Disruption of the Dopamine D2 Receptor Impairs Insulin Secretion and Causes Glucose Intolerance

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The relationship between antidopaminergic drugs and glucose has not been extensively studied, even though chronic neuroleptic treatment causes hyperinsulinemia in normal subjects or is associated with diabetes in psychiatric patients. We sought to evaluate dopamine D2 receptor (D2R) participation in pancreatic function. Glucose homeostasis was studied in D2R knockout mice (*Drd2*$^{-/-}$) mice and in isolated islets from wild-type and *Drd2*$^{-/-}$ mice, using different pharmacological tools. Pancreas immunohistochemistry was performed. *Drd2*$^{-/-}$ male mice exhibited an impairment of insulin response to glucose and high fasting glucose levels and were glucose intolerant. Glucose intolerance resulted from a blunted insulin secretory response, rather than insulin resistance, as shown by glucose-stimulated insulin secretion tests (GSIS) *in vivo* and *in vitro* and by a conserved insulin tolerance test *in vivo*. On the other hand, short-term treatment with cabergoline, a dopamine agonist, resulted in glucose intolerance and decreased insulin response to glucose in wild-type but not in *Drd2*$^{-/-}$ mice; this effect was partially prevented by haloperidol, a D2R antagonist. *In vitro* results indicated that GSIS was impaired in islets from *Drd2*$^{-/-}$ mice and that only in wild-type islets did dopamine inhibit GSIS, an effect that was blocked by a D2R but not a D1R antagonist. Finally, immunohistochemistry showed a diminished pancreatic β-cell mass in *Drd2*$^{-/-}$ mice and decreased β-cell replication in 2-month-old *Drd2*$^{-/-}$ mice. Pancreatic D2Rs inhibit glucose-stimulated insulin release. Lack of dopaminergic inhibition throughout development may exert a gradual deteriorating effect on insulin homeostasis, so that eventually glucose intolerance develops. **(*Endocrinology* 151: 1441–1450, 2010)**

The relationship between dopamine receptors (DR) and glucose homeostasis has not been extensively studied, even though some clinical findings suggest a connection. For example, administration of neuroleptic drugs, which block DR, causes hyperinsulinemia in normal subjects (1) or is associated with diabetes in psychiatric patients (2–4), and among various adverse reactions of atypical antipsychotics, weight gain and impaired glucose tolerance are clinically significant (5). Furthermore, treatment with the dopamine precursor L-DOPA in patients with Parkinson's disease reduces insulin secretion upon oral glucose tolerance test (6). In rodents, L-DOPA injection results in the accumulation of dopamine in pancreatic β-cells and an inhibitory action on insulin response to different secretagogues (7, 8), whereas in male rats treated with a dopamine antagonist, high glucose and insulin levels were ob-

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Abbreviations: BAC, Bacterial artificial chromosome; DR, dopamine receptor; EGFP, enhanced green fluorescent protein; GSIS, glucose-stimulated insulin secretion; IGTT, ip glucose tolerance test; ITT, insulin tolerance test; PCNA, proliferating cell nuclear antigen.

served (9). Insulin secretion is primarily controlled by metabolism-secretion coupling and is mainly regulated by glucose, but this process can be modulated by the central nervous system through parasympathetic and sympathetic nerves (10).

Dopamine exerts its actions by binding to specific membrane receptors, which belong to the family of seven transmembrane domain G protein-coupled receptors. Five distinct DR have been isolated, characterized, and subdivided into the D1- and D2-like subfamilies, on the basis of their biochemical and pharmacological properties (11). The D1-like subfamily comprises D1R and D5R, whereas the D2-like includes the D2R, D3R, and D4R. Some *in vitro* studies performed in isolated pancreatic islets suggested the participation of the D2R in insulin secretion (12–14), although the net effect of D2R stimulation observed in these studies and its functional importance remain controversial. In the present study, we sought to investigate the *in vivo* role of D2R in insulin secretion and glucose homeostasis. To this end, we studied β-cell function and glucose metabolism in mutant mice lacking D2R in comparison with their wild-type siblings. A battery of selective pharmacological tools was used to evaluate the *in vivo* and *in vitro* effects of D2R stimulation and blockade on insulin secretion and glucose metabolism in both wild-type and D2R-deficient mice (*Drd2*$^{-/-}$). The results presented herein show that even though pancreatic D2R are inhibitory to glucose-stimulated insulin secretion, permanent loss of D2R causes glucose intolerance.

Materials and Methods

Animals

Male *Drd2*$^{-/-}$ mice, official strain designation B6;129S2-*Drd2*^{tm1low} by the Induced Mutant Resource at The Jackson Laboratory (Bar Harbor, ME), generated by targeted mutagenesis of the *Drd2* gene in embryonic stem cells (15, 16), were used. The original F_2 hybrid strain (129S2/Sv $\times$ C57BL/6J) containing the mutated *Drd* allele was backcrossed for at least 10 generations to wild-type C57BL/6J mice. Mutant and wild-type mice were the product of heterozygote crossings, and in all cases, control siblings were used. Mice of mixed genotypes were housed in groups of four or five in a temperature-controlled room with lights on at 0700 h and lights off at 1900 h and free access to laboratory chow and tap water. Animals were weighed and used at 2 and 7 months of age. We chose these two age groups to determine whether disruption of the D2R had a developmental effect on glucose homeostasis. Furthermore, prolactin and GH levels are similar between genotypes at 2 months of age (17), whereas at 7 months, prolactin levels are markedly increased, and pituitary GH release is decreased (17, 18).

Transgenic mice carrying an engineered bacterial artificial chromosome (BAC) in which coding sequences of the enhanced green fluorescent protein gene (EGFP) are under the transcriptional control of the mouse D2R gene (*Drd2*) were used to label D2R in pancreatic cells. These *Drd2*-EGFP mice were originally generated by the GENSAT (Gene Expression Nervous System Atlas) project at the Rockefeller University (New York, NY) and the National Institute of Neurological Disorders and Stroke, National Institutes of Health (Bethesda, MD) (19) and maintained in an outbred Swiss-Webster genetic background.

