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Research Article

Sex Differences in Pancreatic β-Cell Physiology and Glucose Homeostasis in C57BL/6J Mice

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Abstract

The importance of sexual dimorphism has been highlighted in recent years since the National Institutes of Health's mandate on considering sex as a biological variable. Although recent studies have taken strides to study both sexes side by side, investigations into the normal physiological differences between males and females are limited. In this study, we aimed to characterized sex-dependent differences in glucose metabolism and pancreatic β-cell physiology in normal conditions using C57BL/6J mice, the most common mouse strain used in metabolic studies. Here, we report that female mice have improved glucose and insulin tolerance associated with lower nonfasted blood glucose and insulin levels compared with male mice at 3 and 6 months of age. Both male and female animals show β-cell mass expansion from embryonic day 17.5 to adulthood, and no sex differences were observed at embryonic day 17.5, newborn, 1 month, or 3 months of age. However, 6-month-old males displayed increased β-cell mass in response to insulin resistance compared with littermate females. Molecularly, we uncovered sexual dimorphic alterations in the protein levels of nutrient sensing proteins O-GlcNAc transferase and mTOR, as well as differences in glucose-stimulus coupling mechanisms that may underlie the differences in sexually dimorphic β-cell physiology observed in C57BL/6J mice.

Key Words: diabetes, sexual dimorphism, islet, insulin secretion, sex differences, beta-cell mass

Abbreviations: AUC, area under the curve; e, embryonic day; FBS, fetal bovine serum; HBSS, Hank's balanced saline solution; Ogt, O-GlcNAc transferase.

What has become clear in the past decade is the firm appreciation to consider sex as a biological variable in both preclinical and clinical research to understand multiple factors contributing to the pathology of metabolic diseases such as type 2 diabetes. Indeed, the very few available studies that are designed to systematically examine sex differences on islets [1, 2] or the fetal origins of obesity and type 2 diabetes have demonstrated that across the lifespan, males and females can differ significantly in their metabolic status and responses to stress [3-7].

Despite the requirement from the National Institutes of Health to study both sexes, islets or metabolic studies reporting side-by-side comparison in males and females remain scant.

However, by cross-referencing available data, strong evidence suggests that glucose homeostasis and metabolic dysfunction are often sex-dependent [8-10]. For example, in healthy humans, females may have a faster clearance of glucose, with higher circulating insulin levels following an oral glucose tolerance test and exhibit greater insulin sensitivity than males. The increased insulin secretion after oral glucose tolerance is in line with reports on pancreatic biopsies that estimate a ~6% increase in β-cell number in females compared with males [11, 12]. Type 2 diabetes has a higher incidence in males compared with females [13]. The preponderance of current data supports this view in many rodent models as well, especially in C57BL/6J mice, a commonly used strain in diabetes research [14]. Although sex differences in insulin sensitivity partially account for this phenomenon, uncharacterized differences in pancreatic β-cell mass or function may also contribute.

Few studies have reported sex differences in islets from various species in parameters from gene expression and function, to number of sensory neurons and mitochondria [1, 15, 16]; however, to our knowledge, sexual dimorphism on β mass and function in mice remains unclear. Thus, a better understanding of mouse physiology and sex differences at the islet level is needed to better understand the pathology of disease and translational capacity of animal models. To our knowledge, a side-by-side comparison of male and female pancreatic β-cell physiology and glucose homeostasis in C57BL/6J mice has not been reported.

Although the profile of male β-cell physiology in age and stress conditions is reported [9, 17-19], there is a lack of

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understanding of normal islet physiology of female mice and how it compares with males. Thus, the goal of this study is to set a baseline comparison on islet physiology between male and female littermates at specific time points when it is commonly tested in metabolic studies: 0, 1, 3, and 6 months of age. In the current study, we aimed to characterize the sex differences in in vivo metabolic parameters as well as β-cell physiology using a C57BL/6J mouse strain, the most used strain in metabolic studies. Comparing littermates side by side, we show that female mice exhibit improved glucose tolerance and insulin sensitivity compared with littermate male mice in adulthood. These data are in line with reduced β-cell mass and islet insulin secretion in females compared with male mice. Mechanistically, our data show that females have altered glucose-stimulus coupling mechanisms, including differences in Ca^{2+} signaling and expression of critical β-cell nutrient sensor proteins, that may underlie the differences in islet mass and function between the sexes in adulthood.

Methodology

Mice

Male and female (12 weeks or 22 weeks of age) naïve C57BL/6J mice were purchased from The Jackson Lab (000664; Jackson Laboratories). Mice used to assess calcium (done at the University of Michigan) and β-cell mass (done at the University of Minnesota) were directly from The Jackson Lab and were tested following a 2-week of period acclimation in our facility. C57BL/6J mice from the Jackson Lab were bred for embryonic, newborn, and 1-month (28-35 days) old studies, and electrophysiology experiments. For embryonic collection, 2 dams were set up for time pregnancies in the evenings and a detection of a vaginal plug indicated embryonic day 0.5 (e0.5). Pancreases were collected on e17.5. Mice in the laboratory were maintained on a normal chow diet (Teklad Global 2018 Rodent Diet). All studies were approved by the Institutional Animal Care and Use Committee at the University of Michigan and University of Minnesota.

Metabolic In Vivo Studies

Body weight, random blood glucose, and serum were collected at indicated time points (at e17.5, birth (newborn), 1 month, 3 months, and 6 months of age). Blood from the tail vein was used to assess glucose using a handheld glucometer and spun down to collect serum for insulin measurements. The Ultrasensitive Mouse Insulin ELISA (ALPCO, AB_2792981) kit was used to measure serum insulin. Insulin sensitivity was assessed following a 6-hour fast with blood glucose measurements before and after (30, 60, and 120 minutes) an IP injection of 0.5 or 0.75 U/kg insulin in 0.9% saline. Glucose tolerance tests were done by fasting mice overnight (14 hours) and, similarly, blood glucose was measured before and after a 2 g/kg IP injection of 30% dextrose solution.

Islet Isolation

Pancreases of animals (nonfasted) were clamped at the ampulla and perfused through the common bile duct with cold Collagenase P in Hank's balanced salt solution (HBSS). Inflated pancreases were dissected from the surrounding viscera and digested for 7 to 10 minutes by incubation in a 37 °C water bath with occasional manual agitation. Digestion was stopped with HBSS (+Ca^{2+}, Mg^{2+}) containing 2% fetal bovine serum (FBS). The digested tissue was filtered through a tea strainer to remove large debris and a 70-μm filter to separate islets from acinar. Captured islets were washed off the screen and handpicked clean into warm RPMI 1640 media (5 mM glucose, 10% FBS, pen/strep), then rested overnight in a humidified 37 °C incubator with 5% CO_2 before testing (perifusion insulin secretion, Ca^{2+} imaging) or collection (Western blot, quantitative PCR). To closely mimic human physiology, we used 5 mM glucose for islet incubation after isolation, whereas other laboratories commonly use 11 mM of glucose found in the readily purchasable RPMI 1640. For specific subsets of experiments (quantitative PCR in Supplementary Fig. S7G-O [20] and electrophysiology in Supplementary Fig. S6A and B [20]), islets were maintained overnight in 10 mM glucose instead of our routine 5 mM glucose. Naïve untested insulin content was measured from 10 islets from each mouse, using Ultrasensitive Mouse Insulin ELISA kits (ALPCO, AB_2792981) and normalized by DNA content with PicoGreen dsDNA assay kit.

