

Melanin Concentrating Hormone Is a Novel Regulator of Islet Function and Growth

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Melanin concentrating hormone (MCH) is a hypothalamic neuropeptide known to play a critical role in energy balance. We have previously reported that overexpression of *MCH* is associated with mild obesity. In addition, mice have substantial hyperinsulinemia and islet hyperplasia that is out of proportion with their degree of obesity. In this study, we further explored the role of MCH in the endocrine pancreas. Both *MCH* and *MCHR1* are expressed in mouse and human islets and in clonal β -cell lines as assessed using quantitative real-time PCR and immunohistochemistry. Mice lacking MCH (MCH-KO) on either a C57Bl/6 or 129Sv genetic background showed a significant reduction in β -cell mass and complemented our earlier observation of increased β -cell mass in *MCH*-overexpressing mice. Furthermore, the compensatory islet hyperplasia secondary to a high-fat diet, which was evident in wild-type controls, was attenuated in MCH-KO. Interestingly, MCH enhanced insulin secretion in human and mouse islets and rodent β -cell lines in a dose-dependent manner. Real-time PCR analyses of islet RNA derived from MCH-KO revealed altered expression of islet-enriched genes such as glucagon, forkhead homeobox A2, hepatocyte nuclear factor (HNF)4 α , and HNF1 α . Together, these data provide novel evidence for an autocrine role for MCH in the regulation of β -cell mass dynamics and in islet secretory function and suggest that MCH is part of a hypothalamic-islet (pancreatic) axis. *Diabetes* 56:311–319, 2007

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ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal-related kinase; HNF, hepatocyte nuclear factor; MCH, melanin concentrating hormone; MCHR, MCH receptor; RIA, radioimmunoassay; TBS, Tris-buffered solution.

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Several neuropeptides that act in the central nervous system to regulate feeding behavior and energy homeostasis are also expressed in the enteric system as part of a potential hypothalamic-pancreatic axis (1). While some of these neuropeptides have an effect on exocrine pancreatic function, their effects on endocrine secretion are not fully understood. Thus, ghrelin, neuropeptide Y, galanin, orexins A and B, leptin, and the agouti gene product are neuropeptides that act in the hypothalamus to regulate feeding behavior and have also been reported to modulate islet function and/or growth (2–11). Melanin concentrating hormone (MCH) is another hypothalamic peptide that is known to regulate energy balance. A previous study reported that mice overexpressing *MCH* have islet hyperplasia, suggesting that MCH may also play a role in islet growth. However, the potential role of MCH and its receptors in the endocrine pancreas and a potential role of MCH in islet biology have not been explored.

MCH is expressed in the lateral hypothalamus and zona incerta and has been shown to be important for feeding and energy homeostasis in rodents (12,13). *MCH* expression in the hypothalamus is upregulated by fasting and suppressed by leptin injection (13–15). Intracerebroventricular injections of MCH acutely stimulate feeding behavior (16). Mice lacking the prohormone are lean and hypophagic, whereas mice overexpressing *MCH* are obese (17,18). In rodents, MCH acts via a high-affinity G-protein-coupled receptor, designated MCH receptor (MCHR)1. In humans and primates, a second receptor, MCHR2, which shares 38% homology with MCHR1 (13), is also expressed (19). Ablation of functional MCHR1 also leads to a lean phenotype in mice consistent with the phenotype of *MCH*-knockout (KO) mutants (20,21). While expression in the brain is limited to the lateral hypothalamus, *MCH* is also expressed in peripheral tissues in mammals including testis and enteric neural system (22), whereas MCHR1 has been detected in muscle, rodent clonal β -cells, adipocytes, and monocytes (23–26). MCH is known to affect cultured cell lines, and a direct effect of MCH on leptin secretion and the leptin promoter has been reported in 3T3-L1 cells; in addition, MCH may affect insulin secretion from β -cell lines (26). However, the physiological role of MCH in most peripheral tissues, including primary human islets, is largely unknown.

We have previously reported that mice overexpressing *MCH* exhibit islet hyperplasia (17,18), suggesting that MCH has the potential to directly or indirectly modulate

islet growth. In this study, we have used primary human and mouse islets and clonal β -cell lines to determine the presence of MCH and its receptors and to explore the direct effect of MCH on islet function. We also examined the significance of MCH in β -cell mass dynamics by evaluating the impact of genetic background and a high-fat diet in MCH-KO mice.

RESEARCH DESIGN AND METHODS

MCH-KO mice and their wild-type littermates were generated by heterozygous breeding as previously described (17). The KO mice were backcrossed to the C57BL/6 or 129Sv genetic backgrounds for at least 10 generations and maintained in the Joslin Animal Facility (27). All mice were housed on a 12-h light/dark cycle with ad libitum access to water and food. Experiments were performed in accordance with recommendations of the institutional animal care and use committee of the Joslin Diabetes Center. For studies on high-fat diet, 8-week-old MCH-KO mice and their wild-type littermates were fed a standard chow (6% g/g fat, 3.5 kcal/g, Lab Diet no. 5008) or a high-fat/hypercaloric diet (23.6% g/g fat, 4.73 kcal/g, Research Diets D12451; Research Diets, New Brunswick, NJ) for 16 weeks before harvesting the pancreas for morphometric analysis.

Islet isolation and insulin secretion experiments. Mouse islets were isolated as previously described (11) using the intraductal liberase technique and handpicked under a stereomicroscope (Stereozoom GZ7; Leica, Deerfield, IL). For ex vivo experiments, islets were washed with RPMI-1640 (containing 3.3 mmol/l glucose; 10% fetal bovine serum, and 1/100 concentration of L-glutamine and penicillin-streptomycin complex) and cultured for 48 h at 37°C before experiments.

