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Glucagon Regulates Hepatic Kisspeptin1 to Impair Insulin Secretion

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Summary

Early in the pathogenesis of Type 2 diabetes mellitus (T2DM), dysregulated glucagon secretion from pancreatic α-cells occurs prior to impaired glucose stimulated insulin secretion (GSIS) from β-cells. However, whether hyperglucagonemia is causally linked to β-cell dysfunction remains unclear. Here we show that glucagon stimulates via cAMP-PKA-CREB signaling hepatic production of the neuropeptide kisspeptin1, which acts on β-cells to suppress GSIS. Synthetic kisspeptin suppresses GSIS *in vivo* in mice and from isolated islets in a kisspeptin1 receptor-dependent manner. Kisspeptin1 is increased in livers and in serum from humans with T2DM and from mouse models of diabetes mellitus. Importantly, liver *Kiss1* knockdown in hyperglucagonemic, glucose intolerant high fat diet fed and $Lepr^{db/db}$ mice augments GSIS and improves glucose tolerance. These observations indicate a hormonal circuit between the liver and the endocrine pancreas in glycemia regulation and suggest in T2DM a sequential link between hyperglucagonemia via hepatic kisspeptin1 to impaired insulin secretion.

Introduction

Glucagon and insulin are secreted respectively, by pancreatic α- and β-cells to precisely control blood glucose homeostasis. An early hallmark of type 2 diabetes mellitus (T2DM) is dysregulated glucagon secretion by pancreatic α-cells. Non-diabetic humans exhibit postprandial suppression of blood glucagon, while individuals with T2DM lack this suppression and may even exhibit increased glucagon levels. In addition, studies in subsets of patients with T2DM suggest that elevated glucagon secretion occurs antecedent to β-cell dysfunction (D'Alessio, 2011) and references therein).

Upon binding to its receptor Gcgr, glucagon activates cellular adenosine-3'-5'-cyclic monophosphate (cAMP) - protein kinase A (PKA) signaling to stimulate hepatic glucose production (HGP) and cause hyperglycemia (Chen et al., 2005). While hyperglycemia stimulates insulin secretion from β-cells, transgenic upregulation of protein kinase A (PKA) activity in hepatocytes in mice results as expected in increased HGP and hyperglycemia but paradoxically in impaired GSIS (Niswender et al., 2005). Consistent with the idea that glucagon may be causally linked to β-cell dysfunction, are findings made during exogenous glucose infusion in rats, where insulin secretion only fails after blood glucagon levels rise, and recovers upon glucagon inactivation by neutralizing antiserum (Jamison et al., 2011).

Based on these considerations for hyperglucagonemia and β-cell dysfunction in T2DM, we reasoned that independent of HGP and hyperglycemia, glucagon signaling in the liver initiates a process, which impacts on GSIS. We tested this hypothesis by comparing a mouse model of liver-specific PKA disinhibition (L-ΔPrkar1a mice, see below) with a model of hyperglycemia resulting from intravenous glucose infusion (D-glucose mice) combined with array-based gene expression analysis for secreted hepatic peptides, and identified *Kiss1*, which encodes the neuropeptide kisspeptin1 to be upregulated in livers of L-ΔPrkar1a but not in D-glucose mice and also to be directly stimulated by glucagon action via Gcgr on hepatocytes.

Kisspeptin1, has been described to be synthesized in the central nervous system and to regulate hypothalamic gonadotropin releasing hormone (GnRH) neurons, and is processed to multiple biologically active N-terminally truncated fragments kisspeptin 54 (K54), K14, K13, K10, of which the latter exerts full biological effects (Seminara and Kaiser, 2005). In experimental conditions individual kisspeptin isoforms are reported to suppress GSIS at nanomolar concentrations but stimulate GSIS at micromolar concentrations (Hauge-Evans et al., 2006; Silvestre et al., 2008). However, a (patho-) physiologic context for circulating levels of kisspeptin1, its effects on β-cell function specifically via kisspeptin1 receptor (Kiss1R), and its interplay with gluco-regulatory hormones are not understood.

Immunometric assays for kisspeptin have provided conflicting information on kisspeptin levels in rodents and humans (Akinci et al., 2012; Cetkovic et al., 2012; Horikoshi et al., 2003; Logie et al., 2012), and mass-spectroscopy-based assays do not provide exact information on circulating concentrations and functional bioactivity of the complement of kisspeptin isoforms in biological samples (Liu et al., 2013). Therefore, we have used - in addition to immunoassays - a bioassay of kisspeptin1 action on GSIS from mouse islets, which either express Kiss1R or selectively lack Kiss1R to reliably measure functional kisspeptin concentrations.

Using this assay, we find that synthetic kisspeptin inhibits GSIS from cultured islets in a dose- and Kiss1R-dependent manner at nanomolar concentrations. Furthermore, mice rendered glucose intolerant by high fat-content diet (HFD) or leptin receptor defective diabetic (*Lepr*$^{db/db}$) mice are hyperglucagonemic, exhibit increased liver kisspeptin1 and harbor in their circulation functional kisspeptin bioactivity equivalent to nanomolar concentrations of synthetic kisspeptin. In these mice, selective liver kisseptin1 knockdown derepresses GSIS and improves glucose tolerance (GT). Importantly, humans with T2DM also exhibit increased liver and plasma kisspeptin1 levels. Furthermore, mice selectively lacking pancreas Kiss1R, when fed a HFD, as compared to control counterparts, show improved GT owing to increased GSIS.

These observations identify the liver as a site of regulated kisspeptin1 synthesis, define a liver to islet endocrine circuit in glucoregulation, and suggest a pathogenic mechanism in T2DM, causally linking hyperglucagonemia via hepatic kisspeptin1 to insufficient insulin secretion. In addition, these findings extend a potential for kisspeptin1 antagonism as a therapeutic means to improve β-cell function in diabetes mellitus.

Results

Disinhibited PKA Activity in Liver Causes Impaired GSIS Independent of Hyperglycemia

To mimic upregulated glucagon-cAMP-PKA signaling *in vivo* in mouse liver independently of glucagon action in other tissues, we selectively disinhibited liver PKA catalytic (PKAc) activity by ablating hepatic protein kinase A regulatory subunit 1A (Prkar1a) using the CRE/LoxP method. Mice homozygous for floxed *Prkar1a* (*Prkar1a*$^{fl/fl}$ mice) (Kirschner et al., 2005) were treated by tail vein injection with adenovirus driving CRE recombinase under control of the CMV promoter (Adv-CRE) to generate mice selectively lacking liver Prkar1a

(L-ΔPrkar1a mice). Control mice received adenovirus expressing green fluorescent protein (Adv-GFP).

Liver extracts harvested four days after injection from Adv-CRE injected mice revealed a 90% reduction in Prkar1a protein (Fig 1A), while other Prkar isoforms and Pkac levels remained unaltered. As expected, L-ΔPrkar1a mice, as opposed to controls, exhibited increased hepatic phosphorylation of cAMP-response element binding protein (CREB) at serine 133 (pCREB), an established PKAc target (Gonzalez and Montminy, 1989) (Fig 1A). Adv-CRE treatment did not affect Prkar1a expression in islet, hypothalamus, adpose tissue and skeletal muscle (Fig. S1A). Liver-specific PKA disinhibition stimulated within 4 days hepatic expression of transcriptional co-activators (*Ppargc1a*, *Src1*) and rate-limiting enzymes (*G6p*, *Pck1*) of the gluconeogenic pathway (Louet et al., 2010), which resulted in fasting hyperglycemia and notably also in insufficient insulin secretion to correct glycemia during intraperitoneal glucose tolerance tests (ipGTT) (Fig 1C,D).

To assess whether hyperglycemia during 4 days is directly associated with impaired GSIS, we generated a model of chronic hyperglycemia without hepatic PKA-CREB activation. Wild-type mice were intravenously infused during 4 days with D-glucose (D-glucose mice) to achieve fasting glucose levels to match those measured in L-ΔPrkar1a mice (Fig 1B). Mice infused with saline served as controls (Sal mice). D-glucose mice exhibited no change in liver pCREB (Fig 1A) and reduced gene expression of the gluconeogenic program (Fig 1D). In contrast to L-ΔPrkar1a mice, D-glucose mice showed increased GSIS and only mildly impaired GT (Fig 1C). Both L-ΔPrkar1a and D-glucose mice showed similar increases in β-cell proliferation, as assessed by Ki67 expression (Fig S1E); albeit, pancreas morphometric parameters or plasma glucagon levels in L-ΔPrkar1a and D-glucose infused mice did not change during the short 4-day protocols (Fig S1B-H), excluding differences in β-, or α-cell mass or in glucagon action to account for the differences in glucose homeostasis.

Selective PKA Disinhibition in Liver Induces Secreted Neuropeptide *Kiss1*

The liver secretes factors, which regulate pancreatic α- or β-cell growth, and which in part have been identified by liver gene expression analyses (El Ouaamari et al., 2013; Longuet et al., 2013; Yi et al., 2013). We reasoned that the liver may also secrete a factor(s) that regulates β-cell function and that may be altered in L-ΔPrkar1a mice. We therefore treated isolated WT mouse islets with serum-free media conditioned with plasma extracted from L-ΔPrkar1a or control mice and examined GSIS at 10 mM glucose. Plasma in 1:10 dilution from L-ΔPrkar1a but not from control mice suppressed GSIS (Fig 1E). 1:100 diluted L-ΔPrkar1a plasma did not suppress GSIS in this functional bioassay. These observations suggested that PKA signaling in the liver generates a secreted molecule(s), which suppresses β-cell GSIS in a dose-dependent fashion (Fig 1E).

To identify this PKA-regulated factor, we analyzed by microarray L-ΔPrkar1a and control mouse livers for differentially expressed genes encoding secreted proteins. We identified a single candidate transcript encoding kisspeptin1 (*Kiss1*) (Fig 1F) to be elevated in liver tissue of L-ΔPrkar1a mice ($P<10^{-11}$, log2FC=2.17). Quantitative RT-PCR (qRT-PCR) and immunoblots (IB) of liver tissue confirmed increased hepatic kisspeptin1 production in L-

ΔPrkar1a mice (Fig 1G). Conversely, liver *Kiss1* was reduced by 50% in D-glucose mice (Fig 1G).

Glucagon Stimulates Hepatic *Kiss1* Expression via Gcgr and cAMP-PKA-CREB Signaling

We next examined whether glucagon directly stimulates hepatic kisspeptin1 production. Glucagon treatment (200 pg/ml) of primary mouse hepatocytes stimulated within 2 hours *Kiss1* expression and protein (Fig 2A,C). As expected, the gluconeogenic gene *Pck1*, an internal control, was also stimulated by glucagon (Fig 2A). Consistent with glucagon activated cAMP-PKA signaling, mouse hepatocytes treated for 2 hours with the adenylyl cyclase activator forskolin (fsk) plus the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) exhibited increased *Kiss1* expression (Fig 2B).

