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Brain region specific glucagon-like peptide-1 receptors regulate alcohol-induced behaviors in rodents

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ABSTRACT

Glucagon-like peptide 1 (GLP-1), an incretin hormone that reduces food intake, was recently established as a novel regulator of alcohol-mediated behaviors. Clinically available analogues pass freely into the brain, but the mechanisms underlying GLP-1-modulated alcohol reward remains largely unclear. GLP-1 receptors (GLP-1R) are expressed throughout the nuclei of importance for acute and chronic effects of alcohol, such as the laterodorsal tegmental area (LDTg), the ventral tegmental area (VTA) and the nucleus accumbens (NAc). We therefore evaluated the effects of bilateral infusion of the GLP-1R agonist exendin-4 (Ex4) into NAc shell, anterior (aVTA), posterior (pVTA) or LDTg on the acute alcohol-induced locomotor stimulation and memory of alcohol reward in the conditioned place preference (CPP) model in mice, as well as on alcohol intake in rats consuming high amounts of alcohol for 12 weeks. Ex4 into the NAc shell blocks alcohol-induced locomotor stimulation and memory of alcohol reward as well as decreases alcohol intake. The *GLP-1R* expression in NAc is elevated in high compared to low alcohol-consuming rats. On the contrary, GLP-1R activation in the aVTA does not modulate alcohol-induced behaviors. Ex4 into the pVTA prevents alcohol-induced locomotor simulation, but does neither modulate CPP-dependent alcohol memory nor alcohol intake. Intra-LDTg-Ex4 attenuates alcohol-induced locomotor stimulation and reduces alcohol intake, but does not affect memory of alcohol reward. Collectively, these data provide additional knowledge of the functional role of GLP-1R in reward-related areas for alcohol-mediated behaviors and further support GLP-1R as a potential treatment target for alcohol use disorder.

1. Introduction

Alcohol use disorder (AUD) is one of the most prevalent psychiatric disorders and the socioeconomic burden of this chronic relapsing brain disorder is substantial (Grant et al., 2015). As patients respond differently to treatments (Heilig and Egli, 2006), the need for new pharmacological treatment options is substantial. Intriguingly, the anorexigenic peptide glucagon-like peptide-1 (GLP-1) has recently been established as a modulator of alcohol reinforcement and has thus been suggested as a novel treatment for AUD (for review (Jerlhag, 2018).

In addition to treatment of diabetes type 2 (for review see (Holst, 2004)), the GLP-1 receptor (GLP-1R) agonist liraglutide is approved as anti-obesity medication in Europe as well as in the US (for review see (Srivastava and Apovian, 2018)), since GLP-1 mimetics reduce food consumption and body weight (Alhadeff et al., 2012; Dickson et al., 2012; Sisley et al., 2014). Various studies report that activation of GLP-1R by various agonists, such as exendin-4 (Ex4) and liraglutide, blocks the ability of acute alcohol to activate the mesolimbic dopamine system as measured by locomotor stimulation, accumbal dopamine release and conditioned place preference (CPP) in mice (Egecioglu et al., 2013c; Shirazi et al., 2013b; Vallof et al., 2016a). In addition, the GLP-1R agonists Ex4, liraglutide and AC3174 reduce alcohol-drinking behaviors in mice as well as rats (Egecioglu et al., 2013c; Shirazi et al., 2013b; Suchankova et al., 2015; Thomsen et al., 2017; Vallof et al., 2016a). Human studies further strengthen a link between GLP-1 and drug-related behaviors, as subjective experience in cocaine is associated with elevated GLP-1 levels (Bouhlal et al., 2017) and alcohol dependence, as well as intravenous-self administration of alcohol in social drinkers are associated to polymorphisms in the *GLP-1R* gene (Suchankova et al., 2015). Albeit a previous study, using a high dose of Ex4, has identified that activation of GLP-1R within the posterior ventral tegmental area (pVTA) reduces alcohol intake, the role of GLP-1R in brain circuitries linked to alcohol reinforcement remains largely unsolved.

The cholinergic projection from the laterodorsal tegmental area (LDTg) to the ventral tegmental area (VTA) together with the

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mesolimbic dopamine projection from the VTA to the nucleus accumbens (NAc) shell, as opposed to core (Bassareo et al., 2003; Cadoni et al., 2000), are crucially involved in the acute rewarding effects of alcohol (Larsson et al., 2005; Larsson and Engel, 2004; Soderpalm et al., 2009; Volkow and Li, 2004). In addition, a disruption within the projections from LDTg, to the VTA and thereafter to NAc shell could constitute one underlying factor of AUD pathophysiology (for review see (Larsson and Engel, 2004; Lodge and Grace, 2006). The findings that GLP-1-containing neurons of the nucleus of the solitary tract (NTS) project to the LDTg, the VTA as well as the NAc shell (Alhadeff et al., 2012; Reiner et al., 2018), which are nuclei known to express GLP-1R (Alvarez et al., 1996; Merchenthaler et al., 1999), further pinpoints these areas as converging the link between alcohol and GLP-1R activation. Providing that the VTA is a heterogeneous area responding differently to both GLP-1R activation and alcohol (Hernandez et al., 2018; Sanchez-Catalan et al., 2014; Schmidt et al., 2016; Shirazi et al., 2013a,b), the response of activation of GLP-1R within the anterior VTA (aVTA) and pVTA on behavioral responses to alcohol may diverge. Hence, this led to the hypothesis that activation of GLP-1R within NAc shell, aVTA, pVTA as well as LDTg regulates various alcohol-mediated behaviors in rodents. Initially, we evaluated the effects of infusion of Ex4 in low doses into the NAc shell, the aVTA, the pVTA or the LDTg, on the ability of acute alcohol to cause locomotor stimulation and induce memory retrieval of alcohol reward in the CPP paradigm in male mice, known to respond profoundly to alcohol in these models (Egecioglu et al., 2013c; Jerlhag et al., 2009; Vallof et al., 2016a, b). As studies reveal a more robust decline in alcohol intake in high compared to low alcohol-consuming rats following administration of GLP-1R agonists (Shirazi et al., 2013b; Vallof et al., 2016a), we evaluated the ability of Ex4 in low doses into the aforementioned areas to regulate alcohol intake in male rats consuming high and stable amounts of alcohol (Simms et al., 2008) for 12 weeks. We also explored the expression levels of *GLP-1R* gene in reward related areas such as prefrontal cortex, amygdala, hippocampus, VTA, NAc and striatum (for review see (Koob and Volkow, 2016)), in high versus low alcohol-consuming rats. Providing that GLP-1 is produced by posttranslational processing of the product from the preproglucagon gene (*GCG*), the expression of *GCG* in the aforementioned areas was evaluated in high versus low alcohol-consuming rats. While previous studies demonstrate that systemic administration of GLP-1R agonists attenuates the reinforcing properties of alcohol (for review see (Jerlhag, 2018)), the present experiments attempt to fill a knowledge gap by demonstrating the functional role of brain region specific GLP-1R that are crucial for acute and chronic alcohol-mediated behaviors in rodents.

2. Material and methods

2.1. Animals

Adult post-pubertal age-matched male NMRI mice (8–12 weeks old and 25–35 g body weight; Charles River, Susfeldt, Germany) were used since we previously have obtained robust locomotor stimulation and memory retrieval of alcohol reward in the CPP model in response to alcohol and other addictive drugs (Egecioglu et al., 2013a; Jerlhag et al., 2009; Vallof et al., 2016c). The mice were group housed and maintained on a 12/12-hour light/dark cycle in rooms at 20 °C with 50% humidity. The experiments in mice were conducted during the light phase since mice are less active during this period and therefore a more reliable alcohol effect is induced. Adult post-pubertal age-matched male outbred Rcc Han Wistar rats (Envigo, Horst, Netherlands) were used for the intermittent access 20% alcohol two-bottle-choice drinking paradigm, since they display a voluntary high and stable alcohol intake causing pharmacological relevant blood alcohol concentrations in this drinking model (Simms et al., 2008). The rats in the intermittent access paradigm were during the entire protocol, housed individually in high Macrolon III cages (Tecniplast, Italy) and maintained on a 12 h reversed light dark cycle (lights off at 8 a.m.) in rooms at 20 °C and 50% humidity. For all experiments tap water and food (normal chow; Harlan Teklad, Norfolk, England) was supplied *ad libitum*. Dose-response tests were carried out in male Wistar rats (Charles River, Germany) that were group housed in rooms at 20 °C with 50% humidity and maintained on a 12/12-hour light/dark cycle. Mice and rats were used herein since previous studies report a robust effect of alcohol in these models. Moreover, different strains of mice and rats show similar response to GLP-1R agonist treatment on alcohol-related behaviors (Egecioglu et al., 2013c; Shirazi et al., 2013b; Thomsen et al., 2017; Vallof et al., 2016a). Each experiment used an independent set of animals. All animals were allowed to acclimatize at least one week before the start of the experiments. The experiments were approved by the Swedish Ethical Committee on Animal Research in Gothenburg. All efforts were made to minimize animal suffering, and to reduce the number of animals used

