Supplementary Information for

Allosteric modulation of β-cell M_3 muscarinic acetylcholine receptors greatly improves glucose homeostasis in lean and obese mice

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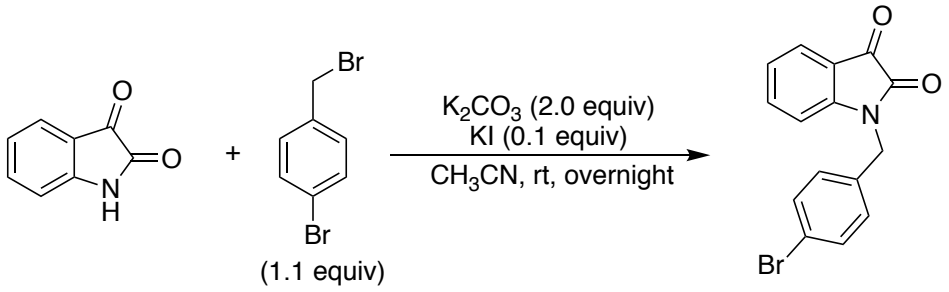
Materials and Methods

Reagents

Insulin (human) was from Eli Lilly (Humulin). Sources of other chemicals, media, and commercial kits are provided in the text below or were from Sigma-Aldrich (St. Louis, MO). VU0119498(1-(4-bromobenzyl)indoline-2,3-dione) was synthesized as described below.



To a solution of isatin (225 mg, 2.28 mmol) in acetonitrile (10 ml) at room temperature was added potassium carbonate (629 mg, 4.55 mmol, 2.0 equiv), potassium iodide (38 mg, 0.23 mmol, 0.1 equiv) and 4-bromobenzyl bromide (626 mg, 2.51 mmol, 1.1 equiv). The resulting mixture was stirred at room temperature overnight and then extracted between EtOAc (3 × 30 ml) and water (30 ml). The combined organic layers were washed with brine, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was purified by silica gel chromatography (EtOAc:hexanes = 1:4, R_f = 0.2) to give the desired compound (1-(4-bromobenzyl)indoline-2,3-dione) as an orange solid (695 mg, 97% yield); mp 179−181 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.58−7.50 (m, 4H), 7.39 (d, J = 8.4 Hz, 2H), 7.10 (t, J = 7.6 Hz, 1H), 6.93 (d, J = 8.0 Hz, 1H), 4.87 (s, 2H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 185.7, 161.1, 152.8, 140.6, 137.8, 134.2, 132.4, 127.2, 126.1, 123.4, 120.6, 113.7, 45.0. IR: ν = 1737, 1613, 1469, 1347, 1169, 1011, 756 cm^{-1}. HRMS (ESI): calculated for $C_{15}H_{11}BrNO_2^+$ $[M+H]^+$, 315.9973; found, 315.9979.

Mouse Maintenance

All animal studies were approved by the National Institute of Diabetes and Digestive and Kidney Diseases/NIH Animal Care and Use Committee. Mice were kept at room temperature (23 °C) on a 12-hr light/dark cycle. Unless stated otherwise, mice consumed a standard chow (7022 NIH-07 diet, 15% kcal fat, energy density 3.1 kcal/g, Envigo Inc.) and had free access to water and food. In a subset of experiments, 4-5-week-old male WT mice (C57BL/6NTac strain) were switched to a high-fat diet (HFD; F3282, 50% kcal fat, energy density 5.5 kcal/gm, Bioserv). Mice consumed the HFD for at least 8 weeks prior to metabolic testing.

Generation of Mice Lacking M3Rs in Pancreatic β-Cells

The generation of homozygous floxed M3R mice (*fl/fl M3R* mice) has been described previously (1). These floxed mice were backcrossed for 10 generations onto the C57BL/6NTac background. *Pdx1-Cre-ER^{TM}* mice with a mixed genetic background were a kind gift by Dr. Doug Melton (Harvard University). After backcrossing the *Pdx1-Cre-ER^{TM}* mice for 10 generations onto the C57BL/6NTac background, we intermated the backcrossed *Pdx1-Cre-ER^{TM}* mice with the *fl/fl M3R* mice to generate *fl/fl M3R-Pdx1-Cre-ER^{TM}* mice and *fl/fl M3R* control littermates. Eight-week old mice were injected for 6 consecutive days with TMX (Sigma) suspended in corn oil (Sigma) (1 mg i.p. per mouse per day). Genotyping protocols for the *fl/fl M3R and Pdx1-Cre-ER^{TM}* mice have been reported previously (1, 2). Throughout the text, we refer to the TMX-treated *fl/fl M3R-Pdx1-Cre-ER^{TM}* mice simply as β-M3R KO mice.

