ONLINE APPENDIX

Hepatic muscarinic acetylcholine receptors are not critically involved in maintaining glucose homeostasis in mice

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SUPPLEMENTAL METHODS

Generation of mutant mice selectively lacking M_3 mAChRs in hepatocytes. Recently, we established a mutant mouse strain harboring a floxed M_3 mAChR allele and a PGK-neo selection cassette ~0.45 kb downstream of the polyA signal of the M_3 mAChR gene (ref. 24 in the main text; Fig. 1B). To exclude the possibility that the presence of the neo marker gene affected the expression of neighboring genes, we eliminated the neo cassette by mating the floxed M_3 receptor mutant mice with EIIaCre transgenic mice which express Cre recombinase in the early embryo (1) (Fig. 1B). The successful removal of the neo gene was confirmed by Southern hybridization (Fig. 1C, D). Male mice heterozygous for the floxed M_3 receptor allele (fl/+) mice lacking the neo cassette were backcrossed to WT female C57BL/6 mice (Taconic). The resulting heterozygous floxed M_3 receptor mice (fl/+) lacking both the neo and the EIIaCre genes were then crossed to AlbCre transgenic mice which express Cre recombinase under the control of the mouse albumin promoter /enhancer (The Jackson Laboratory, Bar Harbor, ME; official strain name: B6.Cg-Tg(Alb-cre)21Mgn/J; genetic background: C57BL/6J). This mouse strain, originally developed by Postic et al. (ref. 25 in the main text), has been used successfully by many laboratories to generate hepatocyte-specific KO mice. The resulting M_3 fl/+ AlbCre mice were then crossed with M_3 fl/+ mice to generate M_3 fl/fl AlbCre mice (referred to as Hep-M3-KO mice throughout the text) and the three corresponding littermate control groups (M_3 fl/fl, +/+, and +/+ AlbCre mice).

Generation of transgenic mice selectively overexpressing M_3 mAChRs in hepatocytes. A liver-specific transgene was created in which the coding sequence of the rat M_3 mAChR was under the transcriptional control of the mouse albumin promoter/enhancer. In addition, a 9-amino acid hemagglutinin (HA) epitope tag was added to the N-terminus of the transgenic M_3 receptor in order to distinguish it more easily from the endogenous mouse M_3 mAChRs. Briefly, a 1.8 kb XhoI-XbaI fragment containing the entire rat M_3 receptor coding sequence was ligated into the XhoI and XbaI sites of the pGEMAlb-SVPA expression plasmid (ref. 29 in the main text), downstream of the 2.5 kb mouse albumin promoter/enhancer and upstream of a 1.2 kb SV40 intron polyadenylation sequence. A 5.5 kb ApaI-MluI fragment containing the transgene was excised, purified, and microinjected into the pronuclei of fertilized ova from C57/BL6 mice (Taconic) by using standard transgenic techniques. We identified four founder mice that stably transmitted the transgene to their progeny. All transgenic mouse lines were maintained on a pure C57BL/6 (Taconic) background.

Genotyping of Hep-M3-KO Mice and control strains. Mouse genotypes were determined via Southern blotting and/or PCR analysis using mouse tail DNA (Fig. 1B-F). The P1 and P2 probes used for Southern blotting studies (Fig. 1B) were generated via PCR using the following primers:

P1 probe (size: 720 bp): forward, 5'-CTCCATTCGTGGATTTGACTCTTG-3';
reverse, 5'-TGCTAAGTGATAGGAGGTAAG-3'.
P2 probe (size: 704 bp): forward, 5'-GGCTGGGAACTTACTGCTTG-3';
reverse, 5'-CAATACATGCCTTTCGCTCA-3'.

The PCR cycling conditions were as follows: 94 ˚C for 10 min, followed by 32 cycles at 94 ˚C for 45 sec, 54 ˚C for 30 sec, and 68 ˚C for 45 sec; 68 ˚C for 7 min.

