Original Research Article

An amylin and calcitonin receptor agonist modulates alcohol behaviors by acting on reward-related areas in the brain

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ABSTRACT

Alcohol causes stimulatory behavioral responses by activating reward-processing brain areas including the laterodorsal tegmental area (LDTg) and ventral tegmental area (VTA) and the nucleus accumbens (NAC). Systemic administration of the amylin and calcitonin receptor agonist salmon calcitonin (sCT) attenuates alcohol-mediated behaviors, but the brain sites involved in this process remain unknown. Firstly, to identify potential sCT sites of action in the brain, we used immunohistochemistry after systemic administration of fluorescent-labeled sCT. We then performed behavioral experiments to explore how infused sCT into the aforementioned reward-processing brain areas affects acute alcohol-induced behaviors in mice and chronic alcohol consumption in rats. We show that peripheral sCT crosses the blood brain barrier and is detected in all the brain areas studied herein. sCT infused into the LDTg attenuates alcohol-evoked dopamine release in the NAC shell in mice and reduces alcohol intake in rats. sCT into the VTA blocks alcohol-induced locomotor stimulation and dopamine release in the NAC shell in mice and decreases alcohol intake in rats. Lastly, sCT into the NAC shell prevents alcohol-induced locomotor activity in mice. Our data suggest that central sCT modulates the ability of alcohol to activate reward-processing brain regions.

1. Introduction

The stimulatory behavioral responses caused by alcohol are attributed to its ability to activate various brain areas processing reward, such as the laterodorsal tegmental area (LDTg), ventral tegmental area (VTA) and nucleus accumbens (NAC) shell (for review see (Engel and Jerlhag, 2014; Jayaram-Lindström et al., 2016; Soderpalm and Ericson, 2013)). These behavioral outcomes of alcohol are closely linked both to direct local activation of the aforementioned areas and to increased activity in these systems (Engleman et al., 2009; Jerlhag and Engel, 2014; Larsson et al., 2005, 2002). The complex mechanisms through which alcohol activates these reward-related areas involve various neurotransmitters and peptides (for review see (Abraha et al., 2017; Vengeliene et al., 2008)). The strong neurochemical relationship between food intake and reward behaviors (Clarke et al., 2014; Volkow et al., 2011, 2012) has led to the identification of appetite regulatory peptides, like amylin, as modulators of artificial rewards (for review see (Jerlhag, 2019)).

The pancreatic hormone amylin, physiologically controls food intake and energy balance by acting on central amylin receptors (AMYRs) (for review see (Hay et al., 2015)). In the literature, salmon calcitonin (sCT), a potent agonist for both the calcitonin receptor (CTR) and AMYR, has been suggested as an important pharmacological tool for investigating the activation of the amylinergic pathway (Braegger et al., 2014). sCT profoundly reduces food intake in rodents (Braegger et al., 2014; Lutz et al., 2001, 2000) and its anorexigenic properties are possibly mediated through AMYR components in the VTA and the LDTg (Mietlicki-Baase et al., 2015; Reiner et al., 2017) among other areas (Lutz et al., 2001). Despite the advantages of sCT in activating the amylin signaling (Lutz et al., 2001), the role of calcitoninergic pathways in alcohol reward behaviors is less evident.

Abbreviations: AMYR, amylin receptor; AUD, alcohol use disorder; CPP, conditioned place preference; CTR, calcitonin receptor; DAPI, 4',6-diamidino-2-phenylindole; EC, electrochemical; FAM, fluorescein amidite; LDTg, laterodorsal tegmental area; MAP2, microtubule-associated protein 2; NAC, nucleus accumbens; NeuN, neuronal nuclei; sCT, salmon calcitonin; TBS, tris-buffered saline; VTA, ventral tegmental area.

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et al., 2000), its action on both CTRs and AMYRs calls for caution as to which type of these receptors mediates sCT’s effects in vivo (Hay et al., 2015).

As recently demonstrated, sCT decreases alcohol-mediated behaviors in various rodent models of alcohol use disorder (AUD) (Kalafateli et al., 2019a, b). However, the brain areas involved in this modulation of alcohol-induced behaviors remain unknown. Behavioral responses to alcohol involve the LDTg, VTA and NAc shell and AMYRs, and therefore CTRs, are expressed in these areas (Baisley et al., 2014; Mietlicki-Baase et al., 2017, 2013; Reiner et al., 2017). Therefore, the present experiments were designed to identify possible central sites of action through which sCT exerts its pharmacological effects against alcohol, possibly by activating the amylin signaling. Firstly, through immunohistochemistry, we investigated whether peripherally administered fluorochrome-labeled sCT can cross the blood-brain barrier and reach the LDTg, VTA and NAc shell. To detect whether the target of the labeled peptide is the same as unlabeled sCT, we tested whether a suprapharmacological dose of sCT administered prior to FAM-sCT antagonizes the FAM-sCT signal in the rat brain. To further identify whether locally administered sCT into these areas modulates alcohol-mediated behaviors, we conducted further behavioral testing in rodents. Thus, we explored the effects of sCT infused into the aforementioned brain areas on locomotor stimulation and reward-dependent memory retrieval in the conditioned place preference (CPP) paradigm, following acute alcohol administration in male mice. Moreover, we evaluated the effects of sCT infused into the LDTg and VTA on acute alcohol-induced dopamine release in the NAc shell in male mice. Lastly, we assessed the effects of sCT infused into the studied brain areas on alcohol, water and food intake in male rats chronically exposed to alcohol.

