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Metabolic characterization of a mouse deficient in all known leptin receptor isoforms

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Abstract

We have characterized a newly generated mouse model of obesity, a mouse strain deficient in all five previously described leptin receptor isoforms. These transgenic mice, named the db_{333}/db_{333} mice, were identified from an ENU mutagenesis screen and carry a point mutation in the seventh exon of the *db* gene encoding the leptin receptor, resulting in a premature stop codon (Y_{333}Stop) and gene product that lacks STAT signaling domains. db_{333}/db_{333} mice have a morbidly obese phenotype, with body weights diverging from wild-type as early as 4 weeks of age ($P<0.05$). To determine the contribution of the short isoforms of the leptin receptor in this metabolic phenotype we performed an extensive metabolic characterization of the db_{333}/db_{333} mouse in relation to the well characterized *db/db* mouse lacking only the long form of the leptin receptor. db_{333}/db_{333} mice have similar endocrine and metabolic parameters as previously described in other leptin receptor transgenic mice including *db/db* mice that lack only the long isoform of the leptin receptor. However, db_{333}/db_{333} mice show a subtle trend towards higher body weight, insulin levels, lower oxygen production, carbon dioxide production, respiratory efficiency ratio and temperature than *db/db* mice suggesting the short isoforms may play an additional role in energy homeostasis.

Keywords

leptin; leptin receptor; Ob-Rb; obesity; *db/db*; db_{333}/db_{333}; diabetes; insulin resistance

Introduction

Leptin signaling is an important mediator of feeding behavior, energy homeostasis and normal immune and reproductive function (Halaas, Gajiwala et al. 1995; Pelleymounter, Cullen et al. 1995; Chehab, Lim et al. 1996; Schwartz, Baskin et al. 1996; Loffreda, Yang et al. 1998; Lord, Matarese et al. 1998). Leptin is a 16 kDa cytokine, secreted by adipocytes that is encoded by the *ob* gene that acts at the hypothalamic level through the leptin receptor (Ob-R) encoded by the *db* gene. Leptin acts as a feedback signal reflecting the nutritional status of the periphery to suppress feeding and to permit neuroendocrine functions (Ahima, Prabakaran et al. 1996; Ahima, Dushay et al. 1997; Heiman, Ahima et al. 1997; Yu, Kimura et al. 1997). Conversely negative energy balance decreases leptin levels, increasing the drive to feed triggering

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neuroendocrine responses that limit energy expenditure (Ahima, Prabakaran et al. 1996; Bates and Myers 2003).

Leptin deficient (*ob/ob*) and leptin receptor deficient (*db/db*) mouse models have provided many insights into leptin signaling. Deficiency in leptin signaling causes a disruption in energy balance resulting in obesity from hyperphagia and decreased energy expenditure followed by the spontaneous development of insulin resistance (Ingalls, Dickie et al. 1950; Zhang, Proenca et al. 1994; Pelleymounter, Cullen et al. 1995). (Frederich, Hamann et al. 1995; Chua 1997). A similar phenotype is observed in humans with very rare mutations in the leptin or leptin receptor genes (Montague, Farooqi et al. 1997; Farooqi, Matarese et al. 2002; Farooqi and O'Rahilly 2006). With the exception of humans with this rare genetic leptin deficiency circulating leptin levels are positively correlated with adiposity (Frederich, Hamann et al. 1995). Obese individuals have elevated circulating leptin levels, but leptin fails to mediate weight loss, suggesting that most human obesity is a form of leptin resistance.

The leptin receptor (Ob-R) belongs to the cytokine receptor class I superfamily (Tartaglia 1997) and in the mouse is alternatively spliced into five known isoforms: Ob-Ra, Ob-Rb, Ob-Rc, Ob-Rd, and Ob-Re (Lee, Proenca et al. 1996). The longest isoform (Ob-Rb) is expressed at a high level in the hypothalamus and at a low level in other tissues including testes, adipose tissues, pancreatic β cells, heart, lung and lymph nodes (Ghilardi, Ziegler et al. 1996; Lee, Proenca et al. 1996; Emilsson, Liu et al. 1997; Fei, Okano et al. 1997; Lollmann, Gruninger et al. 1997; Spanswick, Smith et al. 1997). The Ob-Rb form is highly conserved among species and possesses an intracellular cytoplasmic domain of approximately 300 residues (Chen, Charlat et al. 1996; Lee, Proenca et al. 1996; Chua, Koutras et al. 1997; Tartaglia 1997). Ob-Rb, like other members of the cytokine receptor family mediates tyrosine kinase signaling by means of associated Janus kinase (Jak)/signal transducers and activators of transcription (STAT) pathway. Ob-Ra, Ob-Rc, Ob-Rd and Ob-Re are considered ‘short isoforms’, since they lack the long cytoplasmic domain. Ob-Ra-d contain identical extracellular leptin binding and transmembrane domains as well as the same first 29 intracellular amino acids and diverge in sequence secondary to alternative splicing of 3’ exons (Chua, Chung et al. 1996). The short isoform Ob-Ra is ubiquitously expressed (Lollmann, Gruninger et al. 1997) with particularly high expression in the choroid plexus and microvessels in the CNS and has been suggested to play a role in the transport of leptin across the blood brain barrier (Golden, Maccagnan et al. 1997; Tartaglia 1997; Wu-Peng, Chua et al. 1997). Ob-Re is a secreted isoform (Tartaglia 1997) that forms a complex with circulating leptin suggested to act as a buffering system to regulate leptin availability (Lollmann, Gruninger et al. 1997; Ge, Huang et al. 2002). The other functions of the short isoforms of the leptin receptor have yet to be determined.

The *db/db* mouse has a mutation in the signaling C terminus of the leptin receptor gene that results in a non-functional truncated long form of Ob-Rb (Hummel, Dickie et al. 1966; Chen, Charlat et al. 1996; Lee, Proenca et al. 1996). Central or peripheral administration of leptin in *db/db* mice has no effect on food intake or body weight (Halaas, Gajiwala et al. 1995). Since *db/db* mice express all the other short leptin receptor isoforms besides Ob-Rb, yet still develop the obese phenotype, it has been hypothesized that the weight controlling effects of leptin in mice are mediated by signaling through the long leptin receptor isoform, Ob-Rb. Furthermore, leptin activation of STAT3 occurs only in wild-type (WT) and *ob/ob* mice but not in *db/db* mice (Vaisse, Halaas et al. 1996). However, a recent study on leptin signaling showed that leptin stimulation of interleukin 1 beta (IL-1β) expression in the brain still occurs in *db/db* mice (Hosoi, Okuma et al. 2002). The possibility that leptin has effects in mice lacking the Ob-Rb isoform indicates that the short leptin receptor isoforms could play a yet undetermined role in leptin signaling.

In this present study we have characterized a transgenic mouse model of obesity that lacks all known isoforms of the leptin receptor on a C57BL/6 background. These db_{333}/db_{333} mice, were isolated from an ENU mutagenesis screen and have a T to A transversion in the 7^{th} coding exon of the leptin receptor, causing a premature stop codon: Y_{333}stop. We performed an extensive endocrine and metabolic characterization of this transgenic mouse model of obesity in relation to the well characterized *db/db* mouse lacking just the long form of the leptin receptor.

