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Mechanism of Action of the Glucagon-like Peptide-1 analog Exendin-4 in the Reward System

– A Preclinical Study in the Drug Addiction Field

Degree Project in Medicine

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Abstract

Background: Alcohol use disorder is a great concern for public health and current pharmacological treatment is not sufficient. Over the last years, there has been a growing interest in the role of gut hormones in drug addiction. Glucagon-like peptide 1 (GLP-1), also acting as a neuropeptide, is one of these hormones in focus. Preclinical studies have shown that GLP-1 receptor agonists can reduce alcohol intake, attenuate alcohol-related behaviors and counter the ability of alcohol to increase dopamine levels in the nucleus accumbens (NAc), however, the mechanism of action is unknown. This study aims to elucidate how the GLP-1 analog exendin-4 acts in the reward system, when systemically administered.

Methods: Male Wistar rats were administered an acute systemic injection of exendin-4 or vehicle, and samples of extracellular fluid from the NAc shell were collected using a microdialysis system. The samples were analyzed in an HPLC system to determine extracellular concentrations of the neurotransmitters glutamate, serine, glycine, taurine, beta-alanine and GABA, all involved in the reward process. The levels after injection were compared between treatment group and control group.

Result: Exendin-4 significantly decreased the levels of serine, glycine and taurine in the NAc shell over time, compared to vehicle. No difference between treatments was found for glutamate.

Conclusions: Serine, glycine and taurine are all agonists to the glycine receptor and previous studies have shown that activation of the glycine receptor in the NAc is crucial for alcohol to elevate the dopamine levels in the NAc. In this study we showed that exendin-4 lowers the levels of these neurotransmitters, thus forming the hypothesis that the drug prevents alcohol to activate the reward system through reduced glycine receptor activation in the NAc.

Key words: Mesolimbic dopamine system, nucleus accumbens, alcohol use disorder, gut-brain-axis, GLP-1

1. Background

1.1. The mesocorticolimbic dopamine system

In 1954 James Olds and Peter Milner implanted electrodes in different areas of the brain in rats (Olds and Milner 1954). The animals produced an electrical stimulation by pressing a lever and time spent responding was recorded. Olds and Milner discovered a remarkable difference in responding time depending on the position of the electrode. They concluded that some areas are associated with the positive reinforcement of reward and their findings paved the way for research in the field.

Years of anatomical and physiological studies on reward generated the finding of the mesocorticolimbic dopamine system, that is now a well-established neurocircuit participating in the sensational and motivational aspects of reward (Jayaram-Lindström, Ericson et al. 2016). Central in this network of neurons is the ventral tegmental area (VTA) in the mesencephalon, with its dopaminergic neurons projecting to e.g. the limbic areas of nucleus accumbens (NAc) and amygdala, the prefrontal cortex (PFC) and the hippocampus (for review see e.g. (Koob 1992, Jayaram-Lindström, Ericson et al. 2016) (Figure 1). These pathways can be further divided into two systems; the mesolimbic system (projections from VTA to NAc) and the mesocortical system (from VTA to PFC). The mesolimbic system is considered to be responsible for the rewarding and pleasurable effects, while the mesocortical system seems important to the emotional and motivational aspects (Jayaram-Lindström, Ericson et al. 2016).

Although the mesocorticolimbic dopamine system was evolutionary developed to promote natural rewards, such as food and sex, several drugs of abuse also activate the system (Koob 1992). There is considerable evidence for the connection between alcohol and the mesocorticolimbic dopamine system from preclinical, as well as from clinical and genetic

studies (Koob 1992, Tupala and Tiihonen 2004, Jayaram-Lindström, Ericson et al. 2016). Several microdialysis studies on rats have shown a direct effect of alcohol as measured by increased dopamine (DA) release in NAc (Imperato and Di Chiara 1986, Snape and Engel 1988, Yoshimoto, McBride et al. 1992, Heidbreder and De Witte 1993). Through the introduction of positron emission tomography (PET), this effect was confirmed in human (Boileau, Assaad et al. 2003, Volkow, Fowler et al. 2007). The dopaminergic activity in NAc has been associated with the direct positive reinforcement of alcohol (euphoria, "high") as well as for anticipation and craving (Tupala and Tiihonen 2004, Jayaram-Lindström, Ericson et al. 2016). Regulation of DA levels in the NAc is complex. The DA elevating effect of alcohol in the NAc has been shown to be dependent on the activation of the glycine receptor (GlyR), which is agonized by the amino acid neurotransmitters glycine, taurine and serine (Soderpalm, Lido et al. 2017). Although DA has been the neurotransmitter in focus in addiction research, this monoamine cannot alone explain the neurochemical pathophysiology of addiction. The amino acid neurotransmitter GABA produces postsynaptic inhibition and exhibit an anxiolytic and sedating effect (Koob 1992). These effects are potentiated by alcohol, since alcohol acts on the $GABA_A$ receptor (Koob 1992). Glutamate, also an amino acid neurotransmitter, is excitatory and glutamatergic transmission is inhibited by acute alcohol administration, an effect considered to mediate positive reinforcement (Rao, Bell et al. 2015). Further, data suggest that serotonin and noradrenaline, as well as opioid peptides are involved in the rewarding effects of alcohol (Larsson and Engel 2004). The fact that the action of alcohol in the reward system is complex enables pharmacological research to study alterations in several neurotransmitters. This study focuses on the amino acid group, as these neurotransmitters seem to have key role in the reward system.

