RESEARCH REPORT

A selective role for receptor activity-modifying proteins in subchronic action of the amylin selective receptor agonist NN1213 compared with salmon calcitonin on body weight and food intake in male mice

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Abstract

The role of receptor activity-modifying proteins (RAMPs) in modulating the pharmacological effects of an amylin receptor selective agonist (NN1213) or the dual amylin–calcitonin receptor agonist (DACRA), salmon calcitonin (sCT), was tested in three RAMP KO mouse models, RAMP1, RAMP3 and RAMP1/3 KO. Male wild-type (WT) and knockout (KO) littermate mice were fed a 45% high-fat diet for 20 weeks prior to the 3-week treatment period. A decrease in body weight after NN1213 was observed in all WT mice, whereas sCT had no effect. The absence of RAMP1 had no significant effect on NN1213 efficacy, and sCT was still inactive. However, the absence of RAMP3 impeded NN1213 efficacy but improved sCT efficacy. Similar results were observed in RAMP1/3 KO suggesting that the amylin receptor 3 (AMY3 = CTR + RAMP3) is necessary for NN1213's maximal action on body weight and food intake and that the lack of AMY3 allowed sCT to be active. These results suggest that the chronic use of DACRA such as sCT can have unfavourable effect on body weight loss in mice (which differs from the situation in rats), whereas the use of the amylin receptor selective agonist does not. AMY3 seems to play a crucial role in modulating the action of these two compounds, but in opposite directions. The assessment of a long-term effect of amylin and DACRA in different rodent models is necessary to understand potential physiological beneficial and unfavourable effects on weight loss before its transition to clinical trials.

KEYWORDS
agonist, animal, CTR, obesity, RAMP

1 | INTRODUCTION

Currently, bariatric surgery is the most efficient approach to treat obesity, but it is invasive, expensive and reserved for patients who are critically obese (Peterli et al., 2018). Thus, new therapy approaches to elicit body weight loss need to be developed and a deeper understanding of the underlying mechanisms that control food intake is required (Andreassen et al., 2014; Larsen et al., 2020; Mack et al., 2010; Soudy et al., 2017).

Amylin is a centrally acting peptide hormone coproduced and cosecreted with insulin by pancreatic β cells in response to nutrient influx in the gastrointestinal tract (Bower & Hay, 2016; Cooper, 1994). Amylin's main site of action is located in the area postrema (AP) of the caudal hindbrain (Boyle et al., 2018; Braegger et al., 2014; Lutz et al., 2001). Pharmacologically, the desired effect of amylin's action is to decrease blood glucose and food intake, associated with long-term body weight loss (for review, see Hay et al., 2015). Amylin receptors comprise a calcitonin receptor core (Calcr/CTR a or b) combined with one of the three receptor activity-modifying proteins (RAMP1, 2 or 3). The CTR by itself shows more affinity to calcitonin, but when associated with one of the three RAMPs, producing AMY1, AMY2 and AMY3 (Hay et al., 2018), these receptors present a higher affinity to amylin (Garelja et al., 2018). CTR itself can reach the cell surface, so it is likely that mixed populations of CTR/RAMP complexes and CTR alone are found in the different brain expression sites (Coester et al., 2020; Liberini et al., 2016). In mice, CTR and RAMPs are mostly expressed in the AP and at a lower level in the nucleus of the solitary tract (NTS), in the lateral hypothalamic area, in the ventromedial (VMH) and arcuate (ARC) hypothalamic nuclei and in the ventral tegmental area (Christopoulos et al., 1995; Christopoulos et al., 1999; Coester et al., 2020; Husmann et al., 2000; Sexton et al., 1986; Sexton et al., 1994). The AP contains a high density of AMY (Lutz et al., 2001; Sexton et al., 1994), and all the necessary components have been colocalized at the single-cell level in the rat AP (Liberini et al., 2016) and the mouse ARC (Coester et al., 2020).

Pramlintide, a human amylin analogue, is the only amylin-based approved drug and is used in combination with mealtime insulin to treat glucose excursions in patients with type 1 and type 2 diabetes (Roth et al., 2008; Weyer et al., 2001). Nevertheless, new amylin receptor agonists with improved potency and pharmacokinetics may provide better therapeutics for the treatment of overweight and obesity.

This study aims to investigate the role of RAMPs in mediating the subchronic pharmacological effects and potential differences between the dual amylin–calcitonin receptor agonist (DACRA), salmon calcitonin (sCT) (Hay et al., 2015; Hay et al., 2018; Reidelberger et al., 2001; Reidelberger et al., 2002), and an amylin selective analogue, NN1213.

To assess the effects of sCT and NN1213, knockout (KO) mouse models for a subunit of the amylin receptor were used. RAMP1 KO, 3 KO and 1/3 KO mice allow us to decipher the underlying mechanisms used by these two peptides to decrease body weight. We have shown previously that RAMPs KO mice are good models for the study of amylin signalling (Coester et al., 2019) even though they may present other metabolic alterations due to the fact that RAMPs also affect the action of receptors other than the amylin receptor (Boccia et al., 2020). These mouse models have been extensively characterized, and we showed that acutely, amylin decreased food intake in RAMP1 KO and sCT in RAMP3 KO only, whereas RAMP1/3 KO mice were insensitive to both peptides (Coester et al., 2019).

2 | MATERIAL AND METHODS

2.1 | Animals and husbandry

RAMP1 KO, RAMP3 KO and RAMP1/3 double KO mice on a 129S2/SvEv background were kindly provided by Kathleen Caron (University of North Carolina, USA). RAMP1/3 KO are maintained on a KO breeding scheme, whereas RAMP1 KO and 3 KO are bred by mating heterozygous mice. RAMP3 wild-type (WT) mice served as WT control for the RAMP1/3 KO mice. Animals were genotyped as previously published (Coester et al., 2019).

Male mice were housed in a controlled environment maintained at $21 \pm 2°C$, under a 12-/12-h light–dark cycle (lights off at 19.00 h). Mice had ad libitum access to standard chow (Kliba 3436, 3.14 kcal/g of food) and water, until 4–5 weeks old. Then mice were switched to 45% fat high-fat diet (HFD; D12451 Research Diets, 4.73 kcal/g of food, New Brunswick, NJ, USA) ad libitum from approximately 4–5 to 24–25 weeks old. The same diet was then continued during the 21-day treatment period. The mice were group housed (2–3 mice per cage) until 12 weeks old, when they had to be single-housed due to aggressive behaviour and for food intake measurements. Each cage was equipped with a red plastic house and nest-building material and wood shavings. Prior to the 21-day treatment, mice were injected with vehicle for 1 week to acclimate to the daily subcutaneous injections to reduce stress.

All animal experimental protocols in this study were approved by the University of Zurich Animal Protection Office and Ethics committee, the Veterinary Office of the

Canton of Zurich, and conform to Swiss Animal Protection guidelines and regulations (Swiss Animal Protection and Swiss Animal Act and Ordinance) and in accordance with the EU Directive on the protection of animals used for scientific purposes. These studies adhere to the ARRIVE guidelines.