Experimental Procedures

Animals and surgery

Genetically obese C57BL/6 mice were generated in the laboratory of Dr. Bruce Beutler (The Scripps Research Institute, La Jolla, CA, USA) using ENU mutagenesis (Brown and Peters 1996). Positional cloning was used to determine the location of the point mutation in the leptin receptor gene. Both male db_{333}/db_{333} and male WT littermates were fed *ad libitum* with mouse breeder diet composed of 11% fat, 17% Protein, 3.5% fiber (S-2335 Mouse Breeder, gross energy kcal 4.39kcal/g). $Lepr^{db}/Lepr^{db}$ on a C57BL/6 background (stock number 000697) were obtained from Jackson laboratories. Groups of 5 mice of each genotype were used in all experiments at an age of 6–8 months unless otherwise stated.

Mice were anesthetized with isofluorane (induction 3–5%, maintenance 1.0%) and implanted with transmitter devices (TA10TA-F20; Data Sciences, Arden Hills, MN) into the peritoneal cavity. Transmitter devices contain sensors that measure core body temperature (CBT) and locomotor activity. Mice were allowed to recover for 1 week and then submitted to a second surgery. After anesthesia (induction 3–5%, maintenance 0.9– 1.5%), mice were placed into a stereotaxic apparatus. The skull was exposed, and holes were drilled to accommodate stereotaxic placement of a guide cannula (26 Ga, 1.0-cm length) aiming at the lateral ventricle (anterior–posterior from bregma, 0.3 mm; lateral, 1.0 mm; and ventral, 1.7 mm).

Mice with *ad libitum* access to food and water were monitored using a computer-controlled, open-circuit system (Oxymax System) that is part of an integrated Comprehensive Lab Animal Monitoring System (CLAMS; Columbus Instruments, Columbus, OH). Mice were singly-housed in clear respiratory chambers (20 × 10 × 12.5 cm) with a stainless steel elevated wire floor. Each of these chambers is equipped with a sipper tube delivering water and a food tray connected to a balance. Room air was passed through chambers at a flow rate of ~0.5 L/min. Exhaust air from each chamber was sampled at 20-min intervals for 1 min. Sample air was sequentially passed through O_2 and CO_2 sensors (Columbus Instruments) for determination of O_2 and CO_2 content, from which measures of oxygen consumption (VO_2) and carbon dioxide production (VCO_2) are estimated. Outdoor air reference values are sampled after every 4 measurements. Gas sensors are calibrated prior to the onset of experiments with primary gas standards containing known concentrations of O_2, CO_2, and N2 (Airgas Puritan Medical, Ontario, CA). Respiratory exchange ratio (RER) was calculated as the ratio of carbon dioxide production (VCO_2) to oxygen consumption (VO_2). VO_2 and VCO_2 are corrected for estimated effective metabolic mass per Kleiber's power function. Receiver plates (RPC-1; Data Sciences) positioned inside the chambers continuously monitored core body temperature and locomotor activity by means of a fully automated data acquisition system (Dataquest A.R.T., Data Sciences, Inc.) Mice were acclimated to the respiratory chambers for 4 days before the onset of study. Data are recorded under ambient room temperature clamped at 25°C, beginning from the onset of the dark cycle.

Genotyping

Genotyping of mice was carried out by PCR with 25 ng of genomic DNA isolated from tail clippings in 20 μl reaction mixtures containing 1× PCR buffer-Mg (Invitrogen), 0.2mM each of a mixture of deoxynucleoside triphosphates (Invitrogen), 1.5mM $MgCl_2$ (Invitrogen), 1U of Taq DNA Polymerase (Invitrogen) and 0.5μM of each primer db_{333}/db_{333}-F 5'-GCTGGAAGCCTGTCGTACTCTTCA-3' and db_{333}/db_{333}-R 5'-TACACTGCGTCATAGGTAAACTTCCCTC -3'. After an initial denaturation at 94°C for 3 min, 35 cycles of PCR at 94°C for 45 s, 55°C for 30 s, and 72°C for 1min were performed, followed by a final extension at 72°C for 10 min. The PCR products were purified using a QIAquick PCR purification kit (Qiagen) and sequenced to identify homozygous mutant db_{333}/db_{333} mice.

Analysis of fat depots and organ weights

Mice were anesthesized with 5% isoflurane and subsequently sacrificed. Wet fat pads (omental, mesenteric, subcutaneous and brown adipose tissue) and the liver were weighed immediately.

Measurement of food intake in response to leptin

Mice were given leptin (Peprotech, Rocky Hill, New Jersey) injections (4μg/uL, intracerebroventricular, icv) at 3:00 pm, prior to the onset of the dark cycle at 6:00 pm. An internal cannula (33 Ga, 1.1cm length) connected to plastic tubing and a microsyringe (10 μl) was used for direct delivery into the lateral ventricle. A volume of 1.0 μl (leptin or heat inactivated leptin, 98°C for 30 mins) was injected in a period of 5 min to allow for diffusion. Food intake was measured by weighing the amount of food eaten and normalized to initial body weight using the Kleiber's function of 0.75.

Measurement of endocrine parameters

Mice were anesthetized with 5% isoflurane and blood samples were collected via retro-orbital bleeds with EDTA coated capillary tubes (Drummond, Broomall, PA, USA) into EDTA coated vials (Microvette, Germany). Samples were spun down at 1000 × g for 10 min and plasma was stored at −80 °C until analysis. Circulating levels of insulin, leptin and IL-6 were determined using the mouse serum adipokine kit (Millipore, St. Charles, MO, USA). Circulating levels of interleukin-1 receptor antagonist (IL-1Ra) were determined using the Immunoassay (R&D systems, Minneapolis, MN). All endocrine measurements were performed on plasma samples from mice at 6 months of age.

Insulin tolerance test

The glucose reducing effect of insulin injection was assessed in non fasted mice. Baseline glucose levels are measured by withdrawing ~0.6 uL of blood from the tail from unanesthesized mice before a load of human insulin was administered (1 unit/kg, i.p.; Sigma–Aldrich, St. Louis, MO). Further samples were collected 15, 30, 60, 90 and 120 mins after the insulin challenge. Blood glucose levels (in mg per deciliter) were determined by a glucometer (Glucometer, Rite Aid).

Glucose tolerance test

Mice were fasted for 16hrs overnight and injected intraperitoneally (i.p) with glucose (D-glucose, anhydrous; Sigma–Aldrich, St. Louis, MO) (1.5 mg/g body weight) in sterile water. Blood samples were taken before the glucose administration and then 15, 30, 60, 90 and 120 min after injection). Blood glucose levels were determined by a glucometer (Glucometer, Rite Aid).

Data Analysis

The significance of the difference in values between db_{333}/db_{333}, *db/db* and WT mice was evaluated using a one way ANOVA followed by Tukey's Honestly Significant Difference (HSD) test. For comparison of just db_{333}/db_{333} and WT mice a two tailed independent T test was applied. The statistical significance was at the $P<0.05$ level. Data are expressed as means ± SE.