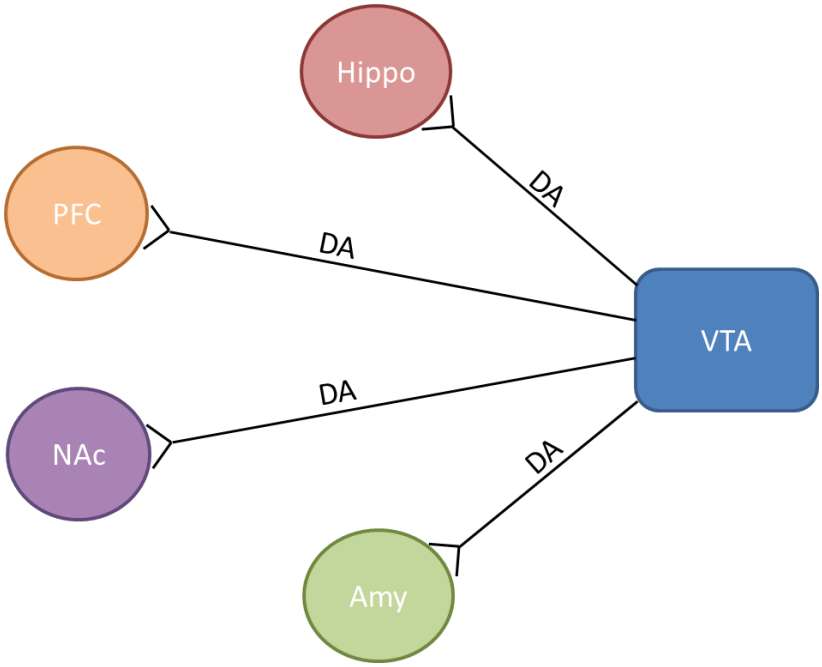

Figure 1
The mesocorticolimbic dopamine system. Dopamine (DA)-producing neurons in the ventral tegmental area (VTA) project to hippocampus (Hippo), the prefrontal cortex (PFC), nucleus accumbens (NAc) and the amygdala (Amy).

1.2. Alcohol – use, disorder, treatment

The consequences of alcohol use are of great concern for public health. A comprehensive meta-analysis of global alcohol use was recently published, claiming alcohol to be the seventh leading risk factor for death, as well as for disability-adjusted life years (DALY) (Collaborators 2018). For people in the age range 15-49 years, use of alcohol was considered the number one risk factor for death (Collaborators 2018). Different types of cancer, tuberculosis, cardiovascular diseases and alcohol use disorder (AUD) itself are a few examples of the causes of DALY's that can be attributed to alcohol (Collaborators 2018). In the same study, the number of current drinkers (defined as having at least one drink in the past year) was estimated to 37.5 % of the global population. In addition to the mortality and

morbidity, alcohol generates great economic costs, in numbers of 2-3 % of gross national product in both high- and middle-income countries (Rehm, Mathers et al. 2009).

Although the above-cited metanalysis estimated the most health-promoting level of alcohol consumption to zero (Collaborators 2018), use of alcohol needs to be distinguished from AUD. In the latest version of Diagnostic and Statistical Manual of Mental Disorders, DSM-5 (2013), the two earlier diagnoses alcohol abuse and alcohol dependence are replaced with one diagnosis, AUD, divided into mild, moderate and severe. The diagnosis is based on different physiological and psychological symptoms as well as problems related to social life but not all three aspects are required to meet the criteria for diagnosis (2013), indicating it is a heterogeneous disorder. Albeit most drinkers do not suffer from AUD, prevalence is high with around 3.6 % globally, and for men in Europe as high as 9.1 % (corresponding number for women 2.0 %) (Rehm, Mathers et al. 2009)

To date, four drugs are approved in the European Union for treatment of AUD. These are (i) disulfiram, this drug disrupts the normal metabolism of alcohol by blocking the enzyme aldehyde dehydrogenase, which in turn causes discomfort such as tachycardia and headache (Heilig and Egli 2006), (ii) acamprosate, acting as a functional glutamate antagonist, yet its mechanism of action is not completely understood (Heilig and Egli 2006), (iii) naltrexone, an opioid receptor antagonist (Heilig and Egli 2006) and (iv) nalmefene, an opioid receptor modulator (Soyka and Muller 2017). The still high prevalence of AUD and variations in treatment results between individuals (Heilig and Egli 2006) leads to the conclusion that current pharmacological treatment is not sufficient. Considering the heterogenicity of the disorder and the complexity of the reward system, this is not unexpected. Over the last years, the gut-brain-axis (i.e. the endocrine and neural connection between the gastrointestinal system and the central nervous system (Romijn, Corssmit et al. 2008)) has been in focus for several research fields, including the addiction field. Glucagon-like peptide-1 (GLP-1) is one

of the hormones of the gut-brain-axis and preclinical, as well as clinical data report that GLP signaling affect alcohol use (Jerlhag 2018). The indications of GLP-1 regulating alcohol intake, and the fact that GLP-1 receptor agonists already exist as approved drugs, make this peptide an appropriate focus in the search for new treatment of AUD.

