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Serotonin regulates glucose-stimulated insulin secretion from pancreatic β cells during pregnancy

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In preparation for the metabolic demands of pregnancy, β cells in the maternal pancreatic islets increase both in number and in glucose-stimulated insulin secretion (GSIS) per cell. Mechanisms have been proposed for the increased β cell mass, but not for the increased GSIS. Because serotonin production increases dramatically during pregnancy, we tested whether flux through the ionotropic 5-HT3 receptor (Htr3) affects GSIS during pregnancy. Pregnant *Htr3a*$^{-/-}$ mice exhibited impaired glucose tolerance despite normally increased β cell mass, and their islets lacked the increase in GSIS seen in islets from pregnant wild-type mice. Electrophysiological studies showed that activation of Htr3 decreased the resting membrane potential in β cells, which increased Ca^{2+} uptake and insulin exocytosis in response to glucose. Thus, our data indicate that serotonin, acting in a paracrine/autocrine manner through Htr3, lowers the β cell threshold for glucose and plays an essential role in the increased GSIS of pregnancy.

Pregnancy places unique demands on the metabolism of the mother. As the pregnancy progresses and the nutrient requirements of the fetus increase, rising levels of placental hormones reduce maternal insulin sensitivity, thereby maintaining the maternal/fetal gradient of glucose and the flow of nutrients to the fetus. The mother balances the resulting increase in insulin demand with structural and functional changes in the islets that generate increased and hyperdynamic insulin secretion. β cell numbers increase, the threshold for glucose decreases, and glucose-stimulated insulin secretion (GSIS) increases (1–3). Failure to reach this balance of insulin demand with insulin production results in gestational diabetes (4).

However, the changes in the maternal islets are not simply a response to increased insulin demand, as they precede the development of insulin resistance. Instead, these changes correlate more closely with levels of circulating maternal lactogens (prolactin and placental lactogen) that signal through the prolactin receptor on the β cell (5–9). Downstream of the prolactin receptor, multiple pathway components have been identified that contribute to the maternal increase in β cell mass (10–16), but not the changes in GSIS.

In response to the lactogen signaling during pregnancy, levels of both isoforms of tryptophan hydroxylase, the rate-limiting enzyme in the synthesis of serotonin (5-hydroxytryptamine; 5-HT), rise dramatically in the islet (13, 17, 18). Islet serotonin acts in an autocrine/paracrine manner through the $G\alpha_q$-coupled serotonin receptor 5-HT2b receptor (Htr2b) to increase β cell proliferation and mass at midgestation and through $G\alpha_i$-coupled 5-HT1d receptor (Htr1d) to reduce β cell mass at the end of gestation (13). These dynamic changes in 5-HT receptor (Htr) expression can explain the shifts in β cell proliferation during pregnancy.

In addition to *Htr2b* and *Htr1d*, β cells also express *Htr3a* and *Htr3b* (13). Unlike the 12 other Htr genes in the mouse genome, which encode G-protein coupled serotonin receptors, *Htr3a* and *Htr3b* encode subunits of the serotonin-gated cation channel Htr3 (19, 20). Five identical Htr3a subunits or a mixture of Htr3a and Htr3b make up a functional Htr3 channel (21). The channel is predominantly Na^+- and K^+-selective, and its opening in response to serotonin actives an inward current and depolarizes the cell membrane (22, 23). Glucose also depolarizes β cells: Rising ATP from glucose catabolism depolarizes the cell by closing ATP-sensitive K^+ channels, which causes Ca^{2+} to enter the cell through voltage-gated Ca^{2+} channels and trigger insulin granule exocytosis (24).

Therefore, we tested the possibility that Htr3 may regulate β cell insulin secretion during pregnancy. We found that lactogen-induced serotonin in the pregnant islet acts through Htr3 to depolarize β cells, thereby lowering the threshold for glucose and enhancing GSIS during pregnancy.

Results

Htr3 Affects Glycemic Control During Pregnancy Without Altering β Cell Mass. Because functional Htr3 channels require Htr3a, we used *Htr3a*$^{-/-}$ mice (25) to examine the role of Htr3 in pancreatic β cells. *Htr3a*$^{-/-}$ mice did not differ significantly in body weight or number of progeny relative to wild-type control littermates (Figs. S1 and S2), but they had reduced glucose tolerance during pregnancy (Fig. 1*A*). In contrast, nonpregnant female *Htr3a*$^{-/-}$ mice had normal glucose tolerance (Fig. 1*B*). This difference between pregnant and nonpregnant mice was not a result of changes in *Htr3a* expression during pregnancy (Fig. 1*C* and Fig. S3).