All experimental procedures were reviewed and approved by the institutional animal care and use committee of the Instituto de Biología y Medicina Experimental, Buenos Aires [in accordance with the Division of Animal Welfare, Office for Protection from Research Risks, National Institutes of Health, (A#5072-01)].

Reagents

Unless otherwise specified, all chemicals were purchased from Sigma (St. Louis, MO).

Intraperitoneal glucose tolerance test (IGTT)

IGTT was performed in conscious male *Drd2*$^{-/-}$, wild-type, and *Drd2*$^{-/+}$ mice at 2 and 7 months of age. Briefly, after overnight fasting (12 h), an ip injection of glucose (2 mg/g body weight) was administered. Blood glucose levels (2 μl obtained from the tail of each mouse) were examined at 0, 15, 30, and 60 min after glucose injection with a hand-held glucose monitor (Ascensia Breeze, Bayer, Toronto, Ontario, Canada).

IGTT was also performed in cabergoline-pretreated mice of both genotypes. To this effect, cabergoline (2 mg/kg ip; Beta Laboratories, Buenos Aires, Argentina) was administered 1 h before IGTT (2 mg/g ip glucose). Glucose levels were measured before glucose administration (time 0) and 30 and 60 min thereafter. Insulin was measured at time 0 and 30 min. A group of animals was pretreated with haloperidol (3 mg/kg, ip) 30 min before cabergoline. The difference between pre- and postglucose levels was calculated as glucose level at 30 or 60 min minus glucose basal level (time 0). The doses of haloperidol and cabergoline were selected from our own previous experience and reports in the literature detailing their effect on DR subtypes (16, 20–22).

Glucose-stimulated insulin secretion (GSIS)

To examine glucose-stimulated insulin secretion, 12-h-fasted mice were used. Blood was collected from the tail vein before (0 min) and 5, 15, and 30 min after glucose administration (3 mg/g). Serum samples were immediately obtained by centrifugation at 3000 rpm for 10 min and stored at −20 C. Insulin secretion levels were assessed by a sensitive rat insulin ELISA kit (Crystal Chem, Chicago, IL).

GSIS was studied on isolated islets from 7-month-old male mice of both genotypes. Hand-picked islets were isolated after an intraductal collagenase V injection, as previously described (23). Pools of five islets were incubated in 250 μl RPMI 1640 medium, supplemented with 10% fetal bovine serum containing 2.8 mM glucose for 2 h, and then incubated with 0, 6.25, 12.5, or 25 mM glucose; 25 mM glucose plus dopamine hydrochloride (10^{-5} or 10^{-8} M) or 25 mM glucose plus dopamine hydrochloride (10^{-5} M) plus the D2R antagonist (−)sulpiride (10^{-5} M Vipral; Laboratorios Roemmers, Buenos Aires, Argentina); or 25 mM glucose plus dopamine hydrochloride (10^{-5} M) plus the D1R antagonist SCH 23390 (1-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5 tetrahydro-^{1}H-3-benzazepine (10^{-5} M) for 1 h. Insulin secretion levels were assessed by RIA. Insulin output was normalized to

the respective insulin content of the islets. To measure insulin content, tubes were centrifuged for 10 min at 800 × *g*, the supernatant was discarded, and acid-ethanol was added (250 μl/sample; 87.5% vol/vol ethanol plus 12.5% vol/vol 2 N HCl). Samples were kept overnight at 4 C, centrifuged for 10 min at 800 × *g*, and the pellet was discarded. Tris base (85 μl, 0.85 M) was added, and samples were stored at −20 C until measurement.

Intraperitoneal insulin tolerance test (ITT)

Mice were fasted for 2 h and then injected ip with human insulin (Humulin 1 U/kg body weight; Eli Lilly, Toronto, Canada). Blood glucose levels were measured at 0, 15, 30, 60, and 120 min.

RIA

Prolactin was measured by RIA using a kit provided by the National Institute of Diabetes and Digestive and Kidney Diseases (Dr. A. F. Parlow, National Hormone and Pituitary Program, Torrance, CA). Results are expressed in terms of mouse prolactin standard RP3. Intra- and interassay coefficients of variation were 7.2 and 12.8%, respectively.

For IGF-I RIA, serum samples (15 μl) and IGF-I standards were subjected to the acid-ethanol cryoprecipitation method as previously described (24). IGF-I was determined using antibody (UB2-495) provided by Drs. L. Underwood and J. J. Van Wyk and distributed by the Hormone Distribution Program of the National Institute of Diabetes and Digestive and Kidney Diseases. Recombinant human IGF-I (Chiron Corp., Emeryville, CA) was used as radioligand and unlabeled ligand. The assay sensitivity was 6 pg/tube. Intra- and interassay coefficients of variation were 8.2 and 14.1%, respectively.

A specific insulin RIA was used as described previously (25) using human insulin for iodination and standard (Beta Laboratories), and anti-bovine insulin antibody (Sigma). The minimum detectable concentration was 2 ng, and the intra- and interassay coefficients of variation were 6.8 and 9.1%, respectively. Pancreatic tissue, 50 mg, was homogenized using a Polytron in 1 ml ice-cold acidic alcohol (0.18 N HCl/70% ethanol), incubated overnight at 4 C, and centrifuged at 12,000 rpm for 5 min. The supernatants were used for determination of insulin concentration by RIA. Results were normalized to the protein content of samples determined by Qubit Quant it protein assay kit (Invitrogen, Buenos Aires, Argentina) following manufacturer's instructions.