1.3. GLP-1 – physiology and pharmacological use

GLP-1 is an anorexigenic peptide hormone produced both in L-cells of the intestines (Novak, Wilks et al. 1987) and in the nucleus of the solitary tract (NTS) of the hindbrain (Larsen, Tang-Christensen et al. 1997). Physiological functions of GLP-1 include inhibition of gastric emptying, stimulation of glucose-dependent insulin secretion and inhibition of glucagon release (for review see e.g. (Holst 2007)). Based on these attributes, GLP-1 receptor agonists were approved for pharmacological treatment of type 2 diabetes mellitus over a decade ago (Holst 2007). A few years later, the same group of drugs were approved for obesity (Zhang, Tong et al. 2015), as it was shown that in addition to regulating the glucose homeostasis in energy balance, these drugs mediate satiety signals and thereby reduce food intake and body weight (for review see e.g. (Hayes, De Jonghe et al. 2010)). A large number of preclinical studies show that GLP-1 signaling not only affect the energy aspect of food intake, but also the rewarding effect and/or the food-seeking motivation (Alhadeff, Rupprecht et al. 2012, Dickson, Shirazi et al. 2012, Alhadeff and Grill 2014, Richard, Anderberg et al. 2015, Alhadeff, Mergler et al. 2017). Growing data from preclinical, clinical and genetic research show that several peptides, including GLP-1, that regulate food intake also control drug use (Thiele, Stewart et al. 2004, Engel and Jerlhag 2014).

1.4. GLP-1 and alcohol

Clinical research on the role of GLP-1 in alcohol consumption is to date limited. However, in a human genetic study, an association between a polymorphism in the gene coding for the GLP-1 receptor and alcohol dependence was found (Suchankova, Yan et al. 2015). In addition, carriers of the risk allele had a higher response in a fMRI reward model and self-administered alcohol to a higher degree than non-carriers (Suchankova, Yan et al. 2015). Further, a preliminary cross-section report noted an extensive reduction in alcohol consumption in patients with diabetes mellitus type 2 treated with the GLP-1 analog liraglutide (Kalra 2011).

The preclinical evidence is more comprehensive, and several studies have shown that GLP-1 receptor activation affects alcohol-related behavior, consumption volume, as well as the alcohol-induced DA release in the NAc (Jerlhag 2018). In conditioning place preference tests (CPP, a model used for studying behavior related to the rewarding effect of drugs) in rodents, the rewarding effect of alcohol is attenuated by systemic administration of the GLP-1 receptor agonists liraglutide (Vallof, Maccioni et al. 2016) and exendin-4 (ex4) (Shirazi, Dickson et al. 2013, Egecioglu, Steensland et al. 2013c). In other words, when administered liraglutide or ex4, the rodents stopped seeking alcohol reward through spending more time in the compartment of the cage that was coupled to administration of alcohol. Repeated ex4 administration further blocked the ability to produce a CPP, i.e. to form a reward-related memory paired with alcohol (Egecioglu, Steensland et al. 2013c). Another way to study drug-related behaviors is the self-administration model (testing the drug-seeking behavior), where rodents get a reward (i.e. a dose of the drug) through pressing a lever. Systemic administration of ex4 decrease the number of lever presses, both when alcohol is ingested (Egecioglu, Steensland et al. 2013c) and administered intravenously (Sorensen, Caine et al. 2016), as to show that the effect is not related to general ingestive behavior. A third model commonly

used in preclinical alcohol studies is the two-bottle-choice model, where the animals get free access to both water and alcohol. The volume of consumed alcohol decreased after administration of ex4 (Shirazi, Dickson et al. 2013, Egecioglu, Steensland et al. 2013c) or liraglutide (Vallof, Maccioni et al. 2016). Relapse-drinking after period of abstinence in human can be reflected in a preclinical model where animals first are trained to drink alcohol, then alcohol is withdrawn for a period to later be reintroduced (Spanagel 2000). Ex4 (Thomsen, Dencker et al. 2017) and liraglutide (Vallof, Maccioni et al. 2016) counter the deprivation-induced increase in alcohol consumption seen in rodents in the control groups. To expand the knowledge of this neuropeptide's role in AUD, one research group studied anxiety in rats and showed that liraglutide prevented both withdrawal-induced anxiety and the development of tolerance against the anxiolytic effect of alcohol (Sharma, Pise et al. 2015). Further, the GLP-1 analog exhibited an anxiolytic effect *per se* (Sharma, Pise et al. 2015). These data showing that GLP-1 receptor activation affects different alcohol-related behaviors and consumption, short-term as well as long-term, indicates that this peptide might have an important role in the pathophysiology of AUD. In addition to the observed behavioral effects of GLP-1 receptor activation, the alcohol-induced DA release in NAc has been studied, showing that GLP-1 analogs attenuate the elevated DA release, both when ex4 (Egecioglu, Steensland et al. 2013c) and liraglutide (Vallof, Maccioni et al. 2016) is tested. Albeit studies have established that GLP-1 receptor agonists block alcohol-related behaviors and the alcohol-induced DA-release, the underlying mechanisms and pathways leading this effect are still unknown.