Significance

During pregnancy, maternal insulin secretion increases markedly. This increase is not simply a response to increased demand, as it precedes the insulin resistance that develops late in pregnancy, nor is it solely a result of increased β cell mass, as secretion per beta cell increases as well. Here we show that the increased islet serotonin induced by pregnancy signals through the 5-HT3 receptor (Htr3) to increase insulin secretion dramatically. Htr3 signaling increases the excitability of the β cell membrane, thereby decreasing the threshold for insulin secretion. These studies elucidate the mechanism for pregnancy-induced increase in insulin release.

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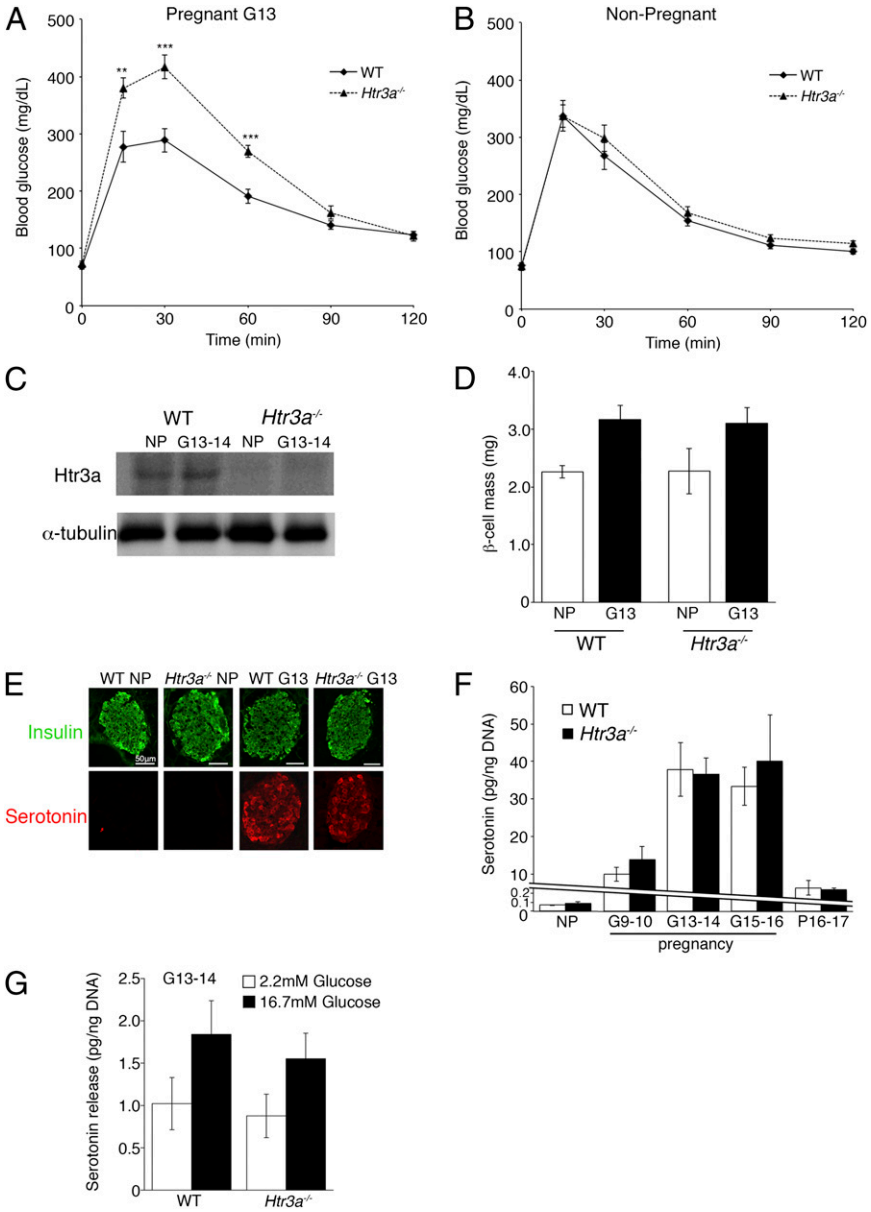