Immunohistochemistry

Pancreata from 2- and 7-month-old animals fixed in formalin were embedded in paraffin, and immunohistochemistry was performed using a modified avidin-biotin peroxidase method as previously described (26). Antibodies for insulin (polyclonal guinea pig antiinsulin antibody, 1:200 dilution; Abcam, Cambridge, MA), glucagon (rabbit antiglucagon, 1:200 dilution; Santa Cruz Biotechnologies, Santa Cruz, CA). Proliferating cell nuclear antigen (PCNA) (monoclonal mouse anti-PCNA antibody 1:20; Dako, Carpinteria, CA) and EGFP (Abcam, Cambridge, UK) were used. Controls included substitution of primary antiserum with nonimmune serum. Appropriate secondary antibodies were chosen. Insulin and PCNA immunoreactivity were visualized using an avidin-biotin kit coupled to alkaline phosphatase (Vector Laboratories, Burlingame, CA), and glucagon and EGFP immunoreactivities were visualized using an avidin-biotin kit coupled to peroxidase (Vector). As chromogens, diaminobenzidine was used for glucagon and EGFP, Vector Blue AP substrate kit III (SK 5300) for PCNA, and Vector Red AP substrate kit I (SK 5100) for insulin. Tissue sections were counterstained with hematoxylin.

Morphometric analysis

Morphometric analysis was performed using a Carl Zeiss transmitted light microscope at a magnification of ×250 and ×400. Image analysis of pancreatic sections for calculation of tissue areas was performed by Northern Eclipse, version 6.0 software (Empix, Imaging, Mississauga, Ontario, Canada). The number of islets (defined as insulin-positive aggregates of at least 20 μm diameter) was scored and used to calculate the islet numerical density (number of islets per square centimeter of tissue). Islets less than 5000 μm^2 were defined as small, those ranging from 5000–10,000 μm^2 as medium, and those exceeding 10,000 μm^2 as large. Mean islet size was calculated as the ratio of the total insulin cell area to the total islet number on the sections. The β-cell fraction was determined as the ratio of the insulin-positive cell area to the total tissue area on the entire section. The α-cell area was calculated by counting glucagon-positive cell area. The β-cell mass was obtained by multiplying the β-cell fraction by the pancreas weight. The number of cells that showed nuclear staining for PCNA was related to the total β- or α-cell area within the same sections. Data were calculated from three sections of each pancreas, representing the entire pancreas for each animal (head, body, and tail). Approximately 70–120 islets per section were analyzed. Four animals were studied per genotype and age, unless otherwise stated in the figure legends.

Statistical analysis

Plasma glucose, insulin, prolactin, and IGF-I titers and all morphometric data are expressed as means ± SEM. The differences between means were analyzed by the unpaired Student's *t* test (in the case of only two groups) or by two-way ANOVA followed by Newman-Keuls test or Tukey's honestly significant difference test for unequal n. Two-way ANOVA with repeated-measures design was used to analyze GSIS (effects of drug and genotype). $P < 0.05$ was considered significant.

Results

Body weight was decreased in 7-month-old $Drd2^{-/-}$ male mice compared with their normal wild-type littermates (23.4 ± 0.08 *vs.* 27.1 ± 0.5 g, $P = 0.045$). Serum prolactin levels were significantly increased in $Drd2^{-/-}$ *vs.* wild-type mice (154.1 ± 29.1 *vs.* 6.0 ± 0.2 ng/ml, $P = 0.023$), and even though there were no significant differences in circulating levels of GH, IGF-I concentration was lower in $Drd2^{-/-}$ mice (815 ± 61 *vs.* 1325 ± 89 ng/ml, $P = 0.035$).

Glucose and insulin responses to glucose overload *in vivo* in $Drd2^{-/-}$ mice and wild-type littermates

We investigated the impact of the lack of D2R on glucose homeostasis *in vivo* in male mice. Fasting glucose

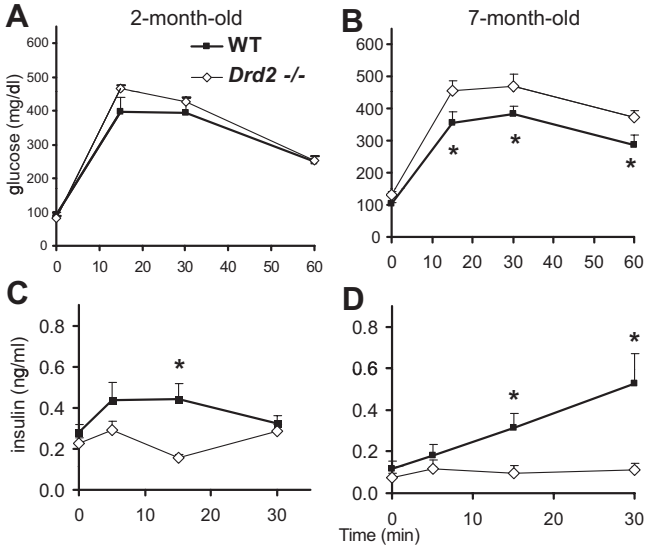

FIG. 1. A and B, Glucose and insulin response *in vivo*. IGTT (2 mg/g) in mice of both genotypes (WT, wild-type) at 2 (A) and 7 (B) months of age. n = 5–8 for each group. Two-way ANOVA with repeated-measures design: *, $P < 0.05$ *vs.* time-matched $Drd2^{-/-}$ mice. C and D, Insulin secretion in response to glucose (3 mg/g) in mice of both genotypes at 2 (C) and 7 (D) months of age. n = 4 and 4 (C) and 11 and 13 (D) for wild-type and $Drd2^{-/-}$ mice. *, $P < 0.05$ *vs.* time-matched $Drd2^{-/-}$ mice.

levels in 7-month-old $Drd2^{-/-}$ mice were significantly higher than in wild-type mice (127 ± 6 *vs.* 105 ± 6 mg/dl, $P = 0.040$; n = 12 and 13, respectively), whereas no significant differences were found in fasting insulin levels (0.07 ± 0.02 *vs.* 0.12 ± 0.04 ng/ml, $P = 0.36$; n = 15 and 14). In 2-month-old $Drd2^{-/-}$ mice, glucose response to IGTT was similar to that observed in wild-type mice (Fig. 1A). However, 7-month-old $Drd2^{-/-}$ mice showed relative glucose intolerance; blood glucose levels were significantly higher than those observed in wild-type littermates 15, 30, and 60 min after the ip glucose load (Fig. 1B, $P = 0.0012$). On the other hand, basal glucose levels and IGTT in 7-month-old heterozygotes ($Drd2^{-/+}$) were similar to those of wild-type mice (Supplemental Fig. 1, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org), and therefore, the rest of the experiments were performed using only $Drd2^{-/-}$ and wild-type mice.