2.2. Drugs

For studies investigating alcohol-induced activation of the mesolimbic dopamine system in mice, 96% alcohol (VWR International AB, Stockholm, Sweden) was diluted in saline (0.9% NaCl) to 15% v/v for intraperitoneal (ip) injections and was administered at a dose of 1.75 g/kg five minutes prior to initiation of the experiments. For the intermittent access alcohol two-bottle-choice drinking paradigm alcohol was diluted to a 20% vol/vol solution using tap water. Ex4, a selective GLP-1R agonist with similar binding to GLP-1 (Goke et al., 1995; Thorens et al., 1993), was used instead of other GLP-1R agonists, since it robustly blocks various alcohol- and drug-related behaviors in rodents (Egecioglu et al., 2013a, b; Egecioglu et al., 2013c; Erreger et al., 2012; Graham et al., 2013; Shirazi et al., 2013b; Sorensen et al., 2016, 2015; Thomsen et al., 2017). Ex4 (Tocris Bioscience, Bristol, England) was in each experiment diluted in Ringer solution (NaCl 140 mM, Ca Cl_2 1.2 mM, KCl 3.0 mM and $MgCl_2$ 1.0 mM; Merck KGaA, Darmstadt, Germany). Each infusion was conducted bilaterally at a volume of in 0.5 µl per side. In mice, Ex4 was infused at a dose of 0.0025 µg per side into the NAc shell, aVTA, pVTA or LDTg. In rats a dose of 0.05 µg per side were selected for NAc shell and the dose of 0.025 µg per side were selected for aVTA, for pVTA and for LDTg. These doses were selected since our dose response studies established that these doses neither effect locomotor activity nor visually alters gross behavior (Supplementary results 1) and that doses in a similar dose range locally infused to these brain areas have been used by others (Alhadeff et al., 2012; Hernandez et al., 2018, 2017; Mietlicki-Baase et al., 2013; Reiner et al., 2018; Schmidt et al., 2016). Ex4 was always administered ten minutes prior to the behavioral test or alcohol injection.

2.3. Guide implantation

The rodent was anesthetized with isofluran (Isofluran Baxter, Apoteket AB, Gothenburg, Sweden) using a pump (Univentor 400 Anaesthesia Unit, Univentor Ldt., Zejtun, Malta), placed in a stereotaxic frame (David Kopf Instruments; Tujunga, CA, USA) and kept on a heating pad to prevent hypothermia. Xylocain (10 mg/ml) adrenalin (5 µg/ml) (Pfizer Inc, Apoteket AB, Gothenburg, Sweden) was used as local anesthetics and carprofen (Rimadyl®, 5 mg/kg ip, Astra Zeneca; Gothenburg, Sweden) was used to relieve pain. The skull bone was exposed and holes for the guide and anchoring screw were drilled. In order to administer Ex4 or vehicle solution, guide (stainless steel, length 10 mm, with an o.d./i.d. of 0.6/0.45 mm) were implanted 1 mm below the surface of the brain and anchored to the screw and the skull bone with dental cement (DENTALON® plus; Agntho's AB, Lidingö, Sweden). The rodents were kept in individual cages for four days until the experiment. This procedure, as opposed to implantation of guide targeting the selected brain region directly, has through experience been shown to impact less brain structures and further minimally

influence the rodent's behavior.

In mice, the following coordinates were used: NAc shell, +1.4 mm and lateral ± 0.6 mm; aVTA, −3.4 mm and lateral ± 0.5 mm; pVTA, −3.6 and lateral ± 0.5 mm; LDTg -5.0 mm and lateral ± 0.5 mm (Franklin and Paxinos, 1996). At the time of the experiment, the cannula was extended another 3.7 mm, 3.3 mm, 3.2 or 2.2 mm ventrally beyond the tip of the guide aiming for drug administration into the NAc shell, aVTA, pVTA or LDTg (Franklin and Paxinos, 1996). The selected coordinates for the NAc shell, aVTA and LDTg in mice are identical to previous studies demonstrating a close link to alcohol-mediated behaviors and other appetite-regulatory peptides (Jerlhag et al., 2009; Larsson et al., 2004; Prieto-Garcia et al., 2015; Vallof et al., 2016a, c).

As dopamine signaling is not influenced by activation GLP-1R in NAc core (Cork et al., 2015; Mietlicki-Baase et al., 2014), an area expressing GLP-1R (Alhadeff et al., 2012; Merchenthaler et al., 1999) and not responding to acute alcohol with dopamine release (Bassareo et al., 2003; Cadoni et al., 2000), the effects of Ex4 into NAc core on alcohol related behaviors was not investigated.

In rats, the present coordinates were used: NAc shell, +1.85 mm and lateral ± 1.0 mm; aVTA, -5.3 mm and lateral ± 0.5 mm; pVTA, -6.8 mm and lateral ± 0.5 mm; LDTg -8.8 mm and lateral ± 1.0 for LDTg. At the time of the experiment, the cannula was extended another 6.8 mm, 7.3 mm, 7.6 mm or 6.0 mm ventrally beyond the tip of the guide aiming for drug administration in the NAc shell, aVTA, pVTA or LDTg in rats (Paxinos and Watson, 1998). The coordinates for NAc shell were selected since acute alcohol injection and alcohol intake in high alcohol-consuming rats increases dopamine release in this area (Larsson et al., 2005). LDTg coordinates in rats correspond to a part of the LDTg responsive to the orexigenic peptide ghrelin (Jerlhag et al., 2012). The division of the heterogeneous VTA into subregions, such as aVTA, pVTA, varies substantially. However, most studies identify -5.5 mm from bregma as the boundary between the anterior and posterior regions of the VTA in rats (for review see (Sanchez-Catalan et al., 2014)). Coordinates for aVTA were selected since infusion of a low dose of alcohol into this area causes a release of dopamine in NAc shell and nicotinic acetylcholine receptors within this part of the VTA modulate the ability of alcohol to activate the mesolimbic dopamine system (Ericson et al., 2008; Jerlhag and Engel, 2014). For the pVTA, the coordinates were selected to be similar to studies demonstrating that Ex4 into this part attenuates cocaine self-administration (Schmidt et al., 2016), cocaine seeking (Hernandez et al., 2018) and reduces palatable high-fat food intake (Mietlicki-Baase et al., 2013). It should however be mentioned that this part of the pVTA is often referred to as the "tail" of the pVTA (Sanchez-Catalan et al., 2014), and GLP-1R in other parts of the pVTA may regulate alcohol-mediated behaviors differently. In both VTA experiments, the medial part was targeted since a previous study reported that alcohol enhances the firing frequency of a subset of dopamine neurons in the medial, but not lateral part of the VTA (Mrejeru et al., 2015).

One hour before initiating the experiment, a dummy cannula was carefully inserted into the guide to remove clotted blood and to hamper spreading depression. At the proceeding drug challenge, the drug was administered over one minute at a volume of 0.5 µl and the cannula was left in place for another minute and was then retracted (5 µl Kloehn, microsyringe; Skandinaviska Genetec AB, V. Frölunda, Sweden). This infusion rate allows minimal diffusion of the drug into surrounding areas. The injection sites were verified following the termination of the experiment. The rodents were decapitated and the brains were mounted on a vibroslice device (752 M Vibroslice; Campden Instruments Ltd., Loughborough, UK). The brains were cut in 50 µm sections, and the location was determined (Franklin and Paxinos, 1996; Paxinos and Watson, 1998) by observation using light microscopy. Only animals with correct placements were included in the statistical analysis (Supplementary Fig. 1A-H).

2.4. Locomotor activity experiments

Locomotor activity was performed as previously described (Jerlhag et al., 2009). In brief, locomotor activity was registered in eight sound attenuated, ventilated and dim lit locomotor boxes (420 × 420 × 200 mm, Kungsbacka mät- och reglerteknik AB, Fjärås, Sweden). Five by five rows of photocell beams, at the floor level of the box, creating photocell detection allowed a computer-based system to register the activity of the mice. Locomotor activity was defined as the accumulated number of new photocell beams interrupted during a 60-minute period. In all experiments the rodents were allowed to habituate to the locomotor activity box one hour prior to drug challenge.

The first series of experiment in mice and rats were designed to select a dose of Ex4, without any effect *per se,* following local and bilateral infusion into the NAc shell, the aVTA or the LDTg. The mice, vehicle (Ringer) or Ex4 (0.00025, 0.0025 or 0.005 µg per side) was infused bilaterally into the i) NAc shell, ii) aVTA or iii) LDTg. The rats were infused with either vehicle (Ringer) or Ex4 (0.0025, 0.025 or 0.05 µg) bilaterally into the i) NAc shell, ii) aVTA or iii) LDTg.