Fig. 1. Htr3 affects glycemic control during pregnancy without altering β cell mass. Blood glucose concentrations were measured after i.p. injection of glucose (2 g/kg body weight) in pregnant G13 (*A*) and nonpregnant (NP) female mice (*B*) with the indicated genotypes. (*C*) Htr3a protein levels in NP and G13 islets were determined by Western blotting. (*D*) Relative β cell mass was calculated as the area of insulin$^+$ cells per total pancreatic area. (*E*) Pancreatic sections from NP and G13 mice were stained for insulin (green) and serotonin (red). (*F*) Serotonin concentrations in NP and G13–G14 islets were assayed by HPLC. (*G*) Serotonin in media from islets cultured at the glucose concentrations shown for 30 min was assayed by HPLC. All data points represent mean ± SEM of at least 5 independent experiments. Statistical significance vs. wild-type (*A* and *B*) was analyzed by Student *t* test. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

To understand the defect in glucose metabolism in pregnant *Htr3a*$^{-/-}$ mice, we measured β cell mass but found no differences from pregnant wild-type mice (Fig. 1*D*). Serotonin production (Fig. 1 *E* and *F*) and release (Fig. 1*G*) were also unchanged in islets from *Htr3a*$^{-/-}$ mice.

Htr3 Increases GSIS During Pregnancy. Because β cell mass was unchanged in *Htr3a*$^{-/-}$ mice, we looked for changes in GSIS at different stages of pregnancy. In islets isolated from wild-type mice, GSIS increased after gestational day 9 (G9) (Fig. 2*A*), peaking at G13–G14 and paralleling the increase in islet serotonin production (13). This increase in GSIS was almost completely blunted in *Htr3a*$^{-/-}$ islets (Fig. 2*B*).

In a glucose dose–response experiment, the wild-type G13–G14 islets released more insulin at both low and high glucose concentrations relative to nonpregnant islets (Fig. 2*C*). The GSIS dose–response curve for G13–G14 *Htr3a*$^{-/-}$ islets, in contrast, had a much smaller increase in GSIS relative to nonpregnant *Htr3a*$^{-/-}$ islets (Fig. 2*C*). Unlike GSIS, KCl-induced insulin secretion was well preserved in *Htr3a*$^{-/-}$ islets (Fig. 2*D*).

To assess GSIS in the intact pancreas, we measured insulin secretion from perfused pancreata of G13–G14 pregnant mice (Fig. 2 *E* and *F*). In nonpregnant females, loss of Htr3a had no effect on GSIS from the pancreas. Pregnancy increased GSIS by threefold in wild-type mice, but substantially less in *Htr3a*$^{-/-}$ mice (Fig. 2*F*), despite the normal increase in β cell mass. Thus, Htr3 signaling affects glycemic control during pregnancy by increasing GSIS, not by increasing β cell mass.

