The calcitonin receptor plays a major role in glucose regulation as a function of dual amylin and calcitonin receptor agonist therapy

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Running title page

Running title: DACRA effects are mediated through both AMY-R and CTR

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Abbreviations:

ANOVA: analysis of variance

AMY-R: amylin receptor

CTR: calcitonin receptor

DACRA: dual amylin and calcitonin receptor agonist

ELISA: Enzyme-linked immunosorbent assay
HbA1c: glycated haemoglobin type A1c

HFD: high fat diet

OGTT: oral glucose tolerance test

RAMP: receptor-activity modifying protein

rAMY: rat amylin

rCT: rat calcitonin
Abstract

Amylin treatment improves body weight and glucose control, though limited by a short action and need for high doses. DACRAs are dual amylin and calcitonin receptor agonists with beneficial effects beyond those of amylin. However, to which extent the additional benefits reside in their higher potency or their targeting of the calcitonin receptor remains unclear. Here we deconstruct the receptors involved in the effects of a DACRA, KBP-088, by comparing it to rat amylin (rAMY), rat calcitonin (rCT) and their combination in obese HFD and diabetic ZDF rats. HFD fed Sprague Dawley rats and ZDF rats were treated for four weeks with KBP-088 (5 µg/kg/day), rAMY (300 µg/kg/day), rCT, (300 µg/kg/day) and the combination of rAMY and rCT (300+300 µg/kg/day) using infusion pumps. Body weight, food intake, fasting glycemia, HbA1c levels and glucose tolerance were assessed. In obese HFD fed rats, KBP-088, rAMY and the combination of rAMY and rCT significantly reduced body weight and improved glucose tolerance, while rCT alone had no effect. In diabetic ZDF rats, rCT was efficient in lowering fasting glycemia similar to rAMY, while dual activation by KBP-088 and the combination of rAMY and rCT were superior to activating either receptor alone. In conclusion, calcitonin therapy regulates fasting blood glucose in a diabetic rat model, thereby underscoring the importance of calcitonin receptor activation, as well as the known role of amylin receptor agonism in the potent metabolic benefits of this group of peptides.

Significance statement

We deconstruct the receptors activated by dual amylin and calcitonin receptor agonist (DACRA) therapy to elucidate through which receptor the beneficial metabolic effects of the DACRAs are mediated. We show that calcitonin receptor activation is important for blood glucose regulation in diabetes. This is in addition to the known metabolic beneficial role of amylin receptor activation.
These data help in understanding the potent metabolic benefits of the DACRAs and underline the potential of DACRAs as treatment for diabetes and obesity.
Introduction

The amylin analogue pramlintide is approved as adjunct therapy to insulin for both type 1 and type 2 diabetes (Weyer et al., 2001; Hollander, Priscilla A; Levy, Philip; Fineman, Mark S; Maggs, 2003; Ratner et al., 2004; Riddle et al., 2007; Ryan et al., 2008). Pramlintide works by reducing post-prandial blood glucose excursions and appetite, thereby leading to improved glucose control, as well as a weight loss (Aronne et al., 2007; Ryan et al., 2008; Smith et al., 2008; Singh-Franco et al., 2011; Traina and Kane, 2011; Herrmann et al., 2014). On the other hand, pramlintide is limited by its short time of action and need for high doses (Dunican et al., 2010; Younk et al., 2011), and therefore peptides with potent amylin-receptor agonistic properties are being carefully investigated.

Dual amylin and calcitonin receptor agonists (DACRAs) are a novel group of peptides in this family, and studies of these have clearly demonstrated that they possess metabolic efficacy beyond that of amylin on classical amylin-related effects, such as weight loss and post-prandial glucose control (Andreassen, Feigh, et al., 2014; Gydesen et al., 2016; Hjuler et al., 2016). Importantly, several DACRA studies using pair-fed groups have shown that DACRA treatment induces body weight loss, delay of gastric emptying and improved glucose tolerance superior to that explained by a reduced food intake (Andreassen, Feigh, et al., 2014; Gydesen et al., 2016; Hjuler et al., 2016; Gydesen, Hjuler, et al., 2017). Furthermore, DACRAs elicit markedly positive effects on fasting hyperglycaemia and HbA1c levels in animal models of type 2 diabetes (Andreassen, Feigh, et al., 2014; Gydesen, Hjuler, et al., 2017; Hjuler et al., 2017), effects not seen with amylin therapy alone. At the molecular level, DACRAs differ from amylin as they activate both the amylin receptor (AMY-R) and the calcitonin receptor (CTR), and do so potently (Andreassen, Hjuler, et al., 2014; Gydesen et al., 2016). Importantly, the CTR and AMY-R share the core 7TM GPCR, though the AMY-R is formed by interaction of a CTR with a receptor-activity modifying protein (RAMP). The interaction with a RAMP switches the receptor from a high-affinity CTR to a high-affinity AMY-R
(Christopoulos et al., 1999; Muff et al., 1999; Armour et al., 2000) (Figure 1). Despite the AMY-R being a CTR with a RAMP, the known effects of their corresponding ligands are diverse, with amylin working primarily on several metabolic parameters, while CT is mostly known to inhibit osteoclast-mediated bone resorption and reduce plasma calcium (Findlay and Sexton, 2004; Inzerillo et al., 2004; Tankó et al., 2004; Henriksen et al., 2016).