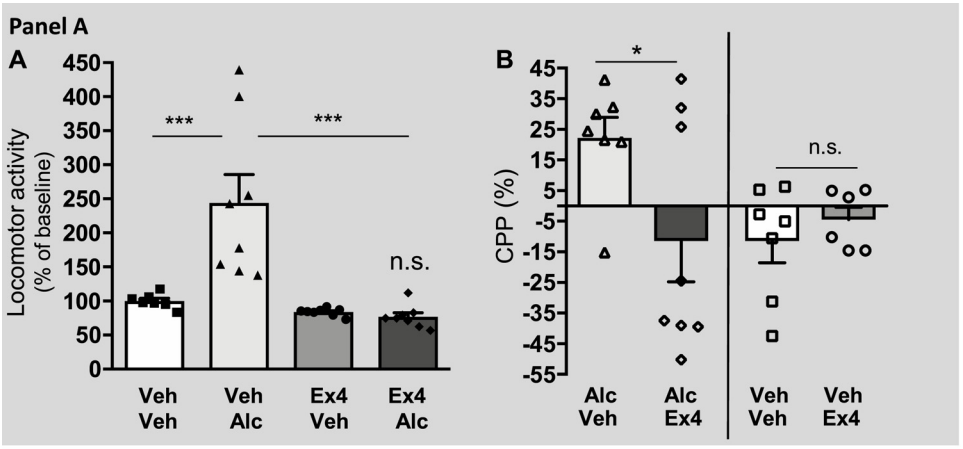
Thereafter, the role of GLP-1R activation in the aforementioned areas for alcohol-induced locomotor stimulation was evaluated in mice. Therefore, the effects of bilateral and local infusion of Ex4 (0.0025 µg per side), a dose with no effect *per se*, or an equal volume of vehicle (Ringer) into the i) NAc shell, ii) aVTA, iii) pVTA or iv) LDTg on alcohol-induced (1.75 g/kg, ip) locomotor stimulation were investigated. Each mouse received one treatment combination (Veh-Veh, Veh-Alc, Ex4-Veh or Ex4-Alc) and was only subjected to one experimental trial.

2.5. Conditioned place preference

Three distinct and separate CPP experiments were conducted to evaluate the role of GLP-1R in the NAc shell, aVTA, pVTA and LDTg for the evaluation memory retrieval of alcohol reward in the CPP paradigm.

The CPP test was performed in mice as previously described (Jerlhag et al., 2009) and the procedure was conducted similarly in all the experiments. In brief, a two-chambered CPP apparatus (45 lx) and distinct visual and tactile cues was used. Each CPP test consisted of pre-conditioning (day 1), conditioning (day 2–5), and post-conditioning (day 6). At pre-conditioning, the mouse was placed in the middle of the CPP chamber with free access to both compartments during 20 min, allowing determination of the initial compartment preference. Conditioning (20 min per session) to alcohol was conducted for four subsequent days using a biased procedure. During conditioning, the two CPP compartments were separated with a divider, allowing conditioning to one specific compartment. Each of these conditioning days, alcohol (1.75 g/kg, ip) was paired with the least preferred compartment and vehicle with the preferred compartment. The mouse then received one alcohol and one vehicle injection every day and the injections were altered between morning and afternoon in a balanced design. At the post-conditioning, the mouse was either infused with Ex4 (0.0025 µg per side) or an equal volume of vehicle (Ringer) locally and bilaterally into the i) NAc shell, ii) aVTA, iii) pVTA or iv) LDTg ten minutes prior to exposure to the CPP box. The mouse was then placed on the midline between the two compartments allowing 20 min of free access to both compartments. This CPP design permits investigations for the retrieval of alcohol reward memory and creates the following treatment groups for each of the three experiments; Alc-Veh and Alc-Ex4. Investigation of alcohol-reward, by conditioning with local infusion of Ex4 prior to alcohol on each conditioning day, would be an interesting study; however, such experiment cannot be conducted since the mice behavior is influenced negatively following two local infusions on each conditioning day for four days. As the aim of these CPP test is to investigate the effect of Ex4 on alcohol-induced CPP, the CPP response is only compared between alcohol-vehicle and alchol-Ex4 treated mice.

In addition, three separate control experiments were conducted to



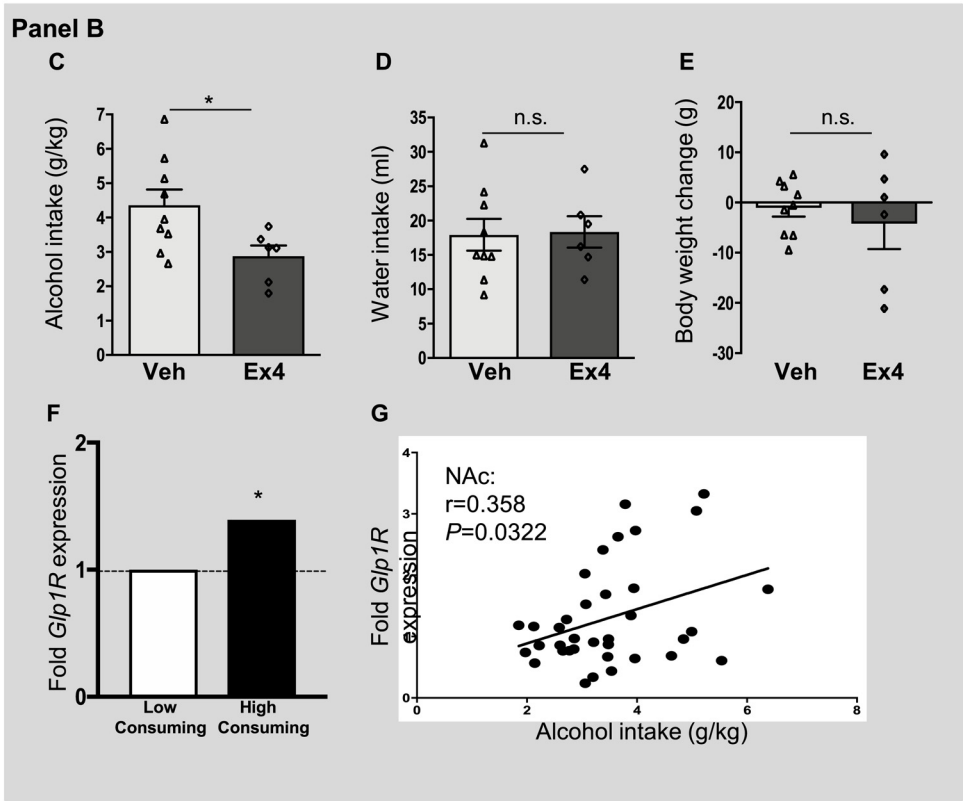


Fig. 1. Effects of microinjections of Ex4 into nucleus accumbens shell on alcohol-related behaviors in rodents and effects of high alcohol consumption on the expression levels of *Glp1R* in rats.

Panel A reveals effects of exendin-4 (Ex4) into nucleus accumbens (NAc) shell on the acute effects of alcohol in male mice. (A) Alcohol (Alc, 1.75 g/kg, ip) caused a locomotor stimulation compared to vehicle (Veh) in mice. This stimulation was blocked by a microinjection of Ex4 into NAc shell (0.0025 µg per side), at a dose with no effect *per se*. There was no difference in response between vehicle treated mice and those treated with Ex4 in combination with alcohol. (***$P < 0.001$, $P > 0.05$ n.s.= non-significant, one-way ANOVA followed by a Bonferroni post-hoc test). (B) NAc-Ex4 (0.0025 µg per side) blocked the alcohol-induced (1.75 g/kg, ip) memory of alcohol reward in the conditioned place preference (CPP) test in mice (*$P < 0.05$, unpaired *t*-test). However, there was no difference in CPP between Ex4 and vehicle treated control mice. Panel B shows GLP-1R related effects in NAc shell in male rats consuming high amounts of alcohol for 12 weeks. (C) In rats, Ex4 into NAc shell (0.05 µg per side) reduced the 24-hour alcohol intake, but did not affect (D) water intake or (E) body weight change. (*$P < 0.05$, $P > 0.05$ n.s.=non significant, unpaired *t*-test). These data are presented as mean ± SEM. (F) Expression of the *Glp1R* in NAc is increased in high compared to low alcohol-consuming rats (values are represented as $2^{-\Delta\Delta CT}$, *$P < 0.05$, unpaired *t*-test of ΔC_T values). (G) Expression of *Glp1R* in the NAc is positively correlated to alcohol intake (values are represented as $2^{-\Delta\Delta CT}$, Pearson correlation test).

evaluate the effect *per se* of Ex4 into the aforementioned areas on CPP. Therefore, the CPP response is compared between vehicle-vehicle and vehicle-Ex4 mice. These mice were subjected to the same procedure as described above (i.e. one pre-conditioning day, four conditioning days and one post-conditioning day), but received vehicle injections instead of alcohol throughout the four conditioning days. At post-conditioning the mice were injected locally and bilaterally with Ex4 (0.0025 µg per side) or an equal volume of vehicle (Ringer) into the i) NAc shell, ii) aVTA or iii) LDTg. Ten minutes later the mice were placed on the midline between the two compartments with free access for 20 min, creating the following two treatment groups Veh-Veh and Veh-Ex4 (for each and one of the three experiments).

The expression of CPP was calculated as the difference in percent of total time spent in the drug-paired (i.e., less preferred) compartment during the post-conditioning and the pre-conditioning session.

2.6. Intermittent access 20% alcohol two-bottle-choice drinking paradigm

This paradigm was used as it induces voluntary intake of high amounts of alcohol with pharmacological relevant blood alcohol concentrations (Simms et al., 2008). In brief, the rats were given free access to one bottle of 20% alcohol and one bottle of water during three 24-hour-sessions per week (Mondays, Wednesdays and Fridays) in a reversed light/dark cycle room, where the bottles were introduced right before the dark cycle onset (Simms et al., 2008). The rats had unlimited access to two bottles of water between the alcohol-access-periods.

