Effects of a selective long-acting amylin receptor agonist on alcohol consumption, food intake and body weight in male and female rats

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Abstract
Alcohol use disorder is a complex neuropsychiatric disorder affecting both males and females worldwide; however, the efficacy of current pharmacotherapies varies. Recent advances show that gut-brain peptides, like amylin, regulate alcohol behavioural responses by acting on brain areas involved in alcohol reward processes. Thus, the activation of amylin receptors (AMYRs) by salmon calcitonin (sCT) decreases alcohol behaviours in male rodents. Given that sCT also activates the sole calcitonin receptor (CTR), studies of more selective AMYR agonists in both male and female rodents are needed to explore amylinergic modulation of alcohol behaviours. Therefore, we investigated the effects of repeated administration of a selective long-acting AMYR agonist, NNC0174-1213 (AM1213), on alcohol, water and food intake, as well as body weight in male and female rats chronically exposed to alcohol. We confirm our previous studies with sCT in male rats, as repeated AM1213 administration for 2 weeks initially decreased alcohol intake in both male and female rats. However, this reduction ceases in both sexes on later sessions, accompanied by an increase in males. AM1213 reduced food intake and body weight in both male and female rats, with sustained body weight loss in males after discontinuation of the treatment. Moreover, AM1213 administration for 3 or 7 days, differentially altered dopamine, serotonin and their metabolites in the reward-related areas in males and females, providing tentative, but different, downstream mechanism through which selective activation of AMYR may alter alcohol intake. Our data provide clarified insight into the importance of AMYRs for alcohol intake regulation in both sexes.

Keywords
alcohol intake, alcohol use disorder, amylin analogue, amylin receptors, body weight, sex differences
1 | INTRODUCTION

Although alcohol abuse can ultimately lead to the development of alcohol use disorder (AUD), a great burden both for the ailing and the society, current pharmacotherapies for the treatment of this disorder vary in efficiency between patients. In attempts to identify new neurochemical alcohol targets that could serve as potential treatments, studies have evaluated the neurochemical mechanisms through which alcohol activates brain areas involved in reward. These mechanisms include activation of interconnected brain areas like the lateral dorsal tegmental area (LDTg), the ventral tegmental area (VTA) and the nucleus accumbens (NAc). Despite the complexity of these mechanisms, recent advances have pinpointed gut-brain peptides as modulators of alcohol-mediated behaviours and the development of AUD.

The gut-brain peptide amylin decreases food intake and acts as a satiation signal via central amylin receptors (AMYRs) in male rats. The amylinergic pathway is involved not only in food regulation but also in the expression of alcohol-related behaviours (for review). Accordingly, differential expression of the components of the AMYR1, that is, of the calcitonin receptor (CTR) and the receptor activity-modifying protein (RAMP) 1, has been shown in the NAc of male rats consuming high amounts of alcohol compared to low alcohol–consuming rats. Furthermore, salmon calcitonin (sCT), an AMYR agonist, inhibits alcohol induced reward-related behaviours in male mice. Likewise, sCT decreases alcohol intake, prevents relapse drinking and reduces operant self-administration of alcohol in male rats chronically exposed to alcohol. In addition to AMYR1s, sCT also activates the (CTR) and has a relatively short half-life, increasing the need of testing a more selective and long-acting AMYR agonist in the investigation of amylinergic regulation of alcohol-mediated behaviours. Furthermore, the recent studies showing that sCT attenuates alcohol-mediated behaviours in rodents have been solely conducted on male animals, despite AUD’s prevalence for both males and females. Hence, we investigated the effect of repeated administration of the long-acting, and selective over the calcitonin receptor (CTR), AMYR agonist NNC0174-1213 (AM1213) at a dose without behavioural effects per se, on alcohol, water, food intake and body weight in both male and female rats exposed to the intermittent alcohol access paradigm. Additionally, we performed ex vivo biochemical experiments investigating monoamines and their metabolites in the LDTg, VTA and NAc following short- and long-term administration of AM1213. Although preliminary, these data could identify possible neurochemical mechanisms involved in the ability of AM1213 to regulate alcohol intake in male and female rats.

2 | MATERIALS AND METHODS

2.1 | Animals

Male and female RccHan Wistar rats (Envigo, Horst, The Netherlands) weighing 180 g at the day of arrival were used for the intermittent alcohol access and ex vivo biochemistry experiments. In the intermittent alcohol access experiment, the individually housed (high Macrolon III cages) rats were maintained on a 12-h light/dark cycle (a reversed cycle with the lights off at 9 AM was applied only for the intermittent alcohol access experiment) in rooms with 20°C and 50% humidity with ad libitum food access. For the ex vivo biochemical experiments, the same rat strain was used, and the animals were maintained under the same conditions as described above. Individually housed male Sprague Dawley rats (Charles River Laboratories, Calco, Italy) were used for the Irwin test (Supporting Information).

The female rats used in the study were intact and not controlled for estrous cycle as recent literature suggests that the female estrous cycle does not have any effect in voluntary alcohol intake paradigms similar to the one used in our studies. Moreover, in the context of more complex behavioural tasks, like operant sucrose self-administration, the female estrous cycle does not appear to influence data variability. Taken together, the observed response differences in female rats in this study are likely not attributed to hormonal differences.

2.2 | Drugs

The long-acting recombinant amylin analogue, AM1213 was used in the present studies. The compound was diluted in vehicle solution (sodium acetate, glycerol and sterile water solution; pH 4.00 ± 0.05). It was administered subcutaneously (SC) at the dose of 0.3 mg/kg 120 min prior to dark cycle onset. Alcohol (95%, Solveco, Stockholm, Sweden) was diluted in tap water to 20% v/v for oral consumption. As shown by the in vitro binding affinity and functional properties of AM1213 on rat AMYR and CTR, AM1213 has higher selectivity for the AMYR over the CTR (Supporting Information).

2.3 | Irwin test

The Irwin tests evaluate the main central and peripheral nervous system functions and were used in the present studies for dose selection purposes. For this test, 24 male Sprague-Dawley rats, approximately 9 weeks of age on the day of treatment, were used. AM1213 or vehicle was administered by a single subcutaneous injection at the dose levels of 0, 0.03, 0.3 and 3 mg/kg to groups of 6. Each animal was weighed before dosing and 2 days after dosing. Irwin test observations in the home cage, during handling and in an open field were made in a blinded fashion before dosing and at 1, 6, 9, 11, 26 and 51 h after dosing.

None of the doses tested showed any effects on behavioural scores whereas a substantial body weight loss ascribed to the pharmacological effect of AM1213 at 3 mg/kg was unsuitable for the purpose of the studies. Thus, a dose of 0.3 mg/kg was used further in the present studies. The extensive data obtained from the Irwin test are not shown in the present study.
2.4 | Intermittent access 20% alcohol two-bottle-choice drinking paradigm

This experiment was designed to identify the effects of AM1213 (0.3 mg/kg, SC) on alcohol, water and food intake as well as body weight in rats that have been consuming alcohol for 10 weeks.

2.4.1 | Ten-week baseline alcohol drinking

After 1 week of habituation to the animal facilities, the rats were given free access to one bottle of 20% alcohol and one bottle of water during three 24-h sessions per week (Mondays, Wednesdays and Fridays). The rats had unlimited access to two bottles of water on the non-alcohol days (Tuesday, Thursday and weekends). All bottles were weighed daily, 24 h after their presentation to the cages. The rats’ body weight was registered once weekly, prior to bottle presentation. In these rats, a stable alcohol intake was established and was maintained for a 10-week period prior to the initiation of the treatments.