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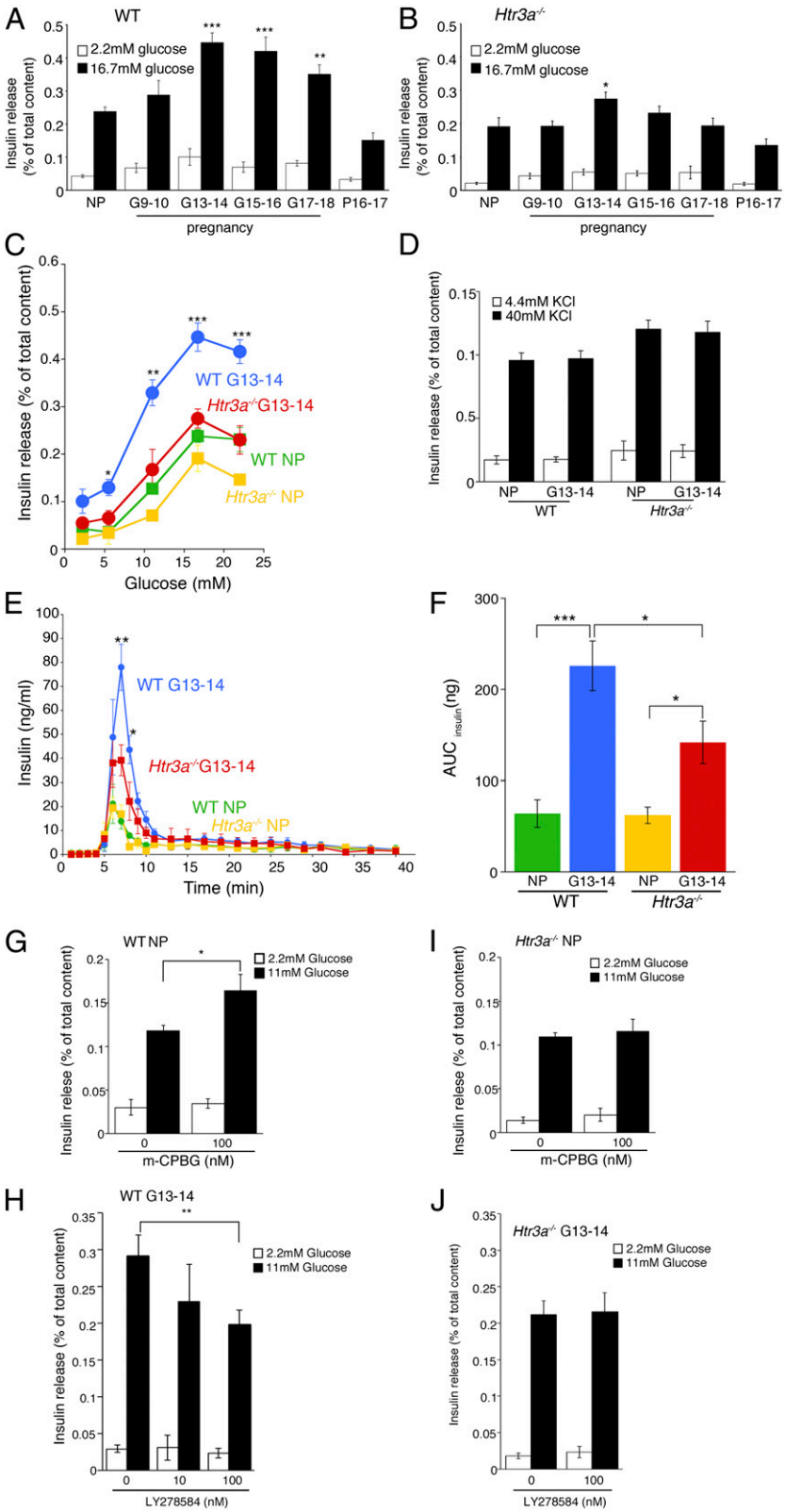

Fig. 2. Activation of Htr3 increases GSIS. After wild-type (*A*) and *Htr3a*$^{-/-}$ (*B*) mouse islets at the gestational (G) or postpartum (P) ages indicated were stimulated with 2.2 or 16.7 mM glucose for 30 min, insulin secreted into the media was calculated as a percentage of the total cellular content. In *C*, the same insulin secretion assay was performed with a range of glucose concentrations. In *D*, insulin secretion was assayed from islets in 2.2 mM glucose stimulated for 10 min with 4.4 or 40 mM KCl. In *E*, insulin secretion was measured from perfused NP and G13–G14 pancreases as glucose concentration was shifted from 2.8 to 16.7 mM, and the area under the curve (AUC) is shown in *F*. Wild-type (*G* and *H*) or *Htr3a*$^{-/-}$ (*I* and *J*) islets were stimulated with 11 mM glucose for 15 min with or without 100 nM Htr3 agonist m-CPBG (*G* and *I*) or for 30 min with or without 100 nM Htr3 antagonist LY278584 (*H* and *J*). All data points represent mean ± SEM of at least 10 independent experiments. Statistical significance versus NP (*A* and *B*), versus *Htr3a*$^{-/-}$ G13–G14 (*C* and *E*), or as indicated (*F*–*J*) was analyzed by Student *t* test. **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

Htr3 Agonists Increase GSIS in Nonpregnant Islets. Next we tested the effect of Htr3 receptor ligands (22) on GSIS from isolated islets. In wild-type nonpregnant islets, Htr3 agonist m-chlorophenylbiguanide (m-CPBG) increased GSIS (Fig. 2*G*), whereas in pregnant islets, Htr3 antagonist LY278584 inhibited GSIS in a dose-dependent manner (Fig. 2*H*); neither affected insulin secretion at a glucose level of 2.2 mM. In *Htr3a*$^{-/-}$ islets, however, neither m-CPBG nor LY278584 altered GSIS, demonstrating the specificity of the two drugs (Fig. 2 *I* and *J*). These data demonstrate that signaling through Htr3 both is necessary for the increase in GSIS in pregnancy and is sufficient to increase GSIS in nonpregnant islets.

