Low Dopamine D2 Receptor Increases Vulnerability to Obesity Via Reduced Physical Activity Not Increased Appetitive Motivation

Supplemental Information

Supplemental Results

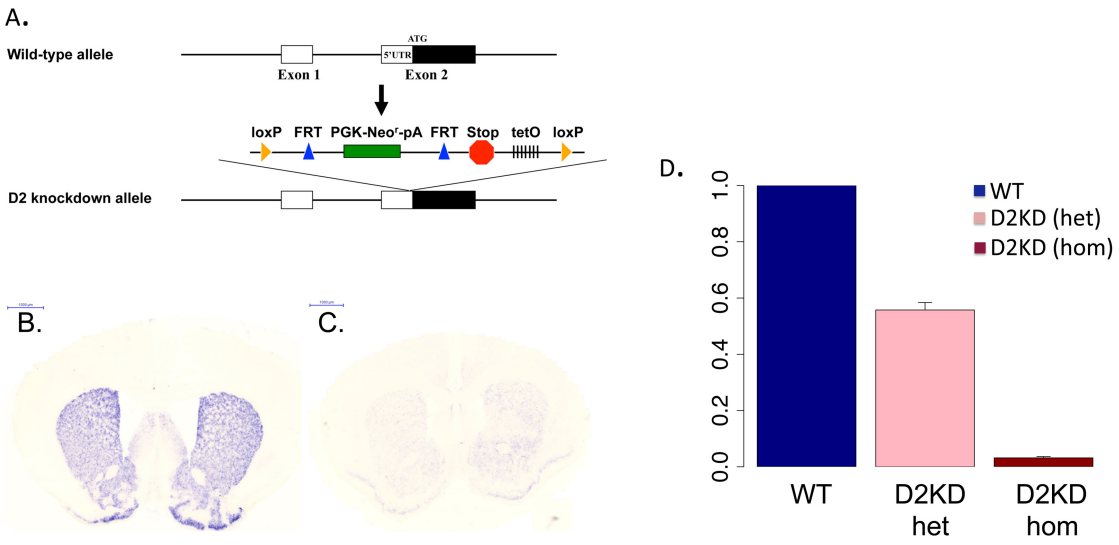

Figure S1. Generation and characterization of D2R expression KD mice. (**A**) Schematic of construct inserted into the *Drd2* locus. In situ hybridization for D2R mRNA in the striatum in (**B**) WT mice and (**C**) KD littermates. (**D**) Quantitative PCR for *Drd2* mRNA comparing mice heterozygous and homozygous for D2R KD with WT littermates *n* = 7, WT; 9, hom; 8, het.

Generation and Verification of D2R Knock Down Mouse Line

The KD line was generated by targeted insertion of a loxP-neo^r-STOP-tetO-loxP cassette into the 5'UTR in the second exon of the *Drd2* gene (Fig S1A). To examine the extent of D2R expression, sections were prepared from the animals and stained by *in situ* hybridization with a riboprobe for *Drd2* transcripts. In WT mice, *Drd2* mRNA expression was high in the striatum and barely detectible in the cortex (Fig S1B). In KD homozygote littermates, D2R mRNA expression was detectible and had the same expression pattern as in WT. However, the expression level was too low to be quantified reliably (Fig S1C). We quantified *Drd2* mRNA using quantitative

PCR (Fig S1D) comparing mice heterozygous and homozygous for the KD with WT littermates. The KD heterozygotes and homozygotes exhibited 55% and 3%, respectively, of WT *Drd2* mRNA expression (Fig S1D). The minimal promoter in tetO and the intact coding sequence in KD mice are likely the reasons for this dramatically reduced expression (knockdown) rather than a complete knockout.