To examine the insulin secretory response to glucose *in vivo*, 2- and 7-month-old mice of both genotypes were used after a 12-h fasting period. Serum insulin concentrations were measured before (time 0) and 5, 15, and 30 min after glucose injection (3 mg/g, ip). In wild-type 2-month-old mice plasma insulin concentrations rose 1.75-fold at 15 min, whereas no increase was observed in $Drd2^{-/-}$ mice (Fig. 1C; $P = 0.028$, $Drd2^{-/-}$ *vs.* wild-type at 15 min). In 7-month-old wild-type mice, there was a 2.7- and 4.6-fold increase in serum insulin concentration 15 and 30 min after glucose administration, whereas $Drd2^{-/-}$ mice displayed nonsignificant changes in insulin levels at all times [$P = 0.049$ and 0.020, wild-type *vs.* time-matched (15 and 30 min) $Drd2^{-/-}$ mice; Fig. 1D].

Adult female $Drd2^{-/-}$ mice also evidenced glucose intolerance and reduced insulin release evoked by glucose (Supplemental Fig. 2). Nevertheless, because time course of glucose elevation was slightly different and cycling pattern of ovarian hormones might modify responses in the wild-type group, we performed the rest of the studies in male mice.

Glucose and insulin response to IGTT *in vivo* in both genotypes in the presence of dopamine agonists and antagonists

We next sought to determine whether short-term administration of the D2-like agonist cabergoline could modify glucose homeostasis *in vivo*. Cabergoline (2 mg/kg) did not modify blood glucose levels significantly in fasted wild-type or $Drd2^{-/-}$ mice (not shown). Nevertheless, administration of cabergoline caused relative glucose intolerance in wild-type mice (Fig. 2A) but not in $Drd2^{-/-}$ mice (Fig. 2B). Blood glucose levels after an IGTT were increased by pretreatment with cabergoline in wild-type but not in $Drd2^{-/-}$ mice in comparison with saline-pretreated mice ($P = 0.042$ and 0.00040, cabergoline *vs.* time matched control at 30 and 60 min, respectively, in wild-type mice). Glucose levels decreased 60 min after glucose overload in $Drd2^{-/-}$ mice, either saline- or cabergoline-treated ($P = 0.000038$), and in saline-treated wild-type mice ($P = 0.014$) but remained elevated in wild-type cabergoline-treated mice ($P = 0.83$, Fig. 2, A and B).

Haloperidol, a dopamine antagonist, partially blocked glucose intolerance evoked by cabergoline at 60 min in wild-type animals; glu-

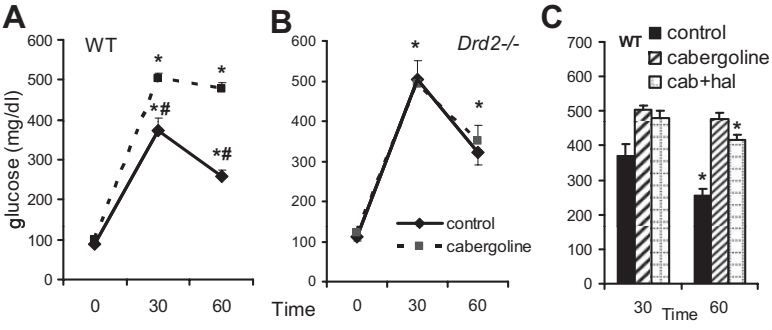

FIG. 2. IGTT in the presence of cabergoline and haloperidol. A and B, Effect of cabergoline (2 mg/kg) pretreatment on IGTT in wild-type (A) or $Drd2^{-/-}$ (B) mice. *, $P < 0.05$ *vs.* respective control; #, $P < 0.05$ *vs.* time-matched cabergoline-treated mice. n = 5 for wild-type mice and 6 for $Drd2^{-/-}$ mice. C, IGTT in wild-type mice in the presence of cabergoline or cabergoline plus haloperidol (3 mg/kg) (cab+hal). *, $P < 0.05$ *vs.* cabergoline-treated mice at 60 min. n = 4 for each group. WT, Wild type.

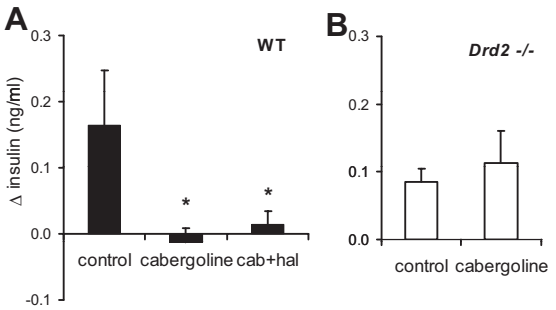

FIG. 3. Insulin response to IGTT and pretreatment with cabergoline. Insulin response (Δ insulin) 30 min after a glucose overload of 2 mg/g in wild-type (A) or *Drd2*$^{-/-}$ (B) mice in the presence or absence of cabergoline (2 mg/kg), or cabergoline plus haloperidol (3 mg/kg) (cab+hal). *, $P < 0.05$ *vs.* control; n = 4 for wild-type mice and 5 for *Drd2*$^{-/-}$ mice. WT, Wild type.

cose levels decreased at 60 compared with 30 min in wild-type and haloperidol- plus cabergoline-treated and not in cabergoline-treated mice ($P = 0.0014$, 0.0026, and 0.11 for time 60 compared with 30 min in wild-type, haloperidol- plus cabergoline-, and cabergoline-treated mice, respectively; Fig. 2C).