For secretion experiments, mouse islets of similar sizes (120–150 μ m) were handpicked from a single harvest pool and cultured for 48 h. Secretion experiments were performed either in 12-well plates or in 1-ml eppendorf tubes (15–25 islets per well or tube) in RPMI-1640 (3.3 mmol/l glucose, unless otherwise indicated). To evaluate the effects of MCH on insulin secretion, islets were stimulated with different concentrations of MCH for 2 h (5.5 mmol/l glucose), media were collected and centrifuged, and supernatants were stored at -20°C for insulin enzyme-linked immunosorbent assay (ELISA) or C-peptide or glucagon radioimmunoassay (RIA). To measure insulin content, islets were extracted with acid ethanol overnight and the supernatants centrifuged to discard cell debris and stored at -20°C for insulin ELISA. For signaling studies, islets were cultured overnight in 7 mmol/l glucose. On the day of the experiment, healthy islets were cultured in 3.3 mmol/l glucose for 2 h. Subsequently, the islets were incubated in glucose (3.3 mmol/l) with increasing concentrations of MCH (0.01–1 mmol/l) for 5 min. Islets were lysed and subjected to Western blotting using anti-p-ERK (extracellular signal-related kinase) or anti-p-Akt antibodies (Cell Signaling) using standard methods (28,29).

Human islets were obtained from Islet Cell Resource or from the Mayo Clinic. Briefly, islets isolated from cadaver pancreata obtained with research intent were performed at the Harvard Center for Islet Transplantation Mayo Clinic Human Islet Isolation Facility based on isolation protocols developed at the University of Alberta (Edmonton, Alberta, Canada). Isolated islets were suspended in CMRL media, preserved in ice, and transported by courier to the Joslin Diabetes Center. Following culture for 48 h in CMRL media, the islets were processed in a manner similar to mouse islets (30). Briefly, 100–150 islet equivalents were handpicked and subjected to secretion studies in 12-well plates in media containing 5.5 mmol/l glucose. Following the stimulation period, media was collected, centrifuged, and processed as described for mouse islets.

Hormonal measurements. Mouse insulin was assayed by ELISA (Crystal Chem, Downers Grove, IL) using mouse insulin standard for rodent samples and human insulin by RIA for human samples (Joslin Diabetes and Endocrinology Research Center Specialized Assay Core). C-peptide and glucagon were measured by RIA (Linco).

Ca²⁺ flux measurements. Single islets adhered to glass coverslips were loaded with 2 μ mol/l fura-2AM for 45 min before experiments. The glass coverslip was then transferred to a holder and positioned on the stage of an inverted Nikon Diaplot microscope. Islets were maintained in buffer containing 20 mmol/l HEPES, 118 mmol/l NaCl, 5.4 mmol/l KCl, 1.2 mmol/l Mg²⁺, 2.4 mmol/l Ca²⁺, and 1.2 mmol/l KH₂PO₄ at 37°C containing 3 mmol/l glucose. Fura-2 complexed to Ca²⁺ and free dye were excited by 340 and 380 nm, respectively, using a Xe-arc lamp equipped with a filter wheel (Lambda 10-2; Sutter Instrument, Novato, CA), and emission at 510 nm was collected with an air-cooled charged-coupled device camera (Quantix; Photometrics, Tucson, AZ). Using the ratio of emission at 340 and 380 nm, together with a calibration of the system, intracellular calcium concentration ([Ca²⁺]_i) was determined as

previously described (31). All fluorescence data were processed using MetaMorph software (Universal Imaging, Downingtown, PA).

Gene expression analysis. RNA was extracted using the RNeasy kit supplemented with DNase treatment according to manufacturer's instructions (Qiagen, Valencia, CA). For detection of mouse *MCH* and *MCHR1* gene expression, 50 ng of RNA was subjected to real-time RT-PCR using TaqMan One Step RT-PCR reagents (Applied Biosystems, Foster City, CA) and the following primers: MCH forward primer 5'-ATTCAAAGAACACAGGCTC CAAAC-3', reverse primer 5'-CGGATCCTTTCAG AGCAAGTA-3', and probe 6FAM-AATCTT-GTAACTCACGGGCTGCCACTGAGT-TAMRA and MCHR forward primer 5'-GCCACCTCCTGCACAA-3', reverse primer 5'-CTTCAC CACGGCAAAAATGAC-3', and probe 5'-FAM-CTACATCAACATCATCATGC TTTCAGTGTGGTA-3' TAMRA. Reverse transcriptase minus reactions were run to exclude residual genomic DNA contamination. For detection of human MCH and MCHR1, cDNA was synthesized from 1 μ g RNA using a kit (Advantage RT for PCR) and oligo(dT) primers (Clontech, Palo Alto, CA). cDNA was diluted 1:10 and used in a real-time PCR including SYBR Green (Applied Biosystems) and the following primers: MCH forward 5'-cattcaggt ggggaaag-3' and reverse 5'-gaaattatgttggagcctgtgt-3' and MCHR1 forward 5'-ggctggatggacctggaa-3' and reverse 5'-gcaagatgacctggag-3'. At the end of the amplification, PCR products were run in an agarose gel, and amplified bands were sequenced in order to confirm the authenticity of the product (data not shown). For real-time PCR analysis of transcription factors, total RNA was reverse transcribed using SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) and cDNA samples amplified using SYBR Green PCR Master Mix (Applied Biosystems). (Primer sequences are available from the authors upon request.) All reactions were run in triplicate in a 7700 Sequence Detection System (Applied Biosystems), and results were normalized by glyceraldehyde-3-phosphate dehydrogenase expression. For insulin mRNA experiments, MIN6 cells were seeded in six-well plates and incubated for 36 h before overnight serum starvation with either 3.3 or 11 mmol/l glucose. The medium was then removed and cells washed twice with PBS. Cells were then incubated with Dulbecco's modified Eagle's medium, 3.3 mmol/l glucose, and various concentrations of MCH peptide for 2 h. Cells were harvested, and total RNA was extracted and reverse transcribed before quantitative real-time PCR.