2.6.1. The role of GLP-1R activation in the NAc shell, aVTA, pVTA or LDTg for alcohol intake in outbred rats

Previously it was established that the reduction in alcohol consumption by systemic Ex4 was more evident in high compared to low alcohol-consuming rats (Shirazi et al., 2013b; Vallof et al., 2016a). Therefore, high alcohol-consuming rats subjected to 12 weeks of intermittent access to alcohol were therefore treated locally and

bilaterally with Ex4 into the i) NAc shell (0.05 µg per side), ii) aVTA (0.025 µg per side) or iii), pVTA (0.025 µg per side) or iv) LDTg (0.025 µg per side) or vehicle (Ringer). In addition, the effect of Ex4 into NAc shell or LDTg on alcohol intake was evaluated in low alcohol-consuming rats. No low alcohol-consuming rats were observed in the groups later subjected to Ex4 into the aVTA or pVTA. Since the focus of the present studies was high rather than low alcohol-consuming rats, additional tests in low alcohol-consuming rats were not included. This also contributes to the reduction of the number of rats used. For NAc shell and LDTg, rats were infused locally just once. For the aVTA and pVTA, there were two infusions, with one day break between each administration, thus each animal served as its own control.

In all these experiments the drug was administered 10 min prior to the onset of dark cycle/bottle presentation, since rats display the highest intake during the first period of the dark cycle. In each test, the effect on alcohol, water, total fluid and preference for alcohol over water (the ratio of alcohol to total fluid intake) as well as food intake as and body weight change was registered 24 h after bottle presentation, as previous studies have established a robust reduction on the 24 h alcohol intake following systemic administration of GLP-1R agonists (Egecioglu et al., 2013c; Shirazi et al., 2013b; Vallof et al., 2016a).

2.6.2. Glp1R and GCG expression in high and low alcohol-consuming rats

Following 12 weeks of intermittent access to alcohol, rats from a separate intermittent access experiment were decapitated and the brains were removed and immediately placed on a cold glass plate. The following areas were then rapidly dissected: NAc, VTA, amygdala, hippocampus, prefrontal cortex and striatum. Only the entire NAc and VTA, rather then shell/core and aVTA/pVTA parts, can be obtained from the present dissections. Directly after each dissection, the brain areas were transferred into a plastic tube and stored in −80 °C until further analysis conducted by TATAA Biocenter AB (Gothenburg, Sweden). For RNA tissue preparation, the brain samples were homogenized by adding a TissueLyser II (Qiagen). The total RNA was extracted using the RNeasy Lipid Tissue Mini kit (Qiagen) and samples were loaded on the QIAcube™ (Qiagen) for automated RNA extraction, following the manufacturer's protocols. The quantity and concentration of RNA were assessed in 1 µl of the final RNA solution, using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). RNA concentration was calculated equally for all samples at 1000 ng per sample. After dilution with Milli-Q™(Millipore Corporation, Billerica, MA, USA) water, the samples were loaded in duplicates in a 96 well plate (Sarstedt AG & Co, Nümbrecht, Germany) and were prepared for a 20 µl reverse transcription reaction into cDNA using the QuantiTect Reverse Transcription kit (Qiagen) as per manufacturer's instructions. The quantitative real-time PCR (qRT-PCR) analysis was performed in the facilities of TATAA Biocenter AB Gothenburg, Sweden. Briefly, the samples were corrected for gDNA contamination using the ValidPrime™ (TATAA Biocenter AB) technology and gene expression analysis was performed using the qRT-PCR instrument IntelliQube™ (Douglas Scientific, Alexandria, MN, USA). For the analysis, the selected reference genes (RG) were *HMBS* and *YWHAZ* and the primary gene of interest (GOI) were *Glp1R* (TaqMan™ assay ID Rn00562406_m1) and *GCG* (TaqMan™ assay ID Rn00562293_m1). The latter was selected as an indicator of GLP-1 expression. The corrected C_T values raw data were analyzed using the comparative C_T method as previously described (Livak and Schmittgen, 2001). The low alcohol-consuming rats were set as the internal calibrator. In brief, the individual ΔC_T values were calculated as: $C_{T\ (GOI)} - C_{T\ (average\ of\ RG)}$. The $\Delta\Delta C_T$ values were calculated as the average ΔC_T of the internal calibrator (low alcohol-consuming rats) subtracted from the individual ΔC_T of each sample. The data is represented as fold change in the form of $2^{-\Delta\Delta CT}$.

2.7. Statistical analysis

The locomotor activity experiments were evaluated by a one-way ANOVA followed by Bonferroni post-hoc test for comparisons between different treatments. The data from the CPP, intermittent access 20% alcohol two-bottle-choice drinking paradigm for NAc shell and LDTg were evaluated by an unpaired *t*-test. Data for Ex4 treatment into anterior and posterior VTA in the intermittent alcohol access model was evaluated by a paired *t*-test. For the gene expression data, an unpaired *t*-test was performed to compare the ΔC_T values between the high and low alcohol-consuming rat groups. The Pearson correlation test was performed to analyze the correlation between fold change (individual $2^{-\Delta\Delta CT}$ values) of gene expression and the mean values of alcohol intake in rats.

3. Results

3.1. Effects of infusion of Ex4 into NAc shell on the acute effects of alcohol in mice and on alcohol intake in rats, as well as NAc-Glp1R expression in high compared to low alcohol-consuming rats

An overall main effect of treatment was found on locomotor activity in mice following infusion of Ex4 into NAc shell and systemic alcohol administration ($F(3,27) = 13.57$, $P < 0.0001$; n = 7 in the Veh-Veh group and n = 8 in each of the following groups; Veh-Alc, Ex4-Veh and Ex4-Alc). As shown in Fig. 1A, post-hoc analysis revealed that alcohol significantly increased locomotor activity compared to vehicle ($P < 0.001$). This alcohol-induced locomotor stimulation was significantly blocked by pre-treatment with bilateral Ex4 into NAc shell ($P < 0.0001$). There was no difference in locomotor activity in vehicle treated mice and Ex4-alcohol treated mice ($P > 0.05$). The selected dose of Ex4 had no effect *per se* on locomotor activity compared to vehicle treatment ($P > 0.05$).

The memory of alcohol reward (n = 7) in the CPP paradigm was attenuated by an infusion of Ex4 (n = 8) into NAc shell in mice ($t(13) = 2.15$, $P = 0.0257$, Fig. 1B). Fig. 1B further reveals no differences in CPP response in control mice treated with vehicle (n = 7) or Ex4 (n = 6) into NAc shell ($t(11) = 0.47$, $P = 0.3224$).

Rats were divided into high and low alcohol-consuming rats from the present intermittent access paradigm. All the rats included in the analysis were infused locally into the NAc shell only once, as observation and analysis of the second infusion revealed that rats were more affected on the second infusion day and therefore did not display reliable data.

In the rats consuming higher amounts of alcohol during baseline, there was no difference ($t(13) = 0.29$, $P = 0.7787$) in the 12-weeks of baseline alcohol intake (g/kg/24hrs) in rats later treated with vehicle (3.9 ± 0.3, n = 9) or Ex4 (3.8 ± 0.3, n = 6). In comparison to vehicle, Ex4 into NAc shell decreased alcohol intake ($t(13) = 2.10$, $P = 0.0319$, Fig. 1C) in these high alcohol-consuming rats. There were no differences in water intake ($t(13) = 0.12$, $P = 0.9043$, Fig. 1D), alcohol preference ($t(13) = 1.17$, $P = 0.2615$, Supplementary Fig. 2 Fig. 2A), total fluid intake ($t(13) = 1.00$, $P = 0.3328$, Supplementary Fig. 2B), food intake ($t(13) = 0.79$, $P = 0.4438$, Supplementary Fig. 2 Fig. 2C) or body weight change ($t(13) = 0.70$, $P = 0.4969$, Fig. 1E), between Ex4 and vehicle treatment.

In the rats consuming low amounts of alcohol during baseline, there was no difference ($t(5) = 0.76$, $P = 0.4836$) in the 12-weeks of baseline alcohol intake (g/kg/24hrs) in animals later treated with vehicle (1.9 ± 0.4, n = 3) or Ex4 (1.6 ± 0.4, n = 4). In comparison to vehicle, Ex4 into NAc shell did not alter alcohol intake (P t $(5) = 0.20$, = 0.8518, Supplementary Fig. 2D) in these low alcohol-consuming rats. There were no differences in water intake ($t(5) = 0.83$, $P = 0.4440$, Supplementary Fig. 2E), alcohol preference ($t(5) = 0.28$, $P = 0.7910$, Supplementary Fig. 2F), total fluid intake ($t(5) = 0.73$, $P = 0.4985$, Supplementary Fig. 2G), food intake ($t(5) = 0.07$,

