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$GABA_B$ receptors and glucose homeostasis: evaluation in $GABA_B$ receptor knockout mice

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Bonaventura MM, Catalano PN, Chamson-Reig A, Arany E, Hill D, Bettler B, Saravia F, Libertun C, Lux-Lantos VA. $GABA_B$ receptors and glucose homeostasis: evaluation in $GABA_B$ receptor knockout mice. *Am J Physiol Endocrinol Metab* 294: E157–E167, 2008. First published October 30, 2007; doi:10.1152/ajpendo.00615.2006.— GABA has been proposed to inhibit insulin secretion through $GABA_B$ receptors ($GABA_BRs$) in pancreatic β-cells. We investigated whether $GABA_BRs$ participated in the regulation of glucose homeostasis in vivo. The animals used in this study were adult male and female BALB/C mice, mice deficient in the $GABA_{B1}$ subunit of the $GABA_BR$ ($GABA_B{}^{-/-}$), and wild types (WT). Blood glucose was measured under fasting/fed conditions and in glucose tolerance tests (GTTs) with a Lifescan Glucose meter, and serum insulin was measured by ELISA. Pancreatic insulin content and islet insulin were released by RIA. Western blots for the $GABA_{B1}$ subunit in islet membranes and immunohistochemistry for insulin and $GABA_{B1}$ were performed in both genotypes. BALB/C mice preinjected with Baclofen ($GABA_BR$ agonist, 7.5 mg/kg ip) presented impaired GTTs and decreased insulin secretion compared with saline-preinjected controls. $GABA_B{}^{-/-}$ mice showed fasting and fed glucose levels similar to WT. $GABA_B{}^{-/-}$ mice showed improved GTTs at moderate glucose overloads (2 g/kg). Baclofen pretreatment did not modify GTTs in $GABA_B{}^{-/-}$ mice, whereas it impaired normal glycemia reinstatement in WT. Baclofen inhibited glucose-stimulated insulin secretion in WT isolated islets but was without effect in $GABA_B{}^{-/-}$ islets. In $GABA_B{}^{-/-}$ males, pancreatic insulin content was increased, basal and glucose-stimulated insulin secretion were augmented, and impaired insulin tolerance test and increased homeostatic model assessment of insulin resistance index were determined. Immunohistochemistry for insulin demonstrated an increase of very large islets in $GABA_B{}^{-/-}$ males. Results demonstrate that $GABA_BRs$ are involved in the regulation of glucose homeostasis in vivo and that the constitutive absence of $GABA_BRs$ induces alterations in pancreatic histology, physiology, and insulin resistance.

γ-aminobutyric acid; insulin; glycemia; pancreas histology

γ-AMINOBUTYRIC ACID (GABA) is the main inhibitory neurotransmitter in the central nervous system. It acts on three types of receptors, $GABA_A$, $GABA_B$, and $GABA_C$ receptors ($GABA_ARs$, $GABA_BRs$, and $GABA_CRs$, respectively). $GABA_{A/C}Rs$ are ionotropic and belong to the superfamily of ligand-gated ion channels. Metabotropic $GABA_BRs$ are functionally coupled to $G_{i/o}$ proteins. $GABA_BRs$ have been described to assemble as heteromers formed by a $GABA_{B1}$ and a $GABA_{B2}$ subunit (3, 31, 32, 34, 61). Two major isoforms of $GABA_{B1}$, $GABA_{B1a}$ and $GABA_{B1b}$, arising from differential promoter usage of the $GABA_{B1}$ gene were isolated (31), coding for proteins of 130 and 100 kDa, respectively. Recently, two strains of mice deficient in either the $GABA_{B1}$ subunit (52) or the $GABA_{B2}$ subunit (18) were developed, both of which suffer from spontaneous seizures, hyperalgesia, hyperlocomotor activity, and severe memory impairment. This demonstrates that most $GABA_B$ functions depend on heterodimerization between the $GABA_{B1}$ and $GABA_{B2}$ subunits. However, $GABA_{B2}{}^{-/-}$, but not $GABA_{B1}{}^{-/-}$, mice still exhibit atypical electrophysiological $GABA_B$ responses, indicating that, in vivo, $GABA_{B1}$ can be functional in the absence of the partner subunit (4).

GABA is also found at high concentrations in other nonneuronal peripheral tissues, such as the endocrine pancreas (15, 20, 37, 58), in which its synthetic and degrading enzymes, glutamate decarbolylase (GAD) and GABA transaminase, respectively (59), its transporters proteins (13, 17), and $GABA_A$ (48) and $GABA_BRs$ (11) are also located. In general, GAD and $GABA_BRs$ have been detected in insulin-producing β-cells in mice, whereas $GABA_ARs$ are located mainly in glucagon-producing α-cells (11, 53). Nevertheless, a recent study (9) showed, by RT-PCR, presence of $GABA_BR$ subunits also in rat α-cells, suggesting that species differences in expression may also exist. It should be noted that local GABA may not originate only from endocrine cells, as GABAergic neurons are closely associated and even penetrate the islet mantle (49, 55). Although a complete GABA system had been characterized in the endocrine pancreas, its role in the regulation of pancreatic physiology has remained elusive. GABA can, via the formation of succinic semialdehyde and succinic acid, be introduced into the tricarboxylic acid cycle and has therefore been suggested to serve as an energy source within the β-cell (55). A role for GABA modulating pancreatic exocrine secretion has recently been suggested (43). In addition, regulated exocytosis of GABA-containing synaptic-like microvesicles in β-cells has been demonstrated (8, 25, 35). Various in vitro studies have postulated an autocrine/paracrine role for GABA in the regulation of insulin, glucagon, and somatostatin secretion, although in some cases contradictory results were obtained. Wendt et al. (60) elegantly demonstrated that GABA released from β-cells inhibits glucagon release from α-cells in rat pancreas, confirming previous results in α_2 cells (48), mouse islets, and perfused rat pancreas (19). Moreover, Xu et al. (62) proposed that insulin sensitized α-cells to β-cell-secreted GABA by phosphorylation and translocation of $GABA_ARs$ to

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the cell surface. Although some authors have proposed that GABA was not involved in somatostatin release (19, 48), others have proposed that GABA inhibited somatostatin secretion through $GABA_A$Rs (47). Regarding insulin, some authors have proposed that GABA had no effect on insulin release under various glucose concentrations in mouse or rat islets (19). Interestingly, expression of transgenic GAD65 and consequently elevated GABA levels in β-cells resulted in impaired insulin secretion and diabetes in transgenic mice (53), in agreement with results proposing an inhibitory role for GABA on insulin secretion in the perfused rat pancreas (23). Recently, it was demonstrated (9, 11) that GABA inhibits insulin secretion specifically through $GABA_B$Rs in the presence of high glucose in MIN6 cells and in rat islets. Moreover, $GABA_B$Rs in the presence of calcium have been shown to enhance metabotropic glutamate signaling in the absence of GABA in cerebellum (56, 57), turning a possible regulation by these receptors more complex, since glutamate is another important autocrine/paracrine islet-signaling molecule (38). Interestingly, in addition to the participation of GABA in these physiological processes, an overexpression of $GABA_A$ receptor pi subunit in pancreatic adenocarcinomas has also been demonstrated (30).

In the present work, the participation of $GABA_B$Rs in the physiology of the endocrine pancreas, in particular regarding the regulation of in vivo mouse glucose homeostasis, was evaluated by a pharmacological approach using a $GABA_B$R agonist, baclofen, in $GABA_B{}^{-/-}$ mice lacking the $GABA_{B1}$ subunit and presenting a constitutive loss of $GABA_B$ responses.

MATERIALS AND METHODS

Animals. $GABA_B{}^{-/-}$ mice lacking the $GABA_{B1}$ subunit of the $GABA_B$R generated in the BALB/C inbred mouse strain (52) were obtained by intercrossing heterozygous animals. Fingertip biopsies (performed for identification purposes) were used to isolate DNA for animal genotyping by PCR as described (52). In addition, 2- to 3-mo-old male and female BALB/C mice from the Instituto de Biología y Medicina Experimental colony were used. All animals were housed in groups in air-conditioned rooms, with lights on from 0700 to 1900, and given free access to laboratory chow and tap water. Studies were performed according to protocols for animal use and approved by the Institutional Animal Care and Use Committee and by the National Institutes of Health (NIH). Female and male mice from both genotypes, $GABA_B{}^{-/-}$ and wild types (WT), were used at 2–3 mo of age. At this age mice used within the same sex did not differ in body weight [body weight (g): WT females 20.2 ± 0.6 (n = 15), $GABA_B{}^{-/-}$ females 22.1 ± 1.0 (n = 14), WT males 27.6 ± 0.3 (n = 46), $GABA_B{}^{-/-}$ males 27.5 ± 0.7 (n = 44)], in agreement with previous data from the colony (12). For each experimental design animals were age-matched littermates, and body weights were recorded.

Basal blood glucose titers and glucose tolerance tests. Blood glucose was measured by a One-Touch Ultra glucose meter (Lifescan) from tail blood. For the glucose tolerance test (GTT), intraperitoneal glucose [2 g/kg body wt (22, 33)] was injected in overnight-fasted mice (15–18 h) and blood glucose evaluated at 0, 10, 20, 30, 40, 60, and 75 min postinjection in WT and $GABA_B{}^{-/-}$ age- and sex-matched mice. Body weights (g) were as follows: WT females 20.2 ± 0.6 (n = 15), $GABA_B{}^{-/-}$ females 22.1 ± 1.0 (n = 14), WT males 27.1 ± 0.3 (n = 16), $GABA_B{}^{-/-}$ males 26.2 ± 0.9 (n = 16).

Baclofen, a $GABA_B$ agonist (Lioresal, a gift from Novartis), was tested at various doses (2.5, 5.0, or 7.5 mg/kg body wt ip) administered 20 min before the 2 g/kg body wt glucose injection in preliminary experiments performed in BALB/C male mice. The dose of 7.5 mg/kg was selected, as results were more consistent, in agreement with previous experiments performed in mice (28). Blood glucose was evaluated at 0, 30, 60, and 75 min postglucose administration in baclofen- or saline-pretreated BALB/C mice. Body weights (g) were as follows: saline-treated males 26.2 ± 0.9 (n = 7), baclofen-treated males 26.0 ± 0.7 (n = 8), saline-treated females 21.1 ± 0.5 (n = 10), baclofen-treated females 21.1 ± 0.4 (n = 10).