2. Material and methods

2.1. Animals

Both mice and rats were used in the present study as they present robust responses to alcohol, as well as to different gut-brain peptides (for review see (Jerlhag, 2019)). The mice and rats were allowed to acclimatize in rooms with 20 °C and 50 % humidity for at least one week before the initiation of the experiment and had food and water ad libitum (normal chow, Harlan Teklad; England). In this study, male rodents were used to allow for reproducibility and comparison between previously acquired data, where systemic sCT administration attenuates various alcohol-mediated behaviors in male animals (Kalafateli et al., 2020a, a; Kalafateli et al., 2019b). The inclusion of female rodents is arguably beneficial, as they may respond differently to sCT. However, the repetition of previously conducted systemic sCT-alcohol experiments with only female rodents would be necessary before initiating experiments with local infusions.

To investigate the effect of sCT on the acute effects of alcohol on locomotor activity, CPP and microdialysis paradigms, adult postpubertal age-matched male NMRI mice, 8–12 weeks old and 20–25 g body weight at the time of arrival (Charles River; Sulzfeld, Germany) were used. NMRI mice were used as they respond robustly to alcohol and peripheral sCT attenuates the acute behavioral responses to alcohol in this strain (Kalafateli et al., 2019b). The mice were group-housed (8 per cage), in rooms with a 12/12 h light/dark cycle. Following surgery, each mouse was single-housed in an individually ventilated cage. An independent set of mice was used in each experiment.

For studies investigating the effects of sCT on chronic alcohol intake in the two-bottle 20 % alcohol intermittent access experiments, adult postpubertal age-matched male outbred Rcc Han Wistar rats (Envigo; Horst, The Netherlands) were used. This rat strain displays voluntary high and stable alcohol intake causing pharmacologically relevant blood alcohol concentrations in the intermittent access model (Momeni et al., 2015; Sims et al., 2008); moreover, systemic sCT reduces alcohol intake in this strain of rats (Kalafateli et al., 2019b). The individually housed rats (high Macrolon III cages), for the duration of the experiment, were maintained on a 12 -h reversed light/dark cycle (lights off at 10 a.m.).

A separate set of male Rcc Han Wistar rats was used for the immunohistochemistry experiments. Those rats were group-housed (4 per cage), under the same conditions described above.

We practice the 3R (refine, reduce, replace), by using only the number of animals that prior power analysis has confirmed and using the same group of animals when the experimental setup allows. We have followed and adapted to the ARRIVE (Animal Research: Reporting of in vivo Experiments) and PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) guidelines. Each experiment was approved by the Swedish Ethical Committee on Animal Research in Gothenburg, Sweden (ethical numbers: 1457/18, 1556/18, 207/14).

2.2. Drugs

For the behavioral studies, sCT (Tocris Bioscience; Bristol, United Kingdom) was diluted in vehicle (Ringer solution: NaCl 140 mM, CaCl2 1.2 mM, KCl 3.0 mM and MgCl2 1.0 mM, Merck KGaA; Darmstadt, Germany) and was administered locally and bilaterally at a volume of 0.5 μl per side into the LDTg (0.005 μg per side), VTA (0.4 μg per side) and NAc shell (0.02 μg per side). The injection volume of 0.5 μl, as well as the infusion procedure per se, have been extensively used previously in mice and rats. This volume is well-tolerated and does not cause diffusion of the drug in surrounding areas (Jerlhag et al., 2007; Kalafateli et al., 2017; Vallof et al., 2019). The aforementioned doses were selected as the highest dose that did not affect baseline locomotor activity, in experiments that were conducted separately for each brain region. The results were statistically analyzed to determine the highest dose that does not differ significantly from the vehicle values. If after analysis none of the doses had a significant effect on locomotor activity, the dose that did not appear to visually affect baseline locomotion in the tested animals was selected. The doses used in the present are not expected to cause nausea or malaise, as previous studies have identified similar and higher doses in the same nuclei to not present such adverse effects (Baisley et al., 2014; Mietlicki-Baase et al., 2013; Reiner et al., 2017). sCT was always given 30 min prior to alcohol exposure, as this time frame has been found to be necessary for attenuation of behavioral responses of alcohol (Kalafateli et al., 2019a, b).

For the acute effects of alcohol (ethanol 95 %, Solveco; Stockholm, Sweden) was diluted in vehicle (0.9 % sodium chloride) to 15 % v/v and was administered at a dose of 1.75 g/kg (intraperitoneally, i.p.). For the chronic alcohol oral administration, alcohol (95 %, Solveco; Stockholm, Sweden) was diluted in tap water to 20 % v/v.