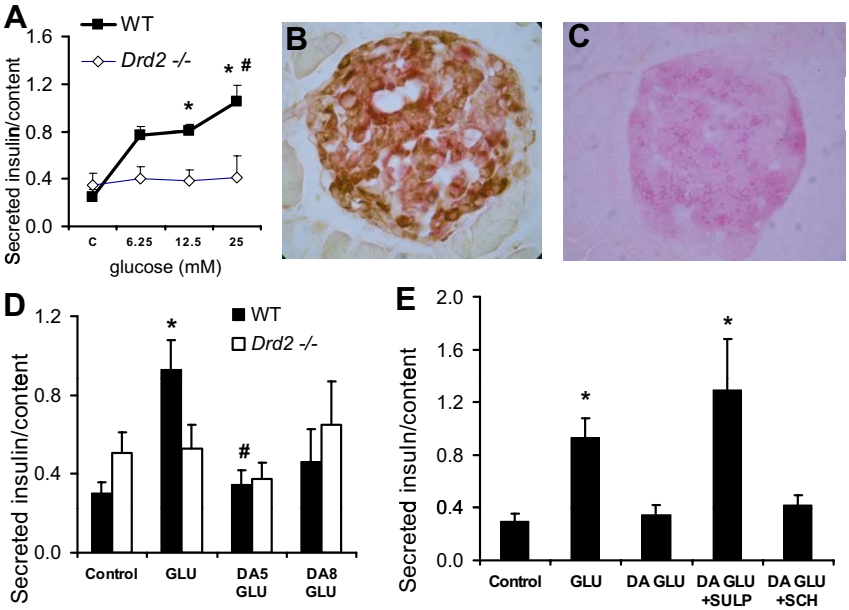

FIG. 4. *In vitro* GSIS. A, GSIS in isolated islets from wild-type (WT) and *Drd2*$^{-/-}$ mice. Results are expressed as insulin secretion/insulin content per incubation vial containing five islets each (n = 5 independent experiments). *, $P < 0.05$ *vs.* respective control value (no glucose added); #, $P < 0.05$ *vs.* respective concentration-matched islets obtained from *Drd2*$^{-/-}$ mice. B and C, Immunohistochemistry showing D2R in islets: B, Islet from a transgenic mouse with EGFP expression coupled to the D2R promoter (*Drd2*-EGFP mouse) with cells labeled with anti-EGFP antibody (and stained with diaminobenzidine) and cells stained with Vector red (labeled with insulin antibody); C, EGFP and insulin immunohistochemistry in pancreas from a wild-type animal with no EGFP expression. D, Effect of 10^{-5} and 10^{-8} M dopamine (DA5 and DA8) on 25 mM glucose (GLU)-induced insulin secretion in mouse islets from both genotypes. Results are the means of six experiments performed in triplicate. *, $P < 0.05$ *vs.* respective genotype-matched control; #, $P < 0.05$ *vs.* genotype-matched GLU. E, Effect of pretreatment with 10^{-5} M sulpiride (DA+GLU+SUL) or 10^{-5} M SCH23390 (DA+GLU+SCH) on 10^{-5} M dopamine inhibition (DA+GLU) of glucose-induced insulin secretion in mouse islets from wild-type animals. *, $P < 0.05$ *vs.* control; n = 5 experiments performed in triplicate.

Furthermore, the insulin response observed in wild-type mice after the glucose load (2 mg/g) was attenuated by cabergoline in wild-type mice ($P = 0.045$, Fig. 3A) but not in *Drd2*$^{-/-}$ mice (Fig. 3B). Pretreatment with 3 mg/kg haloperidol could not significantly prevent the cabergoline-induced decrease in insulin response (Fig. 3A).

In vitro insulin response to glucose

We next investigated insulin secretion in isolated islets from 7-month-old male mice of both genotypes. Glucose (12.5 and 25 mM) increased insulin secretion in islets from wild-type animals ($P = 0.049$ and 0.0011, respectively, *vs.* control; Fig. 4A) and not in *Drd2*$^{-/-}$ mice (P interaction for the effects of dose and genotype = 0.032). Insulin release in response to 25 mM glucose was significantly higher in islets obtained from wild-type compared with *Drd2*$^{-/-}$ mice ($P = 0.015$). Collectively, the *in vivo* and *in vitro* results show that *Drd2*$^{-/-}$ male mice have an impaired insulin response to glucose stimulation.

In vitro insulin response to glucose in the presence of dopamine agonists and antagonists

To determine the presence of D2R in pancreatic islets, we analyzed *Drd2*-EGFP transgenic mice, which express EGFP under the transcriptional regulation of the *Drd2* locus. We detected EGFP immunoreactivity colocalizing with insulin in pancreatic islets of *Drd2*-EGFP transgenic mice (Fig. 4B) and not in islets from wild-type mice, as expected (Fig. 4C).

We next studied the physiological impact of the absence of functional pancreatic D2R in pancreatic islets *in vitro*. GSIS was compared in islets obtained from mice of both genotypes and in the presence or absence of dopamine (10^{-5} and 10^{-8} M). A two-way ANOVA showed significant genotype × drug interaction ($P = 0.0033$). In islets from wild-type mice, glucose increased insulin secretion ($P = 0.0019$), and dopamine prevented this effect ($P = 0.0099$ for glucose *vs.* glucose plus 10^{-5} M dopamine, Fig. 4D). In contrast, neither glucose nor dopamine had any effect on GSIS in islets taken from *Drd2*$^{-/-}$ mice. Finally, we tested the effect of the D2R antagonist sulpiride or the D1R antagonist SCH 23390 on dopamine inhibition of GSIS in islets obtained from wild-type mice.