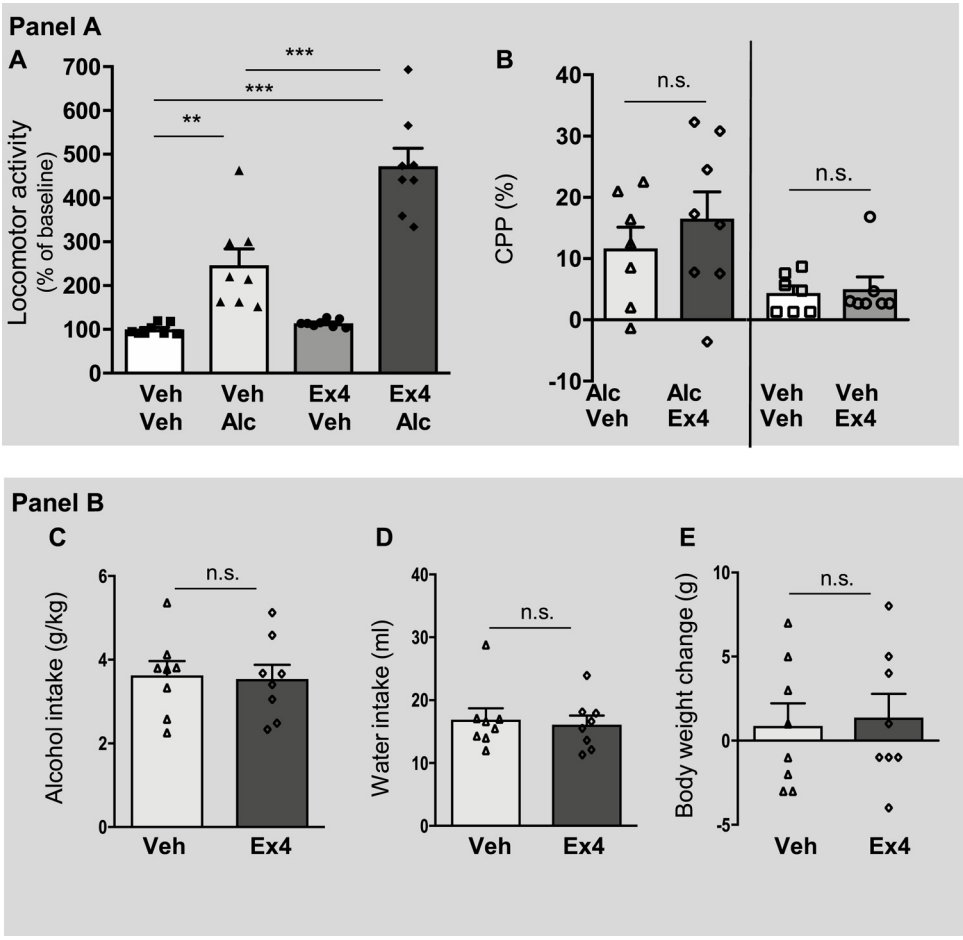


Fig. 2. Effects of Ex4 into the anterior ventral tegmental area on alcohol-mediated behaviors in rodents.
Panel A demonstrates the acute effects of alcohol in male mice. (A) Alcohol-induced (1.75 g/kg, ip) locomotor stimulation was not attenuated by exendin-4 (Ex4) (0.0025 µg per side) into the anterior ventral tegmental area (aVTA), at a dose with no effect *per se* (**$P < 0.01$, ***$P < 0.05$, one-way ANOVA followed by a Bonferroni post-hoc test). (B) There was no difference in conditioned place preference (CPP) response to alcohol (1.75 g/kg, ip) between mice infused with vehicle or Ex4 (0.0025 µg per side) into aVTA. In addition, there was no difference in CPP between Ex4 and vehicle infused control mice. ($P > 0.05$ n.s. = non significant, unpaired *t*-test). Panel B displays consumption data from high-alcohol consuming male rats previously consuming alcohol for 12 weeks. (C) Alcohol intake, (D) water intake and (E) body weight change was not altered by Ex4 (0.025 µg per side) into the aVTA in these high alcohol-consuming rats. ($P > 0.05$ n.s. = non significant, paired *t*-test).

$P = 0.9438$, Supplementary Fig. 2H) or body weight change (t(5) = 0.12, $P = 0.9058$, Supplementary Fig. 2I) between Ex4 and vehicle treatment.

Another set of rats that had voluntarily consumed alcohol for 12 weeks was divided in low- and high-consumers based on their level of previous alcohol consumption (cut-off > 3.5 g/kg per 24 h). Thereafter, the effects of long-term alcohol consumption on the expression of *Glp1R* in NAc were evaluated. In the NAc, a significant effect of alcohol consumption was noted, with elevated *Glp1R* expression in the high compared to the low consuming rats (low consumers: 1.0 ± 0.1 n = 20; high consumers: 1.7 ± 0.3, n = 16; t(34) = 2.61, $P = 0.0134$, Fig. 1E). Separate analysis revealed a significant positive correlation between *Glp1R* expression in the NAc, and alcohol intake (g/kg/24 h) (r = 0.358; n = 36; $P = 0.0322$, Fig. 1F). Table 1 shows that there was no significant differences on *Glp1R* expression in the prefrontal cortex, VTA, amygdala, hippocampus or striatum.

3.2. Effects of Ex4 infusion into the aVTA on the acute effects of alcohol in mice as well as on alcohol consumption in rats

An overall effect of treatment was found on locomotor activity in mice following systemic administration of alcohol and Ex4 into the aVTA (F(3,28) = 39.37 $P < 0.0001$; n = 8 in each group, Fig. 2A). Post-hoc analysis revealed that alcohol significantly increased the locomotor activity in vehicle ($P < 0.01$) as well as Ex4 ($P < 0.0001$) treated mice. The alcohol response was enhanced in Ex4 mice in comparison to

Table 1
Glp1R and *GCG* expression in reward related areas of low and high alcohol-consuming rats after twelve weeks of voluntary alcohol consumption (cut of 3.5 g/kg/24 h).

Brain region	Low alcohol- consuming rats	High alcohol- consuming rats	t	df	*P*-value[1]
Glp1R					
Prefrontal cortex	1.2 ± 0.2; n = 13	1.1 ± 0.2; n = 9	0.185	20	0.855
Ventral tegmental area	1.1 ± 0.1; n = 25	1.1 ± 0.1; n = 21	0.058	44	0.954
Amygdala	1.1 ± 0.1; n = 25	1.1 ± 0.1; n = 18	0.015	41	0.988
Hippocampus	1.0 ± 0.1; n = 18	1.0 ± 0.1; n = 18	0.259	34	0.797
Striatum	1.1 ± 0.1; n = 26	1.1 ± 0.0; n = 21	0.513	45	0.611
GCG					
Nucleus accumbens	1.1 ± 0.3; n = 16	1.8 ± 0.5; n = 14	1.145	28	0.262
Ventral tegmental area	1.1 ± 0.3 n = 14	0.6 ± 0.1; n = 10	1.601	22	0.124
Amygdala	1.1 ± 0.1; n = 27	1.4 ± 0.2; n = 19	1.366	44	0.179
Hippocampus	0.7 ± 0.2; n = 10	2.4 ± 0.9; n = 9	2.002	17	0.062
Striatum	1.4 ± 0.2; n = 20	1.8 ± 0.6; n = 13	0.776	31	0.444

Data are represented as ΔC_T values. [1]unpaired *t*-test.

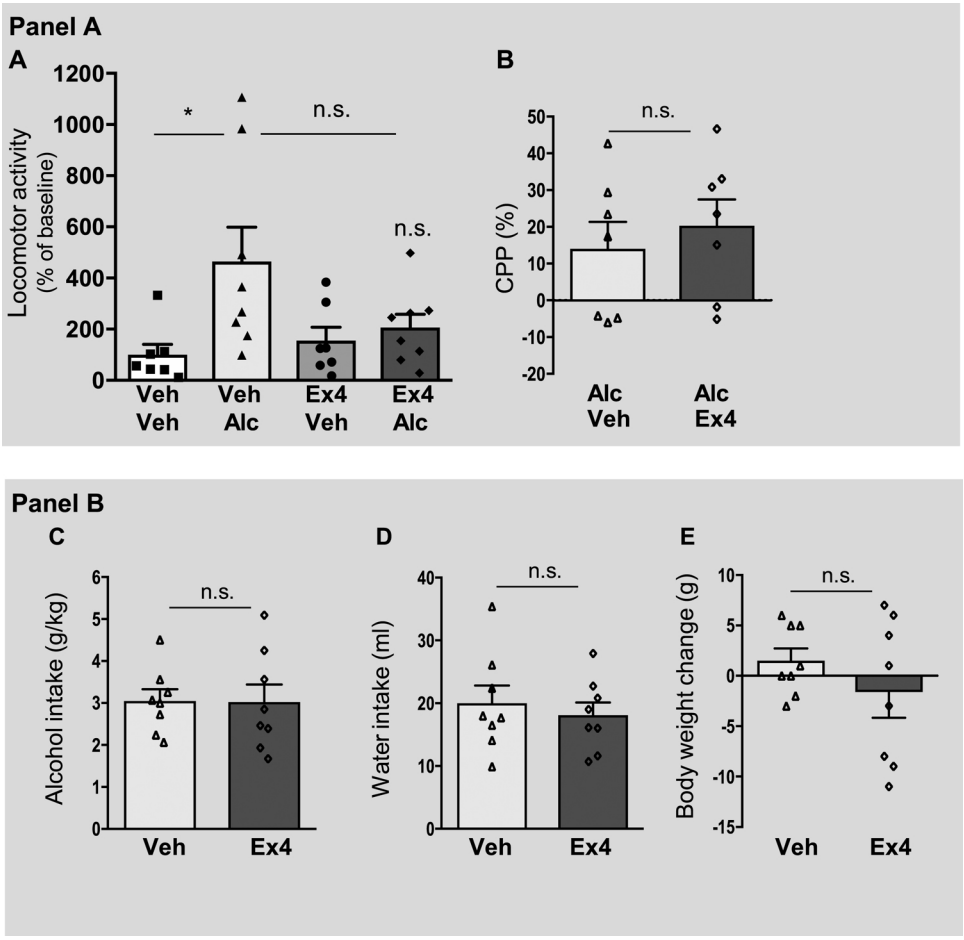


Fig. 3. Effects of Ex4 into the posterior ventral tegmental area on alcohol-mediated behaviors in rodents.
Panel A shows the acute effects of alcohol in male mice. (A) Alcohol-induced (1.75 g/kg, ip) locomotor stimulation was reduced by exendin-4 (Ex4) (0.0025 µg per side) into posterior ventral tegmental area (pVTA). Albeit the locomotor response was lower in Ex4 compared to vehicle treated mice, there was no significant difference between the groups. The selected dose of Ex4 into pVTA had no effect *per se* on locomotor activity. (*$P < 0.05$, $P > 0.05$ n.s. = non significant, one-way ANOVA followed by a Bonferroni post-hoc test). (B) There was no difference in conditioned place preference (CPP) to alcohol (1.75 g/kg, ip) between mice treated with Ex4 (0.0025 µg per side) or vehicle into the pVTA ($P > 0.05$ n.s. = non significant, unpaired *t*-test). Panel B demonstrates consumption data from male rats in the intermittent access model (C) Alcohol intake, (D) water intake or (E) body weight change was not affected by Ex4 (0.025 µg per side) into the pVTA ($P > 0.05$ n.s. = non significant paired *t*-test) in high alcohol-consuming rats. Data are presented as mean ± SEM.

