glucose homeostasis.

To better understand the impaired glucose homeostasis in OB2C mice, we focused our attention on 2-month-old mice, the age at which their diabetes is maximal. We did not see any differences between 2-month-old OB and OB2C mice in pancreas morphology or the ability of islets to respond in vitro to glucose (fig. 5), suggesting that the OB2Cs' enhanced diabetes is not due to a defect in pancreatic development or intrinsic islet cell function. However, this does not rule out the possibility that central regulation of pancreatic function may differ in OB and OB2C mice. While OB2C mice have higher fasting serum glucose than OB mice, the two groups do not differ in their fasting serum insulin levels, suggesting that altered insulin sensitivity in OB2C mice may contribute to their enhanced diabetes.

While null mutation of the htr2c does not result in impairment of glucose homeostasis in young, lean mice, Nonogaki et al. showed that obese 2C mutant mice do develop impaired glucose homeostasis, however, in that study it was unclear whether the mutants' glucose homeostasis was directly influenced by the htr2c mutation or whether it was simply a secondary consequence of their obesity, which is in and of itself a major risk factor for type II diabetes (Kahn et al.). Our pharmacologic studies with fenfluramine and SB242084, showing that these drugs' effects on glucose tolerance are blunted in 2C mutant mice (fig. 6)
indicate that manipulation of the 5HT2CR can play a role in glucose regulation in the absence of obesity. This suggests that young, lean 2C mutant mice, which have normal glucose homeostasis, are somehow able to compensate for their chronic lack of 5HT2CR. This compensation appears to fail in the face of diet-induced obesity, which is accompanied by leptin resistance (Nonogaki et al.) or when crossed onto the OB background (figs. 2-4). These data suggest that the mechanism by which 2C mutant mice maintain normal glucose homeostasis may be leptin-dependent, and that impairment of the leptin system may be required for the 2C mutant glucose homeostasis phenotype to be revealed.

In summary, our findings indicate that serotonin and leptin interact synergistically to regulate glucose homeostasis. These effects appear to be independent of body weight, however, disruption of the leptin system can uncover the effects of 5HT2CR mutation on glucose homeostasis. These findings may have important clinical implications, as diabetes and obesity resulting from atypical antipsychotic drug treatment are a significant obstacle in the treatment of patients with schizophrenia and bipolar disorder. The large number of studies linking atypical antipsychotics to diabetes and obesity has led the FDA to require labeling of these drugs disclosing these risks and suggesting careful monitoring of blood glucose for all patients taking the drugs (Rosack). Atypical antipsychotic drugs are high-affinity antagonists of the 5HT2CR (Di Matteo et al.), and 5HT2CR occupancy has been found to be positively correlated with the obesogenic and diabetogenic effects of these drugs (Matsui-Sakata et al.). Our finding that the 5HT2CR interacts with the leptin system to regulate glucose homeostasis may aid in the development of improved atypical
antipsychotics or strategies for mitigating their metabolic side effects, and further establishes the serotonin system as a possible target for the treatment of diabetes itself.
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