2.4.2 | Two-week AM1213 intervention

After 10 weeks of alcohol intake, the males and females were assigned to treatment groups in a balanced design. Rats were injected with AM1213 or vehicle solution (sodium acetate, glycerol and sterile water solution; pH 4.00 ± 0.05, SC) Monday through Saturday. Measurements of bottles, food intake and body weight were done daily. On alcohol days (Monday, Wednesday and Friday), values for the time points of 1 and 4 h were additionally registered. Preference for alcohol over water (the ratio of alcohol to total fluid intake), water, total fluid and food intake was calculated for all time points.

To evaluate the effects of AM1213 on the calories obtained from alcohol and food, we calculated the caloric content of alcohol (1.8 kcal/g) and food (3.2 kcal/g) for all the measured time points of 1, 4 and 24 h. Moreover, in order to explore the degree of responsiveness to AM1213 between males and females in regards to alcohol intake, food intake and body weight, we analysed the area under the curve (AUC). The AUC was calculated as the 24-h AM1213 values of each measured parameter subtracted from the vehicle values. For the alcohol intake AUC analysis, we compared the AUC between sexes for sessions 1–3 and for 4–6 separately, because the initial decrease in alcohol intake (sessions 1–3) was followed by return to the baseline (sessions 4–6).

2.4.3 | Two-week washout period

Following the injection period, the rats were not given any drugs for two more weeks and all 24-h values were registered as described above.

2.5 | Ex vivo biochemical analysis of monoamines and their metabolites

For this experiment, separate groups of alcohol-naïve male and female rats were used, and the aim was to preliminarily identify the possible role of monoamines modulating the behavioural outcome of AM1213. Based on the outcome of the intermittent access experiment, two separate setups were included in the ex vivo biochemical test. The first group received AM1213 (0.3 mg/kg, SC; males: N = 8, females: N = 8) or vehicle (males: N = 8, females: N = 8) for 3 days (corresponding to the second alcohol session and referred to as short-term treatment). This time frame was selected as we observed reduced alcohol intake in both sexes in the intermittent access experiment sessions during the first three sessions. The second group was injected with either AM1213 (0.3 mg/kg, SC; males: N = 8, females: N = 8) or vehicle (males: N = 8, females: N = 8) for 7 days (corresponding to the fourth alcohol session, and referred to as long-term treatment), because the alcohol intake returns to baseline at this treatment session in the intermittent access experiment. The rats were euthanized and decapitated on the fourth or eighth day, respectively.

In addition, in order to explore possible basal differences between sexes, we analysed the levels of monoamines and their metabolites in the aforementioned brain regions, in the vehicle treated male versus female rats of the short-term administration experiment.

2.5.1 | Tissue isolation

The brains were isolated, rapidly transferred into plastic tubes and were snap frozen in dry ice (stored in −80°C). The frozen brains were placed in a cold rat brain matrix (Zivic instruments, Pittsburgh, PA, USA) and coronally sectioned in 1-mm slices rostral and caudal to the fusion of the optic nerves with the optic chiasm according to the brain atlas.24 The desired section was placed under a stereoscope on a very cold glass plate (mix of dry ice and regular ice) to avoid tissue degradation. The LDTg, VTA and NAc were subsequently isolated from both sides, using a tissue biopsy punch.

2.5.2 | HPLC detection of monoamines and metabolites

Dissected brain tissue samples were homogenized by ultrasound homogenization (Sonifier Cell Disruptor B30, Branson Sonic Power Co. Danbury, CT, USA) in a solution of 0.1 M perchloric acid, 5.37 mM EDTA and 0.65mM glutathione. After centrifugation (10 000 rpm, 5°C, 10 min), the supernatant was collected and analysed for noradrenaline (NA), dopamine (DA) and its metabolites 3-methoxytyramine (3-MT), homovanillic acid (HVA) and 3,4-dihydroxyphenylacetic acid (DOPAC), as well as serotonin (5-HT) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA), using a split fraction HPLC-ED system. The monoamines were
analysed in an ion-exchange column (Nucleosil, 5 μ SA 100 A, 150 × 2 mm, Phenomenex, Torrance, CA, USA) with a mobile phase consisting of 13.3 g citric acid, 5.84 g NaOH, 40 mg EDTA, and 200 mL methanol in distilled water to a total volume of 1000 mL. DOPAC, HVA, 5-HIAA and 3-MT were analysed in a reverse phase column (Nucleosil, 3 μ, C18, 100 A, 50 × 2 mm, Phenomenex) with a mobile phase consisting of 11.22 g citric acid, 3.02 g dipotassium phosphate, 40 mg EDTA, and 60 mL methanol in distilled water to a total volume of 1000 mL. Electrochemical detection was performed by two amperometric detectors (Waters 460), and the currents were recorded with the Dionex Chromelone software package (Dionex, Sunnyvale, CA, USA).

2.6 Statistical analysis

The baseline alcohol intake values between treatment groups and per sex were calculated with an unpaired t test. The data from the 2-week treatment period in the intermittent alcohol access paradigm were assessed with two-way repeated measures ANOVA, followed by a Bonferroni’s test for multiple comparisons for the effect of treatment and measured time points (1, 4 and 24 h). While using similar statistics for the washout period data, only the 24-h values were analysed. The levels of monoamines and their metabolites were evaluated by an unpaired, two-tailed test, for comparisons between vehicle and drug conditions per monoamine, brain region and sex. The AUC was calculated as the 24-h AM1213 values subtracted from the vehicle values for each measured parameter. The alcohol intake, food intake and body weight AUC data were further analysed using a t test for differences between sexes. A probability value of \( P < 0.05 \) was considered as statistically significant. Data are represented as mean ± SEM.

3 RESULTS

3.1 AM1213 decreases alcohol consumption, food intake and body weight in male rats

The average daily alcohol intake values during the 10 weeks prior to vehicle or treatment administration did not differ between rats later assigned to AM1213 (4.1 ± 1.3 g/kg; \( N = 10 \)) and vehicle (4.2 ± 1.3 g/kg; \( N = 10 \)) groups (\( P = 0.9752 \)).

3.1.1 Two-week AM1213 intervention

The overall statistical analysis of all the measured parameters in all time points in male rats is shown in Table 1. A significant interaction effect was noted for alcohol intake in all measured time points (1, 4 and 24 h). Post hoc analysis revealed that AM1213 (0.3 mg/kg, SC), increased alcohol intake at alcohol session 4 (\( P = 0.0064 \)) for the 1-h time point (Figure 1A). For the 4-h time point, AM1213 decreased alcohol intake at alcohol session 1 (\( P = 0.0400 \); Figure 1B). AM1213 treatment decreased alcohol intake at alcohol session 1 (\( P = 0.0009 \)) and increased the intake at alcohol session 4 (\( P = 0.0328 \)) for the 24-h time point (Figure 1C).

The alcohol preference scores were not altered by treatment at the 1-h (Figure 1D) or 4-h (Figure 1E) time points. Albeit, a significant interaction effect at the 24-h (Figure 1F) time point, post hoc analysis did not reveal differences between specific alcohol sessions.