Dynamic Glucose-stimulated Insulin Secretion

Perifusion experiments were performed using a PERI4-V2 machine (Biorep Technologies, Miami, FL, USA). Perifusion buffer (24 mM $NaHCO_3$, 120 mM NaCl, 4.8 mM KCl, 2.5 mM $CaCl_2$ $2H_2O$, 1.2 mM $MgCl_2$ $6H_2O$, 10 mM HEPES, 0.25% BSA) was freshly prepared for each perifusion run. Batches of 65 to 70 islets were handpicked and loaded into perifusion chambers between layers of sedimented microbead suspension (Bio-Gel P-4, BioRad Laboratories), and on top of a fiberglass filter. Perifusion buffer containing selected concentrations of glucose (3 mM, 6 mM, 8 mM, 10 mM, 16.7 mM) or KCl (30 mM) was circulated through the columns and collected in 96-well plates. For each run, a 45 to 60 minutes of incubation of low glucose (3 mM) washing at a rate of 100 μL/min served to stabilize islets before stimulation in 1 of the following sequences: (1) 8 minutes of low glucose (3 mM), 16 minutes of moderate glucose (10 mM), 16 minutes of high glucose (16.7 mM), 10 minutes of low glucose (3 mM) rest, 10 minutes of KCl (30 mM), and 10 minutes of low glucose (3 mM) washout; (2) 8 minutes of low glucose (3 mM), 16 minutes of moderate glucose (8 mM), 10 minutes of low glucose (3 mM) rest, 10 minutes of KCl (30 mM), and 10 minutes of low glucose (3 mM) washout; or (3) 8 minutes of low glucose (3 mM), 16 minutes of mild glucose (6 mM), 10 minutes of low glucose (3 mM) rest, 10 minutes of KCl (30 mM), and 10 minutes of low glucose (3 mM) washout. Samples (100 μL) were collected every minute from the outflow tubing into a 96-well plate. Islets and perifusion solutions were kept at 37 °C in a temperature-controlled chamber, whereas the perfusate was kept at <4 °C. Insulin concentrations were determined using commercially available Ultrasensitive Mouse Insulin ELISA kits (ALPCO, AB_2792981). Any timepoint below the threshold of detection in insulin ELISA were substituted as 0. Islets within perifusion chambers were recovered, washed with HBSS, and stored in −80 °C until lysis (1× RIPA buffer + 1% protease inhibitors) and sonication. Insulin concentrations across time were normalized to DNA as determined from undiluted lysate using a PicoGreen dsDNA assay, per kit instruction. Supplementary Fig. S8 [20] shows individual traces across all experiments.

Endocrine-cell Mass Analysis

Paraffin-embedded pancreases were sectioned at 5 micron thickness representing 5 to 7 areas spaced 200 microns apart across the entire pancreas. For each mouse, pancreas area was stained for insulin (guinea pig, Agilent DAKO IR002, AB_2800361) and glucagon (rabbit, Abcam ab92517, AB_10561971) and imaged for immunofluorescence at 10 × magnification using the Keyence BZ-x800 microscope for a total of 5 to 7 slides per mouse. Using Fiji/ImageJ, insulin-positive and glucagon-positive area was assessed and divided over pancreas area. This value was averaged across all sections for each mouse to obtain a global insulin/pancreas or glucagon/pancreas ratio. This global ratio was multiplied by either pancreas weight to obtain β-cell mass or α-cell mass; or by pancreas weight/body weight to obtain corrected β-cell mass or α-cell mass.

Protein Extraction and Western Blotting

Isolated islets from 6 male and 6 female mice were sonicated in 1 × RIPA buffer (CST) with protease and phosphatase inhibitor cocktails (Abcam). Following BCA assay for protein quantification, lysates were resolved by SDS-PAGE, transferred to polyvinylidene fluoride membrane, and probed using primary antibody for RL2 (Abcam, AB_303264), O-GlcNAc transferase (Ogt; CST, AB_2716710), pS6 S240 (CST, AB_10694233), S6 (SCBT, AB_1129205), pAkt S473 (CST, AB_2315049), Akt (CST, AB_1147620), Kir6.2 (SCBT, AB_2721772), Pdx1 (Millipore, AB_10617514), and Glut2 (Millipore, AB_2890623). Using horseradish peroxidase-conjugated secondary antibody (GE), the blots were imaged with chemiluminescence. The protein bands were quantified using Fiji image software. Representative image of 1 male and 1 female are presented in the figure, along with quantitated data.

RNA Extraction and Quantitative Real-time PCR

RNA was isolated from islets using the Rneasy Plus Micro Kit (Qiagen) following the manufacturer's instructions. cDNA was synthesized from total RNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA, USA). Relative gene expression was assessed on an Applied Sciences QuantStudio Flex 6 Real Time PCR System using Power SYBR Green (Applied Biosciences, Salt Lake City, UT, USA), according to the ΔΔCT method, normalized to 36B4 and Actin housekeeping genes. Primer sequences are listed in Table 1.

Calcium Imaging

Isolated islets were allowed to recover overnight (RPMI, 5 mM glucose, 10% FBS) from islet isolation before measurement of intracellular Ca^{2+}. Islets were preloaded with 2.5 μM Fura2-AM (Invitrogen) in 0.1% DMSO for 30 minutes at 37 °C and washed for 10 minutes before recording commenced. The recording solution contained (in mM): 145 NaCl, 5 KCl, 1.2 $MgCl_2$, 2.6 $CaCl_2$, 10 HEPES, and glucose as indicated; pH 7.4. Ca^{2+} levels are reported as the ratio of alternative excitation at 340 nm/380 nm, with emission measured at 510 nm. All calcium experiments were done at the University of Michigan.

Electrophysiology

Isolated islets were stored in a CO_2 incubator at 37 °C overnight in RPMI supplemented with FBS, pen/strep, and 5 or 10 mM glucose); cells were recorded over the next 2 days. Islets were transferred to a perfused recording chamber on a fluorescence microscope. An extracellular solution containing (in mM) 140 NaCl, 3 $CaCl_2$, 5 KCl, 2 $MgCl_2$, 10 HEPES, pH 7.4 was supplemented with glucose concentration of 3, 6, 8, or 10 mM. Recordings were begun in 10 mM glucose to verify β-cell identity by the appearance of oscillatory activity. Recording electrodes were pulled using a Sutter P-97 puller and firepolished using a Narishige, to 4 to 8 MΩ. Electrodes were filled with an intracellular solution containing (in mM) 90 KCH_3SO_4, 10 NaCl, 10 KCl, 1 $MgCl_2$, 5 HEPES, 0.5 EGTA, pH 7.35, and amphotericin. Current clamp recordings were performed using an Axon Multiclamp 700B, digitized using a Digidata 1320A and analyzed in pClamp. All electrophysiology experiments were done at the University of Minnesota.

Supplemental Figures

Supplementary Figs. S1–S9 are deposited at https://doi.org/10.6084/m9.figshare.23712954.v2

Statistical Analysis

Data are presented as mean ± SEM and were analyzed using 2-tailed unpaired Student *t*-test. Multiple outcome data were assessed using repeated measures 2-way ANOVA. Statistical analyses were performed in GraphPad Prism version 7 with a significance threshold of $P < .05$.

Results

Females Have Improved Glucose Tolerance and Insulin Sensitivity Compared With Males in Adulthood

To establish physiological baseline comparisons between sexes, we characterized in vivo metabolic parameters as well as pancreatic islet physiology of C57BL/6J male (circle) and female (triangle) mice at different ages (Supplementary Fig. S1A [20]). At embryonic day 17.5 (e17.5), there were no differences in body weight and pancreas mass between male and female mice (Fig. 1A-B, Supplementary Fig. S1B [20]). Pancreatic β-cell mass, α-cell mass, and their ratios over pancreas area were comparable between male and female embryos (Fig. 1C-F, Supplementary Fig. S1C-F [20]). Similar results were found between male and female newborn mice (Fig. 1G-L, Supplementary Fig. S1G-K [20]). At 1 month of age, female mice demonstrated smaller body weight (Supplementary Fig. S2A [20]) without significant differences in nonfasted blood glucose or serum insulin (Supplementary Fig. S2B-C [20]). One-month-old female mice demonstrated comparable glucose tolerance and insulin sensitivity as males (Supplementary Fig. S2D-E [20]).