In addition, intraperitoneal GTTs with baclofen (7.5 mg/kg) or saline preinjection were also performed in WT and $GABA_B{}^{-/-}$ age-matched male mice. Body weights (g) were as follows: saline-pretreated WT males 25.0 ± 1.3 (n = 5), baclofen-pretreated WT males 25.8 ± 0.6 (n = 5), saline-pretreated $GABA_B{}^{-/-}$ males 26.0 ± 1.6 (n = 5), baclofen-pretreated $GABA_B{}^{-/-}$ males 24.7 ± 1.8 (n = 4).

Insulin tolerance test. Blood glucose was measured as above in male age-matched WT and $GABA_{B1}{}^{-/-}$ mice fasted for 2–4 h after 0, 10, 20, 30, and 60 min of an intraperitoneal injection of 1 U/kg body wt of human insulin (a gift from Laboratorios Beta, Buenos Aires, Argentina). Body weights (g) were as follows: WT 28.2 ± 0.7 (n = 15), $GABA_{B1}{}^{-/-}$ 28.2 ± 1.3 (n = 15).

Insulin determination. Serum insulin was measured with a mouse insulin ELISA kit (Crystal Chem, Chicago, IL) at 0, 10, 20, 30, and 60 min after the intraperitoneal injection of 3 g/kg body wt of glucose, as described (22, 33), in age-matched WT and $GABA_B{}^{-/-}$ male mice fasted for 15–18 h. Body weights were as follows (g): WT 27.3 ± 0.7 (n = 15), $GABA_{B1}{}^{-/-}$ 28.2 ± 1.4 (n = 13).

In fasted BALB/C male mice preinjected with either saline or baclofen (7.5 mg/kg body wt ip, 20 min before glucose administration), insulin was determined at 0, 5, 15, 30, and 60 min after 3 g/kg body wt ip glucose injection. Body weights (g) were as follows: saline-treated males 25.4 ± 0.6 (n = 9), baclofen-treated males 25.6 ± 0.8 (n = 10).

To measure pancreas insulin content, age- and sex-matched WT and $GABA_{B1}{}^{-/-}$ adult mice were killed in the morning, pancreases were weighed, and insulin was extracted from pancreas homogenates with acid-ethanol. Pancreas weight in each sex did not differ between genotypes [pancreas wet weight (mg): WT females 179 ± 16 (n = 5), $GABA_B{}^{-/-}$ females 214 ± 25 (n = 5), WT males 231 ± 14 (n = 10), $GABA_B{}^{-/-}$ males 249 ± 11 (n = 12)]. Briefly, tissues were homogeneized in 40 vol of acid-ethanol [75% ethanol, 25% acetic acid (25% vol/vol)] and extracted overnight at 4°C. The homogenates were centrifuged (10 min, 2,000 g, 4°C) and the supernatants neutralized with 1 volume of 0.85 M Tris. After a second incubation (60 min, −20°C) and centrifugation (30 min, 3,000 g, 4°C), the supernatants were stored at −70°C until they were used. Pancreatic insulin content, islet insulin content, and insulin secreted from cultured islets were measured by RIA using human insulin for iodination and standard, provided by Laboratorios Beta, and anti-bovine insulin antibody (Sigma, St. Louis, MO) (14). All samples were evaluated in the same RIA. The minimum detectable concentration was 2 ng, and the intra-assay coefficient of variation was 6.8%. Numbers of animals were 10–12 for males and 5 for females.

Islet isolation from WT and $GABA_{B1}{}^{-/-}$ male mice. Pancreatic islets were isolated from 2- to 3-mo-old male mice from each genotype, as described by Asghar et al. (2), with minor modifications. Briefly, 3 ml of collagenase (0.6 mg/ml; Sigma) was injected into the pancreatic duct, and pancreatic tissue was gently removed and digested in collagenase solution at 37°C for 10–15 min. The digestion was stopped by ice-cold RPMI 1640 supplemented with 10% fetal bovine serum. Islets were then handpicked under a dissecting microscope. Islets were either incubated overnight in RPMI 1640 supplemented with 10% fetal bovine serum and antibiotics for insulin secretion studies or homogenized in 10 volumes of ice-cold 0.32 M sucrose containing 1 mM $MgCl_2$ and 1 mM K_2HPO_4 for membrane protein extraction (42) for Western blot analysis.

Glusose stimulated insulin secretion in cultured islets from WT and $GABA_{B1}^{-/-}$ male mice. After the overnight incubation, islets were picked into Krebs-Ringer bicarbonate (KRB) buffer containing 2.8 mM glucose for 1-h preincubation at 37°C. They were then transferred into 1.5-ml microcentrifuge tubes (5 islets/tube) containing 500 μl of KRB buffer with 2.8 mM or 20 mM glucose in the presence or absence of 10 μM baclofen. After 1-h incubation, 300 ul supernatant/sample was collected and frozen at −20°C for insulin assay by RIA. The microcentifuge tubes were centrifuged for 10 min at 3,000 rpm, the rest of the supernatant was discarded, and acid-ethanol was added (250 μl/sample; ethanol-H_2O-HCl = 150:47:3), and the samples were kept overnight at 4°C. They were then speed-vacuumed to evaporate. Thereafter, 60 μl H_2O/sample was added, and samples were used for measuring total insulin content. Insulin results are expressed as the relationship between secreted insulin and insulin content per microcentrifuge tube.

Western Blot analysis. Western blot analysis for the $GABA_{B1}$ subunit was performed in WT and $GABA_{B1}^{-/-}$ male mice islet membrane proteins, obtained as previously described (5, 6, 46). Briefly, 50 μg of islet membrane protein preparations from each genotype and 5 μg of a cerebellum membrane protein preparation of WT animals, used as positive control, were subjected to 8% SDS-PAGE. Proteins were transferred onto nitrocellulose membranes. Blots were blocked for 2 h in PBS-0.2% Tween 20–5% nonfat milk and incubated overnight at 4°C with a guinea pig anti-$GABA_{B1}$ antibody (antibody GP311, 1:4,000 in PBS-0.2% Tween 20–5% nonfat milk; a gift from Dr. Marta Margeta, Jan Lab, University of California San Francisco, San Francisco, CA) that specifically binds the $GABA_{B1a}$ (130 kDa) and $GABA_{B1b}$ (100 kDa) isoforms of the $GABA_{B1}$ subunit of the $GABA_B$ receptor, as described by Margeta-Mitrovic et al. (36). Secondary antibody was horseradish peroxidase coupled (1:1,000 in PBS-0.1% Tween 20–1% bovine serum albumin for 60 min at room temperature; Sigma). Detection was performed using an enhanced chemiluminiscence Western blot analysis system (Western blotting chemiluminescence luminol reagent; Santa Cruz Biotechnology).

Immunohistochemistry. Histological sections of pancreas (5 μm) were cut from paraffin blocks and mounted on glass microscope slides. Immunohistochemistry was performed to localize cells staining for insulin by the avidin-biotin peroxidase method. Sections were incubated in 0.3% (vol/vol) hydrogen peroxidase to block endogenous peroxidase activity for 20 min at room temperature, followed by an incubation with 10% goat serum for 20 min at 37°C to reduce nonspecific binding. Thereafter, slides were incubated with guinea pig anti-bovine insulin (1:1,000; Sigma, MO) overnight at 4°C. After being washed with PBS, slides were incubated with biotinylated goat anti-guinea pig IgG (1:300; Santa Cruz Biotechnology) for 60 min at room temperature and finally incubated with Vectastain ABC system (Vector Laboratories) for 45 min at room temperature. Insulin immunoreactivity was visualized by incubation with diaminobenzidine tetrahydrochloride (Boehringer Mannheim) for 13–15 min. Tissue sections were counterstained with hematoxylin. Controls included substitution of primary antiserum with nonimmune serum or the omission of the secondary antiserum.

For $GABA_{B1}$ immunohistochemistry the same guinea pig anti$GABA_{B1}$ antibody GP311 was used (1:5,000), and procedure was as described above, with minor modifications; incubations with goat serum and diaminobenzidine tetrahydrochloride lasted 30 and 3 min, respectively.

Morphometric analysis. Pancreata from six male and five female adult mice from each genotype were examined. Morphometric analysis was performed using a Nikon-transmitted light microscope at a magnification of ×40, ×100, or ×400. Image analysis was performed with ImageJ software (NIH). The number of islets, β-cell area, taken as the insulin-positive area, and total pancreas area were calculated from three sections of each pancreas, representing the entire pancreas for each animal (head, body, and tail). Approximately 70–120 islets/animal, defined as insulin-positive aggregates of an area larger than 500 μm^2 (16), were analyzed. The number of islets were scored, and islets ranging from 501 to 5,000 μm^2 were defined as small, those of 5,001–10,000 μm^2 were defined as medium, those of 10,001–25,000 μm^2 were defined as large, and those >25,000 μm^2 were defined as very large.

Statistical analysis. Plasma glucose, insulin titers, and all morphometric data are expressed as means ± SE. All statistical analysis was performed with Statistica '99 edition. The differences between means were analyzed by the unpaired Student's *t*-test or by two-way ANOVA followed by Newman-Keuls test or Tukey's honestly significant difference test for unequal N. For multiple determinations in the same animal, two-way ANOVA with repeated-measures design was used, followed by planned comparisons. $P < 0.05$ was considered statistically significant.