A luciferase reporter plasmid containing 1kb of the mouse *Kiss1* promoter element transfected into mouse H2.35 hepatoma cells showed transcriptional activation in response to glucagon or to fsk/IBMX treatment (Fig. S2A). The mouse *Kiss1* promoter contains two putative functional cAMP response element (CRE) half-sites (TGACT) (Zhang et al., 2005) located at −27 and −758 base pairs upstream of the *Kiss1* transcription start site. Mutation of either of the CRE half-sites decreased the transcriptional responses to glucagon or to fsk/IBMX, and combined mutation of both CRE half-sites further decreased the responses (Fig. S2A).

In accordance with CREB mediating cAMP-stimulated *Kiss1* transcription, co-transfection in H2.35 cells with constitutively active CREB Y134F stimulated *Kiss1* reporter activity as long as CRE1 and 2 sites were intact, while dominant negative CREB inhibitor A-CREB blocked reporter stimulation by fsk/IBMX (Fig. S2B,C). Consistent with these findings is robust cAMP-PKA-CREB responsiveness of the human *Kiss1* promoter, which contains a functional CRE half-site at −45 bp proximal to the transcription start site (Zhang et al., 2005). Chromatin of mouse liver extracts using non-specific versus CREB-specific antiserum combined with qPCR of CRE half-site sequences (*in vivo* ChIP), confirmed *in vivo* CREB occupancy of both CRE half-sites within the *Kiss1* promoter (Fig. S2D).

Intraperitoneal glucagon (16 μg/kg) but not PBS treatment in mice increased hepatic kisspeptin1 production 30 minutes after injection (Fig. 2D,E). Physiologic endogenous glucagon secretion provoked by overnight fasting (Fig. S2E) resulted in increased hepatic kisspeptin1 production when compared to *ad libitum* fed mice (Fig. 2F,G). Fasted and subsequently refed mice exhibited a reduction in blood glucagon levels (Fig. S2D) and also of liver *Kiss1* mRNA and kisspeptin1 protein (Fig. 2F,G). *Kiss1* transcript was also detectable by qRT-PCR at low levels in spleen, kidney, skeletal muscle and epididymal fat tissue. In these tissues, *Kiss1* expression remained unchanged during fasting (not shown) indicating that the liver is the main site where *Kiss1* expression is regulated by metabolic cues.

To confirm the role of the liver glucagon receptor in mediating *in vivo* effects of glucagon on hepatic kisspeptin1 production, we generated floxed glucagon receptor ($Gcgr^{fl/fl}$) mice (Fig. S3A), in which we conditionally ablated liver Gcgr by Adv-CRE delivery (L-ΔGcgr mice) (Fig. S3B). Adv-GFP injected $Gcgr^{fl/fl}$ mice served as controls. Adv-CRE treatment

did not ablate Grgr in islet, hypothalamus, and adpose tissue (Fig. S3B). Deprivation of hepatic glucagon signaling in L-ΔGcgr mice resulted in reduced fasting glucose levels, improved GT and unchanged insulin tolerance (Fig. S3C,D). L-ΔGcgr mice showed, as compared to controls, slightly but not significantly reduced liver kisspeptin1 expression (Fig. 2H,I). Importantly, in L-ΔGcgr mice, kisspeptin production did not change in response to intraperitoneal glucagon treatment or after overnight fasting (Fig. 2J,K). Plasma kisspeptin levels in *Gcgr*$^{fl/fl}$ and L-ΔGcgr mice reflected respectively, changes in liver kisspeptin production in response to glucagon treatment or fasting (Fig. S2G,H). Furthermore, L-ΔGcgr mice showed, as compared to controls, significantly dampened liver CREB phosphorylation and CREB occupancy of the *Kiss1* promoter CRE half-sites in response to glucagon treatment or to overnight fasting (Fig. S3E,F).

Glucagon and Insulin Counter-regulate Liver Kisspeptin1 Expression

In the liver, insulin counteracts glucagon action on cAMP-CREB regulated genes (He et al., 2009). Accordingly, *in vitro* insulin treatment (2000 pg/ml) of mouse hepatocytes reduced basal *Kiss1* expression, and as expected, also reduced *Pck1* expression (Fig. 2A). Insulin treatment dampened glucagon-stimulated *Kiss1* and *Pck1* expression in mouse hepatocytes (Fig. 2A). Changes in *Kiss1* expression were qualitatively reflected in corresponding changes in kisspeptin1 protein levels in response to glucagon and insulin treatment, respectively (Fig. 2C).

To verify that *in vivo* insulin effects on liver *Kiss1* expression are directly mediated by liver insulin receptors and to examine whether isolated hepatic insulin resistance may modulate *Kiss1* expression, we generated mice with liver-specific insulin receptor deficiency by Adv-CRE treatment of floxed insulin receptor (*Insr*$^{fl/fl}$) mice (Bruning et al., 1997) (L-ΔInsr mice; Fig. 2L). Importantly, Insr expression in islets, hypothalamus, adipose tissue and skeletal muscle was not different between *Insr*$^{fl/fl}$ and L-ΔInsr mice (Fig S2F). Hepatic ablation of insulin receptor by Adv-CRE in mice did not change liver *Kiss1* mRNA or protein levels (Fig. 2M,O). Conversely, glucagon treatment of L-ΔInsr mice, as compared to *Insr*$^{fl/fl}$ mice, dramatically increased liver *Kiss1* transcript and kisspeptin protein (Fig. 2N,O). Plasma kisspeptin1 levels in *Insr*$^{fl/fl}$ and L-ΔInsr mice, respectively, reflected the changes in liver kisspeptin production (Fig. S2I).

Consistent with these observations of insulin counter-regulation of PKA mediated *Kiss1* stimulation, *in vivo* intraperitoneal insulin (1 IU/kg) administration in L-ΔPrkar1a mice to supplement relatively deficient endogenous serum insulin concentrations and to achieve blood glucose reduction, decreased hepatic *Kiss1* mRNA and protein levels within 60 minutes (Fig. S1I-K).

These findings indicate that – as is established for cAMP-CREB responsive gluconeogenic genes- insulin at sufficiently high concentrations antagonizes glucagon stimulation of *Kiss1* expression. Further, *in vivo* disruption of the hepatic insulin receptor (i.e. liver insulin resistance) alone does not de-repress liver kisspeptin1 production absent additional glucagon signaling. These findings also indicate that the liver is the predominant source of circulating kisspeptin1, which is subject to hormonal regulation.

Kisspeptin1 Knockdown in L-ΔPrkar1a Mice Ameliorates GSIS Despite Continued Gluconeogenesis

To verify that in L-ΔPrkar1a mice hepatic kisspeptin1 is directly linked to impaired GSIS, we knocked down hepatic kisspeptin1 in L-ΔPrkar1a mice by administering adenovirus expressing *Kiss1*-specific shRNA (Adv-Kiss1 shRNA) or a control scrambled shRNA adenovirus (Adv-scr shRNA). Within 3 days of treatment, Adv-Kiss1 shRNA treated mice showed reduced hepatic *Kiss1* mRNA and plasma kisspeptin (Fig. 3A). In contrast, gluconeogenesis, as reflected by mRNA of the gluconeogenic genes (Fig. 3B) as well as functional conversion of intraperitoneally administered gluconeogenic precursor pyruvate to glucose in the fed (non-fasting) state (ip pyruvate conversion test = ipPCT) was similar between Adv-Kiss1 shRNA and control Adv-scr shRNA treated L-ΔPrkar1a mice and also significantly increased as compared to WT mice (Fig. 3D). Furthermore, liver CREB phosphorylation and CREB occupancy of the endogenous *Kiss1* promoter was similar in Adv-scr shRNA and Adv-Kiss1 shRNA treated L-ΔPrkar1a mice (Fig. 3B,C), indicating that PKA signaling per se is not differentially affected by *Kiss1*-specific versus scr shRNA treatment.

Despite ongoing upregulated hepatic gluconeogenesis (Fig. 3B,D), *Kiss1* knockdown in L-ΔPrkar1a mice increased *in vivo* GSIS and improved GT (Fig. 3F,G). ipITT (Fig. 3E), food intake and body weight (Fig. 3H,I), were similar in Adv-Kiss1 and -scr shRNA treated L-ΔPrkar1a mice excluding differences in insulin sensitivity or caloric intake in Adv-Kiss1 versus -scr shRNA treated animals as mechanisms, respectively, for improved GT or for a compensatory increase in insulin secretion in the face of altered insulin resistance.

Kisspeptin impairs GSIS at nanomolar concentrations via Interaction with its Receptor Kiss1R on Pancreatic β-cells

The kisspeptin1 receptor (Kiss1R) shares 82% homology between humans and mouse (Ohtaki et al., 2001). IB confirmed Kiss1R expression in protein extracts of mouse islets, in INS1 and Min6 insulinoma cells as well as in human islets (Fig. 4A). Immunohistochemistry combined with confocal imaging of mouse pancreas localized Kiss1R expression to insulin-producing pancreatic β-cells but not to α-cells (Fig. 4B), indicating that kisspeptin1-Kiss1R signaling likely occurs directly on β-cells.

To specifically investigate the functional role of Kiss1R on β-cells in mediating kisspeptin1 action on GSIS, we generated mice lacking pancreatic Kiss1R by interbreeding PDX1-CRE (Lammert et al., 2001) and floxed Kiss1R (*Kiss1r*$^{fl/fl}$) (Novaira et al., 2013) mice to yield Panc-ΔKiss1R mice (Fig. S4A). Analysis of pancreata revealed similar morphometric parameters and insulin content in control and Panc-ΔKiss1R mice (Fig. S4B). Panc-ΔKiss1R mice, as compared to *Kiss1r*$^{fl/fl}$ controls, exhibited similar plasma glucagon levels (Fig. S4B), slightly elevated fasting serum insulin levels, similar glucose levels, and similar GT during ipGTT (Fig. 4C). In contrast, treatment with K54 (10 nmol ip) suppressed GSIS in *Kiss1r*$^{fl/fl}$ mice, but did not affect GSIS in Panc-ΔKiss1R mice (Fig. 4D). Together with the confocal microscopic observation, which localized Kiss1R within the endocrine pancreas restricted to β-cells (Fig. 4B), these results indicate that kisspeptin1 suppresses GSIS by direct action via its receptor on β-cells.