2.2 | Genotyping

Briefly, DNA was extracted from toe biopsies: 200 μl of 50-mM NaOH was added, heated at 95°C and shaken at 800 rpm for 35 min; samples were then neutralized with 200 μl of 500-mM Tris–HCl, pH 5.5. One microliter of DNA was then combined by the respective primers, and GoTaq polymerase was used (Promega A6001). Animals were genotyped using the following primers: RAMP1 forward TCATGGGGACCTTTAGGTAAGC, RAMP1 reverse ACAGCAATCCTTCT ACCTCAACAC, RAMP3 WT band is detected using R3–1: GTGCTCAAGGGTTCTG TCTG and R3–10: GACCTGGTTCATCTCTGGCTCC and RAMP3 null band is detected using R3–10: GACCTGG TTCATCTCTGGCTCC and neo-60: GCTTCCTCTTG CAAAACCACA (Dackor et al., 2007).

2.3 | Food intake, body weight and non-fasted blood glucose level during 20 weeks on 45% HFD

Weekly body weight and food intake were measured manually. Blood was harvested from the tail, and non-fasted glucose was measured using Contour Next Blood Glucose Meter (Bayer Consumer Care AG, Basel,

Switzerland) every 2 weeks at 14.00 h, that is, in the second half of the light phase.

2.4 | Peptides

All experimental drugs were provided by Novo Nordisk Pharma AG. Vehicle: 5-mM acetate, 249-nM propylene glycol, 0.007% Tween 20; NNC0174-1213 (NN1213): 5 nmol/ml, 30 nmol/kg; sCT 0186: 27.7 nmol/ml, 150 nmol/kg. The structural description of NN1213 (= AM1213) is presented in Figure 1a.

2.5 | BacMam receptor expression and cAMP potency assay

HeLa cells were grown in DMEM supplemented with glucose, sodium pyruvate, GlutaMax™ and phenol red, 10% fetal calf serum and 1% penicillin/streptomycin (ThermoFisher Scientific). The BacMam system (Life Technologies) was used to transduce the HeLa cells with the mouse CTRb family receptors. Recombinant mouse CTRb or the respective AMY1–3b receptors expressing cells were plated to a density of 3×10^5 cell/ml in medium containing either mCTRb (1% v/v) alone or mCTRb (1% v/v) with one of the three RAMPs (15% v/v) and cultured at 37°C, 5% CO_2 and 95% relative humidity. To determine potencies, the cells were stimulated with NN1213, sCT or rat/mouse amylin. Stimulation of CT family receptors activates adenylyl cyclase, leading to accumulation of cyclic AMP (cAMP) when 3-isobutyl-1-methylxanthine (IBMX) is added. Increasing levels of endogenous cAMP were measured
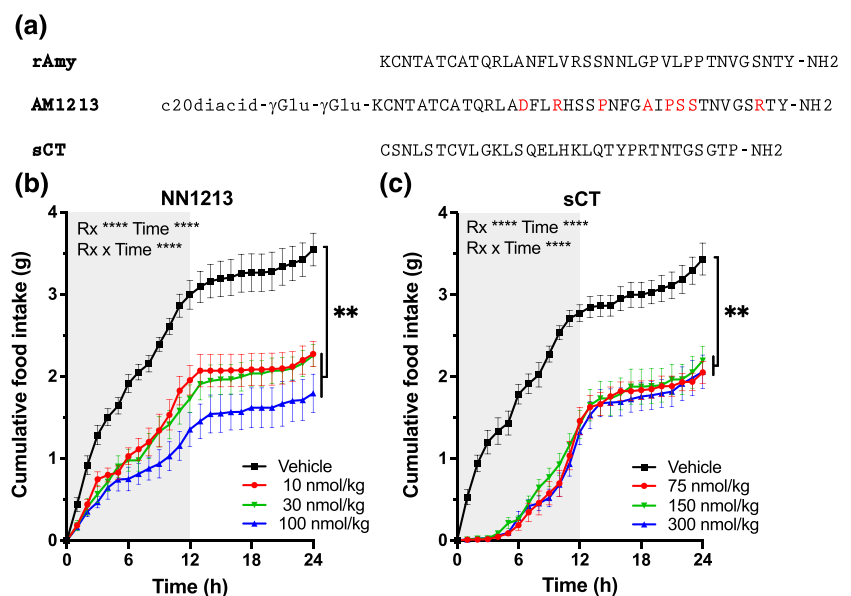

FIGURE 1 Peptide amino acid structure and 24-h food intake in chow-fed C57Bl6 male mice in response three doses of NN1213 and salmon calcitonin (sCT). (a) Amino acid structure of rat amylin, NN1213 and sCT; (b) 24-h cumulative food intake after NN1213 s.c. injection; (c) 24-h cumulative food intake after sCT s.c. injection. The grey area represents the dark phase. Data are expressed as mean ± SEM, N = 8/group. Statistics: two-way analysis of variance (ANOVA) with repeated measured followed by Tukey post hoc comparison; **P < 0.01; Rx: treatment

(a)

rAmy   KCNTATCATQRLANFLVRSSNNLGPVLPPTNVGSNTY-NH2

AM1213   c20diacid-γGlu-γGlu-KCNTATCATQRLADFLRHSSPNFGAIPSSTNVGSRTY-NH2

sCT   CSNLSTCVLGKLSQELHKLQTYPRTNTGSGTP-NH2

as a reduction in fluorescence resonance energy transfer (FRET) between Europium (Eu3+)–cryptate-conjugated anti-cAMP antibody and d2-conjugated cAMP. The fluorescence ratio was plotted as a function of the concentration of compound (cAMP HTRF® Assay kit, Cisbio). Data were analysed in GraphPad Prism 7, and EC50 values were calculated by nonlinear regression analysis of sigmoidal dose response curves. Mean pEC50 with the 95% confidence interval are shown in Figure S1.

2.6 | Acute food intake dose response study in C57BL/6J male mice

C57BL/6J male mice ($n = 8$, Taconic, Germany, 10 weeks of age) were housed in the BioDAQ cages (Research Diets, Inc, New Brunswick, NJ, USA) to continuously monitor their food intake over a 24-h period. After a week of adaptation to new environment and a standard low fat diet (D12450B, Research Diets Inc), a 24-h baseline food intake measurement was performed before study initiation, and mice were fasted for 4 h before dosing. Mice were dosed subcutaneously (<20 min) prior to the onset of the dark cycle, with a single SC injection with three doses of sCT (75, 150 and 300 nmol/kg), three doses of NN1213 (10, 30 and 100 nmol/kg) or vehicle, resp.

2.7 | Mouse subchronic treatment

At the age of 24–25 weeks and after 20 weeks on 45% HFD, male RAMP1/3 KO, RAMP1 KO and RAMP3 KO mice and their respective WT littermates were randomized by body weight and assigned to their treatment group ($n = 8$/group): vehicle; NN1213 (30 nmol/kg/d); sCT (150 nmol/kg/d). Mice were injected subcutaneously once a day for 3 weeks in the middle of the light phase between 11.00 and 12.00 h. Prior to injection, daily body weight and food intake were measured manually.

