PAM haploinsufficiency does not accelerate the development of diet- and human IAPP-induced diabetes in mice

Yi-Chun Chen[1], **Richard E. Mains**[2], **Betty A. Eipper**[2], **Brad G. Hoffman**[1], **Traci A. Czyzyk**[3], **John E. Pintar**[4], **C. Bruce Verchere**[1,5,6]

[1]Department of Surgery, University of British Columbia and BC Children's Hospital Research Institute, 950 West 28th Avenue, Vancouver, BC V5Z 4H4, Canada

[2]Department of Neuroscience, University of Connecticut Health Center, Farmington, CT, USA

[3]Division of Cardio-renal and Metabolic Disease, Merck Research Laboratories, San Francisco, CA, USA

[4]Department of Neuroscience and Cell Biology, Rutgers Robert Wood Johnson Medical School, Piscataway, NJ, USA

[5]Department of Pathology and Laboratory Medicine, University of British Columbia and BC Children's Hospital Research Institute, Vancouver, BC, Canada

[6]Centre for Molecular Medicine and Therapeutics, University of British Columbia, Vancouver, BC, Canada

Abstract

Aims/hypothesis—Peptide hormones are first synthesised as larger, inactive precursors that are converted to their active forms by endopeptidase cleavage and post-translational modifications, such as amidation. Recent, large-scale genome-wide studies have suggested that two coding variants of the amidating enzyme, peptidylglycine α-amidating monooxygenase (PAM), are associated with impaired insulin secretion and increased type 2 diabetes risk. We aimed to elucidate the role of PAM in modulating beta cell peptide amidation, beta cell function and the development of diabetes.

Methods—PAM transcript and protein levels were analysed in mouse islets following induction of endoplasmic reticulum (ER) or cytokine stress, and PAM expression patterns were examined in human islets. To study whether haploinsufficiency of PAM accelerates the development of diabetes, $Pam^{+/-}$ and $Pam^{+/+}$ mice were fed a low-fat diet (LFD) or high-fat diet (HFD) and glucose homeostasis was assessed. Since aggregates of the PAM substrate human islet amyloid

C. Bruce Verchere bverchere@bcchr.ca.

polypeptide (hIAPP) lead to islet inflammation and beta cell failure, we also investigated whether PAM haploinsufficiency accelerated hIAPP-induced diabetes and islet amyloid formation in $Pam^{+/-}$ and $Pam^{+/+}$ mice with beta cell expression of hIAPP.

Results—Immunostaining revealed high expression of PAM in alpha, beta and delta cells in human pancreatic islets. Pam mRNA and PAM protein expression were reduced in mouse islets following administration of an HFD, and in isolated islets following induction of ER stress with thapsigargin, or cytokine stress with IL-1β, IFN-γ and TFN-α. Despite $Pam^{+/-}$ only having 50% PAM expression and enzyme activity as compared with $Pam^{+/+}$ mice, glucose tolerance and body mass composition were comparable in the two models. After 24 weeks of HFD, both $Pam^{+/-}$ and $Pam^{+/+}$ mice had insulin resistance and impaired glucose tolerance, but no differences in glucose tolerance, insulin sensitivity or plasma insulin levels were observed in PAM haploinsufficient mice. Islet amyloid formation and beta cell function were also similar in $Pam^{+/-}$ and $Pam^{+/+}$ mice with beta cell expression of hIAPP.

Conclusions/interpretation—Haploinsufficiency of PAM in mice does not accelerate the development of diet-induced obesity or hIAPP transgene-induced diabetes.

Introduction

Neuroendocrine hormones are biological messengers secreted by neurons or endocrine glands, acting on target tissues and coordinating whole-body homeostasis, including metabolism. Peptide hormones are a class of neuroendocrine hormones that are made as larger and inactive precursors; they are processed to smaller and functionally active hormones via sequential peptide cleavage mediated by endopeptidases. Many peptide hormones also undergo post-translational modifications, including C-terminal amidation, which occurs in more than 50% of neuroendocrine peptides and is mediated exclusively by the enzyme peptidylglycine α-amidating monooxygenase (PAM) [1].

The essential role of PAM has been demonstrated in multiple model organisms. For example, the absence of Pam in mice and zebrafish leads to embryonic lethality [2–4]. Pam whole-body heterozygous knockout mice have a myriad of behavioural defects and develop increased adiposity and mild glucose intolerance with age [2, 5]. Two PAM coding variants (rs35658696 and rs78408340) were recently reported to be associated with reduced insulinogenic index and elevated risk of type 2 diabetes in humans [6–10]. Notably, pancreatic islets from PAM variant rs35658696 donors were found to have mildly impaired glucose-stimulated insulin secretion, possibly due to defective granule trafficking and exocytosis [9]. The authors implicated decreased amidation and function of chromogranin A as a possible mechanism underlying the impaired beta cell function seen in carriers of PAM variants. Together, the available data suggest that PAM activity is important for normal beta cell function.

PAM has two enzymatically active subunits, peptidylglycine α-hydroxylating monooxygenase (PHM) and peptidyl-α-hydroxyglycine α-amidating lyase (PAL). Peptides that have a COOH-terminal glycine residue are first converted into hydroxyl-glycine intermediates by PHM; these intermediates are then cleaved by PAL, yielding COOH-terminally amidated peptides [1]. Although the catalytic cores of PHM and PAL have been structurally characterised [11, 12], how the PAM variants rs35658696 and rs78408340 (both of which reside in the PAL domain) alter enzyme structure and reduce PAM activity or stability is not fully understood. PAM activity is also regulated by a number of hormonal and environmental stimuli. For example, mice fed a copper-deficient diet have increased serum PAM activity [5] and neuroendocrine cells cultured under hypoxic conditions have lowered abundance of amidated peptides [13]. Whether pathological stresses present in diabetes may affect PAM expression or activity remains unclear.

In pancreatic islets, there are a number of amidated peptides that are thought to play roles in coordinating intracellular communication, maintaining beta cell function, and supporting cell survival, including chromogranin A, glucagon-like peptide-1 (GLP-1), cholecystokinin (CCK), neuropeptide Y (NPY) and islet amyloid polypeptide (IAPP). Among these PAM product peptides, IAPP is the second most abundant peptide made in islets. Human, but not rodent, IAPP aggregates to form islet amyloid deposits in type 2 diabetes, likely contributing to islet inflammation and beta cell failure [14–16]. It is plausible that PAM-mediated IAPP amidation could alter IAPP aggregation kinetics and, by extension, that PAM deficiency could influence IAPP-related islet pathology [17].

We aimed to determine whether and how PAM haploinsufficiency might contribute to insulin secretory defects and the development of diabetes. We examined PAM expression in human islet cells, and evaluated PAM expression in rodent islets following exposure to endoplasmic reticulum (ER) or cytokine stress. We studied the impact of PAM deficiency on glucose homeostasis and diabetes development using two mouse models: mice challenged with a high-fat diet (HFD) or mice with beta cell expression of amyloidogenic human IAPP (hIAPP). These studies may provide insight into the role of PAM and islet peptide amidation in beta cell function and glucose homeostasis.

Methods

Human pancreas and islet tissue

Paraformaldehyde-fixed, paraffin-embedded pancreatic tissue sections were received from the Network for Pancreatic Organ Donors with Diabetes (nPOD) via coordination through the University of Florida (Gainesville, FL, USA) [18]. Human islets were acquired from the Clinical Islet Laboratory at the University of Alberta [19]. Upon arrival, islets were hand-picked, washed in Dulbecco's phosphate-buffered saline and stored at − 20°C. The use of human pancreatic tissue was approved by the BC Children's Hospital Institutional Review Board. Details of the organ donor characteristics are presented in electronic supplementary material (ESM) Table 1; the checklist for reporting human islet preparations used in research is also presented in the ESM.

Animal studies

C57BL/6J and hIAPP transgenic mice (hIAPP Tg/0; FVB/N-Tg(Ins2-*IAPP*)RHFSoel/J) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). *Pam*^{+/−} mice (backcrossed more than ten generations onto the C57BL/6J background) were obtained from the University of Connecticut Health Center (Farmington, CT, USA), and maintained on a 14/10 h light/dark cycle, with a chow diet (6% fat; Teklad 2918, Huntingdon, UK) and water ad libitum, in the animal facility of BC Children's Hospital Research Institute (Vancouver, BC, Canada) [2]. hIAPP Tg/0 *Pam*^{+/−} mice and littermate control mice (hIAPP Tg/0 *Pam*^{+/+}, hIAPP 0/0 *Pam*^{+/+} and hIAPP 0/0 *Pam*^{+/−}) were generated by crossing *Pam*^{+/−} males with hIAPP Tg/0 females (all experimental mice were on a mixed first filial generation (F1) FVB/N × C57BL/6J background). For metabolic studies, mice were assigned to receive either a low-fat diet (LFD; 10% fat; D12450H; Research Diets, New Brunswick, NJ, USA) or an HFD (45% fat; D12451; Research Diets), through a simple randomisation method, starting at 8 weeks of age. Metabolic assays were performed in a blinded fashion and are described in the ESM Methods; briefly these included body weight, blood glucose (by glucometer), intraperitoneal glucose tolerance test (IPGTT), intraperitoneal insulin tolerance test (IPITT), plasma insulin (by ELISA), and lean and fat mass (using a body composition analyser). All protocols were approved by the Animal Care and Use Committee at the University of British Columbia.