For the immunohistochemistry experiments, fluorescein amidite (FAM)-labeled sCT (custom labeling; Phoenix Pharmaceuticals Inc, Burlingame, CA, USA) was injected i.p. in order to determine whether systemic administration of the drug is detectable in the brain and specifically in reward-related areas (LDTg, VTA and NAc shell). FAM-sCT was also infused intracerebroventricularly (i.c.v.) as a positive control, to test the activity of the fluorescent label. The powder form of the peptide was diluted in 10 % DMSO in PBS buffer (pH 7.5) and served as the stock solution. This stock was further diluted in 0.9 % sodium chloride for the 5 μg/kg (intraperitoneally, i.p.). For the control experiment regarding the specificity of FAM-sCT in binding on sCT’s pharmacological target, a hundredfold higher dose of unlabeled sCT (500 μg/kg, i.p.) was administered prior to FAM-sCT (5 μg/kg, i.p.). These control experiments were conducted on a separate occasion in a separate group of rats, where no vehicle-FAM-sCT rats were included. The vehicle solution for i.p. injections was 75 % of 0.9 % sodium chloride and 25 % of 10 % DMSO in PBS and for the i.c.v. injections 75 % of Ringer and 25 % of 10 % DMSO in PBS. The sections were stained for neuronal nuclei (Rabbit Anti-NeuN conjugated with Alexa Fluor® 647 in 1:50, ab190565),
30 min following a FAM-sCT (i.p. or i.c.v.) or vehicle injection (i.p.), the rats were perfused and their brains were isolated. For the control experiment regarding the specificity of FAM-sCT in binding to sCT’s pharmacological target, the hundredfold higher dose of unlabeled sCT (i.p.) was administered 30 min prior to the FAM-sCT injection (i.p.). 30 min following this second injection, the rats were perfused and their brains isolated. For perfusion, the rats were deeply anesthetized with sodium pentobarbital (Pentothal, Electra-box Pharma, Tyreso, Sweden) and were transcardially perfused with 0.9 % saline, followed by 6 % formaldehyde solution and stabilized with methanol (Histofix; Histolab products AB, Gothenburg, Sweden). The brains were post-fixed for 24 h in Histofix after perfusion and then immersed in 30 % sucrose solution containing 100 mM phosphate buffer, pH 7.5 for five days. After fixed with a cryo-gel (Tissue-Tec® O.C.T. compound, Sakura® Finetek, Alphen aan den Rijn, The Netherlands) one hemisphere was sagitally cut into 40 μm sections with a sliding microtome (Leica SM2000R, Leica Microsystems; Nussloch, Germany). The sections were stored in cryoprotectant solution (TCS; 25 % ethylene glycol, 25 % glycerine and 0.05 M phosphate buffer) at 4 °C until further processed.

Immunohistochemistry was performed on free-floating sagittal brain sections containing all the brain areas of interest using a rat brain atlas at the lateral level 0.40 mm (Figure 80 of the atlas) (Paxinos and Watson, 2017). For the staining, the brain sections were washed with TBS (Tris-buffered saline) and later blocked in TBS with 3% normal goat serum (Abcam; Cambridge, UK) and 0.1 % Triton-X 100 for 1 h at room temperature. The sections were incubated overnight at 4 °C with the primary antibodies. The second day, after rinsing in TBS, the secondary antibody was incubated at room temperature for 1 h. In between the last washing steps, the sections were incubated in the DAPI solution for 15 min. The sections were washed 3 more times in TBS and were mounted on adhesion glass slides (SuperFrost Plus™, ThermoFisher Scientific, Waltham, MA, USA) and coverslips using a few drops of mounting media (ProLong™ Gold Antifade, Life Technologies Europe BV). The samples were analyzed using a ZEISS LSM 800 confocal microscope with a 63x oil-immersion objective and the ZEN microscope software. The labeled images comprising the whole sagittal brain section were acquired with a Nikon Eclipse Ti-E confocal microscope and analyzed with the NIS Elements software. The brain areas were identified according to the brain atlas by observation (Paxinos and Franklin, 2012) using light microscopy. Only data from animals with correct placements were included in the statistical analysis (Supplementary Fig. S1A–F).

2.6. Locomotor activity

Locomotor activity was recorded in six sound-attenuated, ventilated and dim lit locomotor boxes (420 × 420 × 200 mm; Open Field Activity System; Med Associates Inc; Georgia, Vermont, USA). In this system, 15 × 15 infrared beams at the bottom of the floor detected and a personal computer program recorded distances travelled (cm per 5 min) of each mouse during the entire test session.

Each independent locomotor activity test followed the same design: the mice were allowed to habituate to the locomotor activity boxes for 60 min and then sCT or an equivalent of vehicle was infused. Alcohol or an equivalent of vehicle was administered and the subsequent 60-minute cumulative locomotor activity was registered for each test.

The first set of locomotor activity tests was performed to identify an sCT dose without affecting locomotor activity in mice, when injected into (a) the LDTg, (b) the VTA or (c) the NAc shell. The results of the dose-response studies for all tested sCT doses in each area are described in Table 1A–C.

The second set of locomotor activity tests was performed to investigate the effects of sCT infusion into the (a) LDTg, (b) VTA or (c) NAc shell on alcohol-induced locomotor stimulation in mice.