However, to what extent the superior activity of the DACRAs resides in their potency and to what extent it resides in the dual receptor activation is presently unknown, due to the difficulties of separating the AMY-R mediated effects from the putative CTR-mediated effects. In a recent study, we showed that, even when correcting for the different activity profiles, the DACRA KBP-088 is superior to amylin therapy in terms of body weight loss and glucose tolerance in high fat diet fed rats (Larsen et al., 2019). However, the contribution of the CTR in DACRA therapy has not been studied previously, hence to what extent the CTR is involved in the DACRA mediated effects, particularly on glucose homeostasis and insulin sensitivity, is unknown. Furthermore, whether a DACRA response can be obtained by combination of the selective ligands, amylin and calcitonin, when delivered by continuous infusion, is not known.

In this study, we utilize infusion pumps to compensate for differences in potency and effect time between the peptide ligands. Through this we deconstruct the receptors involved in the effect of a highly potent DACRA, KBP-088, by comparing it to rat amylin (rAMY), rat calcitonin (rCT) and the combination of rAMY and rCT on long term efficacy in rats fed high-fat diet and as intervention in ZDF rats suffering from obesity and hyperglycaemia.
Materials and Methods

Peptide therapy

Synthetic KBP-088, rat calcitonin (lot#JT-71548 (HFD) and JT-76457 (ZDF)) and rat amylin (lot#JT-74056 (HFD) and JT-76456 (ZDF)) (SynPeptide, Shanghai, China) were dissolved in saline (NaCl 0.9%) for subcutaneous (s.c.) delivery. The amino acid sequence of KBP-088 have previously been published head to head with the amino acid sequence of rat amylin (Larsen et al., 2019). The dose chosen for KBP-088 administration was based on previous studies using KBP-088 (Gydesen et al., 2016) and other DACRAs (Hjuler et al., 2015, 2016, 2017; Gydesen, Andreassen, et al., 2017). The doses chosen for amylin and calcitonin were based on a previous study testing increasing doses of amylin in a similar setup (Larsen et al., 2019).

Animal experiments

All animal procedures were performed in accordance with guidelines from the Animal Welfare Division of the Danish Ministry of Justice under the institutional license issued to Nordic Bioscience (2016-15-0201-00910). Animals were housed pair-wise in standard type IV cages (Scanbur A/S, Karlslunde, Denmark) under controlled temperature (21-23 °C, 55-65% relative humidity) and a normal 12-hour light-dark cycle with ad libitum access to food and water. Two studies were performed. In both studies animals received dosing of KBP-088 (5 μg/kg/day s.c.), rat amylin (300 μg/kg/day s.c.), rat calcitonin (300 μg/kg/day s.c.), a combination of rat amylin and rat calcitonin (300 μg/kg/day s.c.) and vehicle (saline) for four weeks. Group size was determined based on previous studies with similar experimental setup (Hjuler et al., 2015, 2016, 2017; Gydesen, Andreassen, et al., 2017). Peptides and vehicle were delivered by continuous s.c. infusion through Alzet osmotic pumps (Model 2ML4, AgnTho’s, Lidingö, Sweden). Under isoflurane anaesthesia, animals were surgically equipped with an osmotic pump s.c. in the neck region.
Initially the skin was locally anesthetized at the site of incision (Marcaine® injection) and animals received analgesia (Norodyl® injection) 3 days post-surgery.