Islet studies

Islets from mice and Lewis rats (purchased from Charles Rivers Laboratory, Sherbrooke, QC, Canada) were isolated and cultured as described [20], and insulin secretion was assessed via ELISA, as described in the ESM Methods. Prior to measurement of PAM protein and mRNA levels, mouse or rat islets were treated for 72 h with 25 mmol/l glucose, or 16 h with 1 μmol/l thapsigargin (stock solution dissolved in DMSO; Sigma-Aldrich, St Louis, MO, USA), or 16 h with a proinflammatory cytokine cocktail (50 U/ml IL-1β, 1000 U/ml IFN-γ and 1000 U/ml TNF-α; R&D systems, Oakville, ON, Canada). PAM, amidated IAPP and non-amidated IAPP protein expression levels were analysed by immunoblotting using anti PAM (JH1761; Eipper and Mains laboratories, Farmington, CT, USA) [21], anti-amidated IAPP (F025; MedImmune, San Diego, CA, USA), and anti-non-amidated IAPP (F084; MedImmune) antibodies, respectively (see ESM Table 2 for antibody information). *Pam, Ins2, Iapp, Scg5, Ddit3, Nos2, rplP and B2m* transcript levels were analysed using SYBR Green-based (Fast SYBR; Invitrogen, Burlington, ON, Canada) quantitative real-time PCR (ViiA7 Real-Time PCR System; Applied Biosystems, Waltham, MA, USA). Primer sequences are listed in ESM Table 3.

Immunostaining

PAM expression in human islet cells was analysed by immunofluorescence staining using primary antibodies against PAM (JH1761), insulin, glucagon and somatostatin, and secondary Alexa Fluor-conjugated antibodies (ESM Table 2). Islet amyloid was analysed by thioflavin S staining (Sigma), as described previously [22]. Images were acquired using a Leica TCS SP5 confocal microscope (Leica Microsystems, Concord, ON, Canada). Amyloid

severity was quantified in a blinded fashion by CellProfiler (Cambridge, MA, USA) and presented as per cent thioflavin S$^+$ area/insulin$^+$ area.

PHM and PAL activity assays

Islet PHM and PAL enzyme activity were assayed as described [23]. In brief, 100 hand-picked islets were washed in Dulbecco's phosphate-buffered saline (Thermo Scientific, Waltham, MA, USA), frozen, and lysed in 20 mmol/l *N*-[Tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid sodium salt (sodium TES; pH 7.4), 10 mmol/l mannitol, 1 mg/ml BSA, 1% (vol./vol.) Triton X-100 (Surfact-Amps X-100; Thermo Scientific) buffer. PHM activity was measured using in-house generated- and ^{125}I-labelled acetyl-Tyr-Val-Gly as a substrate and PAL was measured using in-house generated- and ^{125}I-labelled acetyl-Tyr-Val-α-hydroxyglycine as substrate. Specific activity is expressed as picomoles of the product formed per h (pmol/h) per μg protein in islet lysates.

Identification of PAM transcript isoforms in human and mouse beta cells

Reads from human and mouse beta cell RNA sequencing (RNA-Seq) libraries (GSE116559 [www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE116559] and GSE73289 [www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc= GSE73289]) [24] were assembled into transcripts using StringTie (https://ccb.jhu.edu/software/stringtie/; accessed 4 July 2019), with reference transcriptomes (GRCh38.89 [[ftp://ftp.ensembl.org/pub/current_gtf/homo_sapiens/; accessed 4 July 2019] and University of California Santa Cruz [UCSC] Genome Browser known genes [https://genome.ucsc.edu/cgi-bin/hgGateway, accessed 17 May 2016]) used to guide the assembly and allow for the identification of known and unknown PAM isoforms [25, 26]. Diagrams of human and mouse PAM isoforms were generated using Gene Structure Display Server 2.0 (GSDS; http://gsds.cbi.pku.edu.cn/) [27], with domains highlighted based on UniProt (www.uniprot.org/; accessed 15 July 2019) protein domain annotation and a reference publication [28].

Statistical analysis

Datasets were first analysed for normal distribution. For datasets that passed the normality test, differences between groups were examined for significance (p value <0.05) using either a two-tailed Student's t test, or one-way ANOVA followed by Tukey's post test. For datasets that failed the normality test, non-parametric tests (Mann–Whitney or Kruskal–Wallis) followed by Dunn's post test were performed. Diabetes incidence was displayed as survival curves and analysed by logrank test. All statistical analysis was performed using Prism 8 (GraphPad Software, San Diego, CA, USA).

Results

PAM is expressed in human and mouse islet cells

Coding variants of *PAM* are associated with reduced insulinogenic index, suggesting that PAM defects might have an impact on islet function. We first analysed PAM expression patterns in human and mouse islets by double-immunofluorescence staining for PAM and islet hormones. We found immunoreactivity for PAM in insulin$^+$, glucagon$^+$ and somatostatin$^+$ cells in pancreases from non-diabetic donors (Fig. 1a), in keeping with

immunostaining findings of others [9] and publicly available transcriptome data indicating expression of *PAM* in human islet cells [29]. The pattern of PAM expression in mouse islets was similar (Fig. 1b), with robust PAM immunoreactivity observed in all murine insulin[+], glucagon[+] and somatostatin^{+} cells. To compare and identify novel PAM transcript isoforms expressed in human and mouse beta cells, we used RNA-Seq data from an online database [24] and an in-house database. Sequence alignment and protein domain prediction showed that one of the major *PAM* isoforms, isoform A (which encodes the PAM3 protein), does not contain the transmembrane domain. We also identified the novel *PAM* isoform B, which lacks part of the PHM domain (Fig. 1c). The *Pam* transcript sequence is well-conserved in rodent beta cells, with all three isoforms encoding the full-length PAM protein with small sequence variations. Isoform A encodes a 979 amino-acid-long PAM1 protein, isoform B is an in-frame deletion of PAM1 and encodes a 978 aminoacid-long protein, and isoform C encodes PAM2 protein (lacking exon 14 in the linker region) (Fig. 1d). To evaluate PAM protein expression, we analysed isolated islets from mouse, rat and a non-diabetic human donor by immunoblotting, using an antibody raised against rat PHM domain (JH1761). Because the JH1761 antibody has higher affinity toward rat PAM, we adjusted the amount of islet lysate loaded to reveal PAM expression patterns in mouse, rat and human islets. We found that islet PAM protein expression patterns are comparable among all three species (Fig. 1e).

Cell stresses present in diabetes could have an impact on PAM expression and activity. We found that 3 day culture of mouse islets in high (25 mmol/l) glucose induced a 2.4-fold increase in *Pam* mRNA levels (Fig. 2a). By contrast, islets from mice fed an HFD for 14 weeks had reduced *Pam* transcript levels (Fig. 2b). Treatment of islets ex vivo with the ER stress inducer thapsigargin led to a significant increase in the expression of the ER stress gene *Ddit3*, whereas proinflammatory cytokines led to increased expression of the cytokine-responsive element *Nos2* (Fig. 2d, e). Thapsigargin and cytokines both caused moderate reductions in *Pam* transcript (Fig. 2c) and cytokine treatment reduced levels of PAM protein (Fig. 2f). Of note, the expression of islet peptide hormones were also significantly reduced upon ER stress (*Ins2*) or cytokine treatment (*Ins2, Iapp* and *Scg5*) and significantly upregulated by glucose treatment (*Scg5*), suggesting that PAM expression may be a reflection of beta cell activity and health (ESM Fig. 1).

Normal glucose metabolism in PAM haploinsufficient mice

To determine whether reduced expression of *Pam* in vivo contributes to islet dysfunction and the development of diabetes, we performed metabolic tests on *Pam*^{+/−} mice. Using antibodies against PAM, amidated IAPP and non-amidated IAPP (Fig. 3a), we found that both PAM levels (Fig. 3b) and enzyme activity were reduced by 50% (Fig. 3c, d), and non-amidated IAPP was elevated by approximately 2.7-fold (Fig. 3b) in *Pam*^{+/−} islets. Of note, amidated IAPP levels were comparable in *Pam*^{+/−} and *Pam*^{+/+} islets, suggesting that one *Pam* allele is sufficient for normal peptide amidation to occur, as for hypothalamic thyrotropin releasing hormone (TRH) [30].

When fed an LFD, both male (Fig. 4a, b) and female (Fig. 4e, f) *Pam*^{+/−} mice and their *Pam*^{+/+} littermates displayed comparable weight gain and fasting blood glucose levels over a 6

month period. In addition, male (Fig. 4c, d) and female (Fig. 4g, h) $Pam^{+/-}$ mice fed an LFD for 8 or 16 weeks displayed similar glucose tolerance compared with $Pam^{+/+}$ mice. Interestingly, despite having comparable adiposity (Fig. 4i, j; also shown as per cent body weight in ESM Fig. 2a, b), when fed an LFD, male $Pam^{+/-}$ mice had slightly improved insulin sensitivity vs $Pam^{+/+}$ mice (Fig. 4k). No differences were observed in female $Pam^{+/-}$ and $Pam^{+/+}$ LFD-fed-mice (Fig. 4l–n and ESM Fig. 2e, f). There were no differences in peripheral organ tissue weights (heart, liver, pancreas, epididymal white adipose tissue and inguinal white adipose tissue) in 32-week-old LFD-fed $Pam^{+/-}$ and $Pam^{+/+}$ mice (ESM Fig. 3a–e, males; ESM Fig. 3f–j, females).

PAM haploinsufficiency does not accelerate the development of diet-induced glucose intolerance

Next, we challenged $Pam^{+/-}$ and $Pam^{+/+}$ mice with an HFD to determine whether loss of one *PAM* allele promotes the development of diet-induced obesity and glucose intolerance. We found that HFD-fed $Pam^{+/-}$ and $Pam^{+/+}$ mice had comparable weight gain, fasting blood glucose levels and glucose tolerance over the 6 month diet regimen, in both males (Fig. 5a–d) and females (Fig. 5e–h). Body mass composition, as well as tissue weights, were similar in HFD-fed $Pam^{+/-}$ and $Pam^{+/+}$ mice (males: Fig. 5i, j, ESM Fig. 2c, d, ESM Fig. 4a–e,; females: Fig. 5l, m, ESM Fig. 2 g, h, ESM Fig. 4f–j). The improved insulin sensitivity observed in $Pam^{+/-}$ male mice fed an LFD (Fig. 4k) was not present when mice were fed HFD (Fig. 5k). Thus, PAM haploinsufficiency does not accelerate HFD-induced obesity and glucose intolerance.