The presence of the AlbCre transgene was detected by using the following primer pair: Cre1, 5'-CCTGGAAAATGCTTCTGTCCG (forward); Cre2, 5'-CAGGGTGTTATAAGCAATCCC (reverse). The size of the amplified PCR product was 391 bp. To detect the presence of the two loxP sites in the floxed M_3 receptor allele, PCR primers p1-p4 were used (Fig. 1B, E). The presence of the 5' loxP site was detected by using primers p1 (5'-GTTTAGCTGCCTGGAAGTTTGC; forward) and p2 (5'-TCTACATTTTCGTTCTGAGG; reverse). The presence of the 3' loxP site was detected by using primers p3 (5'-AAGGCAATGGTGTCTGGAAC; forward) and p4 (5'-TGTACAGGTGTACAGTGAGAC; reverse). The sizes of the resulting PCR products are indicated in Fig. 1E. All PCR reactions were run under the following conditions: 94°C for 5 min followed by 32 cycles at 94°C for 45 s, 55°C for 30 s, and 72°C for 30 s.

Genotyping of transgenic mice overexpressing M_3 mAChRs in hepatocytes (Hep-M3-Tg mice). The presence of the M_3 receptor transgene in Hep-M3-Tg mice was confirmed via PCR. Mouse tail DNA was amplified by using the following transgene-specific PCR primers: M_3HA-F (forward), 5'-CCTACGACGTCCCCGACTAC; M_3-R (reverse), 5'-TGATGTAGGTCGTGAACAGG (size of PCR product: 405 bp). Note that the M_3HA-F primer anneals to the sequence encoding the HA tag present at the N-terminus of the transgenically expressed M_3 receptor. The PCR conditions used were as follows: 94°C for 5 min followed by 32 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s.

RT-PCR analysis of M_3 receptor transgene expression. cDNA prepared from different tissues of Hep-M3-Tg mice was amplified via PCR using a primer pair specific for the M_3 receptor transgene (M_3HA-F, 5'-CCTACGACGTCCCCGACTAC [this primer anneals to the HA epitope tag sequence present at the N-terminus of the M_3 receptor transgene]; M_3-R: 5'-TGATGTAGGTCGTGAACAGG; size of the PCR product: 405 bp). The PCR cycling conditions were as follows: 94 ^{o}C for 3 min followed by 32 cycles at 94 ^{o}C for 25 s, 55 ^{o}C for 40 s, and 72 ^{o}C for 30 s. PCRs were carried out in a final volume of 50 µl containing 2 µl of the RT reaction product (corresponding to ~0.1 µg RNA), 5 µl 10x Taq buffer containing 200 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 1 mM of each dNTP, 200 nM of each PCR primer, and 1 unit of recombinant Taq DNA polymerase (Fermentas). Mouse GAPDH mRNA was amplified as an internal control (forward primer, 5'-CGTGGAGTCTACTGGTGTCTTCACC; reverse primer, 5'-GATGGCATGGACTGTGGTCATGAGC; size of the PCR product: 258 bp).

Real-time qRT-PCR analysis of gene expression. cDNA was prepared from mouse liver and other mouse tissues as described above. Gene expression levels were measured by real-time qRT-PCR analysis using an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems). PCR reactions (20 µl total volume) included cDNA (~10 ng initial RNA), 100 nM of each primer, and 10 µl of 2 x SYBR Green MasterMix (Applied Biosystems). The PCR cycling

conditions were as follows: 50°C for 2 min, 95°C for 10 min, and 40 cycles at 95°C for 15 s, and 60°C for 1 min, respectively. The specificity of each RT-PCR product was verified via gel electrophoresis. The expression of cyclophilin A served as an internal control. Three to five independent samples prepared from three to five different mice were used per genotype. PCR reactions were carried out in triplicate in 96-well plates. Data were expressed as percent change in gene expression in M_3 receptor mutant mice relative to their control littermates (100%). Primer sequences are provided in supplemental Table 1.

Preparation of mouse liver membranes for radioligand binding studies. Radioligand binding studies were carried out with membranes prepared from freshly isolated mouse livers. In brief, fresh livers were homogenized for 20 s by using a Polytron homogenizer (setting 5,2) in ice-cold buffer A (25 mM sodium phosphate and 5 mM $MgCl_2$; pH 7.4) containing 0.25 M sucrose, 0.5 mM DTT, 2 mM AEBSF (4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride), 1 μg/ml aprotinin, and 0.1 mM leupeptin. Homogenates were centrifuged at 500 x g for 10 min and the supernatants were transferred to new tubes. This centrifugation step was repeated twice. Supernatants were collected and re-centrifuged at 50,000 x g for 30 min. The pellets were resuspended in 20 ml stock buffer (buffer A containing 0.5 mM DTT, 2 mM AEBSF, 1 μg/ml aprotinin, and 0.1 mM leupeptin) and re-centrifuged at 50,000 x g for 30 min. The resulting membrane pellets were resuspended in 2 ml stock buffer. All centrifugation steps were carried out at 4 ^oC. Membrane protein concentrations were determined by using Bio-Rad protein assay reagent.