2.8 | Sacrifice

On the day of sacrifice, mice were fasted for 2 h during the light phase and sacrificed under pentobarbital anaesthesia (100 mg/kg, IP injection, Cantonal Pharmacy of Zurich, Switzerland). Blood was sampled using cardiac puncture, and brains were harvested and frozen on dry ice. The mice were stored at −20°C until body composition analysis was performed.

2.9 | Measurement of body composition

Quantitative microcomputed tomography of WT mice and RAMP1/3 KO mice was performed using La Theta LCT-100A (LaTheta LCT-100A scanner, Hitachi-Aloka Medical Ltd., Tokyo, Japan) on the carcasses of the animals placed supine in the plexiglass holder after sacrifice (Coester et al., 2019; Senn et al., 2019). The region between the vertebrae L1–L6 was evaluated for lean and fat mass. Although the software LaTheta already automatically distinguished between visceral and subcutaneous fat, each image was manually examined, and in some cases, an image-by-image correction was required. Adipose tissue weights were then computed using the commonly used density factor of 0.92 g/cm^3 to determine the absolute values in gram (Bray, 1999).

2.10 | Biochemical analysis

Blood samples were collected from the right ventricle of the heart during the sacrifice in 1-ml EDTA-coated tubes containing DPP4 inhibitor (10 μl/ml blood) and proteinase inhibitor (P2414; Sigma Aldrich, 30 μl/ml blood). After centrifugation, plasma was kept at −80°C. Plasma levels of leptin and insulin were measured with a Mouse Metabolic Biomarkers kit (MesoScale Discovery, Gaithersburg, MD, USA).

2.11 | Statistical analysis

All analyses were performed using GraphPad Prism software version 8.0 (GraphPad, San Diego, CA). In experiments comparing independent treatment groups, significance was tested using one-way analysis of variance (ANOVA). When more than one factor was compared, two-way ANOVA was performed, followed by Sidak or Tukey's multiple comparisons test as recommended. A P value of <0.05 was considered statistically significant. All data are presented as mean $\pm$ SEM.

3 | RESULTS

3.1 | Efficacy of NN1213 compared with that of sCT at the mCTR compared to AMYs

NN1213 and mouse amylin showed decreased affinity to mCTR compared with sCT (Figure S1a). However, at the level of the amylin receptor AMY1, NN1213 and amylin showed increased potency compared with mCTR alone.

Overall, NN1213 is more potent than amylin but less potent than sCT (Figure S1b–d). The potency of NN1213 and amylin was lower at the AMY2 and AMY3 than at the AMY1 ($P < 0.001$, Figure S1c,d). Thus, these results confirm that NN1213 is an amylin receptor agonist whereas sCT is a DACRA.

3.2 | Acute food intake dose response study in mice

Ten-week-old C57BL/6J mice were tested for their food intake response to single injections of increasing doses of sCT and NN1213 (Figure 1b,c). All doses of NN1213 and sCT decreased food intake compared with vehicle, and no statistical difference was observed among the three doses. The doses of 30 and 150 nmol/kg of NN1213 and sCT, respectively, were chosen because they suppressed food intake to a similar extent at 24 h.

3.3 | 20 weeks of 45% HFD feeding in RAMP1/3 KO, 1 KO, 3 KO and WT mice

From 4 to 24 weeks old, WT and RAMP1/3 KO mice body weight and food intake on 45% HFD were followed weekly (Figure S2). Baseline body weight was similar across both groups with no significant difference at the start of the experiment (Figure S2a). From Week 8 onward, RAMP1/3 KO mice displayed a significantly lower body weight ($P < 0.001$; Figure S2a) and gained 27% less weight over the 20-week period ($P < 0.001$; Figure S2d) compared with the WT cohort. The higher body weight gain in the WT mice is consistent with a 10% increase in cumulative food intake ($P < 0.001$; Figure S2g) and a 30% increase of blood glucose level compared to RAMP1/3 KO mice ($P < 0.001$; Figure S2j).

In RAMP3 WT and KO mice, baseline body weight average was similar at the beginning of the study (Figure S2b). After 20 weeks on HFD, no difference in body weight (Figure S2b), body weight gain (Figure S2e) or food intake (Figure S2h) was observed between WT and RAMP3 KO littermates. However, biweekly tail blood glucose was temporarily increased by 26% and 20% in RAMP3 KO mice at Weeks 14 and 16, respectively, compared with RAMP3 WT mice ($P < 0.05$; Figure S2k). As for the RAMP1 KO, no significant difference during the HFD period was observed, with exception of Week 16, where RAMP1 WT mice showed a significantly higher body weight compared with the KO (Figure S2c). Similarly to the RAMP3 KO, 45% HFD had the same effect on body weight gain in WT and RAMP1 KO mice throughout the entire HFD period (Figure S2f). However, food intake was significantly increased by 10% in RAMP1 WT ($P < 0.001$; Figure S2i), whereas no significant difference in blood glucose level was detected between both genotypes (Figure S2l). Overall, RAMP1 KO/WT mice gained less weight and ate less over the 20 weeks of HFD than RAMP3 KO/WT mice (Strain $P < 0.0001$; Figure S2e,f,h,i), but food intake was similar to that of RAMP1/3 KO.

3.4 | Efficacy of the DACRA sCT in RAMP3 KO mice whereas the amylin-selective agonist NN1213 is efficient in WT but not in RAMP3 KO mice

Baseline body weight was not significantly different at the start of the injection period (Figure 2a) and body weight remained similar among the groups during the treatment period (Figure 2a). Body weight gain decreased by 7% in NN1213-treated RAMP3 WT mice relative to vehicle-treated RAMP3 WT controls ($^{\%}P < 0.05$; Figure 2b,c). RAMP3 KO mice also responded to NN1213 but not to the same extent as WT mice during the first 2 weeks, and at the end of the study, the two groups displayed the same body weight loss (Figure 2b,c). Similar to RAMP1/3 KO (Figure 4), a marked decrease in body weight gain was observed in RAMP3 KO mice treated with sCT between Day 3 and Day 14 ($^{X}P < 0.05$; Figure 2b,c) but not in RAMP3 WT mice. At the conclusion of the study, the effect of sCT in RAMP3 KO mice was not significantly different from WT and KO mice treated with the amylin-selective agonist NN1213, but the initial drop in body weight gain was more marked, more rapid and longer lasting with sCT in KO mice. Overall, a decrease in body weight gain in response to NN1213 was observed in RAMP3 WT mice during the first 2 weeks of treatment whereas RAMP3 KO mice responded to sCT (Figure 2b,c). No significant difference in food intake between the groups was observed (Figure 2d). At the time of sacrifice, blood glucose levels were similar regardless of genotype or treatment (Figure 2e). Thus, the absence of RAMP3 impedes NN1213 efficacy and improves sCT weight loss efficacy.

