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# A critical role for β cell $M_3$ muscarinic acetylcholine receptors in regulating insulin release and blood glucose homeostasis in vivo

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## Summary

**One of the hallmarks of type 2 diabetes is that pancreatic β cells fail to release sufficient amounts of insulin in the presence of elevated blood glucose levels. Insulin secretion is modulated by many hormones and neurotransmitters including acetylcholine, the major neurotransmitter of the peripheral parasympathetic nervous system. The physiological role of muscarinic acetylcholine receptors expressed by pancreatic β cells remains unclear at present. Here, we demonstrate that mutant mice selectively lacking the $M_3$ muscarinic acetylcholine receptor subtype in pancreatic β cells display impaired glucose tolerance and greatly reduced insulin release. In contrast, transgenic mice selectively overexpressing $M_3$ receptors in pancreatic β cells show a profound increase in glucose tolerance and insulin release. Moreover, these mutant mice are resistant to diet-induced glucose intolerance and hyperglycemia. These findings indicate that β cell $M_3$ muscarinic receptors play a key role in maintaining proper insulin release and glucose homeostasis.**

## Introduction

One characteristic pathophysiological feature of type 2 diabetes is that glucose fails to stimulate adequate release of insulin from pancreatic β cells (Kahn, 1994, 2001; Moller, 2001). Glucose-dependent insulin secretion is modulated by several neurotransmitters released from peripheral autonomic nerves (Satin and Kinard, 1998; Ahren, 2000; Gilon and Henquin, 2001). The major neurotransmitter of the peripheral parasympathetic nervous system, acetylcholine, is known to facilitate the release of insulin in a glucose-dependent fashion (Ahren, 2000; Gilon and Henquin, 2001). This activity has been shown to be mediated by activation of muscarinic acetylcholine receptors located on the pancreatic β cells (Satin and Kinard, 1998; Ahren, 2000; Gilon and Henquin, 2001).

Food intake is known to trigger an increase in efferent parasympathetic outflow involving signals of different origins that are integrated in the brain (Niijima, 1989; Gilon and Henquin, 2001). Acetylcholine is released from intrapancreatic parasympathetic (vagal) nerve endings during the preabsorptive and, most likely, also during the absorptive phase of feeding (Ahren, 2000; Gilon and Henquin, 2001). At present, the importance of the parasympathetic innervation of the endocrine pancreas in the maintenance of normal glucose homeostasis remains controversial (Ahren, 2000; Gilon and Henquin, 2001). Much of this controversy is thought to arise from the fact that peripheral parasympathetic (vagal) nerves release at least five different neurotransmitters (Ahren, 2000; Gilon and Henquin, 2001) and that increased parasympathetic outflow does not only affect the function of the endocrine pancreas but also that of many other organs and tissues that have important metabolic functions (Hoffman and Taylor, 2001; Wess, 2004).

The administration of atropine and other muscarinic antagonists has also led to conflicting results regarding the potential role of muscarinic receptors in the regulation of insulin release and glucose homeostasis (Ahren, 2000; Gilon and Henquin, 2001). These agents are known to block all five muscarinic receptor subtypes ($M_1$–$M_5$) which are widely expressed, in a complex, overlapping pattern, in many peripheral tissues and most brain regions (Caulfield and Birdsall, 1998; Wess, 1996, 2004). As a result, the in vivo metabolic effects of muscarinic antagonists are the sum of a multitude of activities involving many different tissues and receptor subtypes. For example, it is well known that atropine impairs the function of essentially all endocrine and exocrine glands and all smooth muscle organs and modulates a very large number of central functions (Caulfield and Birdsall, 1998; Wess, 1996, 2004).

Thus, the present study was designed to assess the importance of β cell muscarinic acetylcholine receptors in glucose homeostasis and insulin release in vivo. Specifically, we focused on the potential metabolic role of the $M_3$ muscarinic receptor subtype which represents the major muscarinic receptor that is functional in pancreatic β cells (Henquin and Nenquin, 1988; Ahren, 2000; Gilon and Henquin, 2001; Duttaroy et al., 2004;

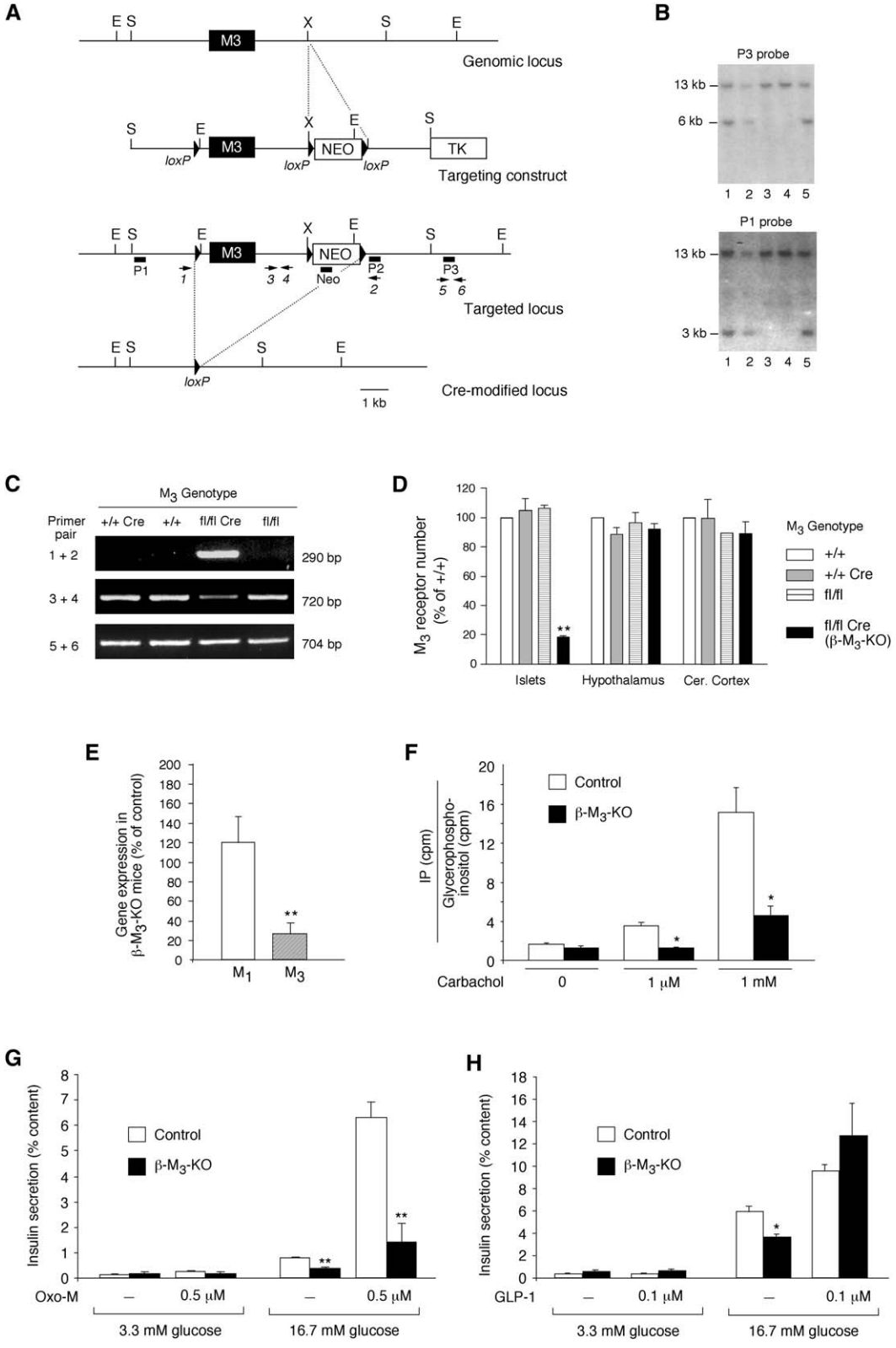


**Figure 1.** Targeted disruption of the mouse $M_3$ muscarinic receptor gene

**A)** Structures of the WT $M_3$ muscarinic receptor allele, gene targeting vector, targeted allele, and Cre-modified locus. The $M_3$ receptor coding region is represented by the filled box. The approximate location of the probes (filled bars) and primers (arrows) used for Southern analysis and PCR genotyping studies, respectively, are indicated. Abbreviations are as follows: E, EcoRV; S, SmaI; X, XhoI.

**B)** Southern blot analysis of EcoRV-digested genomic DNA from three properly targeted ES cell clones (1, 2, and 5). The 13 kb band indicates the presence of the WT $M_3$ receptor allele (both panels), whereas the 6 kb band (upper panel) and the 3 kb band (lower panel) are diagnostic for the proper integration of the targeting construct.

Zawalich et al., 2004). $M_3$ receptors are not only expressed by pancreatic β cells but are present in essentially all glandular and smooth muscle tissues and most regions of the CNS (Levey et al., 1994; Eglen et al., 1996; Caulfield and Birdsall, 1998; Wess, 2004).