Even though a significant treatment effect at the 1-hour (Figure 1G) time point, where the water intake for the vehicle is higher than in AM1213 treated rats, post hoc analysis did not reveal any differences at any specific alcohol session. There were no differences in water intake at the 4-h (Figure 1H) time point. There was a significant interaction effect at the 24-h (Figure 1I) time point, where the water intake values were higher in the vehicle compared to AM1213 treated group of rats; post hoc analysis showed no differences at any specific alcohol session.

There were no differences in total fluid intake at the time point of 1 h (Figure 1J), but a decrease was noted on the 4-h total fluid intake at alcohol sessions 1 (\( P = 0.0235 \)) and 2 (\( P = 0.0032 \)), after AM1213 treatment (Figure 1K). A significant interaction effect on 24-h values of total fluid intake was evident; however, post hoc analysis did not reveal any differences at any specific alcohol session (Figure 1L).

AM1213 significantly decreased food intake at alcohol sessions 2 (\( P < 0.0001 \)) and 3 (\( P < 0.0001 \)) for the 1-h time point (Figure 1M) and at alcohol sessions 1 (\( P = 0.0090 \)), 2 (\( P < 0.0001 \)) and 3 (\( P < 0.0001 \)) for the 4-h time point (Figure 1N). AM1213 significantly decreased food intake at alcohol sessions 1 (\( P < 0.0001 \)), 2 (\( P < 0.0001 \)), 3 (\( P < 0.0001 \)), 4 (\( P = 0.0139 \)) and 5 (\( P = 0.0281 \)) for the 24-h time point (Figure 1O).

An overall interaction effect was noted for the alcohol caloric intake in all measured time points (1, 4 and 24 h). Post hoc analysis revealed that AM1213 treatment increased alcohol caloric intake in the 1-h time point at alcohol session 4 (\( P = 0.064 \); Figure S1A), did not affect alcohol caloric intake at the 4-h time point (Figure S1B) and decreased alcohol caloric intake at alcohol sessions 1 (\( P = 0.009 \)) and 4 (\( P = 0.0328 \)) in the 24-h time point (Figure S1C).

There was a significant effect of treatment and time x treatment interaction for the food caloric intake in all the time points (1, 4, 24 h). AM1213 decreased food caloric intake at alcohol sessions 2 (\( P < 0.0001 \)) and 3 (\( P = 0.0001 \)) in the 1-h time point (Figure S1D), at alcohol sessions 1–3 (\( P = 0.0009 \), \( P < 0.0001 \) and \( P < 0.0001 \), respectively) in the 4-h time point (Figure S1E) and at alcohol sessions 1–5 (\( P < 0.0001 \) for 1–3, \( P = 0.0139 \) for 4 and \( P = 0.0281 \) for 5) in the 24-h time point (Figure S1F).

Body weight was affected by AM1213 treatment and by time x treatment interaction in the 24-h time point. Specifically, AM1213 decreased body weight at alcohol sessions 4 (\( P = 0.0099 \)), 5 (\( P = 0.0031 \)), 6 (\( P = 0.0012 \)), 7 (\( P = 0.0045 \)), 8 (\( P = 0.0031 \)), 9 (\( P = 0.0011 \)), 10 (\( P = 0.0021 \)), 11 (\( P = 0.0178 \)), 12 (\( P = 0.0041 \)) and 13 (\( P = 0.0102 \)) (Figure 1P).
|                | Treatment | Time               | Interaction |
|----------------|-----------|--------------------|-------------|
| **Alcohol intake** | 1 h       | $F_{1, 18} = 2.19$ | $F_{2.011, 36.19} = 7.88$ | $F_{5, 90} = 4.01$ |
|                |           | $0.1563$           | $0.0014$     | $0.0025$ |
|                | 4 h       | $F_{1, 18} = 0.24$ | $F_{2.984, 53.71} = 12.47$ | $F_{5, 90} = 6.96$ |
|                |           | $0.6294$           | $P < 0.0001$ | $0.0001$ |
|                | 24 h      | $F_{1, 18} = 0.65$ | $F_{3.58, 64.50} = 35.67$ | $F_{5, 90} = 19.91$ |
|                |           | $0.4290$           | $P < 0.0001$ | $0.0001$ |
| **Alcohol preference** | 1 h       | $F_{1, 18} = 2.83$ | $F_{3.56, 64.03} = 0.55$ | $F_{5, 90} = 0.59$ |
|                |           | $0.1096$           | $0.6798$     | $0.7108$ |
|                | 4 h       | $F_{1, 18} = 1.81$ | $F_{3.54, 63.78} = 1.56$ | $F_{5, 90} = 1.19$ |
|                |           | $0.1948$           | $0.2003$     | $0.3225$ |
|                | 24 h      | $F_{1, 18} = 1.18$ | $F_{3.63, 65.31} = 8.00$ | $F_{5, 90} = 7.65$ |
|                |           | $0.2907$           | $P < 0.0001$ | $0.0001$ |
| **Water intake** | 1 h       | $F_{1, 18} = 5.12$ | $F_{3.80, 64.43} = 3.66$ | $F_{5, 90} = 0.61$ |
|                |           | $0.0362$           | $0.0104$     | $0.6941$ |
|                | 4 h       | $F_{1, 18} = 4.18$ | $F_{3.93, 7.75} = 3.53$ | $F_{5, 90} = 0.72$ |
|                |           | $0.0559$           | $0.0114$     | $0.6106$ |
|                | 24 h      | $F_{1, 18} = 2.11$ | $F_{53.22, 58.02} = 13.87$ | $F_{5, 90} = 13.87$ |
|                |           | $0.1632$           | $0.9779$     | $P < 0.0001$ |
| **Total fluid intake** | 1 h       | $F_{1, 18} = 2.96$ | $F_{3.57, 66.49} = 11.40$ | $F_{5, 90} = 2.21$ |
|                |           | $0.1027$           | $P < 0.0001$ | $0.0601$ |
|                | 4 h       | $F_{1, 18} = 4.95$ | $F_{3.70, 64.49} = 11.40$ | $F_{5, 90} = 2.87$ |
|                |           | $0.0391$           | $P < 0.0001$ | $0.0189$ |
|                | 24 h      | $F_{1, 18} = 2.14$ | $F_{3.12, 56.13} = 4.18$ | $F_{5, 90} = 1.94$ |
|                |           | $0.1612$           | $P < 0.0001$ | $0.0018$ |
| **Food intake** | 1 h       | $F_{1, 18} = 34.35$ | $F_{4.18, 74.88} = 5.91$ | $F_{5, 90} = 8.80$ |
|                |           | $P < 0.0001$       | $0.0003$     | $P < 0.0001$ |
|                | 4 h       | $F_{1, 18} = 38.25$ | $F_{3.54, 63.71} = 9.42$ | $F_{5, 90} = 20.04$ |
|                |           | $P < 0.0001$       | $P < 0.0001$ | $P < 0.0001$ |
|                | 24 h      | $F_{1, 18} = 15.66$ | $F_{3.27, 58.79} = 48.11$ | $F_{5, 90} = 30.75$ |
|                |           | $0.0009$           | $P < 0.0001$ | $P < 0.0001$ |
| **Alcohol caloric intake** | 1 h       | $F_{1, 18} = 0.65$ | $F_{3.58, 64.50} = 35.67$ | $F_{5, 90} = 19.91$ |
|                |           | $0.4290$           | $P < 0.0001$ | $0.0001$ |
|                | 4 h       | $F_{1, 18} = 1.27$ | $F_{3.84, 69.05} = 11.32$ | $F_{5, 90} = 7.14$ |
|                |           | $0.2733$           | $P < 0.0001$ | $P < 0.0001$ |
|                | 24 h      | $F_{1, 18} = 0.65$ | $F_{3.58, 64.50} = 35.67$ | $F_{5, 90} = 19.91$ |
|                |           | $0.4290$           | $P < 0.0001$ | $P < 0.0001$ |
| **Food caloric intake** | 1 h       | $F_{1, 18} = 29.35$ | $F_{3.49, 62.79} = 2.72$ | $F_{5, 90} = 10.01$ |
|                |           | $P < 0.0001$       | $0.0442$     | $P < 0.0001$ |
|                | 4 h       | $F_{1, 18} = 31.90$ | $F_{2.87, 51.70} = 5.97$ | $F_{5, 90} = 13.13$ |
|                |           | $P < 0.0001$       | $0.0016$     | $P < 0.0001$ |
|                | 24 h      | $F_{1, 18} = 40.37$ | $F_{3.27, 58.79} = 48.11$ | $F_{5, 90} = 30.75$ |
|                |           | $P < 0.0001$       | $P < 0.0001$ | $P < 0.0001$ |
| **Body weight** | 24 h      | $F_{1, 18} = 15.66$ | $F_{3.81, 32.60} = 9.02$ | $F_{12, 216} = 30.34$ |
|                |           | $0.0009$           | $0.0001$     | $P < 0.0001$ |

