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Y5 receptor signalling counteracts the anorectic effects of PYY3-36 in diet induced obese mice

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Abstract

Peptide YY 3-36 (PYY3-36) is known as a critical satiety factor reducing food intake both in rodents and humans. While the anorexic effect of PYY3-36 is thought to be mediated mainly by the Y2 receptor, the involvement of other Y-receptors in this process has never been conclusively resolved. Amongst them the Y5 receptor (Y5R) is the most likely candidate to also be a target for PYY3-36 which is thought to counteract the anorectic effects of Y2R activation. Here we show that short term treatment of diet induced obese WT and Y5R knockout mice (Y5KO) with PYY3-36 leads to significantly reduced food intake in both genotypes, which is more pronounced in Y5R KO mice. Interestingly, chronic PYY3-36 infusion via minipumps to WT mice causes increased cumulative food intake, which is associated with increased body weight gain. In contrast, lack of Y5R reversed this effect. Consistent with the observed increased body weight and fat mass in WT treated mice, glucose tolerance was also impaired by chronic PYY3-36 treatment. Again this was less affected in Y5KO mice suggestive of a role of Y5R's in the regulation of glucose homeostasis. Taken together, our data suggests that PYY3-36 mediated signalling via Y5 receptors may counteract the anorectic effects that it mediates via the Y2 receptor (Y2R) consequently lowering bodyweight in the absence of Y5 signalling. These findings open the potential of combination therapy using PYY3-36 and Y5R antagonists to enhance PYY3-36's food intake reducing effects.

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Introduction

The gut-brain axis plays an important role in the regulation of food intake and targeting molecules within this axis has emerged as a promising strategy for treating obesity. Peptide YY (PYY), a member of the neuropeptide Y (NPY) family, is released in response to food intake particularly of dietary lipids, and is among the most studied satiety signals. PYY is primarily synthesized by endocrine L cells of the lower gastrointestinal tract and is released into the circulation in two forms: the full-length PYY1-36 and the truncated form PYY3-36, which is cleaved from PYY1-36 via dipeptidyl peptidase-IV (DPP-IV). PYY levels increase within 15 minutes of ingesting a meal, peak at approximately 60 minutes after ingestion and remain elevated for up to six hours.

Peripheral administration of PYY3–36 reduces food intake in rodents and humans (1-3). Furthermore, over-expression of PYY, in an adult-onset conditional PYY transgenic mouse model, leads to a significant reduction in fasting-induced food intake without altering body weight or adiposity (4, 5). It has been shown that obese individuals have a deficiency in circulating PYY (6), indicating the potential critical role of PYY in the development of obesity (7). In keeping with this, lean, healthy people with a genetic predisposition for future obesity – on account of a family history of type 2 diabetes – are known to have lower circulating PYY levels, consistent with low PYY levels predisposing to the development of obesity (7).

PYY signals through a set of G-protein-coupled Y-receptors, Y1, Y2, Y4, Y5 and y6, which are widely expressed in central nervous system, including areas of the hypothalamus that are critical for the control of food intake and energy homeostasis (8). While PYY1-36 binds to all known Y- receptors, albeit with varying affinity, PYY3-36 predominantly binds to Y2

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receptor and with an approximately 10 fold reduced affinity also to Y5 receptors (9). Y2 receptors are mostly expressed pre-synaptically, with their activation by PYY3-36 leading to the reduction of neurotransmitter release such as orexigenic acting NPY or GABA in the hypothalamic Arc, thereby inducing satiety (8). This anorectic effect of PYY3-36 is absent in Y2KO mice (1). As such, the activation of Y2R in the hypothalamic arcuate nucleus is primarily responsible for PYY3-36's hypophagic effect. However, there are inconsistent data regarding PYY3-36's effects on food intake and body weight (10), and evidence has emerged that one of the explanations for that could be that PYY3-36's anorectic effects may be antagonized by other Y-receptors, such as the Y5 receptor (Y5R).

The Y5R, is widely expressed in the hypothalamus and mostly colocalized with the Y1 receptor, both receptors known to increase food intake (11), (12). Pharmacological intervention studies have shown that Y5 receptor antagonism can produce weight-lowering effects in diet-induced obese (DIO) mice (13) but not in Y5 deficient mice (10). However, it is not clear whether this reduction in body weight could produce metabolic benefits on whole body energy and glucose homeostasis. Thus, in order to determine whether the beneficial anorectic and gluco-regulatory effects of pharmacologically administered PYY3-36 are antagonized by Y5R signalling under nutritional excess or obese condition, we treated Y5R KO and control mice with high fat diet for 12 weeks followed by PYY3-36 administration and measurement of food intake, body weight and glucose metabolism over the course of 3 weeks.

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Materials and Methods

Animals

All research and animal work was conducted under the regulation of Garvan Institute/St Vincent's Hospital Animal Ethics Committee and were in agreement with the Australian Code of Practice for the Care and Use of Animals for Scientific purpose. Mice were housed under conditions of controlled temperature (22°C) and illumination (12-hour light cycle, lights on at 07:00). 6-7 weeks old male Y5 receptor knockout (Y5KO) and their littermates on a mixed background of C57BL/6JAusb were fed on a high fat diet (HFD) *ad libitum* (60 % calories from fat, research diet, D12492) for 12 weeks. Water was available *ad libitum*. After 12 weeks on HFD, the mice are individually housed for the purpose of accurate food intake measurements. Y5KO mice and control littermates were randomly distributed between the 2 treatments.

Generation of germline Y5 receptor knockout model

The same procedures that were used to generate a Y1/Y5 double targeted allele in embryonic stem (ES) cells were followed using instead WT Bl6 ES cell. The same targeting construct was used and the same screening procedures where applied as described in a previous paper (11).