Synthetic K54 suppressed GSIS in a dose-dependent manner and at concentrations as low as 0.1 nM from *Kiss1R*$^{fl/fl}$ mouse islets that were cultured in serum free media containing either 10 or 20 mM glucose (Fig. 4E **top**), whereas Panc-ΔKiss1R mice were impervious to K54-mediated GSIS suppression (Fig. 4E **bottom**) K10 tested at concentrations equimolar to K54 was equally effective in suppressing GSIS *in vivo* and *in vitro* (not shown).

Kisspeptin at different concentrations has been reported to either suppress or stimulate GSIS. Kisspeptin isoforms at nM concentrations suppress GSIS (Silvestre et al., 2008; Vikman and Ahren, 2009). In contrast, GSIS stimulation has been reported only at kisspeptin concentrations in the range of 10^3 nM (= 1 μM) (Bowe et al., 2012; Hauge-Evans et al., 2006), which are unusually high for a hormone.

To examine the effects of such high kisspeptin concentrations on GSIS, we treated islets with 10^3 nM (= 1 μM) K54 or K10 and found GSIS stimulation from both *Kiss1R*$^{fl/fl}$ and Panc-ΔKiss1R islets (Fig. S4C). These findings, using selective genetic Kiss1R ablation suggest that kisspeptin at very high concentrations may stimulate GSIS in a Kiss1R-independent mechanism raising the possibility of off-target effects on GSIS of supraphysiologic kisspeptin concentrations.

Kisspeptin Suppresses Islet cAMP Synthesis and Antagonizes Incretin Hormone Glucagon-like peptide-1 Receptor mediated GSIS Potentiation

GSIS is potentiated by increased cAMP concentrations in β-cells (Drucker, 2006). Kiss1R belongs to Class I/A of G-protein coupled receptors and shares structural similarities with the galanin receptor, activation of which suppresses cAMP synthesis in β-cells (Lee et al., 1999; Tang et al., 2012). We reasoned that kisspeptin1 may also modulate β-cell cAMP levels. WT mouse islets kept at 10 mM glucose and treated with K54 (10 nM) contained lower cAMP concentrations as compared to PBS treated islets (Fig. 4F). K54 also impaired islet cAMP production in response to the long-acting incretin hormone analogue exendin-4 (E4) (Fig. 4F) – a widely used antidiabetic agent that binds and activates on β-cells the receptor for the incretin hormone glucagon-like peptide-1 (GLP-1) and potentiates GSIS by stimulating β-cell cAMP synthesis (Drucker, 2006). Conversely, Panc-ΔKiss1R islets exhibited slightly increased baseline and E4-induced cAMP concentrations, which were not affected by K54 treatment (Fig. 4F). Consistent with an antagonism between K54 and E4, respectively on β-cell function, K54 dose-dependently suppressed E4-potentiated GSIS from cultured mouse islets cultured at 10 mM glucose (Fig. 4F **bottom**). Perifusion studies of isolated mouse islets revealed that both K54 and K10 suppressed first (0–10 min after glucose stimulation) and second phases of GSIS (Fig. 4G,H) as well as E4-potentiated GSIS (Fig. 4G,H). KCl-induced (30 mM) depolarization of *Kiss1R*$^{fl/fl}$ and Panc-ΔKiss1R islets after perifusion stimulated release of equal amounts of insulin, indicating that insulin exocytosis mechanisms distal to the regulatory β-cell ATP-dependent potassium channel (K_{ATP} channel) are not impaired in Panc-ΔKiss1R islets (Fig 4G).

These observations indicate that kisspeptin1 reduces β-cell cAMP production and renders β-cells resistant to incretin action on cAMP synthesis and GSIS potentiation.

Mouse Models of Impaired GT and DM exhibit Increased Liver Kisspeptin1 Production

We next examined the relevance of glucagon and kisspeptin1 production in the context of DM. Mice receiving for 8 weeks a HFD developed glucose intolerance and insulin resistance as compared to standard diet (SD) fed controls (Fig. S5A,B) and importantly, exhibited increased plasma glucagon levels in the fed state (Fig. S5C) and an increase in liver *Kiss1* expression (Fig. 5A)

In the hypothalamus, *Kiss1* expression is modulated by leptin signaling (Smith et al., 2006). Furthermore, leptin inhibits GSIS via its receptor on β-cells (Kieffer et al., 1997). Therefore, to elucidate the relevance of hyperglucagonemia on liver *Kiss1* and GSIS independent of leptin effects, we also examined mice homozygous for the inactivating leptin receptor db mutation (*Lepr*$^{db/db}$ mice).

Lepr$^{db/db}$ as compared to WT mice exhibited hyperglucagonemia in the fed state (Fig. S5D) and significantly increased liver *Kiss1* expression (Fig. 5A). Both plasma glucagon and liver *Kiss1* mRNA levels were greater in magnitude in *Lepr*$^{db/db}$ as compared to those found in HFD mice. Accordingly, kisspeptin1 immunoreactivity was detectable at low levels in liver of SD mice and was increased both in HFD and in db/db liver tissue (Fig. 5A). Both HFD fed mice and *Lepr*$^{db/db}$ liver tissue showed higher CREB phosphorylation (Fig S5F) and *in vivo* CREB occupancy on both CRE half-sites within the *Kiss1* promoter (Fig. S5G).

Plasma from HFD fed and diabetic *Lepr*$^{db/db}$ mice - as compared to SD fed WT mice - exhibited higher kisspeptin1 concentrations as determined by ELISA (Fig. 5B). *Kiss1R*$^{fl/fl}$ islets cultured in serum-free media conditioned with plasma from HFD or from *Lepr*$^{db/db}$ mice exhibited impaired GSIS. In contrast, Panc-ΔKiss1R islets resisted GSIS inhibition by plasma of HFD or *Lepr*$^{db/db}$ mice. GSIS suppression from WT islets cultured in serum-free media conditioned with HFD or *Lepr*$^{db/db}$ plasma (Fig. 5C) - when compared with GSIS suppression by synthetic K54 (Fig. 4H) - indicated that the functional plasma kisspeptin concentrations in HFD and *Lepr*$^{db/db}$ mice to be equivalent to 0.5–1 nM and 7–10 nM of K10, respectively.

We further examined the relevance of liver kisspeptin in the context of human T2DM. Kisspeptin1 immunoreactivity was detectable at variable intensity by immunoblot of liver samples from humans with T2DM but not from non-diabetic humans (Fig. 5D). Accordingly, circulating kisspeptin1 immunoreactivity was increased in serum from humans with T2DM, as compared to non-diabetic individuals (Fig. 5E). Media conditioned with serum from T2DM but not from non-diabetic individuals, suppressed GSIS from *Kiss1R*$^{fl/fl}$ but not from Panc-ΔKiss1R islets (Fig 5F). These observations indicate that - akin to rodent models of DM - in human T2DM, liver kisspeptin1 expression is increased and that circulating kisspeptin suppresses GSIS.

Liver *Kiss1* Upregulation is Linked to Hyperglucagonemia in DM

We determined whether hyperglucagonemia is linked to liver kisspeptin production in HFD and *Lepr*$^{db/db}$ mice by administering a single dose of the selective Gcgr antagonist (GAI) or an inactive analog (GAC) (Qureshi et al., 2004) 60 minutes before an ipGTT. GAI - as compared to GAC - treated (Fig. 6F,M) mice exhibited improved basal glycemia as well as

GT. GAI treatment led to a reduction in liver gluconeogenic gene (Fig 6C,I) and in *Kiss1* expression (Fig 6D,J) and to corresponding reductions in liver pCREB (Fig 6B,H), and CREB occupancy of CRE 1 and 2 in the *Kiss1* promoter (Fig. 6E,K). Circulating plasma glucagon levels remained unchanged after GAI or GAC treatment in both HFD fed (Fig. 6A) and $Lepr^{db/db}$ mice (Fig. 6G), respectively.

Thus, in the context of glucose intolerance and DM, hyperglucagonemia significantly contributes to hepatic kisspeptin1 production. Further, as demonstrated in $Lepr^{db/db}$ mice, hepatic *Kiss1* regulation occurs independently of leptin.

Liver Kisspeptin1 Knockdown in Diabetic Mice Ameliorates GSIS and GT

We next examined the contribution in DM of hepatic kisspeptin1 towards impaired GSIS by liver selective shRNA-mediated *Kiss1* knockdown. Adv-Kiss1 but not -scr shRNA treatment in HFD or $Lepr^{db/db}$ mice (5–6 weeks of age), respectively, reduced within 3 days liver kisseptin1 production (Fig. 7A,H) and *Kiss1* mRNA (Fig. 7B,I) as well as plasma kisspeptin1 (Fig. 7C,J). Plasma glucagon (Fig. 7D,K), liver pCREB (Fig. 7A,H), liver CREB occupancy on *Kiss1* promoter CRE sites (Fig. S6A,C) remained similar in Adv-Kiss1 and -scr shRNA treated littermates. Liver kisspeptin1 knockdown in both HFD and $Lepr^{db/db}$ mice resulted in improved *in vivo* GT (Fig. 7E,M) and increased GSIS (Fig 7F,N). Caloric intake (Fig 7E,K), body weight (Fig. S6B,D) and insulin tolerance (Fig 7G,O), were not different in Adv-Kiss1 and -scr shRNA treated counterparts, ruling out changes in insulin sensitivity after *Kiss1* knockdown as a mechanism for improvements in GSIS and GT.

These observations indicate that in DM, liver kisspeptin1 negatively impacts GSIS, which can be de-repressed by inhibiting hepatic kisspeptin1 production.

Selective Pancreas Kiss1R Ablation Ameliorates Insulin Secretion and GT in HFD Mice

To directly assess the relevance of kisspeptin-Kiss1R signaling on GSIS in the context of DM, we examined the effects of conditional pancreas Kiss1R ablation in HFD fed mice. $Kiss1R^{fl/fl}$ and Panc-ΔKiss1R mice, placed on a HFD for 8 weeks, exhibited similar weight gain (Fig. S7). Liver kisspeptin1 immunoreactivity, plasma kisspeptin and glucagon levels were similar in HFD mice independent of pancreas Kiss1R status (Fig. S7). Importantly, Panc-ΔKiss1R showed, as compared to $Kiss1R^{fl/fl}$ littermates, slightly lower fasting blood glucose and improved GT (Fig. S7). HFD fed Panc-ΔKiss1R showed relative to controls, both increased fasting insulin and *in vivo* GSIS. No differences were found in insulin tolerance, islet, β-cell or α-cell mass, or pancreas insulin content between Panc-ΔKiss1R and $Kiss1R^{fl/fl}$ littermates (Fig. S7), excluding changes in peripheral insulin action or β-cell mass to account for improved GSIS and GT in HFD fed Panc-ΔKiss1R mice.