1.5. GLP-1 and other drugs

The growing knowledge of the role of GLP-1 signaling in the reward system, raises the possibility that GLP-1 receptor agonists can modulate the behavioral effects of other addictive drugs. The effect of GLP-1 receptor activation on cocaine, amphetamine and nicotine administration has all been preclinically tested with results comparable to the alcohol studies. For cocaine, drug-seeking behavior is attenuated by GLP-1 receptor agonists as shown in a CPP model (Egecioglu, Engel et al. 2013b) and self-administration experiments (Sorensen, Reddy et al. 2015, Schmidt, Mietlicki-Baase et al. 2016, Hernandez, O'Donovan et al. 2017, Hernandez, Ige et al. 2018). Further, the cocaine-induced increase in locomotor activity is weakened by GLP-1 receptor activation (Egecioglu, Engel et al. 2013b, Sorensen, Reddy et al. 2015). Microdialysis measures report that ex4 reduces the ability of cocaine to increase the levels of DA in the NAc shell (Egecioglu, Engel et al. 2013b, Sorensen, Reddy et al. 2015, Reddy, Pino et al. 2016). In addition, GLP-1 receptor activation also attenuates the stimulatory effects of amphetamine (Egecioglu, Engel et al. 2013b) or nicotine (Egecioglu, Engel et al. 2013a) in CPP, locomotor activity and DA-release. Clinical data is spare, but one recent study found that serum levels of GLP-1 are decreased after acute intravenous administration of cocaine on experienced users (Bouhlal, Ellefsen et al. 2017).

Taken together, the increasing amount of data showing that GLP-1 signaling regulates the intake and effect of several addictive drugs indicate that GLP-1 has a general role in reward pathways that is not drug-specific. Further, the effect shown on non-caloric drugs diminish the possibility that the reported effects on alcohol are dependent on energy homeostasis.

2. Aim

The overall aim of this present study was to elucidate the mechanism of action of the GLP-1 analog ex4 in the reward system. More specifically, the study aimed to investigate the effect of an acute systemic injection of ex4 on the extracellular levels of the neurotransmitters glutamate, serine, glycine, beta-alanine, taurine and GABA in the NAc shell of rats.

3. Methods

The present study used an in vivo microdialysis system in rats. This is a well-established technique for measurement of extracellular levels of neurotransmitters in awake and freely moving animals (Chaurasia, Muller et al. 2007).

3.1 Animals

Post-pubertal age-matched male outbred Wistar rats, *Rattus norvegicus*, (Charles River Laboratories, Italy) were used. After arrival to the facility, all animals were allowed to acclimatize at least one week before surgery. The rats were initially group housed and were following surgery kept in individual cages (high Macrolon III, covered with grid tops), to prevent them from gnawing the implanted material. Until the day of the experiment, tap water and food (normal chow; Harlan Teklad, Norfolk, England) were supplied *ad libitum* and the animals were maintained on a 12-hour light/dark cycle (lights on at 8 am) in rooms at 20°C and 50% humidity. A total number of 30 animals started the microdialysis procedure, 4 of these were unable to complete the experiment due to non-functioning probes and another 4 were excluded by cause of incorrect probe placement or by producing remarkedly outstanding

data, so that a number of 22 remained for the final analysis; treatment group (ex4, n=11) and control group (veh, n=11).

3.2. Drugs

Ex4 is a high potency GLP-1 receptor agonist (Goke, Fehmann et al. 1993) with a half-life of 2.4 h (Finan, Clemmensen et al. 2015). Ex4, purchased from Tocris Bioscience (Bristol, England), was diluted in saline (0.9 % NaCl). The dose selected for i.p. injection was 1.2 µg/kg in 2 ml/kg since this dose does not create an aversion yet shows an effect on alcohol-mediated behaviors (Egecioglu, Steensland et al. 2013c). An equal volume of vehicle (veh) (0.9 % NaCl) was administered i.p. in control animals.

3.3. Microdialysis

Preparations - implantation of probe and guide cannula

The probe of the microdialysis setup was unilaterally placed with its dialyses membrane in the NAc shell, allowing neurotransmitters in the nucleus to pass the membrane. The probes were implanted through a surgical procedure performed by laboratory technicians. Rats were anesthetized with isoflurane (Isoflurane Baxter; Univentor 400 Anaesthesia Unit, Univentor Ltd., Zejtun, Malta) and placed in a stereotaxic frame (David Kopf Instruments; Tujunga, CA, USA). Throughout the procedure, rats were kept on a heating pad to avoid hypothermia. Carprofen (5 mg/kg s.c., Rimadyl®; Astra Zeneca, Gothenburg, Sweden) was injected to relieve pain and saline (1 ml/kg s.c.) to prevent dehydration. Head was shaved, and the skin was cut to expose the skull bone. For the NAc shell, following coordinates (Paxinos and Watson 1943- (1998)) were used: + 1.85 mm anterior to the bregma, ± 1.0 mm lateral to the midline and 7.8 mm below the brain surface. Holes were drilled; one for the probe, one for the anchoring screw. The probe was then placed with its membrane in the position for the NAc

shell. Probes were randomly alternated to either the right or the left side, in a balanced matter. To fixate the probe, anchoring screws were fastened, and the skull bone was covered with dental cement (DENTALON® plus; Agntho´s AB, Lidingö, Sweden).