RESULTS

db_{333}/db_{333} mice lack all leptin receptor isoforms due to a premature stop codon

db_{333}/db_{33} mice were identified from an ENU mutagenesis screen and a positional cloning strategy was used to determine the location of the point mutation in the leptin receptor gene (Fig. 1A). The specific mutation is a T to A transversion in the 7th coding exon, causing a premature stop codon: Y_{333}Stop (TAT→TAA). This premature stop codon causes biosynthesis of a truncated leptin receptor. Since the stop codon is upstream of the leptin receptor isoform splice sites, the mice lack earlier described long and short isoforms of the leptin receptor, see Fig.1B.

db_{333}/db_{333} mice are obese and hyperphagic and exhibit altered endocrine and inflammatory parameters associated with obesity

db_{333}/db_{333} mice develop an extreme obese phenotype on normal chow (Fig. 2A). At 6 mths of age db_{333}/db_{333} mice have significantly higher body weights compared with WT littermates (db_{333}/db_{333} 65.0 ± 5.9g, WT 36.25 ± 1.70g, $P<0.05$) (Fig. 2B). db_{333}/db_{333} mice have significantly increased quantities of omental, mesenteric and subcutaneous fat, brown adipose tissue (BAT) and higher liver mass than WT littermates (Fig. 2C). db_{333}/db_{333} mice are hyperphagic and consume significantly more food than WT littermates throughout the 24hr monitoring period (Fig 2D), with the most significant increase in food intake in db_{333}/db_{333} mice in the dark cycle (db_{333}/db_{333} 3.95g ± 0.12, WT 2.24 ± 0.05g, $P<0.05$). (Fig. 2E). db_{333}/db_{333} mice had significantly elevated circulating levels of leptin, insulin and IL-1Ra compared with WT littermates at 6 mths of age, (Table 1). db_{333}/db_{333} also display significantly elevated insulin levels and show a trend towards higher IL-1Ra levels when compared with *db/db* mice. Levels of the pro-inflammatory cytokine interleukin 6 (IL-6) was also elevated in db_{333}/db_{333} and *db/db* mice relative to levels in WT, lean littermates.

db_{333}/db_{333} mice display a trend of consistently higher body weights than *db/db* mice

Both db_{333}/db_{333} and *db/db* mice have significantly greater body weights than WT littermates from as early as 4 weeks of age and throughout this monitoring period of 24wks (db_{333}/db_{333} 65.0 ± 5.9g; *db/db* 60.25 ± 1.38g, WT 36.25 ± 1.70g at 24wks), (Fig. 3). db_{333}/db_{333} mice display consistently higher body weights than *db/db* mice from 8 wks to 24wks of age but this trend only reached statistical significance at one time point (12wk).

db_{333}/db_{333} and *db/db* mice show decreased insulin sensitivity & impaired glucose tolerance relative to lean mice

Intraperitoneal administration of insulin (1 unit per kg of body weight) revealed that both db_{333}/db_{333} and *db/db* mice have an impaired glucose disposal ability relative to the hypoglycemic response to insulin observed in the WT littermates (Fig 4A). The ability of insulin to acutely stimulate glucose mobilization was significantly blunted at 30 mins in db_{333}/db_{333} and *db/db* mice relative to WT littermates. Both db_{333}/db_{333} and *db/db* mice exhibit impaired glucose disposal when challenged with glucose (1.5 mg of glucose per gram of body weight), Fig. 4B. Significant differences in glucose disposal between db_{333}/db_{333} mice and WT littermates were observed at 15 mins and 60 mins after glucose injection.

db$_{333}$/*db*$_{333}$ mice have a lower core body temperature and decreased locomotor activity relative to WT littermates

db$_{333}$/*db*$_{333}$ and *db/db* mice display a significantly lower core body temperature, as determined by radiotelemetry, relative to lean mice (Fig 5A). Average core body temperature over both the 12hr light or dark cycle in *db*$_{333}$/*db*$_{333}$ and *db/db* mice is significantly lower when compared with lean mice (Fig 5B). *db*$_{333}$/*db*$_{333}$ and *db/db* mice display decreased locomotor activity relative to WT mice although there is a modest difference in activity between *db*$_{333}$/*db*$_{333}$ and *db/db* mice towards the end of the dark cycle (Fig 5C). During the dark cycle *db/db* and *db*$_{333}$/*db*$_{333}$ mice show significantly reduced activity relative to WT littermates ($p < 0.05$) Fig 5D.

Functional loss of the leptin receptor in *db*$_{333}$/*db*$_{333}$ mice

The functional loss of leptin signaling in *db*$_{333}$/*db*$_{333}$ and *db/db* mice was determined by comparison with leptin induced reduction in food intake in WT littermates. Injection of leptin (icv 4 μg/μL) caused a significant reduction in food intake in WT mice while no significant appetite suppression was observed in *db*$_{333}$/*db*$_{333}$ or *db/db* mice (Fig 6).

db$_{333}$/*db*$_{333}$ and *db/db* mice showed significantly lower oxygen consumption, carbon dioxide production and respiratory efficiency ratio compared with lean mice

Measurements of O_2 production (VO_2) and CO2 production (VCO_2) were acquired from each metabolic chamber every 20mins and sent to a computer which collects and processes the data continuously over a 24hr monitoring period. Both *db/db* and *db*$_{333}$/*db*$_{333}$ mice displayed significantly lower oxygen consumption (Fig. 7A) and carbon dioxide production (Fig. 7B) at all time points throughout both the light and dark cycle relative to lean mice ($P<0.05$). Respiratory Efficiency Ratio (RER) (VCO_2/ VO_2) in both *db*$_{333}$/*db*$_{333}$ and *db/db* showed a trend of lower RER relative to WT mice that is most pronounced in the dark cycle indicating a trend to the use of lipids as energy substrates in these obese mutants. *db*$_{333}$/*db*$_{333}$ mice also show a consistently lower RER relative to *db/db* although this trend was not deemed statistically significant (Fig 7C).

DISCUSSION

We have characterized a newly generated mouse model of obesity, the *db*$_{333}$/*db*$_{333}$ mice, deficient in all known leptin receptor isoforms in mice. *db*$_{333}$/*db*$_{333}$ mice have a point mutation in the seventh exon of the *db* gene encoding the leptin receptor resulting in a premature stop codon (Y_{333}Stop) upstream of all the known splice sites. *db*$_{333}$/*db*$_{333}$ mice have a morbidly obese phenotype, with body weights diverging from WT as early as 4 weeks of age. The obese phenotype of the *db*$_{333}$/*db*$_{333}$ mice is caused not only by hyperphagia but also by disrupted neuroendocrine regulation of energy expenditure. *db*$_{333}$/*db*$_{333}$ mice had significantly elevated circulating levels of leptin, insulin and interleukin-1 receptor antagonist as also observed in human diabetics and mouse models of obesity (Ludvik, Kautzky-Willer et al. 1997; Luheshi, Gardner et al. 1999; Meier, Bobbioni et al. 2002; Juge-Aubry, Somm et al. 2003; Somm, Henrichot et al. 2005; Somm, Cettour-Rose et al. 2006).

This study describes an extensive *in-vivo* metabolic comparison of the *db*$_{333}$/*db*$_{333}$ mice lacking all forms of the leptin receptor with the *db/db* mice lacking just the Ob-Rb long isoform of the receptor (Chen, Charlat et al. 1996). The functional loss of leptin signaling in *db*$_{333}$/*db*$_{333}$ and *db/db* mice was confirmed by icv administration of leptin which resulted in significant reduction in food intake in WT mice while, as expected, no significant appetite suppression was observed in *db*$_{333}$/*db*$_{333}$ or *db/db* mice. Both *db*$_{333}$/*db*$_{333}$ and *db/db* mice are obese, hyperphagic and insulin resistant. The metabolic comparison of these obese leptin receptor deficient mice with lean mice showed that *db*$_{333}$/*db*$_{333}$ and *db/db* mice consume significantly

