Striatal dopamine 2 receptor upregulation during development predisposes to diet-induced obesity by reducing energy output in mice

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Dopaminergic signaling in the striatum, particularly at dopamine 2 receptors (D2R), has been a topic of active investigation in obesity research in the past decades. However, it still remains unclear whether variations in striatal D2Rs modulate the risk for obesity and if so in which direction. Human studies have yielded contradictory findings that likely reflect a complex nonlinear relationship, possibly involving a combination of causal effects and compensatory changes. Animal work indicates that although chronic obesogenic diets reduce striatal D2R function, striatal D2R down-regulation does not lead to obesity. In this study, we evaluated the consequences of striatal D2R up-regulation on body-weight gain susceptibility and energy balance in mice. We used a mouse model of D2R overexpression (D2R-OE) in which D2Rs were selectively up-regulated in striatal medium spiny neurons. We uncover a pathological mechanism by which striatal D2R-OE leads to reduced brown adipose tissue thermogenesis, reduced energy expenditure, and accelerated obesity despite reduced eating. We also show that D2R-OE restricted to development is sufficient to promote obesity and to induce energy-balance deficits. Together, our findings indicate that striatal D2R-OE during development persistently increases the propensity for obesity by reducing energy output in mice. This suggests that early alterations in the striatal dopamine system could represent a key predisposition factor toward obesity.

development | dopamine D2 receptor | metabolism | obesity | striatum

Dopaminergic (DA) signaling in the striatum, particularly at dopamine 2 receptors (D2R), is one of the most widely studied neurotransmitter systems in obesity (for a review see refs. 1–3). However, despite two decades of research in the field, the contribution of D2Rs to human obesity remains unclear. Pioneer genetic linkage studies showed that the A1 allele of the Taq1A polymorphism, which is thought to associate with a 20–30% reduction in striatal D2R levels, is correlated with higher risk of obesity. Several other reports, however, did not confirm this linkage (reviewed in refs. 3 and 4).

PET studies, which provide a more direct picture of striatal D2R function, have also yielded inconsistent results. A landmark PET study initially reported lower striatal D2/D3R availability in obese subjects (5), a finding later replicated by others (6, 7). Most of these studies, however, employed severely obese subjects [body mass index (BMI) >45] (see ref. 8). In contrast, more recent investigations found an absence of correlation (9–11) or a positive correlation (8, 9, 12–14) between striatal D2/D3R availability and BMI. Accordingly, one way to reconcile these findings would be that low D2R might be a consequence of chronic obesity, rather than a cause, while instead high D2R could act to increase the risk for obesity.

The available animal literature corroborates these possibilities. Obesogenic diets generally lead to reduced striatal D2R function in rodents (15–18). Down-regulation of striatal D2Rs reduces locomotion but does not lead to obesity in mice (16). Thus, one key question is whether high levels of striatal D2R might be causally involved in obesity development.

In the present study, D2Rs were selectively overexpressed (D2R-OE) in medium spiny neurons (MSNs) (19, 20), the main output neurons of the striatum. We examined whether D2R-OE increased body weight (BW) (and related metabolic endpoints) at basal state or on a high-fat diet (HFD). Striatal DA, D2Rs, or D2-MSNs are involved in eating behavior (17, 21, 22) and locomotor activity (16, 23–25). Systemic or constitutive manipulations of D2Rs modulate energy expenditure (26) as well as brown adipose tissue (BAT) thermogenesis (27), a mechanism that is gaining increasing interest with respect to energy-balance regulation (28, 29). Thus, due to the potential multimodal effects of D2Rs on metabolism, we examined all sides of the energy-balance equation. Finally, because developmental processes may play an important role in obesity etiology (30), and based on the established role of D2Rs in development (31–34), we also determined whether D2R up-regulation restricted to development was sufficient to modulate BW-gain susceptibility.

Significance

The need to understand the early determinants for obesity has never been greater with currently over 700 million obese people worldwide. Several decades of research have suggested that dopamine 2 receptors (D2R) in the striatum might be particularly important for obesity etiology, but no study has thus far outlined an unambiguous causal relationship. Here we show that striatal D2R upregulation induces obesity in mice fed a high-fat diet and this is due to reduced energy expenditure rather than increased eating. Importantly, we show that elevated D2R during development is sufficient to persistently increase obesity susceptibility. This work identifies excess D2Rs early in life as a potential key predisposition factor toward obesity and therefore may help uncover strategies for early interventions.