RESULTS

Glucose tolerance tests and insulin secretion in BALB/C mice after baclofen or saline pretreatment. During the intraperitoneal GTT, preinjection with baclofen (7.5 mg/kg ip) induced a significant impairment in the recovery of blood glucose to baseline levels after the glucose overload without modifying either basal titers or the maximal glucose levels attained in BALB/C male mice [blood glucose (mg/dl) 75 min postglucose injection: saline pretreatment 121.0 ± 9.6 ($n = 7$) vs. baclofen pretreatment 180.5 ± 10.7 ($n = 8$), $P < 0.02$; Fig. 1*A*]. Similar results were observed in female BALB/C mice [blood glucose (mg/dl) 75 min postglucose injection: saline pretreatment 138.6 ± 8.3 ($n = 10$) vs. baclofen pretreatment 180.0 ± 6.2 ($n = 10$), $P < 0.01$].

Insulin secretion was also clearly affected by 7.5 mg/kg baclofen pretreatment, inducing a significant decrease in serum levels 5 and 15 min after a 3 g/kg glucose overload, without affecting basal levels (Fig. 1*B*). In addition, when the area under the insulin curve (AUC) was calculated, a significant reduction induced by baclofen administration was observed [AUC ($ng \cdot ml^{-1} \cdot min$): saline pretreatment 19.2 ± 1.50 ($n = 10$) vs. baclofen pretreatment 12.0 ± 2.6 ($n = 10$), $P < 0.03$]. In addition, at this glucose overload an impairment in the recovery of blood glucose was also observed (Fig. 1*B*, *inset*).

Western blot analysis and immunohistochemistry for the $GABA_{B1}$ subunit of the $GABA_BR$ in WT and $GABA_B^{-/-}$ mice. Both Western blot and immunohistochemical analyses demonstrated the presence of $GABA_B$Rs in islets of WT animals and absence of these receptors in islets of $GABA_B^{-/-}$ mice. Previously, the presence of $GABA_B$R proteins had been demonstrated in MIN and HIL cells (11) and also their mRNA in rat islets (9).

Western blots showed the presence of the characteristic bands corresponding to $GABA_{B1a}$ (130 kDa) and $GABA_{B1b}$ (100 kDa) isoforms of the $GABA_{B1}$ subunit in WT islet membranes and in cerebellum membranes, used as positive controls, and absence of bands in islet membranes from $GABA_B^{-/-}$ mice (Fig. 2*A*). As expected, positive immunostaining for the $GABA_{B1}$ subunit was observed in islets from WT mice pancreatic sections (×400; Fig. 2*B*, *right*). No specific staining was observed in $GABA_B^{-/-}$ mice (Fig. 2*B*, *left*). Staining for insulin in a contiguous section showed a large coincidence of immunopositive areas (not shown), in agreement with the β-cell localization proposed for this receptor by RT-PCR and immunoblotting experiments in mice and rats (11).

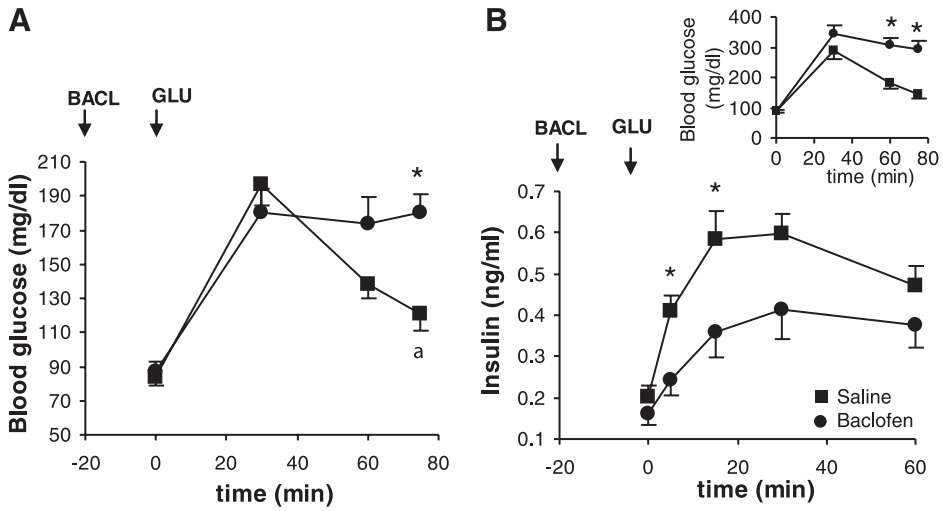

Fig. 1. Glucose tolerance tests (GTT) and insulin secretion in BALB/C mice after baclofen or saline pretreatment. *A*: GTT, carried out with a 2 g/kg ip glucose (GLU) injection, in fasted BALB/C male mice after ip preinjection with baclofen [BACL; 7.5 mg/kg (●), 20 min before glucose administration] or saline (SAL; ■); $n = 8$ for each group. Two-way ANOVA with repeated-measures design: interaction, $P < 0.001$. [a]Significantly different from levels at 30 min in saline-pretreated mice; *significantly different from saline-pretreated animals. *B*: insulin secretion under 3 g/kg ip glucose overload after ip preinjection of 7.5 mg/kg BACL (●) or saline (■) as described above; $n = 10$ for each group. Two-way ANOVA with repeated-measures design: interaction, not significant (NS); treatment factor, $P < 0.001$; time factor, $P < 0.001$. *Significantly different from BACL by planned comparisons, $P < 0.01$ at 5 min and $P < 0.03$ at 15 min. *Inset*: blood glucose levels (mg/dl) after a 3 g/kg ip glucose overload in BACL- or saline-pretreated animals. *Significantly different from saline.

Basal glucose levels and glucose tolerance tests in WT and $GABA_B^{-/-}$ male and female mice. Fasting and fed glucose levels were similar between both genotypes in each sex (Fig. 3, *A* and *B*). In the fed condition, male glucose levels were significantly higher than in females [two-way ANOVA: main effect genotype, not significant (NS); main effect sex, $P < 0.01$]. During the GTT, with a glucose challenge of 2 g/kg body wt, both male and female $GABA_B^{-/-}$ mice showed a blunted elevation of blood glucose compared with WT (Fig. 3, *C* and *D*). This effect was more marked in males than in females, as also evidenced by a significant decrease in the area under the glucose curve in knockout males compared with WT ($P < 0.006$; Fig. 3*C*, *inset*), which did not reach statistical significance in females ($P = 0.09$; Fig. 3*D*, *inset*). As expected, whereas baclofen (7.5 mg/kg) preinjection impeded normal glycemia reinstatement in WT male mice, as shown for BALB/C mice, in $GABA_B^{-/-}$ mice baclofen administration had no effect [blood glucose (mg/dl) 75 min postglucose injection: saline-pretreated WT 150.4 ± 9.4 ($n = 5$), baclofen-pretreated WT 249.2 ± 32.9 (significantly different from saline-pretreated WT mice;$n = 5$), saline-pretreated $GABA_B^{-/-}$ 158.0 ± 9.0 ($n = 5$), baclofen-

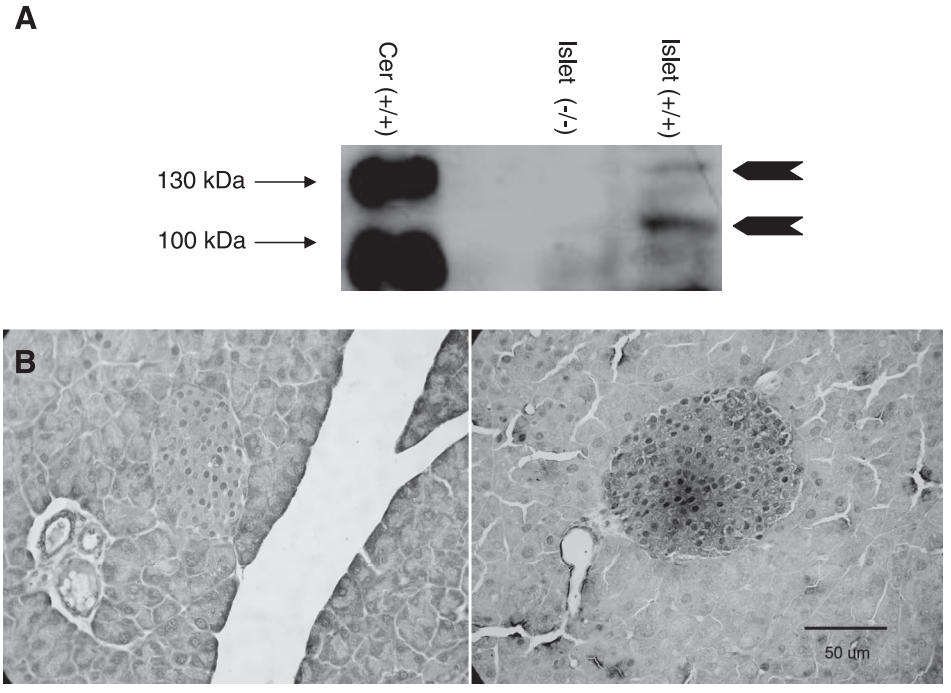

Fig. 2. *A*: Western blot for the $GABA_{B1}$ subunit of the $GABA_B$ receptor in $GABA_B^{+/+}$ and $GABA_B^{-/-}$ mice Langerhans islets and $GABA_B^{+/+}$ cerebellum (Cer) membranes. Note the presence of the characteristic bands of 130 ($GABA_{B1a}$) and 100 kDa ($GABA_{B1b}$) in the wild-type (WT) tissues (Cer, islets) and absence of bands in the $GABA_B^{-/-}$ islet membranes. *B*: immunohistochemistry for the $GABA_{B1}$ subunit of the $GABA_B$ receptor in pancreata of WT and $GABA_B^{-/-}$ mice. Representative microphotographs of pancreas sections of $GABA_B^{-/-}$ (*left*) and WT (*right*) male mice immunostained for the $GABA_{B1}$ subunit of the $GABA_B$ receptor (×400). Note the positive staining in the WT section and the absence of specific staining in the $GABA_B$ knockout section. Magnification bar, 50 μm.