less O_2 and produce significantly less CO_2 in comparison with lean controls as observed in other obese mutants (Pelleymounter, Cullen et al. 1995; Overton, Williams et al. 2001). Neither *db*$_{333}$/*db*$_{333}$ or *db/db* mice display the classical circadian rhythm of lower oxygen demand and CO_2 production in the light cycle and markedly higher O_2 demand and CO_2 production in the dark cycle as seen in lean controls. The reduced Respiratory Efficiency Ratio (RER) in both *db*$_{333}$/*db*$_{333}$ and *db/db* mice indicates the use of lipids as energy substrates in these mice as opposed to carbohydrates in WT mice as suggested by an RER closer to 1. The activity levels observed in *db*$_{333}$/*db*$_{333}$ and *db/db* mice are dramatically reduced compared with WT animals. *db*$_{333}$/*db*$_{333}$ and *db/db* mice also maintain a consistently lower core body temperature of approximately 35°C consistent with previous findings in these Ob-Rb deficient mice (Trayhurn and James 1978; Harris, Mitchell et al. 2001). However a modest increase in activity was observed in *db*$_{333}$/*db*$_{333}$ mice relative to *db/db* mice towards the end of the dark cycle in comparison with *db/db* mice. This trend of increase in activity has previously been described in the s/s mutant mouse that has a Tyr to Ser mutation at position 1138 of the *db* gene that specifically disrupts the activation of the transcription factor STAT3 (Bates, Dundon et al. 2004). In both the *db*$_{333}$/*db*$_{333}$ and s/s mutant mouse neither the core body temperature, VO_2 consumption or CO_2 production were different from that observed in *db/db* mice at this time point suggesting this modest increase in activity in the dark phase does not significantly alter overall energy expenditure.

Ob-Rb is believed to function through a Jak/STAT signal transduction pathway (Ghilardi, Ziegler et al. 1996) to promote fat oxidation (Hwa, Ghibaudi et al. 1996) satiety (Satoh, Ogawa et al. 1998) and the homeostasis of lipids (Unger, Zhou et al. 1999). Leptin signaling in hypothalamic neurons results in a decrease in feeding and an increase in energy expenditure in order to maintain energy homeostasis. (Campfield, Smith et al. 1995; Halaas, Gajiwala et al. 1995; Levin, Nelson et al. 1996). In states of negative energy balance such as starvation, a decrease in leptin levels causes an increase in food intake and a decrease in energy expenditure (Elmquist, Maratos-Flier et al. 1998; Friedman and Halaas 1998). Therefore, lack of the leptin signaling in these mice results in early onset obesity secondary to the increase in feeding and decreased energy utilization.

db$_{333}$/*db*$_{333}$ mice have altered endocrine and metabolic parameters consistent with previously described leptin receptor deficient mouse models of obesity including *db/db* mice that lack only the Ob-Rb isoform (Chen, Charlat et al. 1996; Myers 2004) and several other mutants described that lack all known isoforms of the leptin receptor (Aubert, Herzog et al. 1985; Lee, Li et al. 1997; Li, Ioffe et al. 1998). For example the *db*^{3J}/*db*^{3J} mouse has a frameshift mutation at amino acid 625 (Leiter, Coleman et al. 1980) (Lee, Li et al. 1997) (Luo, Liu et al. 2006). and the *db*^{Pas}/*db*^{Pas} mouse has a duplication of exons 4 and 5 of Ob-R which introduces a premature stop codon at residue 281 (Li, Ioffe et al. 1998). Both mutations result in a truncated receptor without a transmembrane domain and results in complete ablation of all known leptin receptor splice variants (Fig. 1B).

The *db*$_{333}$/*db*$_{333}$ mice display very similar obese phenotypes to the other leptin receptor deficient mouse models of obesity. The C57BL/6 *db*^{3J}/*db*^{3J} mice gain weight in an identical manner to *db*$_{333}$/*db*$_{333}$ mice, for example at 12 wks of age C57BL/6 *db*^{3J}/*db*^{3J} mice weigh 56g ± 7.4 (Luo, Liu et al. 2006) and *db*$_{333}$/*db*$_{333}$ mice weigh 55.5g ± 2.3. The *db*^{Pas}/ *db*^{Pas} mice have a similar weight gain profile as the *db*$_{333}$/*db*$_{333}$ mice, at 24wks of age, *db*^{Pas}/ *db*^{Pas} weigh 67.3g ± 1.71 compared with *db*$_{333}$/*db*$_{333}$ that weigh 68.6g ± 5.96 (Aubert, Herzog et al. 1985).

The *db*$_{333}$/*db*$_{333}$ and *db/db* mice display very similar metabolic phenotypes in this study. Therefore, this study further confirms the finding that the long cytoplasmic domain of the leptin receptor is the main isoform by which leptin exerts its effects on energy homeostasis (Ghilardi,

Ziegler et al. 1996). However, there is trend towards higher body weight, higher insulin and IL1Ra levels, lower CO_2, VO_2, RER and temperature in db_{333}/db_{333} mice when compared with *db/db* mice which raises the question whether the short forms of the receptor play an additional role in energy homeostasis. Transgenic replacement of the long form of the leptin receptor (Ob-Rb) that is restricted to neurons in db^{3J}/db^{3J} animals has been shown to partially rescue the obesity/diabetes phenotype while fully correcting the impaired thermoregulatory thermogenesis phenotype (Kowalski, Liu et al. 2001). This partial attenuation of the obesity/diabetes phenotype (including body weight, circulating leptin, glucose and insulin) without a full restoration of WT levels, may be reflective of a role of the shorter isoforms in energy homeostasis.

In summary, we have characterized a novel transgenic mouse model of obesity, the db_{333}/db_{333} mice, with a mutation that ablates expression of all leptin receptor variants. This db_{333}/db_{333} mouse may prove useful in future studies on the roles of the long form and the short form of leptin receptors respectively.

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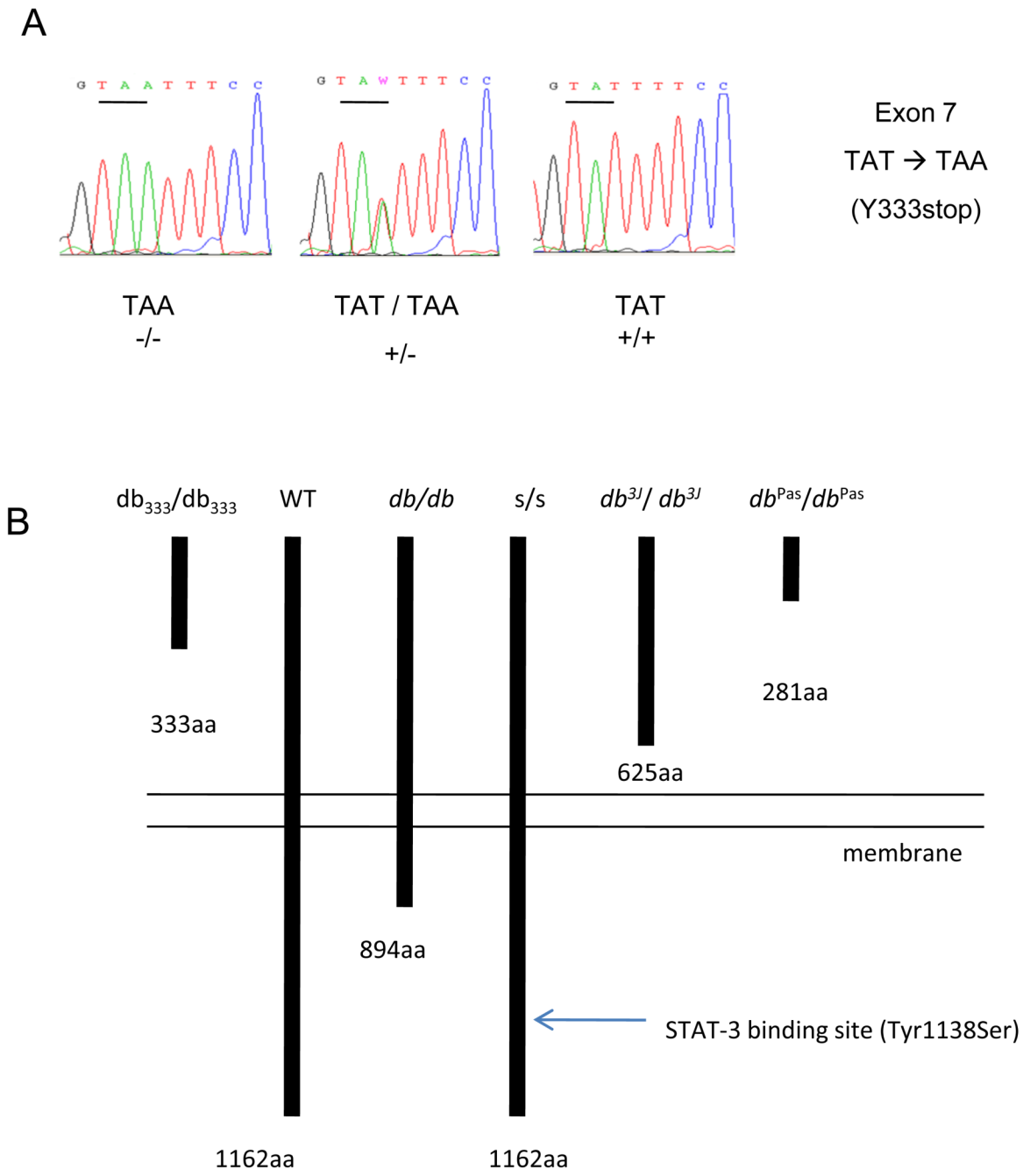