Note: Data shown as $F_{DFn, DFd}$ after repeated measures ANOVA analysis; significance level of $P < 0.05$. The values emphasised in Bold are values were $P < 0.05$ (statistically significant effect).
Repeated AM1213 administration decreases voluntary alcohol intake, food intake and body weight in male rats. Repeated AM1213 treatment (0.3 mg/kg, SC) (A) increased 1-h alcohol intake in male rats (N = 10) on alcohol session 4 in the intermittent access 20% alcohol two-bottle-choice drinking paradigm compared to vehicle (N = 10), but (B) decreased 4-h alcohol intake on session 1. (C) AM1213 treatment decreased 24-h alcohol intake on session 1 and increased alcohol intake on session 4. The treatment did not affect alcohol preference in male rats at any time point of (D) 1, (E) 4 or (F) 24 h. Water intake was unaffected by AM1213 at all measured time points of (G) 1, (H) 4 and (I) 24 h. Treatment (J) did not affect 1-h total fluid intake, but (K) decreased 4-h total fluid intake on sessions 1 and 2, without affecting (L) 24-h values. (M) 1-h food intake was decreased after AM1213 repeated treatment on sessions 2 and 3, similarly to (N) 4-h food intake on sessions 1 to 3 and (O) 24-h food intake on session 1 to 5. (P) 24-hour body weight was decreased in males by AM1213 on treatment days 5 to 13. (Data are presented as mean ± SEM; *P < 0.05, **P < 0.001, ***P < 0.001)
The overall statistical analysis of all the measured parameters in all time points in male rats is shown in Table 2.

As shown in Figure S2A-E, during washout, previous AM1213 treatment had no effect on alcohol intake and alcohol preference. Although a significant interaction effect on water intake, post hoc analysis revealed no significant differences at any specific alcohol session. There were no differences between the previous treatment groups on total fluid intake or food intake after treatment discontinuation.

There was an overall effect of treatment on water intake in the 1- and 4-hour time points, accompanied by an effect of interaction in the 24-hour time point. Further analysis showed that the 1-hour (Figure 2G) and 4-hour (Figure S3F) alcohol preference at any specific alcohol session.

There was an overall effect of treatment on water intake in the 1- and 4-hour time points, accompanied by an effect of interaction in the 24-hour time point. Further analysis showed that the 1-hour (Figure 2G) and 4-hour (Figure S3F) alcohol preference at any specific alcohol session.

An effect of treatment was noted on total fluid intake in all measured time points. Specifically, AM1213 decreased total fluid intake at alcohol sessions 2 (P = 0.0184; Figure 2B). AM1213 also decreased alcohol intake at alcohol sessions 1 (P = 0.0238) for the 24-hour time point (Figure 2C).

Despite a significant interaction effect on alcohol preference in the 24-hour time point, treatment did not affect 1-hour (Figure 2D), 4-hour (Figure 2E) or 24-hour (Figure 2F) alcohol preference at any specific alcohol session.

The treatment and the time × treatment interaction had an overall effect on food intake in all measured time points. Post hoc analysis demonstrated that AM1213 decreased food intake at alcohol sessions 2 (P = 0.0016) and 3 (P = 0.0211) at the time point of 1 hour (Figure 2J). The drug also decreased total fluid intake at alcohol sessions 1 (P = 0.0045), 2 (P = 0.0024) and 3 (P = 0.0403) for the 4-hour time point (Figure 2K) and at alcohol sessions 1 (P = 0.0329) and 3 (P = 0.0382) for the 24-hour time point (Figure 2L).

The overall statistical analysis of all the measured parameters in all time points in female rats is shown in Table 3.

In females, the treatment had no overall effect on alcohol intake in the 1-hour time point (Figure 2A). There was a significant interaction effect in the 4- and 24-hour time point. Post hoc analysis showed that AM1213 decreased 4-hour values of alcohol intake at alcohol session 2 (P = 0.0184; Figure 2B). AM1213 decreased alcohol intake at alcohol sessions 1 (P = 0.0028) and 2 (P = 0.0238) for the 24-hour time point (Figure 2C).

Despite a significant interaction effect on alcohol preference in the 24-hour time point, treatment did not affect 1-hour (Figure 2D), 4-hour (Figure 2E) or 24-hour (Figure 2F) alcohol preference at any specific alcohol session.

There was an overall effect of treatment on water intake in the 1- and 4-hour time points, accompanied by an effect of interaction in the 24-hour time point. Further analysis showed that the 1-hour (Figure 2G) and 4-hour (Figure S3F) alcohol preference at any specific alcohol session.

An effect of treatment was noted on total fluid intake in all measured time points. Specifically, AM1213 decreased total fluid intake at alcohol sessions 2 (P = 0.0184; Figure 2B). AM1213 also decreased alcohol intake at alcohol sessions 1 (P = 0.0238) for the 24-hour time point (Figure 2C).

Despite a significant interaction effect on alcohol preference in the 24-hour time point, treatment did not affect 1-hour (Figure 2D), 4-hour (Figure 2E) or 24-hour (Figure 2F) alcohol preference at any specific alcohol session.

The treatment and the time × treatment interaction had an overall effect on food intake in all measured time points. Post hoc analysis demonstrated that AM1213 decreased food intake at alcohol sessions 2 (P = 0.0016) and 3 (P = 0.0211) at the time point of 1 hour (Figure 2J). The drug also decreased total fluid intake at alcohol sessions 1 (P = 0.0045), 2 (P = 0.0024) and 3 (P = 0.0403) for the 4-hour time point (Figure 2K) and at alcohol sessions 1 (P = 0.0329) and 3 (P = 0.0382) for the 24-hour time point (Figure 2L).