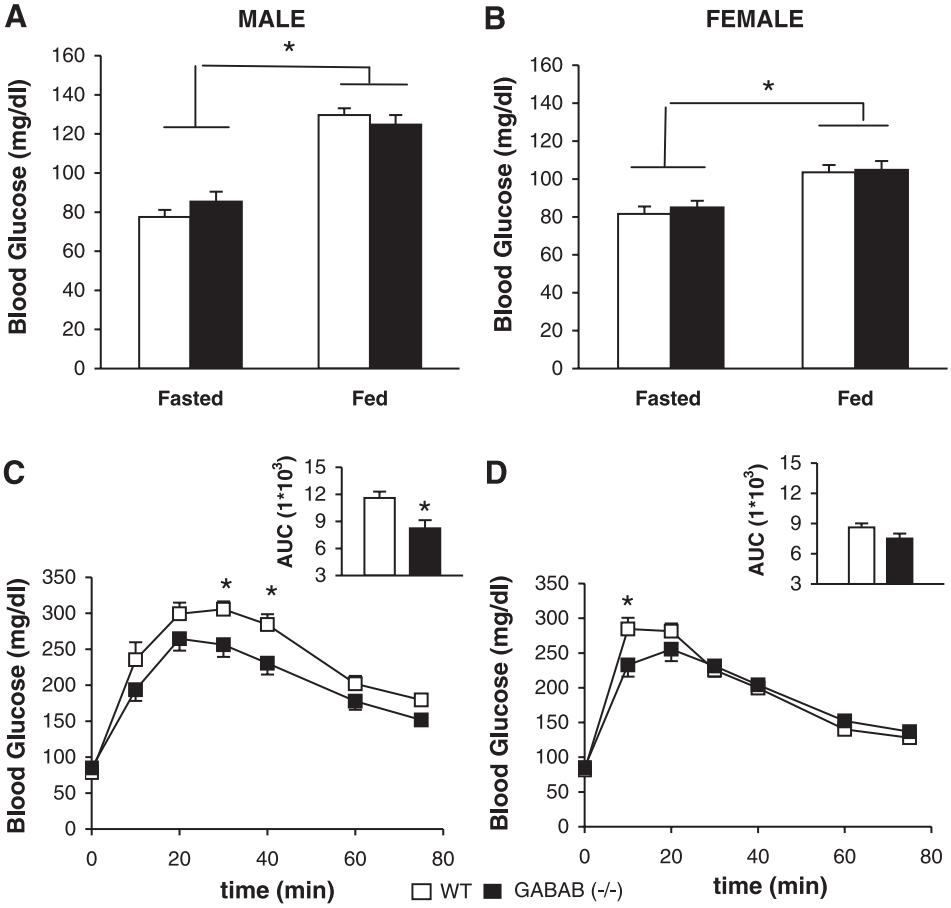

Fig. 3. Basal glucose levels in WT (open bars) and GABA$_B^{-/-}$ (filled bars) mice and GTT in WT (□) and GABA$_B^{-/-}$ (■) mice. *A* and *C*: males; *B* and *D*: females. Basal fed blood glucose levels were determined in the middle of the light period. Fasted blood glucose was determined in the morning after overnight fasting. *A* and *B*: results were analyzed by 2-way ANOVA in each sex: interaction, NS; genotype, NS; feeding condition, $P < 0.001$. Males: $n = 26–29$; females: $n = 14–21$. For GTT, mice were fasted overnight and injected ip with 2 g/kg glucose. *C*: 2-way ANOVA with repeated-measures design ($n = 16$ for each group): interaction, $P < 0.03$. *Significantly different from GABA$_B^{-/-}$ males. *Inset*: area under the curve (AUC). Open bars, WT; filled bars, GABA$_B^{-/-}$. *$P < 0.01$. *D*: 2-way ANOVA with repeated-measures design ($n = 14–15$): interaction, $P < 0.01$. *Significantly different from GABA$_B^{-/-}$ females. *Inset*: AUC. Open bars, WT; filled bars, GABA$_B^{-/-}$, NS.

pretreated GABA$_B^{-/-}$ 169.3 ± 22.3 ($n = 4$); two-way ANOVA: interaction, $P = 0.05$].

Pancreas insulin content and insulin response to a glucose challenge of 3 g/kg ip in WT and GABA$_B^{-/-}$ mice. Because GABA$_B^{-/-}$ mice showed improved GTTs, we next evaluated pancreatic insulin content in these mice compared with WT. Insulin content was increased in GABA$_B^{-/-}$ males (Fig. 4*A*), whereas it did not achieve statistical significance in females ($P = 0.07$; Fig. 4*B*). Because insulin content differed only between male genotypes, we evaluated insulin secretion in WT and GABA$_B^{-/-}$ males. Under a glucose challenge (3 g/kg ip), insulin increased in both genotypes, but overall insulin titers were significantly augmented in GABA$_B^{-/-}$ mice (2-way ANOVA: interaction, NS; main effect genotype, $P < 0.05$; main effect time, $P < 0.001$) (Fig. 4*C*). Although no differences were observed in fasting glucose levels (see above), planned comparisons showed that insulin was significantly increased basally ($P < 0.03$) and after 10 min of glucose administration ($P < 0.04$) in GABA$_B^{-/-}$ mice. Normal fasting glucose levels with increased basal insulin were a first index of insulin resistance in GABA$_B^{-/-}$ males. Although the absolute insulin levels achieved were higher in GABA$_B^{-/-}$ males, the AUCs did not differ between genotypes (not shown). In addition, the percent increase over basal levels 10 min after glucose administration was similar in both genotypes [%increase after 10 min: WT 368.3 ± 53.5 ($n = 15$) vs. GABA$_B^{-/-}$ 298.9 ± 44.3 ($n = 13$), NS]. Under this glucose challenge the blood glucose titers attained were significantly higher in knockouts than in WT (Fig. 4*C*, *inset*), notwithstanding the trend toward higher insulin levels observed in knockouts.

Effect of baclofen on glucose-stimulated insulin secretion in cultured islets from WT and GABA$_{B1}^{-/-}$ mice. To characterize the physiological impact of the absence of functional GABA$_B$Rs in pancreatic islets from GABA$_B^{-/-}$mice, glucose-stimulated insulin secretion (GSIS) assays were performed in islets from both genotypes in the presence or absence of baclofen (10 μM). The GABA$_B$ agonist significantly inhibited GSIS in WT islets without modifying basal levels, as previously described (9, 11); baclofen had no effect on GSIS in islets from GABA$_B^{-/-}$ mice (Fig. 5).

Assessment of insulin resistance in GABA$_B^{-/-}$male mice and WT controls. GABA$_B^{-/-}$ mice showed an altered response during an insulin tolerance test (ITT; Fig. 6*A*), with the glucose levels 60 min after the insulin injection being significantly higher than in controls ($P = 0.04$), indicating that these mice were insulin resistant. As an additional parameter to assess insulin sensitivity, the inverse area under the glucose curve was calculated, demonstrating a significant decrease in knockout animals (Fig. 6*A*, *inset*). When the homeostatic model assessment of insulin resistance (HOMA-IR) was calculated, this index showed a twofold increase in the knockout males (Fig. 6*B*).

Histological analysis of pancreas in adult male and female WT and GABA$_B^{-/-}$ mice. Because we had shown an increase in pancreatic insulin content and increased insulin secretion in GABA$_B^{-/-}$ male mice, we next examined whether this was

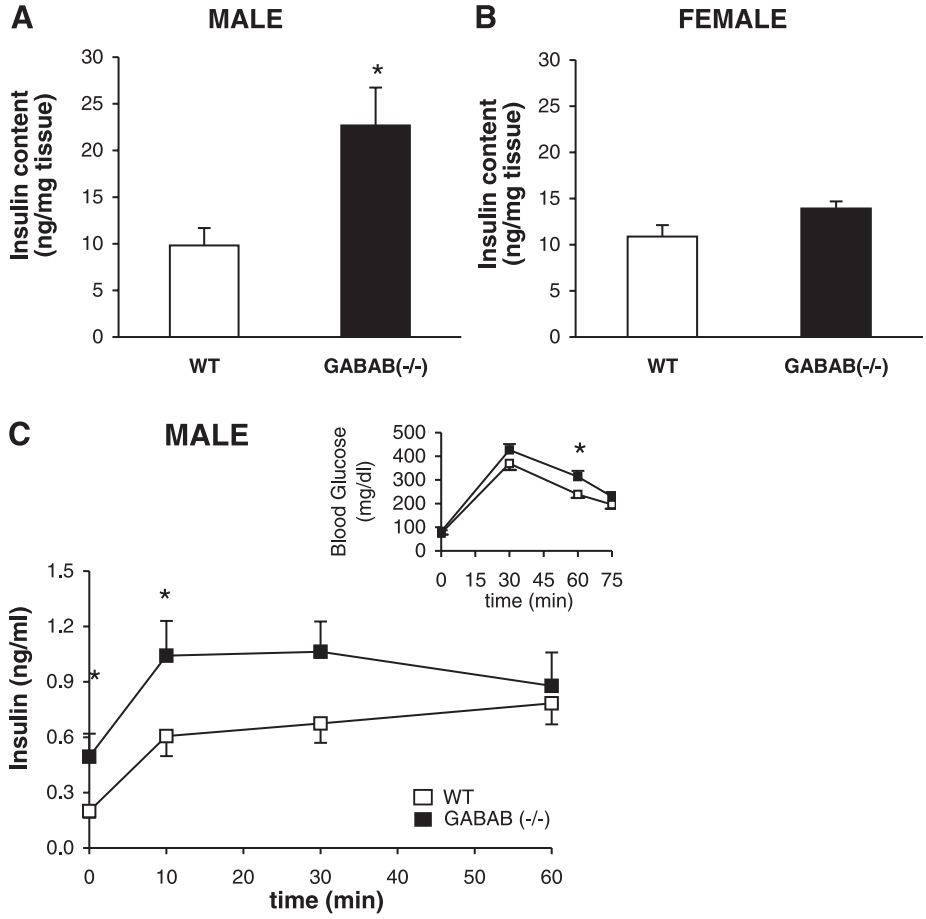

Fig. 4. Pancreas insulin content and insulin response to a glucose overload in WT and $GABA_B^{-/-}$ mice. Pancreas insulin content (ng/mg tissue) in WT (open bars) and $GABA_B^{-/-}$ (filled bars) male (*A*) and female (*B*) mice was determined by RIA after animals were killed in the morning and insulin extracted from pancreas; $n = 10–12$ males and 5 females. *$P < 0.01$ compared with WT. *C*: the insulin response to a glucose overload (3 g/kg ip) was determined in overnight-fasted WT (□; $n = 15$) and $GABA_B^{-/-}$ males (■; $n = 13$). Two-way ANOVA with repeated-measures design: interaction, NS; main effect genotype, $P < 0.05$; main effect time, $P < 0.001$. *Significantly different from WT by planned comparisons, $P < 0.03$. *Inset*: glucose excursion in both genotypes: 2-way ANOVA with repeated-measures design: interaction, NS; main effect genotype, $P < 0.02$; main effect time, $P < 0.001$. *Significantly different from WT by planned comparisons, $P < 0.02$.