$PAM^{+/-}$ and $PAM^{+/+}$ mice have similar beta cell function

To determine whether reduced expression of PAM leads to changes in plasma insulin levels, we analysed fasting plasma insulin levels in mice fed an LFD (males, Fig. 6a; females, Fig. 6b) or an HFD (males, Fig. 6c; females, Fig. 6d). Mice fed an HFD displayed moderate, but non-significant, elevation of fasting plasma insulin levels, compatible with diet-induced insulin resistance [31]; however, insulin levels did not differ between $Pam^{+/-}$ and $Pam^{+/+}$ mice. To directly assess the effect of PAM deficiency in islet insulin secretion, we measured glucose-stimulated insulin secretion in isolated $Pam^{+/-}$ and $Pam^{+/+}$ islets. Islets from $Pam^{+/-}$ and $Pam^{+/+}$ mice had comparable glucose-stimulated insulin secretion and insulin content (Fig. 6e, f).

PAM haploinsufficiency does not have an impact on glucose intolerance induced by beta cell hIAPP overexpression

The amyloid-forming beta cell peptide hIAPP is a PAM substrate. To understand whether PAM loss affects hIAPP fibril formation and hIAPP-induced beta cell dysfunction and diabetes we next generated $Pam^{+/-}$ mice with beta cell expression of hIAPP (hIAPP Tg/0 $Pam^{+/-}$). While hIAPP introduction induced mild yet statistically significant glucose intolerance in $Pam^{+/+}$ and $Pam^{+/-}$ mice (Fig. 7a), it did not significantly alter islet PAM levels in $Pam^{+/+}$ mice, nor did it alter the ~50% reduction of PAM protein typically seen between $Pam^{+/-}$ and $Pam^{+/+}$ mice (Fig. 7b). Overexpression of hIAPP did not appear to alter PAM enzyme activity, since we observed similar PHM and PAL enzyme activity in hIAPP Tg/0 $Pam^{+/-}$ and control hIAPP 0/0 $Pam^{+/-}$ islets (Fig. 7c, d). As observed in mice with a

C57BL/6J back-ground (Fig. 3b), non-amidated IAPP levels were increased in hIAPP 0/0 $Pam^{+/-}$ compared with hIAPP 0/0 $Pam^{+/+}$ mouse islets, while levels of amidated IAPP were unchanged among the groups (Fig. 7b), suggesting that PAM levels do not limit the amidation of IAPP.

To determine if PAM haploinsufficiency accelerates the development of hIAPP-induced diabetes, we placed 10-week-old hIAPP Tg/0 $Pam^{+/-}$ mice and their littermates on a LFD for 24 weeks. While gaining similar weight (Fig. 8a), mice carrying the hIAPP transgene displayed significantly elevated fasting plasma glucose levels (Fig. 8b; $p < 0.05$) and, by the age of 28 weeks, mice had developed mild glucose intolerance (Fig. 8c, 18-week-old mice; Fig. 8d, 28-week-old mice) compared with wild-type littermates, as we have previously observed [32]. The degree of glucose intolerance was comparable between hIAPP Tg/0 $Pam^{+/-}$ and hIAPP Tg/0 $Pam^{+/+}$ mice. We also found no differences in insulin sensitivity or body fat composition between groups of mice (Fig. 8e–g).

To determine whether *Pam* expression impacts susceptibility to HFD-induced diabetes in mice with beta cell expression of hIAPP, we fed 10-week-old hIAPP Tg/0 $Pam^{+/-}$ mice and their littermates an HFD for 24 weeks. While displaying similar weight gain (Fig. 9a), mice carrying the hIAPP transgene had significantly higher fasting blood glucose levels (Fig. 9b) and a higher incidence of diabetes (fasting blood glucose >15 mmol/l for two consecutive measurements) than non-transgenic littermates (Fig. 9c). There was no significant difference in glucose tolerance, insulin sensitivity or body mass composition between hIAPP Tg/0 $Pam^{+/-}$ and hIAPP Tg/0 $Pam^{+/+}$ mice (Fig. 9d–h).

PAM haploinsufficiency does not impact hIAPP-induced beta cell dysfunction and islet amyloid formation

hIAPP Tg/0 $Pam^{+/-}$ mice displayed similar fasting plasma insulin levels compared with their littermates (Fig. 10a, b, 16 weeks and 24 weeks post-LFD, respectively; Fig. 10c, d, 16 weeks and 24 weeks post-HFD, respectively). To gain insight into whether PAM deficiency leads to changes in insulin secretion or amyloid formation, we also studied islets ex vivo. Islets from hIAPP Tg/0 $Pam^{+/-}$ and hIAPP Tg/0 $Pam^{+/+}$ mice released similar amounts of insulin in response to glucose and had comparable insulin content (Fig. 10e, f). Following culture in high (25 mmol/l) glucose medium for 3 or 7 days, thioflavin S$^+$ islet amyloid [22] was readily observed in both hIAPP Tg/0 $Pam^{+/-}$ and hIAPP Tg/0 $Pam^{+/+}$ islets (Fig. 10g, h). hIAPP Tg/0 $Pam^{+/-}$ and hIAPP Tg/0 $Pam^{+/+}$ islets developed similar amyloid burden at both time points (Fig. 10i, j). These data suggest that haploinsufficiency of PAM does not alter insulin secretion or amyloid formation in hIAPP-expressing mouse islets.

Discussion

The neuroendocrine system secretes a variety of hormones that mediate physiological and behavioural responses, including food intake and metabolism [25]. In the endocrine pancreas, islet cells release peptide hormones to maintain glucose homeostasis, and defects in the processing or post-translational modification of these hormones can lead to a shortage of biologically active peptide and result in metabolic dysregulation and diabetes [26]. Polymorphisms in *PAM*, the gene encoding the peptide amidating enzyme PAM, are

associated with disease risk. The *PAM* variant rs13175330 was associated with hypertension in a Korean population [33] and the *PAM* rs35658696 SNP is associated with reduced insulinogenic index [6]. Both *PAM* rs35658696 and rs78408340 variants are associated with increased type 2 diabetes risk [7, 8]. Importantly, the missense variant rs35658696 (encoding p.Asp563Gly) of *PAM* has a minor allele frequency close to 5%, suggesting that approximately one in ten people are *PAM*-variant carriers. PAM may play a direct role in regulating insulin secretion, because islets from carriers of the *PAM* variant rs35658696 have modestly reduced glucose-stimulated insulin secretion [9]. In the same report, the authors suggested that the mutation of PAM likely leads to loss of protein expression (rs78408340) or reduced enzyme activity (rs35658696). In order to elucidate the pathophysiological mechanism underlying PAM deficiency and the development of diabetes, we studied *Pam* whole-body heterozygous knockout mice (to mirror the partial loss of PAM activity in all tissues, analogous to human *PAM*-variant carriers [9]). We found that $Pam^{+/-}$ mice have similar glucose tolerance compared with $Pam^{+/+}$ littermates, suggesting that haploinsufficiency of PAM does not affect whole-body glucose utilisation. Although we observed no differences in body weight between $Pam^{+/-}$ and $Pam^{+/+}$ mice, male (but not female) $Pam^{+/-}$ mice fed an LFD had modestly improved insulin sensitivity. PAM may play a role in the regulation of insulin sensitivity through amidation of neuropeptide substrates, including α-melanocyte-stimulating hormone (αMSH), CCK, NPY or chromogranin A [34–38].

We observed no difference in beta cell function in vivo or in isolated islets in mice that were haploinsufficient for PAM, as compared with controls. While Thomsen et al recently described a mild decrease in glucose-stimulated insulin secretion in human islets from donors that were carriers of the *PAM* variant rs35658696, and silencing *PAM* in EndoC-βH1 cells led to mild reduction of insulin content/secretion [9], our data in mice would suggest that the modest impairment in insulin secretion and increased type 2 diabetes risk in *PAM*-variant carriers is unlikely to be explained by reduced PAM activity. Species differences in glucose metabolism and the capacity of islets to adapt to environmental challenges could also contribute to the lack of metabolic phenotype in $Pam^{+/-}$ mice. C57BL/6 mice, used in most metabolic studies (including this study), do not develop diabetes spontaneously and, even on an HFD, rodent beta cells have higher proliferative capacity to compensate for increased metabolic demands. In contrast, in humans type 2 diabetes is a polygenic disorder that often develops in middle age, when metabolic and inflammatory stresses may increase and islets have reduced tissue remodelling capacity. In this setting, *PAM* polymorphism carriers may display insulin secretion defects that confer increased type 2 diabetes risk.

Thomsen et al proposed that deficient amidation of a secretory granule protein, chromogranin A, might be one contributor to the observed beta cell dysfunction [9]. However, the precise mechanism linking PAM to beta cell function remains an open question. We found that islets from $Pam^{+/-}$ mice contained amounts of amidated IAPP similar to that in islets of $Pam^{+/+}$ mice, suggesting that one copy of *Pam* is sufficient to catalyse normal levels of peptide amidation. Levels of non-amidated IAPP immunoreactivity, which are much lower than levels of amidated IAPP, were elevated in $Pam^{+/-}$ mice (Fig. 3b). In agreement with our findings, $Pam^{+/-}$ mice have been shown to be able to amidate CCK and TRH normally in the hypothalamus [2, 30]. Nonetheless, it is

unclear whether *PAM*-variant carriers produce amidated peptides at physiologically appropriate levels. In aggregate, our data suggest that PAM haploinsufficiency alone seems unlikely to explain susceptibility to type 2 diabetes and beta cell dysfunction, even in the presence of the increased metabolic and secretory stresses of obesity.