The treatment and the time × treatment interaction had an overall effect on food intake in all measured time points. Post hoc analysis demonstrated that AM1213 decreased food intake at alcohol sessions 2 (P = 0.0016) and 3 (P = 0.0211) at the time point of 1 hour (Figure 2J). The drug also decreased total fluid intake at alcohol sessions 1 (P = 0.0045), 2 (P = 0.0024) and 3 (P = 0.0403) for the 4-hour time point (Figure 2K) and at alcohol sessions 1 (P = 0.0329) and 3 (P = 0.0382) for the 24-hour time point (Figure 2L).

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TABLE 3  Effects of 2-week AM1213 versus vehicle treatment in female rats

| Treatment                      | Time | Interaction |
|--------------------------------|------|-------------|
| Alcohol intake                 |      |             |
| 1 h                            | $F_{1, 18} = 2.17$ | $F_{2, 15, 38.61} = 10.36$ | $F_{5, 90} = 1.86$ |
|                               | 0.1581 | 0.0002 | 0.1088 |
| 4 h                            | $F_{1, 18} = 0.24$ | $F_{3, 84, 49.05} = 12.47$ | $F_{5, 90} = 7.14$ |
|                               | 0.6294 | $P < 0.0001$ | $P < 0.0001$ |
| 24 h                           | $F_{1, 18} = 1.75$ | $F_{2, 98, 53.68} = 38.88$ | $F_{5, 90} = 10.08$ |
|                               | 0.2029 | $P < 0.0001$ | $P < 0.0001$ |
| Alcohol preference             |      |             |
| 1 h                            | $F_{1, 18} = 0.33$ | $F_{2, 33, 41.87} = 7.26$ | $F_{5, 90} = 0.35$ |
|                               | 0.5743 | 0.0012 | 0.8809 |
| 4 h                            | $F_{1, 18} = 1.85$ | $F_{2, 91, 52.37} = 5.21$ | $F_{5, 90} = 2.04$ |
|                               | 0.1902 | 0.0035 | 0.0803 |
| 24 h                           | $F_{1, 18} = 1.05$ | $F_{3, 44, 61.89} = 10.64$ | $F_{5, 90} = 7.70$ |
|                               | 0.3187 | $P < 0.0001$ | $P < 0.0001$ |
| Water intake                   |      |             |
| 1 h                            | $F_{1, 18} = 4.22$ | $F_{2, 69, 48.39} = 2.45$ | $F_{5, 90} = 0.74$ |
|                               | 0.0547 | 0.0805 | 0.5939 |
| 4 h                            | $F_{1, 18} = 6.82$ | $F_{2, 82, 50.83} = 1.39$ | $F_{5, 90} = 0.66$ |
|                               | 0.0176 | 0.2567 | 0.6528 |
| 24 h                           | $F_{1, 18} = 7.73$ | $F_{3, 96, 71.20} = 7.25$ | $F_{5, 90} = 2.44$ |
|                               | 0.0124 | $P < 0.0001$ | 0.0402 |
| Total fluid intake             |      |             |
| 1 h                            | $F_{1, 18} = 8.38$ | $F_{2, 45, 44.18} = 3.76$ | $F_{5, 90} = 1.30$ |
|                               | 0.0097 | 0.0235 | 0.2712 |
| 4 h                            | $F_{1, 18} = 9.53$ | $F_{3, 30, 59.42} = 2.55$ | $F_{5, 90} = 1.07$ |
|                               | 0.0063 | 0.0584 | 0.3815 |
| 24 h                           | $F_{1, 18} = 9.98$ | $F_{3, 75, 67.54} = 20.36$ | $F_{5, 90} = 0.80$ |
|                               | 0.0054 | $P < 0.0001$ | 0.5542 |
| Food intake                    |      |             |
| 1 h                            | $F_{1, 18} = 29.35$ | $F_{3, 49, 62.79} = 2.72$ | $F_{5, 90} = 10.01$ |
|                               | $P < 0.0001$ | 0.0003 | $P < 0.0001$ |
| 4 h                            | $F_{1, 18} = 31.90$ | $F_{2, 87, 51.70} = 5.97$ | $F_{5, 90} = 14.70$ |
|                               | $P < 0.0001$ | 0.0016 | $P < 0.0001$ |
| 24 h                           | $F_{1, 18} = 44.73$ | $F_{3, 70, 66.67} = 34.30$ | $F_{5, 90} = 14.70$ |
|                               | $P < 0.0001$ | $P < 0.0001$ | $P < 0.0001$ |
| Alcohol caloric intake         |      |             |
| 1 h                            | $F_{1, 18} = 2.19$ | $F_{2, 01, 36.10} = 7.88$ | $F_{5, 90} = 4.00$ |
|                               | 0.1563 | 0.0014 | 0.0025 |
| 4 h                            | $F_{1, 18} = 0.24$ | $F_{2, 98, 53.71} = 12.47$ | $F_{5, 90} = 6.96$ |
|                               | 0.6294 | $P < 0.0001$ | $P < 0.0001$ |
| 24 h                           | $F_{1, 18} = 1.75$ | $F_{2, 98, 53.68} = 38.88$ | $F_{5, 90} = 10.08$ |
|                               | 0.2019 | $P < 0.0001$ | $P < 0.0001$ |
| Food caloric intake            |      |             |
| 1 h                            | $F_{1, 18} = 34.35$ | $F_{4, 16, 74.88} = 5.91$ | $F_{5, 90} = 8.80$ |
|                               | $P < 0.0001$ | 0.0003 | $P < 0.0001$ |
| 4 h                            | $F_{1, 18} = 38.25$ | $F_{3, 54, 63.71} = 9.42$ | $F_{5, 90} = 20.04$ |
|                               | $P < 0.0001$ | 0.0001 | $P < 0.0001$ |
| 24 h                           | $F_{1, 18} = 44.73$ | $F_{3, 70, 66.57} = 34.30$ | $F_{5, 90} = 14.70$ |
|                               | $P < 0.0001$ | $P < 0.0001$ | $P < 0.0001$ |
| Body weight                    |      |             |
| 24 h                           | $F_{1, 18} = 9.88$ | $F_{2, 54, 45.80} = 6.33$ | $F_{13, 216} = 8.44$ |
|                               | 0.0056 | 0.0109 | $P < 0.0001$ |

Note: Data shown as $F_{DFn, DFd}$ after repeated measures ANOVA analysis; significance level of $P < 0.05$. The values emphasised in bold are values were $P < 0.05$ (statistically significant effect).
Repeated AM1213 administration decreases voluntary alcohol intake, food intake and body weight in female rats. Repeated AM1213 treatment (0.3 mg/kg, SC) (A) did not affect 1-h alcohol intake in female rats \( (N = 10) \) in the intermittent access 20% alcohol two-bottle-choice drinking paradigm compared to vehicle \( (N = 10) \), but (B) decreased 4-h alcohol intake on session 2. (C) AM1213 treatment decreased 24-h alcohol intake on sessions 1 and 2. The treatment did not affect alcohol preference in male rats at any time point of (D) 1, (E) 4 or (F) 24 h. Water intake was unaffected by AM1213 at the time points of (G) 1, and (H) 4 h, but (I) treatment decreased 24-h water intake on sessions 4 and 6. Treatment (J) decreased 1-h total fluid intake on sessions 2 and 3, (K) decreased 4-h total fluid intake on sessions 1 to 3 and (L) decreased 24-h values on session 1 and 3. (M) 1-hour food intake was decreased after AM1213 repeated treatment on sessions 2 and 3, similarly to (N) 4-h food intake on sessions 1 to 3. (O) 24-h food intake was decreased in females by AM1213 on treatment days 5 to 12. (Data are presented as mean ± SEM; *\( P < 0.05 \), **\( P < 0.001 \), ***\( P < 0.001 \))
alcohol sessions 1 \((P = 0.0028)\) and 2 \((P = 0.0236)\) for the 24-hour time point (Figure S3C).