3.5 | Efficacy of the amylin-selective agonist NN1213 in WT and RAMP1 KO mice whereas sCT has no effect in both strains

During the first week of treatment, RAMP1 WT and KO treated with NN1213 similarly responded to the
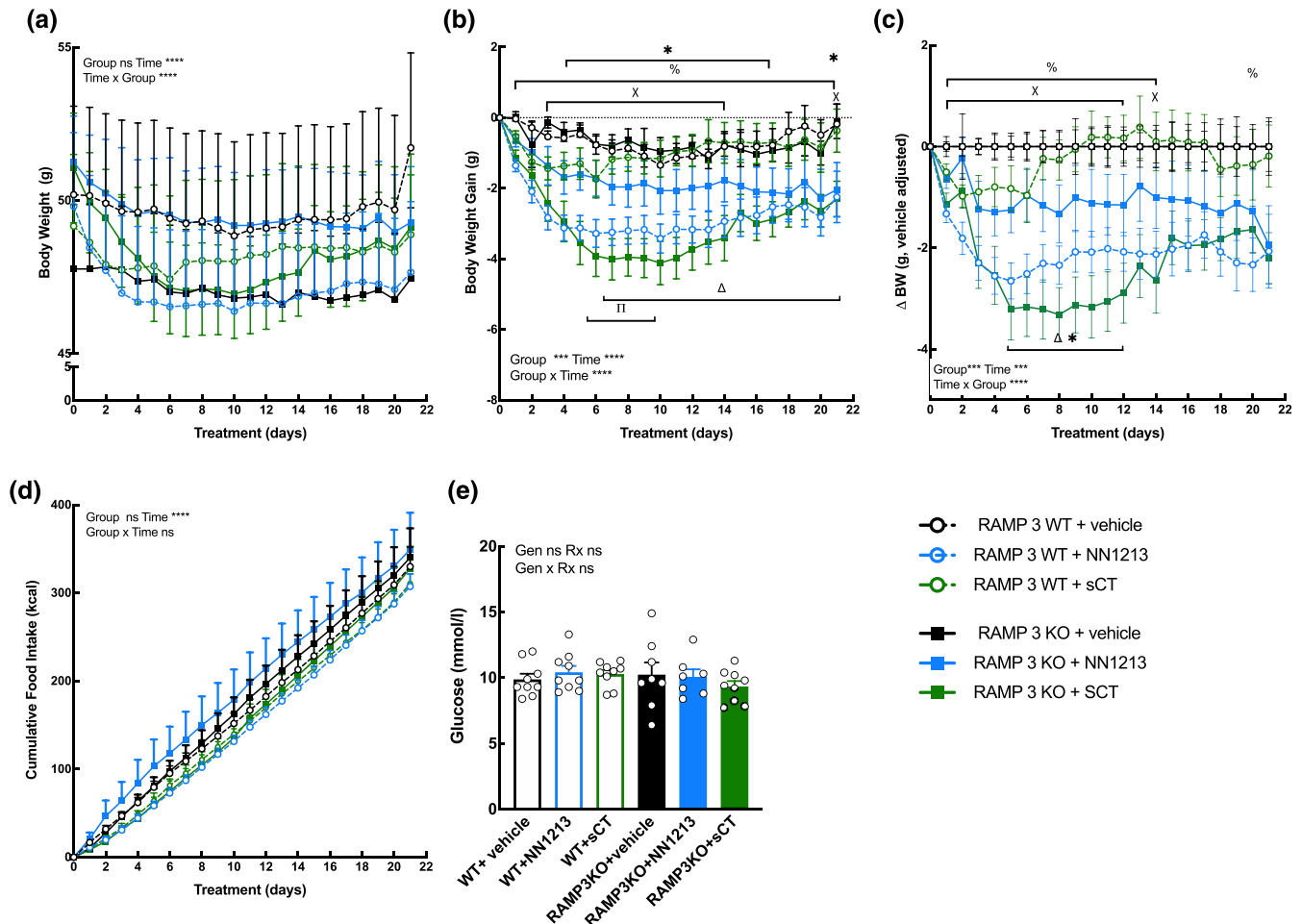
FIGURE 2 Effect of amylin-selective agonist NN1213 (30 nmol/kg) and sCT (150 nmol/kg) subchronic treatment (21 days) in receptor activity-modifying protein (RAMP) 3 wild-type (WT) and knockout (KO) mice after an induction obesity period of 20 weeks on 45% HFD. (a) Daily body weight (g) of RAMP3 WT and KO mice treated for 21 days with vehicle, NN1213 (30 nmol/kg) and salmon calcitonin (sCT) (150 nmol/kg); (b) daily body weight gain (g); (c) body weight gain subtracted from respective vehicle group (g); (d) daily food intake; (e) glucose (mmol/L) at sacrifice. Data are presented as mean ± SEM; number of mice per group WT + veh = 8; WT + NN1213 = 8; WT + sCT = 7; RAMP3 KO + veh = 8; RAMP3 KO + NN1213 = 8; RAMP3 KO + sCT = 7. Statistics: two-way analysis of variance (ANOVA) with repeated measured followed by Tukey post hoc comparison; $^{\%}P < 0.05$ WT + veh versus WT + NN1213, $^{\Delta}P < 0.05$ WT + NN1213 versus WT + sCT, $^{*}P < 0.05$ WT + sCT versus RAMP3 KO + sCT, $^{X}P < 0.05$ RAMP3 KO + veh versus RAMP3 KO + sCT, $^{\Pi}P < 0.05$ RAMP3 KO + sCT versus RAMP3 KO + NN1213

treatment and gained 7% less weight, compared with their respective vehicle-treated group ($^{\%,\ \beta}P < 0.05$; Figure 3b,c). In the second and third week of treatment, NN1213-treated RAMP1 WT and KO mice maintained their reduced body weight loss in comparison with the vehicle control group. No difference in NN1213 weight loss efficacy is observed between WT and RAMP1 KO mice when subtracting from their respective vehicle-treated group (Figure 3c). In contrast, sCT had no effect in RAMP1 WT and KO mice (Figure 3b,c). Starting at Day 14 of treatment, cumulative food intake was significantly increased by 22% and 15% in sCT-treated WT and KO compared with their respective NN1213-treated

group, and this difference remained significant until the end of the treatment period ($^{\Delta,\ \Pi}P < 0.05$; Figure 3c). No genotype or treatment effect on blood glucose level was observed at sacrifice (Figure 3d).