associated with a difference in the histology of the endocrine pancreas. Neither the number of islets relative to pancreas area (Fig. 7*A*) nor β-cell area relative to pancreas area (Fig. 7*C*) differed between genotypes in males. Nevertheless, when the percent distribution of islet number according to size was evaluated, a significant difference between genotypes was evident due to an increase in the number of very large islets (>25,000 μm^2) in the $GABA_B^{-/-}$ males (Fig. 7*B*), representing ~10% of total islet number compared with only 1% in WT ($P < 0.01$). Interestingly, when the percent distribution of islet area according to islet size was evaluated, we observed that 42% of the insulin-positive area in $GABA_B^{-/-}$ mice corresponded to very large islets compared with only 11% in WT ($P < 0.03$; Fig. 7*D*). A representative image of these very large islets (×100) seen in $GABA_B^{-/-}$ animals is shown in Fig. 7*E* compared with a large islet that is more characteristic of WT males (Fig. 7*F*).

In females, no differences between genotypes in any of the morphometric parameters were observed (Fig. 8).

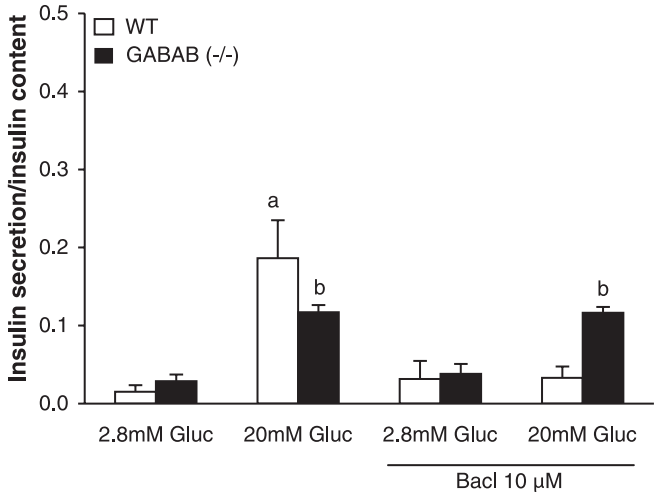

Fig. 5. Glucose-stimulated insulin secretion in isolated islets from WT and $GABA_B^{-/-}$ male mice in the presence or absence of BACL. Results are expressed as insulin secretion/insulin content per incubation vial containing 5 islets each ($n = 4$ independent experiments). [a]Significantly different from 2.8 mM glucose in WT islets; [b]significantly different from 2.8 mM glucose in knockout islets.

DISCUSSION

The presence in the endocrine pancreas of a complete GABAergic system with synthesizing and catabolizing enzymes, receptors, and transporters has been amply documented (11, 15, 17, 20, 37, 48, 58, 59). Nevertheless, an in-depth comprehension of the importance of pancreatic GABA in the control of glucose homeostasis is still lacking. Recent studies have pointed to an autocrine inhibitory action of β-cell-secreted GABA on insulin secretion by acting on $GABA_B$ receptors (9, 11), in agreement with previous data in the perfused rat pancreas (23). An intraislet paracrine inhibitory role for GABA

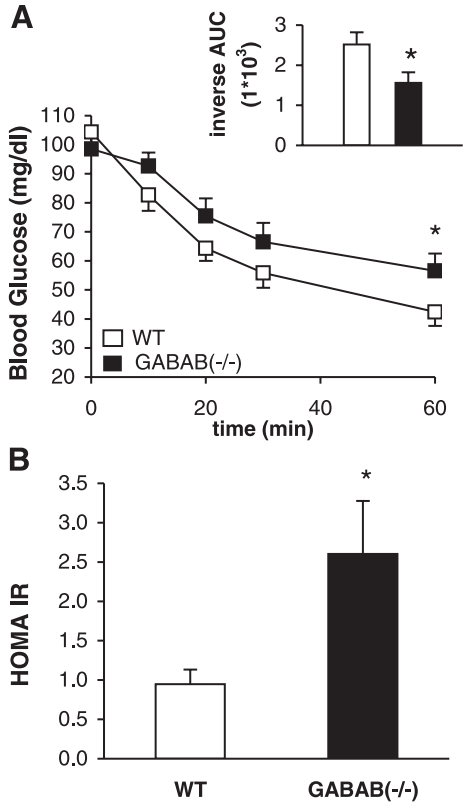

Fig. 6. Assessment of insulin resistance in $GABA_B^{-/-}$ male mice and WT controls. *A*: insulin tolerance tests (ITT): 2-h fasted WT (□) and $GABA_B^{-/-}$ (■) males were injected with 1 U/kg body wt of human insulin, and blood glucose was measured at different time points; $n = 15$ for each genotype. Results were analyzed by 2-way ANOVA with repeated-measures design: interaction, $P < 0.01$. *Significantly different from WT. *Inset*: inverse area under the glucose curve in the ITT (inverse AUC) in WT (open bar) and $GABA_B^{-/-}$ (filled bar) males. *Significantly different from WT, $P < 0.02$. *B*: the homeostatic model assessment of insulin resistance (HOMA-IR) index was calculated for WT (open bar) and $GABA_B^{-/-}$ (filled bar) mice. *$P < 0.03$. HOMA-IR = fasting insulin (μU/ml) × fasting glucose (mmol/l)/22.5.

on glucagon secretion by acting on $GABA_A$ receptors in α-cells was also reported (48, 60, 62), confirming similar results in mouse islets and perfused rat pancreas (19). Although these in vitro studies point to a specific role for GABA in the control of insulin/glucagon release, the importance of this neurotransmitter in the in vivo condition, where all the complex signals regulating glucose homeostasis converge, remained to be elucidated. In addition to a pharmacological approach, the $GABA_B$ receptor knockout mouse was an interesting model to assess this subject.

Baclofen, a specific $GABA_B$ receptor agonist, did not affect the maximal glucose level attained in a GTT but dose-dependently inhibited the reinstatement of basal serum glucose titers 75 min after the glucose challenge in BALB/C mice. In fact, a baclofen-induced inhibition of insulin secretion was also demonstrated. To our knowledge, these are the first data suggesting the participation of $GABA_B$ receptors in the in vivo regulation of glucose homeostasis, and they are in agreement with in vitro results obtained by others (9, 11, 23). Previous works (21) had shown a lack of effect of baclofen on blood glucose after a glucose overload, although a low dose and a single time point were evaluated; in addition, those results were obtained in rats, and species differences may also occur. Next, glucose homeostasis was investigated in the $GABA_B^{-/-}$ mice, in which functional $GABA_B$ receptors are absent, as demonstrated by the absence of the $GABA_{B1}$ subunit in Langerhans islets by immunohistochemical and Western blot analyses. $GABA_B^{-/-}$ animals showed similar weight gain curves from birth to early adulthood to their wild-type littermates within the same sex (12), indicating that profound alterations in their development were not present, at least at the ages tested, and suggesting the need to evaluate this parameter at later time points in life. In the fed condition, serum glucose was significantly higher in males than in females, as had been previously described in other mice strains (1, 22). No differences in either fasting or fed blood glucose levels were observed between genotypes in either sex. Females generally manifested better glucose tolerance than males, which has been suggested to be due to greater insulin sensitivity in liver and adipose tissue in this sex (1, 22). Interestingly, the blood glucose excursion after an intraperitoneal glucose load was significantly smaller in $GABA_B^{-/-}$ mice than in controls in both sexes, with this difference being more pronounced in males. A significant increase in pancreatic insulin content with regard to wild-type controls was observed only in $GABA_B^{-/-}$ males. Furthermore, blood insulin was higher in $GABA_B^{-/-}$ males, both basally and after a 3 g/kg glucose challenge compared with wild-types, although the percent increase over basal levels was similar in both genotypes, indicating a similar response capacity.

To determine whether this difference in insulin secretion between genotypes could be the consequence of an altered physiological response to local GABAergic stimulation due to the absence of functional $GABA_B$ receptors, Langerhans islets from wild-type and $GABA_B^{-/-}$ males were cultured in vitro and subjected to GSIS studies in the presence or absence of the $GABA_B$ agonist baclofen. As demonstrated before (9, 11), baclofen inhibited the GSIS in wild-type islets without altering basal levels. Loss of GABA signal input through $GABA_B$ receptors was demonstrated in the islets of knockout mice, as the $GABA_B$ agonist did not modify the GSIS in these islets. It has been described previously (60) that glucose-induced electrical activity of the β-cell leads to the opening of voltage-gated calcium channels with subsequent Ca^{2+} influx and Ca^{2+}-dependent exocytosis of both insulin and GABA. In addition, it has been proposed that this GABA released from β-cells functions as an autocrine inhibitor of insulin secretion in pancreatic islets (9). Therefore, the increased insulin secretion observed in vivo in $GABA_B^{-/-}$ mice may be the consequence of the loss of this local GABAergic regulation.