Figure 1.

A. The *db*$_{333}$/*db*$_{333}$ mutation. DNA sequence chromatograms from homozygous mutant *db*$_{333}$/*db*$_{333}$ mice (TAA/TAA), heterozygous *db*$_{333}$/*db*$_{333}$ mice (TAA/TAT) and C57Bl/6 WT mice (TAT/TAT). *db*$_{333}$/*db*$_{333}$ mice have a point mutation in the leptin receptor gene causing a premature stop codon. The *db*$_{333}$/*db*$_{333}$ mouse has a T to A transversion (TAT→TAA) in the 7th coding exon of the leptin receptor gene, resulting in a premature stop codon at Tyr$_{333}$ **B. Schematic Presentation of Leptin Receptor Mutations in Mouse Models of Obesity with Leptin Signaling Deficiencies.** The predicted protein length is shown with the numbers at the end of each receptor representing the amino acid residue at the carboxy terminus. The STAT-3

binding site is located at amino acid 1138 and has an important role in the regulation of energy balance through the JAK/ STAT signaling pathway.

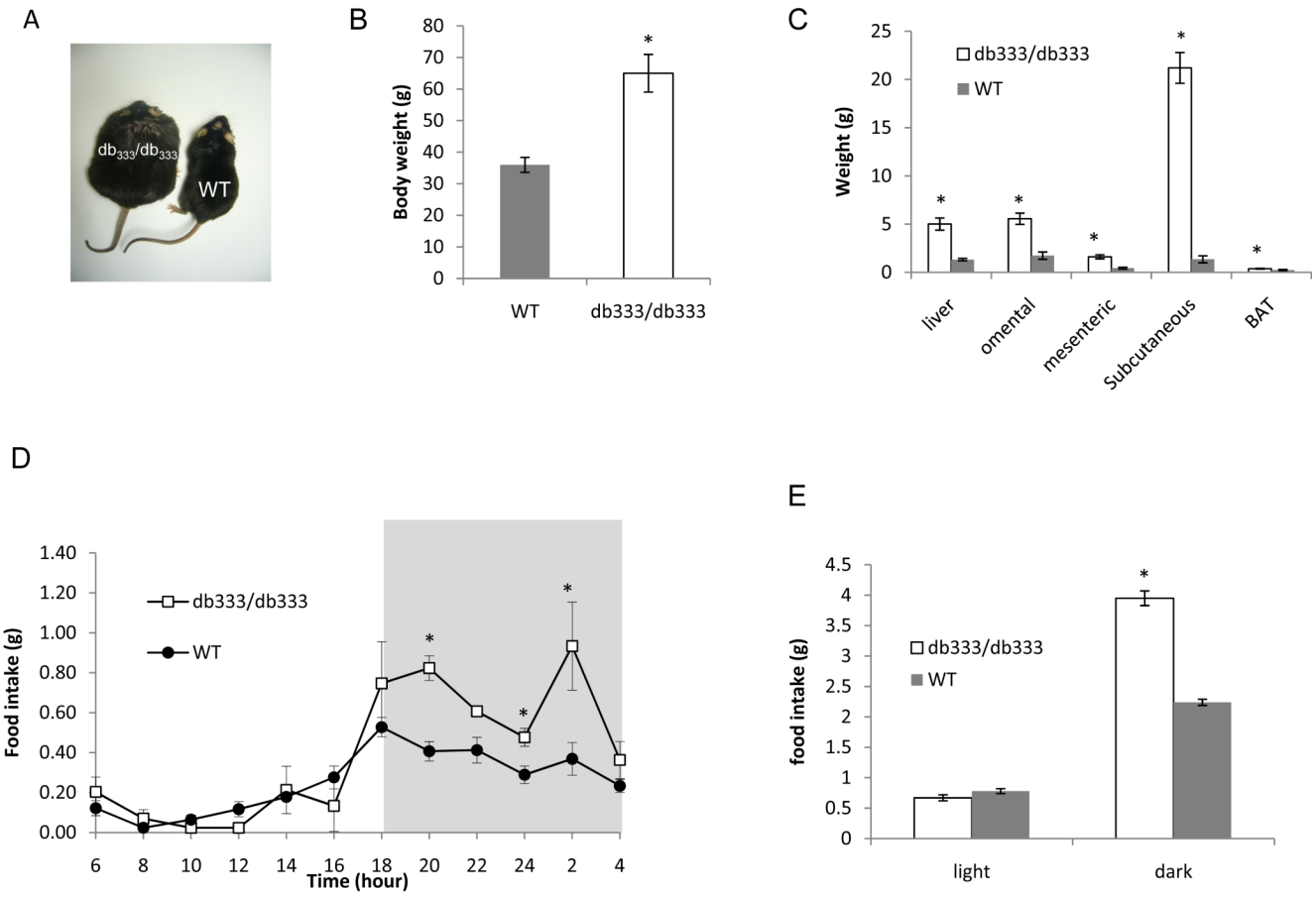

Figure 2. Phenotypic characterization of the *db*$_{333}$/*db*$_{333}$ mice

A. Photograph of *db*$_{333}$/*db*$_{333}$ and WT littermate. **B**. Body weights of *db*$_{333}$/*db*$_{333}$ and WT mice at 6 mths of age. **C**. *db*$_{333}$/*db*$_{333}$ mice have significantly increased omental, mesenteric and subcutaneous fat, increased liver and brown adipose tissue (BAT) weight compared with WT littermates. **D**. Food intake in *db*$_{333}$/*db*$_{333}$ and WT mice during a 24hr monitoring period. **E**. *db*$_{333}$/*db*$_{333}$ mice are hyperphagic and consume significantly more food than WT littermates in the dark cycle (*db*$_{333}$/*db*$_{333}$ 3.95g ± 0.05, WT 0.78 ± 0.05g). Significant ($P<0.05$) differences between group mean were determined by a two tailed independent Ttest and denoted by *asterics*. Data are presented as means ± SE.