Amyloid deposition in pancreatic islets is a prominent feature of type 2 diabetes [39]. To determine whether PAM haploinsufficiency has an impact on islet amyloid formation and diabetes susceptibility, we crossed mice with beta cell expression of hIAPP with *Pam*$^{+/-}$ mice. IAPP is a PAM substrate and synthetic hIAPP variants with a free (non-amidated) COOH-terminus aggregate more slowly than COOH-terminally amidated forms [17, 40]. We hypothesised that impaired amidation of hIAPP in *Pam*$^{+/-}$ mice may lead to production of hIAPP forms with altered fibril-forming kinetics, morphology and cytotoxicity [41]. Both hIAPP Tg/0 *Pam*$^{+/-}$ and hIAPP Tg/0 *Pam*$^{+/+}$ mice developed hyperglycaemia and diabetes upon HFD challenge, yet insulin sensitivity, glucose tolerance and plasma insulin levels were comparable, suggesting that reduced expression of PAM is insufficient to promote hIAPP-induced islet failure and diabetes. It is plausible that any mild impact of PAM haploinsufficiency on glucose tolerance was masked by the more marked glucose intolerance in mice expressing the hIAPP transgene. Since non-amidated hIAPP immunoreactivity is detectable in islets of hIAPP Tg/0 *Pam*$^{+/+}$ mice and haploinsufficiency of PAM does not further increase non-amidated hIAPP levels (Fig. 7a), it is possible that PAM cannot fully amidate hIAPP under conditions of supraphysiological transgenic expression, or that the conditions for optimal PAM activity (i.e. copper or ascorbate concentration and pH) are not present in granules of hIAPP-expressing mice. Future experiments using models with more closely related physiological expression of hIAPP (i.e. hIAPP knockin mice [42]), or exposing islets to synthetic hIAPP peptides ex vivo, may provide insight into the impact of amidation, if any, on the toxicity of hIAPP.

While deleting one copy of *Pam* in mice did not result in significant metabolic abnormalities, the potential importance of PAM in modulating insulin secretion in humans (and in mice) should not be overlooked. Mutation or small hairpin RNA-mediated silencing of *PAM* leads to a modest reduction in glucose-stimulated insulin secretion from human islet cells [9]. In the present study, we found that PAM expression is sensitive to islet stressors. Whereas high glucose increased *Pam* expression in isolated islets, chronic metabolic stress induced by HFD, as well as ER- or cytokine-induced stress, reduced *Pam* transcript levels. Our data suggest that PAM expression closely reflects the secretory burden and health of beta cells. In neuroendocrine cells, it has also been reported that peptide amidation is impaired under conditions of hypoxia, and PAM-mediated peptide amidation may act as an oxygen sensing system [13]. Because hypoxia is deleterious to islets, restoring PAM may potentially reduce loss of islets in pre- and post-transplant settings in type 1 diabetes, when hypoxia is thought to be present.

In summary, we found that PAM haploinsufficiency does not accelerate development of diet- or hIAPP transgene-induced obesity and diabetes. We further showed that 50% PAM deficiency in mouse islets does not have a major impact on peptide amidation, beta cell function or islet amyloid deposition. PAM expression is, however, highly subject to the changes in the islet milieu seen in diabetes, including elevated glucose and the presence of

ER or cytokine stress. This study contributes to our understanding of the role of *PAM* polymorphisms in type 2 diabetes. We showed that PAM haploinsufficiency alone is insufficient to trigger islet dysfunction or glucose intolerance in mice, implying that other mechanisms in addition to PAM haploinsufficiency may be involved in conferring type 2 diabetes risk in humans. Further studies are needed to understand how changes in PAM expression or PAM variants may affect beta cell function and whether restoring PAM expression or activity may be a treatment strategy for improving islet function in diabetes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

Data availability

The datasets generated and analysed during the current study are available from the corresponding author upon request.

Abbreviations

CCK	Cholecystokinin
ER	Endoplasmic reticulum
HFD	High-fat diet
hIAPP	Human islet amyloid polypeptide
hIAPP Tg/0	hIAPP transgenic mice
IAPP	Islet amyloid polypeptide
LFD	Low-fat diet
NPY	Neuropeptide Y
PAL	Peptidyl-α-hydroxyglycine α-amidating lyase
PAM	Peptidylglycine α-amidating monooxygenase
PHM	Peptidylglycine α-hydroxylating monooxygenase

| **RNA-Seq** | RNA sequencing |
| **TRH** | Thyrotropin releasing hormone |

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Research in context

What is already known about this subject?

- Peptidylglycine α-amidating monooxygenase (PAM) is the only enzyme that amidates neuroendocrine peptides (such as islet amyloid polypeptide (IAPP])

- Aged Pam$^{+/-}$ mice display mild glucose intolerance

- Whole genome sequencing studies have shown that two coding variants of PAM are associated with impaired insulin secretion and increased type 2 diabetes risk

What is the key question?

- Does PAM modulate IAPP amidation and beta cell function, and the development of diet-induced obesity and human IAPP (hIAPP)-transgene-induced diabetes?

What are the new findings?

- Expression of PAM mRNA and protein are reduced in islets exposed to endoplasmic reticulum (ER) and cytokine stress

- Glucose-stimulated insulin secretion is not affected by PAM haploinsufficiency in mice

- PAM haploinsufficiency does not accelerate the development of diet- or hIAPP-induced diabetes in mice, suggesting that the increased type 2 diabetes susceptibility associated with PAM variants is not likely to be explained by decreased PAM expression

How might this impact on clinical practice in the foreseeable future?