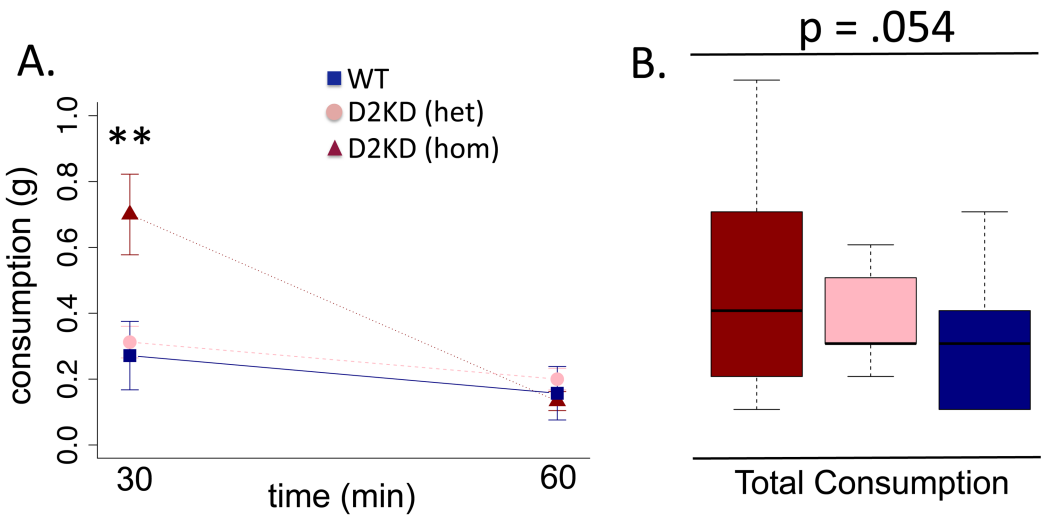

Figure S2. Rate of re-feeding after fasting. (**A**) Amount consumed (g) at 30- and 60- minute time points following 12 hour fast (KD homozygote, red; heterozygote, pink; WT littermates, blue) and (*B*) boxplot of total consumption during 1 hour re-feeding period. *n* = 7, WT; 9, hom; 8, het.

KD Mice Show No Impairment in Feeding Following Period of Fasting

It is possible that when the KD mice become energy depleted they are less capable of robustly feeding to replenish energy supplies, for example, following a bout of wheel running. To test this, we fasted mice and then measured their consumption during a subsequent feeding opportunity. At the 30-minute time point, the KD homozygotes consumed more than the WT or KD heterozygotes (Fig S2, $F_{(2,21)} = 3.35$, $p = .054$). At the 60-minute time point there was no difference in the groups (Fig S2, $F_{(2,21)} = .50$, $p = .61$). Thus, the KD mice show no impairment in pursuing food to replenish depleted energy supplies.

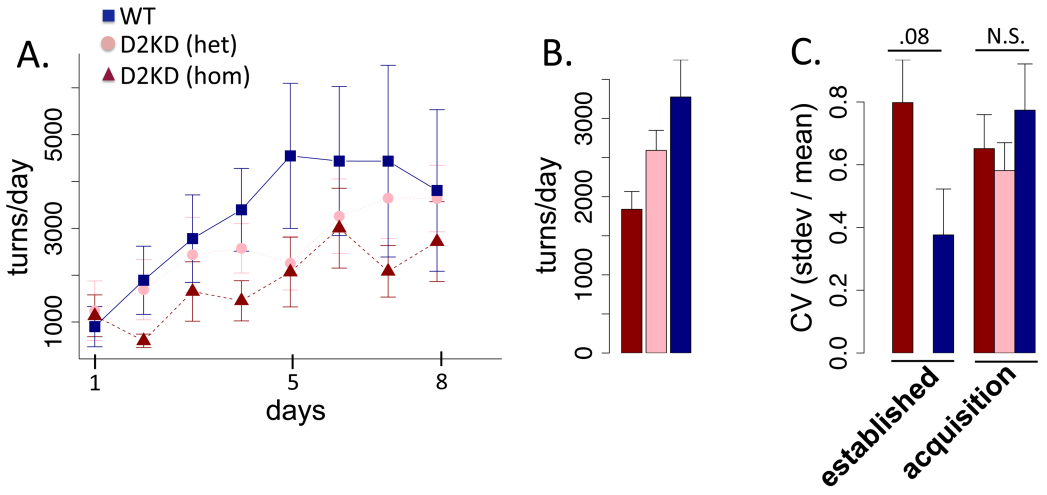

Figure S3. Initial acquisition of wheel running. (**A**) Daily turns recorded across 8 days and (**B**) average daily running in KD homozygotes (red), heterozygotes (pink) and WT littermates (blue) during first exposure to wheels and high-fat (HF) diet. (**C**) Coefficient of variation in running calculated across days for each mouse and averaged by genotype comparing established, long-term exposure (DIO experiment, figure 2) with initial exposure. *n* = 7, WT; 9, hom; 8, het.

Initial Acquisition of Wheel Running Similar Between KD and WT Mice

To assess differences in acquisition of wheel running, we provided running wheels and HF diet to singly housed male mice without prior exposure to either, comparing initial acquisition of running between WT littermates, KD homozygous for the knockdown, as well as KD heterozygotes to examine potential gene-dose effects. There were no significant differences in body weight between the genotypes ($F_{(2,21)}$ = 2.0, p = .15). Although a stair-stepping difference between genotypes was apparent, these differences were not statistically significant (FigS3A and B, $F_{(2,21)}$ = .937, p = .407; WT vs homozygotes, $F_{(1,14)}$ = 1.6, p = .22), suggesting that initial acquisition between the groups was not significantly different and that the difference emerges over time with continued exposure to wheel access and HF diet. WT running was considerably less than seen after months of wheel exposure in the prior experiment (Fig 1D), suggesting the mice in the prior experiment had, over months of wheel access, developed consistent patterns