Determination of liver glycogen content. Glycogen was extracted from liver samples as described (2, 3). In brief, 100 mg liver tissue samples were homogenized in 500 ml 30% KOH and incubated at 97 ^oC for 15 min. Ice-cold 95% ethanol containing 0.8% Na_2SO_4 (3 ml) was added into each tube, followed by a 60 min incubation at -20 ^oC. After centrifugation at 1,200 x g for 30 min, pellets were washed once with ice-cold 95% ethanol containing 0.25% Na_2SO_4 and twice with ice-cold 95% ethanol, and then briefly dried at 97 ^oC. Pellets were resuspended in 200 ml distilled water and digested with 10 mg amylase at 37 ^oC for 30 min. Glucose concentrations in the reaction mixtures were measured by a glucokinase radiometric assay (4). Briefly, samples were incubated in 100 μl of a solution containing 1 U/ml glucokinase, 50 mM triethanolamine hydrochloride (pH 9.0), 2 mM $MgCl_2$, 1 mg/ml BSA, and 40 μM [γ-^{32}P]ATP (30,000 dpm per reaction; Perkin Elmer) at 30 ^oC for 30 min. Subsequently, 100 μl of 2 N $HClO_4$ containing 0.2 mM H_3PO_4 were added, and samples were incubated at 90 ^oC for 40 min. After addition of 100 mM ammonium molybdate and 200 mM triethylamine (50 μl each), samples were centrifuged at 1,200 x g for 30 min. Tissue glucose was measured as incorporation of [γ-^{32}P]ATP and calculated using a standard curve with various glucose concentrations. Four different samples were taken from each mouse liver and analyzed independently. All reagents used for this assay were purchased from Sigma.

Western blotting studies. Mouse liver tissue was homogenized in 20 mM MOPS, 2 mM EGTA, 5 mM EDTA, 30 mM sodium fluoride, 40 mM β-glycerophosphate, 10 mM sodium pyrophosphate, 2 mM orthovanadate, 0.5% NP-40 and complete protease inhibitor cocktail (Roche) and centrifuged at 13,000 x g for 20 min at 4°C. Supernatants were then collected and protein concentrations were measured with a BCA protein quantification kit (Pierce). Protein extracts were separated on 4-12% NuPAGE gels (Invitrogen) and blotted onto Immobilon-FL

PVDF membranes (Millipore). Membranes were blocked at room temperature for 1 h in Odyssey LI-COR blocking buffer (LI-COR) diluted 1:1 in TBS and incubated with primary antibodies in the same buffer overnight at 4°C. The antibodies used were directed against phospho-Erk (Thr202/Tyr204), total Erk (both from Cell Signaling Technology; CS#9101 and CS#9102 respectively), and β-actin (Abcam). After three consecutive 5-min washes in TBS-T (0.1%), blots were incubated with Dylight 680-conjugated goat anti-rabbit IgG and Dylight 800-conjugated goat anti-mouse IgG (both Thermo Scientific) for 1 h at room temperature in blocking buffer containing 0.1% TBS-T and 0.1% SDS. After three washes in TBS-T and a final wash in TBS, blots were scanned with the LI-COR Odyssey (LI-COR), and bands were quantified with Odyssey 3.0 software on the basis of direct fluorescence measurements.

Measurements of in vivo glucose kinetics and insulin clamp procedures. Euglycemic clamps were performed in conscious, unrestrained, catheterized mice as previously described (5; also see ref. 4 in the main text). Food was removed for 5 hr prior to the start of *in vivo* studies. The infusion studies lasted for a total of 90 min. At t = 0 min, mice began receiving a constant infusion of HPLC-purified [^{3}H] glucose (0.1 μCi/min/kg body weight; New England Nuclear), and insulin (3.6 mU/min/kg body weight). A solution of glucose (10% wt/vol) was infused at a variable rate as required to maintain euglycemia (8 mM). Thereafter, plasma samples were collected to determine glucose levels (at t = 10, 20, 30, 40, 50, 60, 70, 80, and 90 min) as well as [^{3}H]glucose SA **(**at t = 40, 50, 60, 70, 80, and 90 min). Steady-state conditions for both plasma glucose levels and SA were achieved by 40 min in these studies.