To assess the physiological role of β cell $M_3$ muscarinic receptors in the regulation of glucose homeostasis and insulin release in vivo, we employed Cre/loxP technology to generate mutant mice lacking $M_3$ receptors in pancreatic β cells only. We anticipated that this strategy would eliminate potential confounding factors caused by the widespread distribution of $M_3$ receptors. Moreover, to study the metabolic effects of enhanced signaling through β cell $M_3$ muscarinic receptors, we also generated mutant mice selectively overexpressing $M_3$ muscarinic receptors in pancreatic β cells.

We found that the absence of β cell $M_3$ muscarinic receptors led to glucose intolerance and significantly reduced plasma insulin levels. On the other hand, mice that selectively overexpressed $M_3$ muscarinic receptors in pancreatic β cells showed a pronounced increase in glucose tolerance and enhanced plasma insulin levels. Strikingly, these mutant mice were resistant to diet-induced glucose intolerance and hyperglycemia. Our findings indicate that β cell $M_3$ muscarinic receptors play a key role in maintaining proper insulin release and glucose homeostasis in vivo.

## Results

### Generation of β cell-specific $M_3$ muscarinic receptor knockout mice

To generate β cell-specific $M_3$ muscarinic receptor knockout (KO) mice (β-$M_3$-KO mice) (also referred to as Cre fl/fl mice; "fl" stands for "floxed" indicating that the $M_3$ receptor coding sequence was flanked by loxP sites; Figure 1A), we crossed mutant mice carrying the "floxed" $M_3$ receptor gene with transgenic mice expressing Cre recombinase under the control of the rat insulin promoter II (RIP-Cre mice; Postic et al., 1999). The structure of the targeting construct used to generate floxed $M_3$ receptor mutant mice is shown in Figure 1A. The proper genomic integration of the targeting vector was verified by PCR and Southern blot analysis (Figures 1A and 1B).

To generate β-$M_3$-KO mice (Cre fl/fl mice), we crossed fl/+ mice with fl/+ mice that were hemizygous for the RIP-Cre transgene. This mating strategy also produced three littermate control groups, fl/fl, +/+, and +/+ Cre mice. All mice were born at the expected Mendelian frequency, and the body weight of the β-$M_3$-KO mice did not differ significantly from that of the three control groups (data not shown). Moreover, β-$M_3$-KO mice were fertile and showed no obvious developmental, behavioral, or morphological deficits.

To assess the efficiency of Cre-mediated deletion of the $M_3$ receptor gene, we employed a PCR strategy using DNA prepared from isolated islets of β-$M_3$-KO mice and the three littermate control groups (Figure 1C; see Figure 1A for the location of the PCR primers). As shown in Figure 1A, PCR primers 1 and 2 were predicted to yield a 290 bp band only following complete Cre-mediated excision of the $M_3$ receptor gene. As expected, use of this primer pair resulted in a 290 bp band only in the case of islet DNA isolated from β-$M_3$-KO mice (Figure 1C). PCR primers 3 and 4 which anneal to the 3′-untranslated region of the $M_3$ receptor gene still gave a weak 720 bp signal using islet DNA from β-$M_3$-KO animals as a template, indicative of the presence of some nonrecombined $M_3$ muscarinic receptor gene. This remaining $M_3$ receptor signal may be due to the presence of genomic DNA derived from non-β islet cells, including cells producing glucagon, somatostatin, and pancreatic polypeptide (Kulkarni et al., 1999).

To confirm the absence of $M_3$ receptor protein in islets from β-$M_3$-KO mice, we used a previously developed immunoprecipitation strategy (Yamada et al., 2001). This strategy involved the labeling of all muscarinic receptors by the non-subtype-selective muscarinic antagonist, [$^3$H]quinuclidinyl benzilate ([$^3$H]QNB), followed by selective immunoprecipitation of $M_3$ receptors by an $M_3$ receptor-specific antiserum (Yamada et al., 2001). This analysis demonstrated that $M_3$ receptor expression was dramatically reduced (by ~80%) in islets from β-$M_3$-KO mice (Figure 1D). In contrast, islets derived from the three control groups showed similar $M_3$ receptor expression levels (Figure 1D). All four analyzed mouse strains (β-$M_3$-KO mice and the three littermate control strains) exhibited similar $M_3$ receptor expression levels in the hypothalamus and cerebral cortex (Figure 1D), two brain regions where $M_3$ receptors are expressed at relatively high levels (Yamada et al., 2001). The relatively small number of islet $M_3$ receptors remaining in β-$M_3$-KO mice may be due to the presence of $M_3$ muscarinic receptors expressed by non-β islet cells (Ahren, 2000; Gilon and Henquin, 2001; Duttaroy et al., 2004) and/or incomplete excision of the $M_3$ receptor gene in β cells. Because of the low expression levels of $M_3$ receptors in mouse pancreatic β cells (<2 fmol/100 islets; see below), combined with the lack of highly sensitive $M_3$ receptor-selective antibodies, we were unable to distinguish between these two possibilities. In fact, previous studies suggest that a small population of β cells may escape Cre-mediated recombination following mating of Rip-Cre transgenic mice with

**C)** PCR analysis of genomic DNA prepared from islets of the indicated $M_3$ receptor genotypes (see text for details).

**D)** Immunoprecipitation of $M_3$ muscarinic receptors from mouse pancreatic islets, hypothalamus, and cerebral cortex. For these studies, 3-month-old littermates of the indicated $M_3$ receptor genotypes were used. [$^3$H]QNB-labeled $M_3$ receptors were immunoprecipitated with an $M_3$ receptor-specific antiserum (Yamada et al., 2001), as described in the Experimental Procedures. Absolute receptor densities for $M_3$ +/+ mice were (fmol/mg protein): islets, 5.6 ± 0.2; hypothalamus, 31.2 ± 1.1; cortex, 38.9 ± 1.2. Data are given as means ± SEM of at least three independent experiments. **$p < 0.01$, as compared to the corresponding +/+ value.

**E)** Analysis of $M_1$ and $M_3$ receptor mRNA expression levels in pancreatic islets. Real-time quantitative RT-PCR studies were carried out using total RNA prepared from islets of β-$M_3$-KO mice and control (fl/fl) littermates (12 weeks old; n = 4 per group). For details, see Experimental Procedures and Supplemental Data. **$p < 0.01$ versus control.

**F)** Carbachol-mediated inositol phosphate (IP) production. IP levels (in cpm) were normalized by glycerophosphoinositol levels (in cpm) which are not affected by the addition of carbachol and can be considered a measure of tissue amount per well (Berridge et al., 1983). Data are given as means ± SEM from three independent experiments. *$p < 0.05$, as compared to the corresponding control (fl/fl) value.

**G and H)** In vitro insulin release studies. Isolated pancreatic islets prepared from β-$M_3$-KO mice and control (+/+) littermates ([**G**], 4-month-old males; [**H**], 11-month-old males) were incubated for 1 hr at 37°C in Krebs solution containing the indicated glucose concentrations, either in the absence of ligands or in the presence of the muscarinic agonist, oxotremorine-M (Oxo-M, 0.5 μM) (**G**), or GLP-1 (0.1 μM) (**H**). 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 expressed as means ± SEM of three independent experiments, each carried out in triplicate. *$p < 0.05$, **$p < 0.01$, as compared to the corresponding WT value.

mutant mice carrying a floxed gene (Postic et al., 1999; Crabtree et al., 2003).

These observations indicate that genomic incorporation of the loxP sites (including the neo selection cassette) had no significant effect on $M_3$ receptor expression levels and that β-$M_3$-KO mice selectively lacked $M_3$ receptors in pancreatic islets (β cells).

The $M_3$ receptor, like the $M_1$ and $M_5$ muscarinic receptor subtypes, is selectively coupled to G proteins of the $G_q$ family (Wess, 1996; Caulfield and Birdsall, 1998). Real-time quantitative RT-PCR studies showed that the dramatic reduction of $M_3$ receptor mRNA levels observed with islets from β-$M_3$-KO mice had no significant effect on $M_1$ receptor expression levels (Figure 1E). We were unable to detect $M_5$ receptor mRNA in islets from β-$M_3$-KO and control mice (also see Duttaroy et al., 2004).

### Islets from β-$M_3$-KO mice display pronounced deficits in muscarinic agonist-induced inositol phosphate production and insulin release

To examine whether the lack of β cell $M_3$ receptors led to impaired signaling at the molecular level, we measured the ability of the muscarinic agonist, carbachol, to stimulate inositol phosphate (IP) production in isolated islets prepared from β-$M_3$-KO mice and control littermates. This analysis showed that carbachol-mediated PI hydrolysis was greatly reduced in islets obtained from β-$M_3$-KO mice (Figure 1F).