DNA and RNA extraction and quantitative real-time PCR

Y5 receptor deletion was confirmed using genomic DNA extracted from the hypothalamic region of the brain and liver. In brief, genomic DNA was isolated from hypothalamic blocks from Y5KO, WT littermate and heterozygous mice. PCR was performed with the following primers: Oligo A, 5′- GCAACTGTTGGGAGGGGCG-3′, Oligo B, 5′- GTGCAGAGCTAATGGTGTGTG-3′ and Oligo C, 5′- CCTCCATACTAGAGTCCTGC-3′

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to distinguish genotype of knockout, heterozygote and wild type. This set of primers produces a PCR product, 400 base pairs long, from DNA in which the Y5-receptor gene has been deleted (Figure 1A). PCR conditions were 5 minutes denaturation at 95°C followed by 45 cycles of 30 seconds at 95°C, 30 seconds at 60°C and 30 seconds at 72°C.

The hypothalamic region of the brain from littermate control and Y5KO mice fed on either a chow diet or high fat diet (HFD) were collected and total RNA was isolated using RNeasy Micro Kit (Qiagen) following the manufacturer's protocol. 40 ng of total RNA was reverse transcribed into cDNA using Superscript III First-Strand Synthesis System (Invitrogen, Mount, Waverley, VIC, Australia). Quantitative real-time PCR using primers for Y1, Y2, Y4 and Y5 receptors (Primer sequences were listed in Table 1) was carried out in at least triplicates from 1:5 dilution cDNA from each sample using the LightCycler® (Light-Cycler® 480 Real-Time PCR system, Roche Applied Science, Germany), SYBR Green I (Molecular Probes) and Platinum Taq DNA Polymerase (Invitrogen). Primers used for assaying housekeeping gene β-actin were previously described (14). The primers 5'-GACTCTCACAGGCTGTCTT-3' and 5'-TTGGTCTCACTGGACCTGT-3' were used for Y1R; 5'-TTTTCGGAGGCTACCAATGT-3' and 5'-AATACAATGGGAGGTCTGCA-3' for Y2R; 5'-ATAGTCGTGTCTGGGCTTTT-3' and 5'-AAGCTTCAAGTCTCTTGCCA-3' for Y4R. The previously described PCR condition was used in all the RT-qPCR experiment, 94°C for 30 seconds, 62°C for 30 seconds, 72°C for 20 seconds for 40 cycles (15, 16).

Surgery for minipump implantation

Mice are anaesthetised with isoflurane and oxygen using a calibrated vaporiser. Reflex testing of the footpad was used to determine depth of anaeathesia. Bupivacaine (8 mg/kg BW, subcutaneous (s.c.) was instilled at the incision site as an analgesic. In addition, carprofen (NSAID) (5mg/kg BW, s.c.) just before surgery and 1-2 days post surgery was

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administrated. Surgical sites were carefully clipped of hair and swabbed with chlorhexidine in alcohol and iodine surgical scrub. Minipumps (pump rate: 0.25 ul/hour, ALZET model 2004) were inserted subcutaneously on the animal's back, between the shoulder blades. Briefly, animal were placed on its chest, and a mid-scapular skin incision was made at the back of the animal adjacent to the site chosen for pump placement. A haemostat was inserted into the incision and a pocket was formed in the subcutaneous tissue by opening and closing the jaws, which housed the pump. The minipumps were filled with either vehicle (50 mM Na_2HPO_4, 70 mM NaCl of 0.05% Tween 80) or PYY3-36 (1 mg/kg/day, 11 mg/ml, diluted with vehicle solution) according to manufacturer's instructions. PYY3-36 peptide diluted in the buffered solution was stable and functional over the period of the experiment. The filled minipump (200 μl) was carefully inserted, delivery port first, into the created pocket. The wound was closed with wound clips. The mice were placed on a heating pad (box half on / half off) after surgery and monitored until they were fully recovered from anaesthesia. They were returned to their home cages and food intake and body weight was monitored as described below.

Determination of food intake, body weight and body composition

HFD fed male mice (10 per group) were housed individually throughout the whole experiment of 3 weeks period. Food intake and body weight was measured once daily at 900h in the morning. At 3 days before and 19 days after treatment, mice were anesthetized with ketamine/xylzine (100 mg/kg and 9 mg/kg, Mavlab, Slacks Creek, Queensland, Australia; Ilium Veterinary Products, Smithfield, New South Wales, Australia) and anal-nasal length and whole body fat mass and lean mass were measured using dual-energy X-ray absorptiometry (DXA; Lunar PIXImus2 mouse densitometer, GE Medical Systems, Madison, WI). Whole body bone mineral density (BMD) and bone mineral content (BMC) were determined at the same time.

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Oral glucose tolerance tests (oGTT)

At 18 days post treatment, mice were fasted for 6 hours before orally feeding with jelly containing 10% D-glucose solution (1.5 g/kg body weight) (17). Blood samples were obtained from the tail tip at 0, 30, 60, 90, 120 and 180 minutes after the oral glucose load, and glucose levels were measured using a glucometer (AccuCheck II; Roche, New South Wales, Castle Hill, Australia).

Tissue collection and analysis

At the completion of the study, mice were fasted for 4 hours and then killed by cervical dislocation and decapitation between 1200h and 1500h, trunk blood was collected in tubes containing EDTA and a buffer for DPP-IV inhibition (10 µl/tube). DPP-IV inhibition buffer was made by dissolving 3.097 g K_3EDTA (MW 406.53, Fluka 03665) in 50 ml Trasyol (10.000 KIE) and adding 0.5 ml 20 mM Val-Pyr (16.48 mg Val-Pyr was dissolved in 4 ml H2O). pH of this solution was then regulated to 7.4 by 1 M HCl. The blood was centrifuged, and the resultant plasma was stored at −20°C for subsequent analysis. In addition, brain, liver, pancreas, kidney, spleen, brown and white adipose tissue (WAT) depots (right inguinal, right retroperitoneal, right epididymal, and mesenteric) were collected, weighed, and stored at -80˚C for further analysis.

Statistical analysis

All values are presented as means ± SEM. One- or two-way ANOVA, repeated measures ANOVA, or Student's *t* test was used to determine the significance of treatment effects and interactions (GraphPad Prism 5, version 6.0f; GraphPad Software, Inc). When there was a significant overall effect or interaction effect, Turkey post hoc tests were performed to identify differences among means. For all statistical analyses, $P < 0.05$ was regarded as significant.