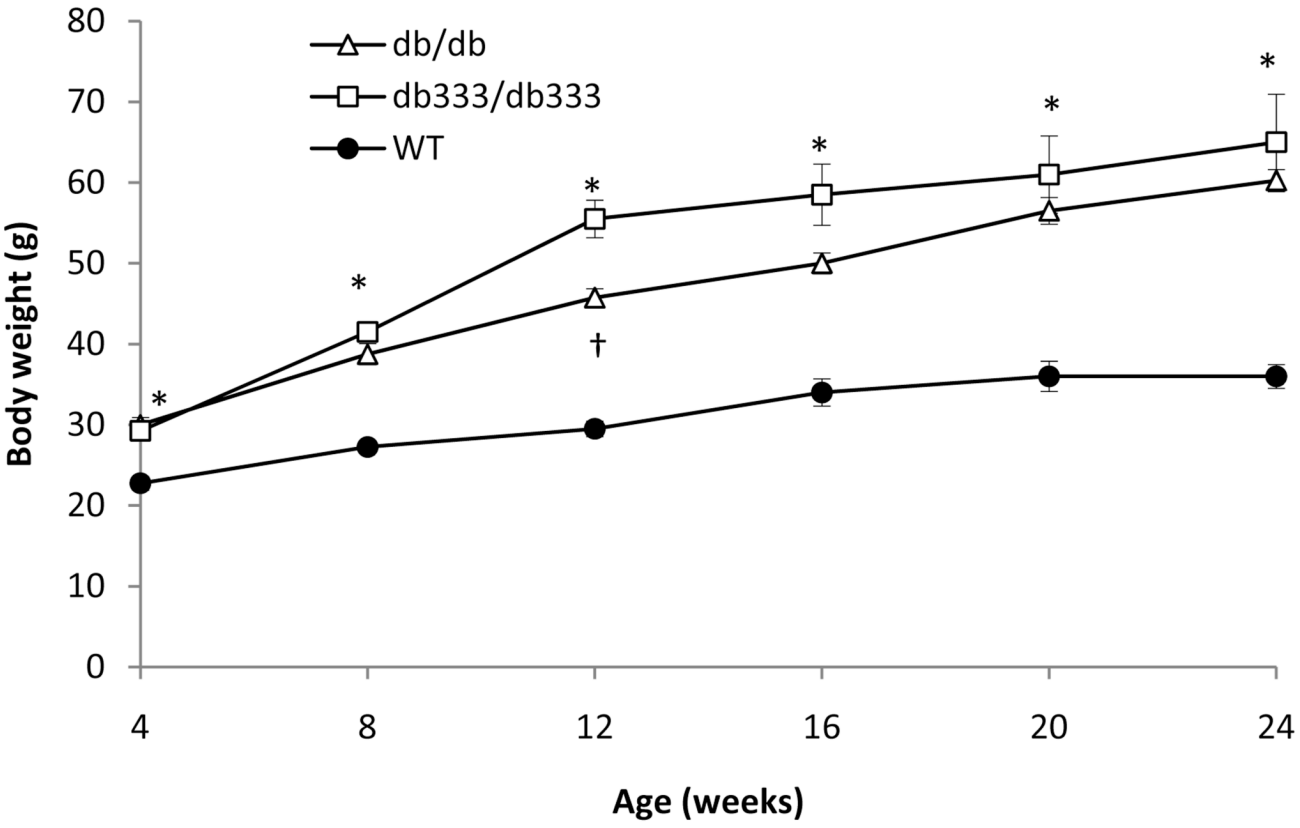

Figure 3. Growth curve of male *db*$_{333}$/*db*$_{333}$, *db/db* and WT mice

Both *db*$_{333}$/*db*$_{333}$ and *db/db* mice have significantly greater body weights than WT littermates from as early as 4 weeks of age and throughout this 24wk monitoring period. *db*$_{333}$/*db*$_{333}$ mice display consistently higher body weights than *db/db* mice from 8 weeks to 24wks of age but this trend only reached statistical significance at one time point († $P<0.05$ at 12wks). At 24wks of age *db*$_{333}$/*db*$_{333}$ mice display a higher body weight than *db/db* mice but this trend is not statistically significant at this time point. At 24wks of age *db*$_{333}$/*db*$_{333}$ and *db/db* mice have significantly greater body weights than WT littermates (*db*$_{333}$/*db*$_{333}$ 65.0 ± 5.9g; *db/db* 60.25 ± 1.38g, WT 36.25 ± 1.70g, $P<0.05$). Data represent the mean of 5 male mice per genotype weighed monthly from 4 weeks to 24 weeks of age. Significant ($P<0.05$) differences between *db*$_{333}$/*db*$_{333}$ and WT group means are denoted by *asterics*, differences between *db/db* and *db*$_{333}$/*db*$_{333}$ mice are denoted by †. All significant differences were determined by one way ANOVA and post hoc Tukey test. Data are presented as means ± SE.

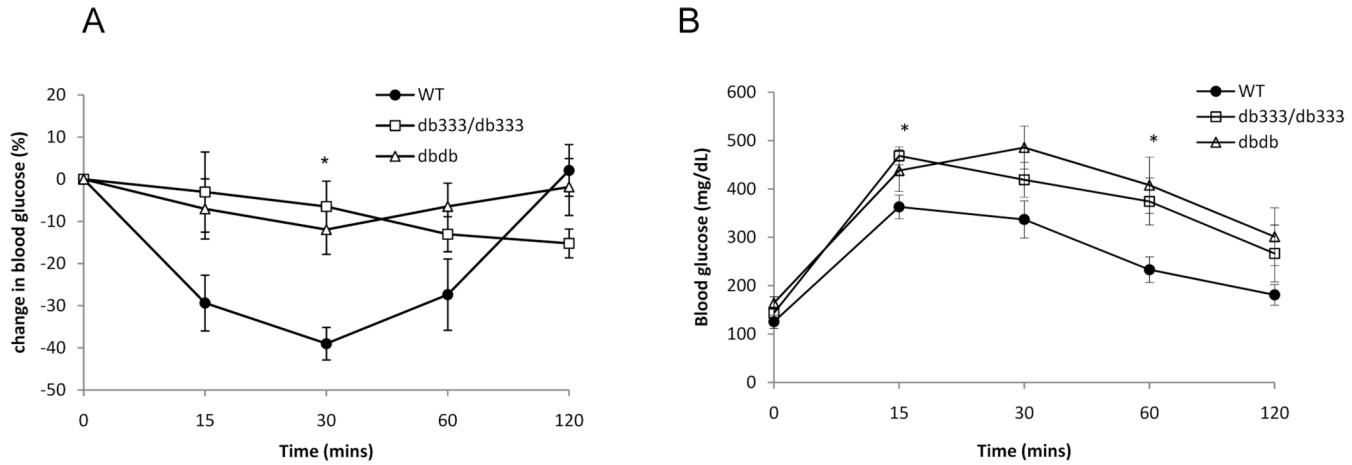

Figure 4.

A. Insulin sensitivity test in *db*$_{333}$/*db*$_{333}$, *db/db* and WT mice. Intraperitoneal insulin administration (1 unit per kg of body weight) revealed that both *db*$_{333}$/*db*$_{333}$ and *db/db* mice have an impaired glucose disposal ability relative to the hypoglycemic response to insulin observed in the WT littermates. Data are presented as percentage change in blood glucose from baseline before and after insulin injection for up to 120 mins. **B. Intraperitoneal glucose tolerance test in *db*$_{333}$/*db*$_{333}$, *db/db* and WT mice.** After glucose challenge *db*$_{333}$/*db*$_{333}$ and *db/db* mice display impaired glucose disposal compared with WT mice. Data are plotted as mean blood glucose ± SE for n = 5 animals of each genotype before and after intraperitoneal glucose injection for up to 120 mins. Significant ($P<0.05$) differences between *db*$_{333}$ and WT group means are denoted by *asterics* and were determined by one way ANOVA and post hoc Tukey test.