Day of microdialysis

Two days after surgery, microdialysis was performed. To avoid possible impact on the neurotransmitter levels, food and water was removed. The inlets of the probes were connected to a microperfusion pump (U-864 Syringe Pump; AgnTho's AB) and continuously perfused with Ringer's solution at a rate of 2.0 µl/minute. The rats were following this allowed one hour of habituation to the microdialysis setup. Samples (i.e. collected extracellular fluid from the outlets of the probes) were then collected in 20-minute intervals. The first four samples were used to create baseline levels, using the average of the three best matching samples. Then, ex4 or veh was intraperitoneally administered (timepoint 0). After injection, nine samples were collected (timepoint 20-180). Sodium azide was added to the samples to maintain stability, and then the samples were quickly freezed in -20 °C. Samples were analyzed in an HPLC system (see below) to detect and quantify the neurotransmitters. The microdialysis was performed during nine different days in four consecutive weeks.

Verification of position of probe and guide cannula

When all samples were collected, the rats were euthanized, and the brains were frozen. Brains were then manually sliced, and the positions of the probes were determined by using a rat brain atlas (Paxinos and Watson 1943- (1998)) for comparison. Only rats with correct placement of the probe were used in the statistical analysis (Figure 2).

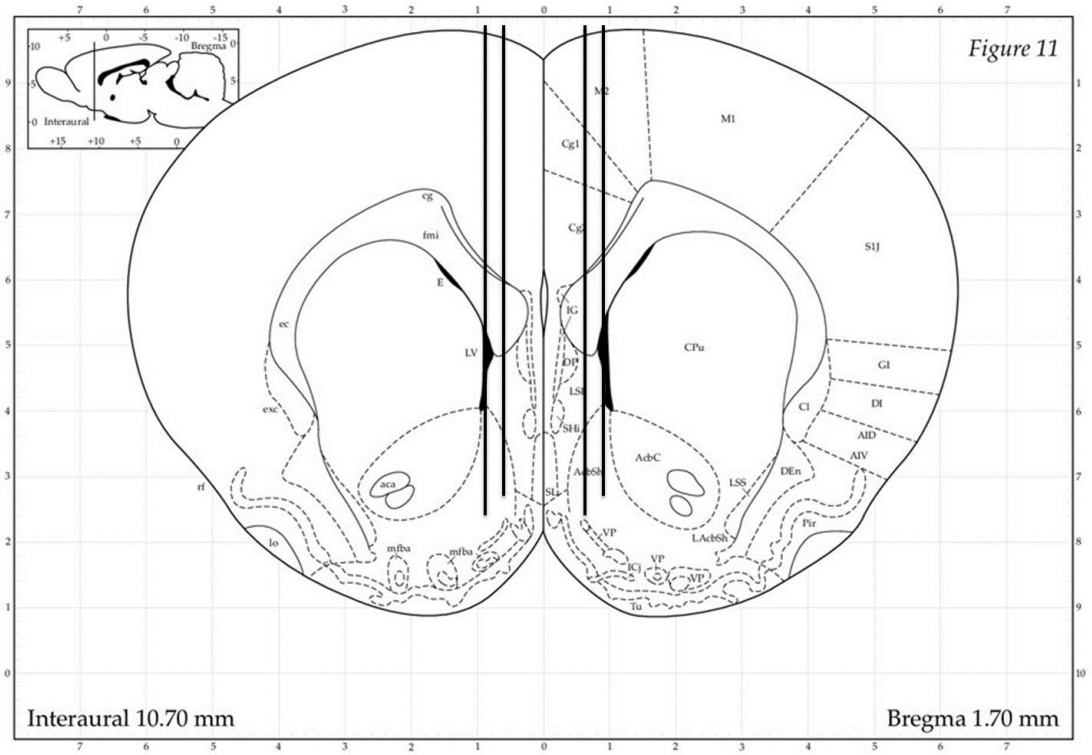

Figure 2
Schematic illustration of probe positions in a coronal rat brain section. Vertical lines are showing four representative probe placements in the nucleus accumbens shell.

3.4. Biochemical assay

Amino acids (glutamate, GABA, serine, glycine, taurine and beta-alanine) were separated and detected using the UltiMate 3000 HPLC system (Thermo Fisher Scientific, Gothenburg, Sweden). First, the system performed a preanalysis derivatization in which the samples were allowed to react for 10 sec with o-phthaldialdehyde⁄2-mercaptoethanol. Next, the amino acids were separated on a 5µM C18 Kinetex® column (4.6 x 150 mm, Phenomenex). The column was maintained at 39°C and contained a SecurityGard (Phenomenex) for protection. To detect the amino acids, a fluorescence detector (Ex: 348 nm, Em: 450 nm) was used. With 2.5 ml/min flow rate, the HPLC pump utilized a gradient in the mobile phase A) 0.1 M sodium phospate, 0.1 mM EDTA, pH 6.38 with phosphoric acid, B) methanol (100%), C) acetonitrile (50%) and D) methanol (4%) and acetonitrile (1%)). The time of analysis for each sample was 14.6 min (injection to injection). To identify the correct peaks in the chromatogram, external standards for the amino acids with two different concentrations (0.5 µM, 1.0 µM) were used. Concentrations were determined using the Chromeleon7 software (Thermo Scientific Chromeleon Chromatography Data System).