2.7. In vivo microdialysis in freely moving animals and dopamine release measurements

The present microdialysis experiments were designed to evaluate the
effects of sCT infusion into the LDTg or VTA on alcohol-induced dopamine release in the NAc shell in mice, using an HPLC-EC (electrochemical detection) system. Due to technical limitations, infusion into the NAc shell together with dopamine measurements in this area were not pursued. After one hour of habituation to the microdialysis set-up, perfusion samples were collected in 20-minute intervals across the entire test session (from –40 to 300 minutes). The baseline dopamine levels were defined as the average of three consecutive samples (-40 to 0 minutes) before local infusion. At 10 min, sCT or an equal volume of vehicle was infused ipsilaterally into the LDTg or the VTA. Thirty minutes later (at 40 min), vehicle or alcohol was injected i.p. and nine additional samples were collected. Collectively, the following four treatment groups were created: vehicle-vehicle (veh-veh), sCT-vehicle (sCT-veh), vehicle-alcohol (veh-Alc), and sCT-alcohol (sCT-Alc).

During the microdialysis experiment, the probe was connected to a microperfusion pump (U-864 Syringe Pump; Agntheta) treatment groups were created: vehicle-vehicle (veh-veh), sCT-vehicle (sCT-veh), vehicle-alcohol (veh-Alc), and sCT-alcohol (sCT-Alc).

3. Conditioned place preference

For the CPP experiments, a two-chambered conditioned place preference apparatus (45 lx) with distinct visual and tactile cues was used. The experiment consisted of the preconditioning phase (day 1), conditioning phase (days 2-5) and a post-conditioning phase (day 6). Mice were injected with vehicle (saline solution, i.p.) at both the preconditioning phase and were placed in the chamber with free access to both compartments for 20 min, to determine the initial place-preference. Conditioning (20 min per session) was done using a biased procedure in which alcohol (1.75 g/kg, i.p.) was paired with the initial least preferred compartment and vehicle (saline solution, i.p.) with the initial preferred compartment. All mice received one alcohol and one vehicle injection every day and the injections were altered between morning and afternoon conditioning in a balanced design, with an equal number of animals per group. CPP was calculated as the difference in % of total time spent in the drug-paired (i.e. less preferred) compartment during the test and pre-conditioning sessions.

The present CPP experiments were designed to assess the effects of local sCT infusion on the retrieval of alcohol-reward dependent memory. Therefore, mice were conditioned to alcohol (alcohol and vehicle injections during the conditioning phase) and at post-conditioning, sCT or an equal volume of vehicle was infused into the (a) LDTg, (b) VTA or (c) NAc. Following infusion, the mice were placed on the midline between the two compartments with free access to both compartments for 20 min (creating the following treatment groups: vehicle-alcohol and sCT-alcohol).

3.1. Intermittent access to 20 % alcohol in a two-bottle-choice drinking paradigm

These experiments were conducted to evaluate the effects of sCT infusion into the (a) LDTg, (b) VTA or (c) NAc shell on alcohol intake in rats exposed chronically to alcohol. The rats were exposed to 12 weeks of alcohol access in an intermittent access model and were subsequently infused with sCT or vehicle bilaterally into the (a) LDTg, (b) VTA or (c) NAc shell 30 min prior to bottle presentation, on an alcohol-drinking day (Monday and Wednesday). Each rat received two infusions (one sCT and one vehicle), serving as its own control in the analysis. For each test day, alcohol, water, and food intake were measured at 1 and 24 h post-alcohol access and body weight was recorded at 24 h. Moreover, the total caloric intake from the alcohol (1.8 kcal/g) and food (chow) (3.2 kcal/g) caloric content was calculated.

The rats were given free-choice access to one bottle of 20 % alcohol and one bottle of water during three 24-hs sessions per week (Mondays, Wednesdays and Fridays). The rats had unlimited access to two bottles of water during the nonalcohol-access periods. All bottles were weighed at 24-hs after the fluids were placed on the rat cages. The body weight of each rat was measured daily prior to bottle presentation, to allow for calculating the grams of alcohol intake per kilogram of body weight (g/kg). The preference for alcohol over water (the ratio of alcohol to total fluid intake) was calculated at all time points. In addition, water and food intake were measured. The alcohol intake experiment was conducted after an initial period of 12 weeks of intermittent access to alcohol.

3.2. Data analysis

All of the treatment groups in both mice and rats in the behavioral studies were designed to include groups of equal size and were randomized and scored blindly. The sample size selection was based on previous power analysis and pilot studies for an effect size of 0.2 standard deviations or more, significance level of 5%, power of study 80% and two-tailed direction of the effect. Analysis of the data was blinded to the statistician. Statistical analysis was performed only when n ≥ 5. For the immunohistochemistry experiment there were n = 3 per experimental condition and no statistical analysis took place. After ANOVA analysis, post hoc tests were performed only if alpha was P < 0.05. All the data sets were assessed for normality and homogeneity and they were normally distributed and subjected to parametric tests where only independent values were included in the analysis. The predefined exclusion criteria were misplaced cannulas. The statistical analyzes were performed using GraphPad Prism 8.3.0 software. Data are presented as mean ± SEM. The probability level for statistical significance was P < 0.05.