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Results

D2R Transgene Expression in Striatal MSNs. D2R-OE mice were generated by crossing mice expressing the tetracycline transactivator (tTA) under the CamKII-α promoter (Tg-CamKII-α-tTA, line B) (35) with mice expressing D2R under the tetracycline operator tetO (Tg-tetO-D2R) (Fig. 1*A*) (20). Although CamKII-α is endogenously expressed in the entire forebrain (35), this cross yielded transgene (Tg) expression restricted to the striatum (Fig. 1*B*), with only very few expressing cells (≤0.52%) in other relevant forebrain regions (*SI Appendix*, Fig. S1), as in refs. 19 and 20. The Tg was not expressed in other CamKII-α–expressing tissues, i.e., the pancreas and skeletal muscle (Fig. 1*C*) (36, 37). Tg expression was further restricted to striatal MSNs (Fig. 1 *D*–*G*), as it was not present in the presynaptic projections from midbrain DA neurons (*SI Appendix*, Fig. S1) or postsynaptic cholinergic neurons expressing choline acetyltransferase (*Chat*) (Fig. 1 *D* and *G* and refs. 19 and 20). The highest expression was found in preproenkephalin-positive (*Penk*$^+$) indirect-pathway MSNs (iMSNs) (39.6%) (Fig. 1 *D* and *E*), but the Tg was also expressed in prodynorphin-positive (*Pdyn*$^+$) direct-pathway neurons (dMSNs) (25.8%) (Fig. 1 *D* and *F*; also see *SI Appendix*, Figs. S2–S4), consistent with ref. 31. Striatal D2R binding is increased by 15% (shown in ref. 20) and thus is in the same physiological range as D2R variations in obese vs. nonobese humans (14).

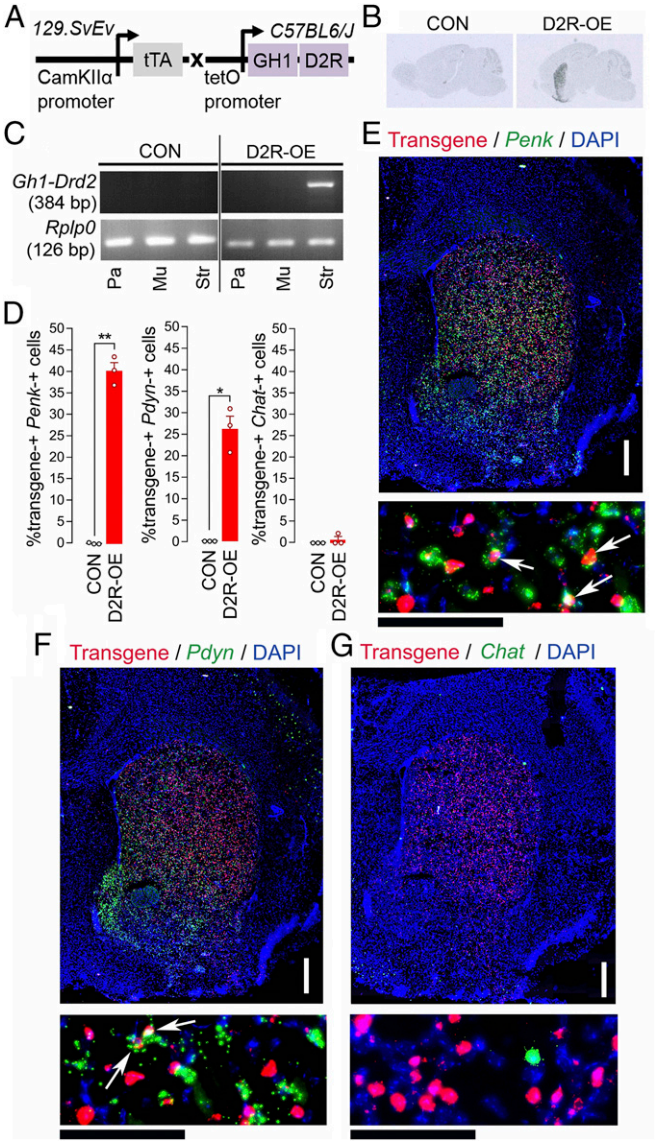

Fig. 1. D2R Tg expression in striatal MSNs. (*A*) Generation of D2R-OE mice. (*B*) Representative images of in situ hybridization for the Tg showing D2R-OE in the striatum. (*C*) The Tg *Gh1-Drd2* was expressed in the striatum (Str) but not pancreas (Pa) or skeletal muscle (Mu) of D2R-OE mice. *Rplp0* expression was constant. All lanes are from same gel. CON: Tg CaMKIIα-tTA mice. (*D*) Tg expression in *Penk*$^+$ and *Pdyn*$^+$ (but not in *Chat*$^+$) cells in D2R-OE but not in CON mice. **P* < 0.05; ***P* < 0.01. All data are means ± SEM. (*E*–*G*, *Upper Images*) Representative images of fluorescent in situ hybridization for the Tg (red) and *Penk* (*E*), *Pdyn* (*F*), or *Chat* (*G*) (green) in the striatum of D2R-OE mice (see CON high-resolution images in *SI Appendix*, Figs. S2–S4). DAPI staining is in blue. (Scale bars: 500 μM.) (*E*–*G*, *Lower Images*) Zoomed-in views. (Scale bars: 100 μM.)