Food caloric intake was affected by treatment and time \(\times\) treatment interaction in all measured time points. Specifically, AM1213 decreased food caloric intake at alcohol sessions 2 \((P < 0.0001)\) and 3 \((P = 0.0010)\) at the 1-h time point (Figure S3D), at alcohol sessions 1 \((P = 0.0136)\), 2 \((P < 0.0001)\) and 3 \((P = 0.0048)\) at the 4-h time point (Figure S3E) and at alcohol sessions 1 \((P < 0.0001)\), 2 \((P < 0.0001)\) and 3 \((P = 0.0001)\) for the 24-hour time point (Figure S3F).

There was an overall effect of treatment and interaction on body weight in female rats. AM1213 decreased the rats' body weight at alcohol sessions 5 to 12 \((P = 0.0424, P = 0.0092, P = 0.0225, P = 0.0252, P = 0.0109, P = 0.0219, P = 0.0402, P = 0.0223\) respectively; Figure 2P).

### 3.2.2 | Two-week washout period

The overall statistical analysis of all the measured parameters in all time points in female rats is shown in Table 4.

| Parameters            | 24 h          | Time          | Interaction   |
|-----------------------|---------------|---------------|---------------|
| Alcohol intake        | \(F_{1, 18} = 0.05\) | \(F_{3.66, 65.82} = 7.01\) | \(F_{5, 90} = 1.58\)  |
| Alcohol preference    | \(F_{1, 18} = 1.55\) | \(F_{3.32, 59.85} = 13.56\) | \(F_{5, 90} = 3.99\)  |
| Water intake          | \(F_{1, 18} = 2.80\) | \(F_{3.51, 63.11} = 13.12\) | \(F_{5, 90} = 4.61\)  |
| Total fluid intake    | \(F_{1, 18} = 2.70\) | \(F_{3.29, 59.17} = 8.20\) | \(F_{5, 90} = 2.99\)  |
| Food intake           | \(F_{1, 18} = 7.52\) | \(F_{2.70, 48.60} = 17.29\) | \(F_{5, 90} = 3.33\)  |
| Body weight           | \(F_{1, 18} = 0.14\) | \(F_{1.39, 24.98} = 35.16\) | \(F_{11, 198} = 0.49\) |

Note: Data shown as \(F_{DFn, DFd}\) after repeated measures ANOVA analysis; significance level of \(P < 0.05\). The values emphasised in Bold are values were \(P < 0.05\) (statistically significant effect).

### 3.3 | Analysis of the degree of responsiveness to AM1213 between male and female rats

The overall statistical analysis is shown in Table 5.

A separate analysis of the AUC revealed no significant differences between alcohol intake AUC for sessions 1-3 between males and female rats, similarly to the alcohol intake AUC comparison for sessions 4–6. However, there was significant difference between food intake AUC in male and female rats, similarly to the body weight AUC.

### TABLE 4  Effects of 2-week washout period after AM1213 treatment in female rats

### TABLE 5  Analysis of the AUC of vehicle—AM1213 treatment in males versus females

Note: Significance level of \(P < 0.05\) after unpaired t test. Comparison of the AUC between males and females; AUC was calculated as the 24-h AM1213 values subtracted from the vehicle values for each measured parameter. The values emphasised in Bold are values were \(P < 0.05\) (statistically significant effect).
3.4 Effects of short-term administration of AM1213 on monoamine levels in male rats

There was no effect of 3-day AM1213 administration on the levels of monoamines in the area of the LDTg (vehicle: N = 8, AM1213: N = 7; Figure 3A). In the VTA (vehicle: N = 6, AM1213: N = 5; Figure 3B), AM1213 increased the DA (P = 0.0141), DOPAC (P = 0.0033), HVA (P = 0.0044), 5-HT (P = 0.0008) and 5-HIAA (P = 0.0109) levels. As shown in Figure 3C, in the NAc (N = 8 for both treatment groups) AM1213 treatment increased 5-HT (P = 0.0043), and it tended to decrease the levels of 3-MT (P = 0.0582) and 5-HIAA (P = 0.0561). The detailed statistical analysis of the effects of short-term AM1213 treatment versus vehicle on the monoamine and their metabolites in the LDTg, VTA and NAc in male rats is shown in Table 6.

3.5 Effects of long-term administration of AM1213 on monoamine levels in male rats

There was no effect of 7-day AM1213 administration on the levels of monoamines in the area of the LDTg (vehicle: N = 8, AM1213: N = 6; Figure S3 A). In the VTA (N = 8 per treatment group; Figure S3B), AM1213 increased the DOPAC/DA (P = 0.0251) and (DOPAC + HVA)/DA ratio levels (P = 0.0458) significantly, and it decreased 5-HT levels (P = 0.0147). In the NAc (Figure S3C), there were no differences in the levels of the monoamines measured in the NAc of male rats (N = 8 per treatment group), after long-term treatment with AM1213. The detailed statistical analysis of the effects of long-term AM1213 versus vehicle on the monoamine and their metabolites in the LDTg, VTA and NAc in male rats is shown in Table 6.

3.6 Effects of short-term administration of AM1213 on monoamine levels in female rats

In the LDTg of female rats (vehicle: N = 8, AM1213: N = 7; Figure 4A), 3-day injections of AM1213 increased 5-HT (P = 0.0172) and 5-HIAA (P = 0.0229) levels. In the VTA (Figure 4B) of female rats (vehicle: N = 7, AM1213: N = 8), short-term AM1213 administration significantly increased 5-HT levels (P < 0.0001) and 5-HIAA (P = 0.0322) levels. In the area of NAc (Figure 4C) of female rats (N = 8 per treatment group), AM1213 treatment significantly decreased the DOPAC/DA (P = 0.0150), HVA/DA (P = 0.0226) and (DOPAC + HVA)/DA (P = 0.0042) ratios and increased 5-HIAA levels (P = 0.0444). The detailed statistical analysis of the effects of short-term AM1213 treatment versus vehicle on the monoamine and their metabolites in the LDTg, VTA and NAc in female rats are shown in Table 7. Figure 4A demonstrates the data obtained from the LDTg. Figure 4B the data from the VTA and Figure 4C the data from the NAc.

3.7 Effects of long-term administration of AM1213 on monoamine levels in female rats

In the LDTg (Figure S4A) of female rats (vehicle: N = 8, AM1213: N = 6), long-term treatment with AM1213 revealed no significant differences in the levels of monoamines. Analysis of the VTA (Figure S4B) data of monoamine levels showed no differences after long-term treatment with AM1213 in any monoamine measured (N = 8 per treatment group). AM1213 significantly increased the levels of 5-HIAA (P = 0.0093) in both the NAc (Figure S4C) of female rats (N = 8 per treatment group). The detailed statistical analysis of the effects of short-term AM1213 treatment versus vehicle on the monoamine and their metabolites in the LDTg, VTA and NAc in female rats are shown in Table 7.