The dose-response locomotor activity experiments were analyzed using one-way ANOVA for independent samples, followed by Tukey’s post-hoc test for all pairwise comparisons. The alcohol-induced locomotor activity experiments were evaluated by two-way repeated measures ANOVA, followed by Tukey’s post-hoc test. The CPP tests were analyzed with an unpaired two-tailed t-test. The microdialysis experiments analysis was done with two-way repeated measures ANOVA, followed by Bonferroni’s post-hoc test for multiple comparisons between treatments and for each time point to control for Type I errors. The alcohol, water, food intake, body weight and total caloric intake data were analyzed using paired two-tailed t-tests.

4. Results

4.1. Systemically administered FAM-labeled sCT crosses the blood brain barrier and is detected in the LDTg, VTA and NAc

A separate group of rats was injected with FAM-sCT i.p. (5 μg/kg, n = 3), FAM-sCT i.c.v. (2 μg, n = 3) or vehicle solution i.p. (n = 3) and 30 min later their brains were removed in order to assess whether FAM-sCT was present in the brain. The FAM-sCT signal was detected in the whole brain as seen in the sagittal section in Supplementary Fig. S2, including the areas of interest (NAc (A), VTA (B) and LDTg (C)) as seen in the lower magnification images in the same figure. Higher magnification images showed more distinct FAM-sCT signal in the areas of the LDTg (Fig. 1A, B), VTA (Fig. 1C, D) and NAc (Fig. 1E, F). The sections were stained for neuronal nuclei, cell nuclei and dendrites. In all three regions, FAM-sCT appeared to be accumulated in areas surrounding neuronal (NeuN positive) and cell nuclei (DAPI positive). When a suprapharmacological dose of unlabeled sCT is administered 30 min prior to FAM-sCT, no FAM signal is detected in the areas of the LDTg (Supplementary Fig. S3A), VTA (Supplementary Fig. S3B) or NAc (Supplementary Fig. S3C). Supplementary Fig. S4 shows that detection of FAM signal is co-localized with the CTR signal at the level of NAc in a brain section of a rat receiving FAM-sCT i.p. injection, indicating that sCT internalizes in CTRs. The orthogonal view of the Z-stack image...
Fig. 1. Peripherally administered FAM-labeled sCT is detected in the LDTg, VTA and NAc. In brain sagittal sections of rats injected i.p., FAM-sCT is detected in the area of the (A, B) LDTg. FAM-sCT (green) shows cellular localization and is detected in neuronal cells (magenta) at the area of the (C, D) VTA. FAM-sCT is also detected at the level of (E, F) NAc.
shows enclosure of the FAM signal in the CTR. Supplementary Fig. S4B shows the lack of FAM signal when CTR is not detected at a neighboring region to the NAc-positive signal in the same rat brain section. Supplementary Fig. S4C shows visualization of the brain region around the third ventricle (i.e. injection site) after i.c.v. FAM-sCT injection. This experiment was used as a positive control to demonstrate positive signal of the FAM-sCT peptide. Supplementary Fig. S4D shows visualization of the NAc in a rat brain after vehicle i.p. injection as the negative control.

4.2. Effects of sCT into the LDTg on acute behavioral responses to alcohol in mice

In mice injected with sCT into the LDTg (0.005 μg per side) and alcohol i.p. (1.75 g/kg), there was an overall effect of alcohol (F(1, 24) = 14.57, P = 0.0008; Fig. 2A) on locomotor activity (vehicle: n = 6, vehicle-alcohol: n = 6, sCT-vehicle: n = 8, sCT-alcohol: n = 8; animals excluded due to guide misplacement), but no effect of sCT (F(1, 24) = 0.19, P = 0.6631) or sCT x alcohol interaction (F(1, 24) = 0.82, P = 0.3741). Alcohol increased locomotor activity in mice LDTg-infused with vehicle when compared to the vehicle group. The alcohol-induced locomotor stimulation was not different between the sCT-treated alcohol mice and the vehicle-treated alcohol mice. sCT alone did not affect locomotion when compared to the vehicle group. sCT into the LDTg (0.005 μg per side) had an overall effect on the alcohol-induced dopamine release in NAc shell (F(3, 50) = 5.35, P = 0.0028; Fig. 2B), as well as time (F(13, 650) = 4.25, P < 0.0001) and time x treatment interaction (F(39, 650) = 3.04, P < 0.0001). Specifically, alcohol (n = 13) increased dopamine in the NAc shell at the time points of 60–120 and 160 min when compared to vehicle (n = 13). sCT into the LDTg (n = 13) blocked this alcohol-induced increase in dopamine in the NAc shell when compared to the alcohol group at 60–120 min. In addition, there was no difference in dopamine release between vehicle-vehicle and sCT-alcohol treated mice. sCT administration (n = 15) alone did not affect dopamine release in the NAc shell at any given time point. The CPP response to alcohol tended to be lower in intra-LDTg sCT (0.005 μg per side)-treated compared to vehicle treated mice (total n = 16, Fig. 2C).