Immunohistochemistry. Frozen sections (7- μ m thick) of mouse pancreatic tissue were stained for MCH or MCHR1. For MCH immunostaining, a rabbit polyclonal antibody was used (14). The MCHR1 antibody was also raised in rabbits (kindly provided by Dr. Pothoulakis, Beth Israel Deaconess Medical Center, Boston, MA). The sections were fixed in cold 80% acetone, air dried, and washed in Tris-buffered solution (TBS). They were preincubated (1 h at room temperature) in TBS with normal goat serum (5%), drained, and incubated (2 h at room temperature) with primary antibody (1:5,000). They were then washed in TBS and incubated with goat anti-rabbit fluorescein isothiocyanate conjugate (Jackson ImmunoResearch Labs, West Grove, PA) (1 h at room temperature, 1:100), washed, and coverslipped with Vectashield anti-fade medium (Vector Labs, Burlingame, CA). In adjacent sections, staining was performed in the absence of primary antibody. Cocktail staining for non- β -cells was performed as previously described in Bouin's fixed paraffin-embedded pancreas sections (30). Images were viewed under a fluorescence microscope (Olympus) using a Phase 3 Imaging System (Media Cybernetics, Silver Spring, MD), and images were captured in the Joslin Advanced Microscopy Core Facility. β -Cell and α -cell mass were estimated by morphometric analysis of pancreas sections, as described earlier (30,32).

Western blotting. MIN6 cells were seeded in six-well plates and incubated for 36 h before overnight serum starvation with either 3.3 or 11 mmol/l glucose. The medium was then removed and cells washed twice with PBS. Cells were then incubated with Dulbecco's modified Eagle's medium with either 3.3 or 11 mmol/l glucose and various concentrations of MCH peptide for 5 min. Cells were washed with ice-cold PBS and lysed on ice with 200 μ l ice-cold radioimmunoprecipitation assay lysis buffer. Protein concentrations were determined using BCA (Pierce). Cell lysates (30 μ g) were subjected to SDS-PAGE, followed by immunoblotting using specific antisera for detection of total and phospho-specific proteins with chemiluminescence (Roche or Pierce).

Statistical analysis. All data, unless otherwise indicated, are presented as means \pm SE. Data were analyzed using an unpaired Student's *t* test or two-way ANOVA with Bonferroni correction for multiple comparisons, as appropriate, using a commercially available statistical program (Statview; Abacus Concepts, Berkeley, CA). The null hypothesis was rejected at the 0.05 level.

RESULTS

MCH and MCHR1 are expressed in mouse and human islets. To examine the presence of MCH and its receptor in islets, we used real-time quantitative RT-PCR and im-

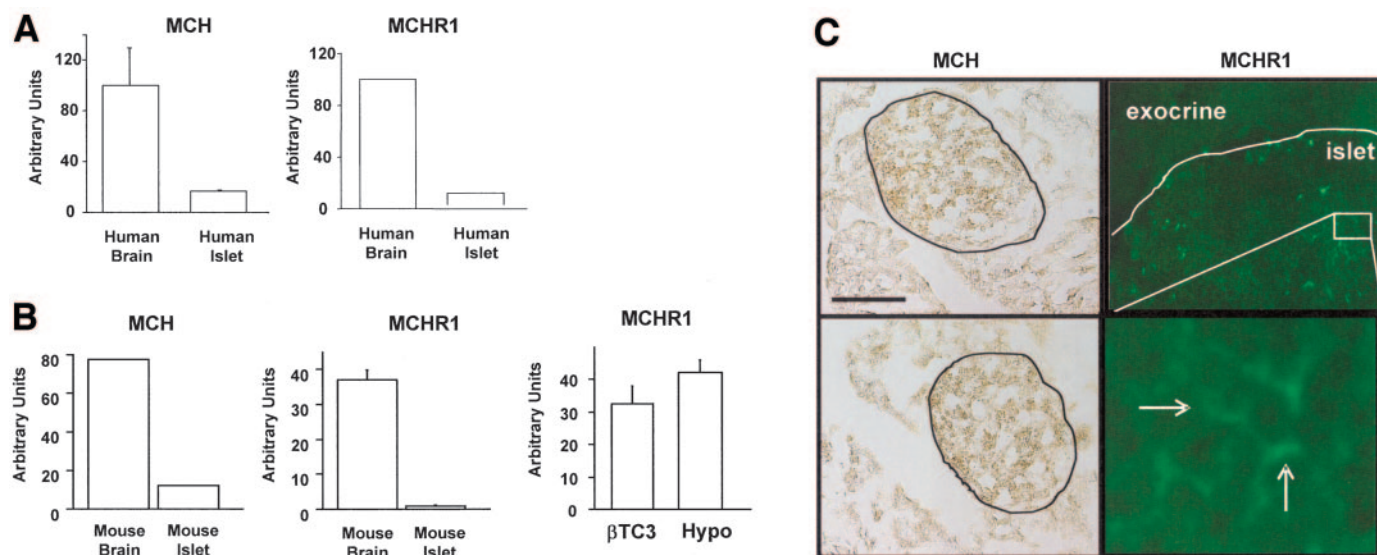


FIG. 1. Human and mouse islets express MCH and MCHR1. Quantitative real-time PCR for MCH and MCHR1 in human islets and human brain (A) and mouse islets, mouse brain, and mouse β -cell line β TC3 and mouse hypothalamus (B). C: Immunohistochemistry of mouse pancreas sections stained for MCH using DAB substrate (two independent islets are shown in left upper and lower panels) and MCHR1 (right upper panel) as described in RESEARCH DESIGN AND METHODS. A magnified image of immunostaining for MCHR1 is shown in the right lower panel. Arrows point to areas of immunostaining.

munohistochemical techniques. Islets were obtained from control C57Bl/6 mice or from cadaveric human donors (Harvard Center for Islet Transplantation, Boston, MA, and Mayo Clinic, Rochester, MN) and cultured for at least 48 h, followed by RNA extraction and preparation of cDNA (33). Mouse hypothalamus and human whole brain were used as positive controls for real-time PCR studies. We detected MCHR1 expression in both mouse and human islets, albeit at a 5- to 10-fold lower concentration compared with expression levels seen in the central nervous system (Fig. 1A and B). The receptor was also identified in a clonal β -cell line, β TC3, consistent with earlier reports (26), suggesting that one of the sources of expression of the receptor in islets is the insulin-secreting β -cell (Fig. 1A and B). We were unable to detect expression of MCH or MCHR1 in mouse pancreatic acinar tissues using real-time PCR (data not shown).