3.8 Differences in basal monoamine levels between male and female rats

In vehicle-treated male rats, the basal 5-HT levels in the LDTg were significantly lower compared to male rats (P < 0.0001, N = 8 per group). In the VTA of male rats (N = 6), the basal levels of DOPAC (P = 0.0434) and HVA (P = 0.0363) were significantly lower compared to female rats (N = 7). In the NAc of male rats (N = 8 per group), the DOPAC/DA (P = 0.0054), HVA/DA (P = 0.0121) and (DOPAC + HVA)/DA (P = 0.0015) levels were lower compared to the basal levels of these monoamines in female rats. In the same area, the basal levels of 3-MT (P = 0.0001) in vehicle-treated male rats were significantly higher, whereas the levels of 5-HT (P = 0.0009) and 5-HIAA (P = 0.0079) in males were significantly lower compared to females. The detailed statistical analysis of the differences in the basal levels of monoamines and their metabolites in the LDTg, VTA and NAc between male and female rats are shown in Table 8.

4 DISCUSSION

Previous data have showed that sCT, an AMYR agonist with affinity for the CTR, reduces alcohol-mediated behaviours in male rodents.13,14 To expand these data, we herein further identify the importance of amylin signaling for alcohol and food intake as well as body weight modulation in both sexes, by using a long-acting selective AMYR analogue. We also indicate a tentative mechanism of action implicated in the outcomes on alcohol intake, by determining the effects of short- and long-term administration of the same compound on ex vivo monoamine levels and their metabolites in reward-related areas in both male and female rats.

AM1213 decreased alcohol intake during the first two alcohol sessions in males and females. Statistical analysis of the AM1213 AUC for alcohol intake revealed a similar response to AM1213 in both sexes. Overall, these data clarify the importance of selective AMYR activation in alcohol intake reduction, which is independent of sex. Previous studies show that both sCT and calcitonin administration
FIGURE 3  Short-term AM1213 administration alters monamine levels and their metabolites in reward-related areas in male rats. (A) AM1213 administration (0.3 mg/kg, SC) for 3 days did not affect the monamine levels or their metabolites in the LDTg of male rats (N = 7) when compared to vehicle (N = 8). (B) AM1213 short-term administration increased DA, DOPAC, HVA, 5-HT and 5-HIAA in the VTA of male rats (N = 7) when compared to vehicle (N = 8). (C) AM1213 increased 5-HT levels in the NAc of male rats (N = 6) when compared to vehicle (N = 8). (Data are presented as mean ± SEM; *P < 0.05, **P < 0.001, ***P < 0.001; n.s., non-significant)
Note: Significance level of $P < 0.05$ after unpaired $t$ test for comparison of AM1213 versus vehicle treatment; N/D, not detected. The values emphasised in bold are values were $P < 0.05$ (statistically significant effect).

Initially reduce alcohol intake.\textsuperscript{13,14,25} Given that these compounds also activate the CTR alone, distinguishing the exact role of AMYRs or CTRs in alcohol intake regulation is challenging. Therefore, future studies including knockout models of the AMYR components (i.e., CTR and RAMPs) will further elucidate the role of the CTR, RAMPs and AMYRs in alcohol-related behaviours.

In male rats, the initial decrease in alcohol intake following AM1213 treatment could possibly be linked to the elevated levels of dopamine, DOPAC, HVA, serotonin and 5-H1AA in the VTA and increased 5-HT in the NAc, as these monoamines in the explored brain areas regulate reward caused by alcohol.\textsuperscript{26–28} In female rats on the other hand, different neurochemical mechanisms may drive this initial reduction in alcohol intake, because short-term AM1213 treatment shows a different pattern of monoaminergic signaling. Indeed, AM1213 increases serotonin and 5-H1AA in both the LDTg and the VTA, reduces the dopamine turnover and increases 5-H1AA in the NAc. Due to the design of the ex vivo experiments the data only give a preliminarily indication that modulation of the serotonergic and/or dopaminergic system by AM1213 drives alcohol reduction by this compound. Additional studies by the means of pharmacological agents and/or microdialysis experiments are warranted, as they can explore in more detail the neurochemical correlates of the ability of AM1213 to reduce alcohol intake. It should however be noted that intra-hypothalamic amylin, at least in pharmacologically suprathreshold high doses, changes the levels of serotonin in the brain\textsuperscript{29} and that CTR mRNA is colocalised with the serotonin transporter mRNA in the mouse brain.\textsuperscript{30} Nevertheless, functional studies indicating that amylin-mediated effects involve the serotonergic system are limited and more studies are needed in order to clarify the role of serotonin in those behaviours.

After the initial decrease, alcohol consumption returned to vehicle baseline on the second alcohol session in male and the third session in female rats. These data are in accordance with our previous studies in male rats, showing that repeated sCT administration decreased alcohol intake during the initial two alcohol sessions, but showed a tolerance effect on the third alcohol drinking session.\textsuperscript{13} Interestingly, a similar alcohol drinking pattern is also observed following repeated calcitonin administration in male rats\textsuperscript{25} and by glucagon-like peptide-1, another gut-brain peptide.\textsuperscript{31,32} During the later alcohol drinking sessions, a visual but not statistically significant biphasic effect on alcohol intake is observed, as we show a discrepancy in response to AM1213 between male and female rats. Interestingly, AM1213 increased alcohol intake on session four in male rats and this consumption visually appears to be elevated on sessions five and six. On the contrary, alcohol intake was at baseline level during sessions 4–6 in female rats. The context of the noted differential effect on alcohol intake between male and female rats remains unknown.

The visually observed difference in the drinking pattern between sexes could possibly be explained by our ex vivo biochemical data. In male rats, which displayed a later increase in alcohol intake, we found an opposite effect on the monoamine levels in the VTA after long-compared to short-term treatment. In fact, in the same area, the dopamine and serotonin turnover are increased, accompanied by reduced serotonin levels. In females, after long-term AM1213 treatment, fewer changes in monoamine levels and their metabolites were noted, which is in line with the observed alcohol-drinking pattern. It should also be noted that after discontinuation of treatment there were no differences in alcohol intake in male or female rats previously treated with AM1213 compared to vehicle.

AM1213 decreased the 24-h water intake, accompanied by reduced total fluid intake in both male and female rats. This is in contrast with previous data showing that single and repeated sCT administration increases water intake in male rats\textsuperscript{13} and can possibly be explained by the potentially different mechanisms of action between AM1213 and sCT, as the latter has been attributed diuretic properties\textsuperscript{33} that might lead to compensatory increased
FIGURE 4  Short-term AM1213 administration alters monoamine levels and their metabolites in reward-related areas in female rats. (A) AM1213 administration (0.3 mg/kg, SC) for 3 days increased 5-HT and 5-HIAA in the LDTg of female rats (N = 7) when compared to vehicle (N = 8). (B) AM1213 short-term administration increased 5-HT and 5-HIAA in the VTA of female rats (N = 8) when compared to vehicle (N = 7). (C) AM1213 decreased DOPAC, HVA/DA, and HVA + DOPAC/DA and decreased 5-HT levels in the NAc of female rats (N = 8) when compared to vehicle (N = 8). (Data are presented as mean ± SEM; *P < 0.05, **P < 0.001, ***P < 0.001; n.s. non-significant)
After discontinuation of treatment, water intake remained lower in females but was slightly elevated in male rats, indicating differences in response between sexes after AM1213 treatment termination.