the alcohol-induced locomotor stimulation in vehicle treated mice ($P < 0.0001$). The selected dose of Ex4 had no effect *per se* on locomotor activity compared to vehicle treatment ($P > 0.05$).

The memory of alcohol reward in the CPP test was not different in mice infused with vehicle (n = 7) or Ex4 (n = 8) into the aVTA (t(13) = 0.85, $P = 0.4111$, Fig. 2B). Control experiments showed that intra-aVTA Ex4 did not induce a CPP *per se* compared to vehicle (t(12) = 0.28, $P = 0.7836$, Fig. 2B).

There were only high alcohol-consuming rats in the present intermittent access test. These rats served as their own control *i.e.* received Ex4 or vehicle day one and the opposite treatment day two and these two infusions did not affect their behavior or consumption pattern.

Following 12 weeks of intermittent alcohol intake, the 24-hour average baseline alcohol consumption of the rats was 3.8 ± 0.2 g/kg (n = 8). Ex4 into aVTA had no effect on alcohol intake (t(7) = 0.36, $P = 0.7324$, Fig. 2C) compared to vehicle treatment. There were no differences in water intake (t(7) = 0.42, $P = 0.6892$, Fig. 2D), alcohol preference (t(7) = 0.13, $P = 0.9023$, Supplementary Fig. 3A), total fluid intake (t(7) = 0.53, P = 0.6088, Supplementary Fig. 3B) or food intake (t(7) = 2.12, P = 0.0722, Supplementary Fig. 3C). No effect on body weight change (t(7) = 0.21, $P = 0.8383$, Fig. 2E) was found following Ex4 compared to vehicle infusion.

3.3. Effects of Ex4 infusion into the pVTA on the acute effects of alcohol in mice as well as on alcohol consumption in rats

In mice, an overall main effect of treatment on locomotor activity was found following systemic administration of alcohol and Ex4 into pVTA (F(3,26) = 3.87, $P = 0.0206$; n = 7 in the Veh-Veh and Ex4-Veh groups and n = 8 in the Veh-Alc and Ex4-Alc groups). As shown in Fig. 3A, post-hoc analysis revealed that alcohol significantly increased locomotor activity compared to vehicle ($P < 0.05$). In comparison to vehicle pre-treatment, alcohol did not increase the locomotor activity in mice pre-treated with Ex4 into pVTA ($P > 0.05$). Albeit the alcohol response was lower in Ex4 compared to vehicle treated mice, there was no significant difference in locomotor activity between the groups ($P > 0.05$). The selected dose of Ex4 had no effect *per se* on locomotor activity compared to vehicle treatment ($P > 0.05$).

The CPP response was not difference in mice infused with vehicle (n = 7) or Ex4 (n = 7) into the pVTA (t(12) = 0.61, $P = 0.5503$, Fig. 3B). As the locomotor activity experiment revealed that Ex4 into any of the studied areas had an effect *per se* and that Ex4 did not affect the CPP *per se*, vehicle control experiments for pVTA were not conducted, aiming at reducing the number of mice used.

There were only high alcohol-consuming rats in the present intermittent access test. These rats served as their own control, *i.e.* received Ex4 or vehicle day one and the opposite treatment day two and these two infusions did not affect their behavior or consumption pattern.

Following 12 weeks of intermittent alcohol intake, the 24-hour average baseline alcohol consumption of the rats was 3.8 ± 0.2 g/kg (n = 8). Ex4 infusion into pVTA had no effect on alcohol intake (t(7) = 0.10, $P = 0.9219$, Fig. 3C) nor on water intake (t(7) = 1.08, $P = 0.3167$, Fig. 3D) compared to vehicle treatment. There were no differences in alcohol preference (t(7) = 0.05, $P = 0.9644$, Supplementary Fig. 4A), total fluid intake (t(7) = 0.92, P = 0.3889, Supplementary Fig. 4B) or food intake (t(7) = 1.82, P = 0.1112, Supplementary Fig. 4C) between vehicle and Ex4 treated rats. There were no differences in body weight change (t(7) = 1.06, $P = 0.3235$, Fig. 3E) between Ex4 and vehicle treatment rats.

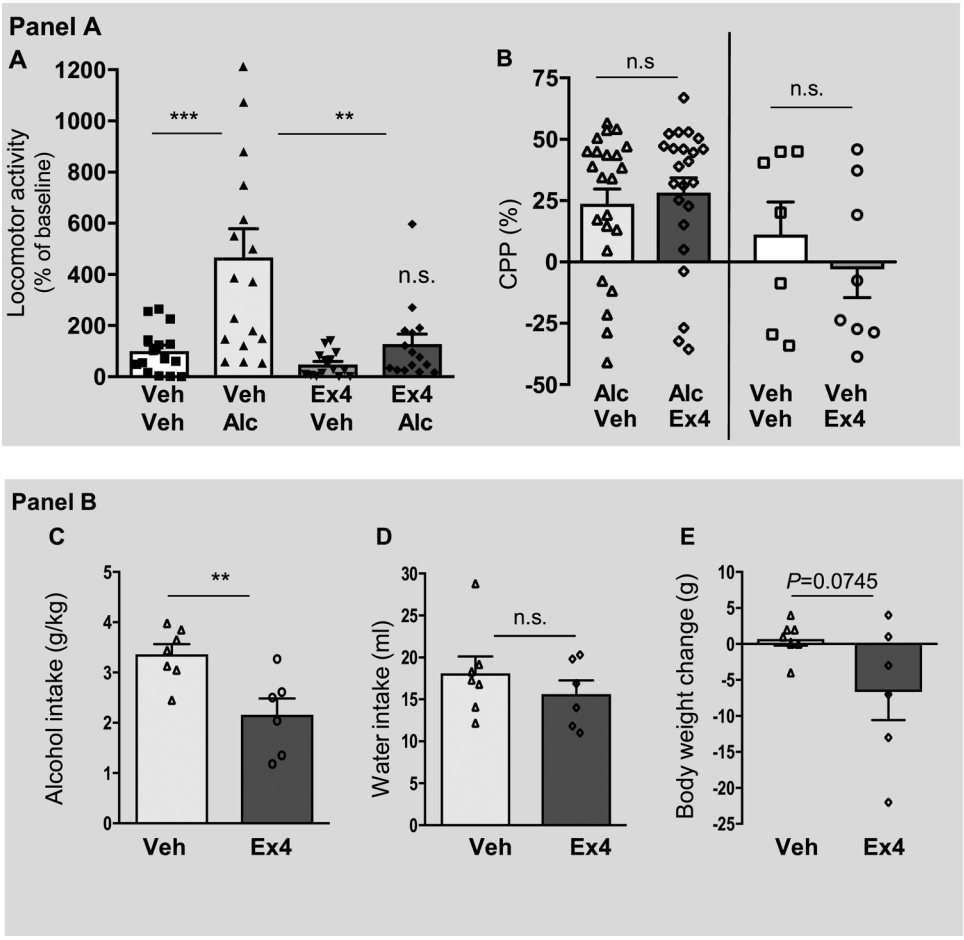


Fig. 4. Alcohol-mediated behaviors following administration into Ex4 into laterodorsal tegmental area in rodents.
Panel A displays the acute effects of alcohol in male mice. (A) Exendin-4 (Ex4; 0.0025 µg per side), at a dose with no effect *per se*, into the laterodorsal tegmental area (LDTg) blocked the alcohol-induced (1.75 g/kg, ip) locomotor stimulation (**$P < 0.01$, ***$P < 0.001$, $P > 0.05$ n.s. = non significant, one-way ANOVA followed by a Bonferroni post-hoc test). (B) LDTg-Ex4 (0.0025 µg per side) did not affect the alcohol-(1.75 g/kg, ip) induced conditioned place preference (CPP). Neither, was there a CPP difference between Ex4 and vehicle infused control mice. ($P > 0.05$ n.s. = non significant, unpaired *t*-test). Panel B reveals data from male rats consuming high amounts of alcohol. (C) Ex4 (0.025 µg per side) into the LDTg reduced alcohol intake, (D) but did not alter water intake. (E) There was a tendency in increased body weight change in Ex4 compared to vehicle treated rats (*$P < 0.05$, $P > 0.05$ n.s. = non significant, unpaired *t*-test). Data are presented as mean ± SEM.