MCH expression in islets was assessed by quantitative PCR on samples of RNA extracted from mouse and human islets. Interestingly, we detected MCH in both mouse and human islets and also localized the neuropeptide in islets by immunohistochemical staining of mouse pancreas sections (Fig. 1). Although it is possible that the peptide is expressed in peri-insular complexes in islets that are known to express other neuropeptides (34,35), the expression of MCHR1 in β -cell lines suggests that the receptor is present on β -cells. Together, these data provide evidence for the presence of MCH in the islets and a potential for modulation of β -cell function by acting via MCHR1.

Effects on β -cell mass. We have previously reported that mice overexpressing MCH manifest islet hyperplasia (18). To evaluate whether the converse effect, i.e., loss of MCH, leads to reduced β -cell mass, we examined MCH-KO mice under two conditions known to independently influence islet growth: genetic background (36,37) and high-fat feeding (38).

Effect of genetic background on alterations in β -cell mass in MCH-KO mice. Genetic background plays a significant role in modulating β -cell replication, mass, and

function (28,29,36). To examine whether mice lacking MCH and bred on different genetic backgrounds display alterations in β -cell mass, we examined wild-type and KO mice backcrossed to C57Bl/6 (B6) and 129Sv (129) strains—two common inbred strains used in the study of diet-induced obesity (28,29). Pancreas, obtained from male KOs and corresponding control littermates on the two backgrounds, were sectioned and stained with a cocktail of antibodies to glucagon, somatostatin, and pancreatic polypeptide; β -cell mass was estimated by morphometric analysis as previously described (30). Mutant mice on both background strains showed small islets and reduced β -cell mass compared with littermate controls (Fig. 2A and C). These data provide evidence that the effects of MCH on modulating β -cell mass are independent of genetic background and imply an important role for the neuropeptide in the regulation of β -cell mass. In pilot studies, females also showed a similar trend, but only data from males is presented.

Effects of a high-fat diet on β -cell mass in MCH-KO mice and on MCH and MCHR1 in wild-type mice.

Most mouse models of obesity exhibit an islet compensatory response to maintain euglycemia in the face of insulin resistance (rev. in 37,38). To begin to dissect the dynamic effects of MCH on modulating β -cell mass, we fed MCH-KO mice and their wild-type littermates with a high-fat diet/hypercaloric diet for 16 weeks. While control mice showed a significant 3-fold increase in β -cell mass, MCH-KO mice failed to manifest a significant islet expansion (~ 1.2 -fold) (Fig. 2B and D). These data suggest that MCH may be a necessary link in the islet compensatory response to insulin resistance. Interestingly, expression of MCH was significantly downregulated in islets from mice on a high-fat diet compared with control mice (Fig. 2E). In contrast, we detected a trend toward an increase in expression of MCHR1 in the high-fat diet group, which did not reach statistical significance (Fig. 2F). These data indicate that high-fat feeding impacts expression of MCH and its receptor in a reciprocal manner in islets and suggest a potential