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Results

Acute effects of PYY3-36 on food intake and body weight in the presence or absence of Y5R

Germline deletion of Y5 receptors leads to a late onset obese phenotype in mice, which is enhanced when fed a high fat diet (18). This is confirmed in our mice where 7-week old Y5KO mice were significantly heavier at the onset of the high fat diet (Figure 1B) and this difference was widened after 12 weeks of HFD (Figure 1B). This is also reflected in the significantly increased body weight gain over the HFD period (Figure 1C). Interestingly, this increase in body weight gain in Y5KO mice was actually occurring in the setting of a slight decrease in average daily food intake (Figure 1D), and reached statistical difference when expressed as a percent of body weight (Figure 1E).

In order to investigate the consequences of PYY3-36 treatment under conditions of DIO on a background of Y5 receptor deficiency, we treated WT and Y5KO mice with a HFD for 12 weeks after which osmotic mini pumps (ALZET model 2004) carrying either vehicle or PYY3-36 were subcutaneously inserted on the back between the scapulae of the mice. The infusion rate of the pumps was 0.25 μl/ hour allowing for a 21 day delivery period. The concentration of PYY3-36 was 11 mg/ml, and food intake and body weight were monitored daily for the next 3 weeks. The acute 24 hour effect of PYY3-36 administration led to a significant reduction in food intake in both WT and Y5KO mice with a pronounced greater effect in Y5KO mice, both in absolute values (Figure 1F) as well as expressed as a percent of body weight (Figure 1G). Interestingly, monitoring the same parameter of the next 24 hours showed a decline in the response to PYY3-36 food inhibition, which was no longer significant in WT mice, however, persisted to be significant in Y5KO mice when expressed in absolute values (Figure 1H) or as a percent of body weight (Figure 1I). However, the reduction in food intake over this 48-hour period did not result in significant changes in body

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weight in either WT or Y5KO mice (Figure 1J), though HFD-fed Y5KO mice were generally heavier than WT mice and the difference almost reached statistically significant in first and second 24h.

Longer term effects of PYY3-36 on food intake and body weight in the presence or absence of Y5 receptor

Food intake and body weight in these mice were then further monitored daily until day 21 post pump implantation. WT mice treated with PYY3-36 showed a transient reduction in food intake. Interestingly however, after rebounding to vehicle levels food intake of PYY3-36 treated mice further increased above levels seen in vehicle treated mice for several days when expressed as absolute values (Figure 2A), as well as percentage of body weight (Figure 2C). In contrast to WT mice, PYY3-36-treated Y5 KO mice showed a more pronounced anorectic effect both in terms of magnitude and duration of the effect when compared to their vehicle treated littermates. Furthermore, unlike WT PYY3-36 treated mice, Y5 KO mice failed to show a compensatory hyperphagic response after the initial reduction in food intake (Figure 2B, 2D). This suggests that the absence of Y5R signalling can enhance the hypophagic effect of PYY3-36 for an extended period until compensatory mechanism may be activated. When comparing cumulative food intake over the entire treatment period, it also appears that WT mice treated with PYY3-36 actually consumed more cumulative food (Figure 2E) while the cumulative food intake in Y5KO mice was similar to vehicle-treated mice due to their initial more pronounced reduction in food intake and lack of hyperphagic response (Figure 2F). This data suggests that Y5 receptor signalling appears to diminish the acute anorectic action of PYY3-36 and promote a subsequent compensatory rebound.

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Consistent with the changes in food intake, there was also a significant body weight gain observed in PYY3-36-treated WT mice compared to vehicle-treated WT controls (Figure 2G). Importantly, weight gain was not seen in the Y5KO mice, which showed a decrease in body weight during the initial phase of treatment but then gradually rebound to the body weight of vehicle-treated mice. (Figure 2H). The increase in body weight gain seen in the WT mice was most likely due to an increase in fat mass which was not significant but show a strong trend both when expressed in absolute values (Figure 3A) or as a percentage of body weight (Supplementary figure 1A). In contrast, in the Y5KO mice, although they started with a significant higher level of fat mass compared to the WT mice, PYY3-36 treatment led to a reduction in fat mass compared to that seen in prior treatment (Figure 3A). The trend to increased fat mass in the DEXA analysis was confirmed when comparing the sum of weights of dissected white adipose tissues depots when expressed as absolute value (Figure 3B), and this became significant when expressed as a percentage of body weight (Supplementary figure 1B). Individual fat depot (WATi, WATe, & WATr) showed a similar trend to an increase as seen for the sum of WATs, again both when expressed in absolute values (Figure 3C-F) or as a percentage of body weight (Supplementary figure 1C-F). The sum of all 4 fat depots only showed a trend of increase in WT mice (Figure 3B), which might be contributed by a slight increase in single depots such as WATe (Figure 3D), WATm (Figure 3E) and WATr (Figure 3F). Interestingly, this was not seen in the Y5 KO mice (Figure 3C-3F, supplementary figure 1C-F). On the other hand, PYY3-36 treatment had no significant effect on brown adipose tissue (BAT) weights in either WT mice or Y5KO mice (Figure 3G, Supplementary figure 1G). Furthermore, lean mass in WT and Y5KO mice was not affected by the PYY3-36 treatment, which both showed slight increases over time consistent with the general increase in body weight (Figure 3H, Supplementary figure 1H). The weights of other

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tissues such as heart, spleen, testis, kidney, and seminal vesicles were also not significantly different between genotype-matched groups (Supplementary Figure 2A-D).