- This study provides insight into the role of PAM in the pathogenesis of type 2 diabetes, and information of potential value for treatment of type 2 diabetes, especially in *PAM*-variant carriers
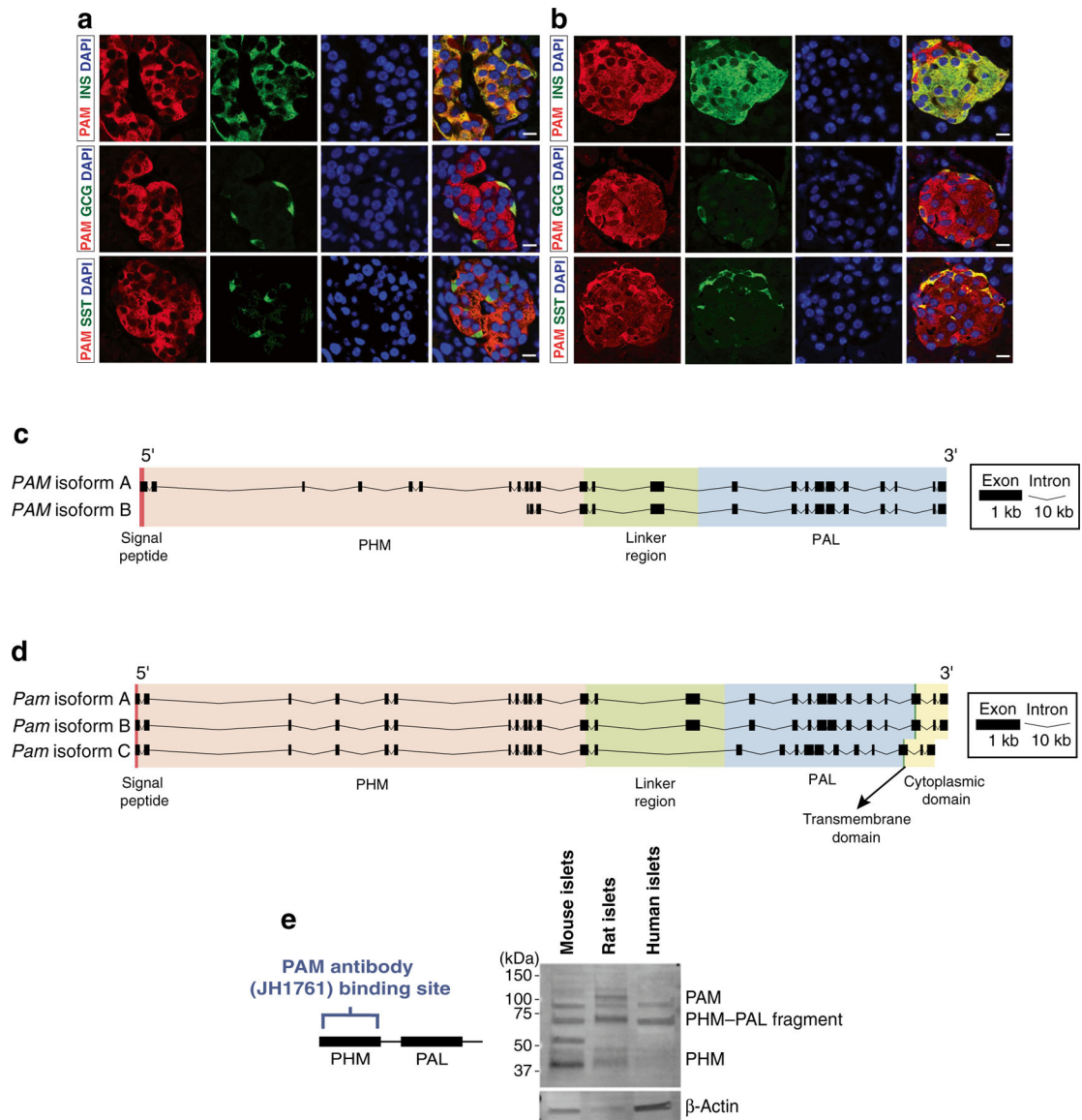
Fig. 1.
PAM expression in human and rodent islets. Representative images of pancreatic sections from (**a**) a non-diabetic donor (nPOD no. 6030) and (**b**) a 16-week-old male C57BL/6J mouse, which were stained for PAM, insulin (INS), glucagon (GCG), somatostatin (SST) and DAPI. Scale bars, 10 μm. (**c, d**) Sequence alignments of major *PAM* transcript isoforms identified in human (**c**) and mouse (**d**) beta cells. (**e**) Islet PAM protein expression in a 16-week-old male mouse, a 24-week-old male Lewis rat and a non-diabetic donor (R2182) were analysed by western blotting using an affinity-purified antibody specific for PAM (JH1761; the position of binding is shown in the schematic)
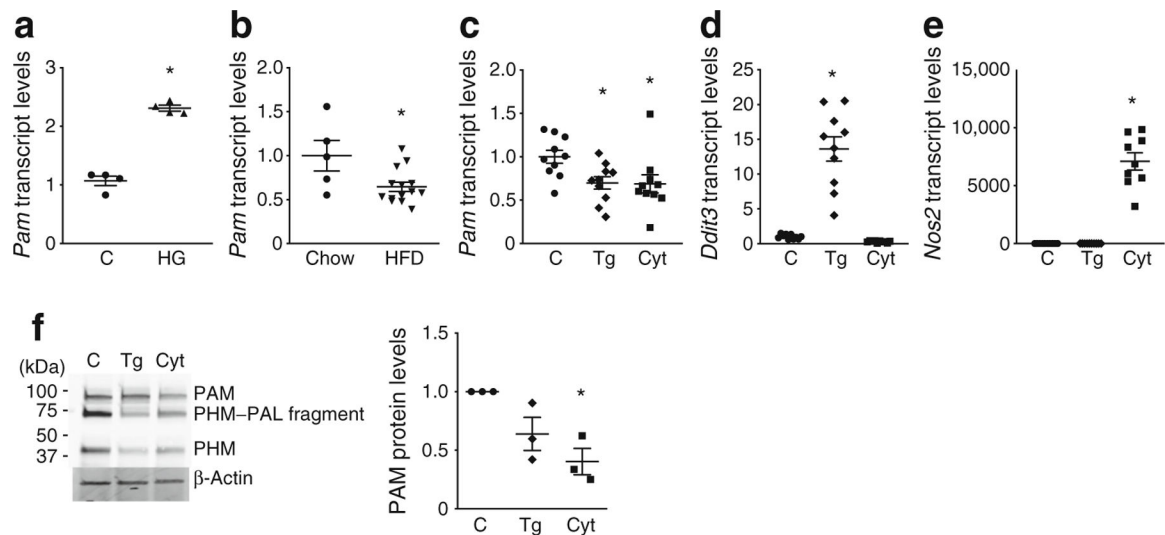
Fig. 2.
PAM expression levels in stress-treated rodent islets. (**a, b**) *Pam* transcript levels in mouse islets from 12–16-week-old male C57BL/6J mice incubated in media containing 11 mmol/l glucose (control [C]) or 25 mmol/l glucose (high glucose [HG]) for 3 days (*n* = 4) (**a**) and islets from 8-week-old male C57BL/6J mice fed for 14 weeks on a 6% fat control (chow) or 45% fat HFD (*n* =5 and *n* = 14, respectively) (**b**) were analysed by quantitative real-time PCR. (**c–e**) *Pam, Ddit3 and Nos2* transcript levels in islets from 12–16-week-old male C57BL/6J mice, treated for 16 h with DMSO (control [C]), 1 μmol/l thapsigargin (Tg), or a cytokine cocktail containing 50 U/ml IL-1β, 1000 U/ml IFN-γ and 1000 U/ml TNF-α (Cyt) (*n* = 10), were analysed by quantitative real-time PCR using *B2m* as reference gene and expressed as fold over control. (**f**) PAM protein expression in isolated islets from 24-week-old male Lewis rats, treated as mouse islets in (**c–e**), was analysed by immunoblotting using an affinity-purified antibody specific for PAM (JH1761) (*n* = 3). PAM protein level was quantified using β-actin as a reference protein and expressed as fold over control. Data are shown as mean ± SEM. *$p < 0.05$ vs control
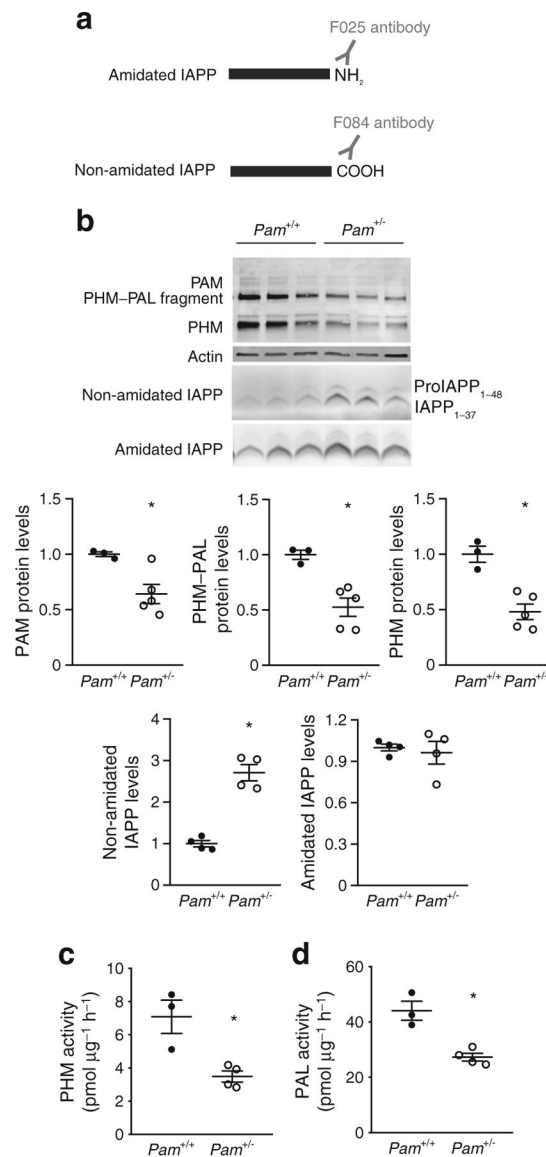
Fig. 3.
PAM expression and enzyme activity are reduced in $Pam^{+/-}$ islets. (**a**) Schematic representation of antibodies used to detect amidated IAPP (F025) and non-amidated IAPP (F084). (**b**) Expression levels of PAM, PAM cleavage products (PHM–PAL fragment and PHM domain), and amidated and non-amidated IAPP in islets from 12–16-week-old male $Pam^{+/-}$ and $Pam^{+/+}$ mice were analysed by immunoblotting, using the antibodies listed in (**a**) and an anti-PAM antibody (JH1761) ($n = 3$–5). (**c, d**) Islet PHM and PAL enzyme-specific activities were analysed by substrate-based assays ($n = 3$–4). Data are expressed as mean $\pm$ SEM. $*p < 0.05$
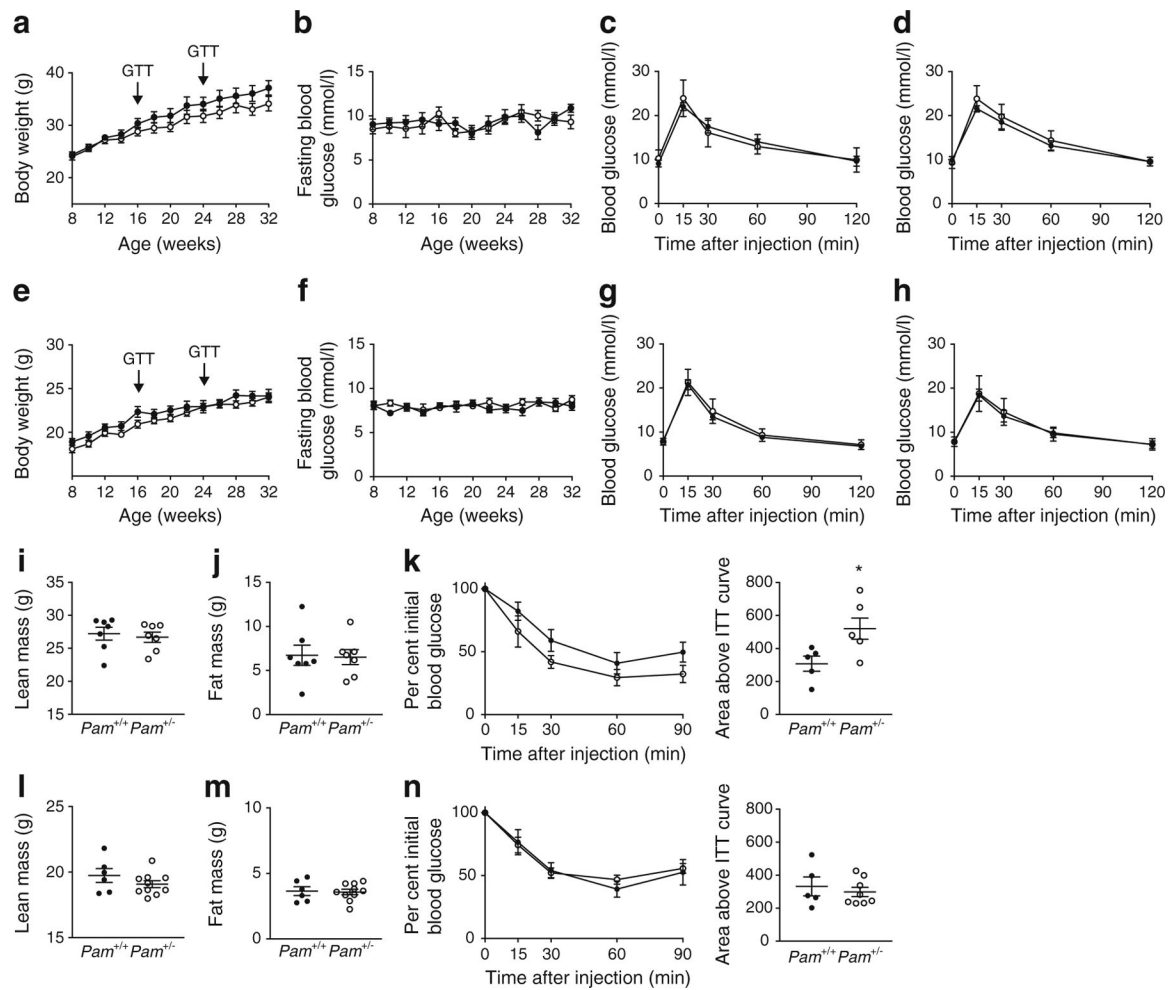
Fig. 4.