Animal study 1) 4-week treatment of high fat diet fed rats

40 male Sprague Dawley rats (Envigo, Venray, The Netherlands) were obtained at 5-6 weeks of age. All rats were fed a 60 kcal% fat high-fat diet (HFD) (#58Y1, TestDiet, London, UK) from arrival and throughout the study period. After 12 weeks on HFD, rats were randomized into treatment groups according to body weight with an average of 507 ± 22 (SD) g (n=8 rats/ treatment group). Body weight and food intake were monitored daily for the initial 10 days of the study and then every third day throughout the study period. At study end an oral glucose tolerance test (OGTT, 2 g/kg, 4 ml/kg) was performed in overnight fasted rats. To assess the effect on gastric emptying, rats received acetaminophen (40 mg/kg) by oral gavage together with the glucose bolus during OGTT and the appearance of acetaminophen in plasma was measured 30 min post administration. At study end rats were euthanized by exsanguination (under isoflurane anaesthesia) followed by dissection. Epididymal, perirenal and subcutaneous inguinal fat depots were surgically removed and weighed.

Animal study 2) 4-week intervention study in ZDF rats

40 male Zucker diabetic fatty (fa/fa) (ZDF) (Charles River Laboratories, Lyon, France) were obtained at 5 weeks of age. This model was selected as it is a common rat model in type 2 diabetes research testing new therapies (Pick et al., 1998; Shibata et al., 2000; Topp et al., 2007; King, 2012). All rats were fed Purina Laboratory Diet (#5008, LabDiet, St. Louis, MO, USA) from arrival and throughout the study period. Rats were allowed to develop diabetes before they were randomized into treatment groups primarily according to fasting blood glucose levels (6 hours) and secondarily according to body weight (n=8 rats/ treatment group). At study start rats weighed 335 ±
12 (SD) g and had a fasting blood glucose level at 11.3 ± 5.7 (SD) mM. Body weight and food intake were monitored daily for the first week of the study and then twice per week throughout the study. Fasting blood glucose levels (6 hours) were measured weekly and HbA1c levels were measured at study end. After 26 days of treatment an OGTT (1 g/kg, 2ml/kg) was performed in rats fasted for 11 hours. To assess the effect on gastric emptying, rats received acetaminophen (20 mg/kg) by oral gavage together with the glucose bolus during OGTT and the appearance of acetaminophen in plasma was measured 30 min post administration. At study end rats were fasted 6 hours, blood was sampled, and rats were euthanized by exsanguinations under isoflurane anaesthesia. Epididymal, perirenal and subcutaneous inguinal fat depots were surgically removed and weighed, and pancreases were surgically removed and stored for analysis of insulin content.

**Glucose tolerance test**

Oral glucose tolerance tests (OGTT) were performed in overnight fasted (11 hours) rats. A glucose bolus (Sigma-Aldrich, Copenhagen, Denmark) were administered p.o. gavage at time 0. EDTA blood samples were collected from the tail vein before glucose challenge (0 min) and the following 15, 30, 60 and 120 min post glucose challenge. Blood glucose was monitored at time 0, 15, 30, 60, 120 and 180 min post glucose challenge.

**Biochemical analysis**

Blood samples were collected in EDTA tubes and centrifuged at 5000 rpm for 10 min at 4 °C and plasma was kept at -20 °C until further analysis. Blood glucose was monitored by Accu-Check® Avia monitoring system (Roche Diagnostics, Rotkreuz, Switzerland). HbA1c levels were measured by DCA Vantage Analyzer (Siemens, Erlangen, Germany). Plasma levels of insulin (Mercodia Rat Insulin ELISA, Mercodia AB, Uppsala, Sweden. RRID: AB_2811229) and acetaminophen (Neogen
Corporation’s Acetaminophen ELISA Kit, Neogen Toxicology, KY, USA) were analysed according to manufacturer’s instructions.

**Tissue analysis**

Pancreases were homogenized and extracted in acid-ethanol (1.5% HCl in 70% EtOH) for determination of insulin content. Protein contents of the extracts were estimated using the Bio-Rad DC Protein Assay (Bio-Rad Laboratories, CA, USA). Insulin contents (Mercodia High Range Rat Insulin ELISA, Mercodia AB, Uppsala, Sweden) were analysed according to manufactures’ instruction.