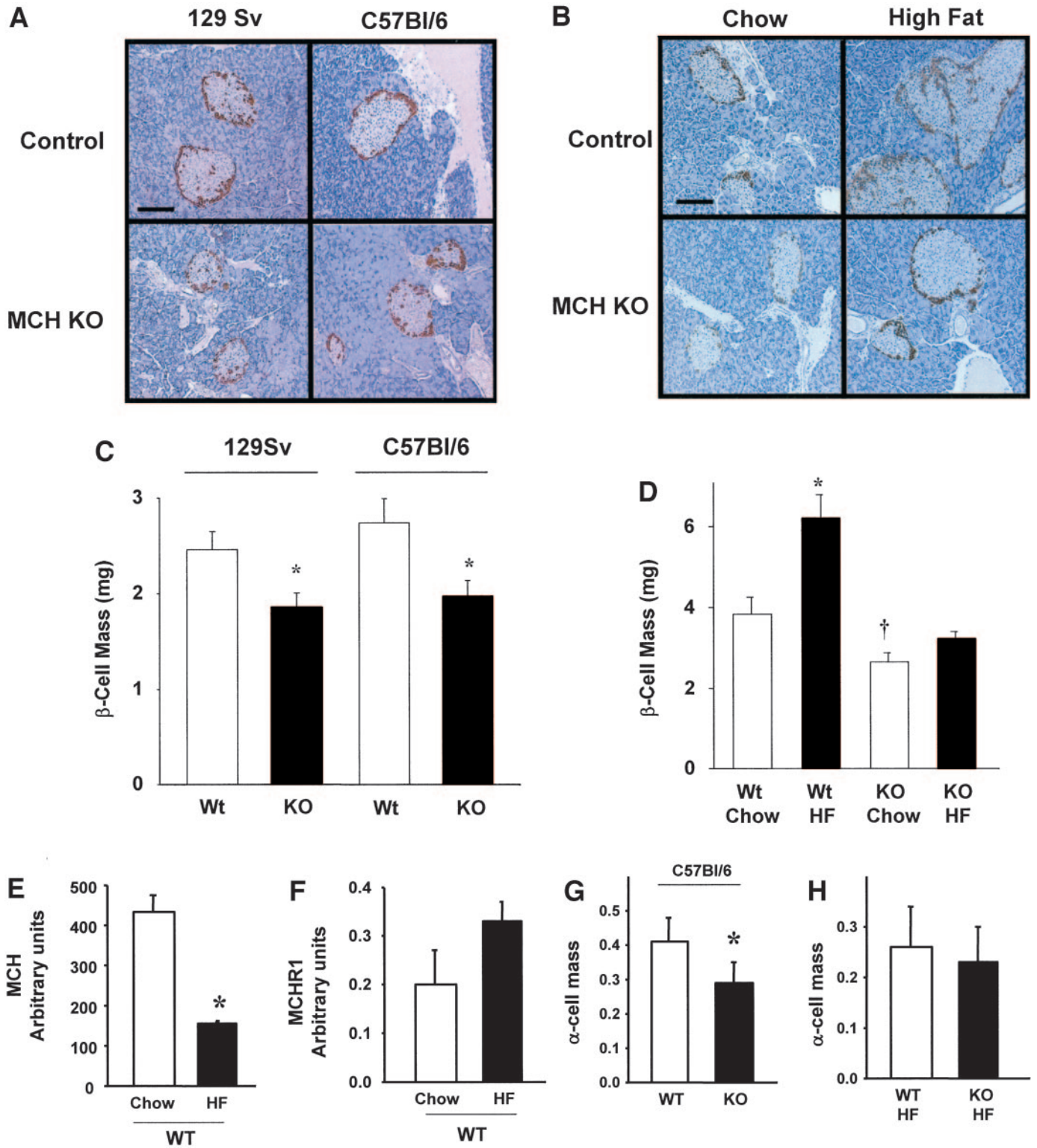


FIG. 2. Reduced β -cell mass in MCH-KO mice on normal chow and high-fat diets. Representative pancreas sections (**A**) and quantitation of β -cell mass (**B**) from control and MCH-KO mice backcrossed on the 129Sv or C57Bl/6 genetic backgrounds. $^*P < 0.05$, wild type vs. KO, $n = 4$. Sections are immunostained with a cocktail of antibodies against non- β -cell hormones (dark brown) and counterstained with hematoxylin (blue) as described in RESEARCH DESIGN AND METHODS. Representative pancreas sections (**C**) and quantitation of β -cell mass (**D**) of control and MCH-KO mice fed normal chow (left panel) or a high-fat (right panel) diet, immunostained with a cocktail of antibodies against non- β -cell hormones (dark brown) and counterstained with hematoxylin (blue). $^*P < 0.05$ wild-type chow vs. wild-type high-fat fed, $n = 4$; $^\dagger P < 0.05$, wild-type chow vs. KO chow fed, $n = 4$. Alterations in expression of MCH (**E**) or MCHR1 (**F**) in wild-type mice on a chow or high-fat diet determined using real-time PCR. For **E**, $P < 0.01$, $n = 4$; for **F**, $P = 0.07$, $n = 4$. Alterations in α -cell mass in wild-type or MCH-KO mice on the B6 background (**G**) or on a high-fat diet (**H**). For **G**, $P < 0.05$, $n = 4$; for **H**, $P = \text{NS}$, $n = 3$. HF, high fat; Wt, wild type.