Morphometric analysis indicated that β cell mass and the average size and number of pancreatic islets were not significantly affected by the lack of β cell $M_3$ muscarinic receptors (Table S1 in the Supplemental Data available with this article online). Previous in vitro studies demonstrated that stimulation of pancreatic (β cell) $M_3$ muscarinic receptors leads to a significant augmentation of glucose-dependent insulin release (Duttaroy et al., 2004; Zawalich et al., 2004). To confirm that islets prepared from β-$M_3$-KO mice were deficient in this activity, we carried out a series of in vitro insulin release studies using isolated islets prepared from wild-type (WT, +/+) and β-$M_3$-KO littermates. Oxotremorine-M (Oxo-M), a non-subtype-selective, hydrolytically stable muscarinic agonist, was used as a muscarinic stimulant throughout all experiments. In the presence of a low concentration of glucose (3.3 mM), Oxo-M (0.5 μM) had no effect on insulin release in islets from either WT or β-$M_3$-KO mice (Figure 1G). However, in the presence of a high concentration of glucose (16.7 mM), Oxo-M (0.5 μM) treatment of WT islets led to a pronounced increase in glucose-dependent insulin release (Figure 1G). In striking contrast, the insulin release-enhancing activity of Oxo-M was dramatically reduced in islets prepared from β-$M_3$-KO mice (Figure 1G). In the absence of Oxo-M, glucose (16.7 mM) also released ~50% less insulin in islets from β-$M_3$-KO mice than in islets from WT mice (Figure 1G). The molecular mechanisms underlying this deficit remain unclear at present. The total insulin content of islets prepared from β-$M_3$-KO mice was similar to that of WT islets (ng insulin per islet: β-$M_3$-KO, 120.4 ± 16.4; WT, 117.1 ± 15.7; means ± SEM of three independent experiments), indicating that the deficits in insulin release observed with islets from β-$M_3$-KO mice were not due to reduced insulin synthesis or storage. Additional control experiments showed that glucagon-like peptide-1 (GLP-1; 0.1 μM), an agent which is able to enhance glucose-dependent insulin release in a fashion similar to acetylcholine (Doyle and Egan, 2001), released similar amounts of insulin in islets from β-$M_3$-KO and control mice in the presence of 16.7 mM glucose (Figure 1H). This observation suggested that the lack of β cell $M_3$ receptors did not result in a generalized impairment in islet function. In sum, these data clearly indicate that glucose-dependent muscarinic stimulation of insulin release is greatly reduced in islets from β-$M_3$-KO mice, consistent with the pronounced reduction in the number of islet $M_3$ muscarinic receptors found with these mutant mice (Figure 1D).

**Table 1.** Glucose and insulin levels in fl/fl Cre mice (β-$M_3$-KO mice) and control littermates

| | +/+ | +/+ Cre | fl/fl | fl/fl Cre |
|---|---|---|---|---|
| Blood glucose (fed, mg/dl) | 136 ± 5 | 122 ± 5 | 129 ± 6 | 125 ± 3 |
| Blood glucose (fasted, mg/dl) | 115 ± 9 | 111 ± 16 | 96 ± 8 | 95 ± 4 |
| Serum insulin (fed, ng/ml) | 1.03 ± 0.16 | 1.66 ± 0.27 | 1.39 ± 0.22 | 1.16 ± 0.11 |
| Serum insulin (fasted, ng/ml) | 0.83 ± 0.22 | 0.72 ± 0.44 | 1.07 ± 0.11 | 0.52 ± 0.21 |

Measurements were carried out with 16- to 20-week old male littermates (n = 6 per genotype). The fed and fasting blood glucose and serum insulin levels of the fl/fl Cre (β-$M_3$-KO) mice did not differ significantly from the corresponding control values ($p > 0.05$).

### β-$M_3$-KO mice display impaired glucose tolerance and insulin release in vivo

Unless stated otherwise, all physiological measurements were carried out with male mice (littermates) that were 4–6 months old. As shown in Table 1, the fed and fasting blood glucose and fed serum insulin levels of β-$M_3$-KO mice did not differ significantly from the corresponding values obtained with the three control strains. β-$M_3$-KO mice showed a clear trend toward reduced fasting serum insulin levels; however, this decrease failed to reach statistical significance in this set of experiments ($p > 0.05$).

To examine whether β-$M_3$-KO mice showed deficits in glucose tolerance, we carried out oral and intraperitoneal (i.p.) glucose tolerance tests (OGTT and IGTT, respectively). Following glucose administration (2 mg/g body weight), the β-$M_3$-KO mice showed significantly higher blood glucose levels than the three control strains (+/+, +/+ Cre, and fl/fl) in both OGTT and IGTT (Figures 2A and 2C). Since the three control strains gave similar responses (this is also true for all other physiological studies described below), the data obtained with these three strains were pooled for the sake of clarity.

To investigate whether the impairment in glucose tolerance displayed by the β-$M_3$-KO mice in the OGTT and IGTT were accompanied by deficits in insulin release, we also monitored serum insulin levels after oral or i.p. administration of glucose (2 mg/g). β-$M_3$-KO mice showed significantly lower serum insulin levels during the first 60 min after glucose administration (OGTT) or during the entire 2 hr observation period (IGTT), respectively, as compared to the corresponding control littermates (Figures 2B and 2D). Consistent with this observation, C-peptide levels were also found to be significantly lower in β-$M_3$-KO mice throughout the entire 2 hr observation period following i.p. administration of glucose (2 mg/g; Figure S1A).

To study the degree of insulin sensitivity of the β-$M_3$-KO mice and their control littermates, we injected mice with a fixed dose of insulin (0.75 U/kg i.p.) and monitored the resulting decreases

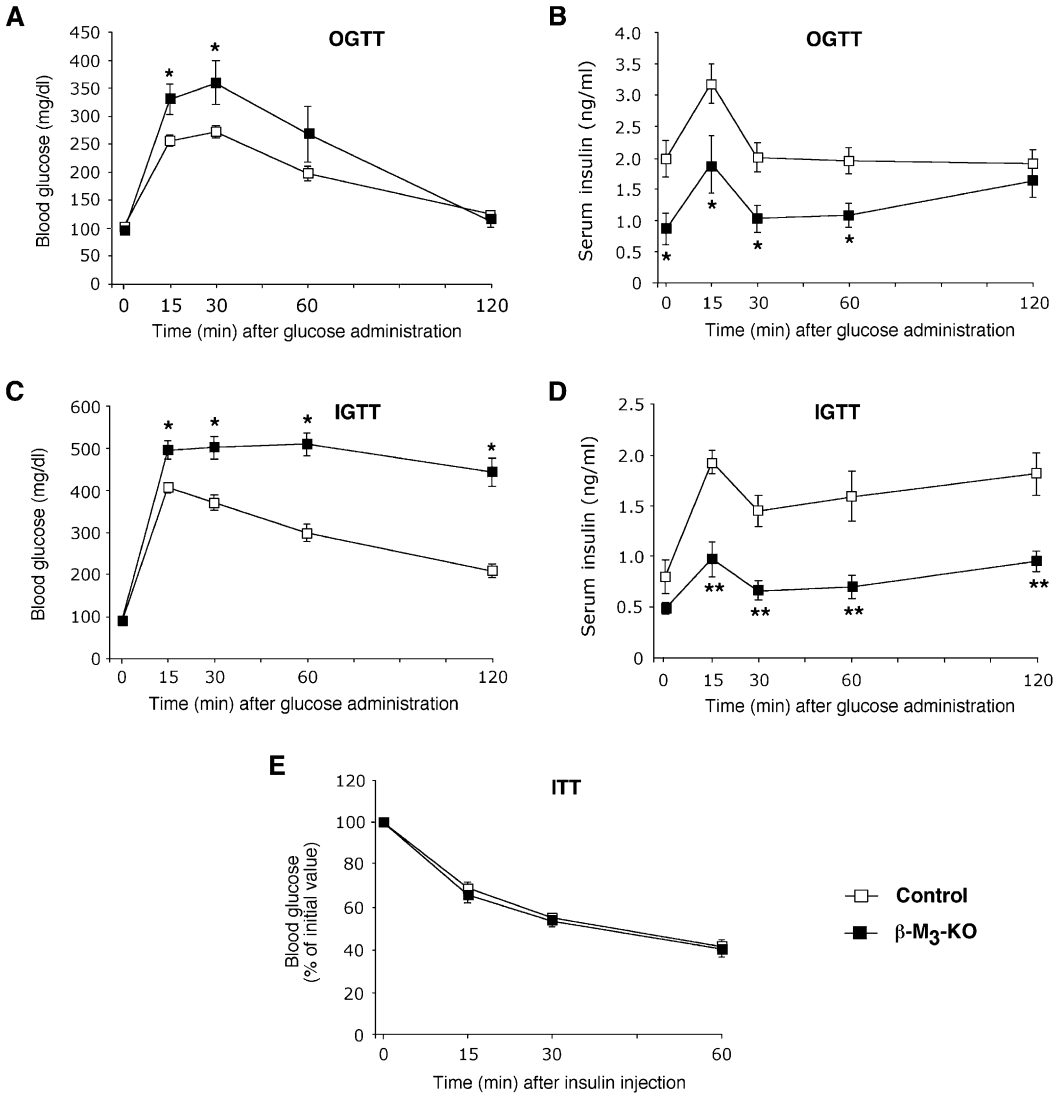


**Figure 2.** Physiological analysis of β-$M_3$-KO mice and control littermates

**A and B)** Blood glucose (**A**) and serum insulin (**B**) levels following application of an oral dose of glucose (2 mg/g; OGTT; 24-week-old male littermates).
**C and D)** Blood glucose (**C**) and serum insulin (**D**) levels following i.p. administration of glucose (2 mg/g; IGTT; 16-week-old male littermates).
**E)** Insulin tolerance test (ITT). Blood glucose levels were measured at the indicated time points following i.p. administration of insulin (0.75 U/kg; 20-week-old male mice). The three control strains (+/+, +/+ Cre, and fl/fl) gave similar results. The data obtained with the three control strains were therefore pooled for the sake of clarity. Data are expressed as means ± SEM (β-$M_3$-KO, n = 6; control, n = 15–18). *$p < 0.05$, **$p < 0.01$, as compared to the corresponding control value.

in blood glucose levels. In these experiments, β-$M_3$-KO mice showed a reduction in blood glucose levels that was very similar to that displayed by the corresponding control littermates (Figure 2E), indicating that the lack of β cell $M_3$ muscarinic receptors has no significant effect on insulin sensitivity.