To determine whether compensatory changes in expression of other Y receptors may underlie the effects observed in Y5KO mice fed in HFD, we then isolated RNA from hypothalamic block of Y5KO mice and their littermates, quantified and compared the receptor expression of Y receptors other than Y5R. Y1R expression was significantly upregulated in Y5KO hypothalamus under a chow diet and shown marked trend towards an increased level under a HFD ((Figure 3I). Y2R expression was not altered by either Y5R deletion or diet conditions (Figure 3J). Interestingly, there was no difference between Y5KO and littermate with regards to Y4R expression under a chow diet, however, under a HFD condition, Y4R expression was almost diminished in Y5WT hypothalamus compared to chow-fed Y5WT hypothalamus (Figure 3K). Y5KO hypothalamus had 9-fold higher Y4R expression level than WT hypothalamus under HFD (Figure 3K).

Effects of short term PYY3-36 treatment on glucose homeostasis

Next, we examined whether continuous PYY3-36 infusion also has an effect on whole body glucose homeostasis in DIO WT or Y5KO mice. For this we performed oral glucose tolerance tests employing a technique developed in our laboratory which allows for the voluntarily administration of glucose in form of a jelly (17), thereby avoiding the stress effect normally associated with gavage, an important consideration when investigating effects dependent on the NPY system which is prone to be strongly influenced by stress (19). In brief, after having been trained to eat the jelly, mice fasted for 6 hours starting at 8 am were orally given glucose-containing jelly at 1.5 g glucose/kg body weight, which mice normally consume within one minute. PYY3-36 treated WT mice displayed significantly worsened

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glucose tolerance compared to vehicle-treated mice in the OGTT (Figure 4A) as well as marked trend towards increased AUC (Figure 4B, $p = 0.09$), which happened on the background of elevated insulin levels during the course of the OGTT (Figure 4C, with no difference in AUC, Figure 4D), indicating a possible impairment in insulin responsiveness. In contrast to WT mice, no adverse effects on glucose tolerance were seen in Y5 KO mice treated with PYY3-36 (Figure 4E-H).

Additionally, we measured basal blood glucose levels before and after PYY3-36 treatment in both genotypes. After 12 weeks on HFD before the commencement of PYY3-36 treatment, basal blood glucose levels after 4 hours fasting were not different between DIO WT and Y5KO mice (Figure 4I). After 3 weeks treatment with either vehicle or PYY3-36, basal glucose levels in WT mice were comparable in the two groups (Figure 4I). Interestingly, PYY3-36-treated Y5KO mice on the other hand displayed a strong trend for decreased blood glucose levels compared to saline treated Y5KO mice (Figure 4I). We then went on to also examining weights of pancreas and liver, two organs that are closely related to whole body glucose homeostasis. No significant differences in pancreas weight in PYY3-36-treated WT or Y5 KO mice were observed when compared to that in vehicle-treated control mice (Figure 4J, Supplementary figure 2E). Liver weights are comparable in genotype-matched DIO mice (Figure 4K, Supplementary figure 2F), suggesting livers are not affected by sub-chronic PYY3-36 treatment.

Effects of PYY3-36 on bone metabolism

Since it is known that central Y2R signalling is also critical for the regulation of bone homeostasis we investigated the effects of short-term treatment of PYY3-36 on bone mass (20). There was no difference in whole body BMD and BMC observed after 12 weeks HFD

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between WT and Y5KO mice (Figure 4L). Furthermore, 3 weeks treatment with PYY3-36 did not have any impact on either bone mineral density (BMD) or bone mineral content (BMC) in either genotype (Figure 4M). We found that BMD and BMC were significantly higher in mice that had osmotic mini -pumps implanted than those prior to treatments, which is most likely due to the fact that pumps themselves being recognized as bone tissues by DXA. There was no difference in body length among groups (Supplementary figure 2G).

Discussion

We demonstrate that PYY3-36 treatment in DIO WT mice leads to acute reduction in food intake, and deletion of Y5 receptor causes further reduction in food intake induced by PYY3-36 within 24h administration, suggesting that an antagonizing role of Y5 receptor in the acute anorectic action of PYY3-36. Our study also reveals that 3-week continuous treatment with PYY3-36 via mini pump displays distinct pattern on daily food intake in the absence or presence of Y5 receptors. Increased cumulative food intake over 3-week course in DIO WT mice treated with PYY3-36 is accompanied by an increase in body weight, which is reversed by the lack of Y5 receptors. In addition, increased fat mass in PYY3-36 treated DIO WT mice is diminished in Y5 receptor deficient mice treated with PYY3-36. Furthermore, PYY3-36-treated DIO WT mice display higher glucose excursion whereas simultaneous deletion of Y5R corrects glucose intolerance, further confirming the involvement of Y5R in PYY3-36 induced effects on food intake and glucose homeostasis.

Our observation that lack of Y5 receptor enhances the body weight lowering effect of PYY3-36, is in agreement with a previous study (21) using PYY3-36 and a selective Y5R antagonist in combination. In that study, acute i.p. PYY3-36 injection dose-dependently reduces spontaneous feeding in DIO WT mice, while combined treatment of PYY3-36 and the Y5R antagonist via subcutaneous chronic infusion for 14 days leads to a greater reduction of body

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weight than treatment with PYY3-36 alone, although this reduced body weight gain was not found to be associated with any significant feeding reduction. We observed that Y5R is not only involved in the acute anorectic action of PYY3-36, but also in the chronic effect of PYY3-36. PYY3-36, a selective Y2R agonist, with a 10-fold lower affinity to Y5R that mediates NPY-induced orexigenic action together with Y1R in the PVN (11). Blockade of Y5 receptor-mediated orexigenic effect could potentiate Y2R-mediated anorectic action of PYY3-36, which may indicate that a combined therapy using Y2R stimulating agent and Y5R blocking agent could result in a greater effect in reducing food intake and body weight.