Because starting body weight was much lower in RAMP1 WT/KO mice compared with the RAMP3 strain (Figures S2c and S3a), nasal–anal length of RAMP1 and RAMP3 mice at the end of the treatment period was measured. Overall, RAMP1 WT and KO mice were significantly shorter than RAMP3 WT and KO mice (data not shown). Thus, the absence of RAMP1 had no significant effect on NN1213 efficacy and did not restore sCT efficacy.
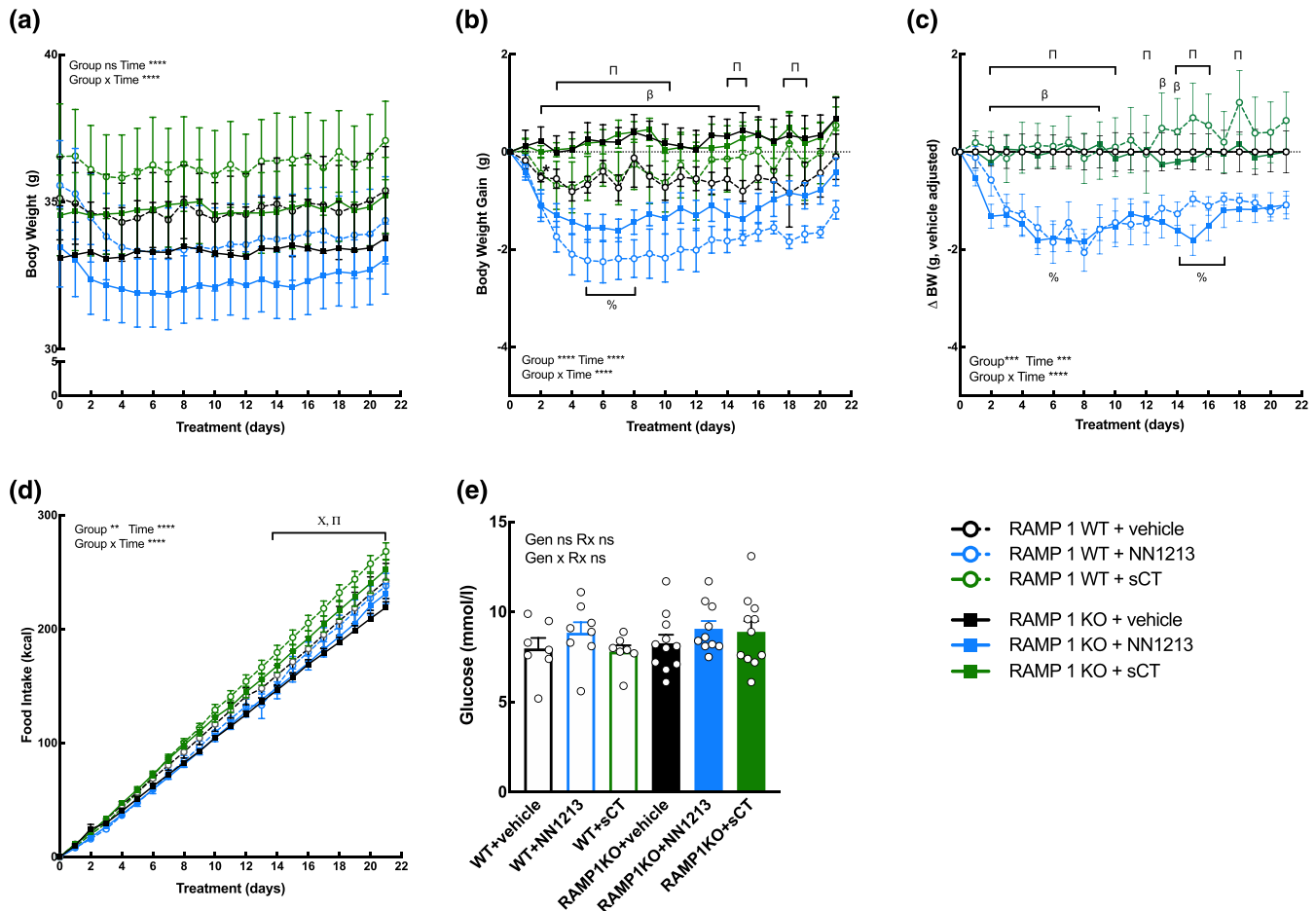
FIGURE 3 Effect of amylin-selective agonist NN1213 (30 nmol/kg) and salmon calcitonin (sCT) (150 nmol/kg) subchronic treatment SC (21 days) in receptor activity-modifying protein (RAMP) 1 wild-type (WT) and knockout (KO) mice after an induction obesity period of 20 weeks on 45% HFD. (a) Daily body weight (g) of RAMP1 WT and KO mice treated for 21 days with vehicle, NN1213 (30 nmol/kg) and sCT (150 nmol/kg); (b) daily body weight gain (g); (c) body weight gain subtracted from respective vehicle group (g); (d) daily food intake; (e) glucose (mmol/L) at sacrifice. Data are presented as mean ± SEM; number of mice per group WT + veh = 9; WT + NN1213 = 9; WT + sCT = 9; RAMP3 KO + veh = 8; RAMP1 KO + NN1213 = 7; RAMP1 KO + sCT = 9. Statistics: two-way analysis of variance (ANOVA) with repeated measured followed by Tukey post hoc comparison; [%]P < 0.05 WT + veh versus WT + NN1213, [β]P < 0.05 RAMP1 KO + veh versus RAMP1 KO + NN1213, [X]P < 0.05 RAMP1 KO + veh versus RAMP1 KO + sCT, [Π]P < 0.05 RAMP1 KO + sCT versus RAMP1 KO + NN1213

3.6 | DACRA sCT decreases body weight in RAMP1/3 KO mice whereas the amylin-selective agonist NN1213 does not

WT and double RAMP1/3 KO animals had a significantly different baseline body weight, and this difference in body weight remained significant until the end of the treatment period (Figure 4a). From Day 3 to Day 8, RAMP1/3 WT injected with the amylin-selective agonist NN1213 showed a 5% weight loss compared with vehicle treated mice ([%]P < 0.05; Figure 4b,c); Figure 4c indicates the body weight gain subtracted from respective vehicle group. After this initial effect, RAMP1/3 WT NN1213-treated mice regained weight until the end of