Pancreata from adult β-$M_3$-KO mice and control (fl/fl) littermates did not differ significantly in weight (control, 278 ± 10 mg, n = 12; β-$M_3$-KO, 250 ± 18 mg; n = 9) and contained similar amounts of insulin (μg/pancreas: control, 343 ± 19, n = 12; β-$M_3$-KO, 302 ± 14; n = 9).

Real-time quantitative RT-PCR studies using total RNA prepared from islets of β-$M_3$-KO and control (fl/fl) littermates showed that the relative lack of β cell $M_3$ receptors had no significant effect on GLUT2 and insulin mRNA expression levels. We noted a small increase (~1.6-fold) in glucagon and glucokinase mRNA levels (Figure S2) in islet RNA samples prepared from β-$M_3$-KO mice. However, plasma glucagon levels were not significantly different in β-$M_3$-KO and control (fl/fl) littermates (β-$M_3$-KO, 78.8 ± 8.1 pg/ml, n = 8; control, 62.0 ± 5.4 pg/ml; n = 11; 32-week-old freely fed males).

### Generation of transgenic mice selectively overexpressing $M_3$ muscarinic receptors in pancreatic β cells

The analysis of β-$M_3$-KO mice indicated that the activity of endogenous β cell $M_3$ muscarinic receptors plays a critical role in mediating glucose-dependent insulin release and maintaining proper glucose tolerance. We therefore speculated that enhanced signaling through β cell $M_3$ muscarinic receptors might facilitate glucose-dependent insulin release and lead to improved glucose tolerance. To test this hypothesis, we generated transgenic mice that selectively overexpressed $M_3$ muscarinic

receptors in pancreatic β cells. To ensure that $M_3$ receptors were selectively expressed by pancreatic β cells, transgene expression was placed under the control of a 0.65 kb fragment of the rat insulin promoter II (Vasavada et al., 1996; Postic et al., 1999; Kulkarni et al., 1999).

To be able to distinguish overexpressed $M_3$ receptors from endogenous $M_3$ receptors, the transgenically expressed $M_3$ receptors contained two modifications, an N-terminal 9 amino acid hemagglutinin epitope tag and a deletion of the central portion of the third intracellular loop (amino acids 274–469). Previous studies have shown that these modifications have no significant effect on the ligand binding and G protein coupling properties of the $M_3$ muscarinic receptor (Schöneberg et al., 1995; Maggio et al., 1996). By using standard transgenic techniques (see "Experimental procedures"), we obtained several mutant mouse lines that had incorporated the $M_3$ receptor transgene into their genomes.

To quantify the number of $M_3$ receptors overexpressed in pancreatic islets (β cells) of the different transgenic lines, we incubated membranes prepared from pancreatic islets with a saturating concentration (2 nM) of the non-subtype-selective muscarinic antagonist,[$^3$H]N-methylscopolamine ([$^3$H]NMS). Control experiments with nontransgenic WT littermates showed that endogenous islet muscarinic receptors were expressed at relatively low levels (~1.6 ± 0.2 fmol/100 islets; n = 7). In contrast, islets from one of the analyzed transgenic lines, hereafter referred to as β-$M_3$-Tg1, showed a pronounced increase in islet muscarinic receptor density (50 ± 2 fmol/100 islets; n = 3). Thus, assuming that the majority of islet muscarinic receptors represent β cell $M_3$ receptors (Ahren, 2000; Gilon and Henquin, 2001; Duttaroy et al., 2004; Zawalich et al., 2004), the β-$M_3$-Tg1 line expressed at least 30-fold more β cell $M_3$ receptors than the corresponding WT control mice.

RT-PCR studies using total RNA prepared from several peripheral and central tissues from β-$M_3$-Tg1 mice confirmed that expression of the $M_3$ receptor transgene could be easily detected using cDNA derived from pancreatic islets (β cells; Figure 3A). In contrast, expression of the $M_3$ receptor transgene remained undetectable in other peripheral tissues and in the hypothalamus (Figure 3A). A very faint transgene-specific RT-PCR signal was observed with cDNA derived from cerebral cortex (Figure 3A).

### Islets from β-$M_3$-Tg1 mice display enhanced muscarinic agonist-induced IP production and insulin release

To determine whether overexpression of β cell $M_3$ receptors led to enhanced signaling at the molecular level, we measured the ability of carbachol to stimulate IP production in isolated islets prepared from β-$M_3$-Tg1 mice and WT littermates. We found that carbachol-mediated PI hydrolysis was greatly enhanced in islets obtained from β-$M_3$-Tg1 mice (Figure 3B). Moreover, basal IP production was also significantly increased in islets from β-$M_3$-Tg1 mice (Figure 3B), consistent with previous findings that increasing the density of G protein-coupled receptors often leads to some degree of ligand-independent G protein activation (Seifert and Wenzel-Seifert, 2002).

In agreement with the outcome of the IP assays, in vitro insulin release studies carried out in the presence of 16.7 mM glucose indicated that the Oxo-M-mediated (0.5 μM) increase in insulin release was significantly greater in islets obtained from β-$M_3$-Tg1 mice, as compared to islets from WT littermates (Figure 3C).

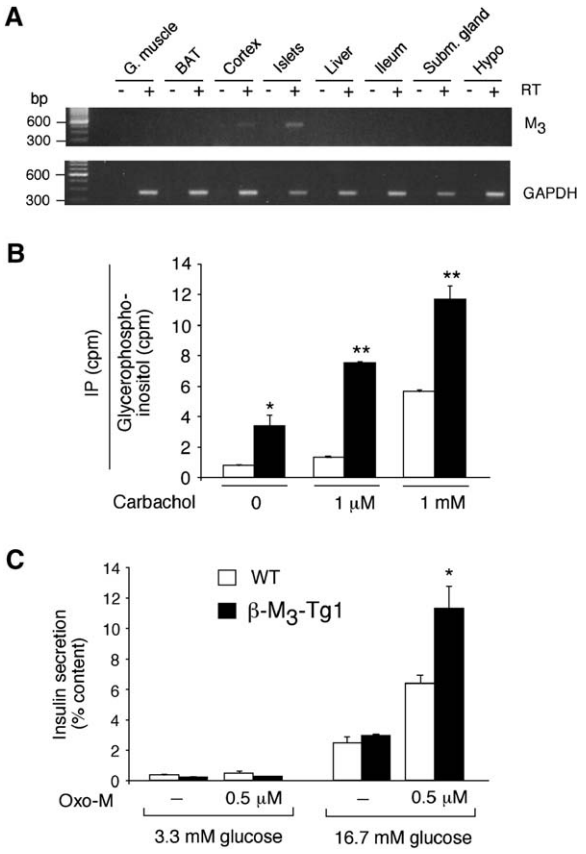


**Figure 3.** In vitro studies with β-$M_3$-Tg1 mice and control littermates maintained on regular mouse chow

**A)** RT-PCR analysis of $M_3$ receptor transgene expression in tissues from β-$M_3$-Tg1 mice.

RT-PCR studies were carried out as described under Experimental Procedures (also see text for details). Abbreviations are as follows: G. muscle, gastrocnemius muscle; BAT, brown adipose tissue; Hypo, hypothalamus; RT, reverse transcriptase.

**B)** Carbachol-mediated inositol phosphate (IP) production. IP levels (in cpm) were normalized by glycerophosphoinositol levels (in cpm), which are not affected by the addition of carbachol and are considered a measure of tissue amount per well (Berridge et al., 1983). Data are given as means ± SEM from three independent experiments.

**C)** In vitro insulin release studies. Isolated pancreatic islets prepared from adult WT and β-$M_3$-Tg1 littermates were incubated for 1 hr at 37°C in Krebs solution containing the indicated glucose concentrations, either in the absence or in the presence of the muscarinic agonist, oxotremorine-M (Oxo-M, 0.5 μM). 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 expressed as means ± SEM of three independent experiments, each carried out in triplicate. *$p < 0.05$, **$p < 0.01$, as compared to the corresponding WT value.