It is noted that our results in cumulative food intake, body weight gain and adiposity in PYY3-36 treated DIO WT mice is different to the study by Pittner et al (22). The discrepancy in the results seen is probably due to several factors, one being housing condition different, with ours being single housed and in the study by Pittner being group housed. Single housing may have changed feeding behaviour and increased energy expenditure, however, it provides more accurate food intake measurement. While acute peripheral administration of PYY3-36 has consistently been shown to reduce food intake, the prolonged exposure to this ligand may cause a desensitisation of Y2 receptors, which then could lose their ability to reduce food intake. This provides some explanation to the paradoxical increase in food intake and body weight as observed between the 4th-9th days in our study. It is also possible that there are other differences with regard to the composition of the HFD, the duration on high fat diet feeding (12 weeks HFD starting from 6-8 weeks of age in our study versus 4 weeks HFD starting from 7 weeks of age in the study by Pittner, as well as the duration of treatment). All these different parameters make a direct comparison difficult. Nonetheless, this study highlights the complexity of PYY3-36's role in food intake regulation.

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Low circulating PYY level has been implicated in the development of obesity and diabetes (23), and PYY3-36 administration to DIO WT mice showed prevention in diet-induced increase in HbA1c (22). Interestingly, we observe reduced glucose intolerance in DIO WT mice after 3-week treatment with PYY3-36, which can be at least partially explained by slightly increased adiposity. However, this impairment was significantly reduced in Y5KO mice treated with PYY3-36, suggesting that Y5 receptor contributes to the worsening of glucose tolerance on HFD, and the removal of Y5R can improve glycemic regulation of PYY3-36. However, it seems if body weight/adiposity were well controlled, there would be less effects on glucose tolerance. Interestingly, Y5R does not seem to affect basal glucose levels in DIO mice in our study.

It is worthwhile mentioning that the differences observed between the various effects of PYY on WT versus Y5KO mice might not only be due to the simple lack of this NPY receptor subtype but also the potential compensatory effects that lack of Y5R alters expression levels of other Y-receptor subtypes. In fact, the expression of Y1 and Y4 receptors in Y5KO hypothalamus was upregulated under high fat diet conditions whereas the expression of Y2 receptors remained unchanged. Significantly elevated expression of anorexigenic Y4 receptor may also contribute to the reduced food intake observed in Y5KO mice. Additionally, the chronic treatment with a HFD, especially duration on HFD may have different effects on modulating expression levels of various Y-receptors further complicating the Y-receptor network, as well as on altering circulating hormonal levels, such as insulin, leptin and adrenaline, etc., which may also, at least partially be responsible for the discrepancies in the anorectic and body weight reducing effects of PYY3-36 in the literature. It is also important to consider that constant infusion of PYY3-36 via mini-pumps does not mimic the natural occurring oscillation of hunger and satiety cycles and this being a main reason for the

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induction of potential compensatory mechanism and a more targeted treatment regime with single doses after a meal might avoid this problem and could still produce long-term beneficial effects on reducing food intake and body weight. Furthermore, in the present study, we showed Y5KO mice are heavier but have reduced food intake, indicating the possibility that the lack of Y5 receptor may affect energy expenditure. However, previous studies have also shown that chronic ICV treatment with a Y5R agonist can lead to great body weight gain with decreased body temperature and BAT uncoupled protein 1 (UCP1) expression indicating decreased energy expenditure (24). The discrepancy in the control of energy expenditure between these studies could be at least partially explained by the compensatory effects of germline Y5KO mice as well as specificity of Y5R antagonists used. More detailed studies, for example, employing Y5KO mice combined with a selective Y5R antagonist or using adult-onset conditional Y5 receptor knock out mice will be required to clarify the specific roles of Y5R in regulating energy expenditure.

In summary, the present study provides new evidence that Y5 receptors antagonize PYY3-36 induced anorectic effects and body weight-reducing effects in diet-induced obese mice. This study also demonstrates that Y5 receptors are involved in PYY3-36's regulation of whole body glucose homeostasis. This highlights the potential that a combined therapy with PYY3-36 and Y5R antagonist could offer improved effectiveness in inhibiting food intake, reducing body weight and maintaining normal glucose homeostasis under HFD condition.

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Disclosure statement

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Figure legends:

Figure 1: **Body weight and food intake of HFD-fed WT and Y5KO mice treated with vehicle or PYY3-36**. Confirmation of Y5R deletion using DNA extracted from the liver and hypothalamus of Y5KO mice and littermate WT (A). WT, wild type; Y5KO, Y5 receptor knockout; Het, heterozygote. Body weight of WT and Y5KO mice fed with HFD for 12 weeks, expressed as absolute value in gram (B) or a percent of initial body weight (C). Daily food intake of WT and Y5KO mice was expressed in gram (D) or a percent of body weight (E) during 12 week HFD feeding. Data are means ± SEM of 19-20 mice per group. ** $p<0.01$ Y5KO versus WT. Food intake of WT and Y5KO mice was measured in first 24 h, expressed as absolute value in gram (F), and as a percent of body weight (G). Food intake of WT and Y5KO mice was measured in the second 24 h, expressed in gram (H) and as a percent of body weight (I). Body weights in these two days (J) after the commencement of vehicle or PYY3-36 treatments. Data are means ± SEM of 9-10 mice per group. * $p<0.05$, ** $p<0.01$ versus respective controls or for the comparisons indicated by horizontal bars.

Figure 2: Daily food intake, cumulative food intake and body weight in WT and Y5KO mice treated with vehicle and PYY3-36 for 20 days. Daily food intake of WT (A, C) and Y5KO mice (B, D) were expressed as an absolute weight in g and as a percent of body weight after 20-day treatment with either vehicle or PYY3-36. Cumulative food intake of WT (E) and Y5KO mice (F) Data are means ± SEM of 9-10 mice per group. * $p<0.05$ versus controls or p value was indicated for the comparison. Daily body weight change of WT (G) and Y5KO mice (H) was measured after vehicle or PYY3-36 treatments over a 20-day period.