Determination of *M3R* mRNA Levels in Mouse Tissues via qRT-PCR

Using standard procedures, *M3R* mRNA expression levels were measured via qRT-PCR using total RNA prepared from different mouse tissues known to express M3Rs (for experimental details, see (3)). The following primers were used: forward, 5'-

GCCACACCATCTGGCAAGT; reverse, 5'-GGTTCATGGAAATGACCCCGA (amplicon size: 186 bp).

Culture of MIN6-K8 Cells

MIN6-K8 mouse insulinoma cells (4) were kindly provided by Dr. Susumu Seino (Kobe University, Japan). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal bovine serum and maintained in a humidified incubator with 95% air and 5% CO_2 at 37 °C.

Measurement of Intracellular Calcium Levels

MIN6-K8 cells were seeded into 96-well plates (10^5 cells per well) 24 hr prior to calcium assays. On the day of the assay, cells were incubated for 1 hr at 37 °C with increasing concentrations of ACh plus 10 μM neostigmine (cholinesterase inhibitor) in the absence or presence of 20 μM VU0119498 (vehicle: 1% DMSO). Changes in intracellular calcium concentrations were determined using FLIPR technology (Molecular Devices) as described (5). Increases in $[Ca^{2+}]_i$ were expressed as changes in fluorescence (peak fluorescence activity minus basal fluorescence activity) divided by basal fluorescence levels. Data were analyzed by using Prism 7 software (GraphPad Software).

Insulin Secretion Studies with MIN6-K8 Cells

MIN6-K8 cells were seeded into 96-well plates (10^5 cells/well). Twenty-four hours later, cells were incubated for 30 min at 37 °C with Krebs-Ringer bicarbonate/HEPES buffer containing 3.3 mM glucose (composition in mM: 120 NaCl, 5 KCl, 24 $NaHCO_3$, 2 $CaCl_2$, 1 $MgCl_2$, 15 HEPES and 0.1% BSA; pH 7.4). The buffer was then removed, and cells were pre-incubated for 30 min at 37 °C in the same buffer containing 3 or 20 μM VU0119498 or vehicle (1% DMSO). After this incubation step, the buffer was replaced again, and cells were stimulated for 1 hr at 37 °C with increasing concentrations of ACh

plus 10 μM of neostigmine and either 3 or 20 μM VU0119498 or vehicle (1% DMSO). Insulin concentrations in the media were measured with an ELISA kit (Crystal Chem). Data were analyzed using Prism 7 software (GraphPad Software).

Insulin Secretion Studies with Isolated Mouse Islets

Mouse islets were isolated as described previously (2). Islets were prepared from 10-14-week-old male or female WT mice (C57BL/6NTac; Taconic) or from male whole body M3R KO mice (6) that had been backcrossed for 10 generations onto the C57BL/6NTac background. Batches of 12 islets were incubated for 30 min in Krebs-Ringer bicarbonate/HEPES buffer containing 3.3 mM glucose at 37 °C. Islets were then incubated in the same buffer in the presence of VU0119498 (20 μM) or vehicle (1% DMSO) at 37 °C for an additional 30 min. Subsequently, islets were incubated for 1 hr at 37 °C in the same buffer containing 16.7 mM instead of 3.3 mM glucose with increasing concentrations of ACh (plus 10 μM neostigmine), in the absence or presence of 20 μM VU0119498. Media were then collected for insulin measurements. Total insulin was extracted by sonication in acid/ethanol from the batch of islets. Insulin concentrations were determined by using an ELISA kit (Crystal Chem, Cat. # 90080).

Human Islets: Source and Culture

Human islets were received from the accredited Human Islet Resource Center at the University of Pennsylvania. Islets were obtained from three deceased donors (two males, one female). Donors were 22, 31, and 57 years old with body mass indexes (BMIs) of 29.8, 24.5, and 25.7 kg/m^2 and HbA1c values of 5.2, 5.4, and 5.1%, respectively. Islet purity ranged from 85 to 95% and viability from 92 to 95%. The cold ischemia time ranged from 4 to 14 hr.