### β-$M_3$-Tg1 mice exhibit hypoglycemia, hyperinsulinemia, and greatly increased glucose tolerance

β-$M_3$-Tg1 appeared healthy and showed no obvious behavioral or morphological abnormalities. Moreover, the body weight of β-$M_3$-Tg1 mice did not differ significantly from that of their WT littermates when mice were fed standard mouse chow (Figure 5A). In striking contrast, β-$M_3$-Tg1 mice displayed an ~35% reduction in blood glucose levels in both the fed and fasting state (Figure 4A; 14-week-old males). The hypoglycemia phenotype displayed by the β-$M_3$-Tg1 mice was accompanied by an ~3-fold increase in fed and fasting serum insulin levels (Figure 4B; 14-week-old males). Plasma glucagon levels were similar in WT

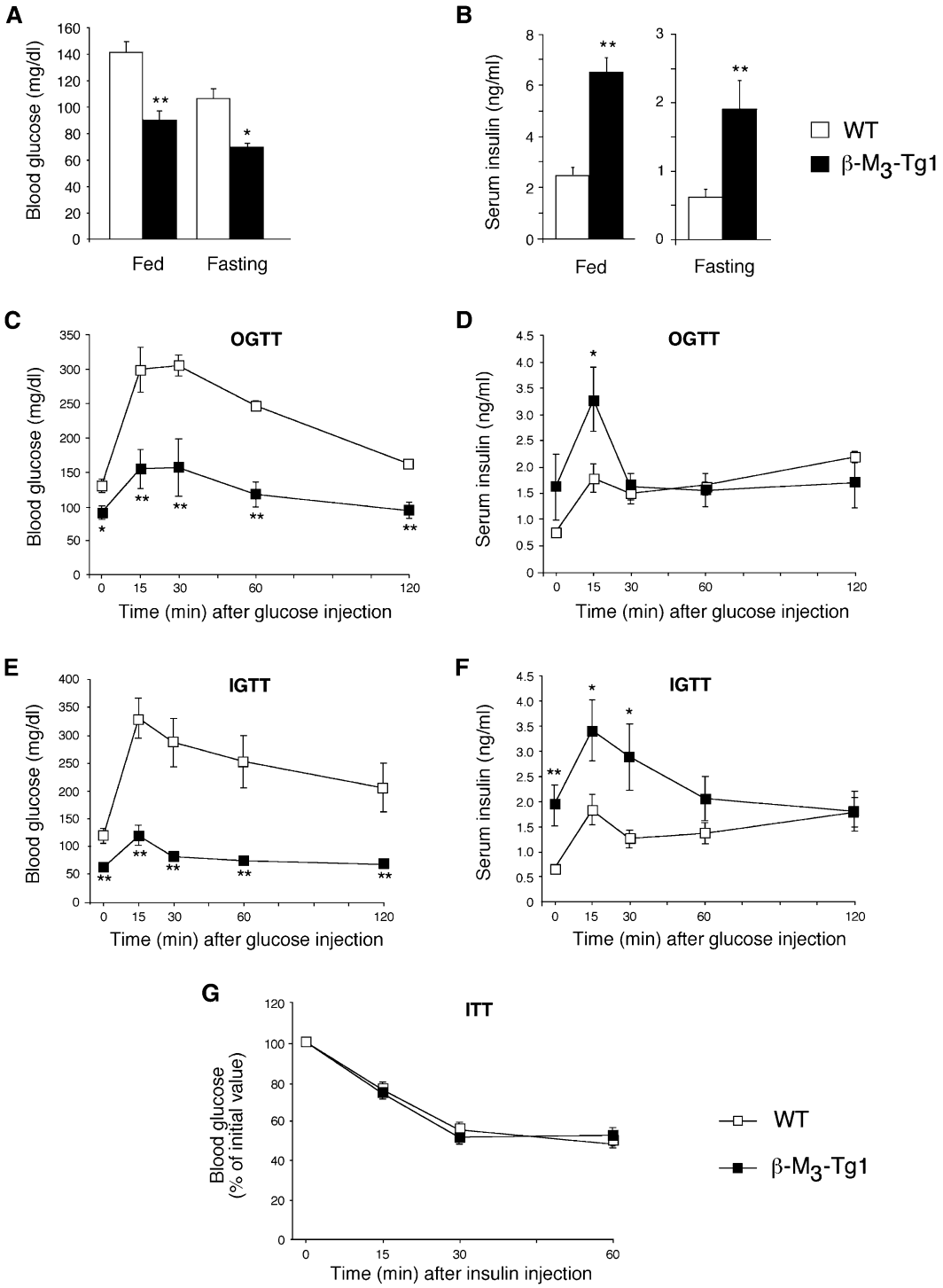


**Figure 4.** Physiological analysis of β-$M_3$-Tg1 mice and control littermates maintained on regular mouse chow

**A)** Fed and fasting blood glucose and **B)** serum insulin levels of β-$M_3$-Tg1 mice and their WT littermates. Blood was collected from male littermates that were 14 weeks old (n = 9–12 per group). *p < 0.05, **p < 0.01, as compared to the corresponding WT value. **C)** Blood glucose and **D)** serum insulin levels following application of an oral dose of glucose (2 mg/g; OGTT; 10-week-old males; WT, n = 6 per group). **E)** Blood glucose and **F)** serum insulin levels following i.p. administration of glucose (2 mg/g; IGTT; 14-week-old males; WT, n = 10; β-$M_3$-Tg1, n = 11). **G)** Insulin tolerance test (ITT). Blood glucose levels were measured at the indicated time points following i.p. administration of insulin (0.75 U/kg; 12-week-old males; WT, n = 7; β-$M_3$-Tg1, n = 7). Data are expressed as means ± SEM. *p < 0.05, **p < 0.01, as compared to the corresponding WT value.

and β-$M_3$-Tg1 mice (WT, 52.8 ± 7.0 pg/ml; β-$M_3$-Tg1, 59.4 ± 10.3 pg/ml; 16-week-old freely fed males; n = 10 per group).

To investigate whether the β-$M_3$-Tg1 mice showed changes in glucose tolerance, we carried out oral and i.p. glucose tolerance tests (OGTT and IGTT, respectively; glucose dose: 2 mg/g body weight). In both OGTT and IGTT, the β-$M_3$-Tg1 mice exhibited dramatically reduced increases in blood glucose levels throughout the entire 2 hr observation period (Figures 4C and 4E). On the

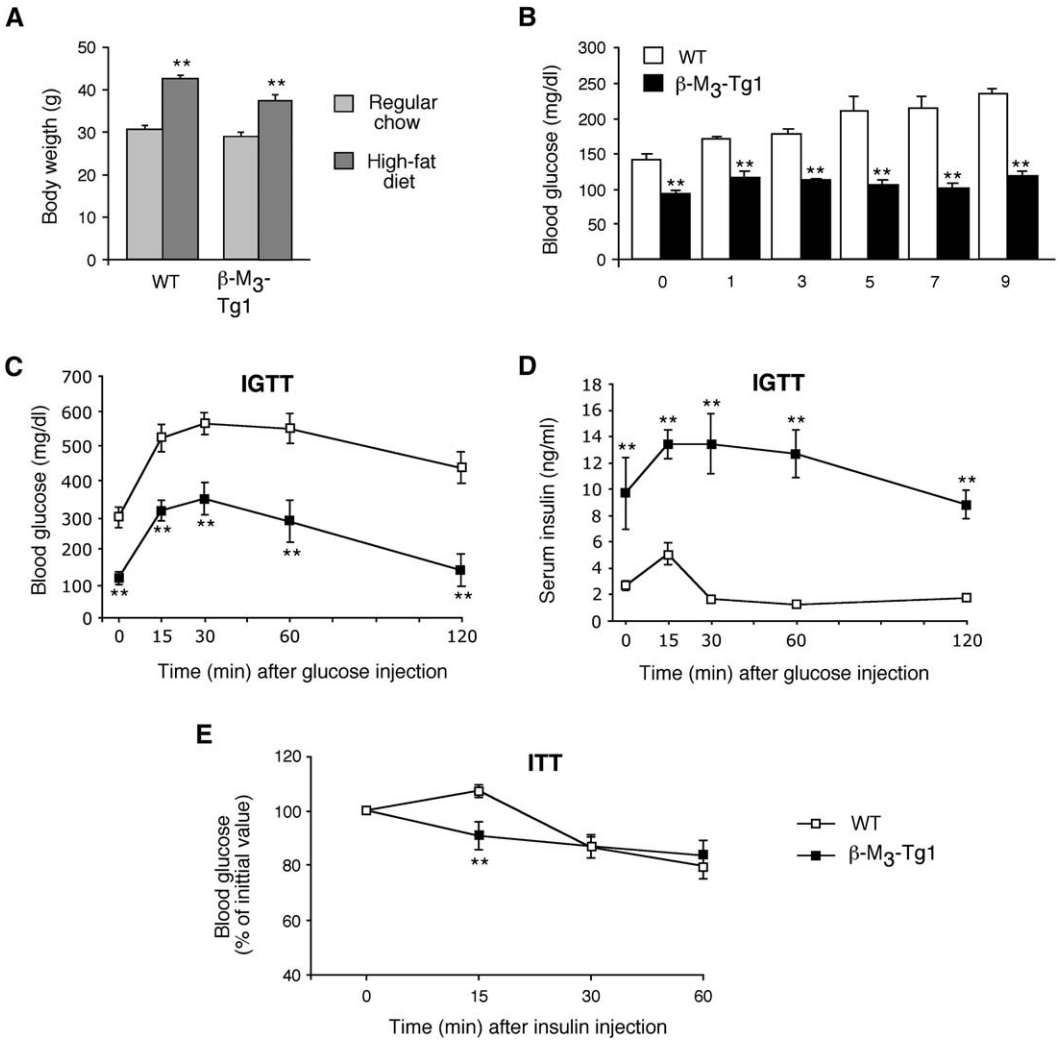


**Figure 5.** Physiological analysis of β-$M_3$-Tg1 mice and control littermates maintained on a high-fat diet

**A)** Body weight of 13-week-old male mice maintained on regular mouse chow or a high-fat diet (n = 7–12 per group).

**B)** Blood glucose levels (fed state) of male mice maintained on a high-fat diet (n = 6 per group). Mice were put on the high-fat diet when they were 4 weeks old.