Discussion

The present findings using genetically defined mouse models suggest a tri-hormonal regulatory circuit between pancreatic α-cells, hepatocytes and β-cells, and assign kisspeptin1 an unexpected role in liver to islet endocrine signaling. In addition, the findings indicate in T2DM a sequential link between hyperglucagonemia and impaired β-cell function via liver-derived kisspeptin1.

Hyperglucagonemia, which occurs early during development of T2DM upregulates kisspeptin1 production by the liver (Fig. 2). Kisspeptin1 in turn functions as a hormone to suppress GSIS (Fig 4). Thus, in T2DM the β-cell is exposed to two counteracting stimuli elicited by glucagon action on the liver. Glucagon-induced HGP and hyperglycemia stimulate, whereas kisspeptin1 production inhibits GSIS.

The physiologic relevance of opposing actions of hepatic cAMP-CREB signaling on β-cell function remains to be fully explored. Teleological considerations render plausible a survival mechanism in that hepatic cAMP-CREB-induced kisspeptin1 serves as a (among other mechanisms) safeguard against insulin secretion and hypoglycemia during fight and flight reactions, should these occur during and interrupt prandial nutrient absorption – when insulin secretion otherwise would be elevated. To this end, it is likely that epinephrine, another fight and flight mediator, that activates liver cAMP signaling (Sherline et al., 1972), may also participate in liver *Kiss1* regulation.

It is important to note that our studies using genetically defined Kiss1R deficient islets reveal that kisspeptin applied at supraphysiologic doses in the micromolar range stimulates GSIS, likely due to effects which are not mediated by the bona-fide kisspeptin1 receptor Kiss1R (Fig. S4C). This observation of Kiss1R-independent effects at high doses of kisspeptin, which are unusually high for a hormone, may in part explain the contradictory observations on GSIS between physiologic and supraphysiologic kisspeptin1 concentrations (Bowe et al., 2012; Hauge-Evans et al., 2006; Silvestre et al., 2008; Vikman and Ahren, 2009). Our studies suggest that Kiss1R signaling in β-cells suppresses cAMP and inhibits GSIS. An intravenous bolus K10 in the non-human primate *Macaca mulatta* is reported to stimulate GSIS, albeit circulating kisspeptin concentrations were not measured in that study (Wahab et al., 2011). Based on the studies herein, humans with T2DM exhibit increased liver kisspeptin1 immunoreactivity, increased circulating kisspeptin and their plasma suppresses GSIS from mouse islets in a Kiss1R-dependent manner (Fig 3, 4). Future studies on the interplay between glucagon and kisspeptin1 in humans will need to carefully examine the dose response of kisspeptin1 on GSIS combined with reliable measurements of circulating functional kisspeptin1 isoforms.

Our observations further suggest that in T2DM GSIS is insufficient to overcome the co-existing inhibition on β-cells exerted by kisspeptin1. This mechanism results in inadequate insulin secretion to meet metabolic demands and aggravates β-cell dysfunction and hyperglycemia. Clinical observations indicate that in T2DM, although incretin GLP-1 is normally secreted, endogenous GLP-1 is insufficient to achieve physiologic GSIS potentiation. Furthermore, treatment with dipeptidyl-peptidase IV inhibitors, which inhibit GLP-1 degradation and increase endogenous GLP-1 concentrations by two-fold, or treatment with long-acting GLP-1 analog E4 restore β-cell function in T2DM. These observations have led to the concept of reduced GLP-1 sensitivity of β-cells in T2DM (Meier and Nauck, 2008). Antagonism by K54 on E4 induced GSIS potentiation (Fig. 4E,F) suggests that increased circulating kisspeptin1 levels in T2DM may at least in part contribute to the diminished response to endogenous GLP-1 in type 2 diabetic subjects.

Thus, our findings uncover the liver as a site of regulated kisspeptin production and provide mechanistic and causal underpinnings for common observations made in clinical T2DM: a) relative hyperglucagonemia, b) insufficient insulin secretion to regulate glycemia, and c) diminished response to endogenously secreted GLP-1 and restoration of β-cell function by pharmacologic GLP-1 receptor agonism.

In a broader context, neutralizing circulating kisspeptin1 or antagonism of Kiss1R on β-cells are appealing avenues to augment GSIS and improve glucose homeostasis in T2DM. In this regard, Kiss1R antagonists, which would not cross the blood brain barrier and interfere with hypothalamic reproductive functions of kisspeptin1 would be particularly advantageous. Another important aspect is that plasma kisspeptin1 activity may serve as a biomarker to identify T2DM patients who would benefit most from aggressive β-cell-targeted therapy.

Experimental Procedures

Animal Studies

Animal studies were approved by the local Institutional Animal use and Care Committee, and were performed in 6–8 week old C57Bl/6 male mice. *Lepr*$^{db/db}$ (*Lepr*$^{db/db}$; B6.BKS(D)-leprdb/J) mice were from Jackson Laboratories. *Gcgr*$^{fl/fl}$ were generated by homologous recombination technology (Supplemental information). Intravenous glucose infusions were performed as described (Alonso et al., 2007). Genotyping PCR primers and dynamic physiologic testing details are provided in Supplemental information.

Adenovirus Injection Studies

Adenovirus (Adv-CRE and Adv-GFP, University of Iowa) were injected into tail vein (10^9 plaque forming units/mouse in 1×PBS). Adenovirus expressing shRNA under U6 promoter was generated (Life Technologies) to target mouse *Kiss1* sequence: GCTCTCTCTCTTTGACCTAGG.

Immunofluorescence Histology and Islet Morphometry

Pancreas immunofluorescence histology and morphometry and pancreas insulin content measurment were conducted as described (Song et al., 2011). Confocal imaging was performed on a Zeiss Axoivert. Antibodies are provided in Supplemental information.

Isolated Islet Studies

Islet isolation was performed by collagenase digestion, gradient centrifugation and three rounds of microscope-assisted manual picking of islets. Static incubation studies were conducted as previously described with 20 hand-picked equal-sized islets were studied in each group (Song et al., 2011).. After overnight culture (37 C, 5% CO2, 95% O2 in humid chamber) of isolated islets in RPMI 1640 (Mediatech) containing 5 mM glucose, 1% each Na-Pyruvate, HEPES, Penicillin/Streptomycin and 0.2% bovine serum albumin (BSA), islets were switched to either 10 or 20 mM glucose containing RPMI 1640. Where indicated, Kisspeptin-10 (0–100 nM, and 1 μM), E4 (10 nM) or vehicle (PBS) was added. After 30 min incubation glucose concentrations, supernatant was taken for insulin measurements and pelleted islets were taken in acid ethanol (0.18M HCl in 70% ethanol) for insulin

measurements in islets (ELISA, Alpco). Islet protein concentration was measured using the BCA method (Thermo Fisher). Dose response curves of GSIS inhibition by Kisspeptin-54 or -10 (0–100 nM) to serve as a functional bioassay for plasma kisspeptin1 activity performed at 6 separate times provided intra-assay and inter-assay coefficient of variations of 7.3% and 9.2%, respectively.

Immunoblots

IB were performed as described (Song et al., 2011) in at least three different separately obtained experimental samples. Luminescence images of representative IBs are shown. Corresponding Actin IB show protein loading control.

Human samples

The Institutional Review Board at Johns Hopkins University approved studies of de-identified human samples. Human tissue and serum samples were obtained from National Disease Research Interchange (NDRI) and Origene. Information on samples is provided in Supplemental information.

Immunoassays

Kisspeptin1 ELISA for mouse (USCN Life Sciences) and human (Phoenix) were used according to manufacturers' instructions. The mouse ELISA kit failed to recognize kisspeptin in human samples and vice versa.

Statistics

Results are presented as average ± standard error of the mean (SEM). Student's T-test was used for single comparisons and ANOVA with Bonferroni adjustment for multiple to calculate differences between groups. A P value of <0.05 was considered significant and indicated with *.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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1. Glucagon stimulates both hepatic kisspeptin1 production and gluconeogenesis.
2. Kisspeptin1 suppresses glucose stimulated insulin secretion (GSIS) from β-cells.
3. Hyperglucagonemia in diabetes impairs insulin secretion via hepatic kisspeptin1.
4. In diabetic mice, liver *Kiss1* knockdown improves GSIS and glycemia