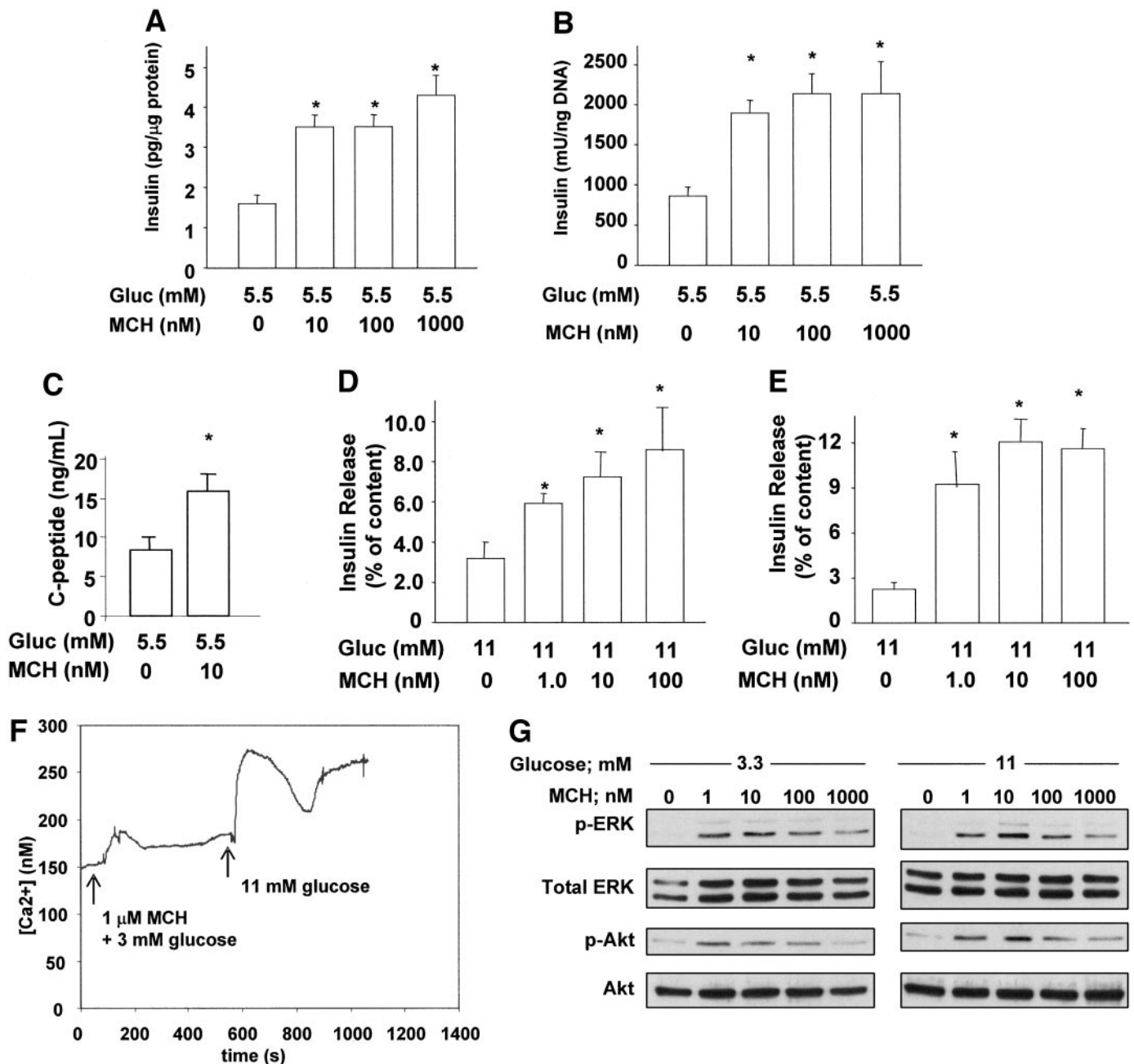


FIG. 3. MCH stimulates insulin secretion and alters Ca^{2+} flux, p-ERK, and p-Akt protein levels. MCH modulates glucose-stimulated insulin secretion in mouse islets (A), human islets (B), glucose-stimulated C-peptide release in human islets (C), and glucose-stimulated insulin secretion in the clonal cell lines β TC3 (D) and MIN6 (E). F: MCH stimulates intracellular Ca^{2+} flux in islets isolated from C57Bl/6 mice after an overnight culture. MCH (1 μ mol/l) was added to islets cultured in 3 mmol/l glucose (arrow on left). The effects of the addition of a higher concentration of glucose (11 mmol/l) are shown by the arrow on the right. G: MCH modulates phosphorylation of ERK and Akt in isolated islets from C57Bl/6 mice. Following an overnight culture, islets were treated with 3.3 mmol/l glucose and increasing concentrations of MCH (0.001–1 μ mol/l). $P < 0.05$ vs. 0, $n = 3$ –5. Glu, glucose.

role for MCH in nutrient-induced regulation of islet growth/function.

Effects of genetic background and high-fat feeding on α -cell mass in MCH-KO mice. To evaluate whether mice lacking MCH are influenced by genetic background or nutritional status, we assessed α -cell mass. A significant decrease in α -cell mass was observed in the KO group on the B6 background (Fig. 2G) but not in wild-type mice on a high-fat diet (Fig. 2H).

MCH stimulates insulin secretion in mouse and human islets and clonal β -cell lines and does not alter insulin mRNA. To directly evaluate whether exogenous MCH has an effect on islet secretory function, we incu-

bated overnight-cultured mouse islets with increasing concentrations of rat MCH in the presence of 5.5 mmol/l glucose. A dose-dependent approximate twofold insulin stimulatory effect was evident at 5.5 mmol/l glucose (Fig. 3A), which did not further increase in the presence of higher glucose concentrations (11 or 16 mmol/l; data not shown). Similarly, we detected a dose-dependent increase in insulin release from human islets in response to MCH in the presence of 5.5 mmol/l glucose, which reached a maximal effect at 10 nmol/l MCH in five independent experiments (Fig. 3B). The increase in insulin was paralleled by a significant rise in C-peptide levels in the culture media (Fig. 3C), providing strong evidence for a physio-

logical role for the neuropeptide in the regulation of insulin release.

To localize the stimulatory effects to β -cells, we performed secretory studies with mouse-derived β -cell lines, β TC3 and MIN6 (39,40). Exogenous rat MCH stimulated insulin release in a dose-dependent manner in both cell lines and showed a greater effect, on a molar basis, in MIN6 cells (Fig. 3D and E).

We have previously reported that mice overexpressing *MCH* manifest hyperinsulinemia (18). The ability of MCH to stimulate insulin release indicates that one source for the high levels of circulating insulin is a direct stimulatory effect of MCH on β -cells. To further explore this possibility, we examined the effects of MCH on insulin release in islets isolated from *MCH*-overexpressing mice. Indeed, islets from *MCH*-overexpressing mice showed greater secretory responses to stimulation with MCH (at a glucose concentration of 5.5 mmol/l) compared with wild-type islets (basal: 2.88 ± 0.82 ; 100 nmol/l MCH: 8.24 ± 1.1 pg/ μ g protein; $P < 0.01$; $n = 3$). These data confirm that high circulating levels of insulin in *MCH*-overexpressing mice is, in part, due to MCH-stimulated insulin release from β -cells.

To explore the effects of MCH on other hormones secreted by islets, we examined glucagon secretion at a glucose concentration of 5.5 mmol/l. We did not detect significant differences between groups, although there was a trend toward an increase in islets treated with MCH (control: basal 36 ± 14 vs. stimulated 37 ± 11 ; MCH [1 mmol/l] treated: basal 36 ± 10 vs. stimulated 43 ± 12 pg/ml; $P = \text{NS}$; $n = 4$).

To examine whether the effects of MCH impact insulin expression at the level of transcription, we measured the effects of increasing concentrations of MCH on insulin mRNA at 3.3 mmol/l glucose. No significant differences were detected between groups after normalizing the data for TATA-binding protein (data not shown).

Effects of MCH on Ca^{2+} flux and downstream signaling proteins in isolated islets. An increase in intracellular Ca^{2+} is a consistent molecular event that precedes insulin secretion in β -cells (41). To begin to identify potential mediators of MCH-stimulated insulin release, we examined alterations in Ca^{2+} flux in response to MCH stimulation (1 mmol/l) in isolated islets from wild-type mice. At a 3 mmol/l glucose concentration, we detected a reproducible increase in Ca^{2+} flux after MCH treatment (Fig. 3F). These data indicate that one pathway utilized by MCH to modulate insulin secretion is by altering Ca^{2+} flux in β -cells.

Although the precise pathways that mediate the actions of MCH after binding to the MCHR1 are not fully understood, some studies indicate a role for ERK (13). To explore whether MCH utilizes a similar pathway in islet β -cells, we examined the effects of exogenous MCH on activation of ERK in the presence of 3.3 mmol/l glucose, similar to the conditions used for Ca^{2+} flux experiments. At both glucose concentrations used (3.3 and 11 mmol/l), MCH enhanced the effects of ERK phosphorylation that peaked between 10 and 100 nmol/l of MCH, suggesting an interaction between glucose and MCH signaling (Fig. 3G). Furthermore, we observed a similar effect on activation of phospho-Akt, indicating a potential cross talk between proteins in MCH signaling with those in insulin/IGF-I signaling pathways (Fig. 3G). These data suggest that MCH activates similar pathways in islets as those in the brain.