D2R-OE in the Striatum Accelerates Diet-Induced Obesity. Mice were fed chow from weaning and then assigned to chow or HFD in adulthood (Fig. 2*A*). D2R-OE did not affect BW on a chow diet across postnatal development (Fig. 2*B*). When fed HFD, however, D2R-OE mice markedly increased BW gain ($P < 0.01$ vs. wild-type and Tg-tetO-D2R mice; $P < 0.001$ vs. Tg-CamKII-α-tTA mice) (Fig. 2*C*), a difference not observed in chow-fed mice (Fig. 2*C*). D2R-OE increased fat mass ($P < 0.001$) (Fig. 2*D*) on both chow and HFD, indicating that metabolic abnormalities existed before exposure to HFD. There was a main effect of the Tg-CamKII-α-tTA Tg on lean mass ($P < 0.001$) (Fig. 2*E*) and on postnatal BW curves (mild effect size; $P < 0.01$) (Fig. 2*B*), while the Tg-tetO-D2R Tg had no influence. We thus chose Tg-CamKII-α-tTA mice as littermate controls to control for Tg-CamKII-α-tTA effects. In a new cohort fed HFD chronically, we confirmed the effects of D2R-OE on diet-induced obesity (DIO) vs. Tg-CamKII-α-tTA controls (CON) ($P < 0.01$) (Fig. 2*F* and *SI Appendix*, Fig. S5) and showed that these effects emerged rapidly (1 wk) upon HFD onset.

We then found that D2R-OE was accompanied by signs of glycemic dysregulation and hyperlipidemia, as evidenced by increased plasma glucose levels in an oral glucose tolerance test (oGTT) ($P < 0.01$ at 30 and 90 min, $P < 0.001$ at 60 min) (Fig. 2*G*) and insulin sensitivity test (IST) ($P < 0.01$ at 15 min) (Fig. 2*H*) and increased fed-state levels of plasma insulin ($P < 0.05$), triglycerides ($P < 0.05$), and cholesterol ($P < 0.01$) but not glucose or leptin (*SI Appendix*, Fig. S6).

D2R-OE in the Striatum Modulates Energy Balance. We aimed at identifying the physiological mechanisms underlying the rapid onset of DIO in D2R-OE mice. D2R-OE markedly reduced gross food intake in metabolic cages both before HFD initiation and 3 wk following ($P < 0.05$) (Fig. 3*A*) but not at HFD initiation, possibly due to its novelty and hedonic properties. We confirmed this effect in a group-housed home-cage setting ($P < 0.001$) (Fig. 3*B*). Daily locomotor activity was reduced in D2R-OE mice after 1 ($P < 0.05$) and 3 ($P < 0.001$) wk on HFD but not before HFD exposure (Fig. 3*C*). Locomotion in the open field was unaltered by D2R-OE (*SI Appendix*, Fig. S7), indicating intact locomotor function. Importantly, D2R-OE led to an overall reduction in energy expenditure (EE) [main genotype (GT) effect: $P < 0.01$] (Fig. 3*D*) which correlated with gross food intake ($r = 0.75$, $P < 0.001$ in CON vs. $r = 0.46$, $P < 0.05$ in D2R-OE mice) (*SI Appendix*, Fig. S8). Reductions in EE appeared before the emergence of BW differences (Fig. 2 *C* and *F*) and thus could represent a major predisposition factor for obesity in D2R-OE mice. Because locomotor activity was unchanged at such time points, we speculated that EE differences arose from defects in BAT thermogenesis, a sympathetically driven mechanism that produces heat through oxidation of lipids (28) and can be recruited by HFD (28, 38, 39). We observed an overall reduction in interscapular skin temperature [an indicator of BAT thermogenesis (40)] in D2R-OE mice (main GT effect: $P < 0.05$) (Fig. 3 *E* and *G*). We observed the