An apparent difference in the degree to which each sex of the $GABA_B$ knockout colony was affected in their glucose homeostasis regulation was observed, with males being more affected than females, similar to sex differences observed in other transgenic/knockout models (16). These may be due specifically to the absence of functional $GABA_B$ receptor expression in the pancreas or to other sex-specific alterations observed in $GABA_B^{-/-}$ mice, such as an increase in basal prolactin secretion, which we have observed only in males (12). Prolactin and its related hormones have been demonstrated (10, 41) to stimulate insulin gene transcription, biosynthesis, and secretion and to increase β-cell proliferation; therefore, a possible participation of this hormone in the more marked increase in insulin content and secretion in males

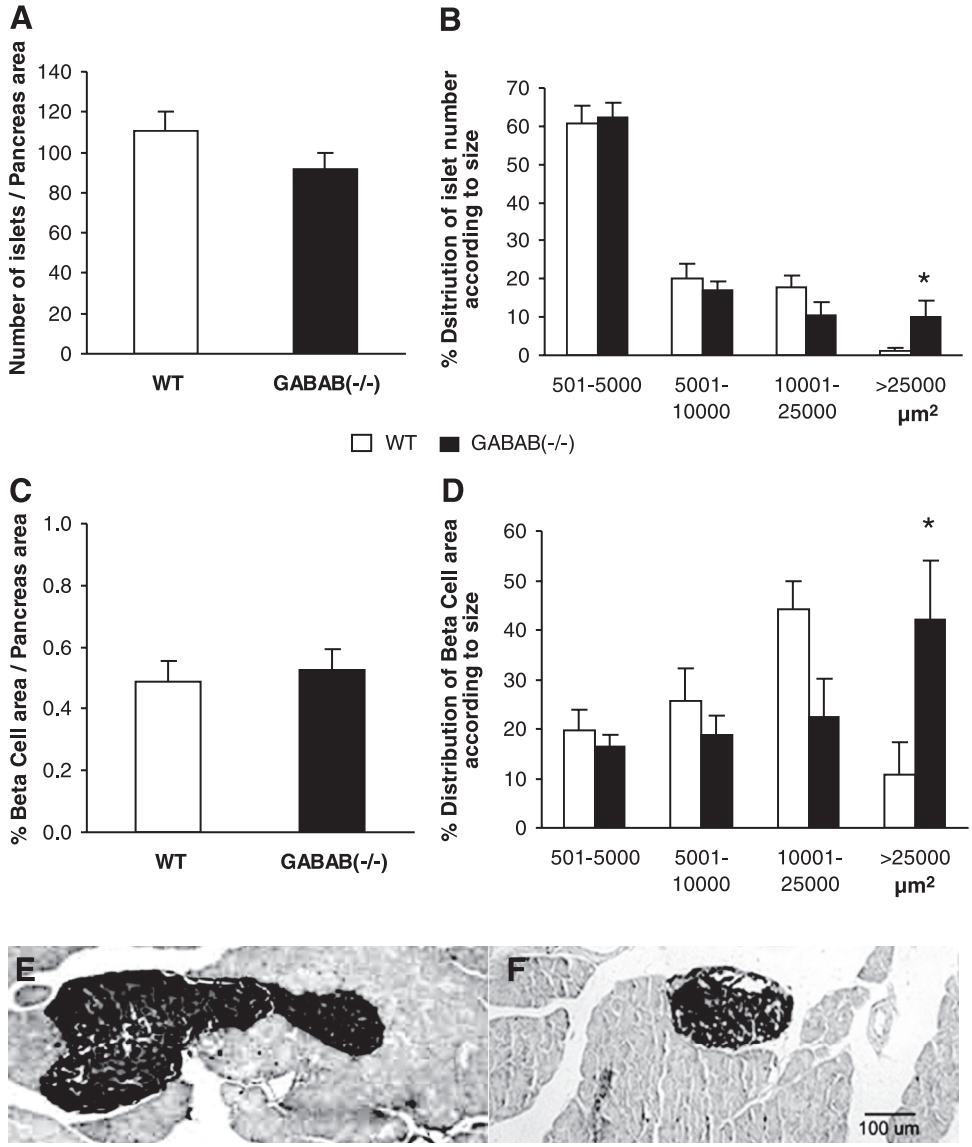

Fig. 7. Histological analysis of pancreas in adult male WT (open bars) and $GABA_B^{-/-}$ mice (filled bars). Three different pancreas sections from each animal ($n = 6$) were immunostained for insulin and subjected to morphometric analysis. *A*: no. of islets relative to pancreas area. *B*: %distribution of islet number according to size, 2-way ANOVA: interaction, $P < 0.01$, *Significantly different from WT. *C*: β-cell area relative to total pancreas area. *D*: %distribution of β-cell area according to islet size, 2-way ANOVA: interaction, $P < 0.01$, *Significantly different from WT. *E*: a representative image of a very large islet from a $GABA_B^{-/-}$ male. *F*: a representative image of a large islet characteristic of WT males. Magnification bar, 100 μm, ×100.

cannot be disregarded. The $GABA_{B1}^{lox511/lox511}$ mice, in which a temporal and spatial conditional inactivation of the $GABA_{B1}$ gene can be obtained, could serve to discriminate between these situations (24).

An alteration in the endocrine pancreas histology was also observed in $GABA_B^{-/-}$ males, with an increase in the population of very large islets, which corresponded to 10% of islet number and 42% of the insulin-positive area. An increase in the proportion of very large islets was also shown in a severe murine model of type 2 diabetes, the muscle IGF-I receptor-lysine-arginine mouse, which expresses a dominant negative IGF receptor in skeletal muscle (2), as well as in NOD mice (29). Whether these very large islets in the $GABA_B^{-/-}$ males have an increased secretion capacity or the increase in secretion is a phenomenon general to all islets remains to be studied. The increase in very large islets is intriguing. It has been demonstrated (44) that the ontogeny of islet cells in early life involves a balance between replication and neogenesis and programmed cell death of β-cells. Which of these phenomena may be altered in $GABA_B^{-/-}$ mice is yet unknown, but it is interesting to note that $GABA_B$ receptors interact directly with transcription factors such as activating transcription factor 4/cAMP response element-binding protein 2 (40) or CCAAT/enhancer-binding protein homologous protein (50), and therefore, the lack of functional $GABA_B$ receptors may alter the normal expression of one or more targets in the islet or the whole animal. In this context, two growth factors known to induce islet cell hyperplasia and decrease apoptosis are IGF-I and IGF-II (26, 27, 45), and their pancreatic expression will be studied in $GABA_B^{-/-}$ mice from birth to adulthood. Conversely, pancreatic histology was similar in both female genotypes, in agreement with minor alterations in glucose homeostasis in $GABA_B^{-/-}$ females.

Interestingly, the higher fasting insulin levels in $GABA_B^{-/-}$ males in the presence of normal glucose suggested a condition of insulin resistance. A significantly increased HOMA-IR index in these animals reinforced this observation. Moreover, insulin tolerance was also significantly impaired in $GABA_B^{-/-}$ males. Therefore, $GABA_B^{-/-}$ mice, particularly the males, show a complex alteration of glucose homeostasis. On the one

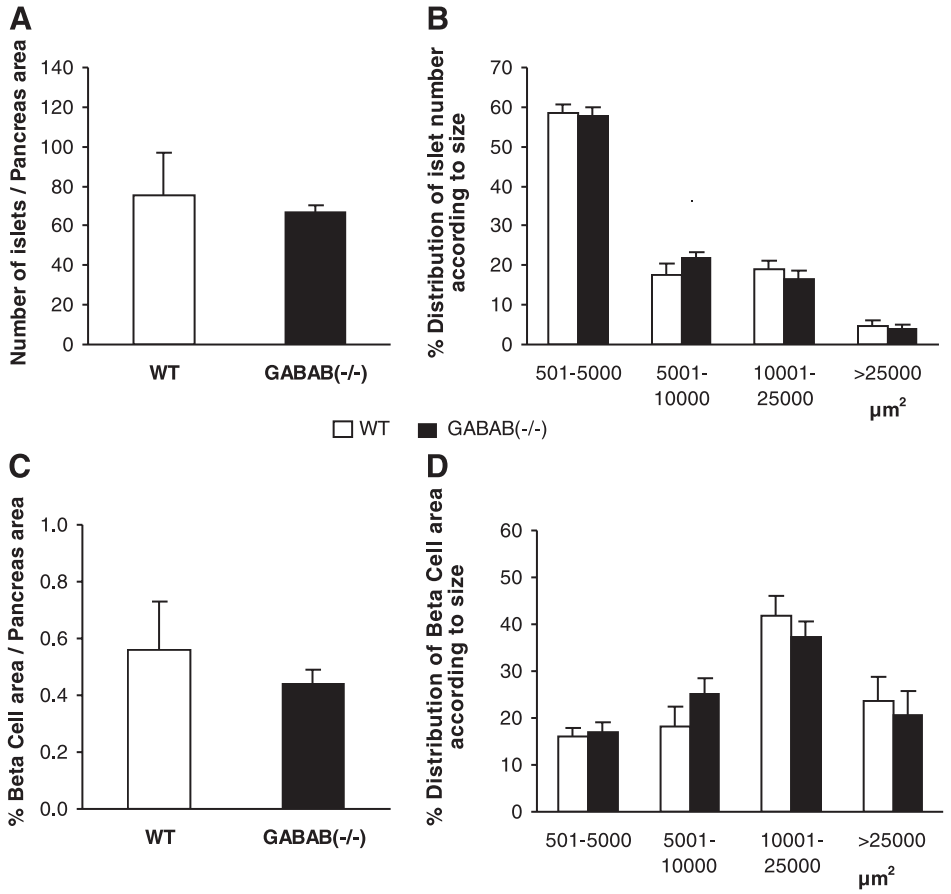

Fig. 8. Histological analysis of pancreas in adult female WT (open bars) and $GABA_B^{-/-}$ (filled bars) mice. Three different pancreas sections from each animal ($n = 5$) were immunostained for insulin and subjected to morphometric analysis. *A*: no. of islets relative to pancreas area. *B*: %distribution of islet number according to size, 2-way ANOVA, NS. *C*: islet area relative to total pancreas area. *D*: %distribution of islet area according to islet size, 2-way ANOVA, NS.