Alterations in islet gene expression patterns in MCH-KO mice. Alterations in insulin secretion and β -cell growth are usually associated with changes in gene expression of key proteins involved in synthesis and/or secretion of insulin. To examine whether absence of MCH influences gene expression patterns in the mutant islets, we evaluated insulin signaling proteins, transcription factors, and glucose-sensing proteins in islet RNA isolated from MCH-KO and control mice. Absence of MCH in the KOs was confirmed by real-time PCR (Fig. 4). Surprisingly, we detected significantly enhanced expression of the hepatocyte nuclear factor (HNF) family of transcription factors, including HNF4 α , HNF1 α , transcription factor 2 (HNF1 β), and the forkhead homeobox A2, whereas pancreatic duodenal homeobox-1 was not altered. While insulin expression was not altered, glucagon mRNA was significantly downregulated, consistent with reduced α -cell mass, suggesting a potential role for MCH in modulating islet α -cell function. These data indicate a previously unidentified role for MCH in modulating islet gene expression and function.

DISCUSSION

In this study, we demonstrate, for the first time to our knowledge, the presence of the neuropeptide MCH and its receptor MCHR1 in primary human and mouse islets, suggesting a potential role for this appetite-regulating neuropeptide in modulating islet mass and function. The ability of exogenous MCH to stimulate insulin secretion, in both human and mouse islet tissue, provides additional evidence that MCH may regulate islet secretory function.

The mechanisms that underlie the ability of β -cells to compensate for insulin resistance are not fully understood (37). Several growth factors, hormones, and nutrients including insulin, IGF-I, and glucose (37,42,43) have been proposed as potential candidates that promote the compensatory islet growth response. We have previously reported that mice overexpressing *MCH* also exhibit islet hyperplasia, which is out of proportion to the very mild obese phenotype. In contrast, mice lacking MCH have substantially reduced insulin levels and normal glucose tolerance (44). This suggests that MCH contributes to modulating islet/ β -cell growth via a pathway that is independent of insulin resistance. Alternatively, MCH may have a permissive effect and activate signaling pathways that cross talk with insulin-dependent pathways to promote islet hyperplasia in the state of insulin resistance. Since high-fat feeding in mammals is consistently associated with islet hyperplasia due to insulin resistance (38), we explored alterations in β -cell mass dynamics in MCH-KO by feeding the mutants a high-fat diet. Surprisingly, the compensatory islet hyperplasia that was evident in wild-type controls was virtually absent in the KOs, indicating that in the absence of MCH, β -cell mass cannot expand appropriately. This effect was seen on both B6 or 129Sv genetic strains, indicating that the impact of MCH on β -cell growth and/or apoptosis is likely independent of background gene modifiers. Furthermore, somewhat surprisingly, we observed a reciprocal effect on expression of *MCH* and *MCHR1* in wild-type mice fed a high-fat diet, with a decrease in peptide levels in the islets. Whether circulating levels of MCH can modulate local islet production of MCH and upregulate MCHR1 expression and/or whether other circulating factors impact expression of the peptide and its receptor in islets requires further study.