**C)** I.p. glucose tolerance test (IGTT; high-fat diet). Blood glucose levels were measured at the indicated time points following i.p. administration of glucose (2 mg/g; 13-week-old males; WT, n = 9; β-$M_3$-Tg1, n = 7).

**D)** Serum insulin levels following i.p. administration of glucose (2 mg/g; high-fat diet; 16-week-old males; WT, n = 7; β-$M_3$-Tg1, n = 5).

**E)** Insulin tolerance test (ITT; high-fat diet). Blood glucose levels were measured at the indicated time points following i.p. administration of insulin (0.75 U/kg; 18-week-old males; WT, n = 9; β-$M_3$-Tg1, n = 7). Data are expressed as means ± SEM. **$p < 0.01$, as compared to the corresponding WT value.

other hand, β-$M_3$-Tg1 mice displayed significantly increased serum insulin levels (by ~2- to ~3-fold as compared to their WT littermates) during the first 15–30 min after glucose administration (Figures 4D and 4F). The measurement of plasma C-peptide levels led to similar results (IGTT; Figure S1B).

Administration of a fixed dose of insulin (0.75 U/kg i.p.) showed that overexpression of β cell $M_3$ receptors had no significant effect on insulin sensitivity (12-week-old males; Figure 4G), indicating that the increase in serum insulin levels displayed by the β-$M_3$-Tg1 mice was not due to peripheral insulin resistance.

### β-$M_3$-Tg1 mice are resistant against diet-induced hyperglycemia and glucose intolerance

As described in the previous paragraph, β-$M_3$-Tg1 mice showed a pronounced increase in glucose tolerance, probably due to enhanced insulin release following glucose administration. It is well known that the consumption of an energy-rich, high-fat diet triggers a number of metabolic changes including impaired glucose tolerance and hyperglycemia. To examine whether the overexpression of $M_3$ receptors in pancreatic β cells could prevent or reduce the severity of these metabolic deficits, 4-week-old β-$M_3$-Tg1 mice and their WT littermates were fed a high-fat diet (fat content: 35.5%, w/w) and then monitored for 9 weeks.

Consumption of the high-fat diet led to significant weight gain in both WT and β-$M_3$-Tg1 mice, as compared to WT and β-$M_3$-Tg1 mice maintained on regular mouse chow (Figure 5A). WT mice consuming the high-fat diet developed hyperglycemia (Figure 5B). In striking contrast, blood glucose levels of β-$M_3$-Tg1 mice remained in the normal range during the entire observation period (Figure 5B). Moreover, after 9 weeks on the high-fat diet, WT mice showed markedly increased fasting blood glucose levels and severely reduced glucose tolerance, as

assessed by an i.p. glucose tolerance test (Figure 5C). On the other hand, at the same point, β-$M_3$-Tg1 littermates exhibited normal fasting blood glucose levels and glucose tolerance (Figure 5C). Following the i.p. glucose load, β-$M_3$-Tg1 mice showed significantly higher serum insulin levels throughout the entire 2 hr observation period (Figure 5D), suggesting that the improved glucose tolerance displayed by the β-$M_3$-Tg1 mice maintained on the high fat diet is due to enhanced insulin release.

Administration of a fixed dose of insulin (0.75 U/kg i.p.) to mice maintained on the high-fat diet showed that both WT and β-$M_3$-Tg1 mice displayed a reduction in insulin sensitivity, as compared to WT and β-$M_3$-Tg1 mice consuming standard mouse chow (compare Figure 5E with Figure 4G). However, blood glucose levels initially declined more rapidly in β-$M_3$-Tg1 than in WT mice (18-week-old males; Figure 5E).

To exclude the possibility that the pronounced metabolic changes displayed by the β-$M_3$-Tg1 mutant mice were caused by a transgene "integration effect", we analyzed another independent transgenic mouse line, referred to as β-$M_3$-Tg2, that showed an at least 8-fold increase in the density of islet (β cell) $M_3$ receptors (number of islet [$^3$H]NMS binding sites: 12.5 ± 1.1 fmol/100 islets). Analysis of blood glucose levels and glucose tolerance tests showed that β-$M_3$-Tg2 mutant mice exhibited qualitatively similar phenotypical changes as β-$M_3$-Tg1 mice (data not shown). This observation strongly supports the concept that the pronounced metabolic changes displayed by the β-$M_3$-Tg1 mice were indeed caused by overexpression of $M_3$ receptors in pancreatic β cells.

Pancreata from adult β-$M_3$-Tg1 mice and WT littermates did not differ significantly in weight (WT, 226 ± 6 mg, n = 7; β-$M_3$-Tg1, 248 ± 9 mg; n = 9) and contained similar amounts of insulin (μg/pancreas: WT, 284 ± 17, n = 7; β-$M_3$-Tg1, 262 ± 13, n = 9).

## Discussion

The primary focus of this study was to examine the potential importance of β cell $M_3$ muscarinic receptors in maintaining normal glucose homeostasis and insulin release in vivo. We found that β-$M_3$-KO mice showed a significant impairment in glucose tolerance, independent of the route of glucose administration (intragastric or i.p.; Figures 2A and 2C). This observation clearly indicates that the activity of β cell $M_3$ muscarinic receptors is essential for maintaining normal glucose homeostasis. This finding also suggests that the glucose-mediated activation of pancreatic efferent parasympathetic nerves is not only a transient phenomenon associated with the vagus-mediated preabsorptive phase of feeding (Ahren, 2000; Gilon and Henquin, 2001), but represents a rather long-lasting effect that persists throughout the absorptive phase of feeding.

In agreement with the in vitro insulin release studies (Figure 1G), we observed that the impairment in glucose tolerance displayed by the β-$M_3$-KO mice was accompanied by significantly blunted increases in serum insulin levels following an intragastric or i.p. glucose load in vivo (Figures 2B and 2D). This observation strongly suggests that glucose-mediated stimulation of efferent parasympathetic nerves triggers the activation of β cell $M_3$ muscarinic receptors, leading to a long-lasting increase in pancreatic insulin release that is critical for maintaining normal glucose homeostasis.

Polymorphisms in the $M_3$ receptor gene that are associated with type 2 diabetes or impaired glucose tolerance in humans have not been reported so far. However, given the metabolic deficits displayed by the β-$M_3$-KO mice, such polymorphisms may exist.

It has been reported that the RIPII promoter is not only functional in pancreatic β cells but may also have some activity in cerebral cortex and hypothalamus (Gannon et al., 2000; Cui et al., 2004). However, by using a combined radioligand binding/immunoprecipitation strategy which can determine the number of functional receptors, we found that $M_3$ receptor expression levels were similar in the hypothalamus and cerebral cortex of β-$M_3$-KO mice and control littermates (Figure 1D). By crossing mice heterozygous for the floxed $M_3$ receptor allele (fl/+ mice) with fl/+ mice that expressed Cre recombinase under the control of the rat nestin promoter and enhancer (Tronche et al., 1999; Brüning et al., 2000), we recently generated brain/neuron-specific $M_3$ receptor KO mice (brain-$M_3$-KO mice). Preliminary studies showed that these mutant mice displayed normal glucose tolerance and glucose-induced insulin release (unpublished data). Taken together, these observations strongly suggest that the metabolic deficits displayed by the β-$M_3$-KO mice are not due to reduced central $M_3$ receptor levels.

Ohnuma et al. (1996) showed that intracerebroventricular (ICV) administration of a muscarinic receptor antagonist (N-methylatropine) reduced glucose tolerance and blunted glucose-dependent increases in plasma insulin levels in rats. This observation is consistent with the concept that central muscarinic receptors also play a role in regulating neuronal pathways critical for proper insulin release and glucose homeostasis. However, given our observations summarized in the previous paragraph, the metabolic deficits described by Ohnuma et al. (1996) are most likely due to the blockade of non-$M_3$ or multiple muscarinic receptor subtypes.