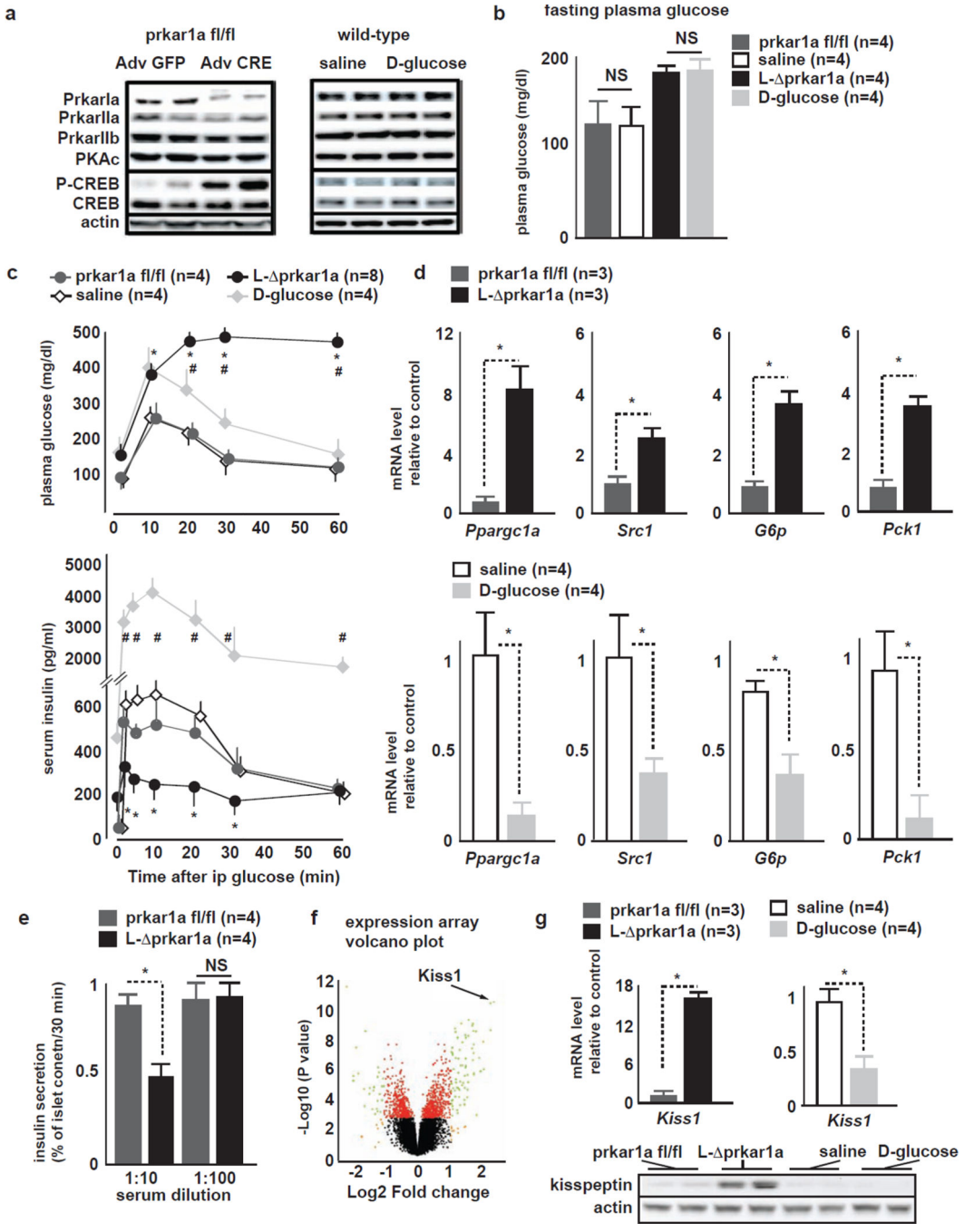

Figure 1. Comparison between L-Δprkar1a and D-glucose mice identifies *Kiss1*

A (left) Representative liver IB of *prkar1a*$^{fl/fl}$ and L-Δprkar1a 4 days after adenovirus treatment. L-Δprkar1a mice show Prkar1a ablation and increased pCREB **(right)** Liver IB from Sal- and D-glucose mice shows unaltered Prkar subtypes, Pkac, pCREB.

B Fasting glucose levels in *prkar1a*$^{fl/fl}$, L-Δprkar1a, Sal- and D-glucose mice. Prkar1a$^{fl/fl}$ and Sal- mice have similar fasting glucose; D-glucose infusion achieves fasting glucose similar to L-Δprkar1a mice (mean±SEM, * P<0.05).

C (top) plasma glucose and **(bottom)** serum insulin during ipGTT in *prkar1a*$^{fl/fl}$, L-Δprkar1a, Sal- and D-glucose mice. L-Δprkar1a mice exhibit impaired GT (**top**) and GSIS (**bottom**). D-glucose mice have mildly impaired GT and robust GSIS. Prkar1a fl/fl and Sal- mice have similar GT and GSIS (mean±SEM, * P<0.05).

D qRT-PCR of indicated genes of gluconeogenic program in *prkar1a*$^{fl/fl}$, L-Δprkar1a, Sal- and D-glucose-mouse livers. **(top)** gluconeogenic program is upregulated in L-Δprkar1a as compared to *prkar1a*$^{fl/fl}$ mice; **(bottom)** gluconeogenic program is downregulated in D-glucose as compared to saline-mice (mean±SEM, * P<0.05)..

E GSIS of WT mouse islets cultured in serum free media conditioned with plasma of *prkar1a*$^{fl/fl}$ or L-Δprkar1a mice. *Prkar1a*$^{fl/fl}$ plasma does not affect GSIS. L-Δprkar1a plasma at 1:10 but not at 1:100 dilution suppresses GSIS (mean±SEM, * P<0.05).

F Volcano plot of gene expression analysis of liver from *prkar1a*$^{fl/fl}$ and L-Δprkar1a mice. Significant upregulation of *Kiss1* transcript is detected in L-Δprkar1a mice.

G (top) qRT-PCR of *Kiss1* transcript and **(bottom)** IB in liver tissue from mice with indicated liver genetic complement or intravenous infusion. L-Δprkar1a liver shows increased *Kiss1* transcript and kisspeptin protein. D-glucose mice show *Kiss1* downregulation as compared to controls (mean±SEM, * P<0.05).

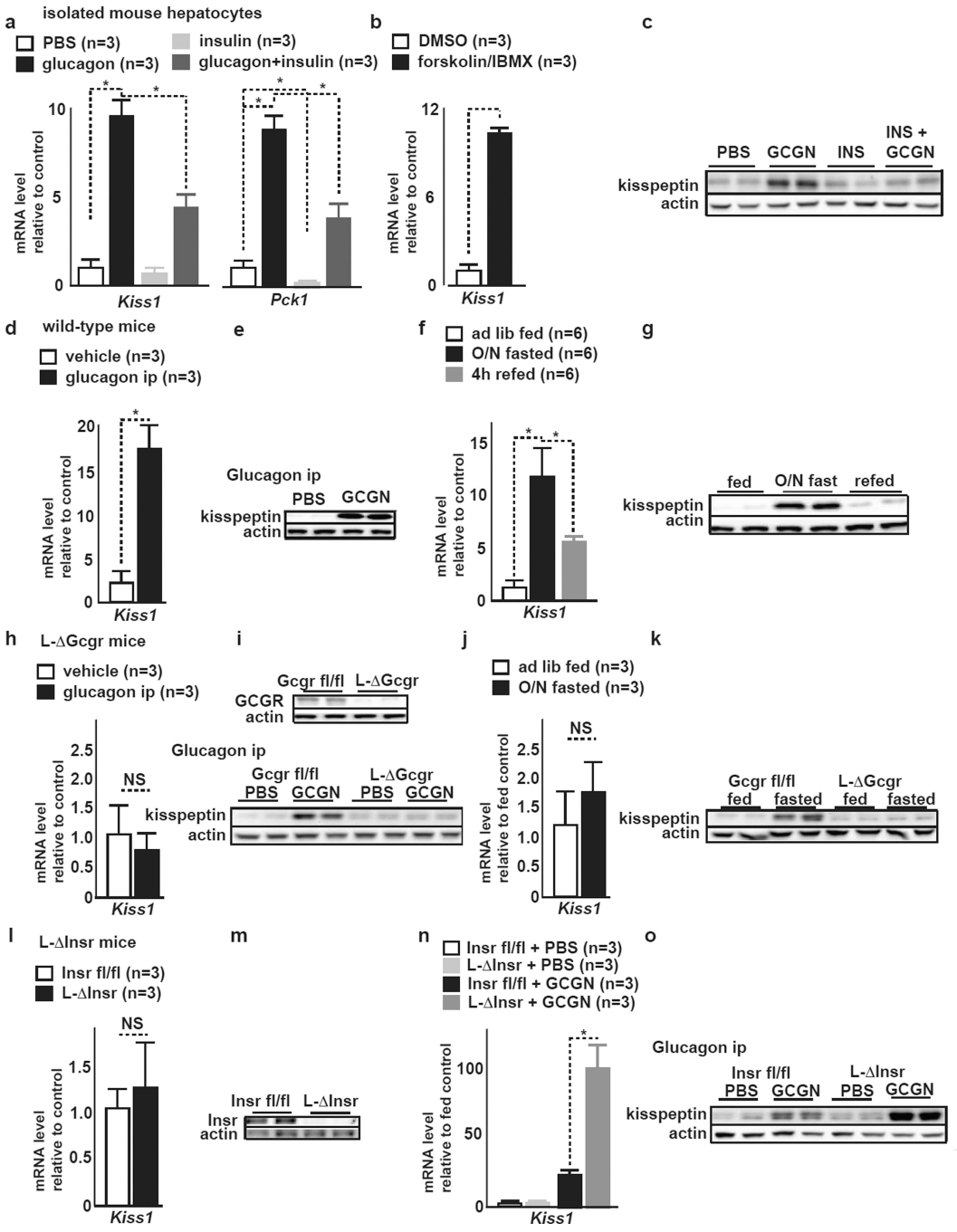

Figure 2. Glucagon and insulin counter-regulate liver *Kiss1* expression

A qRT-PCR of *Kiss1* and *Pck1* in isolated mouse hepatocytes exposed to indicated treatment. Glucagon stimulates, insulin suppresses both genes (mean±SEM, *P<0.05).

B qRT-PCR of *Kiss1* in isolated mouse hepatocytes exposed to vehicle (DMSO) or fsk/IBMX. cAMP stimulation stimulates *Kiss1* expression (mean±SEM, *P<0.05)..

C Representative IB of cultured mouse hepatocytes after treatment with PBS, glucagon, insulin or INS plus GCGN. Glucagon stimulates kisspeptin, insulin treatment has little effect on already low kisspeptin. Insulin counterregulates glucagon stimulation of kisspeptin.

D qRT-PCR of *Kiss1* in liver tissue of WT mice after *in vivo* ip treatment with vehicle (PBS) or glucagon. Glucagon treatment stimulates *Kiss1* in liver (mean±SEM, *P<0.05).
E Representative liver IB in WT mice after *in vivo* ip treatment with PBS or glucagon. Glucagon increases liver kisspeptin1 (mean±SEM, *P<0.05).
F qRT-PCR of *Kiss1* in liver of ad lib fed, O/N fasted, and refed WT mice. Fasting stimulates liver *Kiss1* expression, refeeding supresses elevated *Kiss1* (mean±SEM, *P<0.05).
G Liver IB from ad lib fed, O/N fasted, and refed WT mice. Fasting stimulates liver kisspeptin1.
H qRT-PCR of *Kiss1* in liver of L-ΔGcgr mice after ip PBS or glucagon. Glucagon does to stimulate *Kiss1* in L-ΔGcgr mice (mean±SEM, * P<0.05).
I (top) Representative liver IB from $Gcgr^{fl/fl}$ and L-ΔGcgr mice. L-ΔGcgr lack GCGR. **(bottom)** Liver IB from $Gcgr^{fl/fl}$ and L-ΔGcgr mice after ip treatment with PBS or glucagon. Baseline kisspeptin1 is similar in $Gcgr^{fl/fl}$ and L-ΔGcgr mouse liver. Glucagon treatment stimulates kisspeptin1 in $Gcgr^{fl/fl}$ but not in L-ΔGcgr mice.
J qRT-PCR of *Kiss1* in liver of ad lib fed and O/N fasted L-ΔGcgr mice. O/N fast does not stimulate *Kiss1* in L-ΔGcgr mouse liver (mean±SEM, *P<0.05).
K Representative liver IB from ad lib fed and O/N fasted $Gcgr^{fl/fl}$ and L-ΔGcgr mice. Baseline kisspeptin1 is similar in $Gcgr^{fl/fl}$ and L-ΔGcgr livers. Fasting stimulates kisspeptin1 in Gcgr fl/fl but not in L-ΔGcgr liver.
L qRT-PCR of *Kiss1* in liver of $Insr^{fl/fl}$ and L-ΔInsr mice. Liver Insr ablation does not affect *Kiss1* expression (mean±SEM, *P<0.05).
M Representative liver IB of $Insr^{fl/fl}$ and L-ΔInsr mice. L-ΔInsr liver lacks insulin receptor immunoreactivity.
N qRT-PCR of *Kiss1* in liver of $Insr^{fl/fl}$ and L-ΔInsr mice after ip treatment with vehicle PBS or glucagon. Glucagon stimulates *Kiss1* in $Insr^{fl/fl}$ mice and more so in L-ΔInsr liver (mean±SEM, *P<0.05).
O Representative liver IB of $Insr^{fl/fl}$ and L-ΔInsr mice after ip PBS or glucagon. Glucagon stimulates stronger liver kisspeptin1 production in L-ΔInsr than in $Insr^{fl/fl}$ liver.