At 3 months of age, female mice consistently exhibited lower body weight (Fig. 2A), reduced nonfasted blood glucose, and random serum insulin compared with male mice (Fig. 2B-C). Pancreas mass was also smaller in 3-month-old females (Fig. 2D), but when normalized to body weight, was comparable to age-matched males (Supplementary Fig. S2F [20]). There was no significant difference in β-cell mass at 3 months (Fig. 2E, Supplementary Fig. S2G-H [20]), but in females, we

Table 1. Quantitative PCR primer list

Gene	Forward primer	Reverse primer
Ins1	GCCATGTTGAAACAATGACCT	CAGAGAGGAAGGTACTTTGGACTATAA
Ins2	GAAGTGGAGGACCCACAAGT	AGTGCCAAGGTCTGAAGGTC
Pdx1	GAAATCCACCAAAGCTCACG	CGGGTTCCGCTGTGTAAG
Glut2	ACACCGGAATGTTCTTAGCC	GTGAGAAGCCGAGGAAAG
Kir6.2	GCTGCATCTTCATGAAAACG	TTGGAGTCGATGACGTGGTA
Sur1	CTGGTGGCCATCGACACAA	TGTACAGGAGCCAGCAGAAT
Gck	CCATTTTCCAGGGGTAGCA	CACAAATGCTCCCAGTCCA
Atp1a1	CCTCTGCTTCGTGGGTCTTATC	TCGCTGTGATTGGATGGTCTC
Atp1a3	CAGTCATCTTCCTCATCGGCATCA	TTGGCGGTCAGCGTCAGA
36B4	AGATGCAGCAGATCCGCAT	GTTCTTGCCCATCAGCACC
Actin	GCCCTGAGGCTCTTTTCCAG	TGCCACAGGATTCCATACCC

observed a trend toward increased α-cell mass with a significant increase in α-cell-to-pancreas and α-cell-to-β-cell area ratio compared with males (Supplementary Fig. S2I-L[20]).

At 6 months of age, female mice consistently exhibited smaller body and pancreas weight and lower nonfasted blood glucose and random serum insulin compared with age-matched males (Fig. 2F-I, Supplementary Fig. S2M [20]). Interestingly, β-cell mass is significantly lower in females relative to 6-month-old males (Fig. 2J, Supplementary Fig. SN-O, T [20]). Between the 3- and 6-month time points, female β-cell mass did not change, whereas male β-cell mass was increased (Supplementary Fig. S2U [20]). We observed no significant differences in α-cell mass or ratios at this timepoint (Supplementary Fig. S2P-S [20]).

Females exhibited improved glucose tolerance and insulin sensitivity compared with male mice as early as 3 months of age (Fig. 2L-M). We validated this finding in a separate cohort of mice, where young and adult male and female mice were tested together. Here, we additionally highlight that with age, male mice developed increased insulin resistance, whereas female mice maintained relatively stable insulin sensitivity (Fig. 2N, insulin tolerance test [ITT] area under the curve [AUC]). To understand the sex differences in glucose tolerance and circulating insulin levels that precede the divergence in β-cell mass, we focused on ex vivo islet studies at 3 months of age.

Sex-dependent Dynamic in Vitro Islet Glucose-stimulated Insulin Secretion

We assessed whether there are sex differences in islet insulin secretory capacity by measuring dynamic glucose-stimulated insulin secretion in young (1 month) and adult (3 month) mice. We found no significant differences in basal (3 mM glucose) and glucose-stimulated (10 mM and 16.7 mM glucose) insulin secretion between islets of young males and females (Supplementary Fig. S3A-H; individual traces shown in Supplementary Fig. S4 [20]), evidenced by comparable AUC and average peak insulin secretion.

In adult mice, there was no significant difference in basal insulin secretion between males and females at 3 mM glucose (Supplementary Fig. S3I-J[20]). However, we observed a trend toward increased insulin secretion (2-way ANOVA $P = .0648$; AUC $P = .0624$) by adult female islets compared with male islets on 6 mM glucose stimulation (Fig. 3A-B; individual traces shown in Supplementary Fig. S4[20]). Interestingly, 8 and 10 mM glucose stimulation led to lower peak insulin secretion in female islets compared with males (Fig. 3C-H; individual traces shown in Supplementary Fig. S4 [20]). In response to 16.7 mM glucose or KCl-induced depolarization, there is a trending decrease (16.7 mM Glucose [G] AUC $P = .0802$; KCl AUC $P = .0664$) in insulin secretion by adult female islets compared with male (Fig. 3I-K; Supplementary Fig. S5A; individual traces shown in Supplementary Fig. S4 [20]). These data suggest that female islets may be more glucose sensitive and secrete more insulin in response to 6 mM glucose, but in higher concentrations of glucose (8-16.7 mM glucose), they secrete less insulin than male islets.

Despite differences in insulin secretion at various levels of glucose stimulation between male and female islets, we found no differences in naïve (untested) total islet insulin content between the sexes (Fig. 4A). We also detected no differences in insulin content of islets recovered after perifusion glucose-stimulated insulin secretion experiments (Supplementary Fig. S5B [20]), and male islets still exhibited greater insulin secretion than female islets of less than 10 mM glucose stimulation or KCl-induced depolarization when normalized to insulin content (Supplementary Fig. S5C-F [20]). At the transcriptional level, there were no significant differences in *Ins1* expression between sexes; however, lower *Ins2* expression was observed in female islets (Supplementary Fig. S7A-B; Supplementary Fig. S8A-B [20]). Pdx1 protein, a key transcription factor that regulates insulin gene transcription, interestingly, was significantly decreased in female islets compared with males (Fig. 4B-C) but not at the mRNA level (Fig. 4D, Supplementary Fig. S8C [20]). Altogether, these data suggest that sex differences observed in insulin secretion are not dependent on insulin content, but rather on glucose-stimulus coupling pathways.

Calcium Signaling and Membrane Potential Changes in Response to Glucose

Insulin secretion is triggered by membrane depolarization and the influx of Ca^{2+} into the cytosol. To explore the mechanisms that may account for the differences in glucose-stimulated insulin secretion that we observed for islets from male and female mice, cytosolic Ca^{2+} imaging was performed in isolated islets using the cytosolic Ca^{2+} indicator dye, Fura-2. Exposing