Figure 3: Fat mass and body composition of WT and Y5KO mice after 20-day treatment of vehicle and PYY3-36. Fat mass of WT and Y5KO mice was determined by DXA before and after vehicle or PYY3-36 treatments (A). Total WAT weights (B), inguinal

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white adipose tissue (WATi, C), epididymal WAT (D), mesenteric WAT (E), retroperitoneal WAT (F) and brown adipose tissue (BAT, G) of WT and Y5KO were measured after 20 days treatment with either vehicle or PYY3-36. Lean mass of WT and Y5KO mice was determined by DXA before and after vehicle or PYY3-36 treatments (H). Expression level of Y1R (I), Y2R (J) and Y4R (K) in the hypothalamus of WT and Y5KO mice under a chow and high fat diet conditions. WAT, white adipose tissue. Data are means ± SEM of 9-10 mice per group. * $p<0.05$ and P value was shown for the comparison indicated by horizontal bar.

Figure 4: Glucose metabolism and bone parameters in WT and Y5KO treated with either vehicle or PYY3-36. Oral glucose tolerance test (OGTT) and area under the curve (AUC) during OGTT in WT mice (A, B) as well as blood insulin levels and AUC during the OGTT (C, D) in WT mice treated with either vehicle or PYY3-36. OGTT and AUC (E, F) as well as insulin levels and AUC of Y5KO mice treated with vehicle or PYY3-36 (G, H). Basal blood glucose level (I) and the weights of pancreas (J) and liver (K) measured in HFD-fed WT and Y5KO mice treated with or without PYY3-36. Data are means ± SEM of 5-10 mice per group. Bone mineral density (BMD, L) and bone mineral content (BMC, M) determined by DXA before and after treatments. * $p<0.05$ and P value was shown for the comparison indicated by horizontal bar.

Supplementary figure 1: Fat mass (A), total WAT weights (B), WATi (C), WATe (D), WTm (E), WATr (F), BAT (G) and lean mass (H) of WT and Y5KO mice treated with or without PYY3-36 were expressed as a percent of body weight. Data are means ± SEM of 9-10 mice per group. * $p<0.05$, ** $p<0.01$ versus respective controls or for the comparisons indicated by horizontal bars.

Supplementary figure 2: Tissue weights of WT and Y5KO mice treated with vehicle or PYY3-36. The weights of heart, spleen, testis were expressed as value in gram (A) and as a percent of body weight (B). The weights of kidney and seminal vesicle were expressed as a

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value in gram (C) and as a percent of body weight (D). Pancreas and liver weights were expressed as a percent of body weight were shown in (E) and (F). Body length of WT and Y5KO mice treated with or without PYY3-36 was shown in (G). Data are means ± SEM of 9-10 mice per group. * $p<0.05$, ** $p<0.01$ versus respective controls or for the comparisons indicated by horizontal bars.

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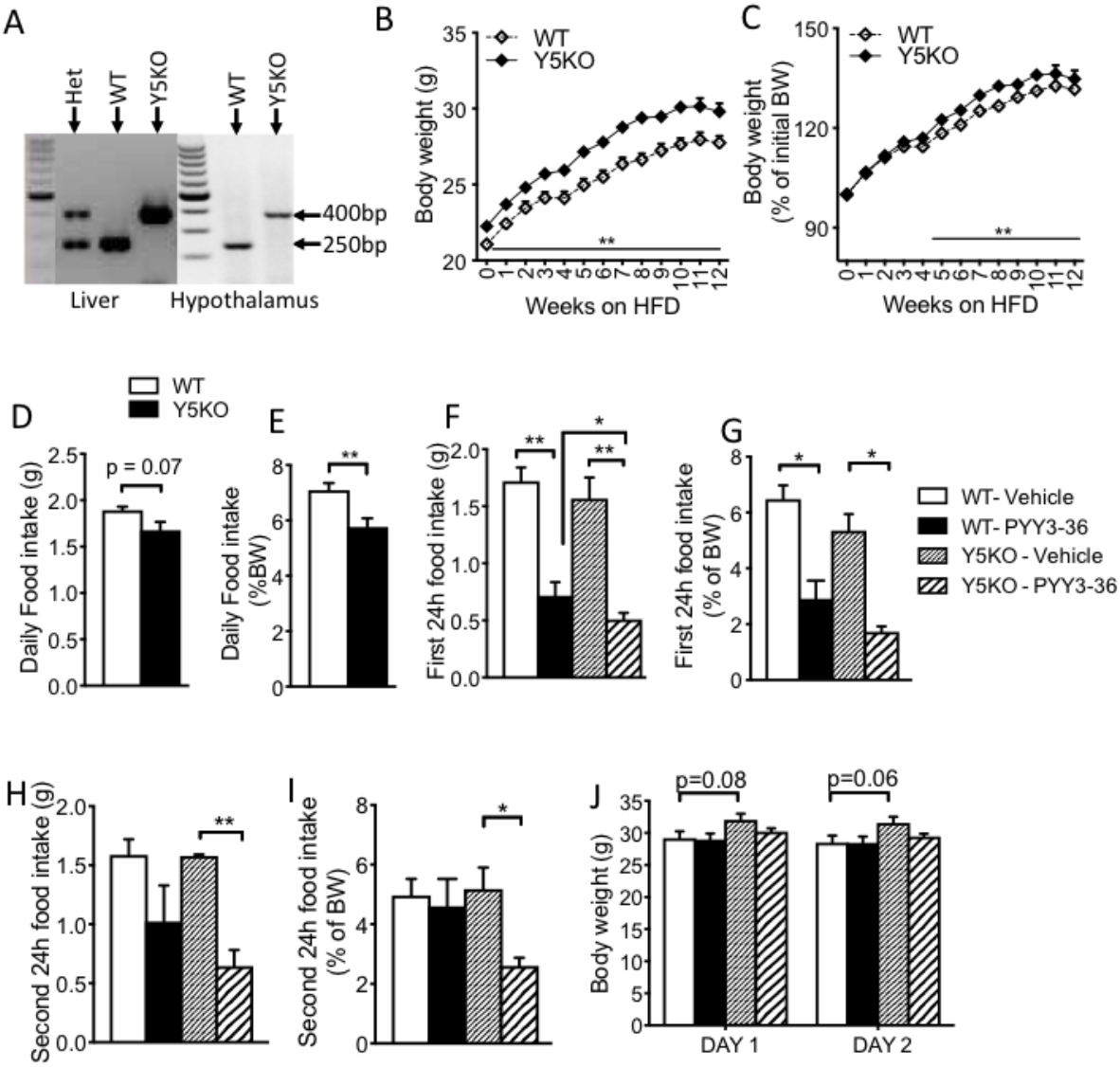