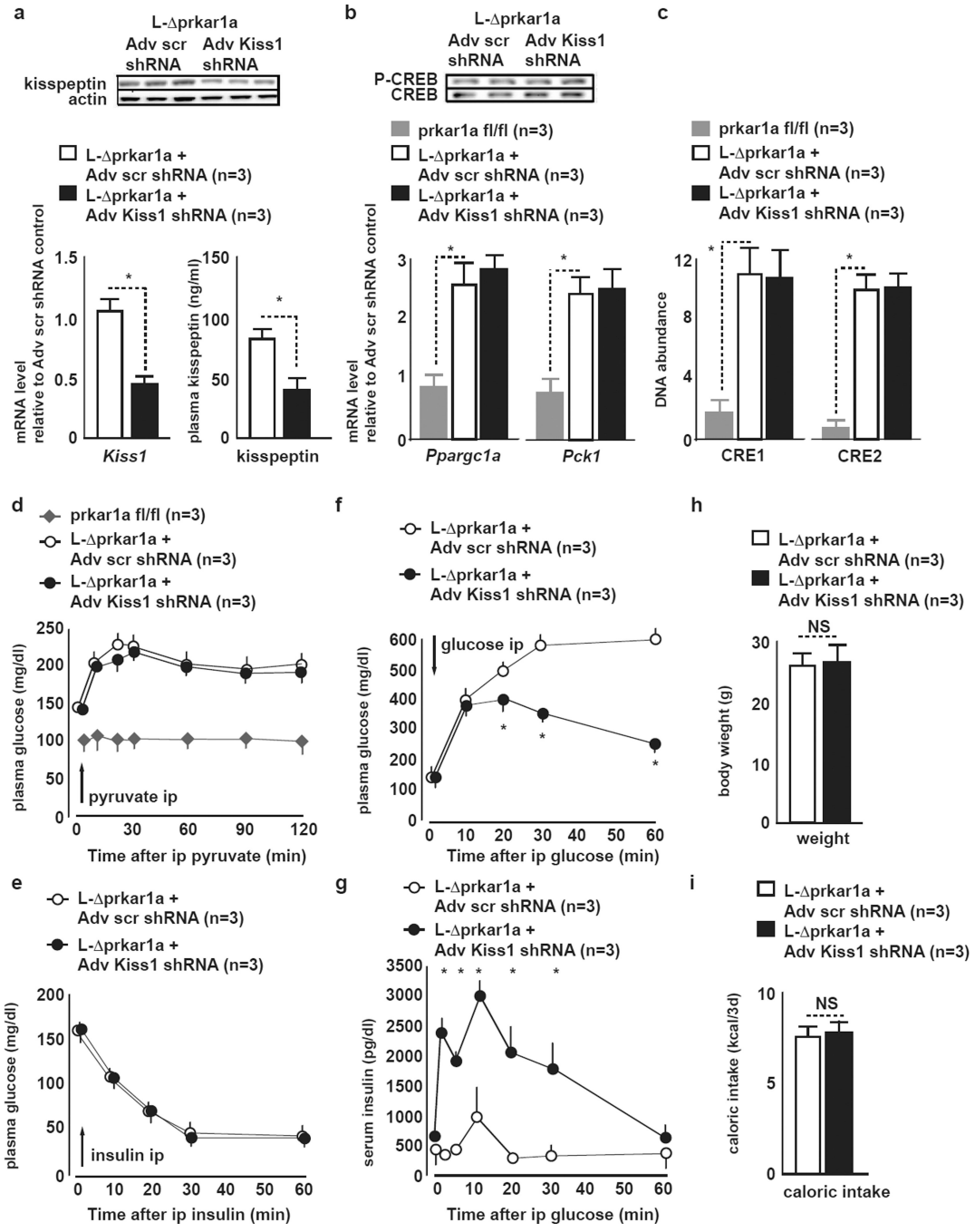

Figure 3. In L-Δprkar1a mice liver *Kiss1* knockdown derepresses GSIS and ameliorates glucose tolerance despite continued upregulated gluconeogenesis

A (top) Liver kisspeptin1 IB, (**bottom left**) liver qRT-PCR of *Kiss1* mRNA, and (**bottom right**) plasma kisspeptin1 in L-Δprkar1a mice 3 days after Adv-scr or -Kiss1 shRNA treatment. Liver kisspeptin1 protein, *Kiss1* mRNA and plasma kisspeptin1 are reduced after Kiss1 *Kiss1* L-Δprkar1a mice (mean±SEM, *P<0.05).

B (top) pCREB and total CREB IB, **(bottom)** qRT-PCR of *Ppargc1a* and *Pck1* in liver of L-Δprkar1a mice 3 days after Adv-scr shRNA or Adv-Kiss1 shRNA treatment. CREB

phosphorylation and total CREB protein are unaffected by Kiss1 knockdown in L-Δprkar1a liver. *Ppargc1a* and *Pck1* mRNA levels are upregulated in L-Δprkar1a as compared to *prkar1a*$^{fl/fl}$ livers and are unaffected by liver *Kiss1* knockdown in L-Δprkar1a mice (mean ±SEM, *P<0.05).

C *In vivo* ChIP of CREB occupancy on CRE1 and CRE2 within the *Kiss1* promoter in liver samples. CREB occupancy on *Kiss1* CRE1 and 2 in L-Δprkar1a liver is increased as compared to *prkar1a*$^{fl/fl}$ liver and unaffected by *Kiss1* knockdown in L-Δprkar1a mice (mean±SEM, *P<0.05).

D ipPCT in fed *prkar1a*$^{fl/fl}$ and L-Δprkar1a mice 3 days after Adv-scr or -Kiss1 shRNA treatment. Gluconeogensis activity is increased in L-Δprkar1a as compared to *prkar1a*$^{fl/fl}$ mice. Gluconeogenesis activity in L-Δprkar1a mice is unaffected by *Kiss1* knockdown (mean±SEM, *P<0.05).

E ipITT in L-Δprkar1a mice 3 days after treatment with Adv-scr or -Kiss1 shRNA. Peripheral insulin sensitivity in L-Δprkar1a mice is unaffected by *Kiss1* knockdown (mean ±SEM, *P<0.05).

F ipGTT in L-Δprkar1a mice 3 days after treatment with Adv-scr or -Kiss1 shRNA. L-Δprkar1a mice with *Kiss1* knockdown show improved GT as compared to controls (mean ±SEM, *P<0.05).

G Serum insulin during ipGTT in L-Δprkar1a mice 3 days after treatment with Adv-scr or -Kiss1 shRNA. GSIS is augmented in L-Δprkar1a mice after liver *Kiss1* knockdown as compared to controls (mean±SEM, *P<0.05).

H Body weight in L-Δprkar1a mice 3 days after treatment with Adv-scr or -Kiss1 shRNA. Body weight in L-Δprkar1a mice is unaffected by *Kiss1* knockdown.

I Caloric intake in L-Δprkar1a mice during 3 days after treatment with Adv-scr or -Kiss1 shRNA. Caloric intake in L-Δprkar1a mice is unaffected by *Kiss1* knockdown.

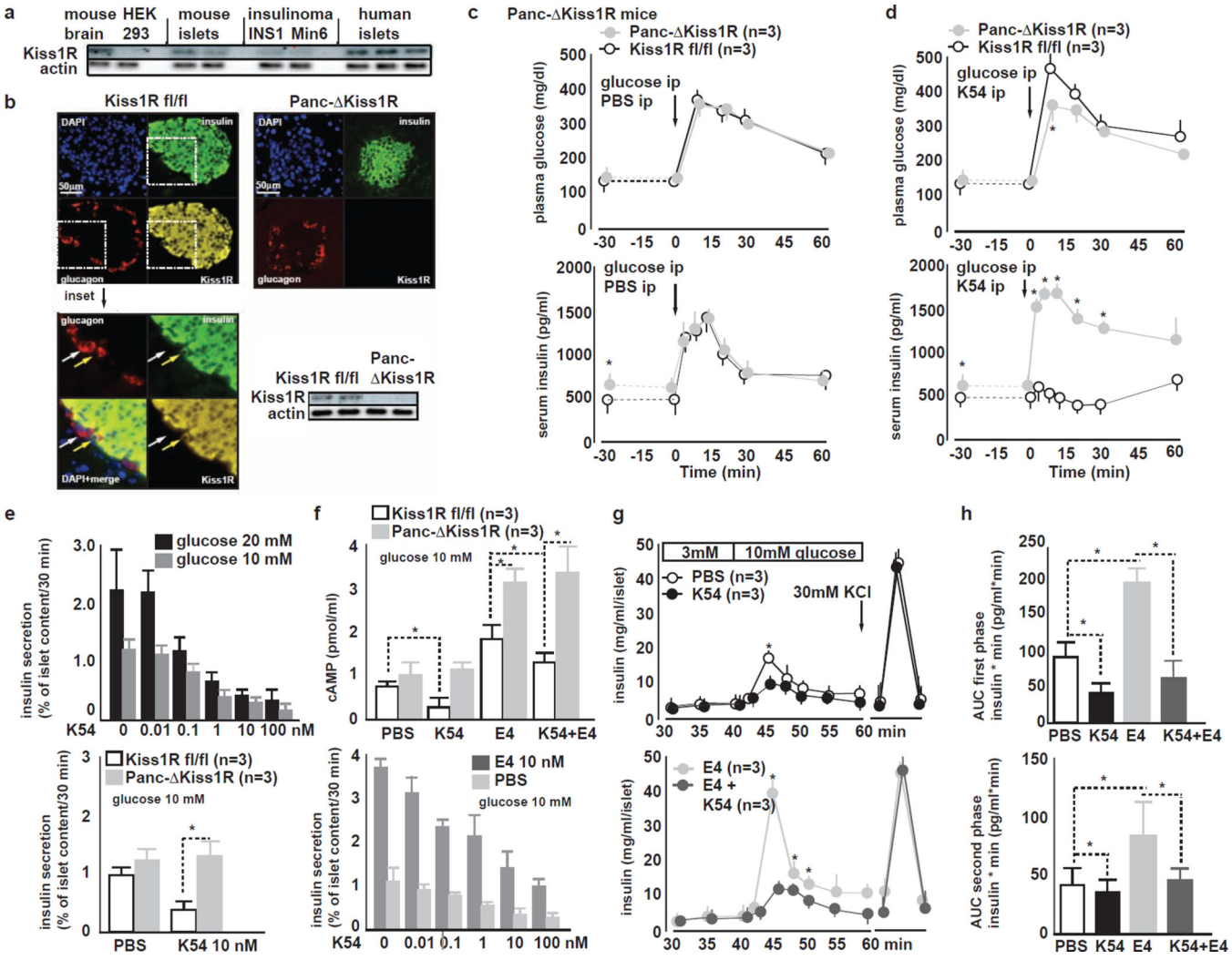

Figure 4. Kisspeptin1 at nanomolar concentrations inhibits GSIS in a Kiss1R-dependent manner. Pancreas Kiss1R is located in β-cells

A Representative IB for Kiss1R mouse brain, HEK 293T cells, mouse islets, INS1 rat insulinoma cells, Min6 mouse insulinoma cells and human islets. Mouse brain, mouse islets, insulinoma cells and human islets express Kiss1R. HEK 293T cells do not expess Kiss1R.