References

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Supplemental Table 1. Primer sequences (mouse) used for real-time qRT-PCR studies

Gene	Primer Sequence	Amplicon size (bp)
M_3 receptor	Forward: 5' GCCACACCATCTGGCAAGT Reverse: 5' GGTTCATGGAAATGACCCCGA	186
PGC1α	Forward: 5' CAGCCTCTTTGCCCAGATCTT Reverse: 5' CTCAAATATGTTCGCAGGCTCA	158
PGC1β	Forward: 5' TTGTAGAGTGCCAGGTGCTGAC Reverse: 5' TCCTCAGATGTGGGATCATAGTCA	205
IRS1	Forward: 5'-GATAGCGAGGCTGAGCAAGA Reverse: 5'-CCTGCCAGACCTCCTTGAA	190
PPARγ	Forward: 5'-TGACCCAATGGTTGCTGATTACA Reverse: 5'-CAATGGCCATGAGGGAGTTAGA	156
Akt2	Forward: 5'-GAATGCCAGCTGATGAAGACTGA Reverse: 5'-CTACATGGAAGGTCCTCTCGATGA	96
Acly	Forward: 5'-GACACCATCTGTGATCTTG Reverse: 5'-GATGGTCTTGGCATAGTCAT	102
G6Pase	Forward: 5'-ATTCCGGTGTTTGAACGTCAT Reverse: 5'-CCACAGCAATGCCTGACAAGA	134
PEPCK	Forward: 5'-GTGTCATCCGCAAGCTGAAGA Reverse: 5'-GGCACTGTGTCTCTCTGCTCTTG	121
PC	Forward: 5'-AGGTGGCCAAAGAGAATGGTATG Reverse: 5'-CAGCAGCATGTTTGGCAAGTAGT	74
FAS	Forward: 5'-CCGAGTCAGAGAACCTACAG Reverse: 5'-CTTCCATCTCCTGTCATCAT	88
CREB	Forward: 5'-GTCCAGGTCCATGGCGTTATC Reverse: 5'-GAACTGTTTGGACTTGTGGAGACTG	70
CPT1a	Forward: 5'-GGATGGACACTGTAAAGGAGACA Reverse: 5'-CACTGCTTAGGGATGTGTCTATGA	110
IR	Forward: 5'-CTCTGTCCGCATCGAGAAGA	122

	Reverse: 5'-CAATGTAGTTGTCCTCCACAGAATC	
GK	Forward: 5'-GGACTTCTCCGAGATGCTATCAAGA Reverse: 5'-GCGGTCTTCATAGTAGCAGGAGATC	108
Cyclophilin A	Forward: 5' AAGGTGAAAGAAGGCATGAGC Reverse: 5' AGTTGTCCACAGTCGGAAATG	100

Acly, ATP citrate lyase
CPT1a, carnitine palmitoyltransferase 1a
CREB, cAMP-response element binding protein
FAS, fatty acid synthase
G6Pase, glucose-6-phosphatase
GK, glucokinase
IR, insulin receptor
IRS1, insulin receptor substrate 1
PC, pyruvate carboxylase
PEPCK, phosphoenolpyruvate carboxykinase
PGC1α, peroxisome proliferator activated receptor gamma coactivator 1α
PGC1β, peroxisome proliferator activated receptor gamma coactivator 1β
PGK, phosphoglycerine kinase
PPARγ, peroxisome proliferator-activated receptor γ

Supplemental Table 2. Serum levels of hepatic enzymes and metabolites in freely fed Hep-M3-KO and Hep-M3-Tg mutant mice and their corresponding control littermates

	Control	Hep-M3-KO	WT	Hep-M3-Tg
Cholesterol (mg/dl)	160 ± 9	169 ± 8	184 ± 9	211 ± 10
Albumin (g/dl)	3.5 ± 0.1	3.5 ± 0.1	3.6 ± 0.1	3.6 ± 0.1
Alkaline phosphatase (IU/l)	42.8 ± 3.6	45.8 ± 3.0	58.7 ± 4.6	58.0 ± 3.6
ALT (IU/l)	48.3 ± 5.1	51.0 ± 5.5	56.0 ± 5.1	60.2 ± 6.5
AST (IU/l)	89.0 ± 17.5	85.8 ± 9.3	77.3 ± 7.3	109.6 ± 14.7
Total protein (g/dl)	4.2 ± 0.3	4.7 ± 0.2	4.8 ± 0.1	4.9 ± 0.1
Uric acid (mg/dl)	1.6 ± 0.2	2.0 ± 0.3	2.3 ± 0.3	2.5 ± 0.2