Htr3 Lowers the β Cell Threshold for Glucose. To assess the effect of Htr3 on Ca^{2+} influx, we measured glucose-induced intracellular Ca^{2+} ($[Ca^{2+}]_i$) increases in β cells in intact islets, using Fluo-3 and confocal microscopy. As shown in Fig. 3*A*, after the addition of 22 mM glucose, Ca^{2+}-induced Fluo-3 fluorescence increased rapidly and reached a peak after ∼3 min in individual β cells in each islet. Using a cutoff of twofold, β cells were classified into high or low glucose-responders on the basis of their increase in Fluo-3 fluorescence in response to glucose. In nonpregnant wild-type islets, high glucose-responders made up a third of the β cells (Fig. 3 *A* and *B*). In wild-type pregnant islets, the peak Ca^{2+} responses (Fig. 3*A*) and the fraction of high glucose-responders both were increased (Fig. 3*B*). However, the fractions of high glucose-responders in both *Htr3a*$^{-/-}$ pregnant islets and LY278584-treated pregnant wild-type islets were similar to those found in nonpregnant wild-type islets.

In a glucose dose–response experiment, increasing glucose concentration increased the fraction of high glucose-responders in nonpregnant wild-type islets (Fig. 3*C*). The percentage of high glucose-responders was increased at all glucose concentrations during pregnancy in wild-type islets, but not in *Htr3a*$^{-/-}$ islets (Fig. 3*C*).

To directly assess insulin secretion from individual β cells, we monitored glucose-induced insulin granule exocytosis from insulin-GFP-expressing β cells, using total internal reflection fluorescence (TIRF) microscopy (26). Fig. S4 shows representative TIRF imaging data from islets during 22 mM glucose stimulation. In wild-type nonpregnant islets, β cell secretory responses ranged from highly responsive cells to cells with only occasional exocytotic fusion events (Fig. S4*A*). In a wild-type pregnant islet, most of the β cells shift to highly responsive (Fig. S4*B*). However, *Htr3a*$^{-/-}$ pregnant islets and wild-type pregnant islet treated with LY278584 displayed a range of secretory responses more closely resembling nonpregnant islets (Fig. S4 *C* and *D*). Fig. 3 *D* and *E* displays the combined data from multiple cells. These data demonstrate that activation of Htr3 in β cells during pregnancy increases their glucose-evoked Ca^{2+} responses, thereby recruiting low-responsive β cells into the pool of highly glucose-responsive β cells and increasing net GSIS.

Htr3 Decreases Resting Membrane Potential in β Cells. Although Htr3 is a ligand-gated cation channel (22), agonists did not induce insulin secretion without glucose stimulation. Because nonselective cation channels can influence membrane excitability through background Na^+ leak conductance (27), we hypothesized that activation of Htr3 may increase membrane excitability and thereby decrease the membrane threshold for insulin secretion. To test this hypothesis, we used perforated whole-cell voltage-clamp experiments. Continuous superfusion of the β cells with Krebs-Ringer buffer (KRB) solution removed the endogenously secreted serotonin (Fig. 4 *A*–*H* and Fig. S5). Exogenous serotonin and the Htr3-specific serotonin agonist m-CPBG significantly increased inward background current in wild-type β cells (Fig. 4 *A*–*C*), and this increase was attenuated by LY278584