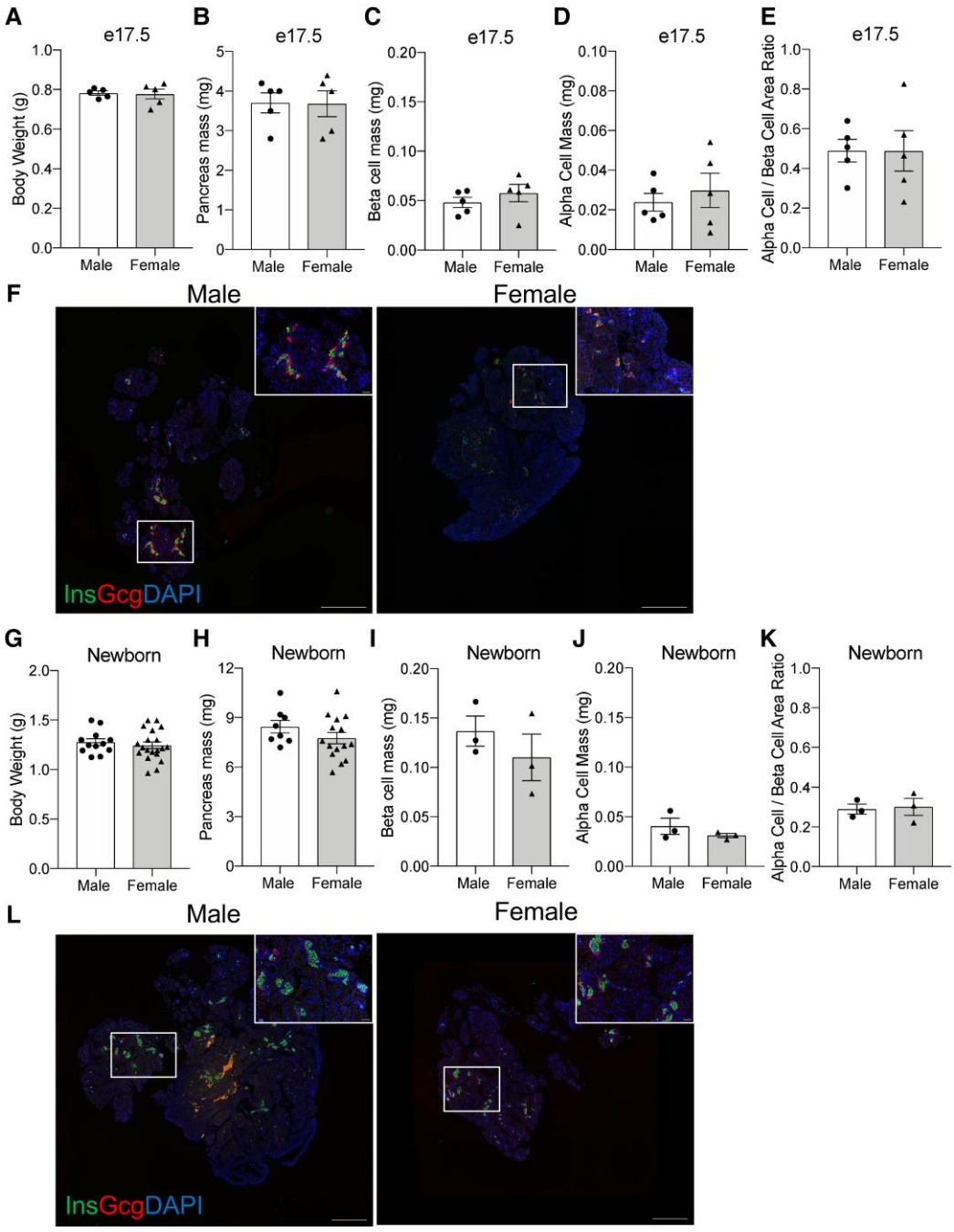

Figure 1. Pancreatic endocrine cell mass between male and female C57BL/6J mice at embryonic day 17.5 and birth Body weight (A), pancreas mass (B), beta cell (C) and alpha cell (D) mass and alpha cell-to-beta cell area ratio (E) of male and female C57BL/6J mice at embryonic day (e) 17.5 (n = 5). Representative image of pancreas section (F) between male and female mouse at e17.5, stained against insulin (green), glucagon (red) and DAPI. Magnification: 10×, 20× (inset). Scale bar: 500 um, 50 um (inset). Body weight (G), pancreas mass (H), beta cell (I) and alpha cell (J) mass and alpha cell-to-beta cell area ratio (K) of male and female C57BL/6J mice at birth (newborn; n = 12-20, n = 3 endocrine cell mass). Representative image (L) of pancreas section between male and female mouse at birth, stained against insulin (green), glucagon (red) and DAPI. Magnification: 10×, 20× (inset). Scale bar: 500 um, 50 um (inset). Statistical analysis performed using unpaired, two-tailed, student T-Test $P \leq .05$.

islets from male or female mice to stimulatory glucose (8 mM) revealed that a smaller proportion of female islets exhibited Ca^{2+} oscillations (Fig. 5A-B). Female islets that oscillated exhibited a decreased amplitude (Fig. 5C) and an increased period (Fig. 5D) compared with oscillating male islets. However, no differences in the plateau fraction of oscillations were observed between sexes (Fig. 5E).

To understand whether these differences reflected changes in β-cell function upstream of Ca^{2+} influx, we next assessed membrane potential oscillations in isolated islets from male and female mice. Following isolation, pancreatic islets were incubated in either 5 mM or 10 mM glucose overnight before experimental testing. In islets incubated in 5 mM glucose overnight, a higher proportion of female islets exhibited oscillations at lower glucose concentrations (eg, 6 and 8 mM) compared with males (Fig. 5F-H). Importantly, these differences did not persist in islets incubated overnight in 10 mM glucose (Supplementary Fig. S6[20])