3.4. Effects of intra-LDTg infusion of Ex4 on the acute effects of alcohol in mice and on alcohol intake in rats

An overall main effect of treatment was found on locomotor activity in mice following systemic administration of alcohol and infusion of Ex4 into the LDTg ($F(3,27) = 8.87$, $P < 0.0001$; n = 15 in the Veh-Veh, Ex4-Veh and Ex4-Alc groups and n = 17 in the Veh-Alc group). As shown in Fig. 4A, post-hoc analysis revealed that alcohol significantly increased locomotor activity compared to vehicle ($P < 0.0001$). This alcohol-induced locomotor stimulation was significantly blocked by pre-treatment with a LDTg infusion of Ex4 ($P < 0.001$). There was no difference in locomotor activity response in vehicle and Ex4-alcohol treated mice ($P > 0.05$). The selected dose of Ex4 had no effect *per se* on locomotor activity compared to vehicle ($P > 0.05$).

In the CPP test the memory of alcohol reward observed in vehicle treated mice (n = 23) was not blocked by infusion of Ex4 (n = 23) into the LDTg ($t(44) = 0.54$, $P = 0.5914$, Fig. 4B). There were no differences in the CPP response between intra-LDTg Ex4 (n = 8) and vehicle (n = 7) infused mice, when conditioned with vehicle ($t(13) = 0.81$, $P = 0.4329$; Fig. 4B).

Rats from the present intermittent access paradigm, were divided into high and low alcohol-consuming. All the rats included in the analysis were infused locally into the LDTg only once, as observation and analysis of day two infusion revealed that rats were more affected the second day and therefore did not display reliable data.

In the high alcohol-consuming rats during baseline drinking, there was no difference ($t(11) = 0.19$, $P = 0.8520$) in the 12-weeks of baseline alcohol intake (g/kg/24hrs) in alcohol consuming rats later treated with vehicle (3.2 ± 0.2, n = 7) or Ex4 (3.1 ± 0.3, n = 6). In these high alcohol-consuming rats, Ex4 infusion into the LDTg decreased alcohol intake ($t(11) = 3.26$, $P = 0.0076$, Fig. 4C) compared to vehicle. There were no differences in water intake ($t(11) = 0.93$, $P = 0.3709$, Fig. 4D), alcohol preference ($t(11) = 1.42$, $P = 0.1847$, Supplementary figure 5A) between the two treatment groups. Compared to vehicle, Ex4 reduced the total fluid intake ($t(11) = 2.24$, $P = 0.0467$, Supplementary figure 5B), as well as the food intake ($t(11) = 3.21$, $P = 0.0083$, Supplementary figure 5C). There was a tendency in increased body weight change by Ex4 ($t(11) = 1.97$, $P = 0.0745$, Fig. 4E) in the high alcohol-consuming rats.

In low alcohol-consuming rats, there was no difference ($t(13) = 0.04$, $P = 0.9713$) in the 12-weeks of baseline alcohol intake (g/kg/24hrs) in animals later treated with vehicle (1.5 ± 0.2, n = 8) or Ex4 (1.5 ± 0.2, n = 7). In low alcohol-consuming rats, Ex4 infusion into the LDTg did not affect alcohol intake ($t(13) = 0.53$, $P = 0.5270$, Supplementary figure 5D) compared to vehicle. There were no differences in water intake ($t(13) = 0.87$, $P = 0.4010$, Supplementary figure 5E), alcohol preference ($t(13) = 0.24$, $P = 0.8128$, Supplementary figure 5F), total fluid intake ($t(13) = 0.98$, $P = 0.3436$, Supplementary figure 5G) or food intake ($t(13) = 0.16$, $P = 0.8728$, Supplementary figure 5H) between the two treatment groups. Ex4 did not alter body weight change by Ex4 ($t(13) = 1.29$, $P = 0.2192$, Supplementary figure 5I) compared to vehicle.

3.5. Expression levels of prefrontal cortex-GCG in high compared to low alcohol-consuming rats

For expression in the prefrontal cortex, there was a significant effect of alcohol consumption with increased *GCG* expression in the high- compared to the low-consuming rats (low consumers: 1.1 ± 0.3 n = 23; high consumers: 2.0 ± 0.4, n = 21; $t(42) = 2.09$, $P = 0.0425$,

Supplementary Fig. 6A). A significant positive correlation was also found between *GCG* expression, in the prefrontal cortex, and alcohol intake (g/kg/24 h) ($r = 0.362$; $n = 44$; $P = 0.0157$, Supplementary Fig. 6B). Table 1 shows no significant differences on *GCG* expression in the NAc, VTA, amygdala, hippocampus or striatum.

4. Discussion

The present data do not only demonstrate that activation of GLP-1R attenuates alcohol-mediated behaviors in rodents, but they also provide an additional insight into the modulatory role of different brain region GLP-1R on such behaviors.

Here, accumbal GLP-1R were pinpointed as regulators of alcohol-mediated behaviors as we demonstrate that infusion of Ex4 into NAc shell inhibits the ability of acute alcohol injection to cause locomotor stimulation and attenuates reward-dependent memory retrieval in the CPP paradigm in mice. Additionally, accumbal GLP-1 signaling modulates the long-term effects of alcohol, as shown by the fact that intra-NAc shell administration of Ex4 reduces alcohol intake in rats consuming high, but not low, amounts of alcohol for 12 weeks. Additionally, *Glp1R* expression is elevated in rats consuming high amounts of alcohol for 12 weeks, as compared to low. Moreover, a significant positive correlation between *Glp1R* expression in the NAc and alcohol intake was observed. The differences in the *Glp1R* expression could suggestively be a result of long-term alcohol consumption and thus possibly contributing to increased vulnerability in the reward system, which is associated with development of AUD. However, as no baseline measurement of *Glp1R* expression in alcohol-naïve state is available, the possibility should be considered that high consuming rats express more *Glp1R* compared to low in an alcohol-naïve state. Moreover, rats with elevated *Glp1R* levels could be more responsive to GLP-1 and therefore display lower alcohol intake, and not higher as reported herein. Studies in the NTS, where GLP-1 is produced centrally and reaches the areas studied herein, have revealed that acute alcohol increases *c-Fos* like immunoreactivity (Lee et al., 2011; Thiele et al., 2000) and enhances presynaptic GABA release (Aimino et al., 2018). Therefore, the effects of chronic alcohol exposure on GLP-1-producing neurons of the NTS are unknown. For further elucidation, future studies should evaluate the effects of chronic alcohol intake on central, as well as circulating levels of GLP-1. The increased number of GLP-1R in NAc in high alcohol-consuming rats, could also provide a tentative explanation to why high compared to low alcohol-consuming rats respond more profoundly to GLP-1R agonists in regards to decreased alcohol intake (Shirazi et al., 2013b; Vallof et al., 2016a), a result reported also herein. In further support for a suppressive effect of accumbal GLP-1R on alcohol-related behaviors are data showing that Ex4 into the NAc shell decreases alcohol intake in female rats (Abtahi et al., 2018). A tentative insight into how activation of accumbal GLP-1R may regulate the reinforcing properties of alcohol is provided by the findings that presynaptic GLP-1R on glutamatergic neurons in NAc inhibits the activity of dopamine afferents (Cork et al., 2015), which are activated by alcohol (Larsson and Engel, 2004; Soderpalm et al., 2009; Volkow and Li, 2004).

Albeit the effects of Ex4 infusion into NAc core was not investigated, as this area does not respond to acute alcohol injection (Bassareo et al., 2003; Cadoni et al., 2000), previous studies have established that activation of GLP-1R in the NAc core reduces consummatory behavior (Alhadeff et al., 2012; Dossat et al., 2013, 2011) and operant responses to cocaine (Hernandez et al., 2017). The findings that Ex4 into the NAc core does not influence chow intake and body weight (Alhadeff et al., 2012), further support the contention that region-specific GLP-1R play different functional roles.

We also found that Ex4 into NAc shell does not affect food intake or body weight, suggesting that accumbal GLP-1R influence alcohol- rather than homeostatic-related behaviors. This is further substantiated by the findings that activation of GLP-1R in NAc shell decreases cocaine self-administration (Hernandez et al., 2017), without altering sucrose intake (Alhadeff et al., 2012; Hernandez et al., 2017) and chow (Alhadeff et al., 2012; Dossat et al., 2011). On the other hand, infusion of Ex4 into NAc shell decreases high fat diet consumption, accompanied by body weight reduction (Alhadeff et al., 2012), further indicating that brain region specific GLP-1R modulate various physiological and behavioral processes differently.