The University of Pennsylvania Institutional Review Board has exempted this research from ethical review because islets were received from deceased, de-identified

organ donors. All pancreata acquired by the Human Islet Resource Center at the University of Pennsylvania were from deceased donors after having obtained consent from their families through UNOS (United Network for Organ Sharing). The islet isolation procedure and culturing conditions have been described in detail previously (7, 8). Isolated human islets were kept under culture conditions in medium at 5 mM glucose and 25 °C prior to transfer to the laboratory. The time that elapsed from islet isolation to transfer to the laboratory ranged from 1 to 3 days.

Perifusion of Human Islets for Insulin Release Measurements

Cultured human islets (180 islets) were placed on a nylon filter in a plastic perifusion chamber (Millipore) and were perfused at a flow rate of 1 ml per min (9). The perifusion apparatus consisted of a computer-controlled low pressure chromatography system (BIO-RAD Econo system) that allowed programmable rates of flow and glucose concentrations in the perfusate, a water bath (set at 37 °C), and a fraction collector (BIO-RAD; model 2128). The perifusate was a Krebs buffer (pH 7.4) containing (in mM): 114 NaCl, 5 KCl, 24 $NaHCO_3$, 1 $MgCl_2$, 2.2 Ca^{2+}, 1 Pi, 10 HEPES (pH 7.4), and 0.25% of BSA equilibrated with 95% O_2 and 5% CO_2. In all experiments, a physiological mixture of amino acids (10) was included in the perfusate. Islets were perifused in the presence or absence of VU0119498 (5 μM). Initially, islets were perifused for 20 min with 4 mM glucose. The glucose concentration was then raised to 8 mM for an additional 20 min. Subsequently, an ACh ramp (0 to 1 μM; 25 nM increment/min) was applied for an additional 40 min. Neostigmine (10 μM), a cholinesterase inhibitor, was included in the perfusate to prevent ACh hydrolysis. At the end of each experiment, KCl (30 mM) was added after a 30-min washout period. The area under curve (AUC) for ACh-stimulated insulin release was calculated using GraphPad software (Prism 6.0.) The baseline at 8 mM glucose (second phase insulin release) was used for the calculation of AUC values.

In vivo Metabolic Tests

In vivo metabolic tests were performed using standard protocols as described previously (2). Oral glucose tolerance tests were carried out as follows. After an overnight (12-14 hr) fast, male mice were injected i.p. with different doses of VU0119498 or vehicle (10% DMSO, 35% PEG 400, 55% PBS). Thirty min later, the mice received glucose via oral gavage (regular chow mice: 2 g glucose/kg; HFD mice: 1 g glucose/kg). Blood glucose levels were measured with a Bayer Contour glucometer using blood collected from the tail vein before and at specific times after glucose administration. To measure glucose-stimulated insulin secretion (GSIS), mice were treated with glucose in the same fashion as described above. Blood samples were collected from the tail vein at defined time points after glucose administration using heparin-coated capillary tubes, and plasma was obtained via centrifugation (10,000 g, 10 min, 4 °C). Plasma insulin levels were determined using a commercial ELISA kit (Crystal Chem).

Saliva Secretion Test

Male WT mice (C57BL/6NTac; Taconic; 10-week-old males) were anesthetized by administration of chloral hydrate (400 mg/kg i.p.). Mice were then placed on a metal board over a temperature-controlled blanket (37 °C), and both front legs were taped to the board to constrict body movement. The mouth was held open with a metal clip, and a Schirmer test strip (Fisher Scientific, cat. # NC9933058) was then placed into the oral cavity to monitor salivation. Subsequently, mice were injected i.p with 0.1, 0.5, or 2 mg/kg of VU0119494 or vehicle (10% DMSO, 35% PEG 400, 55% PBS). For each dose, the length of the moistened (blue) area of the strip was recorded at 5 min intervals for a 30 min time period. At the end of the experiment, the orthosteric muscarinic agonist pilocarpine (1 mg/kg i.p.) was administered (positive control), and pilocarpine-induced salivation was determined over a 10 min time period.

Measurement of Mouse Pupil Size

Male WT mice (C57BL/6NTac; 8-10-week-old males) were handled several days before testing to minimize stress. On the day of testing, mice were dark-adapted for at least 1 hr prior to measuring baseline pupil size. During the procedure, non-anesthetized mice were gently restrained by hand, and pupil size was monitored in the dark using an infrared camera (Sony Handycam camcorder, FDRAX33) and an infrared light source. Mice were then injected i.p. with vehicle (10% DMSO, 35% PEG 400, 55% PBS) or different doses of VU0119494 (0.1, 0.5, and 2 mg/kg). To measure maximal pupillary constriction, 1-2 ul of a 100 μM carbachol solution (sterile-filtered through a 0.22 μm Corning filter) was topically applied to one eye. Mice received only one treatment per day. Video recordings were analyzed by creating screenshots using the VLC media player (https://www.videolan.org/vlc/) of the baseline pupil size prior to any treatment and pupil size at 15, 30, 45 and 60 min after drug/vehicle treatment. Pupil area was then quantified using ImageJ (https://imagej.nih.gov/ij/). To determine the relative pupil area across time, pupil size at each post-injection time point was divided by the baseline pupil size prior to injection.