Metabolic characterisation of *Pam*^{+/−} and *Pam*^{+/+} mice. (**a–h**) Eight-week-old male (**a–d**) and female (**e–h**) *Pam*^{+/−} (white circle) and *Pam*^{+/+} (black circle) littermates were fed a 10% fat diet (LFD) and body weight (**a, e**) and 5 h fasting blood glucose levels (**b, f**) were measured every 2 weeks for 24 weeks. IPGTTs were performed at timepoints indicated by black arrows in (**a**) and (**e**). In: (**a–d**), $n = 7$; (**e–h**), $n = 10$ for *Pam*^{+/−} and $n = 5$ for *Pam*^{+/+}. Following 8 (**c, g**) and 16 (**d, h**) weeks of diet, IPGTTs were performed in 5 h-fasted *Pam*^{+/−} and *Pam*^{+/+} mice using a glucose dose of 1.5 g/kg body weight. (**i–n**) At week 20 of the LFD, male (**i–k**) and female (**l–n**) *Pam*^{+/−} and *Pam*^{+/+} mice were fasted for 5 h and lean mass (**i, l**) and fat mass (**j, m**) were analysed. (**k, n**) IPITTs were performed using an insulin dose of 1.5 IU/kg lean mass. In (**i–n**), $n = 5–10$. Data are expressed as mean ± SEM
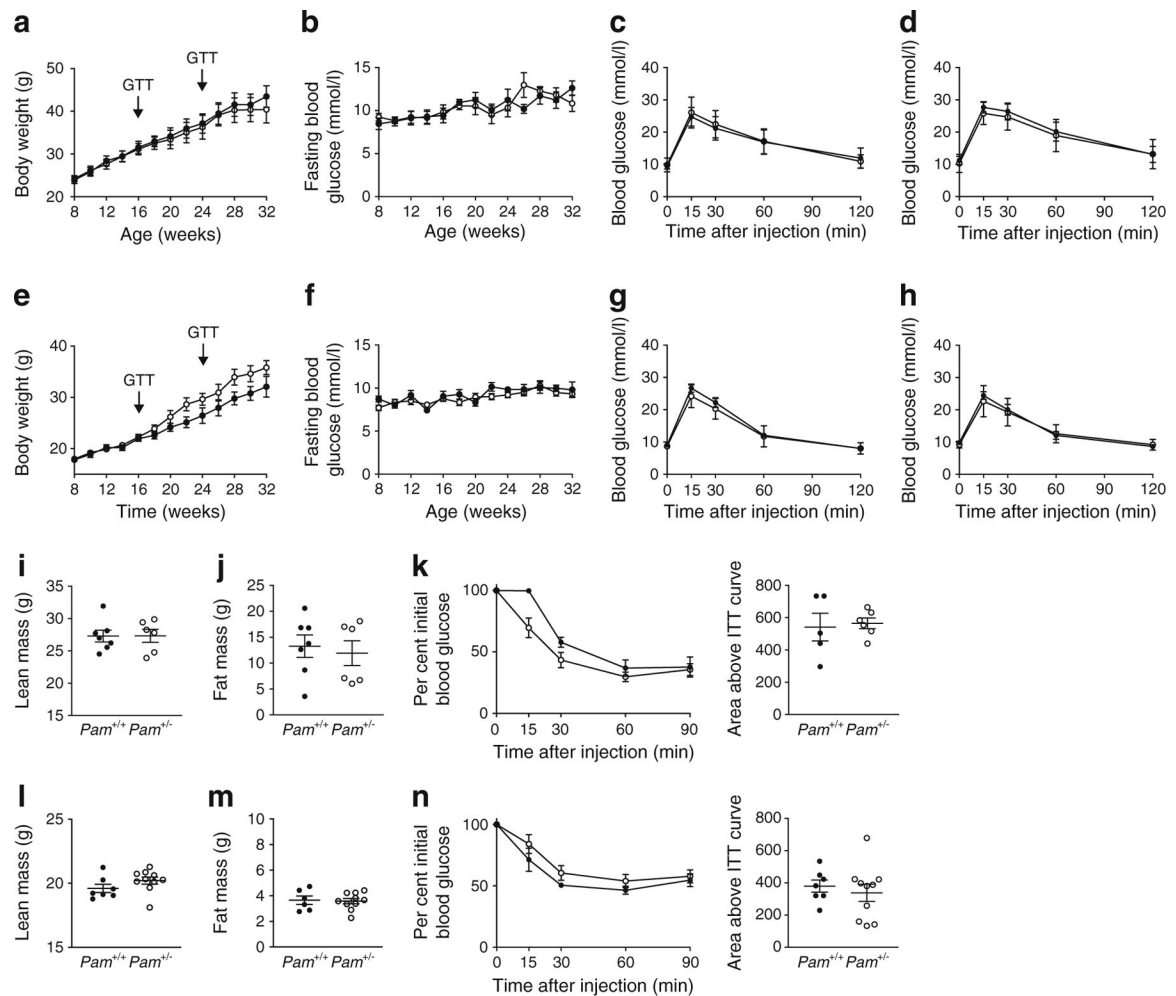
Fig. 5.

Metabolic characterisation of HFD-challenged *Pam*^{+/−} and *Pam*^{+/+} mice. (**a–h**) Eight-week-old male (**a–d**) and female (**e–h**) *Pam*^{+/−} (white circle) and *Pam*^{+/+} (black circle) mice were fed with a 45% fat diet (HFD) and body weight (**a, e**) and 5 h fasting blood glucose levels (**b, f**) were measured every 2 weeks for 24 weeks. IPGTTs were performed at timepoints indicated by black arrows in (**a**) and (**e**). In: (**a–d**) $n = 7$; (**e–h**) $n = 10$. At 8 (**c, g**) and 16 (**d, h**) weeks of HFD, IPGTTs were performed in 5 h-fasted *Pam*^{+/−} and *Pam*^{+/+} mice using a glucose dose of 1.5 g/kg body weight. (**i–n**) After 20 weeks of HFD-challenge, male (**i–k**) and female (**l–n**) *Pam*^{+/−} and *Pam*^{+/+} mice were fasted for 5 h and lean mass (**i, l**) and fat mass (**j, m**) were analysed. (**k, n**) IPITTs were performed using an insulin dose of 1 IU/kg lean mass. In (**i–n**), $n = 5$–10. Data are expressed as mean ± SEM
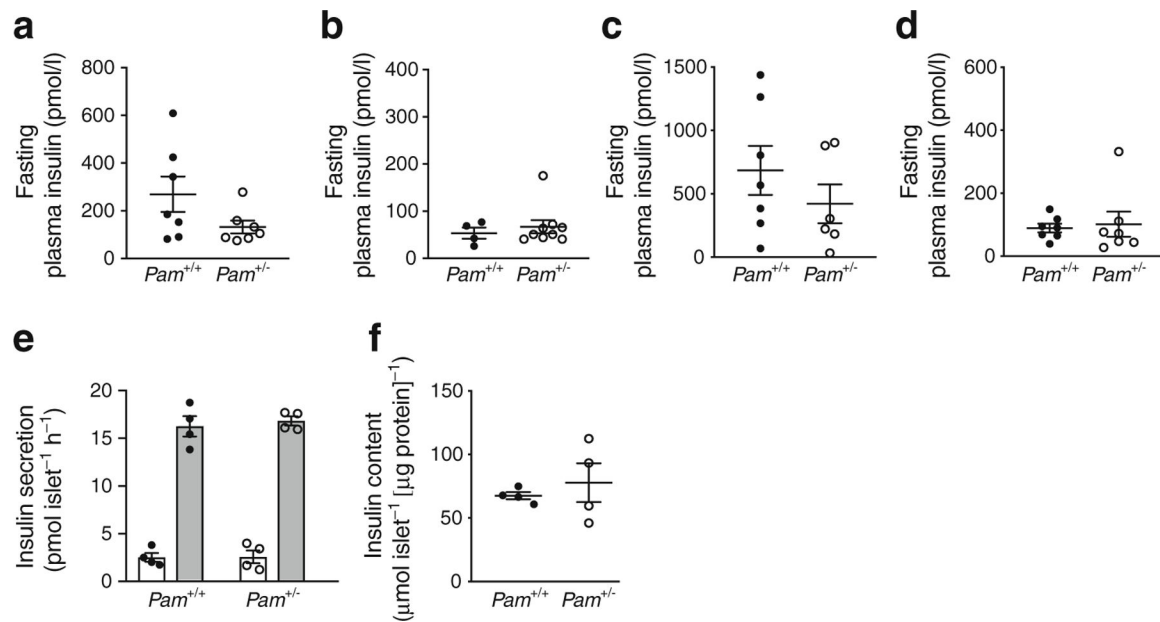
Fig. 6.