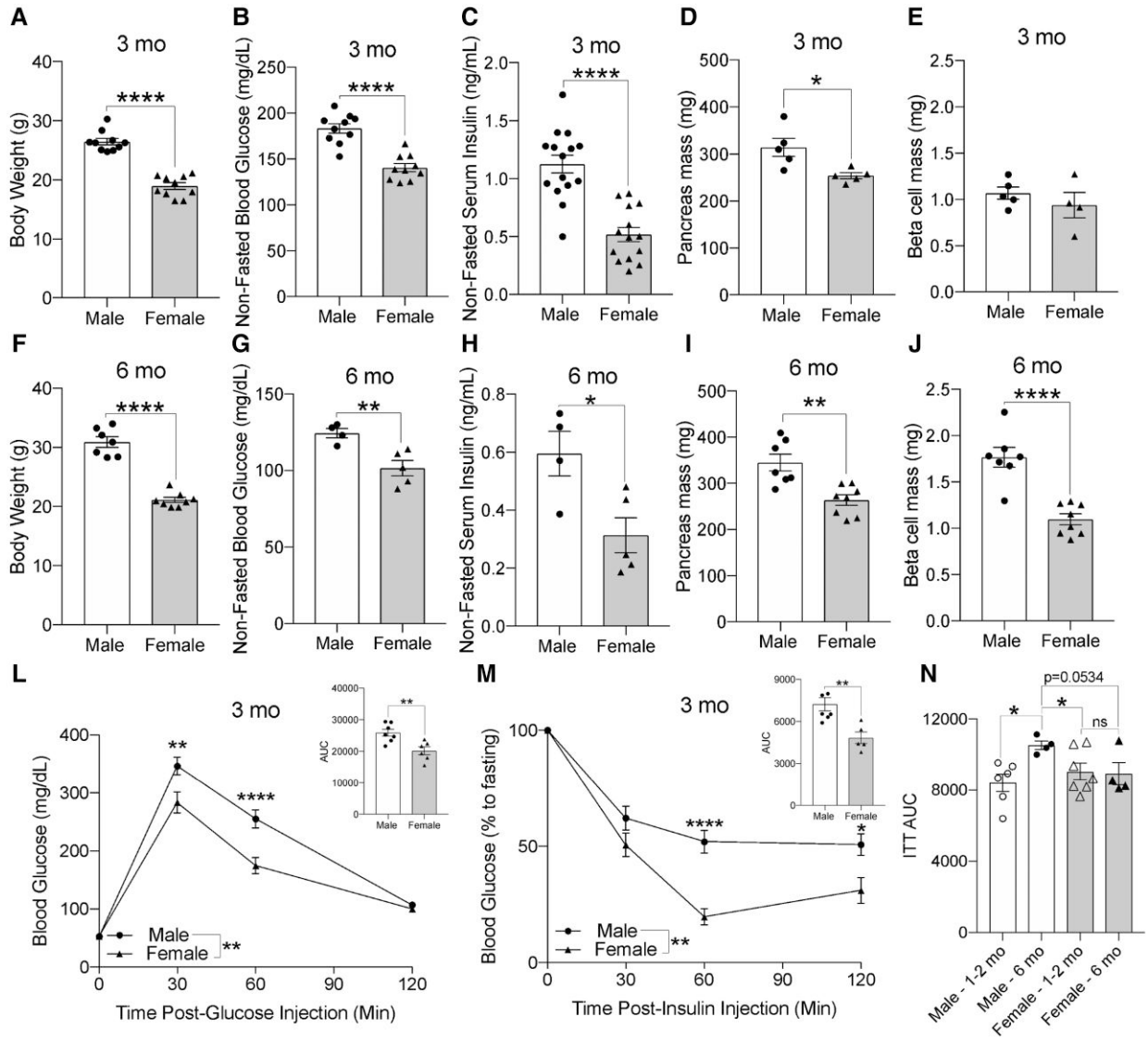

Figure 2. Female C57BL/6J mice exhibit improved glucose tolerance with age with concomitant lower beta-cell mass and circulating insulin levels. Body weight (A), non-fasted blood glucose (B), non-fasted serum insulin (C), beta cell (D) mass and alpha cell mass (E) of male and female C57BL/6J mice at 3-months of age (n = 14-15, n = 4-5 for endocrine cell mass). Body weight (F), non-fasted blood glucose (G), non-fasted serum insulin (H), beta cell (I) mass and alpha cell mass (J) of male and female C57BL/6J mice at 6-months of age (n = 5-8, n = 7-8 for endocrine cell mass). Intraperitoneal glucose tolerance test (IPGTT; ip 2 g/kg glucose/bw) performed at 3 months of age (L, n = 6-7). Intraperitoneal insulin tolerance test (ITT; ip insulin/bw) performed at 3 months of age (0.75 U/kg) (M, n = 5-6). AUC of ITT (0.5 U/kg) performed on separate cohort of mice testing both sex and age across 1-2- and 6-months old mice (N, n = 4-7). Statistical analysis performed using unpaired, two-tailed, student T-Test or 2-way ANOVA with repeated measures. $P \leq .05$, $^{**}P \leq .01$, $^{****}P \leq .0001$.

Alterations in Nutrient Sensing/Signaling/Growth Pathways Between Adult Male and Female Islets

To determine a possible molecular explanation of altered insulin secretion, we first measured gene expression (*Glut2*, *Kir6.2*, *Sur1*, *Gck*, *Atp1a1*, *Atp1a3*; Supplementary Fig. S7C-F, Supplementary Fig. S8D-I) and protein levels (Glut2, Kir6.2) of key molecules involved in glucose-stimulus coupling (Fig. 6A-B; Supplementary Fig. S9H [20]). Female islets had reduced Glut2 glucose transporter protein compared with males, despite having comparable mRNA levels (Fig. 6B; Supplementary Fig. S7F and J; Supplementary Fig. S8D and M). Comparable expressions of *Gck* (glucokinase), *Kir6.2*, *Sur1* (subunits of K^+/ATP channel), and Atp1a1 and Atp1a3 (components of Na^+/K^+ pump) were observed between male and female islets incubated in either 5 mM or 10 mM glucose (Supplementary Fig. S7C-E, K-O; Supplementary Fig. S8E-I, N-R[20]).

Next, we measured protein levels of key upstream nutrient sensors and growth pathways critical in the maintenance of β-cell function and health from isolated islets of 3-month-old mice. We focused on relevant nutrient sensing/signaling/growth pathways in adult islets. mTORC1/S6 is a glucose and amino acid sensitive pathway critical in β-cell proliferation and maturity maintenance [21]. Phospho-S6 relative to total S6 (indicative mTORC1 activity) is significantly elevated then female islets (Fig. 6A and C). However, the total S6 protein level is significantly reduced in the female islets (Fig. 6A and D). Ogt is another critical nutrient sensor protein that integrates macronutrient signaling to regulate β-cell mass and function [22, 23]. Total Ogt expression is not significantly

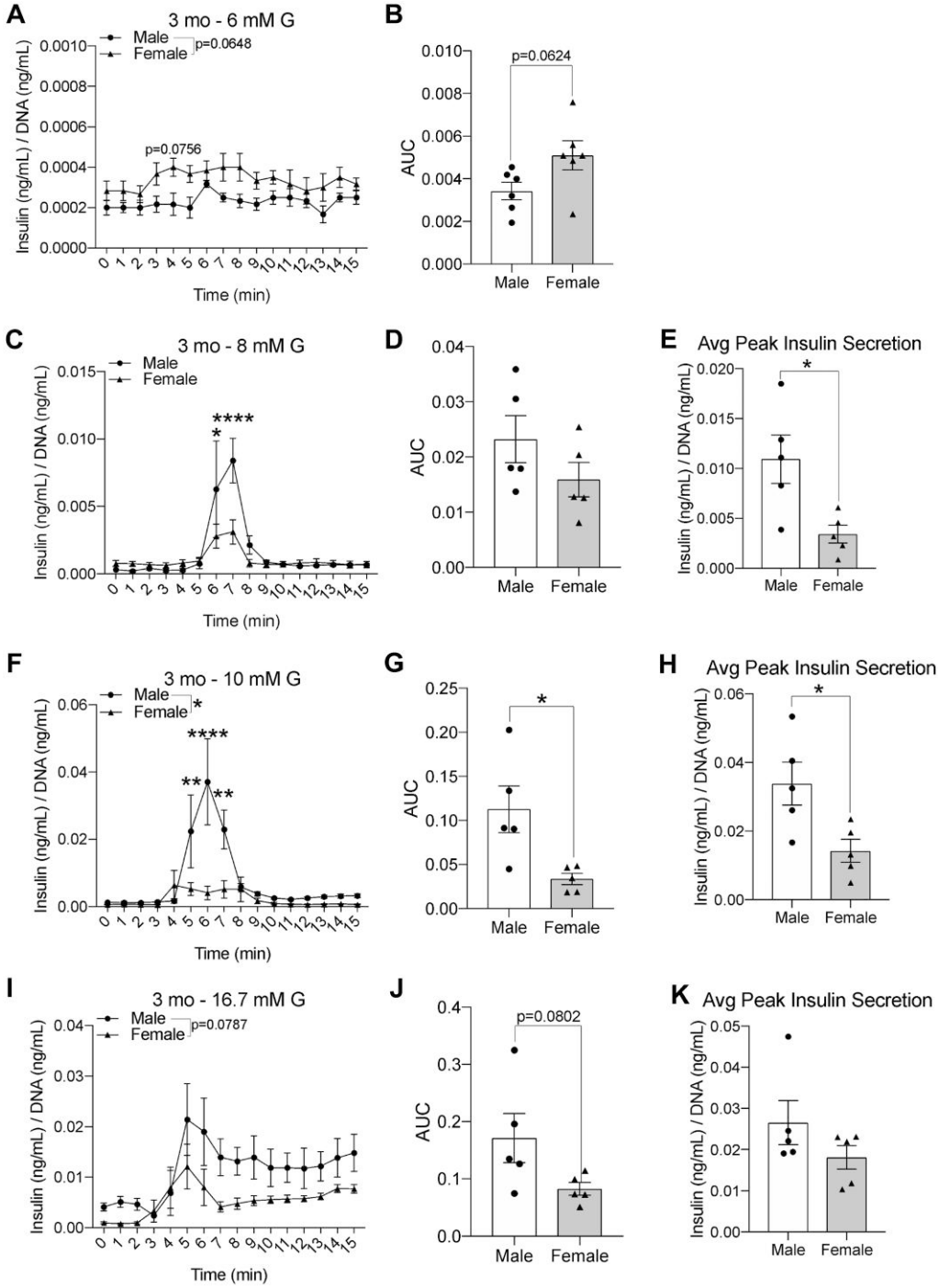

Figure 3. Dynamic glucose stimulated insulin secretion from islets of male and female mice(A) Dynamic GSIS (6 mM glucose) across 15-minute incubation time for male (n = 6) and female (n = 6) 3-month-old mice. Zero time point denotes the start of the 6 mM perfusion. (B) Area under curve analysis for 6 mM glucose dynamic GSIS for 3-month-old mice. (C) Dynamic GSIS (8 mM glucose) across 15-minute incubation time for male (n = 5) and female (n = 5) 3-month-old mice. Zero time point denotes the start of the 8 mM perfusion. (D) Area under curve analysis for 8 mM dynamic GSIS in 3-month-old mice. (E) Analysis of average peak insulin secretion for 8 mM dynamic GSIS for 3-month-old mice. (F) Dynamic GSIS (10 mM glucose) across 15-minute incubation time for male (n = 5) and female (n = 5) 3-month-old mice. Zero time point denotes the start of the 10 mM perfusion. (G) Area under curve analysis for 10 mM dynamic GSIS in 3-month-old mice. (H) Analysis of average peak insulin secretion for 10 mM dynamic GSIS for 3-month-old mice. (I) Dynamic GSIS at high glucose (16.7 mM) across 15-minute incubation time for male (n = 5) and female (n = 5) 3-month-old mice. Zero time point denotes the start of the 16.7 mM perfusion. (J) Area under curve analysis for high glucose (16.7 mM) dynamic GSIS in 3-month-old mice. (K) Analysis of average peak insulin secretion for high glucose (16.7 mM) dynamic GSIS for 3-month-old mice. For all perifusion runs, samples were collected every minute; 0.1 mL/min flow rate. Data presented as insulin concentration at each time point normalized to DNA concentration of recovered islets. Data are average +/– SEM. Statistical analysis performed using unpaired two-tailed student T-Test and 2-way ANOVA with repeated measures. $*P \leq .05$, $**P \leq .01$, $****P \leq .0001$.