of running. To assess consistency of running, we examined the coefficient of variance between days for each mouse for both experiments (Fig S3C). In this experiment, there was no significant effect of genotype on consistency of daily running, with all groups showing considerable daily variability (Fig S3C, $F_{(2,21)} = .671$, $p < .52$). In contrast, in the prior study after months of exposure to running wheels (Fig 1), the WT mice showed a marginally significant decrease in variance in daily running (Fig S3C, $F_{(1,16)} = 3.36$, $p < .08$), while the KD mice continued to show inconsistency in daily running, suggesting that over time the WT mice developed consistent and prolonged running habits while the KD mice did not. Though the apparent gene-dose effect in overall running is intriguing, this did not reach statistical significance (Fig S3B, $p = .457$). There were no significant genotype differences in body weight at the conclusion of the 8-day exposure to wheels and high fat diet ($F_{(2,21)} = 2.0$, $p = .16$).

Body Composition (% Fat) of Mice Used in Calorimetry Experiment

Table S1. DEXA scan results.

	age (weeks)	b.w. (g)	% body fat
WT	23.0 ± 1.2	28.9 ± .97	16.4 ± .91
KD hom	24.0 ± 1.7	26.7 ± .86	16.0 ± .85
KD het	25.3 ± 1.6	29.4 ± .67	18.4 ± .90

Sex Differences

Dietary-induced Obesity: There was no significant genotype difference between WT and KD mice in initial weight, both including and not including age as a factor (data not shown, genotype main effect with age as factor, $F_{(1,42)} = .324$, $p = .57$; without age, $p = .61$); however, there was a significant genotype X sex difference (sex x genotype, $F_{(1,42)} = 12.42$, $p < .01$) where male KD mice weighed less (KD 22.7 ± .83, WT 24.5 ± .46) while female KD mice weighed more (KD 21.37 ± 1.0, WT 18.56 ± .33) than WT.

Although there appears to be an evident difference between sexes where females overall gain more weight (Fig 1B), this was not statistically significant ($F_{(1,31)}$ = 1.7, p = .19). There was no significant effect of age (age, $F_{(1,31)}$ = 2.56, p = .11). There was a significant interaction between genotype and sex in weight gain, presumably because without running wheel effects observed in WT, sex is more predictive in the KD mice (Fig 1B; $F_{(1,31)}$ = 5.7, p = .02). There was also a marginally significant wheel by sex interaction ($F_{(1,31)}$ = 3.38, p = .07), likely reflecting greater benefit from wheel running for males among WT mice. There was no 3-way interaction between genotype, wheel condition and sex ($F_{(1,31)}$ = .30, p = .48).

There were no significant main effects of sex or age or significant interactions with genotype or wheel condition on consumption nor wheel running (main effect, p = .55; sex x genotype, p = .72) or age effect (p = .78).

Open Field: There were no significant sex differences, neither main effect nor interactions (distance, main effect, p = .18).

Fasting Glucose: A genotype by sex interaction was noted ($F_{(1,34)}$ = 4.9, p < .05), likely reflecting an apparent increased fasting glucose among female KD mice, though this may be spurious as no sex difference or interactions with sex were observed with glucose clearance. However, with the insulin clearance, there was a significant interaction between sex and wheel access (data not shown; $F_{(1,33)}$ = 4.97, p < .05) where female WT provided access to wheels did not show improved insulin response like their male counterparts (genotype x wheel x sex; $F_{(1,33)}$ = 3.12, p = .08). Although this might suggest that females benefit less from exercise opportunity, given that, among WT mice, we observe a clear reduction in fasting glucose for both sexes as well as clearly improved glucose clearance, again suggesting this observation be treated with caution.

Summary: Although we generally found few significant sex differences, the studies were not specifically designed to test sex differences and so may be underpowered for that purpose. Both male and female mice were included as samples of convenience and to provide an initial evaluation of potential sex differences. These data indicate that there may be important sex

differences in how voluntary exercise impacts glucose regulation and insulin sensitivity, but follow-up studies directly addressing such sex differences are required.