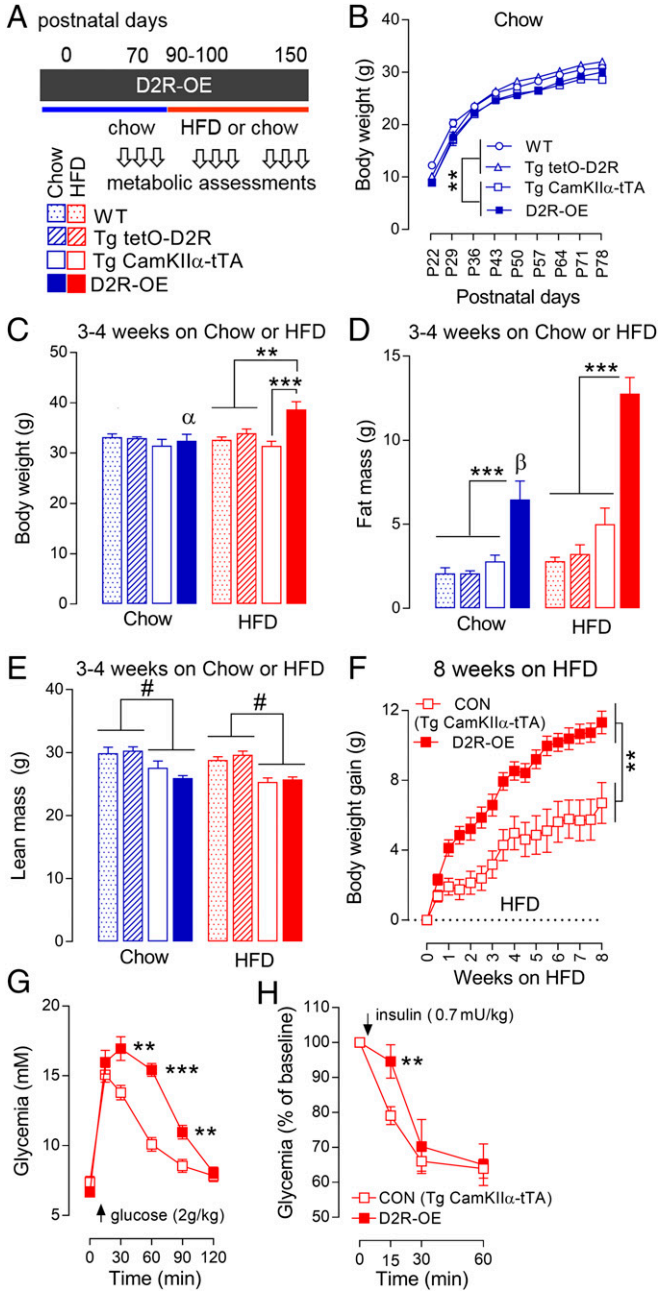

Fig. 2. D2R-OE in the striatum accelerates DIO. (*A*) Experimental design. Mice were exposed to HFD in adulthood. (*B*) D2R-OE did not affect BW across postnatal development (chow diet). Tg CaMKIIα-tTA affected BW. $^{**}P < 0.01$: post hoc GT effects. (*C*–*E*) D2R-OE increased BW (*C*) and fat mass (*D*) on both chow and HFD but did not affect lean mass (*E*). Tg CaMKIIα-tTA affected lean mass. $^{**}P < 0.01$; $^{***}P < 0.001$: post hoc GT effects among a diet. α: $P < 0.05$; and β: $P < 0.01$: post hoc diet effects among a GT. $^{\#}P < 0.001$: main GT effect. (*F*) D2R-OE increased BW gain vs. Tg CaMKIIα-tTA controls (CON). $^{**}P < 0.01$: main GT effect. (*G* and *H*) D2R-OE led to elevated plasma glucose in an oGTT at 6-wk HFD ($^{**}P < 0.01$; $^{***}P < 0.001$: post hoc GT effect) (*G*) and in an IST at 7-wk HFD ($^{**}P < 0.01$: a priori post hoc GT effect) (*H*). All data are means ± SEM.

same trend in whole-body skin temperature (main GT effect: $P = 0.05$) (Fig. 3 *F* and *G*). Histological assessment of H&E-stained BAT samples showed larger lipid droplets in D2R-OE mice (Fig. 3*H*), indicative of reduced thermogenic activity. mRNA levels of uncoupling protein 1 (*Ucp1*), the primary gene for BAT thermogenesis, were also reduced in D2R-OE mice ($P < 0.05$) (Fig. 3*I*), while other thermogenic mRNAs were unaffected (Fig. 3*I*). Taken together, our findings indicate that D2R-OE in the striatum leads to reduced EE and reduced BAT thermogenic capacity, which in turn are likely to accelerate the development of DIO.