**Data and statistical analyses**

All data are presented as mean ± standard error of the mean (SEM). The statistical analyses of group differences were conducted using one-way ANOVA followed by Tukey’s post-hoc test for multiple comparison or two-way ANOVA followed by Dunnet’s test for multiple comparison. Statistical analyses of non-parametric data were conducted using Kruskal Wallis test followed by Dunn’s post-hoc test for multiple comparison. Normality of data distribution is determined by Shapiro-Wilk normality test. All analyses were performed using GraphPad Prism 8 software (San Diego, CA, USA). A value of P < 0.05 was considered statistically significant.
Results

Body weight loss, reduction of food intake and improvement of glucose tolerance

are mainly mediated through the amylin receptor in high-fat diet fed rats

To address how the AMY-R and the CTR each contribute to the beneficial effects of KBP-088, high-fat diet fed rats received KBP-088 therapy in comparison with the selective agonists rAMY and rCT or a combination of the two. KBP-088 (5 µg/kg/day s.c.), rAMY (300 µg/kg/day s.c) and the combination of rAMY and rCT (300 + 300 µg/kg/day s.c.) significantly reduced food intake in the initial phase of the study, while rCT (300 µg/kg/day s.c.) alone only slightly reduced food intake and this was only the first day after study start. In addition, KBP-088 and rAMY resulted in a prolonged reduction of food intake (Figure 2B). Chronic treatment with KBP-088, rAMY and the combination of rAMY and rCT resulted in a 17%, 11% and 8% vehicle-corrected weight loss, respectively, while rCT alone had no effect on body weight in HFD fed rats (Figure 2A, C).

Interestingly, KBP-088 was superior in terms of body weight loss even though rAMY and KBP-088 equally reduced the accumulated food intake (Figure 2D). In addition, KBP-088 and rAMY tends to reduce overall adiposity across different fat depots (Figure 2E-G), while KBP-088 showed a modest reduction of liver weight (Figure 2H); however, when adjusting for body weight no difference was observed.

An OGTT performed at study end showed a trend towards improvement in oral glucose tolerance in response to KBP-088, rAMY and the combination of rAMY and rCT (Figure 3A, C). Underscoring the mode of action, this improvement was achieved with significantly reduced plasma insulin levels compared to vehicle, with KBP-088 being superior (Figure 3B, D). Moreover, KBP-088 significantly reduced gastric emptying rate by 33% compared to vehicle, while rAMY and the combination of rAMY and rCT resulted in gastric emptying rates reduced by app. 20% compared to
vehicle. rCT alone had no effect on the gastric emptying rate (Figure 3E). All beneficial treatment effects observed in HFD fed rats are consistent with known effects of amylin receptor agonism.

Both the calcitonin and amylin receptor are important for improvement of hyperglycaemia in diabetic rats

To elucidate how the AMY-R and CTR contribute to the effects of KBP-088 on blood glucose levels, KBP-088 was compared to the selective agonists rAMY and rCT or a combination of the two in diabetic ZDF rats. ZDF rats were left untreated until the age of 11 weeks where hyperglycaemia had developed, and fasting blood glucose levels had increased to an average of 11.3 ± 5.7 (SD) mM. After development of hyperglycaemia, treatment was initiated by insertion of infusion pumps containing saline (vehicle), KBP-088 (5 µg/kg/day s.c.), rAMY (300 µg/kg/day s.c.), rCT (300 µg/kg/day s.c.) or a combination of rAMY and rCT (300 + 300 µg/kg/day s.c.).

rAMY, the combination of rAMY and rCT and KBP-088 initially reduced the food intake and body weight, though it normalized to vehicle levels during the study. Only KBP-088 treatment resulted in a minor body weight reduction at study end, even though the food intake was normalized (Figure 4A-B). In accordance with the body weights at study end, no marked differences in overall adiposity was observed (Table 1). All treatments resulted in an immediate reduction in fasting blood glucose levels (Figure 4E), independent on the effect on food intake (Figure 4C). rCT and rAMY were efficient in lowering fasting blood glucose levels to the same extent, while dual receptor activation by KBP-088 and the combination of rAMY and rCT was superior to activating either receptor alone (Figure 4E-F). The blood glucose lowering effect induced by dual receptor activation was sustained throughout the study, while the effect of either rCT or rAMY was reduced towards the end of the treatment period (Figure 4E). Furthermore, at study end all treatments tended to lower HbA1c levels compared to vehicle, though only KBP-088 significantly (Figure 4D). These trends corresponded well with the fasting blood glucose levels.
Dual receptor activation through chronic therapy improves glucose tolerance superior to activating either receptor alone