Supplemental Methods & Materials

Generation of D2R Knockdown Mice

We used a novel genetic strategy to generate a D2R knockdown line in which a "loxP-PGK-neo^r-STOP-tetO-loxP" cassette was inserted into the 5'untranslated region (5' UTR) in the second exon of the *Drd2* gene via gene targeting in embryonic stem cells. The gene-targeting vector was constructed from three PCR fragments that were amplified using 129Sv/J ES cell DNA as templates. A 5' fragment (2.5 kb) that ends at -26 bps from ATG (inside the 5'UTR) was used to construct the entire short arm of the gene-targeting vector. The long arm was constructed from two PCR fragments. These two fragments were connected by a HindIII site and covered a 7.9 kb 3' region starting at -21 bps from ATG (inside the 5'UTR). A "loxP-FRT-PGK-neo^r-FRT-STOP-tetO-loxP" cassette was ligated in between the two arms to generate the targeting vector. We sequenced the regions near all the ligation sites to confirm precise construction of the gene-targeting vector.

The minimal promoter in 'tetO' and the intact D2R coding sequence gives low level of D2R expression (knockdown). The inclusion of the loxP sites in the cassette permits rescue of D2R expression by cell type specific expression of the Cre recombinase that will excise the cassette. The inclusion of the tetO promoter permits expression of D2R in cells that express the tetracycline-responsive transactivator. Both rescue strategies were verified in other studies though neither was used in the present study. The knock-in line was originally generated on the 129/SvJ background and was subsequently crossed to the C57BL/6J background for > 10 generations.

Verification of D2R Knockdown

In situ: Mice were anesthetized with isoflurane and transcardially perfused with 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (PBS), postfixed for 1-3 days, and then infiltrated with 30% diethylpyrocarbonate (DEPC)-treated sucrose solution. For *in situ* hybridization, 20-30 micron coronal sections were taken through the striatum with a cryostat (Leica). They were mounted onto glass slides, fixed in a solution of 4% PFA in PBS, treated with Proteinase K (0.4-0.8 mg/mL), then glycine (2 mg/mL), fixed again, and treated with 100 mM triethanolamine containing 25 mM acetic anhydride. Hybridization was performed overnight with an antisense probe labeled by *in vitro* transcription using 11-digoxigenin-UTP (Roche). Signals were visualized with NBT/BCIP after immunohistochemical labeling with an anti-digoxigenin Fab fragment conjugated to alkaline phosphatase at 1:5000 dilution (Roche).

Quantitative PCR: Mice used in the calorimetry, wheel acquisition and refeeding experiments were anesthetized with isoflurane and decapitated. Brains were dissected and placed in ice-cold PBS. Tissue punches (2 mm) were taken from the striatum. Total RNA was extracted using the RNEAsy Lipid Extraction Kit (Qiagen). Two micrograms of RNA from each mouse were used for reverse transcription and subsequent RNAse H digestion with the Superscript III First-Strand Synthesis System (Life Technologies/Invitrogen). Equal volumes of cDNA from the reverse transcription reaction from each mouse were combined for each genotype and diluted to 1:10, 1:100, and 1:1000 for qRT-PCR, which was conducted with the iTaq Universal SYBR Green SuperMix in a CFX-96 qPCR system (BioRad). Each sample was run in triplicate. The D2R primers were ACTCAAGGGCAACTGTACCCA (forward) and GCATCTCCATTTCCAGCTCCT (reverse). D2R CT measures were normalized to GAPDH, then KD homozygotes and heterozygotes were calculated as percentage of WT mRNA. Data represent average across dilution series.

Assessment of Body Composition

Mice were anesthetized prior to DEXA imaging. Mice were injected with a mixture of Ketamine (80 mg/kg body weight) and xylazine (5 mg/kg body weight) solution. Body composition was measured by DEXA (Lunar PIXImus densitometer system, GE Healthcare) using PIXImus 2 software, as described in (1). The system was calibrated according to manufacturer's instructions prior to the start of the experiment.

Indirect Calorimetry

Metabolic cage analysis of energy balance was carried out using metabolic cages (TSE Systems, Chesterfield, MO). Mice were singly housed in a TSE Labmaster System and allowed 2 days of acclimation to the chambers. Following acclimation, food/water intake, physical activity, and O_2 uptake and CO_2 production were monitored for 4 consecutive days. Metabolic rate (kcal/hr/kg) was normalized to body weight.

Fasting-Refeeding

Mice were fasted for 12 hours. For testing, each mouse was individually housed and given a ball of BioServ 60% fat (high sugar/high fat) food, weighing 29-40 grams. Consumption was measured 30 minutes into the experiment and then again at the 60-minute time point. Mice had free access to water during both the fasting and testing periods.

Data Analysis

All statistical analyses were performed using R statistical software (R version 2.12.1 (2010-12-16); The R Foundation for Statistical Computing, http://www.r-project.org).

Supplemental Reference

1. Savic D, Ye H, Aneas I, Park SY, Bell GI, Nobrega MA (2011) Alterations in TCF7L2 expression define its role as a key regulator of glucose metabolism. Genome Res. 2011 Sep;21(9):1417-25. PMID: 21673050