Developmental D2R-OE in the Striatum Is Sufficient to Potentiate DIO. A growing body of evidence indicates that developmental processes play an important role in obesity etiology (30), while D2Rs can modulate brain development and maturation (31–34). We thus evaluated whether obesity in D2R-OE mice emerged from developmental D2R-OE. Tg expression was maintained until early adulthood and then was shut off by doxycycline (DOX) (Fig. 4*A* and *SI Appendix*, Fig. S9). One week of DOX almost completely shut off Tg expression, and 2 wk shut it off fully (Fig. 4*B*), in line with refs. 19 and 20. D2R-OE reduced gross food intake, independently of DOX (main GT effect: $P < 0.01$) (Fig. 4*C*). D2R-OE mice fed HFD also gained BW faster than CON mice, independently of DOX (main GT effect: $P < 0.001$) (Fig. 4*D*), indicating developmental effects of D2R-OE. Of note, BW differences emerged after 1 wk on HFD, i.e., after 2 wk of adult DOX treatment, a time point at which both D2R protein and mRNA have returned to normal (Fig. 4*B* and refs. 19 and 20). Finally, D2R-OE increased fat mass, decreased lean mass, and decreased interscapular skin temperature [main GT effects: $P < 0.001$ and $P < 0.001$) (Fig. 4*E*) and $P < 0.001$ (Fig. 4 *F* and *G*)], phenotypes that were all independent of DOX treatment. Taken together, these data indicate that developmental overexpression of D2Rs in the striatum is sufficient to modulate food intake and BAT thermogenesis and in turn to promote DIO.

Discussion

Striatal D2Rs have been one of the key neurotransmitter markers under investigation in obesity research in the past few decades (1–3). However, it remained unclear whether variations in striatal D2Rs modulate the risk for obesity and, if so, in which direction (3–18). Here we show that overexpressing D2Rs in striatal MSNs robustly increases BW gain and body fat but does so primarily when mice are fed with HFD. These changes are accompanied by signs of glycemic dysregulation and hyperlipidemia. Such findings align with Kim et al. (41) showing that D2R constitutive knockout mice are 20% lighter than controls. Our findings are also consistent with previous work identifying a trend for a positive correlation between preexisting D2R availability levels and future weight gain in mice (16). Hence, elevated, rather than reduced, levels of striatal D2Rs are pathologically involved in BW gain.

This possibility might seem at odds with previous work in D2R knockdown (D2R-KD) mice, which develop obesity upon exposure to HFD and running wheels (26). One main difference is that the D2R-KD effects (26) could be mediated by loss of D2Rs in other striatal cell types (e.g., cholinergic neurons) or outside the striatum (e.g., midbrain, pancreas, muscle); for example, pancreatic D2Rs modulate metabolism (42). Here, in contrast, D2Rs were selectively overexpressed in striatal MSNs; the highest percentage was found in iMSNs (40%), but dMSNs also expressed D2Rs to a lesser extent (26%). A D1R/D2R coexpression of 26% is likely higher than under wild-type conditions [the exact number is still under debate (43, 44)]. Therefore, we cannot exclude the possibility that the obesity phenotype of D2R-OE mice may be related to higher dMSN D2R levels. Future studies using viral overexpression of D2Rs in iMSNs could help rule out this possibility, but viruses would need to be injected during development, given our current developmental findings. In addition, to the best of our knowledge, it is currently unknown whether alterations in D2R levels in human obesity or rodent models are restricted to iMSNs or if pathology-related changes also involve dMSNs. Striatal MSNs have the capacity to coexpress D1Rs and D2Rs (43–45). There is also precedence for pathology-associated