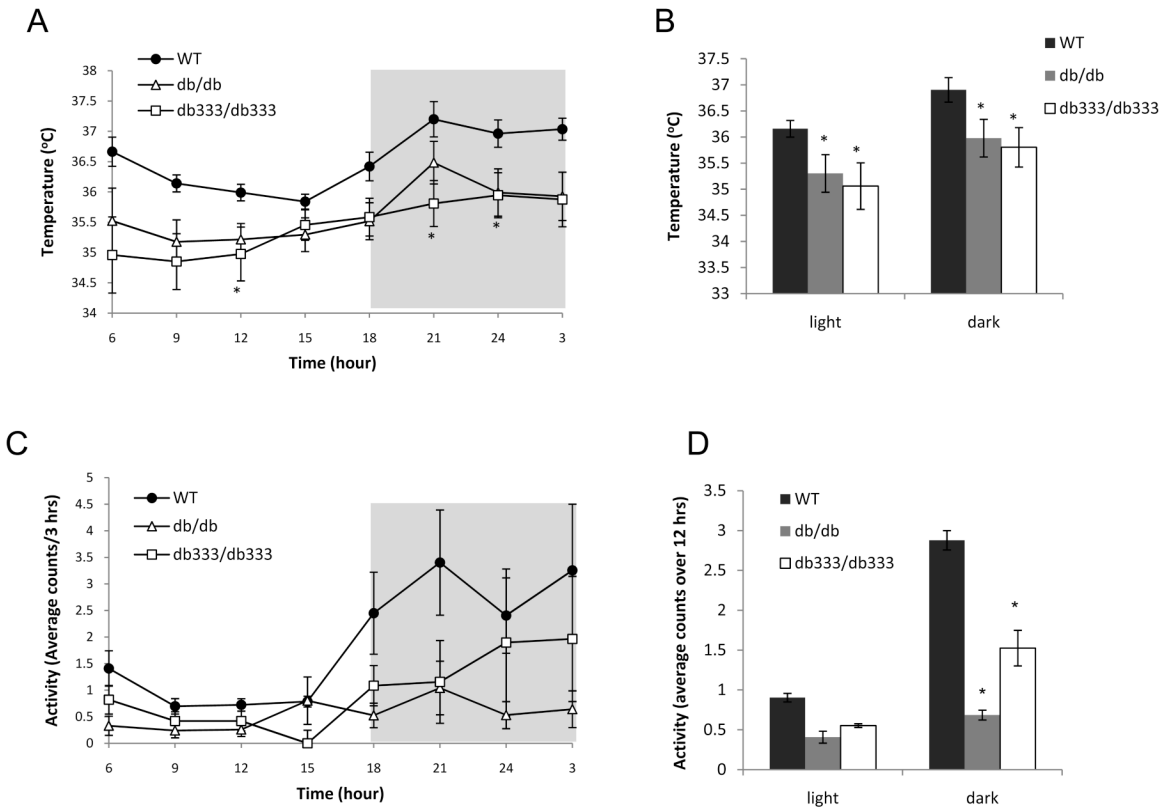

Figure 5. Core Body Temperature and spontaneous locomotor activity in db_{333}/db_{333}, *db/db* and WT mice during a 24hr monitoring period

A. db_{333}/db_{333} and *db/db* mice display a significantly lower core body temperature relative to WT mice **B.** Average core body temperature over the 12hr light or dark cycle in db_{333}/db_{333} and *db/db* mice is significantly lower when compared with WT littermates **C.** Average spontaneous physical activity of db_{333}/db_{333} *db/db* and WT mice during a 24hr monitoring period. db_{333}/db_{333} and *db/db* mice display decreased locomotor activity relative to WT mice. **D.** Averaged activity for db_{333}/db_{333}, *db/db* and WT mice during the light and dark cycle. Both db_{333}/db_{333} and *db/db* mice display lower average activity than WT mice. Data are plotted as mean ± SE for n = 4 animals of each genotype. Significant ($P<0.05$) differences between group means are denoted by *asterics* and were determined by one way ANOVA and post hoc Tukey test.

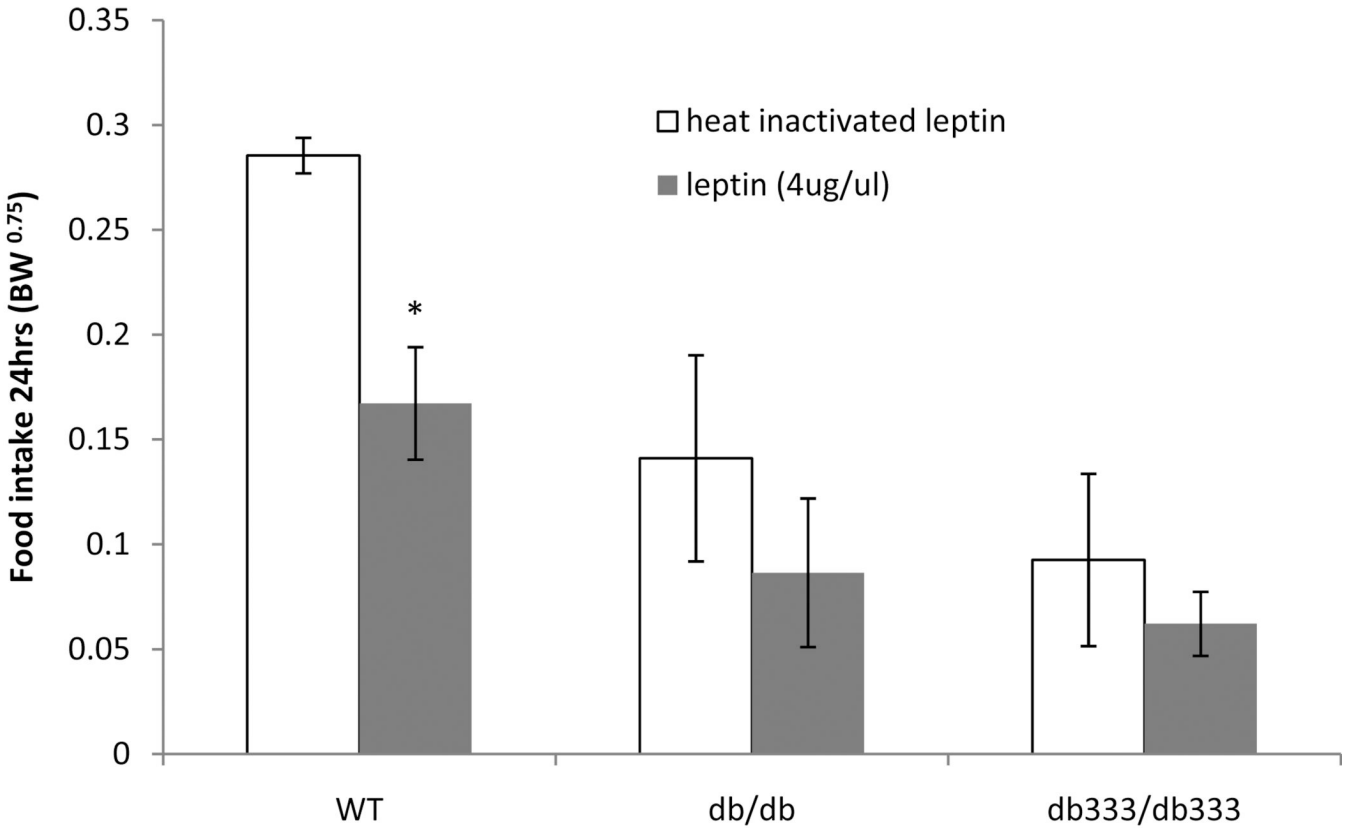

Figure 6. Effects of icv leptin administration on food intake in *db_{333}/db_{333}*, *db/db* and WT mice

Injection of leptin (icv 4 μg/μL) caused a significant reduction in food intake in WT mice (P=0.0038) while no significant appetite suppression was observed in *db_{333}/db_{333}* or *db/db* mice. Data are plotted as mean ± SE for n = 4 animals of each genotype. Significant differences between group means are denoted by *asterics* and were determined by one way ANOVA and post hoc Tukey test.