hand, lack of functional $GABA_B$ receptors results in an increase in pancreatic insulin content that improved the response to a moderate glucose overload (2 g/kg). On the other hand, at higher glucose concentrations, such as 3 g/kg, although insulin is increased vs. wild type, a somewhat impaired GTT was observed. These results suggest that in the first condition the ability of the pancreas to secrete more insulin is dominant, whereas with a higher glucose challenge insulin resistance appears in agreement with an increased HOMA index and an impaired ITT in $GABA_B^{-/-}$ mice. An increase in insulin content and secretion in $GABA_B^{-/-}$ mice would be in agreement with the hypothesis that pancreatic GABA inhibits insulin secretion (53) through $GABA_B$ receptors (9, 11), because in these animals the neurotransmitter would not be able to exert its effect in β-cells, and therefore, an increase in insulin can be expected, suggesting the participation of $GABA_B$ receptors in this regulation in vivo. On the other hand, the increase in the HOMA-IR index and the impaired ITT may be the consequence of a sustained increase in insulin secretion that would induce insulin resistance in peripheral tissues, as has been described in functional insulinomas (39, 51, 54), where alterations in insulin receptor splice variants, signaling, and binding have been demonstrated. Whether other hormones involved in the regulation of glucose homeostasis, such as glucagon and somatostatin, are also affected in the $GABA_B^{-/-}$ mouse will have to be determined. Although previous studies suggested that glucagon is regulated mainly by GABA through $GABA_A$ receptors (60, 62) and somatostatin is either not dependent on GABA (19, 48) or only through $GABA_A$ receptors (47), the lack of $GABA_B$ receptor expression may have caused changes in other components of the islet GABA system and indirectly affected these hormones.

Our observations demonstrate that $GABA_B$ receptors are involved in the regulation of glucose homeostasis in vivo, as established by pharmacological studies and in $GABA_B$ receptor knockout mice. In addition, they show that young adult male $GABA_B^{-/-}$ mice have alterations similar to those determined in the prediabetic stage, with normal fasting glucose levels, an increase in insulin secretion, islet hyperplasia, and an incipient insulin resistance. Some of these results are similar to those obtained in prediabetic NOD mice (1, 29) and therefore suggest that $GABA_B^{-/-}$ mice may be an interesting model to study the characteristics of the prediabetic stage and the potential development of diabetes. Observations will be conducted earlier in life to determine at which developmental stages alterations appear and later in adult life to determine whether these animals eventually become fully diabetic. In addition, the specific intracellular mechanisms underlying the increased insulin production and/or secretion in $GABA_B$ knockout males as well as the alterations in peripheral tissues leading to insulin resistance are already under investigation. Our first set of evidence shown herein demonstrates that islets from $GABA_B$ knockout mice cannot respond to local GABA like their wild-type counterparts.

Furthermore, these results point to the potential side effects of $GABA_B$ drug administration on glucose homeostasis. Both agonists and antagonists are in use or being evaluated for future use. The former, such as baclofen, are used in the treatment of

spasticity and trigeminal neuralgia, and they are also being evaluated for the therapeutic treatment of dependence on drugs of abuse, as baclofen has been shown to decrease cravings for various drugs; the latter, such as the antagonist SGS742, are in advanced clinical trials for cognitive impairment (7).

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REFERENCES

1. **Amrani A, Durant S, Throsby M, Coulaud J, Dardenne M, Homo-Delarche F.** Glucose homeostasis in the nonobese diabetic mouse at the prediabetic stage. *Endocrinology* 139: 1115–1124, 1998.
2. **Asghar Z, Yau D, Chan F, Leroith D, Chan CB, Wheeler MB.** Insulin resistance causes increased beta-cell mass but defective glucose-stimulated insulin secretion in a murine model of type 2 diabetes. *Diabetologia* 49: 90–99, 2006.
3. **Bettler B, Kaupmann K, Mosbacher J, Gassmann M.** Molecular structure and physiological functions of GABA(B) receptors. *Physiol Rev* 84: 835–867, 2004.
4. **Bettler B, Tiao JY.** Molecular diversity, trafficking and subcellular localization of GABA(B) receptors. *Pharmacol Ther* 110: 533–543, 2006.
5. **Bianchi MS, Catalano PN, Bonaventura MM, Silveyra P, Bettler B, Libertun C, Lux-Lantos VA.** Effect of androgens on sexual differentiation of pituitary gamma-aminobutyric acid receptor subunit GABA(B) expression. *Neuroendocrinology* 80: 129–142, 2004.
6. **Bianchi MS, Lux-Lantos VA, Bettler B, Libertun C.** Expression of gamma-aminobutyric acid B receptor subunits in hypothalamus of male and female developing rats. *Brain Res Dev Brain Res* 160: 124–129, 2005.
7. **Bowery NG.** GABAB receptor: a site of therapeutic benefit. *Curr Opin Pharmacol* 6: 37–43, 2006.
8. **Braun M, Wendt A, Birnir B, Broman J, Eliasson L, Galvanovskis J, Gromada J, Mulder H, Rorsman P.** Regulated exocytosis of GABA-containing synaptic-like microvesicles in pancreatic beta-cells. *J Gen Physiol* 123: 191–204, 2004.
9. **Braun M, Wendt A, Buschard K, Salehi A, Sewing S, Gromada J, Rorsman P.** GABAB receptor activation inhibits exocytosis in rat pancreatic beta-cells by G-protein-dependent activation of calcineurin. *J Physiol* 559: 397–409, 2004.
10. **Brelje TC, Stout LE, Bhagroo NV, Sorenson RL.** Distinctive roles for prolactin and growth hormone in the activation of signal transducer and activator of transcription 5 in pancreatic islets of langerhans. *Endocrinology* 145: 4162–4175, 2004.
11. **Brice NL, Varadi A, Aschcroft SJ, Molnar E.** Metabotropic glutamate and GABA(B) receptors contribute to the modulation of glucose-stimulated insulin secretion in pancreatic beta cells. *Diabetologia* 45: 242–252, 2002.
12. **Catalano PN, Bonaventura MM, Silveyra P, Bettler B, Libertun C, Lux-Lantos VA.** GABA(B1) knockout mice reveal alterations in prolactin levels, gonadotropic axis, and reproductive function. *Neuroendocrinology* 82: 294–305, 2005.
13. **Chessler SD, Simonson WT, Sweet IR, Hammerle LP.** Expression of the vesicular inhibitory amino acid transporter in pancreatic islet cells: distribution of the transporter within rat islets. *Diabetes* 51: 1763–1771, 2002.
14. **Diaz-Torga GS, Mejia ME, Gonzalez-Iglesias A, Formia N, Becu-Villalobos D, Lacau-Mengido IM.** Metabolic cues for puberty onset in free grazing Holstein heifers naturally infected with nematodes. *Theriogenology* 56: 111–122, 2001.
15. **Franklin IK, Wollheim CB.** GABA in the endocrine pancreas: its putative role as an islet cell paracrine-signalling molecule. *J Gen Physiol* 123: 185–190, 2004.
16. **Freemark M, Avril I, Fleenor D, Driscoll P, Petro A, Opara E, Kendall W, Oden J, Bridges S, Binart N, Breant B, Kelly PA.** Targeted deletion of the PRL receptor: effects on islet development, insulin production, and glucose tolerance. *Endocrinology* 143: 1378–1385, 2002.
17. **Gammelsaeter R, Froyland M, Aragon C, Danbolt NC, Fortin D, Storm-Mathisen J, Davanger S, Gundersen V.** Glycine, GABA and their transporters in pancreatic islets of Langerhans: evidence for a paracrine transmitter interplay. *J Cell Sci* 117: 3749–3758, 2004.
18. **Gassmann M, Shaban H, Vigot R, Sansig G, Haller C, Barbieri S, Humeau Y, Schuler V, Muller M, Kinzel B, Klebs K, Schmutz M, Froestl W, Heid J, Kelly PH, Gentry C, Jaton AL, Van der Putten H, Mombereau C, Lecourtier L, Mosbacher J, Cryan JF, Fritschy JM, Luthi A, Kaupmann K, Bettler B.** Redistribution of GABAB(1) protein and atypical GABAB responses in GABAB(2)-deficient mice. *J Neurosci* 24: 6086–6097, 2004.
19. **Gilon P, Bertrand G, Loubatieres-Mariani MM, Remacle C, Henquin JC.** The influence of gamma-aminobutyric acid on hormone release by mouse and rat endocrine pancreas. *Endocrinology* 129: 2521–2529, 1991.
20. **Gladkevich A, Korf J, Hakobyan VP, Melkonyan KV.** The peripheral GABAergic system as a target in endocrine disorders. *Auton Neurosci* 124: 1–8, 2006.
21. **Gomez R, Asnis N, Tannhauser SL, Barros HM.** GABA agonists differentially modify blood glucose levels of diabetic rats. *Jpn J Pharmacol* 80: 327–331, 1999.
22. **Goren HJ, Kulkarni RN, Kahn CR.** Glucose homeostasis and tissue transcript content of insulin signaling intermediates in four inbred strains of mice: C57BL/6, C57BLKS/6, DBA/2, and 129X1. *Endocrinology* 145: 3307–3323, 2004.
23. **Gu XH, Kurose T, Kato S, Masuda K, Tsuda K, Ishida H, Seino Y.** Suppressive effect of GABA on insulin secretion from the pancreatic beta-cells in the rat. *Life Sci* 52: 687–694, 1993.
24. **Haller C, Casanova E, Muller M, Vacher CM, Vigot R, Doll T, Barbieri S, Gassmann M, Bettler B.** Floxed allele for conditional inactivation of the GABAB(1) gene. *Genesis* 40: 125–130, 2004.
25. **Hayashi M, Yamada H, Uehara S, Morimoto R, Muroyama A, Yatsushiro S, Takeda J, Yamamoto A, Moriyama Y.** Secretory granule-mediated co-secretion of L-glutamate and glucagon triggers glutamatergic signal transmission in islets of Langerhans. *J Biol Chem* 278: 1966–1974, 2003.
26. **Hill DJ, Petrik J, Arany E, McDonald TJ, Delovitch TL.** Insulin-like growth factors prevent cytokine-mediated cell death in isolated islets of Langerhans from pre-diabetic non-obese diabetic mice. *J Endocrinol* 161: 153–165, 1999.
27. **Hill DJ, Strutt B, Arany E, Zaina S, Coukell S, Graham CF.** Increased and persistent circulating insulin-like growth factor II in neonatal transgenic mice suppresses developmental apoptosis in the pancreatic islets. *Endocrinology* 141: 1151–1157, 2000.
28. **Jacobson LH, Bettler B, Kaupmann K, Cryan JF.** GABAB1 receptor subunit isoforms exert a differential influence on baseline but not GABAB receptor agonist-induced changes in mice. *J Pharmacol Exp Ther* 319: 1317–1326, 2006.
29. **Jansen A, Rosmalen JG, Homo-Delarche F, Dardenne M, Drexhage HA.** Effect of prophylactic insulin treatment on the number of ER-MP23+ macrophages in the pancreas of NOD mice. Is the prevention of diabetes based on beta-cell rest? *J Autoimmun* 9: 341–348, 1996.
30. **Johnson SK, Haun RS.** The gamma-aminobutyric acid A receptor pi subunit is overexpressed in pancreatic adenocarcinomas. *JOP* 6: 136–142, 2005.
31. **Kaupmann K, Huggel K, Heid J, Flor P, Bischoff S, Mickel S, McMaster G, Angst C, Bittiger H, Froestl W, Bettler B.** Expression cloning of GABA B receptors uncovers similarity to metabotropic glutamate receptors. *Nature* 386: 239–246, 1997.
32. **Kaupmann K, Malitschek B, Schuler B, Heid J, Froestl W, Beck P, Mosbacher J, Bischoff S, Kulik A, Shigemoto R, Karschin A, Bettler B.** GABA B receptor subtypes assemble into functional heteromeric complexes. *Nature* 396: 683–687, 1998.
33. **Kulkarni RN, Bruning JC, Winnay JN, Postic C, Magnuson MA, Kahn CR.** Tissue-specific knockout of the insulin receptor in pancreatic beta cells creates an insulin secretory defect similar to that in type 2 diabetes. *Cell* 96: 329–339, 1999.
34. **Kuner R, Köhr G, Grünewald S, Eisenhardt G, Bach A, Kornau H.** Role of heteromer formation in GABAB receptor function. *Science* 283: 74–77, 1999.
35. **MacDonald PE, Obermüller S, Vikman J, Galvanovskis J, Rorsman P, Eliasson L.** Regulated exocytosis and kiss-and-run of synaptic-like microvesicles in INS-1 and primary rat beta-cells. *Diabetes* 54: 736–743, 2005.