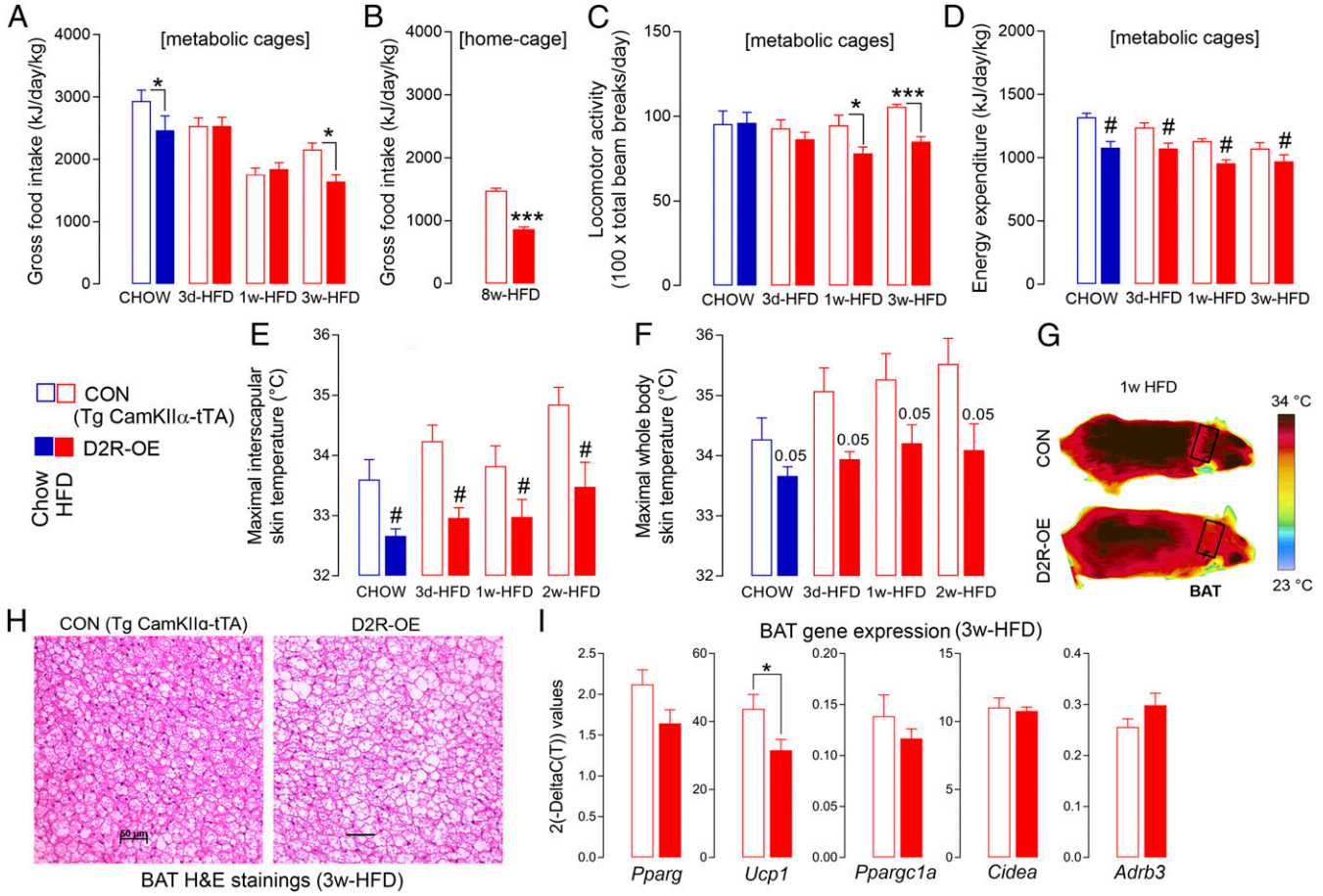

Fig. 3. D2R-OE in the striatum modulates energy balance. (*A*) D2R-OE reduced gross food intake on chow and at 3-wk HFD. $^{*}P < 0.05$: post hoc GT effects. w, weeks. (*B*) D2R-OE reduced daily home cage gross food intake. $^{***}P < 0.001$. (*C*) D2R-OE reduced daily locomotor activity at 1- and 3-wk HFD. $^{*}P < 0.05$; $^{***}P < 0.001$: post hoc GT effects. (*D*) D2R-OE reduced daily EE across time. $^{\#}P < 0.01$: main GT effect. (*E*) D2R-OE reduced maximal interscapular skin temperature. $^{\#}P < 0.05$: main GT effect. (*F*) D2R-OE nonsignificantly reduced maximal body skin temperature: $P = 0.05$: main GT effect. (*G*) Representative infrared images of skin temperature. (*H*) Representative images of H&E-stained BAT sections. Note the larger intracellular lipid droplets in D2R-OE mice. (Scale bars: 50 µM.) (*I*) D2R-OE reduced mRNA expression of *Ucp1* but not of other thermogenic genes. $^{*}P < 0.05$. All data are means ± SEM.

changes in DA receptor expression patterns beyond normal anatomical boundaries (46, 47). Future postmortem studies could investigate the cellular specificity of D2R abnormalities in the obese state.

We also determined the physiological mechanisms responsible for obesity in D2R-OE mice. D2R-OE led to hypophagia, consistent with a previous report in striatal D2R-KD rats (17). Importantly, D2R-OE decreased EE, a reduction that preceded the onset of HFD and BW gain. D2R-OE also decreased locomotor activity at 1 and 3 wk of HFD, which contrasts with the locomotor-inhibiting effects of iMSN-D2R-knockout after longer HFD exposure (16). These observations indicate that (*i*) D2R-OE mice develop obesity due to reduced energy output, (*ii*) EE other than locomotion drives the initial BW gain, and (*iii*) locomotor deficits may contribute to BW gain at later time points.