B (left) Immunohistochemistry for insulin, glucagon and Kiss1R in pancreas from *Kiss1R*$^{fl/fl}$ mice. Kiss1R immunoreactivity colocalizes with insulin-positive β-cells but not with glucagon-positive α-cells. 20× magnification. Pseudocoloring: red: glucagon, green: insulin, yellow: Kiss1R, blue: nucleus counter-stain with DAPI **(left bottom)** inset of previous image at 40× magnification.

(right top) Immunohistochemistry for insulin, glucagon and Kiss1R in pancreas from Panc-ΔKiss1R mice. Kiss1R immunoreactivity is lacking in Panc-ΔKiss1R islet.

(right bottom) Representative islet IB from *Kiss1R*$^{fl/fl}$ and Panc-ΔKiss1R mice. Kiss1R is absent in Panc-ΔKiss1R islets.

C ipGTT in Kiss1R$^{fl/fl}$ and Panc-ΔKiss1R mice during ip co-injection of PBS and glucose. **(top)** GT is similar in *Kiss1R*$^{fl/fl}$ and Panc-ΔKiss1R mice. **(bottom)** baseline fasting glucose

is slightly elevated in Panc-ΔKiss1R mice as compared to *Kiss1R*$^{fl/fl}$ littermates. *In vivo* GSIS is similar in *Kiss1R*$^{fl/fl}$ and Panc-ΔKiss1R mice (mean±SEM, *P<0.05).

D ipGTT in *Kiss1R*$^{fl/fl}$ and Panc-ΔKiss1R mice during ip co-injection of 10 nM K54 and glucose. K54 impairs GT **(top)** and GSIS **(bottom)** in *Kiss1R*$^{fl/fl}$ but not in Panc-ΔKiss1R mice (mean±SEM, *P<0.05).

E (top) Dose response curve of K54 and K10 inhibition of GSIS from WT mouse islets during static at 10 or 20 mM glucose. Both K54 and K10 inhibit GSIS in a dose-dependent manner from 0 to 100 nM at both 10 and 20 mM glucose; **(bottom)** GSIS from Kiss1R fl/fl and Panc-ΔKiss1R islets treated with PBS K10 or K54. K10 or K54 (both 10 nM) inhibit GSIS from Kiss1R fl/fl but not from Panc-ΔKiss1R islets.

F cAMP synthesis and GSIS in response to K54 and to incretin analogue exendin-4 (E4) in Kiss1R fl/fl and Panc-ΔKiss1R islets. **(top)** K54 impairs cAMP synthesis in *Kiss1R*$^{fl/fl}$ but not in Panc-ΔKiss1R islets. E4 stimulates cAMP synthesis similarly in both *Kiss1R*$^{fl/fl}$ and in Panc-ΔKiss1R islets. K10 reduces E4-stimulated cAMP levels in *Kiss1R*$^{fl/fl}$ but not in Panc-ΔKiss1R islets. **(bottom)** During static incubation of mouse islets, K54 impairs GSIS and also E4 potentiation of GSIS from islets in a dose dependent manner (mean±SEM, *P<0.05).

G Islet perifusion assay in WT islets in response to K54 and to E4. **(top)** K54 impairs both first and second phasea of GSIS and **(bottom)** E4-potentiated first and second phase GSIS; End of perifusion shows similar insulin release upon KCL induced depolarization (mean ±SEM, *P<0.05).

H Area under the curve (AUC) of **(top)** first and **(bottom)** second phase GSIS from WT mouse islets treated with PBS, K54, E4 or K54+E4. K54 inhibits both first and second phases of GSIS and E4 potentiated GSIS (mean±SEM, *P<0.05).

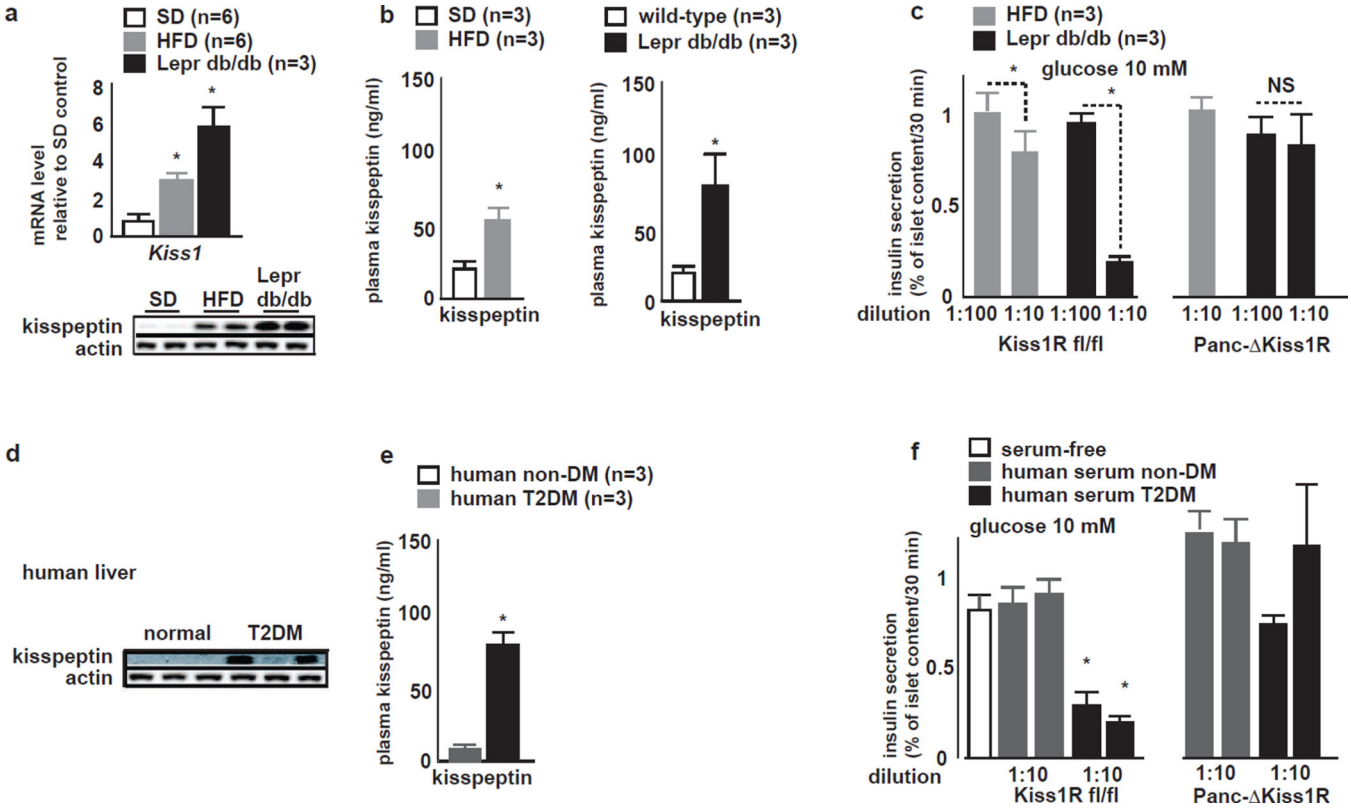

Figure 5. Liver Kisspeptin1 expression and plasma kisspeptin levels are elevated in mouse models of DM and in humans with T2DM

A (top) qRT-PCR for *Kiss1* in liver tissue; and (**bottom**) liver IB for kisspeptin1 in SD, HFD fed and *Lepr*$^{db/db}$ mice. Both *Kiss1* mRNA and kisspepetin1 protein are increased in HFD fed mice and found at higher levels in db/db mouse livers (mean±SEM, *P<0.05).

B Plasma kisspeptin1 in SD, HFD fed and *Lepr*$^{db/db}$ mice. Plasma kisspeptin1 is increased in **(left)** HFD fed and **(right)** in *Lepr*$^{db/db}$ mice as compared to SD fed littermates (mean ±SEM, *P<0.05).

C GSIS from cultured **(left)** Kiss1R$^{fl/fl}$ and **(right)** Panc-ΔKiss1R mouse islets in media conditioned with plasma from HFD fed and Lepr$^{db/db}$ mice. GSIS from *Kiss1R*$^{fl/fl}$ is suppressed during culture in media conditioned with HFD fed or db/db plasma at 1:10 dilution but not at 1:100 dilution. GSIS from Panc-ΔKiss1R islets is unaffected by media conditioned with plasma of HFD fed or *Lepr*$^{db/db}$ mice (mean±SEM, *P<0.05).

D Representative liver IB for kisppeptin1 in humans without DM and humans with T2DM. Humans with T2DM exhibit varying degrees of kisspeptin immunoreactivity in liver tissue.

E Plasma kisspeptin1 in humans without DM and with T2DM. Plasma kisspeptin1 levels are elevated in humans with T2DM as compared to humans without diabetes (mean±SEM, *P<0.05).

F GSIS from cultured **(left)** *Kiss1R*$^{fl/fl}$ and **(right)** Panc-ΔKiss1R mouse islets in media conditioned with plasma from humans with T2DM and without DM. GSIS from Kiss1R$^{fl/fl}$ but not from Panc-ΔKiss1R islets is suppressed during culture in media conditioned with T2DM plasma (mean±SEM, *P<0.05).