Plasma and islets insulin levels in *Pam*^{+/−} and *Pam*^{+/+} mice. (**a–d**) Fasting plasma insulin levels were measured in blood collected from male (**a, c**) and female (**b, d**) *Pam*^{+/−} and *Pam*^{+/+} mice kept for 24 weeks on a 10% fat diet (LFD; **a, b**, *n* = 4–9), or a 45% fat diet (HFD; **c, d**; *n* = 6–7). In vitro insulin secretion (**e**) and insulin content (**f**) assays were performed using islets collected from 12-week-old chow-fed *Pam*^{+/−} and *Pam*^{+/+} mice. Islets were treated with 1.67 mmol/l glucose for 1 h, followed by 16.7 mmol/l glucose for 1 h. Insulin immunoreactivity in the incubation medium and islet lysates was measured by ELISA (*n* = 4). Data are expressed as mean ± SEM
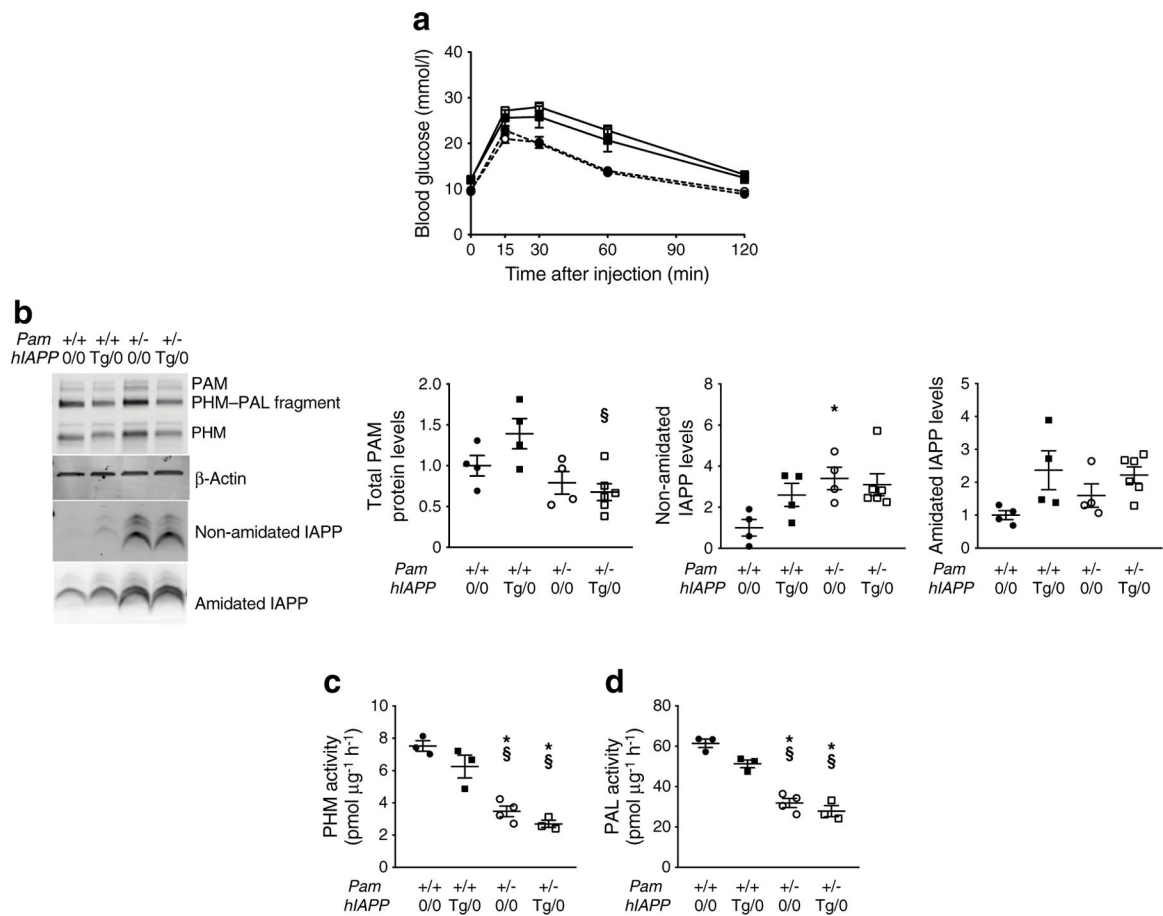
Fig. 7.
Glucose tolerance in hIAPP transgenic PAM heterozygous mice. (**a**) GTTs were performed in 5 h-fasted 8-week-old male hIAPP 0/0 $Pam^{+/+}$ (black circle, $n = 16$), hIAPP Tg/0 $Pam^{+/+}$ (black square; $n = 7$), hIAPP 0/0 $Pam^{+/-}$ (white circle; $n = 10$) and hIAPP Tg/0 $Pam^{+/-}$ (white square; $n = 17$) mice with a glucose dose of 1.5 g/kg body weight. When analysed by two-way ANOVA and corrected with Bonferroni test, $p < 0.05$ for the AUC of IPGTT for both hIAPP Tg/0 $Pam^{+/+}$ vs hIAPP 0/0 $Pam^{+/+}$, and hIAPP Tg/0 $Pam^{+/-}$ vs hIAPP 0/0 $Pam^{+/-}$ (data not shown). (**b**) Expression of PAM (total PAM protein level = sum of PAM, PHM–PAL fragment and PHM expression levels), non-amidated IAPP and amidated IAPP in islets from hIAPP 0/0 $Pam^{+/+}$, hIAPP Tg/0 $Pam^{+/+}$, hIAPP 0/0 $Pam^{+/-}$ and hIAPP Tg/0 $Pam^{+/-}$ mice, analysed by immunoblotting using PAM- and IAPP-specific antibodies ($n = 4$–6) (**c, d**) Islet PHM and PAL specific activities were analysed by substrate-based assays ($n = 3$–4). Data are expressed as mean ± SEM. *$p < 0.05$ vs hIAPP 0/0 $Pam^{+/+}$; §$p < 0.05$ vs hIAPP Tg/0 $Pam^{+/+}$
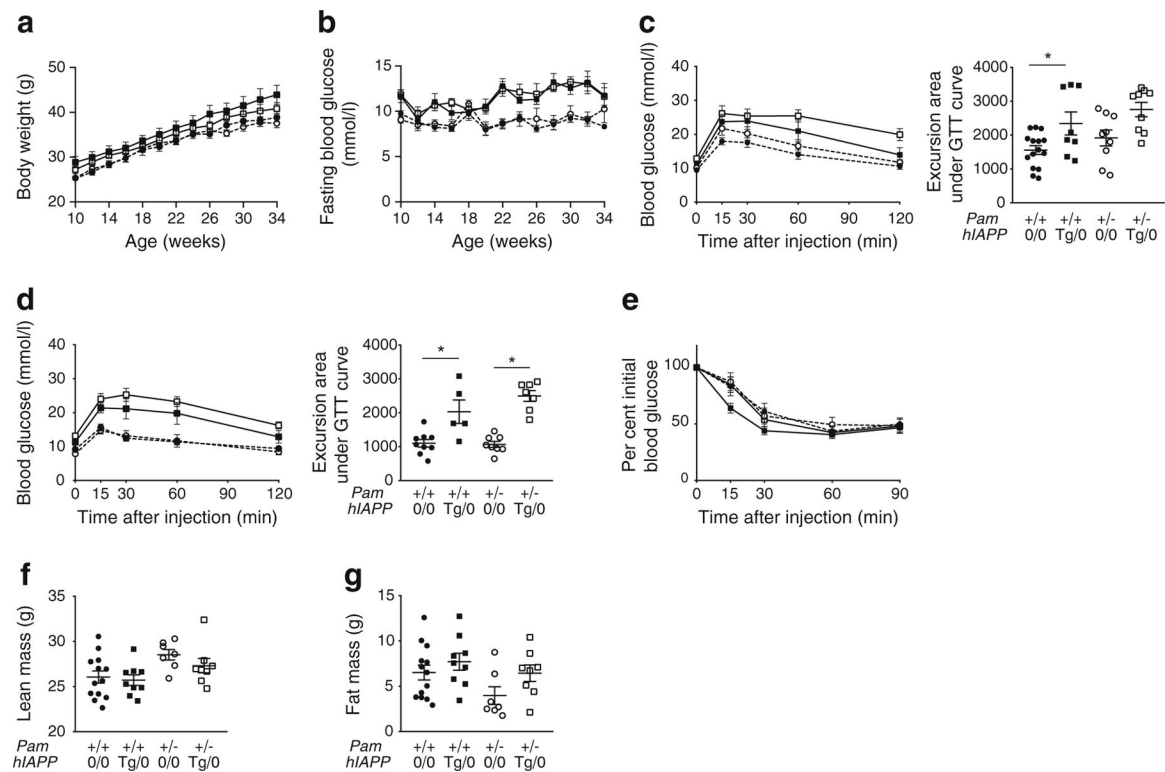
Fig. 8.