References

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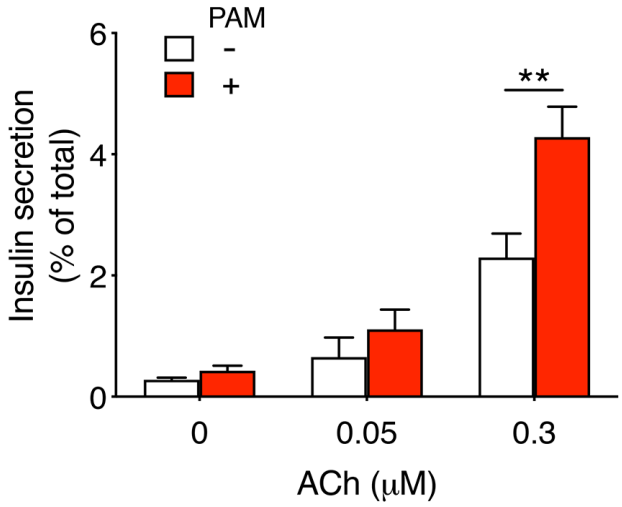

Fig. S1. VU0119498 (PAM) promotes ACh-stimulated insulin secretion in islets isolated from female WT mice. Assays were carried out in the presence of 16.7 mM glucose. ACh-stimulated increases in insulin secretion were studied in the absence or presence of 20 μM PAM. The amount of insulin secreted into the medium during the 1 hr incubation period was normalized to the total insulin content of each well (islets plus medium). Data are presented as means ± SEM of three independent experiments. ***P* <0.01 (two-way ANOVA followed by Tukey's post hoc test).

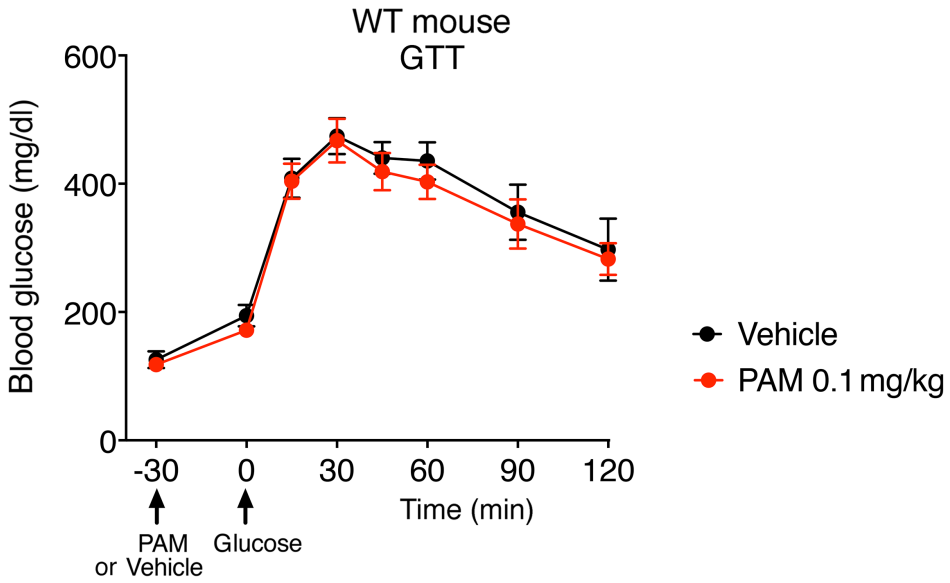

Fig. S2. Glucose tolerance remains unchanged after administration of a low dose of VU0119498 (PAM) to WT mice. Mice (12-week-old males) maintained on regular chow were fasted overnight and then injected i.p. with PAM (0.1 mg/kg) or vehicle. Thirty min later, the mice received glucose via oral gavage (2 g/kg). Blood samples were collected from the tail vein at the indicated time points. Data are given as means ± SEM (n=8 per group).