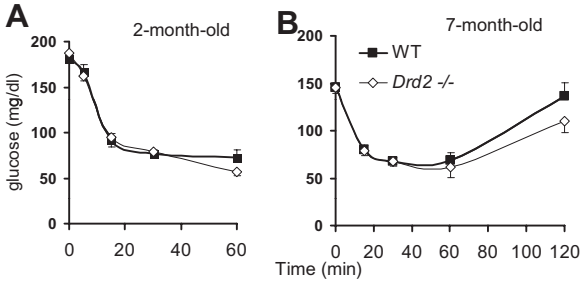

FIG. 5. ITT in fasted male mice of both genotypes at 2 (A) and 7 (B) months of age. Males were injected with 1 U/kg body weight human insulin, and blood glucose was measured at different times; n = 14 (WT) and 15 ($Drd2^{-/-}$) for 2-month-old and 18 and 20 for 7-month-old mice.

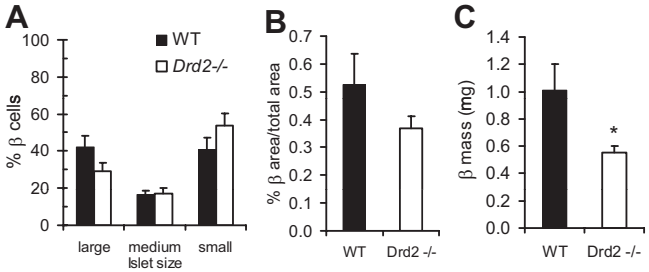

FIG. 6. Immunohistochemistry. A, Percentage of large (>10,000 μm^2), small (<5,000 μm^2), and medium (5,000–10,000 μm^2) islets in pancreas from 7-month-old wild-type (WT) and $Drd2^{-/-}$ mice; B, percentage of insulin immunoreactive area (β area) in relation to total pancreatic area of the section; C, β-cell mass (obtained by multiplying the β-cell fraction by the pancreas weight). Three different pancreas sections from each animal (n = 6 animals per genotype) were immunostained for insulin and glucagon and subjected to morphometric analysis. *, $P < 0.05$ *vs.* wild type.

As shown in Fig. 4E, sulpiride but not SCH 23390 prevented dopamine inhibition, indicating that this effect is mediated by D2-like receptors (glucose and glucose plus dopamine plus sulpiride were significantly different from the control group; $P = 0.0091$ and 0.0025, respectively). Neither dopamine nor sulpiride modified basal glucose release (not shown).

In vivo insulin action

To assess the effects of D2R deficiency on insulin action *in vivo*, we measured the changes in plasma glucose concentrations after a single ip injection of insulin. As shown in Fig. 5, A and B, glucose disappearance curves were comparable in both genotypes in 2- and 7-month-old mice. In 7-month-old mice, glucose was measured up to 120 min to evaluate glucose rebound, and no significant differences were found. In females, a similar glucose response to ip insulin was found in both genotypes (Supplemental Fig. 2D).

Islet and β-cell morphometry in $Drd2^{-/-}$ and wild-type mice and pancreatic insulin content

To further examine the impairment of insulin response to glucose observed both *in vivo* and *in vitro* in $Drd2^{-/-}$ mice, we performed a morphometric analysis of the pancreata of 7-month-old male mice of both genotypes. As shown in Table 1, the ratio of pancreas weight to body weight was not different between both genotypes, nor was islet density or average islet size. Islet density and distribution according to size were unaltered between genotypes (Fig. 6A). Percentage of β-cell area was not different between genotypes (Fig. 6B), but a significant decrease in absolute β-cell mass in 7-month-old $Drd2^{-/-}$ males compared with wild-type males was observed (Fig. 6C), which might be related to the tendency to lower pancreas size in $Drd2^{-/-}$ mice (Table 1). Furthermore, there was a decrease in pancreatic insulin concentration (nanograms per microgram protein) measured by RIA (Table 1). In contrast, we did not detect changes in α-cell mass or insulin to glucagon ratio between genotypes (Table 1).

Pancreatic β-cell proliferation

The preceding results suggested that the absence of D2R might be responsible for reduced β-cell mass and impaired insulin response to glucose, accounting for the subsequent impairment in glucose tolerance. To determine whether D2R might participate in the proliferation of β-cells within the islet, we examined costaining of PCNA with insulin or glucagon in pancreatic cell sections obtained from mice of both genotypes at 2 and 7 months of age. As shown in Fig. 7B, insulin cells undergoing replication, measured by PCNA-positive nuclei per given β-cell area, exhibited a 54% reduction in pancreas from 2-month-old $Drd2^{-/-}$ mice when compared with those of the wild-type pancreas, suggesting a decrease in β-cell proliferative capacity at this early stage. By 7 months, this decrease in β-cell proliferation was not evident (Fig. 7C). No differences in PCNA-positive cells per α-cell area were observed (not shown). Because at 2 months of age there was already a developmental effect related to the lack of D2R, we included immunohistochemistry data of a younger group (less than 1 month old) and could demonstrate that proliferation of β-cells (measured by the percentage of PCNA-stained nuclei in β-cells) was impaired already at 1 month (Fig. 7A).