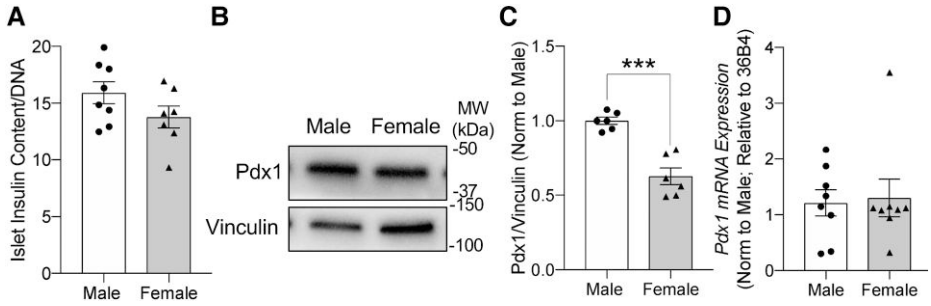

Figure 4. Insulin content between male and female islets Naïve, untested islet insulin content, normalized to DNA (A, n = 7-8). Western blot of islets showing representative image of Pdx1 (B) with quantification (C) (n = 6). Data normalized to loading control Vinculin. Pdx1 gene expression, normalized to 36B4, between male and female islets (D) (n = 8). Islets were isolated from 3-months old male and female mice. Statistical analysis performed using unpaired two-tailed student T-Test. ***$P \leq .001$

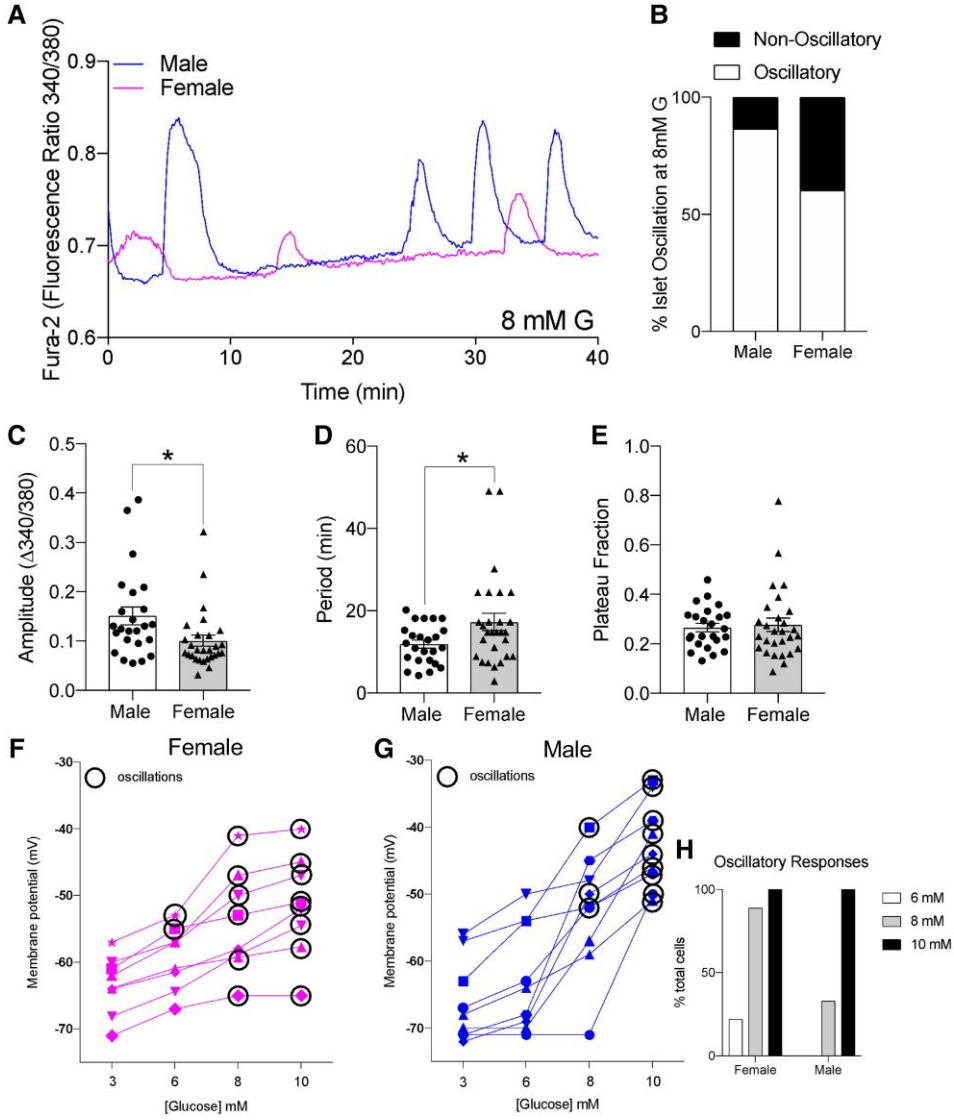

Figure 5. Intracellular calcium and membrane potential oscillation in male and female beta cells Intracellular Ca2+ measurement of islets incubated in 8 mM glucose using Fura-2 dye with representative trace (A) and % of islet cells exhibiting oscillatory behavior (B). Calculated amplitude (C), period (D) and plateau fraction (E) from the Ca2+ traces. Membrane potential (mV) across increasing glucose concentrations (3 mM, 6 mM, 8 mM, 10 mM, 3 mM return to baseline) in islets isolated from (F) female and (G) male mice at 3 months of age. % Cells with oscillatory response at each glucose level (H). Black circles denote the observation of oscillatory activity at indicated glucose concentrations. Column graph in (G) depicts the percentage of total probed cells displaying oscillatory responses at indicated glucose concentrations (6 mM, 8 mM, 10 mM). Islets used in this experiment were incubated overnight in media containing 5 mM glucose post-isolation. Western blot of islets from 3-months old male and female mice showing Glut2 blot (I) and quantification, normalized to Vinculin (J, n = 6). Islet Glut2 mRNA, normalized to housekeeping gene, 36B4 (K, n = 8). Statistical analysis performed using unpaired two-tailed student T-Test. *$P \leq .05$, **$P \leq .01$.