In striking contrast to β-$M_3$-KO mice, whole body $M_3$ receptor KO mice exhibit a reduction in body weight and body fat mass, associated with an increase in glucose tolerance and insulin sensitivity (Yamada et al., 2001; Duttaroy et al., 2004). As discussed by Yamada et al. (2001), it is likely that these metabolic changes are primarily due to the fact that whole body $M_3$ receptor KO mice are hypophagic and lean.

Interestingly, isolated islets prepared from β-$M_3$-KO mice showed a significant reduction in glucose-induced (16.7 mM) insulin secretion (Figure 1G). To explore the molecular basis underlying this phenomenon, we carried out a series of additional experiments. These studies indicated that the reduction in glucose-induced insulin secretion observed in the absence of $M_3$ receptors is not due to decreased islet insulin content, reduced GLUT2 or glucokinase expression levels, or changes of an unknown circulatory factor. Moreover, in vitro insulin release studies using GLP-1 as a secretagogue showed that the lack of β cell $M_3$ receptors did not result in a generalized impairment in islet function (Figure 1H). At present, we therefore do not have a clear explanation for the reduction in glucose-induced insulin secretion displayed by islets prepared from β-$M_3$-KO mice. One possibility is that the lack of β cell $M_3$ receptors causes secondary changes in the expression or functional state of as yet unknown downstream signaling components involved in glucose-stimulated insulin release.

Given the metabolic deficits displayed by the β-$M_3$-KO mice, we tested the hypothesis that enhanced signaling through β cell

$M_3$ muscarinic receptors might facilitate insulin release leading to improved glucose tolerance. Specifically, we analyzed a transgenic mouse line, referred to as β-$M_3$-Tg1, that selectively overexpressed a modified version of the $M_3$ muscarinic receptor in pancreatic β cells (Figure 3A).

Strikingly, β-$M_3$-Tg1 mice displayed an ~35% reduction in blood glucose levels in both the fed and fasting state (Figure 4A), but showed an ~3-fold increase in fed and fasting serum insulin levels (Figure 4B), as compared to their WT littermates. β-$M_3$-Tg1 mice showed similar sensitivity to exogenously administered insulin as their control littermates (Figure 4G). This observation clearly indicates that the increase in serum insulin levels displayed by the β-$M_3$-Tg1 mice is not a secondary effect caused by a state of insulin resistance.

Moreover, β-$M_3$-Tg1 mice showed dramatically reduced increases in blood glucose levels in glucose tolerance tests (Figures 4C and 4E), accompanied by significantly enhanced elevations in serum insulin levels (~2- to ~3-fold as compared to their WT littermates; Figures 4D and 4F). The striking metabolic changes displayed by the β-$M_3$-Tg1 mouse line were also observed with a second transgenic line, β-$M_3$-Tg2, that overexpressed β cell $M_3$ receptors at considerably lower levels than the β-$M_3$-Tg1 mice (data not shown). Taken together, these findings indicate that basal and glucose-dependent insulin release and glucose tolerance can be greatly enhanced by increasing the density of β cell $M_3$ receptors.

We also demonstrated that β-$M_3$-Tg1 mice were protected against diet-induced hyperglycemia and glucose intolerance (Figures 5B and 5C). Insulin measurements suggested that the improved glucose tolerance displayed by the β-$M_3$-Tg1 mice maintained on the high fat diet is most likely due to enhanced insulin release (Figure 5D).

All data presented in the Results section were obtained with male mice. To examine whether the major phenotypes displayed by the male $M_3$ receptor mutant mice were gender-independent, we repeated several key experiments with female β-$M_3$-KO and β-$M_3$-Tg1 mice (and control littermates). In these studies, the female mutant mice showed similar phenotypes as observed with their male counterparts (Tables S2 and S3; Figure S3), indicating that the important metabolic functions of β cell $M_3$ receptors are gender-independent.

Our data therefore support the concept that β cell $M_3$ muscarinic receptors play a critical role in regulating insulin release and glucose homeostasis in vivo. The $M_3$ receptor is not only expressed in pancreatic β cells but is present in many peripheral and central tissues (Wess, 1996). The activity of the $M_3$ receptor, like that of other G protein-coupled receptors (GPCRs), is regulated by several other cellular proteins including GPCR kinases (GRKs), RGS proteins, and various classes of receptor-associated proteins (Wess, 1996; Bockaert et al., 2004). Future studies may therefore lead to the development of therapeutic strategies able to selectively enhance signaling through β cell $M_3$ receptors by targeting receptor-regulatory proteins that are preferentially expressed in pancreatic β cells.

## Experimental procedures

### Generation of mutant mice selectively lacking $M_3$ muscarinic receptors in pancreatic β cells

A mouse $M_3$ muscarinic receptor genomic clone was isolated from a 129Sv/J mouse genomic library (Genome Systems; Yamada et al., 2001). To generate a floxed $M_3$ receptor allele, we created the targeting vector shown in Figure 1A, which is derived from the pLoxpneo vector (Yang et al., 1998). In this construct, a PGKneo selection cassette flanked by loxP sites was introduced ~0.45 kb downstream of the polyA signal of the mouse $M_3$ muscarinic receptor gene. A third loxP sequence, together with a diagnostic EcoRV site, was introduced into an intronic region, ~0.27 bp upstream of the translation start codon. The targeting vector was linearized by NotI and introduced into TC1(129SvEv) embryonic stem (ES) cells (Deng et al., 1996) by electroporation. Clones resistant to G418 and gancyclovir were isolated as described (Yang et al., 1998). The occurrence of homologous recombination was confirmed by Southern hybridization (Figures 1A and 1B). Properly targeted ES cell clones were microinjected into C57BL/6 blastocysts to generate male chimeric offspring, which in turn were mated with female C57BL/6 mice (Taconic) to generate F1 offspring. F1 animals heterozygous for the floxed $M_3$ receptor allele (fl/+) mice were then crossed to hemizygous transgenic mice expressing Cre recombinase under the control of the β cell-specific rat insulin promoter II (RIP-Cre mice; genetic background: C57BL/6; supplier: The Jackson Laboratories; Postic et al., 1999). The structure of the RIP II transgene has been described in detail previously (Postic et al., 1999). The resulting fl/+ Cre and fl/+ mice were then intermated to generate β-$M_3$-KO mice (Cre fl/fl mice) and the three corresponding littermate control groups, fl/fl, +/+, and +/+ Cre mice.

### Generation of mutant mice selectively overexpressing $M_3$ muscarinic receptors in pancreatic β cells

A transgene was constructed in which expression of the rat $M_3$ muscarinic receptor was under the control of a 650 bp fragment of the rat insulin promoter II (RIP II; Vasavada et al., 1996). The transgenic $M_3$ receptor contained two modifications in order to distinguish it more easily from the endogenous mouse $M_3$ muscarinic receptor, an N-terminal 9 amino acid hemagglutinin epitope tag and a deletion of the central portion of the third intracellular loop (Ala274–Lys469). Previous studies have shown that these modifications have no significant effect on the ligand binding and functional properties of the $M_3$ receptor (Schöneberg et al., 1995; Maggio et al., 1996). Briefly, the RIP-$M_3$ transgene was constructed by placing the modified $M_3$ receptor coding sequence downstream of the 650 bp RIP II fragment and upstream of untranslated human growth hormone sequences containing transcriptional termination, polyadenylation, and splicing signals (the vector into which the modified $M_3$ receptor coding sequence was inserted was kindly provided by Dr. Adolfo Garcia-Ocana, University of Pittsburgh). The resulting 4.1 kb transgene was isolated, purified, and microinjected into the pronuclei of ova prepared from C57BL/6 mice (Taconic). Transgenic mice were identified by Southern blot analysis of BlgII-digested mouse tail DNA. A 1.36 kb BamHI-BglII fragment corresponding to most of the 3′ untranslated human growth hormone sequence was used as a probe. Using these procedures, we identified three founder mice that stably transmitted the transgene to their progeny. From these founder animals, we generated two independent transgenic mouse lines, referred to as β-$M_3$-Tg1 and β-$M_3$-Tg2. Both lines were maintained on a pure C57BL/6 background.

### Genotyping studies

#### *β-$M_3$-KO mice and control strains*

Mouse genotypes were determined via Southern blotting and/or PCR analysis (Figures 1A and 1B; for details, see Supplemental Data). The presence of the Cre transgene was detected by using the PCR primers 5′-CCTGGAAAA TGCTTCTGTCCG (forward) and 5′-CAGGGTGTTATAAGCAATCCC (reverse; size of PCR product: 400 bp; PCR conditions, 94°C for 10 min followed by 32 cycles at 94°C for 45 s, 54°C for 30 s, and 68°C for 45 s).

#### *β-$M_3$-Tg mice*

To confirm the presence of the $M_3$ receptor transgene in β-$M_3$-Tg mice, the following PCR primers which anneal to the 3′-untranslated region of the transgene were used: 5′-CTACGGGCTGCTCTACTGCTTCAGG (forward), 5′-GGCACTGGAGTGGCAACTTCCAAGG (reverse) (size of PCR product, 171 bp; PCR conditions, 94°C for 10 min followed by 30 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 60 s).