Discussion

Even though the involvement and importance of dopamine as a neurotransmitter and neuromodulator in the regulation central nervous system function are well known, the effects of dopamine on insulin secretion and pancreatic β-cell function are poorly understood. The par-

TABLE 1. Effects of D2R deficiency on islet density, size, α-cell fraction and mass, insulin to glucagon ratio, and pancreatic insulin concentration in 7-month-old mice

	WT	*Drd2*$^{-/-}$	*P*
Pancreas weight (mg)	0.197 ± 0.014	0.156 ± 0.016	0.075
Pancreas weight/BW	6.5 ± 0.30	7.4 ± 0.39	0.12
Islet density (n/cm^2)	53.2 ± 18.6	45.8 ± 11.3	0.76
Mean islet size (μm^2)	17440 ± 5282	11975 ± 1728	0.38
α-Cell fraction (%)	0.091 ± 0.024	0.059 ± 0.005	0.26
Ratio insulin/ glucagon area	5.4 ± 0.6	5.0 ± 0.4	0.54
Pancreas insulin concentration (pg/μg protein)	8.82 ± 1.25	3.77 ± 0.77	0.017

For the first six parameters, n = 6 and 7; for insulin concentration measured by RIA, n = 7 and 5.

ticipation of the D2R on pancreatic function has been mainly studied using pharmacological agents with limited selectivity *in vivo*, or *in vitro* studies that preclude an integrative physiological analysis. To address the problem more directly, we combined the use of mice carrying targeted deletion of the D2R gene and different pharmacological agonists and antagonists, *in vivo* and *in vitro*. We report that the D2R play a crucial role in insulin secretion and glucose homeostasis; *Drd2*$^{-/-}$ male mice exhibit an impairment of insulin response to glucose overload and high fasting blood glucose levels, are glucose intolerant, and possess a reduced β-cell mass at 7 months of age. Decreased glucose tolerance was observed in *Drd2*$^{-/-}$ mice of both sexes, but we performed most experiments in male mice because cycling sexual hormones in females might influence glucose homeostasis (27, 28).

The reduction in β-cell mass, decreased insulin concentration, and a defect in glucose-sensing or stimulus-secretion coupling in the β-cells in D2R-deficient mice might explain the supra-normal rise in blood glucose levels that is evidenced after a glucose challenge. In turn, this reduction in pancreatic β-cell mass may be due to a decreased replication in earlier ages, as shown by the reduction in PCNA-positive β-cells in 1- and 2-month-old *Drd2*$^{-/-}$ mice. On the other hand, results of the glucose and insulin tolerance tests suggest that glucose intolerance is mainly caused by a blunted insulin secretory response rather than an increase in peripheral insulin resistance. This was confirmed by GSIS *in vivo* and *in vitro*. Nevertheless, the changes in glucose tolerance in *Drd2*$^{-/-}$ mice were modest; fasting blood sugar levels were 21% higher, and postinjection glucose levels were 22% higher than those in wild-type mice. Furthermore, *Drd2*$^{-/+}$ mice did not display an intermediate phenotype. In the absence of insulin resistance, it is likely that severe β-cell hypoplasia or dysfunction is required to produce severe glucose intolerance or overt diabetes.

In wild-type mice, insulin secretion in response to glucose *in vivo* was blocked by cabergoline pretreatment. Therefore, to determine the role of pancreatic D2R in the insulin response to glucose, islets from wild-type and *Drd2*$^{-/-}$ mice were incubated *in vitro* and subjected to GSIS in the presence or absence of dopamine. Glucose stimulated insulin release only in islets obtained from wild-type mice, and dopamine inhibited this effect. Furthermore, a D2R antagonist but not a D1R antagonist blocked the inhibitory effect of dopamine on insulin secretion from islets of wild-type mice. These results demonstrate that pancreatic D2R inhibit insulin secretion in response to glucose.

A basis for the molecular mechanisms mediating dopamine action on glucose-stimulated insulin secretion in pancreatic β-cells has been revealed. DR were described in INS-1E β-cells as well as in dispersed rat, mouse, and human islets (13). In the present study, we confirmed the presence of D2R in mouse islets using *Drd2*-EGFP mice. It has been shown that dopamine exerts a differential effect on glucose-induced insulin secretion depending on the concentration used (14). Consequently, some reports documented inhibition (13, 29, 30), whereas others reported an increase of insulin secretion upon acute dopamine accumulation (30, 31). Because D2R agonists inhibit insulin secretion at lower concentrations, at higher concentrations, these agonists may act on other receptors or may not be absolutely specific for a given DR. Our present data demonstrate that even though pancreatic D2R are inhibitory to glucose-stimulated insulin secretion, permanent loss of D2R results in decreased insulin response to glucose in adult mice and that this defect is progressive because glucose intolerance is not observed in young animals. Our results also point to an important defect in glucose sensing or stimulus-secretion coupling in β-cells, because there was a complete lack of responsiveness of islets to glucose both *in vivo* and *in vitro* in 7-month-old mice. A possible explanation to reconcile results could be that the lack of D2R-mediated insulin inhibition throughout development in *Drd2*$^{-/-}$ mice exerts a gradual deteriorating effect on insulin response to glucose, and therefore, at 7 months of age, islets respond poorly to glucose stimulation, as occurs in type 2 diabetes. This hypothesis is consistent with the fact that in diabetes, the prolonged stimulation of β-cells depletes insulin granule stores, and eventually, β-cells become unable to secrete pulses of insulin and become insensitive to changes in glucose concentration (32).

With regard to the mechanism of action of dopamine, it has been suggested that a down-regulation of D2R could influence the regulation of insulin secretion by releasing