4.3. Effects of sCT into the LDTg on alcohol and food intake in rats chronically exposed to alcohol

The average baseline alcohol intake of the rats prior to injections was 5.8 ± 0.24 g/kg/24 h. sCT into the LDTg (0.005 μg per side) did not affect 1-h alcohol intake (Fig. 2D), but decreased alcohol intake at the 24-h time point (Fig. 2D) in rats (n = 11). Alcohol preference was not affected by sCT at any time point (Fig. 2E). There was a tendency to reduce 1-h food intake after sCT administration, but there was no effect at the 24-h value (Fig. 2F). Water intake was unaffected by sCT at both 1 and 24 h (Fig. 2G), similar to total fluid intake (Fig. 2H). sCT did not affect the body weight of rats (Fig. 2I). Lastly, the total caloric intake was unaffected by sCT in the area of the LDTg (Supplementary Fig. S4A).

4.4. Effects of sCT into the VTA on acute behavioral responses to alcohol in mice

In mice injected with sCT into the VTA (0.4 μg per side) and alcohol i. p. (1.75 g/kg), there was an overall effect of alcohol on locomotor activity (F(1, 26) = 4.49, P = 0.0315; vehicle and sCT-alcohol groups: n = 7, rest groups: n = 8; animals excluded due to guide misplacement; Fig. 3A) and an sCT x alcohol interaction (F(1, 26) = 5.16, P = 0.0315), but not an effect of sCT (F(1, 26) = 5.05, P = 0.0924). Specifically, alcohol increased locomotor stimulation when compared to the vehicle-control group. sCT into the VTA blocked the ability of alcohol to increase locomotion when compared to the vehicle-alcohol group. There was no difference in locomotor activity between the sCT only and vehicle-receiving groups. On alcohol-induced dopamine release in the NAc shell, sCT into the VTA (0.4 μg per side) had an overall effect (F(3, 33) = 3.92, P = 0.0094; Fig. 3B) as well as a time x treatment interaction (F(39, 429) = 3.16, P < 0.0001), but not time (F(13, 429) = 1.57, P = 0.2922; alcohol group: N = 10, rest groups: N = 9). Alcohol increased accumulable dopamine release at the time points of 60–80 and 120–180 min compared to the vehicle-receiving group. Intra-VTA sCT blocked alcohol from increasing dopamine in the NAc shell at 60–140 and 180 min compared to the group receiving vehicle-alcohol. Finally, sCT alone did not affect accumulable dopamine release at any time point when compared to vehicle. The CPP response to alcohol was unaffected by sCT into the VTA (0.4 μg per side) given on the post-conditioning day (vehicle: n = 12, sCT: n = 13; animal excluded due to guide misplacement; Fig. 3C).

4.5. Effects of sCT into the VTA on alcohol and food intake in rats chronically exposed to alcohol

The average baseline alcohol intake of the rats prior to injections was 4.14 ± 0.19 g/kg/24 h. 1-h alcohol intake in rats (n = 9) was not affected by intra-VTA sCT (0.4 μg per side), but 24 h alcohol intake was decreased (Fig. 3D). Alcohol preference over water was not affected at either 1 or 24-h time point (Fig. 3E). 1-h food intake tended to be higher after sCT administration into the VTA at the time point of 1 h, but there was no difference in the 24-h values (Fig. 3F). Water intake was unaffected after sCT administration (Fig. 3G). Total fluid intake was not affected by sCT at 1 or 24 h (Fig. 3H). sCT into the VTA did not affect rat body weight (Fig. 3I). Lastly, the total caloric intake was unaffected by sCT in the area of the VTA (Supplementary Fig. S4B).

4.6. Effects of sCT into the NAc shell on acute behavioral responses to alcohol in mice

In mice injected with sCT into the NAc shell (0.02 μg per side), locomotor activity was overall affected by alcohol (1.75 g/kg) (F(1, 27) = 31.26, P < 0.0001; vehicle: n = 6, rest groups n = 8; animals excluded due to guide misplacement; Fig. 4A), sCT (F(1, 27) = 9.16, P = 0.0054) and sCT x alcohol interaction (F(1, 27) = 7.88, P = 0.0092). Alcohol increased locomotor stimulation when compared to vehicle and sCT-vehicle receiving mice. sCT into the NAc shell blocked alcohol-induced locomotor stimulation when compared to the vehicle-alcohol group, while sCT-treated animals did not show any difference in locomotion when compared to vehicle. As shown in Fig. 4B, the CPP response in mice was not affected by sCT into the NAc shell (0.4 μg per side) on the post-conditioning day (n = 16).

4.7. Effects of sCT into the NAc shell on alcohol and food intake in rats chronically exposed to alcohol

The average baseline alcohol intake of the rats prior to injections was 3.74 ± 0.21 g/kg/24 h. Alcohol intake in rats after intra-NAc shell sCT administration (0.4 μg per side, n = 10) was not affected at the time point of 1 h (Fig. 4C), but there was a tendency for sCT to decrease alcohol intake at 24 h (Fig. 4C). Alcohol preference was not affected by sCT in either time point of 1 or 24 h (Fig. 4D). Food intake was unaffected by sCT into the NAc shell (Fig. 4E). Water intake was not affected by sCT administration at the time point of 1 or 24 h (Fig. 4F), similar to total fluid intake (Fig. 4G) and rat body weight (Fig. 4H). Lastly, the total caloric intake was unaffected by sCT in the area of the NAc (Supplementary Fig. S4C).