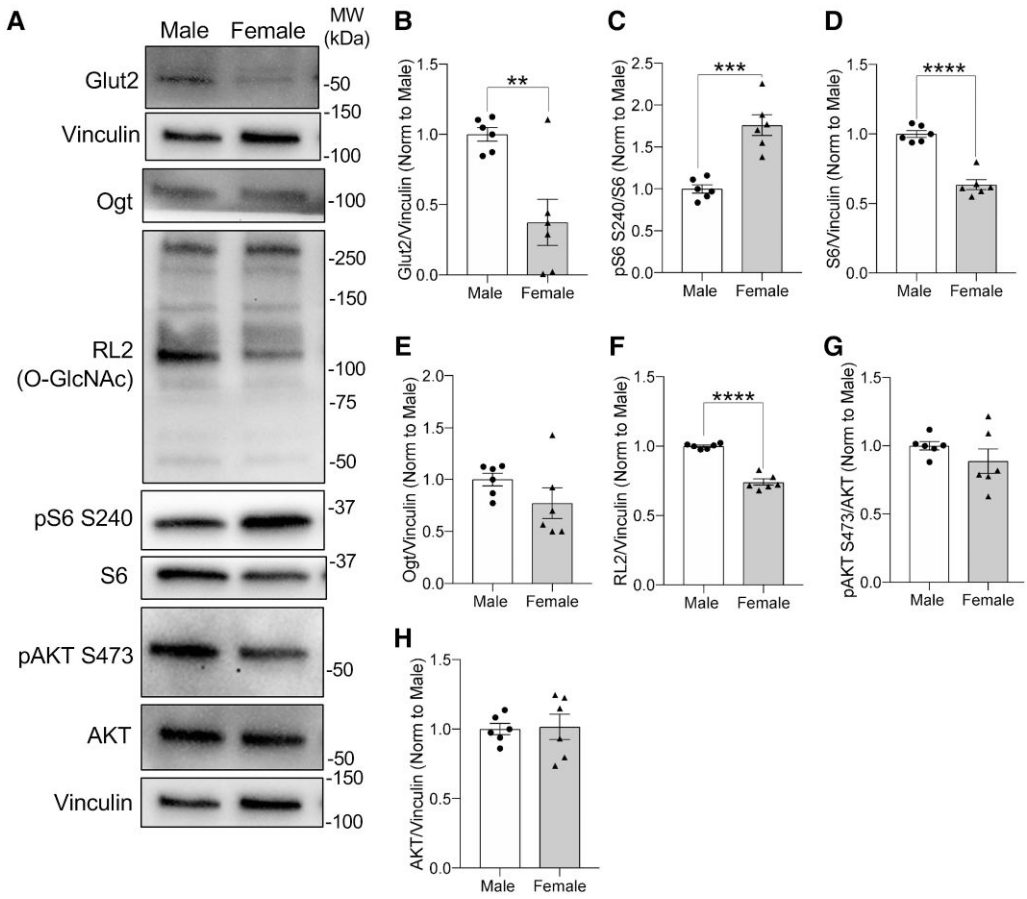

Figure 6. Alterations to nutrient sensor protein expression in male and female islets Western blot of islets isolated from 3-months old male and female mice. Representative image (A) with quantifications of Glut2 (B), pS6 S240 (C), S6 (D), Ogt (E), RL2 (F), pAktS473 (G), and Akt (H) (n = 6 mice). Proteins were normalized to loading control, Vinculin. Phosphorylated versions of proteins were normalized to total/pan-version of the target protein. Statistical analysis performed using unpaired two-tailed student T-Test. $^{**}P \leq .01$, $^{***}P \leq .001$, $^{****}P \leq .0001$.

different between sexes, but RL2 levels, indicative of Ogt activity, O-GlcNAcylation, is reduced in female islets (Fig. 6A and E-F). Akt is a protein kinase that plays a key role in β-cell metabolism, proliferation, and apoptotic signaling [24]. Phospho-Akt relative to total Akt and total Akt levels are not different between sexes (Fig. 6A and G-H). Affirming this, sexually dimorphic changes of protein expressions were observed from islets in a different cohort of mice (control animals from a transgenic mouse model in C57BL/6J background) (Supplementary Fig. S9[20]). Altogether, these data show that major nutrient sensor proteins and their activity are different between male and female islets, which may underlie the differences observed in β-cell function and mass.

Discussion

Since the National Institutes of Health announced its policy on sex as a biological variable, there has been more awareness for systematically studying both sexes side by side in clinical and basic science settings [25]. Although there are more studies that incorporate both sexes and note the sexually dimorphic response to the metabolic stress of interest, there are limited reports that document baseline or normal physiological differences between male and female mice in C57/Bl6J, the most common strain used for metabolic studies. To explore the sex-dependent differences in glucose metabolism and pancreatic islet physiology, we assessed various metabolic, islet physiological, and molecular parameters between male and female C57/Bl6J littermates. In the current study, we found that adult female mice have improved glucose tolerance, with greater insulin sensitivity, compared with males. Lower circulating insulin levels in adult females are consistent with relatively lower β-cell mass and decreased islet insulin secretion compared with males. This reduction in insulin secretion may be, in part, explained by a reduction in total protein levels of nutrient-sensing proteins and altered glucose sensitivity (eg, lower Ogt activity and reduced Ca^{2+} oscillations in response to glucose) in the female mice. Altogether, this study reveals baseline differences in β-cell physiology and metabolism between male and female C57/Bl6J mice.

In young (1 month old) mice, there were no differences observed in body weight or glucose/insulin tolerance in either of the sexes. However, in adult (3 month old) mice, females displayed improved glucose and insulin sensitivity with lower circulating levels of glucose and insulin. This corroborates human data showing females exhibit improved glucose and insulin tolerance [8, 11]. Their basally improved glucose metabolism may be a contributing factor to their resistance to diabetes and metabolic stresses compared with male counterparts [14]. Food intake is reported to be lower in female mice [26, 27]. In terms of circulating insulin levels, there are reports

in human studies that show females have higher circulating insulin levels [11, 13, 28]. However, it is important to note that these clinical studies assess plasma insulin with oral glucose delivery, which is impacted by the gut-incretin response. The reports on isolated islet insulin secretion from human donors vary given the difficulty of procuring samples that properly control for age, body mass index, and other confounding factors that can impact glucose metabolism. Although some report higher stimulation index in females [29], others report a trend toward higher glucose-stimulated islet insulin secretion in male donors [30]. Further studies into the glucose and incretin effect on insulin secretion between sexes across age will supplement our current knowledge on how the dynamics of insulin secretion and glucose metabolism differ between males and females.

Increased insulin resistance is associated with hyperinsulinemia because of increased β-cell mass. In C57Bl/6 mice, we note that β-cell mass is lower in females than males at 6 months of age, with males exhibiting increased β-cell mass over time, whereas female β-cell mass remains relatively stable between 3 and 6 months of age. Our findings on the lower β-cell mass in adult female rodents appear to be consistent with some previous reports, but without a proper direct comparison between sexes and variability in how each laboratories assesses β-cell mass, the data from those reports cannot be compared [31]. Another interpretation of the data is that the β-cell mass remains stable between female mice aged 3 and 6 months because there was no change in insulin resistance. Indeed, the β-cell mass expansion observed in male mice coincides with progressively increasing insulin resistance in males at 3 and 6 months of age. This is in line with the idea that insulin resistance or increased insulin demand can increase β-cell mass in part by proliferation and reduction in cell death in rodents and humans [17, 32, 33]. Insulin demand may stimulate an intrinsic β-cell response to proliferate, such as the unfolded protein response, which has been shown to be a critical regulator of β-cell proliferation [34]. In rats, consistent with our data, β-cell mass remained similar between sexes during the prepubertal and pubertal stage, but males exhibited greater β-cell mass in adulthood (9 weeks) [35], which was, in part, dependent on differences in GH exposure between the sexes.

Additionally, the differences that we observed between male and female glucose metabolism and β-cell function and mass in adulthood were not observed in young mice. This suggests that sexual maturation is a key determinant in the sexually dimorphic physiology of mice. Testosterone is a positive regulator of insulin secretion and β-cell mass [36, 37]. On the other hand, estrogen positively impacts β-cell health in various studies [38, 39]. Further studies are needed to understand the complex role of sex hormones pre-, during, and postpuberty in their relation to glucose metabolism and islet physiology.