The present studies reveal that infusion of Ex4 into the aVTA does not prevent alcohol-induced locomotor stimulation, retrieval of reward memory in the CPP paradigm in mice or reduces alcohol intake in rats consuming high amounts of alcohol. This may indicate that aVTA-GLP-1R activation does not attenuate the ability of alcohol to stimulate dopamine afferents in the NAc shell (Imperato and Di Chiara, 1986; Larsson et al., 2005). This is contradicting to studies showing that nicotinic acetylcholine receptors in the anterior part of the VTA are important for alcohol reinforcement (Jerlhag et al., 2006a; Larsson et al., 2002; Lof et al., 2007). The possibility should however be considered that the lack of effect of Ex4 into aVTA on alcohol-mediated behaviors is related to the possible low selected dose. The selected dose used herein, which has no effect *per se* on locomotor activity and CPP is physiologically relevant as provided by the tendency in food intake reduction observed following Ex4 into aVTA and by the fact that Ex4 in this dose range into the pVTA attenuates various reward-related behaviors in rodents (Alhadeff et al., 2012; Hernandez et al., 2018; Mietlicki-Baase et al., 2013; Schmidt et al., 2016; Wang et al., 2015). On the other hand, the present findings reveal that Ex4 into the aVTA potentiates alcohol-induced locomotor stimulation. As alcohol into the aVTA increases accumbal dopamine in rats (Jerlhag and Engel, 2014), the possibility should be considered that Ex4 further enhances this alcohol response by activating post-synaptic, rather than pre-synaptic, aVTA-GLP-1R, which should be evaluated in upcoming studies.

The physiological heterogeneity of VTA (Hnasko et al., 2012; Holly et al., 2016; Menegas et al., 2017) is supported by the present data showing that infusion of Ex4 into the pVTA attenuates alcohol-induced locomotor stimulation, but does not affect the reward dependent memory retrieval in the CPP in mice or decreases alcohol intake in high alcohol-consuming rats. Moreover, there were no difference in the expression of *GCG*, as an indication of GLP-1, or *Glp1R* in the VTA, in high compared to low alcohol-consuming rats. On the contrary, activation of GLP-1R in the aforementioned part of the VTA, which sometimes is referred to as the “tail” of the pVTA, reduces sucrose, chow as well as high fat diet intake (Alhadeff et al., 2012; Mietlicki-Baase et al., 2013; Wang et al., 2015), decreases body weight (Alhadeff et al., 2012) and prevents cocaine self-administration and seeking (Hernandez et al., 2018; Schmidt et al., 2016). We therefore suggest that activation of GLP-1R on different neuronal afferents, where GLP-1R are expressed on presynaptic glutamatergic afferents rather than within the dopaminergic cells (Cork et al., 2015; Mietlicki-Baase et al., 2014; Wang et al., 2015), regulate some, but not all reward-mediated behaviors. A previous study has established that a high dose of Ex4 into the former part of pVTA (-5.7 mm from Bregma) reduces sucrose, chow (Dickson et al., 2012) and alcohol (Shirazi et al., 2013b) consumption, further supporting that GLP-1R within different parts of the VTA modulate various behaviors. However, as this high dose decreases locomotor activity and water intake (Dickson et al., 2012; Shirazi et al., 2013b), influences the rats’ gross behavior (pilot data) and causes an unselective receptor activation (Barrera et al., 2009; Malendowicz et al., 2003; Sonne et al., 2008), the possibility should be considered that these results are due to unselective behaviors not related to GLP-1R activation. In conclusion, the findings from aVTA as well as pVTA reveal that activation of subregion specific GLP-1R modulate various physiological processes.

We also found that infusion of Ex4 into the LDTg inhibits alcohol-induced locomotor stimulation in mice and decreases alcohol intake in high, but not low, alcohol-consuming rats. This reduction in alcohol intake is complied by decline in total fluid intake, without altering water consumption. A decrease in food intake and a tendency in body

weight reduction in high alcohol-consuming rats is observed following Ex4 into the LDTg, which is in line with previous studies showing that Ex4-LDTg microinjections reduces body weight and cumulative food intake (Reiner et al., 2018). It should be considered that Ex4 activates GLP-1R, possibly on presynaptic GLP-1R on axon terminals from NTS afferents (Reiner et al., 2018), may prevent the ability of alcohol to stimulate the reward related cholinergic LDTg afferents (Lammel et al., 2012; Larsson et al., 2005; Steidl and Veverka, 2015; Steidl et al., 2017) targeting the NAc shell (Dautan et al., 2014) or VTA (Cornwall et al., 1990). As opposed to intra-NAc shell or systemic (Egecioglu et al., 2013c) activation of GLP-1R in LDTg by Ex4 does not affect memory retrieval of alcohol reward in the CPP paradigm. The sparse LDTg-innervation of hippocampus (Cornwall et al., 1990), an area involved in memory function (for review see (Riedel and Micheau, 2001)), may provide a tentative explanation for this.

Despite that the present study collectively suggests that brain region specific GLP-1R modulate alcohol-related behaviors, the role of GLP-1R in other areas not studied herein, remains to be evaluated (Llewellyn-Smith et al., 2011). For instance, the NTS, an area projecting to all areas of the mesolimbic dopamine system (Alhadeff et al., 2012; Merchenthaler et al., 1999) and where Ex4 acts locally to reduce food-mediated behaviors (Richard et al., 2015) should be considered as a potential site where GLP-1R activation may contribute to inhibition of alcohol reinforcement.

Our intermittent access experiments also demonstrated that prolonged exposure to high alcohol consumption may modulate the endogenous GLP-1 system in rodents, as the expression levels of *GCG* in the prefrontal cortex are elevated in high compared to low alcohol-consuming rats and there is a positive correlation between prefrontal cortex-*GCG* expression and increased alcohol intake. Although GLP-1 is one product of the GCG gene, it should be considered that elevated *GCG* expression might reflect increase in alternate products such as glucagon, glicentin, IP-2, and GLP-2. However, the mechanisms leading to significant increase in *GCG* expression in the prefrontal cortex, a region distal to the known site of GLP-1 production (Merchenthaler et al., 1999) and of importance for neuroadaptations occurring during manifestation of AUD (Koob and Le Moal, 2008), remain to be investigated. The present study reveals *GCG* expression in various areas, where *in situ* hybridization did not detect *GCG* mRNA in female rats previously (Merchenthaler et al., 1999). As qPCR allows detection of lower amounts of the gene, the possibility should be considered that the detected *GCG* expression reflects unknown preproglucagon production or possibly axonal transportation of mRNA from the soma (Bramham and Wells, 2007) and this could imply that these levels are not physiologically relevant. Moreover, it should be considered as a limitation that areas with high expression of GLP-1, such as NTS, were not included in this study. To assess this further, upcoming studies should evaluate the protein levels of GLP-1 in high versus low alcohol-consuming rats.

A potential factor influencing the obtained results could be aversion, a common side effect of systemic treatment with GLP-1R agonists (Kanoski et al., 2012). However, this appears less likely, since the selected doses of Ex4 have no effect on water intake *per se*. In further support are the findings that Ex4 into NAc shell, LDTg or VTA does not affect kaolin intake as an indication of aversion (Alhadeff et al., 2012; Dickson et al., 2012; Dossat et al., 2011; Reiner et al., 2018). A reduction in motor activity could also possibly influence the interpretation of the present results. In mice, this appears less likely since the selected dose has no effect *per se* on locomotor activity or CPP. Albeit local infusion of Ex4 did not alter motor behavior in alcohol naïve rats, possibly alcohol-exposed rats respond differently. However, no visual effect on motor behavior was observed following local Ex4 infusions, which we previously have established for systemic Ex4 injections in alcohol-consuming rats (Egecioglu et al., 2013c). Moreover, the obtained results might be influenced by cannula implantations and intracranial injections, though highly unlikely, as all respective controls have been included. Diffusion of Ex4 to surroundings areas could be another influential factor. However, we have tried to minimize this possibility since a low volume was used and as previous studies have established, misplaced infusions doe not influence behaviors similarly to correctly placed (Jerlhag et al., 2008, 2009; Jerlhag et al., 2006a, b; Kuzmin et al., 2009; Prieto-Garcia et al., 2015; Vallof et al., 2016c). Albeit Ex4 into NAc shell and LDTg reduces alcohol intake in rats consuming high amounts of alcohol in the intermitted access paradigm, these rats may not be dependent to alcohol. Even though systemic administration of GLP-1R agonists reduces both alcohol intake in non-dependent male rats, as well as the motivation to consume alcohol in alcohol-dependent selectively bred Sardinian alcohol-preferring rats (Vallof et al., 2016a), possibly other brain region specific GLP-1R modulate drinking in dependent rats. For the anterior and posterior VTA, only high alcohol-consuming rats were studied, raising the possibly that Ex4 might reduce alcohol intake in low alcohol-consuming rats. However, this seems unlikely, since the reduction in alcohol intake by Ex4 administered systemically (Shirazi et al., 2013b; Vallof et al., 2016a) or into the NAc shell and LDTg is selective to high consuming rats. Another limitation could lie in the inclusion of only male rats in this study. Female rats should be included in future studies, since alcohol-mediated behaviors might be regulated by different brain region specific GLP-1R in males and females.

Albeit the present study focuses on the importance of neuronal GLP-1R for alcohol-related behaviors, it provides a tentative mechanism through which circulating GLP-1R agonists known to cross the blood brain barrier (Kastin et al., 2002), may attenuate the development of AUD. Moreover, the present data extend on the previously described role of GLP-1R in modulations of alcohol-related behaviors (Egecioglu et al., 2013c; Sorensen et al., 2016; Suchankova et al., 2015; Thomsen et al., 2017; Vallof et al., 2016a), by demonstrating that such behaviors are driven by brain region specific GLP-1R.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.psyneuen.2019.02.006.

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