### Mouse maintenance and diet

Mice were housed four to five per cage in a specific pathogen-free barrier facility, maintained on a 12 hr light/dark cycle. Unless indicated otherwise, all

experiments were carried out with male littermates that were 4–6 months old at the time of testing.

Mice were fed ad libitum with a standard mouse chow (4% (w/w) fat content; Zeigler, Gardners, PA). When stated, 4-week-old male mice were put on a high-fat diet (35.5% (w/w) fat content; # F3282, Bioserv, Frenchtown, NJ) for 9 weeks.

#### Islet isolation and in vitro insulin secretion studies

Pancreatic islets were isolated from WT and $M_3$ receptor mutant mice and prepared for in vitro insulin release studies as described by Saeki et al. (2002). After purification, islets from β-$M_3$-KO and control mice were incubated for 18–20 hr in RPMI 1640 cell culture medium (Invitrogen/Gibco) supplemented with 5.5 mM glucose, 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. We noted that islets from β-$M_3$-Tg1 mice released significant amounts of insulin into the medium during the 18–20 hr incubation step, consistent with previous findings that overexpression of G protein-coupled receptors can lead to some degree of ligand-independent signaling (Seifert and Wenzel-Seifert, 2002). For this reason, this incubation step was omitted when islets from β-$M_3$-Tg1 and WT control mice were used for insulin secretion studies. Prior to stimulation, islets were preincubated for 1 hr at 37°C in an oxygenated, modified Krebs solution (composition in mM: 3.3 glucose, 120 NaCl, 5 KCl, 1.1 $MgCl_2$, 2.6 $CaCl_2$, 25 $NaHCO_3$, and 0.5% bovine serum albumin; [pH 7.4]). Batches of 10 islets were then incubated in 12-well cell culture plates for 1 hr in a $CO_2$ incubator at 37°C in the same solution supplemented with different amounts of glucose and 0.5 μM of the muscarinic agonist, Oxo-M (Sigma), or 0.1 μM GLP-1 (7–36) amide (Bachem). All experiments were carried out in triplicate. The medium was collected for insulin measurements, and the remaining islet insulin was extracted by sonication in acid/ethanol (Davalli et al., 1995). The amount of insulin secreted during the incubation period was normalized to the total insulin content of each well (islets plus medium). Insulin concentrations were determined by ELISA (Crystal Chem Inc.).

#### In vivo physiological studies

Oral and intraperitoneal (i.p.) glucose tolerance tests (OGTT and IGTT, respectively) were carried out with mice that had been subjected to an overnight (10–12 hr) fast. In the OGTT, mice were administered an oral load of glucose (2 mg/g body weight) via oral gavage. In the IGTT, mice received the same dose of glucose via i.p. injection. In both tests, blood samples were collected via retroorbital sinus puncture before (0 min) and 15, 30, 60, and 120 min after glucose administration. Blood glucose levels were determined using an automated blood glucose reader (Glucometer Elite Sensor, Bayer). Serum insulin concentrations were determined via ELISA (Crystal Chem Inc.). Plasma glucagon concentrations were measured via radioimmunoassay (Linco kit).

For insulin tolerance (sensitivity) tests, human insulin (0.75 U/kg; Eli Lilly) was administered i.p. to mice that had been fasted overnight for 10–12 hr (β-$M_3$-KO and control mice) or for 6 hr (β-$M_3$-Tg and WT mice), respectively. Blood glucose measurements were carried out immediately before and 15, 30, and 60 min after insulin injection. Blood samples were taken from the tail vein.

#### Real-time quantitative RT-PCR studies

Total RNA was isolated from pancreatic islets of β-$M_3$-KO mice and control (fl/fl) littermates (~100–150 islets per mouse) using the RNAqueous-Micro kit (Ambion) and treated with DNase I for 15 min at room temperature. Reverse transcription was performed using SuperScript III RT Super Mix (Invitrogen), and gene expression levels were measured by real-time quantitative RT-PCR (7900HT SDS; Applied Biosystems). PCR reactions (25 μl total volume) included cDNA (~50 ng of initial RNA sample), 100–120 nM of each primer, and 12.5 μl of 2× SYBR Green Master Mix (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 expression of cyclophilin A served as an internal control. Four independent samples prepared from four different mice were used per genotype. PCR reactions were carried out in triplicate. The results were expressed as fold changes in expression of a particular RNA transcript relative to cyclophilin A expression between control and $M_3$ receptor mutant mice (for primer sequences, see Supplemental Data).

#### Carbachol-mediated inositol phosphate (IP) production in isolated pancreatic islets

Islets prepared from control and $M_3$ receptor mutant mice were incubated for 20 hr in 12-well plates (75 islets/well) in RPMI 1640 cell culture medium (Invitrogen/Gibco) supplemented with 5.5 mM glucose, 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 μCi/ml of *myo*-[$^3$H]inositol (specific activity: 20 Ci/mmol; American Radiolabeled Chemicals Inc.) at 37°C in a 5% $CO_2$ atmosphere. After this labeling period, islets were washed five times at room temperature with Krebs-Ringer bicarbonate buffer (120 mM NaCl, 5 mM KCl, 1.1 mM $MgCl_2$, 2.6 mM $CaCl_2$, and 25 mM $NaHCO_3$, 3.3 mM glucose, and 0.5% bovine serum albumin; [pH 7.4]). Islets were then preincubated in 1 ml of the same buffer containing 10 mM LiCl at room temperature for 20 min. After the addition of different concentrations of carbachol, islets were incubated for 1 hr at 37°C. Reactions were terminated and IP and glycerophosphoinositol levels were determined via anion chromatography, as described in detail previously (Berridge et al., 1983; Hamdan et al., 2002).

#### Radioligand binding studies with membranes prepared from pancreatic islets

Membranes were prepared from isolated mouse pancreatic islets as described (Ahren et al., 1999) and incubated with a saturating concentration (2 nM) of the non-subtype-selective muscarinic antagonist, [$^3$H]NMS (specific activity: 83 Ci/mmol; PerkinElmer), essentially as described (Dörje et al., 1991). Binding reactions were carried out for 1 hr at room temperature (22°C). Nonspecific binding was determined in the presence of 10 μM atropine.

#### Quantification of $M_3$ muscarinic receptor expression levels via an immunoprecipitation strategy

To quantitate the expression of $M_3$ muscarinic receptor protein in different mouse tissues, we used a combined radioligand binding/immunoprecipitation strategy (Yamada et al., 2001). Membranes were prepared from mouse islets and different brain tissues as described (Gautam et al., 2005) and then incubated for 1 hr at room temperature (22°C) with a saturating concentration (2 nM) of the non-subtype-selective muscarinic antagonist, [$^3$H]QNB (specific activity: 42 Ci/mmol; PerkinElmer). Subsequently, [$^3$H]QNB-labeled $M_3$ receptors were immunoprecipitated by the use of an $M_3$ receptor-specific rabbit polyclonal antiserum, as described by Yamada et al. (2001). The amount of [$^3$H]QNB-labeled $M_3$ receptors in the immunoprecipitates was determined by liquid scintillography.

#### Determination of pancreatic insulin content

Total pancreatic insulin content was measured by using an acid-ethanol method, as described in detail previously (Duttaroy et al., 2004). Insulin concentrations in pancreatic extracts were measured via ELISA (Crystal Chem Inc.).

#### RT-PCR analysis of $M_3$ receptor transgene expression

Total RNA was extracted from various peripheral and central tissues of β-$M_3$-Tg1 mice using the QIAzol Lysis Reagent (Qiagen). The RNA was then reversed transcribed with MuLV reverse transcriptase, and cDNA was generated using the GeneAmp RNA PCR kit (Applied Biosystems). The reverse transcription step was omitted in control samples to test for the presence of contaminating genomic DNA. The RT products were amplified via PCR using a primer pair specific for the $M_3$ receptor transgene ($M_3$HA-F, 5′-CCTA CGACGTCCCCGACTAC [this primer anneals to the hemagglutinin epitope tag sequence present at the N terminus of the transgenic construct]; $M_3$-R: 5′-TGATGTAGGTCGTGAACAGG) and the following cycling conditions: 94°C for 3 min followed by 30 cycles at 94°C for 25 s, 54°C for 40 s, and 72°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 10× *Taq* buffer containing 200 mM $(NH_4)_2SO_4$ (Fermentas), 2 mM $MgCl_2$, 1 mM of each dNTP, 200 nM of each PCR primer, and 1 unit of recombinant *Taq* DNA polymerase (Fermentas). As an internal control, a mouse GAPDH-specific primer pair (forward, 5′-CGTGGAGTCTACTGGTGTCTTCACC, reverse, 5′-GATGGCATGGACTGTGGTCATGAGC) was used. The sizes of the RT-PCR products were: $M_3$ receptor transgene, 405 bp; GAPDH, 258 bp.

### Statistics

Data are expressed as means ± SEM for the indicated number of observations. p values were calculated using one-way analysis of variance followed by appropriate posthoc tests.

### Supplemental data

Supplemental data include Supplemental Experimental Procedures, three figures, and three tables and can be found with this article online at http://www.cellmetabolism.org/cgi/content/full/3/6/449/DC1/.

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