the treatment period (Figure 4b), but if compared with vehicle-injected group, this weight loss was maintained (Figure 4c). In comparison, NN1213 had no significant effect in RAMP1/3 KO mice on body weight in the first days, and mice continued to gain weight as did the vehicle control RAMP1/3 KO mice (Figure 4a–c). sCT decreased WT mice body weight gain by 2% during the first 2 days (P = 0.03 vs. vehicle WT), and this was followed by weight regain similar to vehicle-treated mice. Conversely, sCT-treated RAMP1/3 KO mice progressively lost weight and reached a maximum 5% weight loss on day 10 ([X]P < 0.05; Figure 4b,c), which was followed by some weight regain, but the two sCT-treated groups remained significantly different throughout the
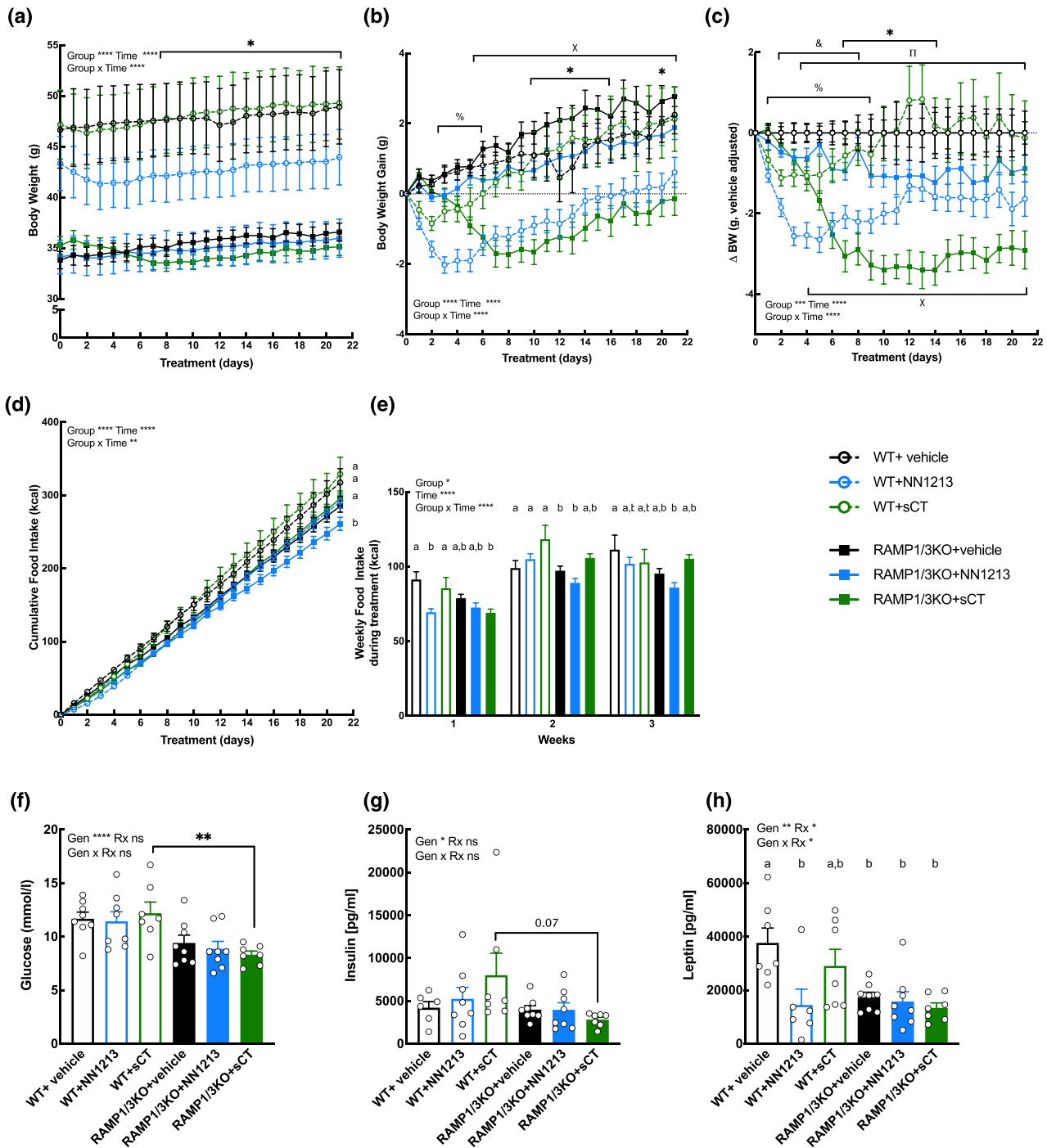
FIGURE 4   Effect of amylin-selective agonist NN1213 (30 nmol/kg) and salmon calcitonin (sCT) (150 nmol/kg) subchronic treatment for 21 days in receptor activity-modifying protein (RAMP) 1/3 wild-type (WT) and knockout (KO) mice after an induction obesity period of 20 weeks on 45% HFD. (a) Daily body weight (g) of RAMP1/3 WT and KO mice treated for 21 days with vehicle, NN1213 (30 nmol/kg) and sCT (150 nmol/kg); (b) daily body weight gain (g); (c) bodyweight gain subtracted from respective vehicle group (g) which was set to 0; (d) daily food intake; (e) weekly food intake; (f–h) glucose (mmol/L), insulin (pg/ml) and leptin (pg/ml) concentration measured in plasma of RAMP1/3 WT and KO mice at time of sacrifice. Data are presented as mean $\pm$ SEM; number of mice per group WT + veh = 8; WT + NN1213 = 8; WT + sCT = 7; RAMP1 KO + veh = 8; RAMP1 KO + NN1213 = 8; RAMP1 KO + sCT = 7. Statistics: two-way analysis of variance (ANOVA) with repeated measured followed by Tukey post hoc comparison; (a–c): $^{\%}P < 0.05$ WT + veh versus WT + NN1213, $^{*}P < 0.05$ WT + sCT versus RAMP1/3 KO + sCT, $^{\&}P < 0.05$ WT + NN1213 versus RAMP1/3 KO + NN1213, $^{X}P < 0.05$ RAMP1/3 KO + veh versus RAMP1/3 KO + sCT, $^{\Pi}P < 0.05$ RAMP3 KO + sCT versus RAMP3 KO + NN1213. (f–h) *Symbols denote significant differences between the two genotypes; $^{*}P < 0.05$, $^{**}P < 0.01$. (d, e, h) Parameters with differing letters (a, b) differ from each other by $P < 0.05$ after two-way ANOVA (group, time) followed by Tukey post hoc test

21 days (*$P < 0.05$; Figure 4b,c); hence, sCT produced lasting weight loss in the RAMP1/3 KO but not in the WT mice.

For the entire duration of the experiment, cumulative food intake as well as weekly food intake was significantly lower in RAMP1/3 KO mice treated with NN1213 than the other groups, which ate a similar amount of food during the treatment period (Figure 4d,e). Thus, even though NN1213-treated RAMP1/3KO mice did not significantly decrease their body weight, food intake was decreased.

No effect of sCT or NN1213 on blood glucose levels was observed between the two genotypes. However, RAMP1/3 KO mice displayed lower glucose levels than WT mice ($P < 0.0001$, $F[1, 40] = 22.18$; Figure 4f) and insulin ($P = 0.03$, $F[1, 38] = 4.617$; Figure 4g). This genotype effect may result from the lower body weight displayed by RAMP1/3 KO mice compared with WT mice (*$P < 0.05$; Figure 4a). NN1213-treated WT mice showed a 60% decrease in leptin plasma concentration compared with WT-vehicle mice (Figure 4h). RAMP1/3 KO mice displayed significantly lower leptin levels compared with WT mice treated with vehicle (Figure 4h) and were similar to that of WT NN1213 mice. sCT-treated RAMP1/3 KO also displayed a 48% decrease in visceral fat mass compared to sCT-treated WT mice ($P < 0.05$; Table 1). Lean mass was significantly decreased by 23% in RAMP1/3 KO mice compared with the WT cohort (Table 1), regardless of the treatment received, which reflects their lower body weight in general ($P < 0.0001$, $F[1, 40] = 21.06$; Figures S2a and 4a).

3.7 | Distribution of CTR, RAMP1, RAMP2 and RAMP3 in the AP and NTS of vehicle-treated WT, RAMP1 KO, RAMP3 KO and RAMP1/3 KO HFD-fed mice

An exploratory staining was performed in the four mouse models to assess the expression of CTR and the three RAMPs in the AP and NTS of these mice and test whether the KO of one or two RAMPs affects the mRNA expression of CTR and the other RAMPs.