5. Discussion and conclusions

We here confirm previous results revealing that sCT, an AMYR and CTR agonist, as well as a novel synthetic amylin analogue, block alcohol-mediated behaviors in rodents (Kalafateli et al., 2019a, b; Kalafateli et al., 2020b). With the present studies, we expand this knowledge by...
Fig. 2. Effects of sCT infusion into the LDTg on acute alcohol behavioral responses in mice and chronic alcohol behavioral responses in rats. (A) An acute alcohol injection increased locomotor stimulation in mice when compared to the vehicle (Veh) group. sCT infusion into the LDTg decreased, but did not block this alcohol-induced locomotion. There was no difference between the Veh-alcohol (Alc) and sCT-Alc groups. (B) An acute alcohol injection caused dopamine release in the NAc shell in mice when compared to the vehicle (Veh) group. sCT into the LDTg blocked this alcohol-induced dopamine release. (C) LDTg-sCT did not affect alcohol reward-dependent memory in the CPP in mice. sCT into the LDTg did not affect (D) alcohol intake at 1-h, but decreased it at 24 h in rats. Same dose of sCT did not affect (E) alcohol preference, tended to decrease (F) 1-h food intake, but did not affect 24-h values. (G) Water intake, (H) total fluid intake and (I) body weight remained unaffected by LDTg-sCT administration. (Data are presented as mean ± SEM; *P < 0.05, **P < 0.001, ***P < 0.001 for Veh-Veh vs Veh-Alc; #P < 0.05 ##P < 0.001, ###P < 0.001 for Veh-Alc vs sCT-Alc comparisons; n.s.: non-significant).
Fig. 3. Effects of sCT infusion into the VTA on acute alcohol behavioral responses in mice and chronic alcohol behavioral responses in rats.

(A) An acute alcohol injection increased locomotor stimulation in mice when compared to the vehicle (Veh) group. sCT infusion into the VTA (0.4 μg per side) block this alcohol-induced locomotion. (B) An acute alcohol injection caused dopamine release in the NAc shell in mice when compared to the vehicle (Veh) group. sCT into the VTA blocked this alcohol-induced dopamine release. (C) VTA-sCT did not affect alcohol reward-dependent memory in the CPP in mice. sCT into the VTA did not affect (D) alcohol intake at 1 -h, but decreased it at 24 h in rats. Same dose of sCT did not affect (E) alcohol preference, tended to increase (F) 1-h food intake, but did not affect 24 -h values. (G) Water intake, (H) total fluid intake and (I) body weight remained unaffected by VTA-sCT administration. (Data are presented as mean ± SEM; *P < 0.05, **P < 0.001, ***P < 0.001 for Veh-Veh vs Veh-Alc; #P < 0.05, ##P < 0.001, ###P < 0.001 for Veh-Alc vs sCT-Alc comparisons; n.s.: non-significant).
indicating brain sites participating in this alcohol-amylin pathway. Firstly, our immunohistochemical results show that peripheral FAM-labeled sCT penetrates the brain and reaches reward-processing areas, including the LDTg, VTA and NAc. The present behavioral experiments further reveal that locally administered sCT into either the LDTg, VTA or NAc shell modulates various acute and chronic alcohol-mediated behaviors in male rodents. Our results collectively propose that the amylin, and potentially calcitonin, signaling in the studied brain areas is involved in both acute and chronic alcohol behavioral responses.

Previous studies have already detected AMYR components in the LDTg, VTA and NAc (Baisley et al., 2014; Mietlicki-Baase et al., 2013;...
et al., 2018). Interestingly, antagonism of the receptors in the VTA prior to sCT administration of the FMNs in the VTA (Mietlicki-Baase et al., 2015). Nevertheless, the exact neuronal mechanisms underlying these events are to-date unknown. In the present study, we did not detect any effect of intra-VTA sCT administration on short- or long-term food intake. This is in contrast to previous studies where the same dose of sCT administered to the VTA reduced 1 -h cumulative food intake in ad libitum fed rats (Mietlicki-Baase et al., 2013). However, this might be due to different amylinergic-mediated regulation of energy balance between alcohol-naive and alcohol-experienced rats. Moreover, further investigation of the effect of sCT on alcohol and food caloric intake would shed more light on the regulation of those behaviors by sCT and is warranted for the future.

Thirdly, we show that sCT into the NAc shell blocks alcohol-evoked locomotor stimulation. A role of AMYRs in the NAc was previously indirectly linked to alcohol intake, as high alcohol-consuming rats have lower expression of the calcitonin receptor gene and higher expression of the receptor activity modifying protein 1 gene in that area, when compared to low alcohol-consuming rats (Kalafateli et al., 2019a). Physiological relevance of AMYRs within the NAc shell is further supported by previous studies where higher doses of amylin infused into the NAc shell of ad-libitum maintained rats, slightly decrease sucrose intake (Baisley and Baldo, 2014). In our hands, intra-NAc shell sCT administration did not affect food intake in rats. This contradicts data showing that a high amylin dose into the NAc shell reduced chow intake in food-deprived rats (Baisley and Baldo, 2014). However, this discrepancy might be related to the fact that the rats in our study were fed ad libitum, that they were injected with sCT and not amylin per se and that they were alcohol-exposed and not naïve.