During OGTT, all treatments led to improved glucose tolerance compared to vehicle, though only KBP-088 and the combination of rAMY and rCT significantly (Figure 5A, C). However, the differences in glucose levels during the OGTT were defined by the fasting blood glucose levels at test start (Figure 5A). These differences in fasting levels are reflected in the tAUC, where treatment with KBP-088 and the combination of rAMY and rCT led to a significant reduction in tAUC (Figure 5C) and are further confirmed as the iAUC show no significant differences between treatments (Figure 5E). The improved glucose tolerance was also reflected in the plasma insulin levels during OGTT, where treatment with KBP-088 and the combination of rAMY and rCT tended to increase the plasma insulin levels compared to the other treatment groups (Figure 5B, D). Gastric emptying rate was reduced by app. 20% compared to vehicle by both KBP-088, rAMY and rCT, while the combination of rAMY and rCT significantly reduced gastric emptying by 30% compared to vehicle (Figure 5F).

Dual receptor activation is important for long-term improvement of hyperglycaemia

KBP-088 and the combination of rAMY and rCT significantly reduced fasting blood glucose levels at study end, while rCT or rAMY alone only tended to reduce blood glucose levels at this time point (Figure 6A). In addition, fasting insulin levels at study end were slightly increased by all treatments, with KBP-088 having the most pronounced effect (Figure 6B). This was reflected in the pancreatic insulin contents (Figure 6C), suggesting that dual receptor activation by KBP-088 improved regulation of blood glucose levels by increased pancreatic insulin content as well as secretion.
Discussion

We demonstrate that the calcitonin-mediated activation of CTR is a critical component in fasting blood glucose regulation by dual amylin and calcitonin receptor agonists in a diabetic rat model. Importantly, these data show that the overall metabolic effects of the DACRAs involve not only potent activation of the AMY-R, but also of the CTR to elicit glucose control.

To understand these data, similarities between AMY-R and CTRs must be kept in mind. The CTR and the AMY-R are highly similar, with the primary difference being the association of a RAMP to the AMY-R and not the CTR (Christopoulos et al., 1999; Muff et al., 1999; Armour et al., 2000). These receptors are highly selective in their ligand binding, with amylin binding the AMY-R and calcitonin binding the CTR (Christopoulos et al., 1999; Muff et al., 1999; Hay et al., 2015).

However, several studies showing additional potential of dual agonists on metabolic effects have been published (Andreassen, Feigh, et al., 2014; Gydesen et al., 2016; Hjuler et al., 2016, 2017; Gydesen, Hjuler, et al., 2017; Larsen et al., 2019), and therefore understanding how these additional effects arise, require a deconstruction of the involvement of the individual receptors. To shed light on the receptor involvement we compensated for differences in activity between the selective and the dual agonists by continuous delivering peptides through osmotic pumps and by use of high doses of the selective agonists.

We found that rCT alone possessed a glucoregulatory effect in the diabetic rat, whereas it neither in the diabetic model, nor in the obese model, possessed the ability to regulate food intake and bodyweight, consistent with these being classical AMY-R mediated effects (Roth et al., 2006; Mack et al., 2007; Lutz, 2012; Hay et al., 2015). Importantly, when combining rCT and rAMY, the full dual agonist response on fasting blood glucose was obtained, clearly illustrating the added benefit of CTR agonism for the treatment of type 2 diabetes. Our data also show that the effects of the
tested peptides are dependent on the disease stage. In HFD fed animals the main effect of KBP-088 was mediated through the AMY-R resulting in body weight loss, reduction in food intake and improved glucose tolerance in animals treated with KBP-088, rAMY or the combination of rAMY and rCT. This improved glucose tolerance was obtained with significantly reduced insulin levels, which corresponds with previous studies showing that both DACRAs (Andreassen, Feigh, et al., 2014; Hjuler et al., 2015, 2016; Gydesen et al., 2016; Gydesen, Andreassen, et al., 2017) and infusion of amylin (Roth et al., 2006; Trevaskis et al., 2010; Larsen et al., 2019) reduce plasma insulin in rat models of obesity. In diabetic ZDF rats, on the other hand, CTR activation was found to be essential for the beneficial effects of KBP-088 by lowering fasting glycemia, HbA1c levels and gastric emptying rate and improving glucose tolerance.