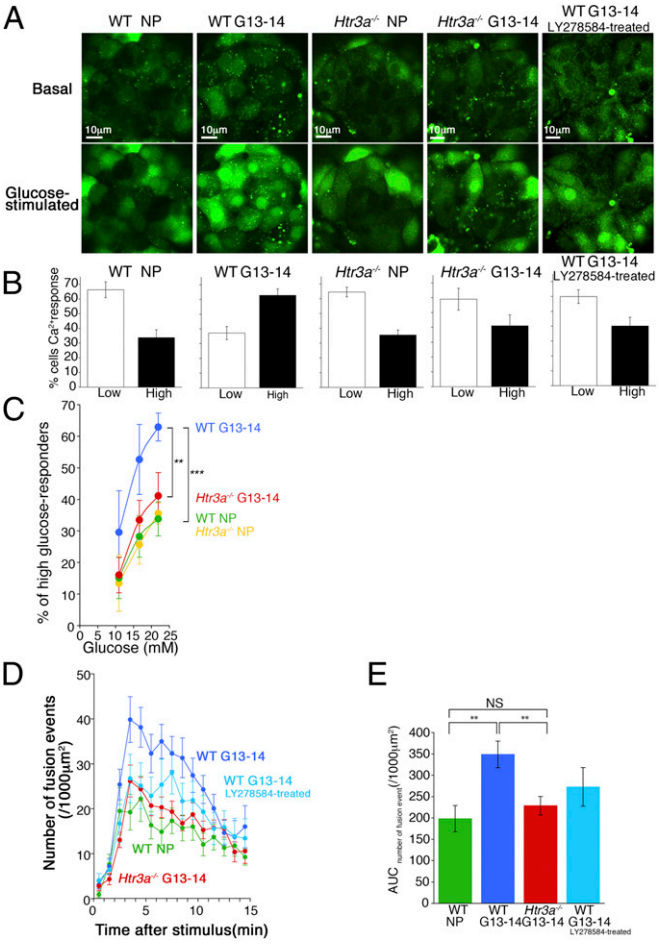

Fig. 3. Htr3 lowers the β cell threshold for glucose. β cell $[Ca^{2+}]_i$ in cultured islets was assayed with Fluo-3:00 AM. Representative images of Fluo-3 fluorescence in β cells after glucose stimulation are shown in *A*. In *B*, graphs show the percentage of high glucose-responders (high, black columns) or low glucose-responders (low, white columns) after glucose stimulation. In *C*, the percentage of high responding β cells is shown as glucose concentration is increased (n = 8–10 islets per group). TIRF imaging is used to measure secretory events during 22-mM glucose stimulation. (*D* and *E*) The number of exocytotic fusion events detected in 1-min intervals after glucose stimulation in individual β cells. The graph in *D* shows the mean number of exocytotic events per 1,000 μm^2 at 1-min intervals after glucose stimulation (n = 10 islets per group), and the AUC is shown in *E*. All data points represent mean ± SEM. Statistical significance was analyzed by Student t test. $^{**}P < 0.01$; $^{***}P < 0.0011$.

(Fig. 4 *E*–*G*). However, neither m-CPBG nor LY278584 changed the membrane current in *Htr3a*$^{-/-}$ mice (Fig. 4 *D* and *H*).

To test whether the increased inward current changed the β cell membrane potential, we performed cell patch current-clamps on single, isolated β cells (Fig. 4 *I* and *J*, and Fig. S6). m-CPBG induced a depolarizing shift in membrane potential in wild-type β cells (Fig. 4*I*, control, −66.6 ± 0.9 mV), but not in *Htr3a*$^{-/-}$ β cells (Fig. 4*J*). Next, we used patch current-clamps on β cells in intact pregnant islets to determine whether the endogenously secreted serotonin can influence β cell membrane potential. We observed the expected decrease in resting membrane potential in β cells in wild-type pregnant islets (Fig. 4*K*, control, −56.2 ± 4.4 mV) relative to a single β cell, but not in *Htr3a*$^{-/-}$ islets (Fig. 4*L*, control, −68.3 ± 1.7 mV). In contrast, LY278584 increased the resting membrane potential of β cells in wild-type pregnant islets (Fig. 4*K*; LY278584, −62.0 ± 2.4 mV), but not in *Htr3a*$^{-/-}$ islets (Fig. 4*L*). Thus, in islets from pregnant