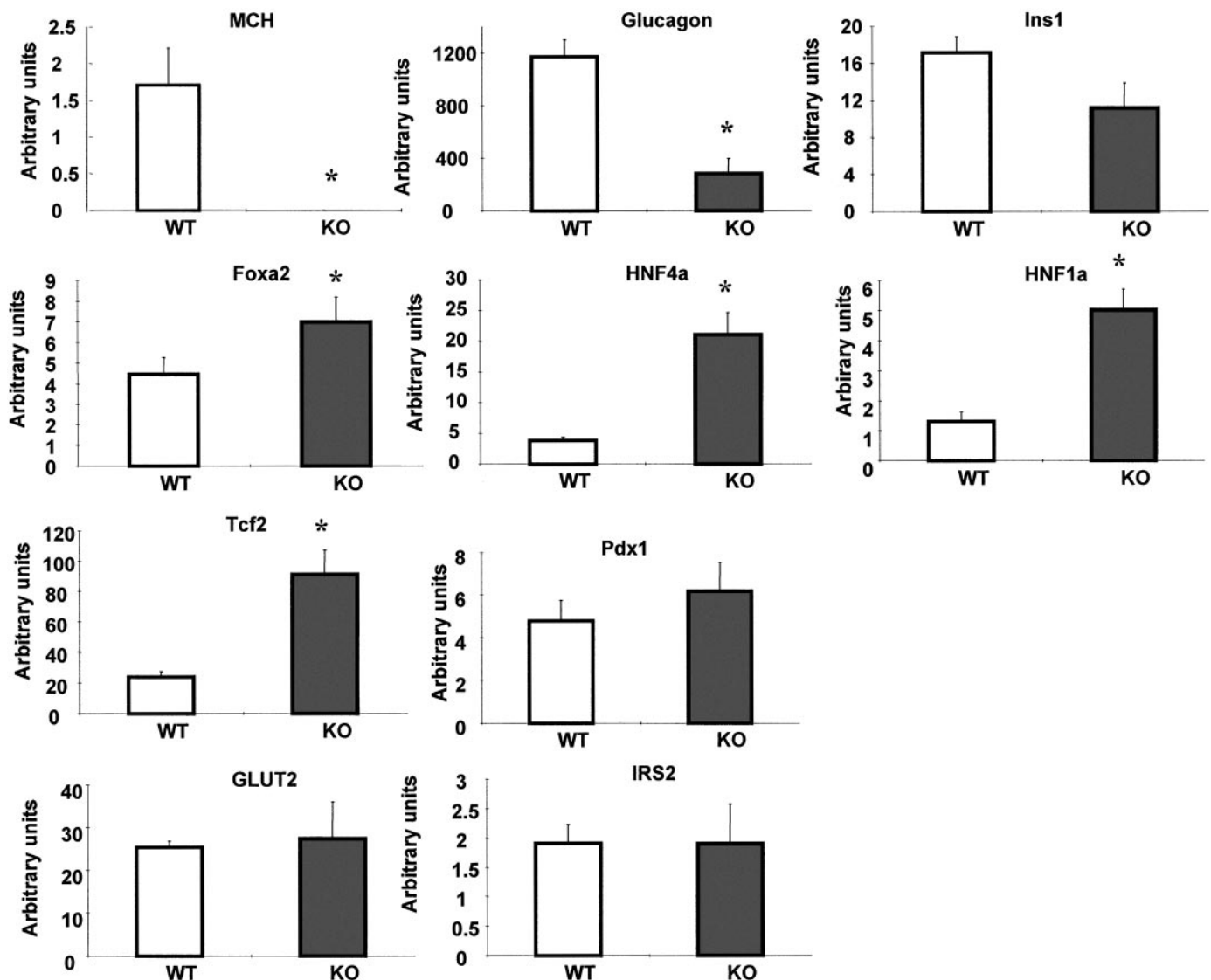


FIG. 4. Loss of MCH leads to alterations in islet gene expression. Islets isolated from MCH-KOs manifest reduced expression of glucagon and enhanced expression of the HNF family of transcription factors. Gene expression analyses was performed as described in RESEARCH DESIGN AND METHODS. * $P < 0.05$ KO vs. wild type (WT), $n = 3-5$. Foxa2, forkhead homeobox A2; IRS2, insulin receptor substrate; PDX-1, pancreatic duodenal homeobox-1.

Together, these data support the possibility that locally produced MCH has a primary effect on the maintenance of β -cell mass.

The precise mechanisms and pathways that mediate MCH action are not fully understood. Recent studies in HEK293 cells and 3T3-L1 adipocytes cells expressing *MCHR1* provide insights into these pathways (45,46). In HEK293 cells overexpressing *MCHR1*, MCH inhibits cAMP levels stimulated by forskolin or by a β -adrenergic receptor agonist and also increases intracellular Ca^{2+} levels (45). These reports are consistent with the association of *MCHR1* with multiple G proteins (13,45,47). Interestingly, MCH and forskolin are able to synergistically activate ERK via pertussis-independent activation pathways (13,45). In 3T3-L1 cells, *MCHR1* has been shown to modulate leptin expression through activation of ERK1/2 and p70s6 kinase, which are implicated in pathways that regulate protein synthesis and cell size control (48,49). Indeed, neuropeptide Y, another appetite-regulating neuropeptide, has been shown to enhance β -cell replication by acting via ERK (50). While some of these downstream signals have been

demonstrated to occur in the brain, suggesting a physiological basis for appetite-regulating effects of MCH (45), our studies suggest that MCH also engages similar downstream pathways to modulate β -cell function. Furthermore, in the islets of MCH-KO mice, the alterations in the HNF family of transcription factors, which are involved in islet cell development and growth, indicate a potential novel link between MCH signaling pathways and islet cell growth.

In addition to having direct effects on islet signaling pathways and insulin secretion, MCH also seems to have effects on insulin sensitivity. Mice overexpressing *MCH* were insulin resistant, and the degree of resistance was significantly out of proportion to the degree of obesity (18). In contrast, mice lacking MCH have normal or improved glucose tolerance, despite having less insulin release in response to a glucose load, an effect that is seen even in old mice (44). Furthermore, mice lacking both MCH and leptin had substantial improvement in glucose tolerance and lower insulin levels, albeit these double-null animals were leaner than *ob/ob* animals (51). However,

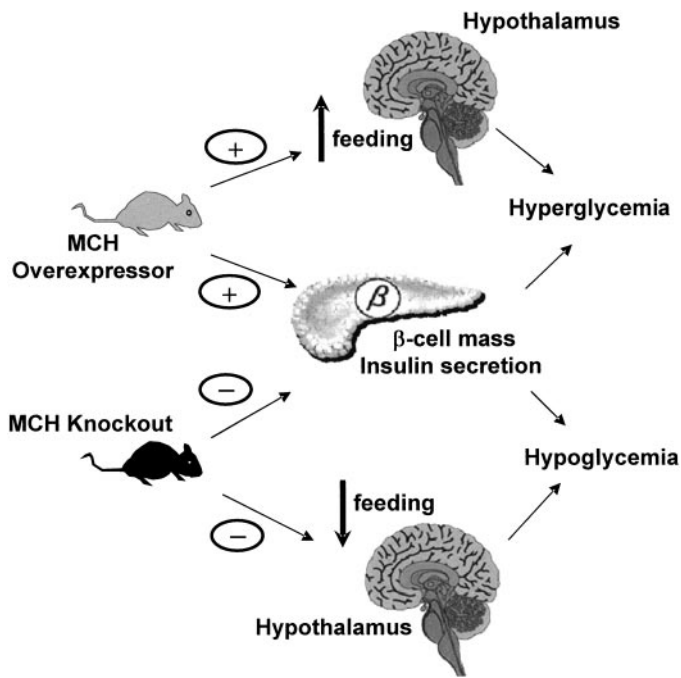


FIG. 5. Schematic representation of the impact of loss or gain of function of MCH in islets on hypothalamic and islet function.

mice lacking leptin and the MCH receptor had a somewhat different phenotype; these animals had substantial improvements in insulin sensitivity, even though these mice were not leaner than *ob/ob* animals (52). This suggests that there may be a relationship between effects of MCH on insulin sensitivity and direct effects on islets, which needs exploration in future studies.

It has been recognized for many years that peptides that control appetite also act at the level of the exocrine pancreas to modulate secretory responses (1). It is possible that a similar feedback loop exists between the hypothalamus and the endocrine pancreas mediated by neuropeptides that can alter feeding behavior on one hand but also regulate the secretion of pancreatic endocrine hormones for the overall maintenance of glucose homeostasis on the other. In this context, neuropeptide Y, galanin, and ghrelin all stimulate appetite but inhibit insulin release, whereas leptin inhibits both appetite control and insulin secretion. In contrast, MCH and insulin (and to a certain extent, orexins A and B) all exert positive effects both at the level of the hypothalamus and in the islet. The role of MCH, exemplified by the phenotypes of *MCH*-overexpressing mice (18) and *MCH*-KO mice (this study), provides an example of a neuropeptide that has broad effects on metabolism by affecting appetite and pancreatic endocrine function (Fig. 5). The effects of neuropeptide members of the hypothalamus-gut axis on modulating β -cell growth with potential for long-term trophic control are not fully explored and are worthy of further investigation, with a view to developing therapeutic approaches to treat type 1 and type 2 diabetes by regenerating β -cells.

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