Notably, our results also indicate a link between striatal D2Rs and BAT function, adding to available pharmacological data (27). BAT thermogenesis is an essential mechanism for the regulation of EE in rodents, in human newborns (28), and potentially also in human adults (29). Although still under debate, BAT thermogenesis is thought to contribute to offsetting calorie excess (28); for example, HFD stimulates BAT thermogenesis (28, 38, 39), and mice lacking functional BAT become obese (38). We observed that changes in EE occurred before and throughout HFD treatments, including at day 3 post-HFD when no differences in other energy-consuming behaviors emerged [i.e., food intake (thermic effect of food) and locomotion]. Changes in EE therefore likely emerged from defective BAT thermogenesis, as supported by our findings showing reduced interscapular skin temperature, enlarged BAT lipid droplets, and reduced BAT *Ucp1* expression.

Future studies should aim at investigating the cellular and circuit mechanisms linking striatal D2Rs, BAT, and EE. One possibility includes the lateral hypothalamus, which receives direct and indirect projections from MSNs (48–50), is known to regulate BAT activity (51), and, when impaired, can promote obesity even in the face of reduced eating (51), similar to D2R-OE. In addition, D2R-OE leads to increased intrinsic excitability of MSNs (31), a phenotype that has been linked to obesity risk (52) and that also could be relevant in this model.

Finally, we show that developmental D2R-OE is sufficient to induce DIO and impair BAT function. This indicates that early disruption of striatal D2Rs can lead to persistent changes in energy metabolism and thus may represent a key predisposition factor toward obesity. Our study also adds to the growing body of evidence indicating that D2Rs can act as developmental modulators (31–34). For example, excess D2R activation during adolescence impairs dendritic spine morphogenesis and working memory (32). Of note, although the CamKII-α protein is mostly specific to postnatal development (53), the D2R Tg was detected at E17.5,

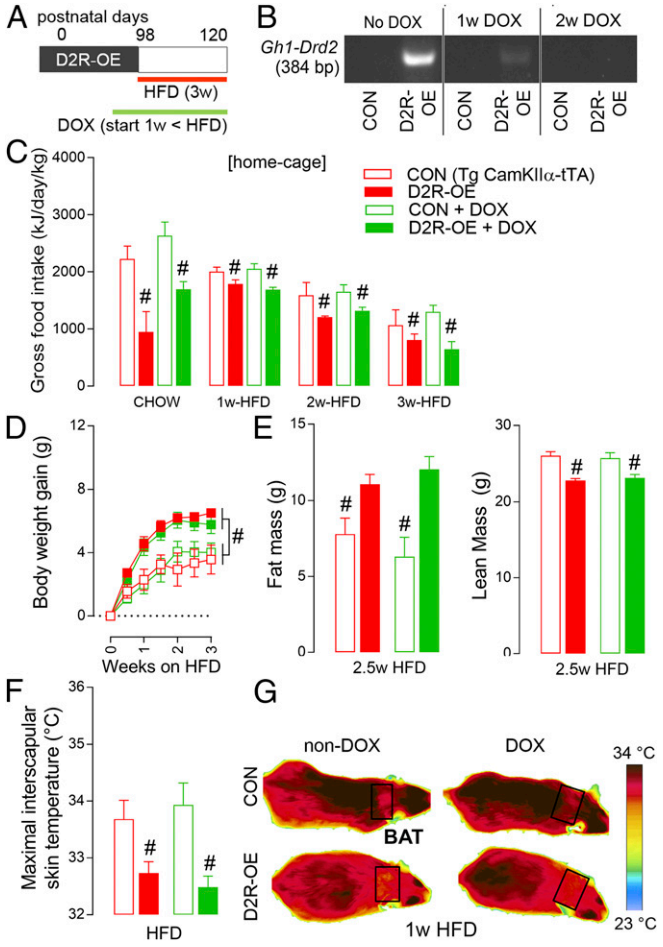

Fig. 4. Developmental D2R-OE in the striatum is sufficient to potentiate DIO. (*A*) DOX treatment timeline. (*B*) PCR (*Gh1-Drd2* Tg) shows that DOX shuts off Tg expression. All lanes are from same gel. (*C*–*F*) The effects of D2R-OE were independent of DOX; *P* values: main GT effects. D2R-OE reduced gross food intake on chow and on HFD ($^{\#}P < 0.01$) (*C*), increased BW gain on HFD ($^{\#}P < 0.001$) (*D*), increased fat mass and reduced lean mass ($^{\#}P < 0.001$) (*E*), and reduced interscapular skin temperature ($^{\#}P < 0.001$) (*F*). (*G*) Representative infrared images of skin temperature. w, weeks. All data are means ± SEM.

albeit at lower levels (20). Therefore, the effects observed herein may reflect late prenatal and/or postnatal brain maturation.