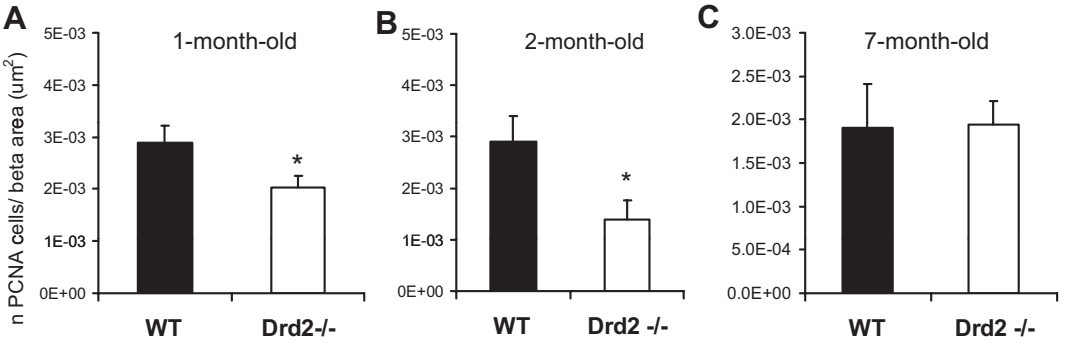

FIG. 7. PCNA-positive β-cells. Number of PCNA-positive cells per insulin immunoreactive area (in square micrometers) in pancreatic sections from mice of both genotypes at 1 (A), 2 (B), and 7 (C) months of age. Three different pancreas sections from each animal (n = 3 animals) were immunostained for insulin and PCNA and subjected to morphometric analysis. *, $P < 0.05$ *vs.* age-matched wild type.

epinephrine and norepinephrine from the adrenal medulla, which leads to the inhibition of insulin secretion in the pancreas (14). On the other hand, it has been reported that glucose-induced electrical activity of β-cells leads to the opening of voltage-gated calcium channels with subsequent Ca^{2+} influx and Ca^{2+}-dependent exocytosis of insulin (33) and that dopamine decreases cell membrane depolarization as well as cytosolic Ca^{2+} entry, and thus insulin secretion evoked by glucose stimulation is blunted (13).

On the other hand, variations in circulating hormones found in *Drd2*$^{-/-}$ mice might participate in altered pancreatic function. To begin with, prolactin levels are chronically elevated in *Drd2*$^{-/-}$ mice due to the lack of dopaminergic inhibition at the pituitary level (17). The physiological significance of prolactin in pancreatic function is mainly indicated by the increased insulin secretion and islet mass during pregnancy in both rodents and humans (34–36). Prolactin increases β-cell proliferation, insulin gene transcription, and glucose-dependent insulin secretion in isolated pancreatic islets and rat insulinoma cells (34–38). Moreover, in rats with chronic hyperprolactinemia, there is an increase in glucose and insulin concentrations after glucose overload. Men and women with chronic hyperprolactinemia have postprandial hyperinsulinemia and an exaggerated insulin secretory response to glucose (39–41). In addition, prolactin receptor deficiency is accompanied by islet and β-cell hypoplasia, reduced pancreatic insulin, and blunted insulin secretory response to glucose (42). Therefore, our present results are not consistent with a main effect of the hyperprolactinemic state on the pancreatic phenotype described, because hyperprolactinemia *per se* would evoke the opposite results in β-cell mass and insulin secretion to those we describe in the *Drd2*$^{-/-}$ mouse.

On the other hand, GH and serum IGF-I are decreased in *Drd2*$^{-/-}$ mice. Both hormones promote islet cell proliferation and increase insulin gene transcription and insulin secretion (43–46). Studies using animal models such as dwarf rats and GH receptor knockout mice show low glucose and insulin concentrations concordant with a state of insulin hypersensitivity (47). In humans, GH-deficient subjects have long been recognized as exhibiting increased insulin sensitivity, decreased insulin secretion, and hypoglycemia (48, 49), even though some reports observed insulin resistance in GH-deficient subjects (50, 51). Therefore, decreased β-cell mass and insulin secretion may be in part related to low GH action found in the *Drd2*$^{-/-}$ mice. Nevertheless, the fact that we did not find increased peripheral sensitivity to insulin or decreased basal glucose levels, which are generally present in animal models with low GH, and our *in vitro* results highlight the importance of pancreatic D2R in the phenotype described.

D2R deficiency on other tissues, particularly hypothalamus, may be influencing the phenotype. To this regard, it has been shown that bromocriptine improves glycemic control and glucose tolerance in obese type 2 diabetic patients (52) and in nondiabetic obese animals and humans and that bromocriptine can reverse many of the metabolic alterations associated with obesity by resetting central (hypothalamic) circadian organization of monoamine neuronal activity. Furthermore, glucose intolerance and insulin resistance result from decreased dopaminergic input to the area of the suprachiasmatic nucleus in animal models (53). Recently, bromocriptine was approved by the U.S. Food and Drug Administration for the treatment of type 2 diabetes. The drug appears to employ central mechanisms in ameliorating hyperglycemia. Nevertheless, our study strongly suggests that pancreatic D2R could also participate in the effect of dopamine agonists. We have recently studied mice lacking neural D2R (*nDrd2*$^{-/-}$) generated by us (54) and found that glucose and insulin responses at 30 min after a glucose overload of 3 mg/g were similar to those of wild-type mice (unpublished results). These results further indicate that the insulin release impairment

found in the *Drd2*$^{-/-}$ mice was not mainly dependent on the lack of D2R in the central nervous system.

Our results are relevant in the analysis of some clinical findings such as the effect of chronic treatment with antipsychotic medications that can induce abnormalities in glucose metabolism that increase risk for cardiovascular disease and diabetes (55–57) or the altered glucose tolerance associated with prolonged treatment with atypical antipsychotics in humans (5) and in animal models (58). Furthermore, older diabetics who take antipsychotic medications have an increased risk of ending up in the hospital with elevated blood glucose levels, or hyperglycemia (59). These data together with our present results clearly demonstrate that D2R are modulators of insulin secretion.

We conclude that mice lacking D2R display an impaired glucose metabolism and that pancreatic islet D2R are involved in this effect. Our finding that the D2R plays an essential role in β-cell proliferation and insulin secretion adds a novel participant to the list of growth factors and hormones that control the fundamental and multifactorial process of glucose homeostasis. It is important to consider that a combination of defects, hormonal and genetic, might aggravate the metabolic dysfunction that accompanies an isolated signaling defect. Furthermore, this study constitutes a contribution to unraveling glucose intolerance found after prolonged treatments with neuroleptic drugs.

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