In WT mice, CTR is colocalized at the mRNA level with all the three RAMPs in one cell as indicated by the full arrows (Figure S3a–d), and RAMPs are also present in AP cells without CTR (Figure S3d). Although CTR seems to be always colocalized with RAMP in the AP (Figure S3c), this is not the case in the NTS (Figure S3a, b). Furthermore, RAMP2 seems to be the preferentially colocalized RAMP with CTR in the AP (Figure S3d). Interestingly, when RAMP1 or RAMP3 is absent, it decreases the transcription of other RAMPs and of CTR in the AP and NTS (Figure S3e–p). In particular, in the absence of RAMP3, RAMP2 and RAMP1 are highly colocalized within the same cells but not with CTR (Figure S3i–l). Furthermore, in RAMP1/3 KO mice, the depletion of RAMP1 and RAMP3 decreased the transcription of RAMP2 and prevented its colocalization with CTR. Thus, the depletion of RAMP3 may prevent the colocalization of CTR with RAMP1 or RAMP2, but this is not the case in RAMP1 KO mice (Figure S3e–h). Therefore, these data might suggest a transcriptional regulation of CTR and RAMP by its endogenous ligands and by RAMP3. Whether the protein expression reflects what we

TABLE 1 Body composition in g or as percent of body weight (% BW) in RAMP1/3 WT and KO injected with vehicle, NN1213 and sCT for 21 days after 20 weeks on 45% HFD

	WT + vehicle	WT + NN1213	WT + sCT	1/3 KO + vehicle	1/3 KO + NN1213	1/3 KO + sCT
Lean	7.13 ± 0.55[a]	6.61 ± 0.38[a,b]	7.41 ± 0.48[a,b]	5.52 ± 0.21[b]	5.26 ± 0.21[b]	5.56 ± 0.22[b]
Lean % BW	15.6 ± 1.06	15.5 ± 1.19	16.1 ± 0.63	16 ± 0.68	15.7 ± 1.09	15.8 ± 0.81
Visceral fat	3.89 ± 0.64[a,b]	3.39 ± 0.6[a,b]	4.10 ± 0.50[a]	2.7 ± 0.28[a,b]	2.89 ± 0.34[a,b]	2.12 ± 0.17[b]
Visceral fat (% BW)	8.2 ± 0.77	7.4 ± 1.13	8.6 ± 0.68	7.74 ± 0.69	8.31 ± 0.7	5.97 ± 0.37
Subcutaneous fat	2.10 ± 0.23	1.72 ± 0.32	1.77 ± 0.28	1.76 ± 0.2	1.75 ± 0.23	1.67 ± 0.17
Subcutaneous fat (% BW)	4.6 ± 0.43	3.7 ± 0.6	3.7 ± 0.5	5.03 ± 0.45	5.04 ± 0.45	4.71 ± 0.43
Total fat	5.99 ± 0.85	5.11 ± 0.9	5.87 ± 0.71	4.46 ± 0.43	4.64 ± 0.55	3.79 ± 0.31
Total fat (% BW)	12.8 ± 1.06	11.1 ± 1.65	12.3 ± 0.98	12.7 ± 0.96	13.3 ± 1.07	10.7 ± 0.69
Fat ratio (%)	44.9 ± 3.66	41.1 ± 5.71	43.7 ± 2.48	44.2 ± 2.7	45.9 ± 3.2	40.3 ± 2.6

Note: Data are presented as mean ± SEM; $N = 8$/group. For each row, groups with differing superscript letters differ from each other at $P < 0.05$ level by Tukey post hoc adjustment after significant intergroup differences were found by two-way ANOVA.

Abbreviations: HFD, high-fat diet; KO, knockout; sCT, salmon calcitonin; WT, wild type.

observed at the mRNA level still remains to be determined.

4 | DISCUSSION

The aim of this study was to assess the weight loss efficacy of the amylin selective agonist (NNC0174-1213) in the three RAMP KO mouse models compared with that of DACRA sCT and to investigate the role of RAMPs in modulating the pharmacological effects and potential differences on body weight and food intake in response to the treatment. We first observed that in WT mice, subchronic sCT does not have any sustained effect on body weight and food intake whereas the amylin agonist NN1213 was able to decrease body weight and to transiently decrease food intake. Second, NN1213 partially decreased body weight in RAMP1 KO mice, but not in RAMP3 and RAMP1/3 KO mice suggesting the necessity of AMY3 for NN1213 body weight loss efficacy. Third, sCT lowered body weight gain in RAMP1/3 and RAMP3 KO mice but not in WT and RAMP1 KO mice suggesting that the DACRA's effects on body weight during subchronic administration is hindered by the presence RAMP3 (Table S1).

The subchronic treatment clearly demonstrates that WT mice are sensitive to NN1213 by decreasing their body weight which is accompanied by a moderate anorectic effect. The effect of NN1213 is stronger in the first 5 days of treatment and is followed by a stabilization period where the mice maintained their body weight loss. On the contrary, and different from the effect in lean or HFD-fed rats (Andreassen et al., 2014; Feigh et al., 2011; Feigh et al., 2012; Gydesen et al., 2016; Hjuler et al., 2015; Larsen et al., 2020), the DACRA sCT had a minimal effect in the different WT mice. WT mice displayed no change in insulin and leptin when treated with sCT, while NN1213 decreased leptin levels. Interestingly, even though total body fat mass analysis is not different between WT groups, visceral fat is decreased in NN1213-treated WT mice compared with vehicle and sCT-treated WT mice. Given the lower leptin levels, it is possible that we may have missed a decrease in subcutaneous fat pads because we only quantified fat mass from L1 to L6. The first main conclusion of this study is that subchronic sCT treatment, different from rats, even at a relatively high dose (150 nmol/kg) does not have any effect in WT mice whereas the amylin agonist NN1213 at a dose of 30 nmol/kg is able to elicit body weight loss by decreasing fat mass in mice.

To examine the contribution of each RAMP in the response to sCT and NN1213, single and double KO mice were tested. NN1213 produced a body weight loss in

RAMP1 KO, whereas in RAMP3 KO and RAMP1/3 KO, it had no effect. This suggests that the presence of AMY3 is necessary to mediate the effect of NN1213. Conversely, sCT induced body weight loss in both RAMP1/3 double KO (−5%) and RAMP3 KO (−8%) mice but no sustained effect in the RAMP1 KO mice. Thus, we may hypothesize that the presence of AMY3 impedes sCT's body weight loss efficacy in mice during subchronic administration. To explain this effect of DACRA in the absence of RAMP3, we have previously shown that, in C57Bl6 mice, CTRa seems to be preferentially colocalized with RAMP3 compared with RAMP1 in the AP (Coester et al., 2020); thus, we may hypothesize that in the absence of RAMP3, CTR is available and preferentially binds sCT. Using in situ hybridization, we here showed that in absence of RAMP3, CTR is less colocalized with the remaining RAMPs. More studies at the pharmacological levels are however needed to understand why AMY3 prevents the subchronic action of sCT in mice but not in rats, and why WT mice do respond to sCT after acute administration.