Figure 1

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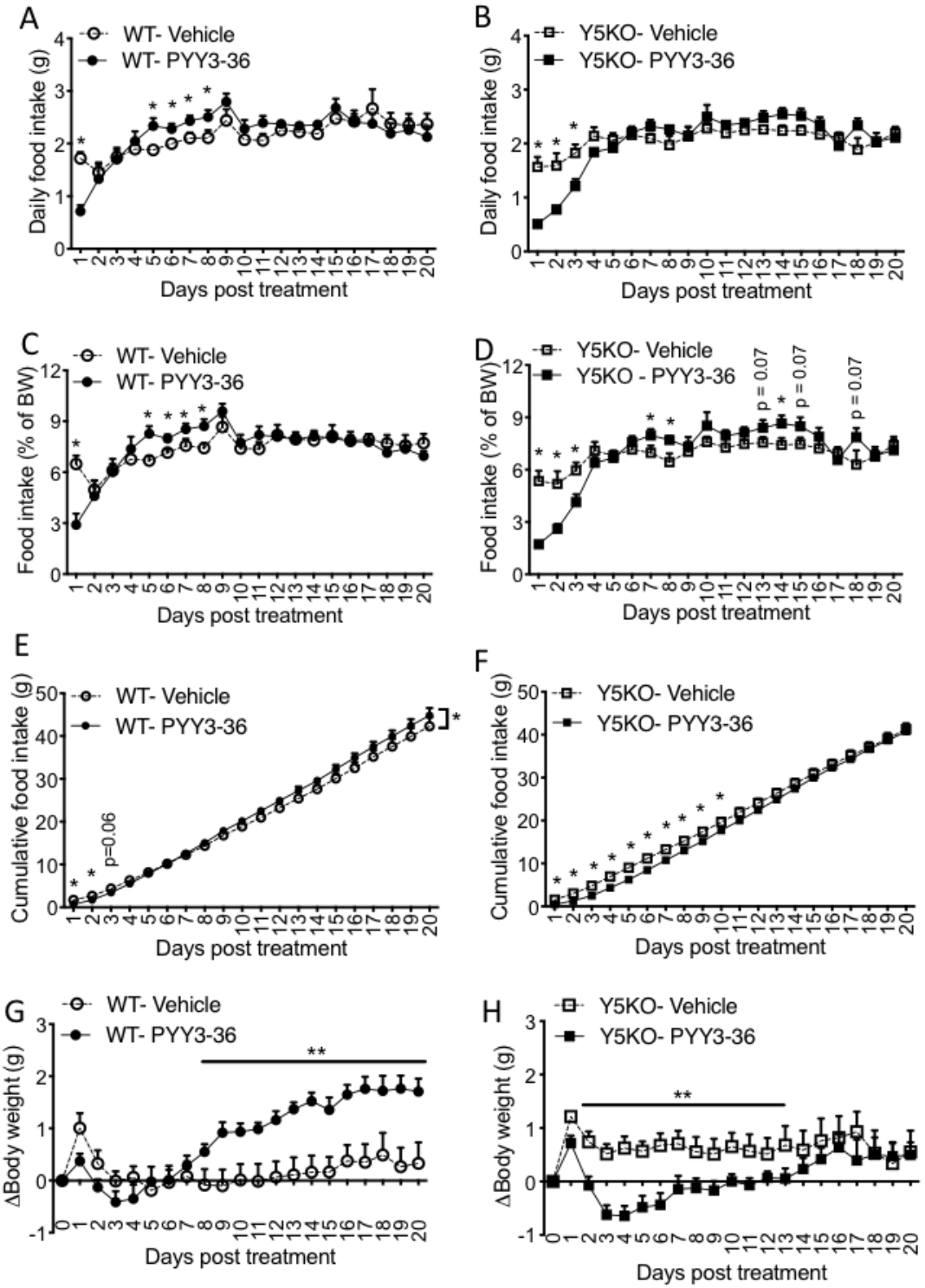