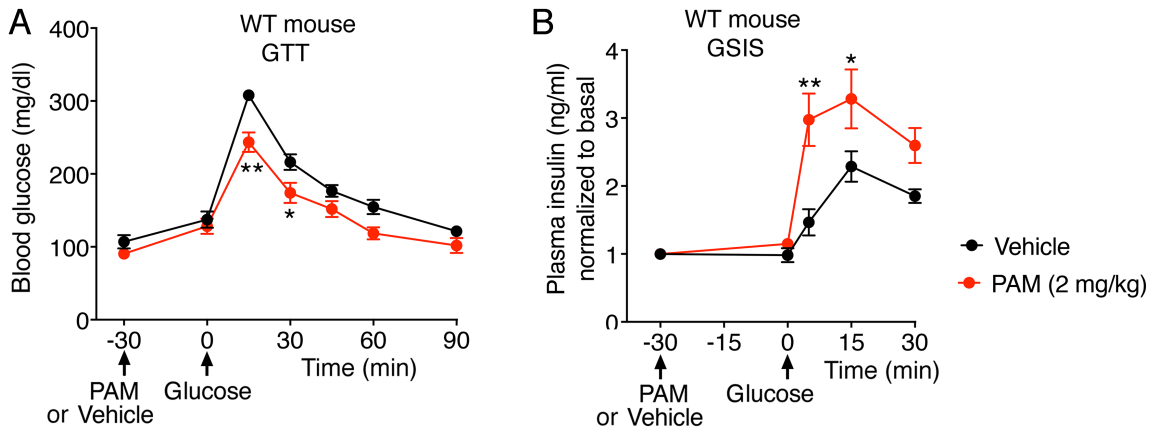

Fig. S3. Glucose tolerance and GSIS are improved after treatment of WT mice with a high dose of VU0119498 (PAM). Mice (14-week-old males) maintained on regular chow were fasted overnight and then injected i.p. with PAM (2 mg/kg) or vehicle. Thirty min later, the mice received glucose via oral gavage (2 g/kg). (*A*) Oral glucose tolerance test (GTT). (*B*) Glucose-stimulated insulin secretion (GSIS) after oral glucose administration. Blood/plasma samples were collected from the tail vein at the indicated time points. Data are given as means ± SEM (n=6 per group). * P <0.05; ** P <0.01 (two-way ANOVA followed by Bonferroni's post hoc test).

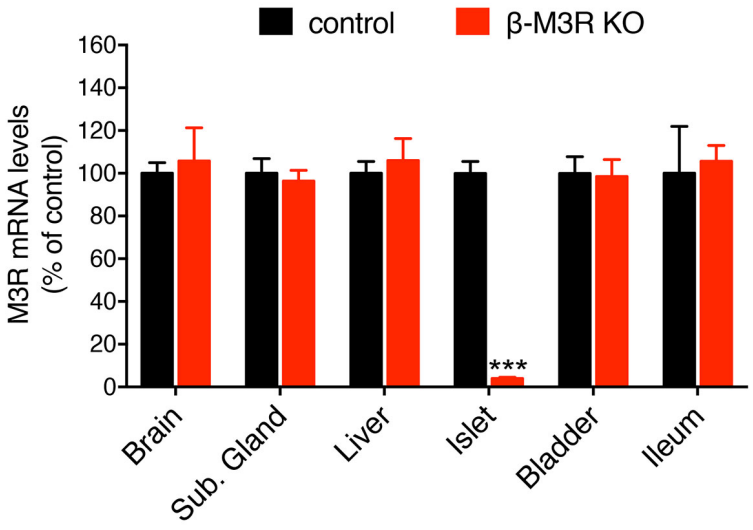

Fig. S4. Generation of β-cell-specific M3R knockout mice (β-M3R-KO mice). Floxed *M3R* mice carrying the *Pdx1-Cre-ER*^{TM} transgene (*fl/fl M3R-Pdx1-Cre-ER*^{TM} mice) and their *fl/fl M3R* control littermates (16-week-old males) were injected with TMX for 6 consecutive days, as described under Methods. *M3R* expression levels were then examined by qRT-PCR using total RNA prepared from the indicated tissues. Sub. gland, submandibular gland. Note that *M3R* mRNA expression is selectively reduced in islets from TMX-injected *fl/fl M3R-Pdx1-Cre-ER*^{TM} mice (β-barr2-KO mice). RNA expression data are given as means ± SEM (3 mice per genotype). *** P <0.001, as compared to the corresponding control group (Student's t-test).