The ability of rAMY but not rCT to reduce food intake in both HFD fed rat and ZDF rats, suggests that the anorectic effect of DACRAs is mainly mediated through activation of the AMY-R. Furthermore, the reduction in food intake in HFD fed rats corresponds to the reduction in body weight induced by both rAMY, KBP-088 and the combination of rAMY and rCT. This is consistent with other studies showing that chronic administration of amylin and amylin analogues reduce body weight and food intake in diet-induced obese rodents (Mack et al., 2003; Roth et al., 2006) and obese subjects (Aronne et al., 2007; Smith et al., 2008). Interestingly, KBP-088 was superior in terms of inducing a sustained weight loss in both rat models despite having an accumulated food intake similar to rAMY treated rats. This corresponds well with previous studies showing that infusion of the dual agonists davalintide (Mack et al., 2011) and KBP-088 (Larsen et al., 2019) is superior to amylin in reducing body weight in obese rat, albeit the mechanism behind this superiority is unclear as it does not appear to entail the CTR. The weight loss observed using the infusion pumps is very consistent with previous reports (Larsen et al., 2019), and has been reported to be through a combination of effects on appetite regulation and an increase in energy expenditure.
(Hjuler et al., 2016; Park et al., 2015; Wielinga et al., 2010; Fernandes-Santos et al., 2013). We speculate that the superiority of KBP-088 in terms of body weight loss may be caused by pharmacological challenges observed with amylin, as increasing doses of amylin does not improve the efficacy (Larsen et al., 2019). The mechanism of action underlying the weight loss is known to include appetite regulation, an effect mediated by amylin receptors expressed in the area postrema (Hay et al., 2015), however, there are also studies indicating direct effects on the adipocytes of amylin, although the effects appear to be related to insulin sensitivity more so than weight loss (Miegueu et al., 2013; Moon et al., 2011). Interestingly, the calcitonin receptor does not appear to be involved in this pharmacological response, as calcitonin receptor knockout mice have no overt adipose tissue phenotype (Bartelt et al., 2017).

To the best of our knowledge, the effect of rCT on fasting blood glucose and HbA1c is an effect not previously reported. An old study found CT infusion to reduce blood glucose levels in patients with diabetes, supporting our observations (Starke et al., 1981). Though, Starke et al. used salmon calcitonin which is now known to be a DACRA and hence also activates AMY-R (Christopoulos et al., 1999; Young, 2005). In line with this, it has been found that CTR knockout mice have impaired glucose tolerance (Heeren et al., 2017). Albeit, a CTR knockout will also affect the AMY-Rs, as the AMY-R is a CTR with a RAMP (Christopoulos et al., 1999; Muff et al., 1999; Armour et al., 2000). Hence it cannot be excluded that the observed effects are related to the AMY-R. In contrast, they found a deletion of the CT gene to improve glucose tolerance and protect from diet-induced obesity in mice (Heeren et al., 2017). Furthermore, another recent paper found a CT gene deletion to prevent obesity and hyperglycaemia in aged obese mice (Nakamura et al., 2018). Even though, these papers suggest that CT has a negative effect on glucose metabolism it confirms an association between CT and glucose metabolism. In addition, the studies were performed in knockout mice making it difficult to compare to the present data, which is focused on the pharmacological
responses of CT. Further, knockout of RAMPs cannot be used to study AMY-R related effects, as RAMPs can associate with a number of other receptors including CGRP-R (Hay and Pioszak, 2016; Hay *et al.*, 2016). Hence several receptors will be affected by a RAMP knockout.

Our data suggest that the beneficial effect of KBP-088 and other DACRAs on fasting blood glucose and HbA1c levels (Feigh *et al.*, 2014; Gydesen, Hjuler, *et al.*, 2017; Hjuler *et al.*, 2017) is mediated though activation of both the CTR and the AMY-R, an effect not driven by body weight loss, and thereby appears to be independent of reductions in adipocyte size and numbers, which are known to be essential for the weight-lowering effects of both DACRAs and amylin (Gydesen *et al.*, 2017; Hjuler *et al.*, 2017; Duffy *et al.*, 2018). With respect to the post-prandial effects on blood glucose, one could speculate that the effect of amylin on this parameter is mainly driven by its ability to lower blood glucose by suppressing inappropriate glucagon secretion and slowing gastric emptying as well as its anorectic effect (Hay *et al.*, 2015; Hay, 2017). In ZDF rats, rCT reduced gastric emptying rate similar to KBP-088 and rAMY which might contribute to the positive effect on blood glucose levels, albeit mainly the post-prandial levels. The beneficial effect on blood glucose might also indicate a preserved beta-cell mass as shown in previous DACRA studies (Andreassen, Feigh, *et al.*, 2014; Hjuler *et al.*, 2017). This is confirmed by increased pancreatic insulin content in rats treated with KBP-088.