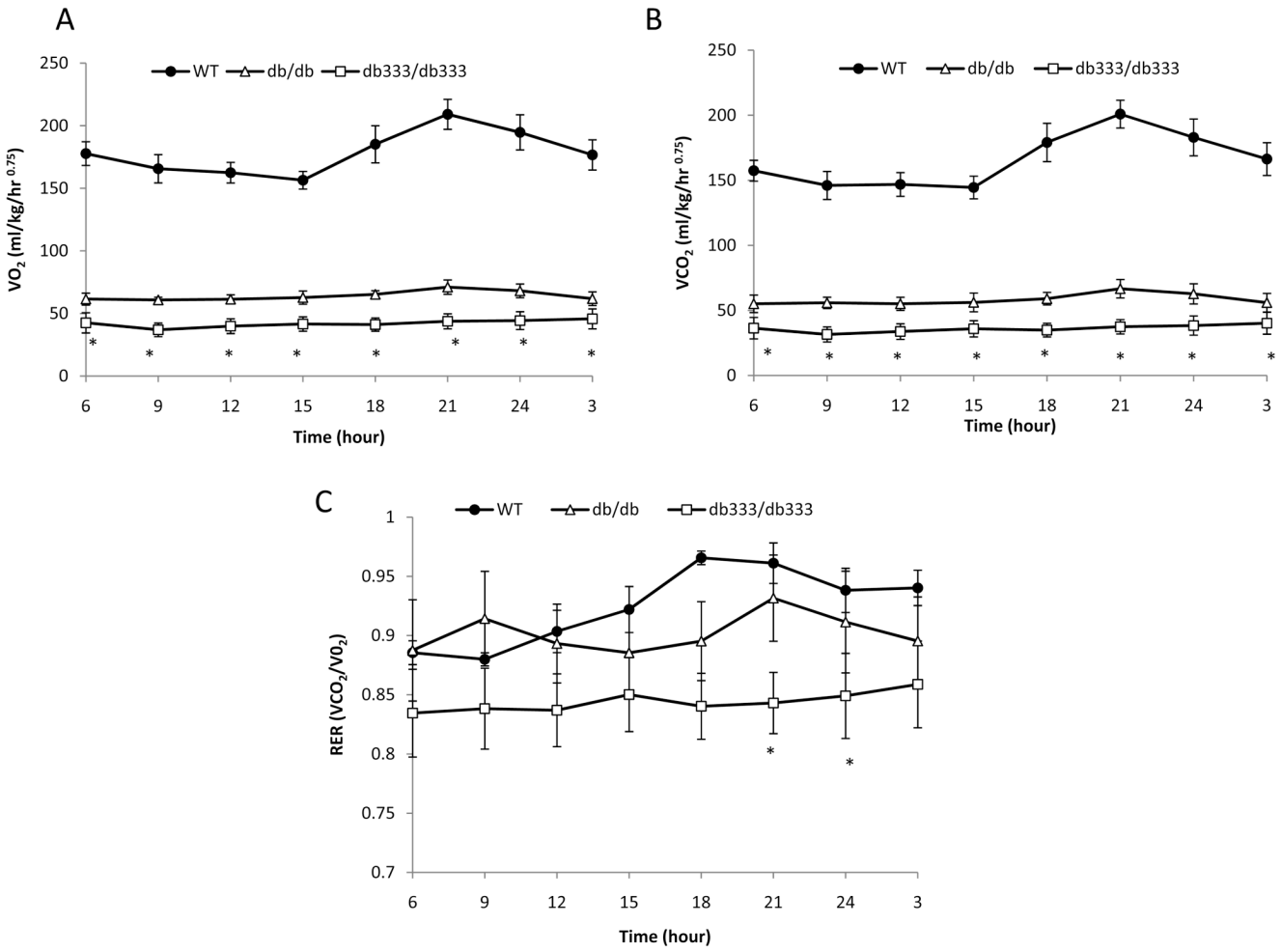

Figure 7. Metabolic comparison of *db*$_{333}$/*db*$_{333}$, *db/db* and WT mice during a 24hr monitoring period

A. Oxygen consumption in *db*$_{333}$/*db*$_{333}$, *db/db* and WT mice monitored for 24hrs. Both *db/db* and *db*$_{333}$/*db*$_{333}$ mice displayed similar oxygen consumption at all time points and these obese mutants showed significantly lower oxygen consumption than WT mice throughout both the light and dark cycle. **B.** CO_2 production in *db*$_{333}$/*db*$_{333}$, *db/db* and WT mice monitored for 24hrs. Both *db/db* and *db*$_{333}$/*db*$_{333}$ mice displayed similar levels of CO_2 production at all time points and these obese mutants showed significantly lower CO_2 production than WT mice throughout both the light and dark cycle. **C.** Respiratory Efficiency Ratio (RER) (VCO_2 / VO_2) in *db*$_{333}$/*db*$_{333}$, *db/db* and WT mice over a 24hr period. Both *db*$_{333}$/*db*$_{333}$ and *db/db* show a trend of lower RER relative to WT mice that is most pronounced in the dark cycle. *db*$_{333}$/*db*$_{333}$ mice show a consistently lower RER relative to *db/db* and WT mice. Data are plotted as mean ± SE for n = 4 animals of each genotype. Significant ($P<0.05$) differences between group means are denoted by *asterics* and were determined by one way ANOVA and post hoc Tukey test.

Table 1

db_{333}/db_{333} mice had significantly elevated circulating levels of leptin, insulin and interleukin-1 receptor antagonist (IL-1Ra) at 6 months of age when compared with WT lean littermates

db_{333}/db_{333} also display significantly elevated circulating insulin levels and show a trend towards higher IL-1Ra levels when compared with *db/db* mice. Levels of the pro-inflammatory cytokine interleukin 6 (IL-6) was also elevated in db_{333}/db_{333} and *db/db* mice relative to levels in WT, lean littermates.

	***db*333/*db*333**	**WT**	***db/db***
Insulin (pg/ml)	6589 ± 341 * †	1159 ± 400	3686 ± 654*
Leptin (pg/ml)	35318 ± 2239 *	5874 ± 707	36248 ± 5639*
IL-1Ra (pg/ml)	3523 ± 118 *	891 ± 324	2501 ± 375
IL-6 (pg/ml)	61 ± 24	41 ± 09	47 ± 18

Significant (P<0.05) differences between db_{333}/db_{333} and WT group means are denoted by *, differences between *db/db* and db_{333}/db_{333} mice are denoted by †.