In the present study, sCT injected into the LDTg, VTA or NAc shell did not affect alcohol reward-dependent memory retrieval in the CPP paradigm. Similar to this, the glucagon-like peptide 1 receptor agonist exendin-4 infused into the LDTg and posterior part of the VTA (Vallø et al., 2019) and neuromedin U into the VTA (Vallø et al., 2019), did not influence alcohol reward-dependent memory retrieval of CPP in mice. This possibly suggests the involvement of different brain areas and neurotransmitter systems in the processing of reward-dependent memory expression, as the latter is formed and expressed through pathways including the prefrontal cortex and hippocampus (Hyman et al., 2006).

Here, we show that sCT infusion into select brain areas differentially influenced alcohol-induced behaviors. A possible explanation could suggest the involvement of additional neuromcircuits in the expression of acute and chronic alcohol behaviors (Mahmoudi et al., 1997; Schulz et al., 1980). Similarly, previous studies have reported variable attenuation of alcohol-related behavioral responses after administration of other gut-brain peptides (Vallø et al., 2019; Vallø et al., 2019), suggesting differential effects of peptide receptor activation in regards to alcohol-related behaviors. The findings that rat body weight remained unaffected after sCT administration in any studied brain area is most likely because metabolically relevant changes are observed during longer time frames than 24 h (Wolden-Hanson et al., 2000) that was studied herein.

Although intracranial infusions could potentially damage brain tissue and alter the obtained behavioral results, the inclusion of vehicle groups in all the experiments diminishes this possibility. Diffusion of the administered drugs into surrounding tissue could also be a potential limiting factor, as diffused drug could pass on to surrounding brain tissue causing off target effects. Animals with misplaced guides were excluded from the present analysis, but the possibility of sCT diffusion into other brain areas cannot be excluded. However, this appears less likely, as animals with misplaced guides did not exhibit the drug behavior in past analyses (Vallø et al., 2019; Vestlund and Jerlhag, 2020) and a low volume of 0.5 μl, which is less likely to spread, was used. This study covers the effects of the activation of amylin and/or
calcitonin pathways in certain brain areas processing reward. Systemic sCT might modulate alcohol-mediated behaviors via several brain regions, like the posterior VTA, NAc core and area postrema (Lutz et al., 2001). Therefore, additional experiments should be conducted to evaluate the ability of systemic sCT to modulate alcohol-mediated behaviors and future studies including these are warranted. In addition, studies including pharmacological antagonists and lesions in the areas of interest would shed more light on the brain areas involved in the modulation of alcohol behaviors by sCT. Although our immunohistochemistry data show that FAM-sCT binds on the CTR, the lack of a validated immunohistochemistry antibody for any of the three receptor activity-modifying proteins, which are essential for the formation of the AMYR complex, challenges the visual identification of AMYRs in the brain. We recently showed that a selective AMYR agonist and amylin analogue, decreases chronic alcohol consumption in both male and female rats (Kalafateli et al., 2020b), similarly to the results obtained in our previous sCT studies. Therefore, further behavioral experiments utilizing such selective AMYR agonists directly infused into the reward areas of the brain are of great interest in the exploration of the type of receptor involved in the modulation of alcohol behaviors. Lastly, the lack of inclusion of female rodents in the present study should be considered as a limitation. Although previous studies show that a selective AMYR agonist (Kalafateli et al., 2020b) and a long-acting GLP1-R agonist (dulaglutide) (Vall¨og et al., 2020) reduces alcohol intake in male and female rats, these studies show some differences in alcohol response between sex following repeated treatment with these drugs. Thus, future behavioral and immunohistochemical studies in female rodents are warranted and will shed more light in the possible differential regulation of alcohol reward by sCT between sexes. However, female rats were not included herein as alcohol studies using systemic sCT should be conducted in female rats prior to local infusions experiments. Indeed, repetition of the current studies including female rodents, although necessary, falls beyond the scope of the current experiments.

The identification of the LDTg, VTA and NAc as essential centers of the amylinergic modulation of different alcohol-mediated behaviors after sCT infusion is a novel aspect for amylin research. Our present results propose a role of AMYR components located in the reward systems as regulators of reward caused by alcohol and hypothetically by other drugs of abuse. There are commercially available drugs that act selectively on AMYRs for the treatment of other diseases, like diabetes. Our results can be of clinical importance, as the aforementioned agents can be tested as pharmacotherapies for AUD and perhaps for other addictive disorders.

Author’s contribution

ALK performed hands on work, analyzed data, wrote the manuscript and managed literature search, TMS performed the imaging analysis and contributed to the manuscript, DV performed hands on work and analyzed data, HZ contributed to the manuscript and EJ designed the study, contributed to the conception and interpretation, managed literature search, analyzed data and wrote the manuscript. All authors contributed to and have approved the final manuscript.

Declaration of Competing Interest

EJ has previously received financial support from the Novo Nordisk Foundation. This does not alter the authors’ adherence to any of the journal’s policies on sharing data and materials. The remaining authors declare no competing interest.

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Appendix A. The Peer Review Overview and Supplementary data

The Peer Review Overview and Supplementary data associated with this article can be found in the online version, at doi:https://doi.org/10.1016/j.pneurobio.2020.101969.

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