With these data in mind, questions about the receptor construction in the different target tissues arise, and future studies will address whether one or both target receptors are present in the tissues of interest for glucose deposition, i.e. muscles, fat and liver. An intriguing finding is that amylin and calcitonin therapy alone appear to lose glucose control over time, while the combination maintains the ability. These data potentially indicate that dual activation evokes a different route of receptor regulation than selective activation, although this has not been studied in further detail.
In conclusion, the calcitonin receptor is essential for blood glucose regulation in a diabetic rat model receiving DACRA therapy, while the amylin receptor affects both blood glucose levels and food intake. Though, dual activation of amylin and calcitonin receptors by KBP-088 or the combination of rAMY and rCT is superior to activating either receptor alone in terms of reducing body weight and improving HbA1c levels and glucose control. Thus, the DACRA KBP-088 offers a promising mode of action as a therapy for metabolic conditions.

**Authorship contributions**

Participated in research design: Larsen, Karsdal, Henriksen

Conducted experiments: Larsen

Performed data analysis: Larsen, Sonne, Andreassen

Wrote or contributed to writing the manuscript: Larsen, Henriksen
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Foot notes

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Disclosure

Figure legends

Figure 1: Schematic overview of the amylin receptor (AMY-R) and the calcitonin receptor (CTR) and their ligands. CTR and AMY-R share the core 7TM GPCR, though the AMY-R is formed by interaction of a CTR with a RAMP. DACRAs can bind and activate both AMY-R and CTR, while AMY and CT are selective for the AMY-R and CTR respectively. AMY: amylin, CT: calcitonin, DACRA: dual amylin and calcitonin receptor agonist, RAMP: receptor activity-modifying protein.

Figure 2: Body weight (A) and food intake (B) of HFD fed rats during the study. Body weight change (C) and accumulated food intake (D) at study end. Weight of inguinal (E), epididymal (F) and perirenal (G) adipose tissue, and liver (H) at study end. Food intake, adipose tissue weight (epididymal and inguinal) and liver weight data are analysed by one-way ANOVA followed by Tukey’s multiple comparisons test. Body weight and perirenal adipose tissue weight data are analysed by Kruskal Wallis followed by Dunn’s multiple comparisons test. *P<0.05, **P<0.01 compared to vehicle and ¤P<0.05, ¤¤P<0.01, ¤¤¤P<0.001 compared to KBP-088. n= 8 rats per treatment group. Data are shown as mean ± SEM.

Figure 3: Blood glucose (A) and plasma insulin (B) levels during the oral glucose tolerance test in HFD fed rats. Total area under the curve (tAUC) of blood glucose (C) and plasma insulin (D). Gastric emptying 30 minutes post administration as percentage of vehicle (E). Insulin data are analysed by one-way ANOVA followed by Tukey’s multiple comparisons test and gastric emptying data are analysed by Kruskal Wallis followed by Dunn’s multiple comparisons test. *P<0.05, **P<0.01, ***P<0.001 compared to vehicle. n= 8 rats per treatment group. Data are shown as mean ± SEM.

Figure 4: Food intake of ZDF rats during the study (A). Body weight during the study shown as percentage of baseline (B). Accumulated food intake (C) and HbA1c levels (D) at study end.
Fasting (6 hours) blood glucose levels during the study as percentage of fasting blood glucose levels at baseline (E). Calculated total area under the curve (tAUC) of fasting blood glucose levels (% of baseline) (F). HbA1c levels and tAUC values are analysed by one-way ANOVA followed by Dunnett’s multiple comparison test and fasting blood glucose levels during the study are analysed by 2-way ANOVA followed by Dunnett’s multiple comparison test. *P<0.05, **P<0.01, ***P<0.001 compared to vehicle. n= 8 rats per treatment group. Data is shown as mean ± SEM.

**Figure 5:** Blood glucose (A) and plasma insulin (B) levels during the oral glucose tolerance test in ZDF rats. Total area under the curve (tAUC) of blood glucose (C) and plasma insulin (D). Incremental area under the curve (iAUC) of blood glucose (E). Gastric emptying 30 minutes post administration as percentage of vehicle (F). Insulin data are analysed by one-way ANOVA followed by Tukey’s multiple comparisons test and gastric emptying data are analysed by Kruskal Wallis followed by Dunn’s multiple comparisons test. *P<0.05, **P<0.01 compared to vehicle. n= 8 rats per treatment group. Data is shown as mean ± SEM.