3.5. Statistical analysis

Concentrations of the neurotransmitters for each timepoint were converted to %-change from baseline. The baseline (100 %) was defined as the average of the three baseline measurements. The levels after injection were compared between treatment group and control group in a two-way ANOVA with repeated measures, (time as one factor, treatment as the other), using the GraphPad software. Pairwise comparisons between treatment group and control group for each timepoint were performed in a Bonferroni post-hoc test.

4. Ethical considerations

Using animals in research always require ethical discussions. Anyone (including students) working with research animals in Sweden, is required to complete a course covering legal, ethical and physiological aspects, as well as practical skills. The present experiment was rather invasive (intracerebral probe and intraperitoneal injection) and the rats were single-housed for two days. Any animal showing signs of suffering or losing more than 15 % in body weight between surgery and the day of microdialysis were immediately euthanatized, in agreement with the defined humane endpoints. However, to answer the research question, this technique was considered the most appropriate and better pharmacological treatment is highly desirable, since AUD is a major cause of mortality and morbidity, as described in the background section.

The experiment was approved by the Swedish Ethical Committee on Animal Research in Gothenburg.

5. Results

Two-way ANOVA with repeated measures revealed an overall effect of time ($F(11,110)=3.97$, $p<0.0001$) on glutamate release in the NAc shell in rats systemically treated with ex4 or veh. However, there was no overall effect of time x treatment interaction ($F(11,110)=1.09$, $p=0.378$) or treatment ($F(1,10)=0.16$, $p=0.695$) (figure 3A). On serine release, an overall effect of time ($F(11,110)=7.97$, $p<0.0001$) and time x treatment interaction ($F(11,110)=2.46$, $p<0.01$) was found, but no overall effect of treatment ($F(1,10)=3.44$, $p=0.094$) (figure 3B). For glycine, an overall effect of time ($F(11,110)=3.12$, $p<0.001$) and time x treatment ($F(11,110)=2.77$, $p<0.01$) was revealed, while there was no effect of treatment alone ($F(1,10)=4.80$, $p=0.053$) (figure 3C). For taurine there was no effect of time ($F(11,110)=1.28$, $p=0.243$), yet an effect of time x treatment ($F(11,110)=3.23$, $p<0.001$), but no effect of treatment alone ($F(1,10)=3.74$, $p=0.082$) (figure 3D). Bonferroni post-hoc test showed no significant difference between the groups at any timepoint for glutamate. For serine, there was a significant difference at the timepoints 20-100 min and at 140 min. Glycine levels were significantly different at the timepoints 20-120 min and taurine at the timepoints 20-80 min.

The study also intended to measure beta-alanine and GABA, but failed to detect these transmitters.