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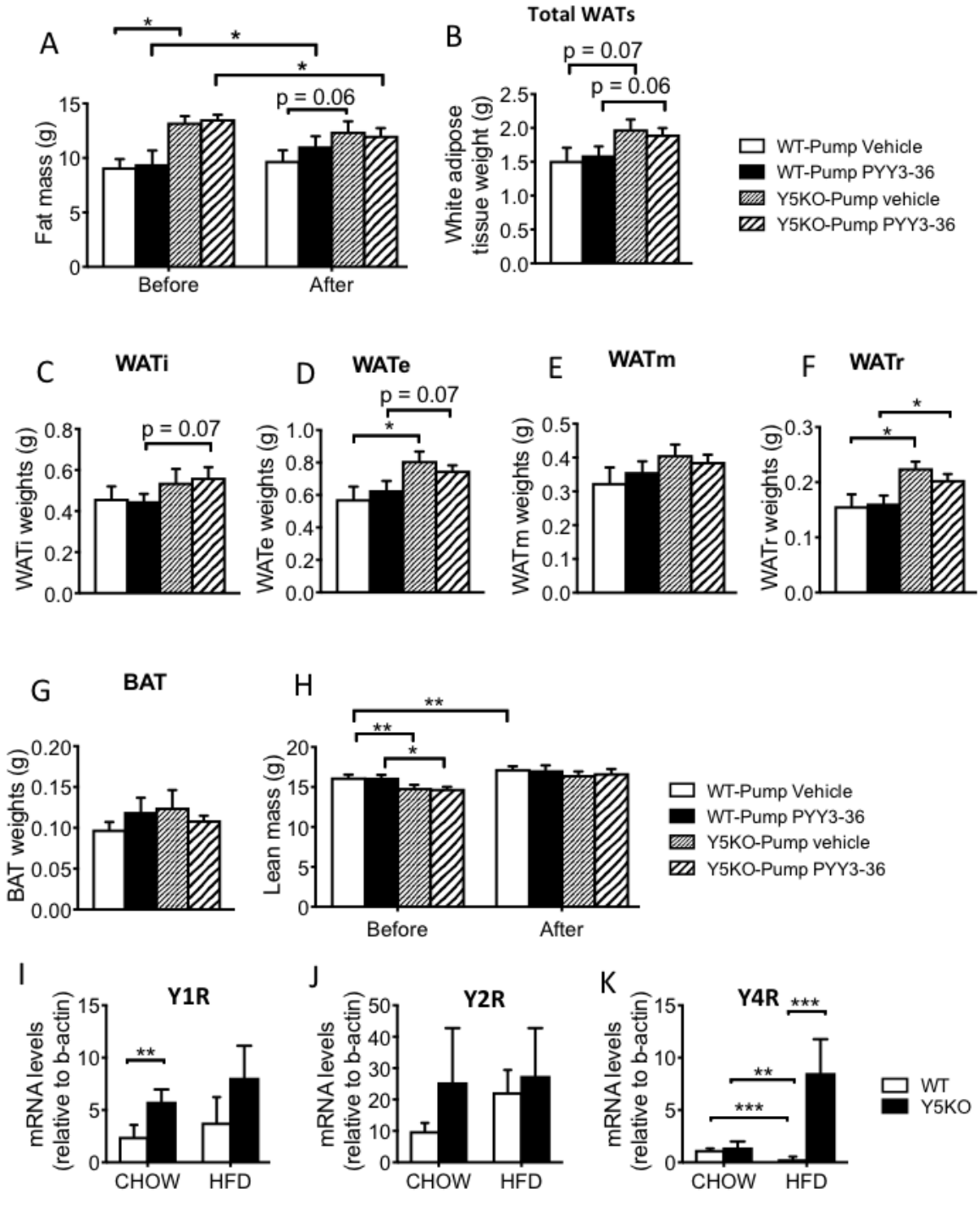

Figure 3

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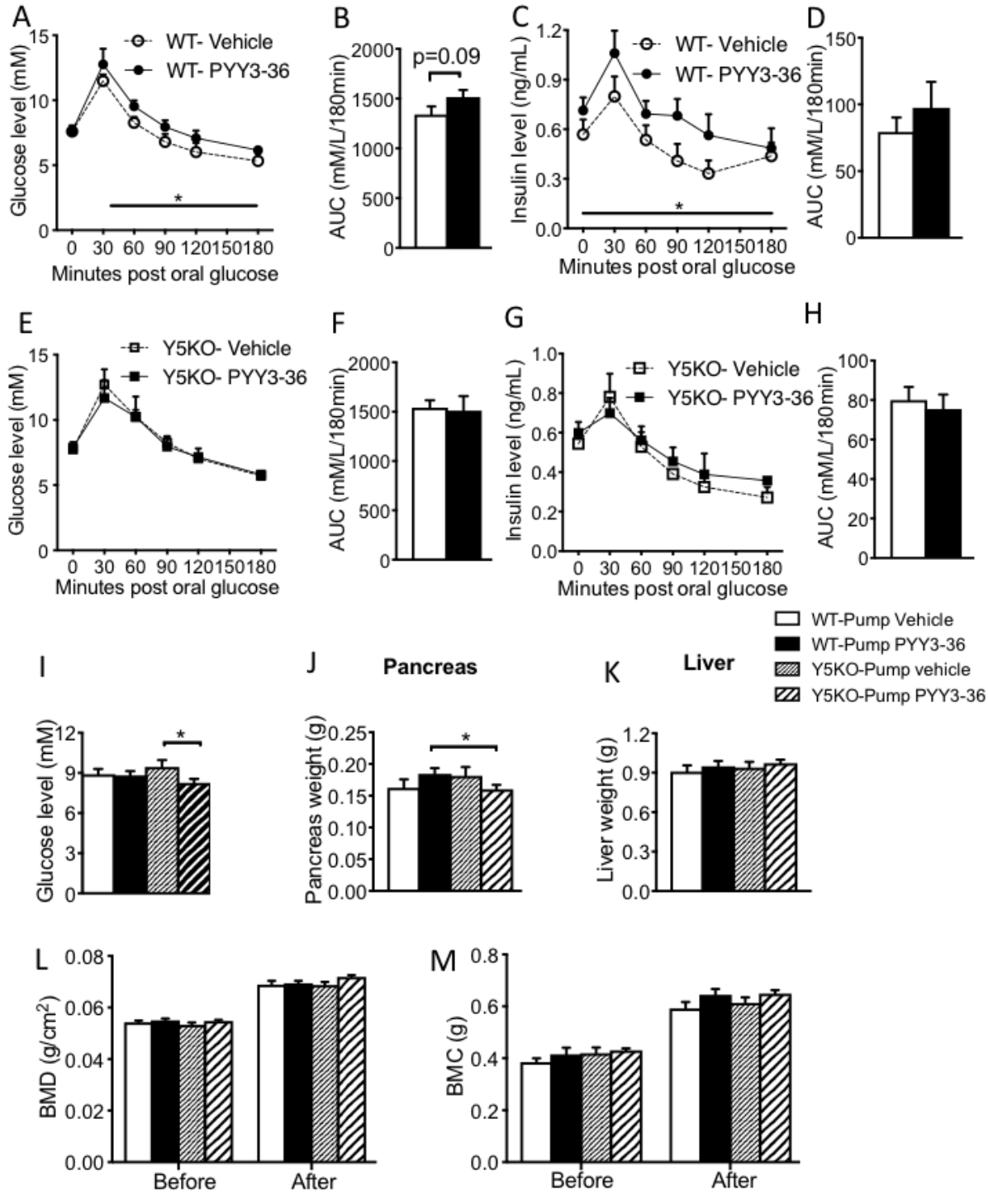

Figure 4

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