Metabolic characterisation of hIAPP transgenic PAM heterozygous mice. (**a, b**) Ten-week-old male hIAPP 0/0 *Pam*^+/+ (black circle, dashed line; $n = 15$), hIAPP Tg/0 *Pam*^+/+ (black square; $n = 13$), hIAPP 0/0 *Pam*^+/− (white circle, dashed line; $n = 9$), and hIAPP Tg/0 *Pam*^+/− (white square; $n = 10$) mice were fed a 10% fat diet (LFD) and body weight (**a**) and 5 h fasting blood glucose levels (**b**) were monitored every 2 weeks. When analysed by two-way ANOVA and corrected with Bonferroni test, $p < 0.05$ for the AUC of blood glucose for both hIAPP Tg/0 *Pam*^+/+ vs hIAPP 0/0 *Pam*^+/+, and hIAPP Tg/0 *Pam*^+/− vs hIAPP 0/0 *Pam*^+/− (data not shown). (**c, d**) After 8 (**c**) and 20 (**d**) weeks of LFD, IPGTT was performed in 5 h-fasted hIAPP Tg/0 *Pam*^+/− and littermate mice using a glucose dose of 1.5 g/kg body weight and area under the GTT curve was determined. In: (**c**) $n = 8$–15; (**d**) $n = 5$–9. (**e**) After 12 weeks of LFD, IPITTs were performed in 5 h-fasted hIAPP Tg/0 *Pam*^+/− and littermate mice using an insulin dose of 1 IU/kg lean mass (hIAPP 0/0 *Pam*^+/+, $n = 12$; hIAPP Tg/0 *Pam*^+/+, $n = 8$; hIAPP 0/0 *Pam*^+/−, $n = 7$; hIAPP Tg/0 *Pam*^+/−, $n = 8$). (**f, g**) Lean (**f**) and fat (**g**) mass compositions were analysed ($n = 7$–13). Data are expressed as mean ± SEM. *$p < 0.05$
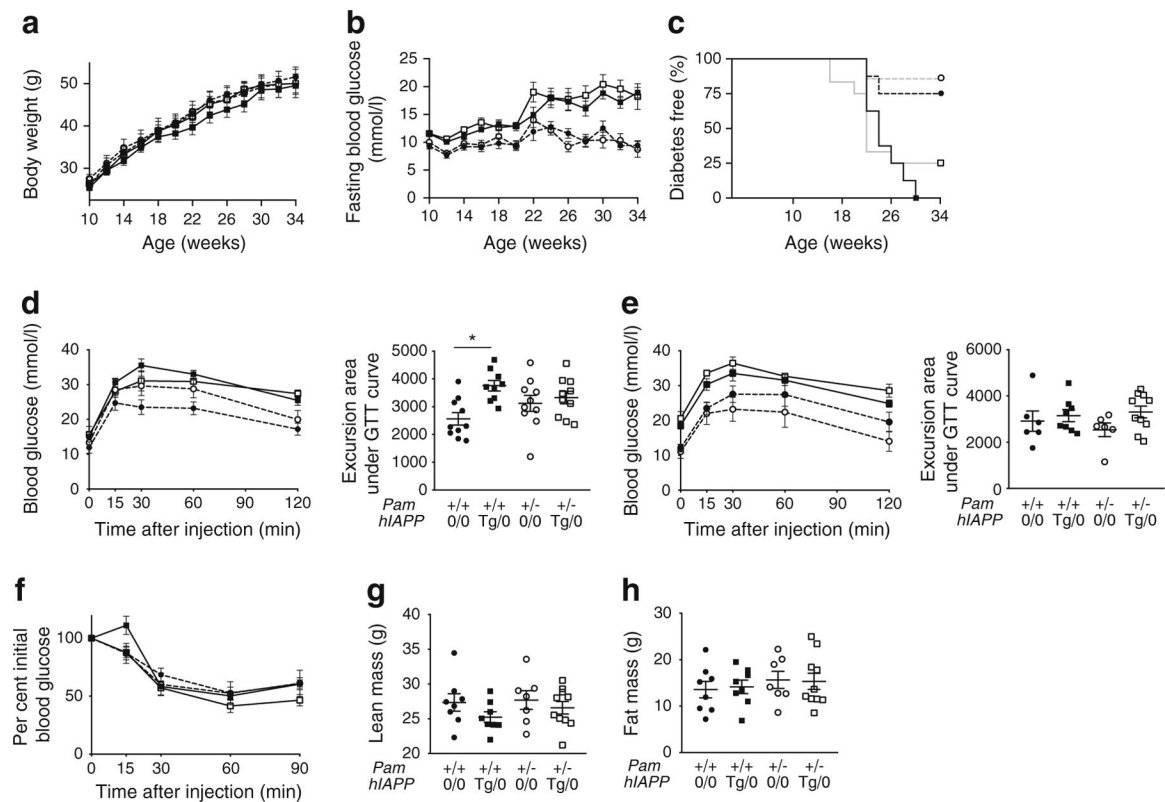
Fig. 9.

Metabolic characterisation of HFD-fed hIAPP transgenic PAM heterozygous mice. Ten-week-old male hIAPP 0/0 $Pam^{+/+}$ (black circle, dashed line), hIAPP Tg/0 $Pam^{+/+}$ (black square), hIAPP 0/0 $Pam^{+/-}$ (white circle, dashed line) and hIAPP Tg/0 $Pam^{+/-}$ (white square) mice were fed an HFD (45% fat diet). (**a–c**) Body weight (**a**) and 5 h fasting blood glucose levels (**b**) were monitored every 2 weeks ($n = 10$), and diabetes-free survival rates were analysed (**c**) (diabetes was defined as two consecutive fasting blood glucose measurements >15 mmol/l). In (**b**) and (**c**), $p < 0.05$ for hIAPP Tg/0 $Pam^{+/+}/Pam^{+/-}$ mice vs hIAPP 0/0 $Pam^{+/+}/Pam^{+/-}$, analysed by two-way ANOVA and corrected with Bonferroni test or logrank test, respectively. (**d, e**) After 8 (**d**) and 20 (**e**) weeks of HFD, IPGTTs were performed in 5 h-fasted hIAPP Tg/0 $Pam^{+/-}$ and littermate mice using a glucose dose of 0.75 g/kg body weight, and area under the GTT curve was analysed. In: (**d**) $n = 9$–10; (*e*) $n = 6$–10. (**f**) After 12 weeks of HFD, IPITTs were performed in 5 h-fasted hIAPP Tg/0 $Pam^{+/-}$ and littermate mice using an insulin dose of 1 IU/kg lean mass (hIAPP 0/0 $Pam^{+/+}$, $n = 7$; hIAPP Tg/0 $Pam^{+/+}$, $n = 8$; hIAPP 0/0 $Pam^{+/-}$, $n = 5$; hIAPP Tg/0 $Pam^{+/-}$, $n = 7$). (**g, h**) Lean (**g**) and fat (**h**) mass composition were analysed ($n = 7$–10). Data are expressed as mean ± SEM. *$p < 0.05$
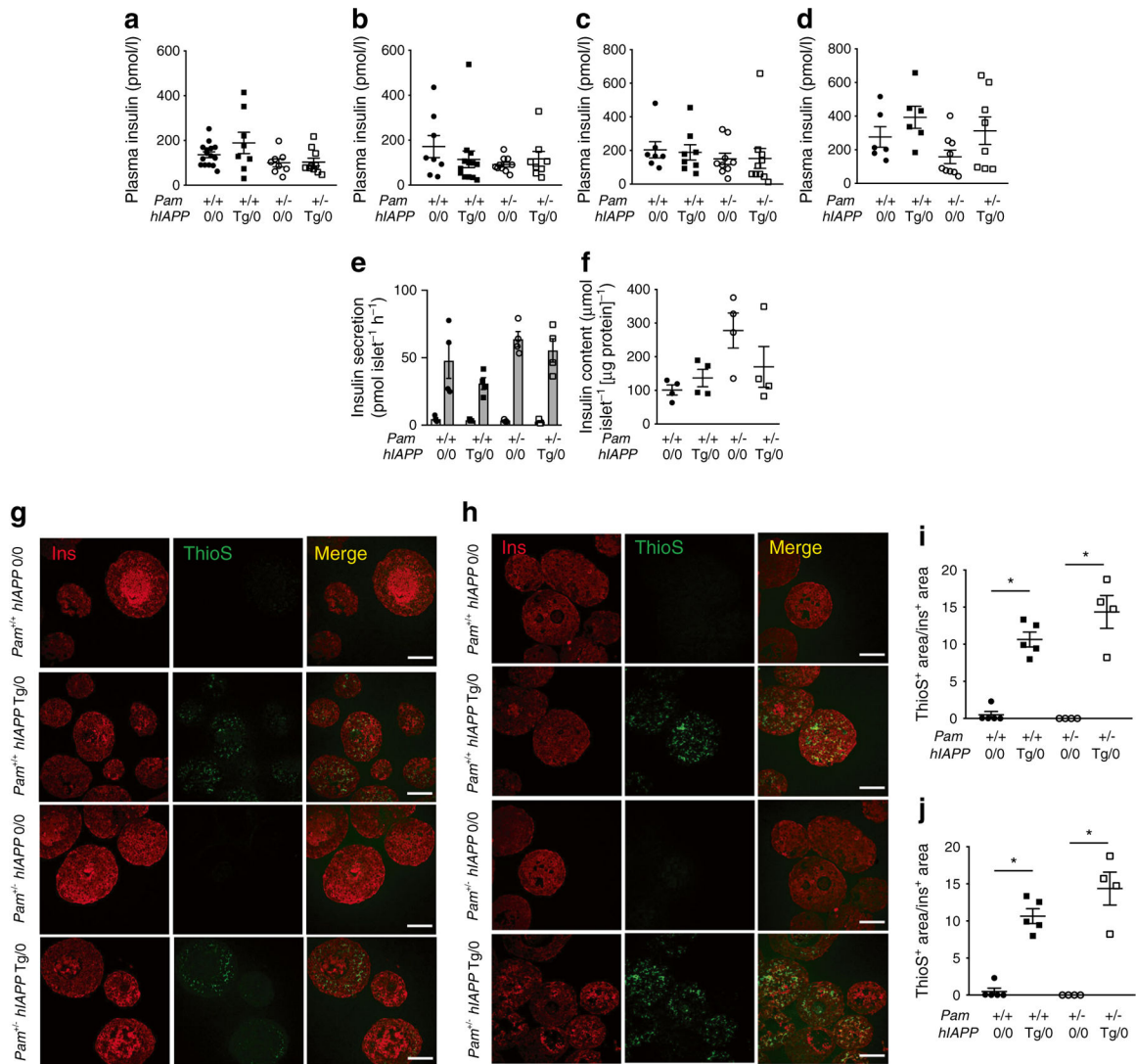
Fig. 10.
Beta cell function and islet amyloid deposition in hIAPP transgenic PAM heterozygous mice. (**a–d**) Plasma insulin levels were determined by ELISA in hIAPP 0/0 *Pam*^{+/+}, hIAPP Tg/0 *Pam*^{+/+}, hIAPP 0/0 *Pam*^{+/−} and hIAPP Tg/0 *Pam*^{+/−} male mice fed a diet with 10% fat for 16 weeks (**a**) or 24 weeks (**b**) ($n = 8$–15) or 45% fat for 16 weeks (**c**) or 24 weeks (**d**) ($n = 6$–10). (**e, f**) In vitro glucose-stimulated insulin secretion (**e**) and insulin content (**f**) were measured using islets collected from hIAPP Tg/0 *Pam*^{+/−} and littermate mice ($n = 4$). (**g–j**) Islets isolated from 16-week-old chow-fed female hIAPP Tg/0 *Pam*^{+/−} and littermate mice were cultured in 25 mmol/l glucose for 3 days (**g, i**) or 7 days (**h, j**). Islet amyloid severity was determined as thioflavin S[+] area/insulin[+] area (**i, j**; $n = 4$–5). Scale bars, 75 μm. Data are expressed as mean ± SEM. *$p < 0.05$. Ins, insulin; ThioS, thioflavin S