These current studies highlight the species-specific effects of DACRA. Opposite to mice, several studies have demonstrated that sCT or other DACRA compounds are able to induce long-term weight loss in diet induced obese rats (Andreassen et al., 2014; Feigh et al., 2011; Feigh et al., 2012; Gydesen et al., 2016; Hjuler et al., 2015; Larsen et al., 2020). Rats treated subchronically with one of the above-mentioned peptides lost weight and transiently reduced their food intake in a dose-dependent manner. For example, rats treated with KBP-089 showed a body weight loss of 17% and presented a reduction in fat depots and fat accumulation in liver and in muscles (Gydesen, Andreassen et al., 2017; Gydesen, Hjuler et al., 2017). Two other DACRAs from the same company, KBP-088 and KBP-042, were also able to reduce body weight when injected or given orally in a sustained and dose-dependent manner; additionally, they reduced white adipose tissue mass and adiposity hypertrophy (Andreassen et al., 2014; Hjuler et al., 2015; Srivastava & Apovian, 2018). These KBP compounds were unfortunately not tested in HFD-fed mice preventing us to make a comparison with our current study.

The reason for this species difference in sCT's and DACRA's actions remains unknown. So far, no basic differences between the roles of RAMPs and the different CTR subtypes in mice versus rats have been reported. We could hypothesize that RAMP1–3 distribution may differ between mice and rats and because sCT's efficacy is improved in the absence of RAMP3, this may play a role in the differential effects in rats versus mice. Even though acute sCT produces an anorectic effect in mice and rats

(Coester et al., 2019), we could also hypothesize that in a subchronic setting, CTR transcription or transport is differentially regulated between mice and rats or that activation of downstream pathways may be different between species leading to this resistance and absence of effect in mice.

As briefly mentioned above, we have previously identified that in the C57Bl6 background mouse AP (Coester et al., 2020), CTRa is mostly colocalized with RAMP3 compared to RAMP1. In the ARC, RAMP1 and RAMP3 are equally colocalized with CTR albeit at a lesser density than in the AP. However, in the ARC and AP, RAMP3 seems more abundant than RAMP1 (Coester et al., 2020). However, in the mice on a 129S2/SvEv background, RAMP2 seems to be most abundant and the preferred associated RAMP subunit to CTR in the AP. For comparison, in the rat AP, at the single-cell level, CTRa+ neurons are colocalized with RAMP1 (3%), RAMP3 (10%) and RAMP1/3 (25%) (Liberini et al., 2016). Furthermore, the metabolic characterization of these RAMP KO mice models suggests that AMY3 is more relevant for eating effects and AMY1 for adiposity (Coester et al., 2019). Thus, although the present study sheds some light on the specific role of RAMP in mediating the effect of DACRA and amylin selective analogue, further studies need to be conducted to investigate the role and the exact expression of AMY1–3 in mice to properly understand their mechanism of action in mouse models. If one RAMP is knocked out, we may expect the other two to be overexpressed. On the basis of this concept, we may expect that RAMP3 KO mice showed an overexpression of RAMP1 and RAMP2 to compensate the loss of function of RAMP3. Opposite to our expectation, however, we here showed that the depletion of one or two RAMPs (in a 129S2/SvEv background mouse) decreases the transcription of the other RAMPs. Furthermore, it seems that RAMP3 plays a crucial role because its absence may prevent the colocalization of the RAMP1 or RAMP2 with CTR.

The role of RAMP2 that forms AMY2 when combined to CTR (Morfis et al., 2008; Tilakaratne et al., 2000), in mediating amylin-related signals, is poorly investigated. Unlike RAMP1 and RAMP3 KO mouse models that survive during embryonic development, RAMP2 KO mice are lethal embryonically, showing generalized edema (Kadmiel et al., 2012). Several studies demonstrated that RAMP2 is essential for angiogenesis and for the development and integrity of a functional lymphatic system (Dackor et al., 2007; Fritz-Six et al., 2008; Ichikawa-Shindo et al., 2008). These findings indicate that endogenous expression of RAMP1 and RAMP3 is not able to compensate for the physiological function of RAMP2. In contrast, global deletion of either RAMP1 or RAMP3 does not affect survival, perhaps because the expression of other RAMPs and particularly RAMP2 is able to compensate for their absence and their loss of function. At present, we do not know whether some effects observed in this study can be explained by amylin agonist or DACRA action at the AMY2 receptor, that is, whether there is a pharmacological role of AMY2 in the control of food intake and body weight when interacting with amylin, amylin agonists and DACRAs. In the rat AP, RAMP2 is present in amylin-induced cFos+ neurons and is upregulated after acute amylin, whereas RAMP1 and RAMP3 are downregulated (Liberini et al., 2016).

4.1 | Limitation of the study

RAMP can dimerize with either CTR to form the amylin receptors 1–3 or with the calcitonin receptor-like receptor (CLR). CLR/RAMP1 is considered as the primary CGRP receptor. CLR/RAMP2 and CLR/RAMP3 are the adrenomedullin 1 and 2 receptors (Lee et al., 2016). Amylin receptors 1 and 3 can also bind CGRP, and AMY1 is considered as the second CGRP receptor. Together, RAMP, CLR and CTR generate at least seven pharmacologically distinct receptors (not including splice variants) making the study of these signalling pathway quite arduous (Boccia et al., 2020; Lee et al., 2016; Lorenzen et al., 2019). Thus, whole-body RAMP KO animal models assess diverse metabolic functions that are not limited to only amylin-related signalling. Furthermore, our in situ hybridization colocalization should be interpreted carefully because we showed the localization of CTR and the different RAMPs at the mRNA level. Whether this is reflected at the protein level remains unknown due to the absence of specific RAMP antibodies. Furthermore, the presence of CTR and RAMPs mRNA within one cell does not guarantee that these two components form a functional receptor subunit. Finally, given the sex difference we previously observed in regard to their metabolism (Coester et al., 2019) and given that obesity is as prevalent in men and women, testing the anorectic effect of NN1213 in female mice may be necessary in the future to fully validate new pharmaceutical drugs.

4.2 | Conclusion

Together these studies highlight the crucial role of AMY3 in mediating DACRA and selective amylin receptor agonist weight lowering effect in mice, albeit in opposite directions. They also emphasize that these compounds may produce species-specific effects that need to be

further investigated. Indeed, these results indicate the need to test preclinical compounds in multiple species and whether negative effects in one species may preclude the absence of effect in a clinical study remains to be further investigated.

CONFLICT OF INTEREST
L.M.J., S.L. and K.R. are full-time employees of Novo Nordisk and hold minor share portions as part of their employment. T.A.L. has received research support from investigator-initiated sponsored proposals from Novo Nordisk. S.A., A.C. and C.L.F. declare no financial and no non-financial conflict of interest.

AUTHOR CONTRIBUTIONS
L.M.J., K.R., T.A.L. and C.L.F. conceptualized the study; L.M.J., S.L. and C.L.F. did the design of methodology; S.A., A.C. and C.L.F. did the investigation; S.A. and C.L.F. wrote the original draft; L.M.J., K.R., T.A.L. and C.L.F. reviewed and edited the final output; K.R. and T.A.L. were responsible for the resources. L.M.J. and C.L.F. are the guarantor of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Full datasets are available upon request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.