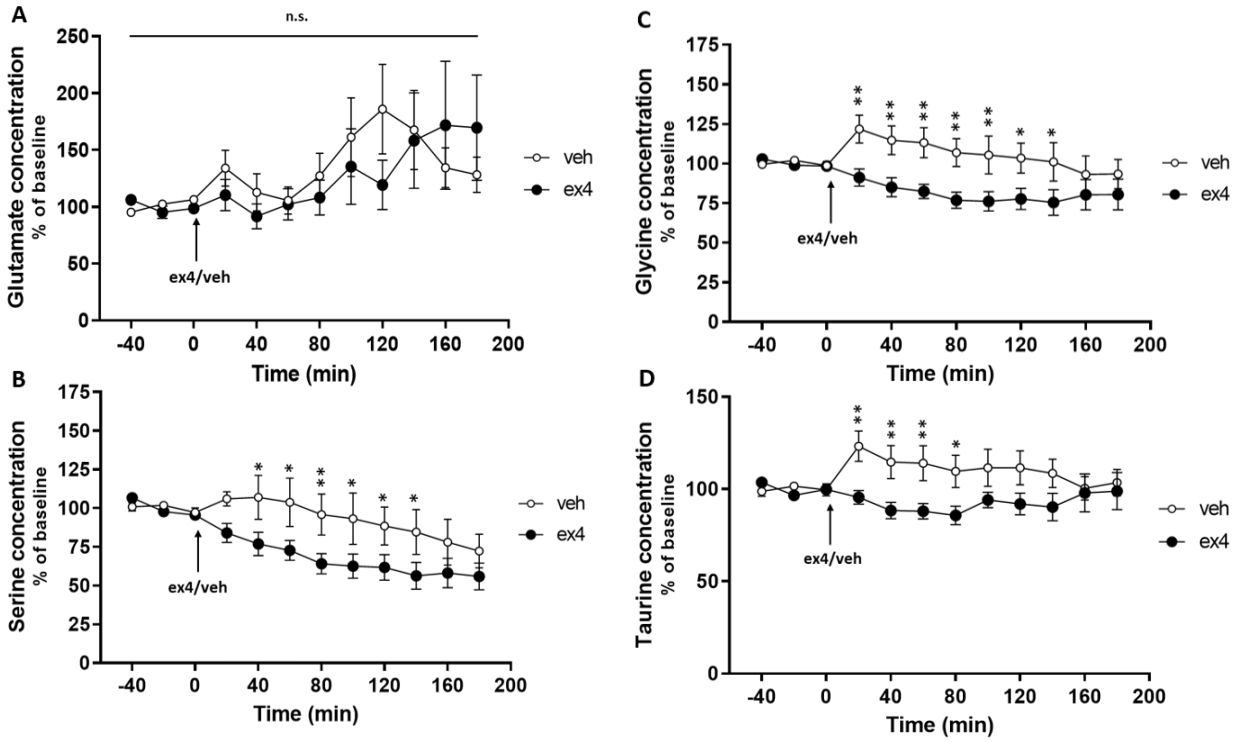

Figure 3

Systemic injection of exendin-4 (ex4) (1.2 µg/kg i.p.) significantly decreases the levels of serine, glycine, taurine, but not glutamate in the nucleus accumbens (NAc) shell in rats, over time compared to vehicle (veh). (A) No significant difference was found on the glutamate level. (B) Ex4 decreases the level of serine in the NAc shell. (C) Ex4 decreases the level of glycine in the NAc shell. (D) Ex4 decreases the level of taurine in the NAc shell. Data was analyzed using two-way ANOVA with repeated measures and is presented as mean (% from baseline) ±SEM. Bonferroni post-hoc test was performed for each timepoint: $^{**}p<0.01$, $^{*}p<0.05$, non-significant (n.s.) $p>0.05$. Arrows indicate time of injection of ex4 or veh.

6. Discussion

It has previously been shown that ex4 attenuates the elevation of DA in the NAc induced by alcohol (Egecioglu, Steensland et al. 2013c), however, the mechanism behind this action has not been described. To our knowledge, the present study is the first to show that a GLP-1 analog *per se* lowers the accumbal levels of neurotransmitters involved in the reward process. In this experiment, we have shown that, over time, a systemic administration of ex4 significantly reduces extracellular levels of glycine, taurine and serine in the NAc shell, compared to vehicle. No significant difference was found for glutamate.

6.1. The action of exendin-4 in the reward system – a hypothesis

Glycine, taurine and serine are all amino acids neurotransmitters with affinity for the GlyR) - glycine as an agonist, taurine as a partial agonist, while serine is less studied (for review see e.g. (Soderpalm, Lido et al. 2017)). Indeed, activation of GlyRs in the NAc has been demonstrated to be crucial for the DA elevating effect of alcohol, since local administration of the GlyR antagonist strychnine in the NAc of rats blocks the alcohol-induced DA increase, both when alcohol is administered systemically and locally in the NAc (Molander and Soderpalm 2005, Jonsson, Adermark et al. 2014, Clarke, Soderpalm et al. 2015). Further, when administered to the NAc, strychnine *per se* decreases the DA levels in the same nucleus, suggesting that GlyRs are tonically activated to maintain basal DA levels in the NAc (Molander and Soderpalm 2005, Adermark, Clarke et al. 2011, Clarke, Soderpalm et al. 2015). Glycine itself has been shown to act in the NAc to elevate the local DA levels (Yadid, Pacak et al. 1993), although in another study, this effect was not present in all tested animals (Molander, Lof et al. 2005). Similarly, taurine itself also exhibit a DA-elevating effect in the

NAc when administered to the nucleus, an effect that is blocked by simultaneous strychnine administration (Ericson, Molander et al. 2006).

The reports of GlyRs in the NAc being crucial for alcohol to elevate DA in the NAc (Molander and Soderpalm 2005, Jonsson, Adermark et al. 2014, Clarke, Soderpalm et al. 2015) in combination with the findings in this study demonstrating that ex4 decreases the levels of glycine, taurine and serine in the NAc form the hypothesis that ex4 prevents activation of the mesolimbic dopamine system through the decrease of these neurotransmitters. Since GlyRs have been suggested to sustain basal DA levels in the NAc, it would be plausible to think that ex4 lowers these levels. However, several microdialysis studies have demonstrated that the basal levels of DA in the NAc remain unaffected by ex4 (Egecioglu, Engel et al. 2013a, Egecioglu, Engel et al. 2013b, Egecioglu, Steensland et al. 2013c). Thus, it is possible that ex4 lowers the GlyR agonist levels in the NAc enough to attenuate alcohol-induced DA release, yet not enough to affect basal levels of DA.

Contrary to the suggestion that lowered levels of GlyR agonists in the NAc can negatively affect alcohol consumption, Söderpalm and colleagues have studied the opposite, i.e. increased GlyR agonist levels as a pharmacological approach on AUD (for review see (Soderpalm, Lido et al. 2017)). Systemic administration of the glycine reuptake inhibitor Org25935, resulting in elevated levels of glycine in the NAc (Lido, Stomberg et al. 2009), has been shown decrease alcohol consumption in rats (Molander, Lido et al. 2007). In accordance with the above-mentioned glycine administration study (Molander, Lof et al. 2005), only a subpopulation of the rats responded to Org25935 with increased accumbal DA, and after pretreatment with Org25935, alcohol did not further elevate DA in responders, still non-responders exhibited a smaller DA increase compared to controls (Lido, Stomberg et al. 2009). Further, the pharmacological AUD treatment acamprosate has been shown to increase basal levels of DA in the NAc in a GlyR dependent manner (Chau, Stomberg et al. 2010) and

block a further DA increase by alcohol (Chau, Lido et al. 2018). In conclusion, it seems possible that both an increase and a decrease of GlyR agonists in the NAc can prevent an alcohol-induced DA release in the NAc, thus blocking drug-reinforcement since the DA release in the NAc correlates with the subjective feeling of "high" in humans (Volkow, Wang et al. 1999).