We can speculate on potential candidate mechanisms. Developmental D2R-OE may induce local remodeling within the striatum in the form of persistent changes in function and/or neuronal rewiring. Striatal circuits continue to mature throughout childhood and adolescence (34, 54–57) and thus could represent sensitive targets following early-life D2R-OE. For instance, MSN intrinsic excitability decreases across postnatal life (55), a parameter that is altered in D2R-OE mice (31). D2R-OE also leads to rewiring of striatal output pathways (58); however, like the functional changes in excitability (31), these effects do not persist after switching off the Tg in adulthood (31, 58) and thus cannot fully explain the obesity phenotype. A second possibility includes proximal alterations within the hypothalamus, i.e., the key area for energy balance, which is not fully mature until week 4 of age in rodents (30). Proximal alterations may also occur within the ventral tegmental area, which participates in energy-balance regulation (59), provides direct projections to the striatum, and is sensitive to the effects of D2R in development (33). Finally, the persistent effects of developmental D2R-OE may involve distal remodeling at the level of the BAT, which continues to mature during postnatal life, reaching full thermogenic capacity only at weaning (30). Future studies should aim at addressing some of these possibilities in developmental D2R-OE mice. One first step could include restricting D2R Tg expression to a shorter developmental window by feeding DOX at different time points (e.g., in late and early adolescence, at weaning, or early postnatally), thus narrowing the critical period.

Obesity and its comorbidities are now the leading causes of preventable deaths in the United States. With 40% of adults and 20% of children meeting criteria for obesity, the need to understand the causes and early determinants has never been greater (60). Many human studies have suggested that striatal D2Rs may be important for obesity etiology (3–14), but thus far no animal study has outlined an unambiguous causal relationship. Our work now indicates that high striatal D2R during development increases the risk for obesity in the mouse. Clinical investigations could thus direct future efforts toward lean or mildly obese children and adolescents, in which D2R levels might be particularly important, whereas extreme obesity might be less relevant for understanding D2R-related disease etiology. Our study also reveals that obesogenic diets are necessary to reveal the full effects of D2R on obesity in the mouse, so that diet could be integrated in future clinical analyses as a cofactor. Finally, there has been considerable focus on eating as the primary cause of obesity (61); our work highlights the importance of also including locomotor activity and EE (62) in future human studies on this topic.

Deciphering cause from consequence in human diseases is a daunting task, in particular in the face of chronic, gradual, and multifactorial diseases such as obesity. Here we provide evidence that developmental D2R-OE in mice persistently increases the propensity for obesity by reducing energy output. We anticipate future work in animals will help identify the underlying mechanisms and hope these findings will contribute to understanding the role of D2Rs in human obesity.

Materials and Methods

Animals and Tg Expression. Transgenic male D2R-OE mice (20) and littermate controls were fed chow (16.1 kJ/g; no. 3436; Kliba Nafag), a 60% HFD (19.3 kJ/g; D12492; Ssniff), or the same diets supplemented with DOX (40 mg/kg). Gross food intake (which includes the nondigestible component) was measured manually. All procedures were approved by the New York State Psychiatric Institutional Animal Care and Use Committees or the Cantonal Veterinarian's Office of Zurich. Transgenic expression was determined by (*i*) oligo in situ hybridization (20), (*ii*) fluorescent multiplex in situ hybridization (RNAscope kit v2.0; Advanced Cell Diagnostics), and (*iii*) PCR for the *Gh1-Drd2* Tg sequence (primers are given in *SI Appendix*, Table S1).

Metabolic Analyses. Body composition was assessed using a quantitative NMR scanner (Echo Medical Systems) (63). Metabolic cages consisted of an automated open-circuit indirect calorimetry system combined with infrared photobeams for locomotion (PhenoMaster; TSE Systems) (63). Data were averaged from 2 d of recording after a 2-d acclimatization period. Exploratory locomotion was assessed in a 30-min open field test. Glycemic regulation was assessed using an oGTT (2 g/kg glucose) and an IST (0.7 mU/kg insulin). Plasma metabolites were measured using enzymatic reaction kits (DiaTools) and plasma hormones using electrochemiluminescence assays (MSD) (63).

BAT Analyses. Surface interscapular and body temperatures were determined using an infrared camera (E60; FLIR Tools) (40). BAT histology was conducted on paraformaldehyde-fixed, paraffin-embedded slices stained with H&E (63). Expression of thermogenic genes was analyzed using the RNeasy Lipid Tissue Mini Kit (QIAGEN) and qRT-PCR (CFX384; Bio-Rad). Primers are given in *SI Appendix*, Table S1.

Statistical Analyses. All data were analyzed with StatView (SAS) using an unpaired Student's *t* test (two-tailed) (Welch's correction if unequal variance) or parametric ANOVAs followed by Fisher's least significant difference post hoc test if interactions were significant. *SI Appendix*, Tables S2–S5 show all statistical results.

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