We further found that repeated AM1213 reduced food intake in both sexes, showing a tolerance pattern in later sessions. AM1213 robustly reduced the initial food intake in both male and female rat. Notably, food intake returned to baseline on the last session in males, whereas this return to baseline was observed on the fourth session in females. Interestingly, the food intake AUC analysis revealed a higher reduction in males than females. The tolerance pattern following the initial food intake reduction is in accordance with our previous data showing that repeated treatment with sCT reduced food intake during the three alcohol session days,

\[ \text{(34,35)} \] and with other studies showing robust food intake drop after acute and chronic activation of AMYRs in rats.\[ \text{(34,35)} \] Similarly to our results, long-term amylin administration reduces food intake in female rats, most profoundly during the first week of treatment, which corresponds to three alcohol sessions in our study, followed by a return to the baseline at later sessions.\[ \text{(36)} \] This tolerant pattern is evident in other studies, where repeated sCT infusions for 7 weeks did not sustain food intake reduction for more than 5 days in male diet-induced obese rats.\[ \text{(37)} \] Interestingly, following discontinuation of treatment, AM1213 treated females showed increase in food intake on discontinuation sessions 4 and 5, not accompanied by body weight differences.

We here found a robust body weight reduction during the entire treatment period in both sexes. Given the tolerance effect on food intake by AM1213, the prolonged body weight reduction in males plausibly reflects long-term metabolic effects on adiposity, rather than food intake changes. Previous studies in obese male rats showed that daily infusions of amylin and leptin are needed in order to sustain body weight loss.\[ \text{(38)} \] Interestingly, in our studies, AM1213 alone sustained body weight loss in male rats even after its discontinuation, demonstrating a robust long-term effect. Supportively, repeated administration of sCT or amylin reduces body weight in male rats\[ \text{(11,13,39)} \] and amylin causes a sustained decrease in body weight in high-fat fed female rats.\[ \text{(36)} \] The body weight decrease was more profound in males as the analysis of the AUC revealed. Moreover, the body weight reduction was sustained during the washout period in male rats, although this is not observed in females. The protracted effects noted in males suggest that AM1213 acts differently on male and female rats. The observed sex differences could also potentially be attributed to the diverse molecular background between sexes, as female rats have higher mRNA levels of endogenous amylin in the

### TABLE 7

Results of short- and long-term AM1213 versus vehicle treatment in monoamines and their metabolites in brain areas in female rats

|                  | Short-term treatment |          |          | Long-term treatment |          |          |
|------------------|----------------------|----------|----------|---------------------|----------|----------|
|                  | LDTg     | VTA      | NAc      | LDTg     | VTA      | NAc      |
| Noradrenaline (NA) | 0.4317 | 0.2813  | 0.4634  | 0.9412 | 0.4870  | 0.5908  |
| Dopamine (DA)     | 0.2532 | 0.7524  | 0.3915  | 0.1217 | 0.2398  | 0.3144  |
| DOPAC             | N/D     | 0.4542  | 0.5643  | N/D     | 0.3187  | 0.2670  |
| DOPAC/DA          | N/D     | 0.1524  | 0.0150  | N/D     | 0.9981  | 0.9200  |
| HVA               | N/D     | 0.6460  | 0.9189  | N/D     | 0.8650  | 0.1660  |
| HVA/DA            | N/D     | 0.7505  | 0.0226  | N/D     | 0.1895  | 0.6352  |
| (DOPAC + HVA)/DA  | N/D     | 0.3511  | 0.0404  | N/D     | 0.7033  | 0.7782  |
| 3-MT              | N/D     | 0.0322  | 0.0396  | N/D     | 0.6990  | 0.0586  |
| Serotonin (5-HT)  | 0.0172 | P < 0.0001 | 0.0663 | 0.9374 | 0.8990  | 0.1660  |
| 5-HIAA            | 0.0329 | 0.0444  | 0.3197  | 0.1528  | 0.0093  |
| 5-HIAA/5-HT       | 0.7979 | 0.7610  | 0.0741  | 0.1486  | 0.2824  |

Note: Significance level of P < 0.05 after unpaired t test for comparison of AM1213 versus vehicle treatment; N/D, not detected. The values emphasised in Bold are values were P < 0.05 (statistically significant effect).

### TABLE 8

Results of basal monoamine levels and their metabolites in brain areas in males versus female rats

|                  |          |          |          |          |          |          |
|------------------|----------|----------|----------|----------|----------|----------|
|                  | LDTg     | VTA      | NAc      | LDTg     | VTA      | NAc      |
| Noradrenaline (NA) | 0.7903 | 0.1966  | 0.3058  |          |          |          |
| Dopamine (DA)     | 0.5483 | 0.9367  | 0.2400  |          |          |          |
| DOPAC             | N/A     | 0.0434  | 0.3557  |          |          |          |
| DOPAC/DA          | N/A     | 0.6555  | 0.0054  |          |          |          |
| HVA               | N/A     | 0.0363  | 0.8763  |          |          |          |
| HVA/DA            | N/A     | 0.9396  | 0.0121  |          |          |          |
| (DOPAC + HVA)/DA  | N/A     | N/A     | 0.0015  |          |          |          |
| 3-MT              | N/A     | N/A     | 0.0001  |          |          |          |
| Serotonin (5-HT)  | P < 0.0001 | 0.4187 | 0.0009  |          |          |          |
| 5-HIAA            | 0.4241 | 0.1831  | 0.0079  |          |          |          |
| 5-HIAA/5-HT       | 0.2008 | 0.2103  | 0.0723  |          |          |          |

Note: Significance level of P < 0.05 after unpaired t test for comparison of monoamines (after vehicle treatment) in brain areas in males versus female rats; N/A, not available comparison. The values emphasised in Bold are values were P < 0.05 (statistically significant effect).
behaviours. AM1213 decreased body weight in both sexes and previous data showing amylinergic regulation of alcohol-mediated rats. The initial reduction in food and alcohol intake is in line with our agonist, initially reduced alcohol and food intake in male and female regards to differential sex responses to AM1213, further behavioural and molecular experiments are warranted in order to further clarify the mechanisms underlying those sex differences.

The present study shows that AM1213, a selective AMYR agonist, initially reduced alcohol and food intake in male and female rats. The initial reduction in food and alcohol intake is in line with our previous data showing amylinergic regulation of alcohol-mediated behaviours. AM1213 decreased body weight in both sexes and this effect is observed even after discontinuation of treatment in male rats. Notably, our present results that AM1213 differentially modulates alcohol, food intake as well as body weight, implies the involvement of distinctive amylinergic modulation of these behaviours, which are different between sexes. Overall, our results expand the significance of AMYRs in the regulation of alcohol intake and pinpoint their importance in alcohol intake reduction in both sexes.

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AUTHORS CONTRIBUTION
ALK performed hands on work, analysed data, wrote the manuscript and managed literature search. JV performed hands on work and analysed data. EE and KR contributed to the manuscript and interpreted data. EJ designed the study, contributed to the conception and interpretation, managed literature search, analysed data and wrote the manuscript. All authors contributed to and have approved the final manuscript.

DATA AVAILABILITY STATEMENT
The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

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

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