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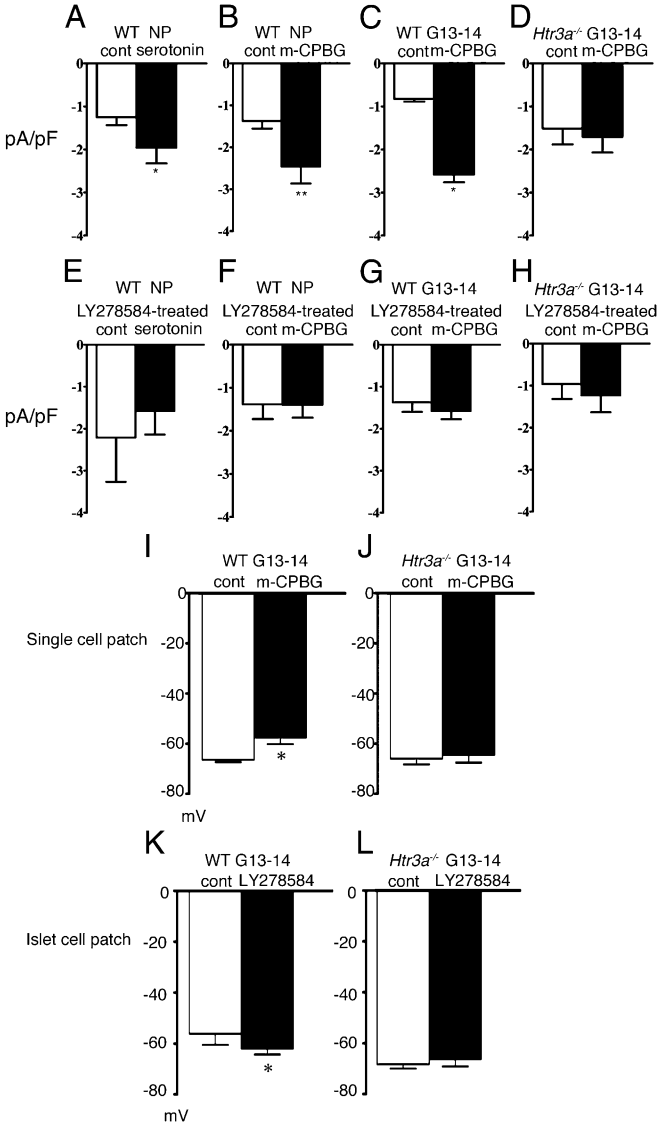

Fig. 4. Htr3 decreases β cell resting membrane potential. Serotonin and Htr3 agonist m-CPBG increased inward currents in wild-type β cells, but not in *Htr3a*$^{-/-}$ β cells (*A–D*). Htr3 antagonist LY278584 attenuated this increase (*E–G*) and had no change in current amplitude in *Htr3a*$^{-/-}$ β cells (*H*). Holding current levels at −70 mV were compared in the absence (control) and presence of m-CPBG from each pregnant β cell. Perforated whole-cell current was divided by cell capacitance to give current density (pA/pF). m-CPBG depolarized the resting membrane of single β cells from wild-type pregnant (*I*) but not *Htr3a*$^{-/-}$ pregnant (*J*) mice. LY278584 hyperpolarized resting potentials in islet-patch mode on islets from wild-type pregnant (*K*) but not *Htr3a*$^{-/-}$ pregnant (*L*) mice. All data points represent mean ± SEM of at least 3 independent experiments. Statistical significance versus control was analyzed by Student *t* test. $*P < 0.05$.

mice, endogenously secreted serotonin acting through Htr3 decreased the resting β cell membrane potential.

Discussion

During pregnancy, maternal lactogens markedly induce islet serotonin production and secretion (13, 17, 18), which in turn acts in an autocrine/paracrine manner through the Htr2b receptor to drive β cell proliferation and increase total β cell mass (13). Here we have established that maternal islet serotonin also acts through Htr3 to increase GSIS during pregnancy. The modest reduction in β cell membrane potential caused by Na^+ leak through Htr3 in response to serotonin was insufficient to produce an action potential and induce insulin exocytosis on its own. However, the reduced membrane potential increased membrane excitability and decreased the threshold for activating voltage-dependent Ca^{2+} entry and insulin exocytosis, thereby increasing the glucose-responsiveness of β cells. Taken together, activation of Htr3 increased GSIS by lowering the glucose threshold for insulin secretion during pregnancy.