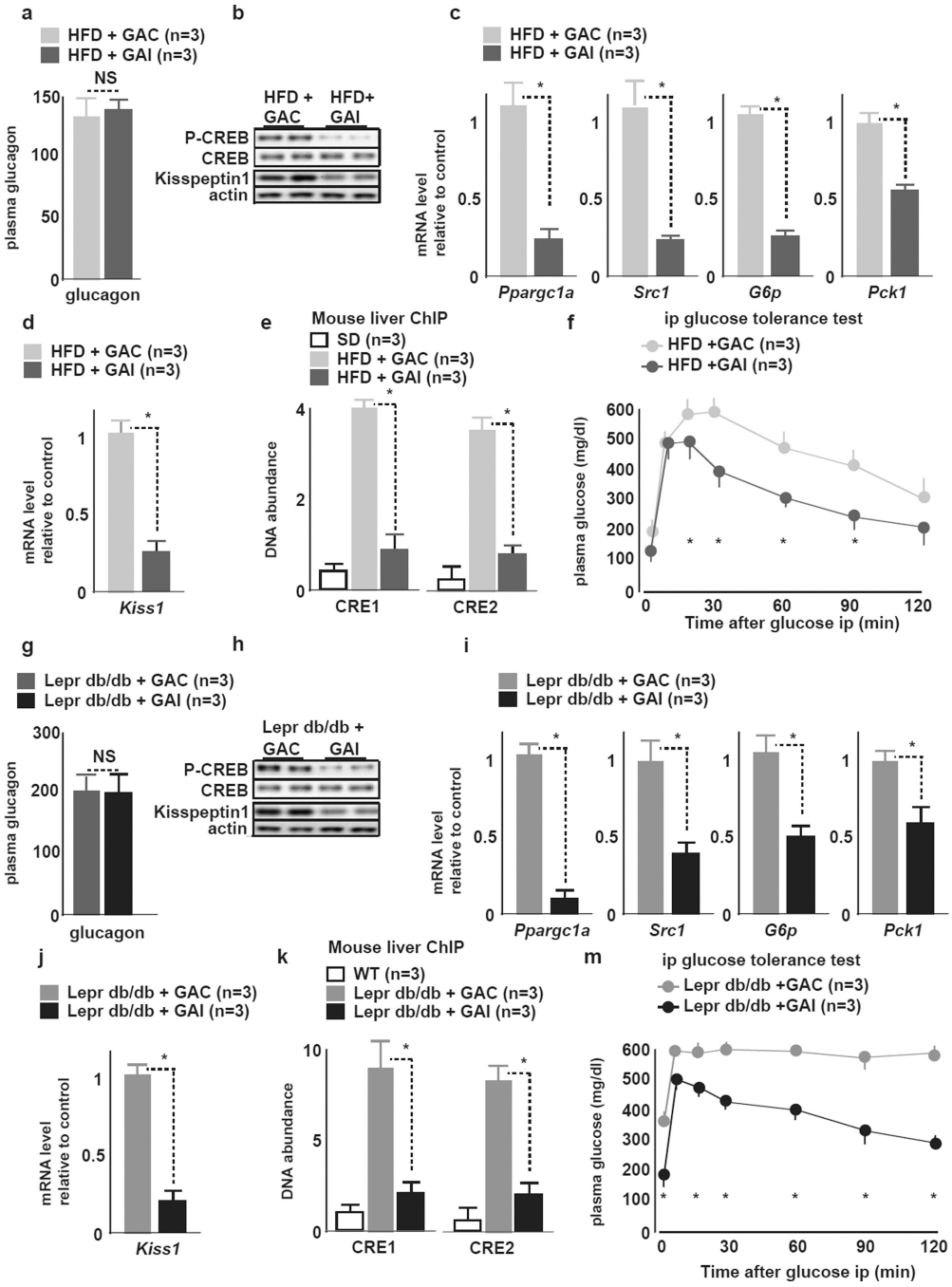

Figure 6. Hyperlucagonemia is linked to liver kisspeptin1 production in HFD fed and *Lepr*$^{db/db}$ mice

HFD fed mice: Panels A–F

Lepr$^{db/db}$ mice: Panels G–M

A, G Plasma glucagon levels in the fed state 60 min after treatment with GAI or GAC. Plasma glucagon levels remain unchanged after GAI or GAC treament.

B, H Representative liver IB for pCREB, total CREB and kisspeptin1 in GAI and GAC treated mice. Phospho-CREB is reduced in mice treated with GAI but not GAC.

C, I qRT-PCR of indicated genes of the gluconeogenic program in livers of GAI or GAC treated mice. GAI but not GAC treatment downregulates *Pparg1a, Src1, G6P* and *Pck1* mRNA (mean±SEM, *P<0.05).

D, J qRT-PCR of *Kiss1* in livers of GAI and GAC treated mice. GAI but not GAC treatment downregulates liver *Kiss1* mRNA (mean±SEM, *P<0.05).

E, K *In vivo* ChIP of CREB occupancy on **(left)** CRE1 and **(right)** CRE2 half-sites of the *Kiss1* promoter in livers of GAI or GAC treated SD mice, HFD and $Lepr^{db/db}$ mice. GAI reduces CREB occupancy on *Kiss1* CRE 1 & 2 to levels similar to those in control mice (mean±SEM, *P<0.05).

F, L ipGTT in GAI or GAC treated mice. GAI treatment improves GT as compared to GAC treatment (mean±SEM, *P<0.05).

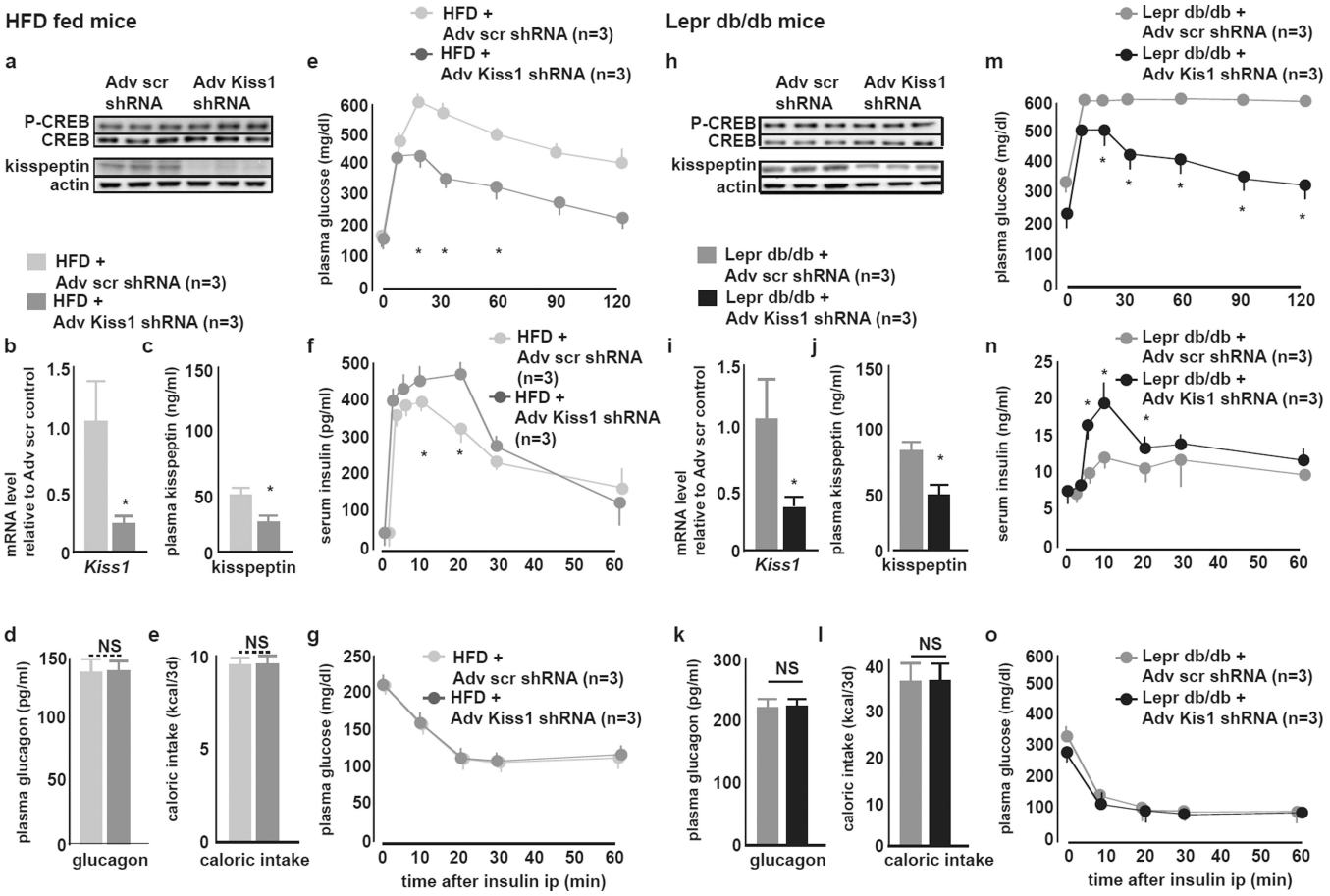

Figure 7. Kiss1 shRNA knockdown *in vivo* in livers of in HFD and *Lepr*$^{db/db}$ mice de-represses GSIS and glucose tolerance

HFD mice: Panels A–H

Lepr$^{db/db}$ mice: Panels I–P

A, I Representative liver IB of pCREB, total CREB and kisspeptin1 3 days after treatment with Adv-scr or -Kiss1 shRNA. Liver pCREB is not affected and liver kisspeptin1 protein is reduced by *Kiss1* knockdown.

B, J qRT-PCR of *Kiss1* in livers 3 days after treatment with Adv-scr or -Kiss1 shRNA. Adv-Kiss1 shRNA downregulates liver *Kiss1* mRNA levels as compared to Adv-scr shRNA treatment (mean±SEM, *P<0.05).

C, K Plasma kisspeptin1 levels 3 days after treatment with Adv-scr or -Kiss1 shRNA. Liver *Kiss1* knockdown reduces plasma kisspeptin1 (mean±SEM, *P<0.05).

D, L Plasma glucagon levels 3 days after treatment with Adv-scr or -Kiss1 shRNA. Liver *Kiss1* knockdown does not change plasma glucagon levels (mean±SEM0.

E, M Caloric intake in during 3 days after treatment with Adv-scr or -Kiss1 shRNA. Caloric intake is unaffected by *Kiss1* knockdown (mean±SEM).

F, N ipGTT 3 days after treatment with Adv-scr or -Kiss1 shRNA. GT is improved after *Kiss1* knockdown (mean±SEM, *P<0.05).

G, O GSIS during ipGTT 3 days after treatment with Adv-scr or -Kiss1 shRNA. GSIS is improved after *Kiss1* knockdown (mean±SEM, *P<0.05).

H, P ipITT 3 days after treatment with Adv-scr or -Kiss1 shRNA. Insulin tolerance is not different after *Kiss1* knockdown.