At the islet level, although we detected no differences in insulin secretion in young mice, we observed sexual dimorphism in the β cells' response to glucose-stimulated insulin secretion in adult mice. In the lower glucose condition (6 mM Glucose [G]), female islets secreted more insulin than male islets. However, exposure to higher glucose concentrations (8, 10, and 16.7 mM Glucose [G]) led to higher insulin secretion by male islets. There were no significant differences in islet insulin content, suggesting the difference in insulin secretion was at the level of glucose-stimulus coupling. Assessing membrane potential oscillation in response to glucose, a greater proportion of female β cells oscillated in 6 mM glucose than in males, which was consistent with our finding of increased insulin secretion in females at lower glucose levels. However, at higher glucose concentrations, the membrane potentials of male islets were more depolarized than those of females, with a concomitant increase in Ca^{2+} influx. Although not directly compared, a previous publication using adult C57BL/6J mice corroborates our data that male islets secrete more insulin in response to glucose than females [40]. In Wistar rats, female islets were reported to have increased insulin secretion and mitochondrial function compared with males [1]. In humans, female islets have also been reported to have higher secretion capacity than male islets [2]. Therefore, sex differences in pancreatic islets may not have conserved molecular processes in diverse species; however, differences in the age of testing and the response to aging between species must also be considered. As summarized in Table 2, depending on the species and glucose used, the influence of these factors on insulin secretion may vary.

At the molecular level, the Glut2 glucose transporter was expressed at a lower level in female islets than males, which may contribute to the membrane potential changes and Ca^{2+} oscillation in female islets in response to lower glucose concentrations. This may also suggest that female β cells may be more immature than their male counterparts. Consistently, a single-cell RNA sequence from 9-month-old mice revealed higher expression of β-cell maturity markers (ie, Ins1, Ins2, Ucn3, CPE, and Pdx1) in male β cells than in females [16]. A unique condition for insulin demand that females, but not males, may experience is pregnancy. In a normal pregnancy, dams transiently increase β-cell mass [46]. In humans, increased insulin secretion is observed in early pregnancy [47]. During pregnancy, an increase in the proportion of insulin-expressing, but Glut2-low, progenitor population in the islet is reported to contribute to β-cell mass expansion [46]. This may suggest that basally low Glut2 expression observed in female islets may allow the plasticity and flexibility that are needed in β-cell expansion during pregnancy.

Additionally, we report that female islets have reduced Ogt activity (O-GlcNAcylation), compared with males. Ogt is a posttranslational modifying enzyme that adds an O-GlcNAc group to serine/threonine residues of target protein to impact cellular health and function. Ogt expression is highly enriched in the pancreas and has been shown to be critical in maintenance of β-cell mass and function through its impact on insulin biosynthesis and processing, mitochondrial function, and intracellular Ca^{2+} regulation [22, 48-50], suggesting that the activity of this master nutrient- and stress-sensor protein may be one of the driving factors of the differences observed. Insulin resistance is positively associated with O-GlcNAcylation [51, 52], providing a potential molecular link between disparities in insulin demand and β-cell function and health between sexes. Further studies into age- and sex-dependent changes to islet O-GlcNAcylation are needed to further test the hypothesis.

Limitations

In this study, we used C57BL/6J mice, the common strain used in obesity and diabetes research. It is important to point out that C57BL/6J mice develop glucose intolerance and obesity, but they do not spontaneously develop type 2 diabetes

Table 2. Reported isolated islet glucose-stimulated insulin secretion

Species	Method	Glucose (mM)	Male/female	Author [reference]
Human	Static	1-16.7	No change (confounded by older age in females)	Lyon et al (2016) [41]
Human	Static (IIDP reported)	Not available	No change	Kong et al (2018) [42]
Human	Perfusion	1-15	No change (trend toward increase in first phase)	Henquin et al (2018) [30]
Human	Perfusion	3-16.7	No change	Brownrigg et al (2023) [43]
Wistar rat[a] 3 months 18 months 18 months	Static Static Perfusion	3.3-16.7	Decreased Decreased Decreased	Li et al (2016) [1]
Wistar rat[b] Weaning (23-24 days) Puberty (37-38 days) Adulthood (64-66 days)	Static	1-8.3-16.7	No change Decreased (1, 16.7) Increased (1, 8, 16.7)	Catell (2022) [35]
Mouse[b] (Ins1-Cre^{Thorens} +/-) C57Bl/6 mixed 16- wk	Perfusion	20-10	Increased	Skovsø et al (2022) [44]
Mouse[b] (Control mice) C57Bl/6 90 days	Static	5.6-11.1	Increased	Santos-Silva et al (2015) [40]
Mouse[b] (control mice) C57Bl/6Crl 12 wk	Perfusion	11	Decreased	Daraio et al (2017) [45]
Mouse[b] Offspring of control pregnancy C57Bl/6J 8 wks	Static	2.8-16.7	Trend increased	Nicholas et al (2020) [31]

[a]Denotes study that directly studied/analyzed between males and females.
[b]Denotes study that did not directly compare males and females but shows the experiments within the same figure.

without genetic manipulation; for instance, removal of critical genes relevant to glucose homeostasis such as Ogt [22] and mTORC1 [53]. Another limitation is that there are strain-dependent variations in response to metabolic stress [54]; therefore, it is important to consider that there may also be differences in β-cell physiology depending on the strain or species used. Additionally, how β-cell mass is analyzed between laboratories should be taken into consideration. Currently, β-cell mass (milligrams per pancreas) is calculated by multiplying relative insulin-positive area (the percentage of insulin positive area over total pancreas area) by pancreas weight. Thus, this method is very labor intensive and limited to cross-sectional observations with restricted sampling area that varies from different laboratories. We and other groups such the Bernal-Mizrachi and Gannon laboratories, have consistently assessed the average of insulin-positive cells/area over 5 minimum regions of the pancreas, about 200 μM apart [17, 22, 55]. It is important to test at least 5 regions of the pancreas given the nonhomogenous and random location of islets within the pancreas. Another important limitation of the study is the sensitivity of the intraperitoneal insulin tolerance test (IPITT) and intraperitoneal glucose tolerance test (IPGTT) for metabolic homeostasis testing. Glucose and insulin tolerance tests are gross physiological measurements and tend to be insensitive to mild changes. Therefore, future research can employ hyperinsulinemic-euglycemic clamp studies to deepen our understanding of sexually dimorphic insulin sensitivity phenotypes.

In summary, this study is a step toward a greater understanding of the normal physiology of sex differences in glucose handling and islet physiology. Sex matters in β-cell mass, and a deeper understanding of baseline sexual differences, is a critical step in understanding the etiology of metabolic diseases. More importantly, this study highlights the critical need for our research community to improve reporting of methods (ie, glucose concentrations of islets incubation before experiments, age, sex, parity, animal strain, and specifics on β-cell mass analysis) in research articles and to include both sexes in all basic science studies and clinical trials to fully optimize drug treatments, dosage, and timing to maximize their efficacy for the entire population. It will also be important to dissect mechanisms from genetics or sex chromosomes that allow for disparities in gene dosage and regulatory mechanisms bestowed to the developing fetus to differential responses of the placenta and gonadal hormones during and following puberty, which may all impose lasting epigenetic changes and hormone-driven cellular signaling responses in different organs and physiology between sexes [9, 56]. Finally, future studies can tackle the importance of developmental windows in how sex differences in the islets may arise and how these differences impact islet function and disease risk across lifespan.

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Author Contributions

S.J., M.B., and E.U.A. developed the study. S.J., M.B., A.W., E.G., E.O., G.C., S.K., L.S., E.B., and E.U.A. designed experiments, generated and analyzed data, assisted with manuscript preparation, and approved the final version. S.J., M.B., and E.U.A. interpreted the data and wrote and edited the manuscript. E.U.A. conceived the study, was in charge of overall direction, and was guarantor of this work.

Disclosures

The authors declare that they have no conflicts of interest with the contents of this article.

Data Availability

Original data generated and analyzed during this study are included in this published article or in the data repositories listed in Reference [20].

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