Interestingly, we found that Htr3 signaling did not affect all β cells equally but, rather, increased overall islet GSIS largely by recruiting low glucose-responsive β cells into the pool of highly glucose-responsive β cells. In contrast, the levels of Ca^{2+} responses and insulin exocytosis rates in response to glucose did not substantially change in the highly glucose-responsive β cells. Thus, the heterogeneity in glucose-responsiveness among β cells observed in normal islets decreased in pregnant islets, where almost all β cells became highly glucose-responsive.

Heterogeneity in β cell glucose sensitivity has previously been attributed to differences in expression and activity of glucose-sensing components such as glucokinase (28, 29). Prior studies also reported that expression of glucokinase and glucose transporter isoform 2 (GLUT2) was increased in pregnant or prolactin-treated islets, which could potentially explain the increase in GSIS (30, 31). However, we did not detect changes in the mRNA encoding glucokinase or GLUT2 in islets at G13–G14 (13) or in islets from pregnant *Htr3a*$^{-/-}$ mice relative to wild-type (Fig. S7). Instead, most of the change in GSIS during pregnancy could be explained by signaling through Htr3.

The physiological effect of serotonin on GSIS in β cells has been debated (32), with reports of both inhibitory and stimulatory effects (32–35). These differences may derive from differences in age, sex, species, and physiologic state of the islets, which may affect the local concentration of serotonin and the combination of serotonin receptor types expressed on the cell surface (13, 36). Analyzing the effects of a receptor-specific agonist or antagonist in combination with data regarding the β cell expression of distinct receptors provides a way to explain the effects of serotonin on insulin secretion in a specific environment. Indeed, we observed that Htr3-specific agonist m-CPBG increased GSIS from nonpregnant β cells, and an Htr3-specific antagonist inhibited the normal increase of GSIS from pregnant β cells, which secrete high levels of serotonin. Furthermore, to remove the complication of local serotonin production, we tested the effects of exogenous serotonin during continuous superfusion of the β cells with KRB solution to remove locally produced serotonin and showed that serotonin depolarizes β cells through the Htr3a receptor. Our results support the conclusion that locally produced serotonin contributes to the normal increase in β cell GSIS during pregnancy.

Other studies have identified an intracellular pathway by which covalent coupling of serotonin (serotonylation) to the small GTPases, Rab3a and Rab27a, can enhance exocytosis directly and augment insulin secretion from β cells (37). However, we observed that the exocytotic steps after activation of voltage-dependent Ca^{2+} channels were not altered during pregnancy. Our results demonstrate that the increase in GSIS during pregnancy resulted from signaling by extracellular serotonin via the cell-surface receptor Htr3, and we found no evidence for the involvement of intracellular serotonylation.

In conclusion, we have identified Htr3 as a key component in a signaling pathway by which serotonin increases the sensitivity of maternal β cells to glucose during pregnancy. Further study of this pathway may provide insights into the genetic causes of gestational diabetes, as well as strategies for detecting, preventing, and treating both type 2 diabetes and gestational diabetes.

Materials and Methods

Animal Experiments. C57BL/6J mice were housed on a 12-h light–dark cycle in climate-controlled, pathogen-free barrier facilities. The institutional animal care and use committees at Kyorin University and the University of California, San Francisco, approved all studies involving mice. *Htr3a* targeted mice were purchased from Jackson laboratory and backcrossed with C57BL/6J mice for more than 10 generations; they were used at the age of 8–16 wk. Mating was confirmed by the presence of a vaginal plug the next morning, designated day 0 of gestation. An i.p. glucose tolerance test was performed as described previously (13).

Islet Preparation, RT-PCR, and Insulin Secretion. Pancreatic islets were isolated from wild-type and *Htr3a*$^{-/-}$ mice by collagenase digestion, as previously described (26). RNA extraction, RT-PCR, and real-time RT-PCR were performed as previously described (13). Primer sequences are available on request. The pancreas perfusion experiments are described previously with slight modifications (38, 39). The insulin release in the perfusate was measured by ELISA.

TIRF Microscopy. To label insulin secretory granules, islets were infected with recombinant adenovirus Adex1CA insulin-GFP, as previously described (26). Experiments were performed 2 d after the final infection. The Olympus total internal reflection system with a high-aperture objective lens was used as described previously (40). The exocytotic fusion events in each corresponding cell were counted on time-course.

SI Materials and Methods gives additional experimental procedures and information.

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