36. **Margeta-Mitrovic M, Mitrovic I, Riley RC, Jan LY, Basbaum AI.** Immunohistochemical localization of GABA(B) receptors in the rat central nervous system. *J Comp Neurol* 405: 299–321, 1999.
37. **Michalik M, Nelson J, Erecinska M.** GABA production in rat islets of Langerhans. *Diabetes* 42: 1506–1513, 1993.
38. **Moriyama Y, Hayashi M.** Glutamate-mediated signaling in the islets of Langerhans: a thread entangled. *Trends Pharmacol Sci* 24: 511–517, 2003.
39. **Nankervis A, Proietto J, Aitken P, Alford F.** Hyperinsulinaemia and insulin insensitivity: studies in subjects with insulinoma. *Diabetologia* 28: 427–431, 1985.
40. **Nehring RB, Horikawa HP, El Far O, Kneussel M, Brandstätter JH, Stamm S, Wischmeyer E, Betz H, Karschin A.** The metabotropic GABAB receptor directly interacts with the activating transcription factor 4. *J Biol Chem* 275: 35185–35191, 2000.
41. **Nielsen JH, Galsgaard ED, Moldrup A, Friedrichsen BN, Billestrup N, Hansen JA, Lee YC, Carlsson C.** Regulation of beta-cell mass by hormones and growth factors. *Diabetes* 50, *Suppl* 1: S25–S29, 2001.
42. **Olpe HR, Karlsson G, Pozza MF, Brugger F, Steinmann M, Van Riezen H, Fagg G, Hall RG, Froestl W, Bittiger H.** CGP 35348: a centrally active blocker of GABAB receptors. *Eur J Pharmacol* 187: 27–38, 1990.
43. **Park YD, Cui ZY, Wu G, Park HS, Park HJ.** Gamma-aminobutyric acid secreted from islet beta-cells modulates exocrine secretion in rat pancreas. *World J Gastroenterol* 12: 3026–3030, 2006.
44. **Petrik J, Arany E, McDonald TJ, Hill DJ.** Apoptosis in the pancreatic islet cells of the neonatal rat is associated with a reduced expression of insulin-like growth factor II that may act as a survival factor. *Endocrinology* 139: 2994–3004, 1998.
45. **Petrik J, Pell JM, Arany E, McDonald TJ, Dean WL, Reik W, Hill DJ.** Overexpression of insulin-like growth factor-II in transgenic mice is associated with pancreatic islet cell hyperplasia. *Endocrinology* 140: 2353–2363, 1999.
46. **Rey-Roldan EB, Bianchi MS, Bettler B, Becu-Villalobos D, Lux-Lantos VA, Libertun C.** Adenohypophyseal and hypothalamic GABA B receptor subunits are downregulated by estradiol in adult female rats. *Life Sci* 79: 342–350, 2006.
47. **Robbins MS, Grouse LH, Sorenson RL, Elde RP.** Effect of muscimol on glucose-stimulated somatostatin and insulin release from the isolated, perfused rat pancreas. *Diabetes* 30: 168–171, 1981.
48. **Rorsman P, Berggren PO, Bokvist K, Ericson H, Mohler H, Ostenson CG, Smith PA.** Glucose-inhibition of glucagon secretion involves activation of GABAA-receptor chloride channels. *Nature* 341: 233–236, 1989.
49. **Saravia-Fernandez F, Faveeuw C, Blasquez-Bulant C, Tappaz M, Throsby M, Pelletier G, Vaudry H, Dardenne M, Homo-Delarche F.** Localization of gamma-aminobutyric acid and glutamic acid decarboxylase in the pancreas of the nonobese diabetic mouse. *Endocrinology* 137: 3497–3506, 1996.
50. **Sauter K, Grampp T, Fritschy JM, Kaupmann K, Bettler B, Mohler H, Benke D.** Subtype-selective interaction with the transcription factor CCAAT/enhancer-binding protein (C/EBP) homologous protein (CHOP) regulates cell surface expression of GABA(B) receptors. *J Biol Chem* 280: 33566–33572, 2005.
51. **Sbraccia P, D'Adamo M, Leonetti F, Caiola S, Iozzo P, Giaccari A, Buongiorno A, Tamburrano G.** Chronic primary hyperinsulinaemia is associated with altered insulin receptor mRNA splicing in muscle of patients with insulinoma. *Diabetologia* 39: 220–225, 1996.
52. **Schuler V, Lüscher C, Blanchet C, Klix N, Sansig G, Klebs K, Schmutz M, Heid J, Gentry C, Urban L, Fox A, Spooren W, Jaton AL, Vigouret J, Pozza M, Kelly PH, Mosbacher J, Froestl W, Käslin E, Korn R, Bischoff S, Kaupmann K, van der Putten H, Bettler B.** Epilepsy, hyperalgesia, impaired memory, and loss of pre- and postsynaptic GABA(B) responses in mice lacking GABA[B(1)]. *Neuron* 31: 47–58, 2001.
53. **Shi Y, Kanaani J, Menard-Rose V, Ma YH, Chang PY, Hanahan D, Tobin A, Grodsky G, Baekkeskov S.** Increased expression of GAD65 and GABA in pancreatic β-cells impairs first-phase insulin secretion. *Am J Physiol Endocrinol Metab* 279: E684–E694, 2000.
54. **Skrha J, Sindelka G, Haas T, Hilgertova J, Justova V.** Comparison of insulin sensitivity in patients with insulinoma and obese Type 2 diabetes mellitus. *Horm Metab Res* 28: 595–598, 1996.
55. **Sorenson RL, Garry DG, Brelje TC.** Structural and functional considerations of GABA in islets of Langerhans. Beta-cells and nerves. *Diabetes* 40: 1365–1374, 1991.
56. **Tabata T, Araishi K, Hashimoto K, Hashimotodani Y, van der Putten H, Bettler B, Kano M.** Ca2+ activity at GABAB receptors constitutively promotes metabotropic glutamate signaling in the absence of GABA. *Proc Natl Acad Sci USA* 101: 16952–16957, 2004.
57. **Tabata T, Kano M.** GABA(B) receptor-mediated modulation of glutamate signaling in cerebellar Purkinje cells. *Cerebellum* 5: 127–133, 2006.
58. **Thomas-Reetz AC, De Camilli P.** A role for synaptic vesicles in non-neuronal cells: clues from pancreatic beta cells and from chromaffin cells. *FASEB J* 8: 209–216, 1994.
59. **Wang C, Kerckhofs K, Van de Casteele M, Smolders I, Pipeleers D, Ling Z.** Glucose inhibits GABA release by pancreatic β-cells through an increase in GABA shunt activity. *Am J Physiol Endocrinol Metab* 290: E494–E499, 2006.
60. **Wendt A, Birnir B, Buschard K, Gromada J, Salehi A, Sewing S, Rorsman P, Braun M.** Glucose inhibition of glucagon secretion from rat alpha-cells is mediated by GABA released from neighboring beta-cells. *Diabetes* 53: 1038–1045, 2004.
61. **White J, Wise A, Main M, Green A, Fraser N, Disney G, Barnes A, Emson P, Foord S, Marshall F.** Heterodimerization is required for the formation of a functional GABAB receptor. *Nature* 396: 679–682, 1998.
62. **Xu E, Kumar M, Zhang Y, Ju W, Obata T, Zhang N, Liu S, Wendt A, Deng S, Ebina Y, Wheeler MB, Braun M, Wang Q.** Intra-islet insulin suppresses glucagon release via GABA-GABAA receptor system. *Cell Metab* 3: 47–58, 2006.