**Figure 6:** Fasting (6 hours) blood glucose (A) and plasma insulin (B) in ZDF rats at study end. Pancreatic insulin content calculated as ng insulin per mg protein (C). Data are analysed by one-way ANOVA followed by Dunnett’s multiple comparison test. *P<0.05 compared to vehicle. n= 8 rats per treatment group. Data is shown as mean ± SEM.
Table 1: Adiposity in the ZDF rats at study end. Weights of inguinal, epididymal and perirenal adipose tissue (AT) at study end. Data is shown as mean ± SEM.

<table>
<thead>
<tr>
<th></th>
<th>Inguinal AT (g)</th>
<th>Epididymal AT (g)</th>
<th>Perirenal AT (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle (n=8)</td>
<td>4.5 ± 0.3</td>
<td>3.9 ± 0.3</td>
<td>5.1 ± 0.3</td>
</tr>
<tr>
<td>KBP-088 5 µg/kg (n=8)</td>
<td>4.3 ± 0.3</td>
<td>3.6 ± 0.1</td>
<td>5.0 ± 0.2</td>
</tr>
<tr>
<td>rAMY 300 µg/kg (n=8)</td>
<td>4.6 ± 0.3</td>
<td>4.1 ± 0.2</td>
<td>5.2 ± 0.2</td>
</tr>
<tr>
<td>rCT 300 µg/kg (n=8)</td>
<td>4.6 ± 0.4</td>
<td>4.0 ± 0.2</td>
<td>5.1 ± 0.3</td>
</tr>
<tr>
<td>rAMY + rCT 300 µg/kg (n=8)</td>
<td>4.8 ± 0.2</td>
<td>4.1 ± 0.2</td>
<td>5.6 ± 0.3</td>
</tr>
</tbody>
</table>
Figure 1
Figure 2

A. Body weight change over time (days) for different treatment groups:
- Vehicle
- KBP-088 5 µg/kg
- rAMY 300 µg/kg
- rCT 300 µg/kg
- rAMY + rCT 300 µg/kg

B. Food intake (g/2 animals) over time (days):
- Vehicle
- KBP-088 5 µg/kg
- rAMY 300 µg/kg
- rCT 300 µg/kg
- rAMY + rCT 300 µg/kg

C. Accumulated food intake day 1-28 (g/2 animal):
- Vehicle
- KBP-088 5 µg/kg
- rAMY 300 µg/kg
- rCT 300 µg/kg
- rAMY + rCT 300 µg/kg

D. Body weight change (g) for different treatment groups:
- Vehicle
- KBP-088 5 µg/kg
- rAMY 300 µg/kg
- rCT 300 µg/kg
- rAMY + rCT 300 µg/kg

E. Inguinal AT (g) for different treatment groups:
- Vehicle
- KBP-088 5 µg/kg
- rAMY 300 µg/kg
- rCT 300 µg/kg
- rAMY + rCT 300 µg/kg

F. Epididymal AT (g) for different treatment groups:
- Vehicle
- KBP-088 5 µg/kg
- rAMY 300 µg/kg
- rCT 300 µg/kg
- rAMY + rCT 300 µg/kg

G. Perirenal AT (g) for different treatment groups:
- Vehicle
- KBP-088 5 µg/kg
- rAMY 300 µg/kg
- rCT 300 µg/kg
- rAMY + rCT 300 µg/kg

H. Liver weight (g) for different treatment groups:
- Vehicle
- KBP-088 5 µg/kg
- rAMY 300 µg/kg
- rCT 300 µg/kg
- rAMY + rCT 300 µg/kg
Figure 3
Figure 4

A. Food intake (g/2 animal) over time (days)

B. Body weight (% of baseline) over time (days)

C. Accumulated food intake day 1-28 (g/2 animal)

D. HbA1c (%) over time (days)

E. Fasting blood glucose (% of baseline) over time (days)

F. tAUC fasting blood glucose (% of baseline)

- Φ - Vehicle
- KBP-088 5 µg/kg
- rAMY 300 µg/kg
- rCT 300 µg/kg
- rAMY + rCT 300 µg/kg
Figure 5
Figure 6