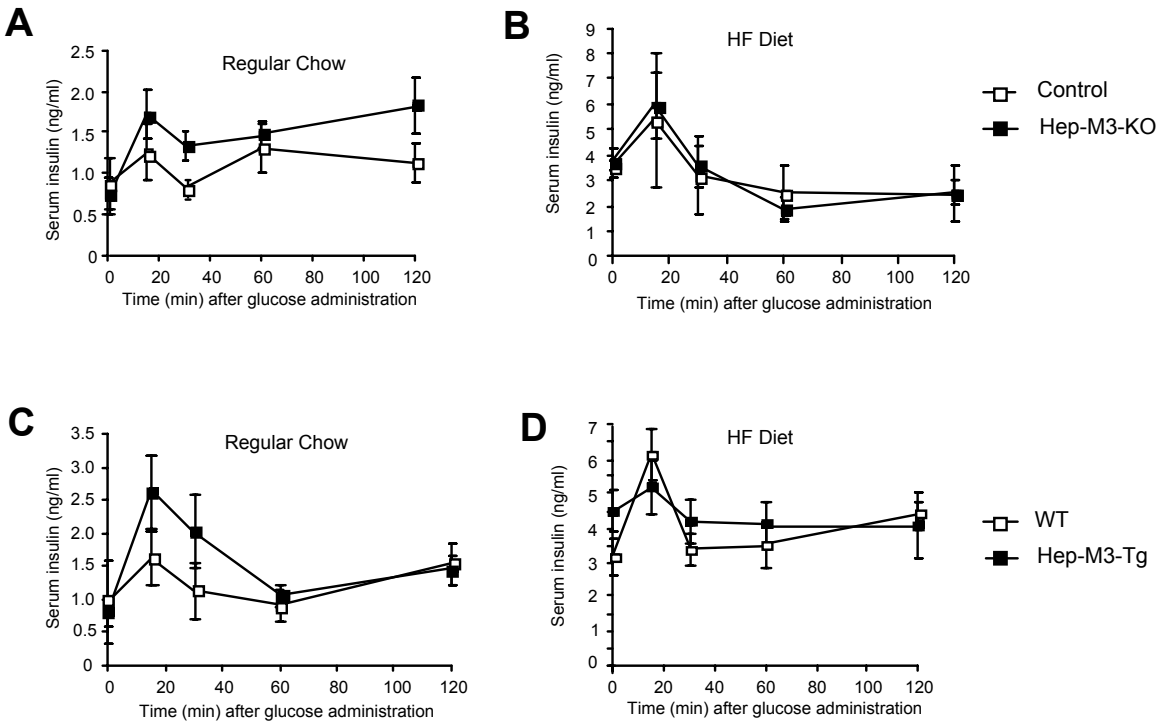
Data expressed as means ± SEM; n = 6-9 per group (8-month-old males). ALT, alanine aminotransferase; AST, aspartate aminotransferase.

Supplemental Table 3. Serum levels of corticosterone, norepinephrine, epinephrine, and glucagon in freely fed Hep-M3-KO and Hep-M3-Tg mutant mice and their corresponding control littermates

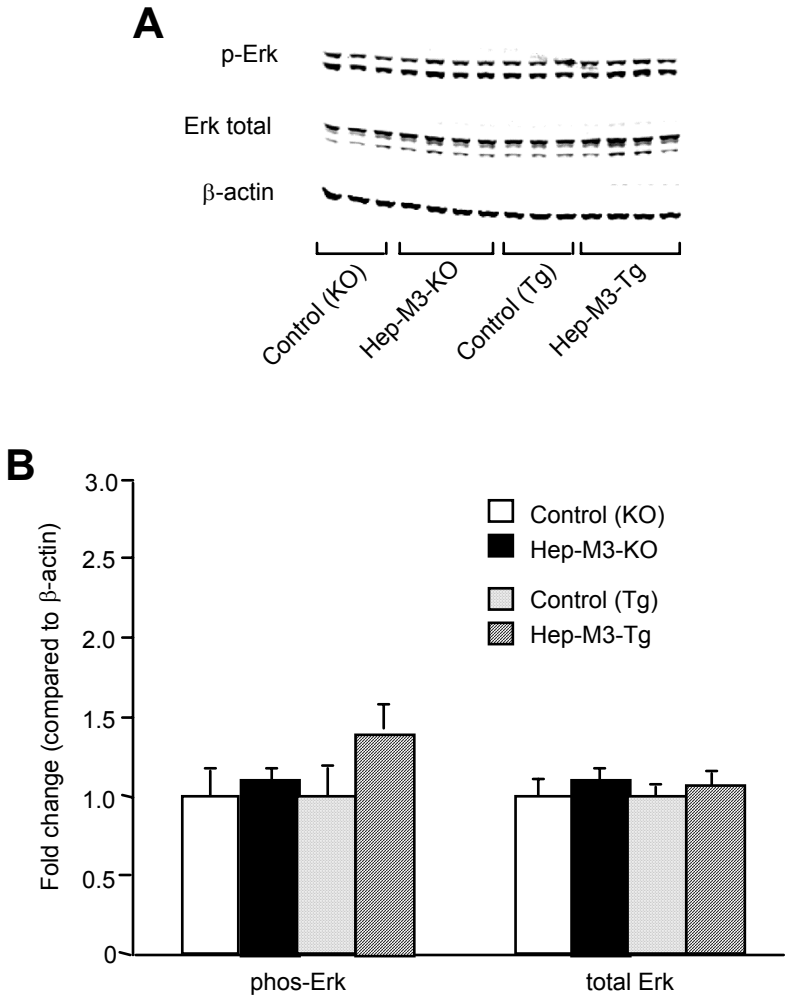
	Corticosterone (ng/ml)	Norepinephrine (ng/ml)	Epinephrine (ng/ml)	Glucagon (pg/ml)
Control	94 ± 26	2.2 ± 0.2	0.71 ± 0.05	69 ± 12
Hep-M3-KO	137 ± 35	2.2 ± 0.2	1.04 ± 0.34	82 ± 2
WT	139 ± 14	7.6 ± 0.4	0.70 ± 0.07	48 ± 3
Hep-M3-Tg	154 ± 22	7.4 ± 0.6	0.66 ± 0.08	62 ± 11

Data expressed as means ± SEM (n = 5-9 per group; 2-month-old males).

Supplemental Figure 1. Glucose-dependent insulin release in Hep-M3-KO and Hep-M3-Tg mutant mice and control littermates. ***A, B:*** Glucose-induced insulin secretion in Hep-M3-KO and control mice maintained on either regular chow (***A***) or a high-fat (HF) diet (***B***). ***C, D:*** Glucose-induced insulin release in Hep-M3-Tg and WT control mice maintained on either regular chow (***C***) or a HF diet (***D***). Serum insulin levels were measured at the indicated time points following oral administration of 2 mg/g of glucose (18-20-week-old males, n=6 per group). Mice had been fasted overnight (12 hr) prior to testing.



Supplemental Figure 2. Hepatic ERK signaling remains unaffected in Hep-M3-KO and Hep-M3-Tg mutant mice. ***A:*** Western blot analysis of mouse liver extracts. Membranes were probed for Erk phosphorylation or total levels of Erk. ***B:*** Quantification of Western blotting data. Data (means ± SEM) were normalized relative to β-actin expression and expressed as fold change compared to the corresponding control mice (2-month-old males).



Supplemental Figure 3. Lack of M_3 receptor expression in mouse Kupffer cells. Total RNA was prepared from Kupffer cells derived from livers of WT C57BL/6 mice and subjected to RT-PCR analysis using a mouse M_3 receptor-specific primer pair (ref. 19 in the main text). Two independently prepared batches of RNA (KC1 and KC2) were analyzed. Control samples (indicated by the '-' signs above the lanes) that had not been treated with reverse transcriptase (RT) did not give any detectable signal, confirming the absence of contaminating genomic DNA. Whereas a clear M_3 receptor signal could be observed with mouse hepatocyte (Hep) cDNA, M_3 receptor expression was undetectable in mouse Kupffer cells (KC1 and KC2). The expression of F4/80 mRNA which codes for a Kupffer cell-specific marker protein (ref. 18 in the main text) was readily detectable in both KC1 and KC2. For the amplification of F4/80 cDNA, the following PCR primers were used: 5'-GGAAAGCACCATGTTAGCTGC-3' (forward), 5'-CCTCTGGCTGCCAAGTTAATG-3' (reverse). PCR cycling conditions were as follows: 94 ºC 30 s, 55 ºC 30s, 72 ºC 30 s for 35 cycles.