6.2 Methodologic considerations

Microdialysis is a valuable technique for studying direct neurochemical action in the central nervous system, yet some limitations can be found. Although efforts were made to minimize the impact of external factors (e.g. removing food, allowing time for habituation before starting the measurements), the possibility that other environmental factors (e.g. handling, smells, sounds) might have affected the animals cannot be ruled out. Due to delivery issues, the size (and age) of the rats varied between batches and we also subjectively observed some variation of stress level for different batches. Since we had a control group for all measurements and that the study was designed so that both control and treatment group were examined on the same days, variations between batches and external factors possibly affecting the result were further minimized. When controlling the placement of the probes in the brains, we found that a few brains had smaller bleedings and that in an even fewer number of brains, the probe was slightly tilted, still, the dialysis membranes were in the NAc shell. Data from rats with remarks considering the probe were carefully studied so that rats that gave markedly different results were excluded in the statistical analysis.

Because of the nature of the experimental technique, one can only draw conclusions about the currently studied areas (i.e. the NAc shell in this case). Since the GLP-1R is expressed in numerous brain areas, including the NAc, the VTA and the amygdala (Merchenthaler, Lane et al. 1999), the effect of ex4 should be tested in more areas. The rats in

the present study were alcohol-naïve and only received one acute injection of ex4. Further studies are needed to examine whether the results remain in alcohol-preferring rats and after repeated administration, to better resemble the clinical situation. Only male rats were used in this experiment and certainly, female animals should also be tested.

6.3 Clinical implications

A conceivable compliance issue for ex4 is the twice-daily injections, however, an extended-release variant for administration once-weekly is now available (Syed and McCormack 2015). The once-weekly ex4 is currently being tested in a randomized controlled trial on alcohol-dependent patients, with a reduction in number of "heavy drinking days" as the primary endpoint (Antonsen, Klausen et al. 2018).

Well-recognized adverse events of GLP-1 receptor agonists are a hypoglycemia and gastrointestinal symptoms (i.e. nausea, diarrhea, vomiting) (Xue, Ren et al. 2016, Htike, Zaccardi et al. 2017), although these are typically occurring at the beginning of the treatment and are usually mild (Horowitz, Aroda et al. 2017). The risk of hypoglycemia is generally low and for most associated with co-treatment with other anti-diabetic drugs (Prasad-Reddy and Isaacs 2015, Xue, Ren et al. 2016, Htike, Zaccardi et al. 2017). There is an ongoing discussion about GLP-1 receptor agonists causing an increased risk of pancreatitis after preclinical and clinical indications (Nachnani, Bulchandani et al. 2010, Elashoff, Matveyenko et al. 2011, Gier, Matveyenko et al. 2012), although there are contradictory data, e.g. from a systematical review of studies on patients with diabetes mellitus type 2 treated with GLP-1 receptor agonists (Drab 2016) and a comprehensive preclinical study (Nyborg, Molck et al. 2012). In addition, some warnings have come from preclinical studies concerning an increased risk of thyroid c-cell neoplasia, although not confirmed in humans (for review see e.g. (Chiu, Shih et

al. 2012)). The potential risks of pancreatic and thyroid diseases need to be examined in long-term safety studies.

7. Conclusions

This study presents the hypothesis that ex4 acts in the reward system to block the DA-increasing effect of alcohol through lowering the extracellular levels of glycine, taurine (and serine) in the NAc shell. Ex4 therefor appears to have a mechanism of action distinct from currently approved AUD drugs. Considering that AUD is a heterogenous disorder, drugs with diverse mechanisms of action is requested to personalize the treatment and possibly combine different medications. Despite the lack of available pharmacological treatment, new drugs cannot alone manage this substantial health and social issue. The majority of AUD patients does not receive any treatment at all (Heilig and Egli 2006) and the reason for this needs to be elucidated and rectified.

Although the focus in this paper has been on AUD, the effect of ex4 was studied in the reward system without the exposure of alcohol and therefore, the results can also be applied to discussions of ex4 as a treatment for other substance use disorders, and possibly the rewarding aspect of food in obesity treatment. We therefor hope that this study can contribute with knowledge to further research in all these fields and in the physiology of the reward system.

8. Populärvetenskaplig sammanfattning

Alkoholberoende är en av våra stora folksjukdomar. Tillståndet orsakar stort lidande och förkortar liv, framförallt på grund av dess följdsjukdomar, såsom hjärt- och kärlsjukdomar samt flera sorters cancer. I tillägg till de direkta konsekvenserna för individen och närstående orsakar alkoholbruket stora samhällsekonomiska konsekvenser (t ex i form av förlorade arbetstimmar och sjukvårdskostnader). Ett mindre antal läkemedel finns idag tillgängliga för behandling av alkoholberoende, men trots det står många utan behandling och antalet drabbade är fortsatt högt. Sjukdomen ter sig olika i olika individer och det finns ett betydande behov av ett större utbud av läkemedel för att kunna individanpassa behandlingen. I den här studien undersöks verkningsmekanismerna av en ny potentiell kandidat.

Under senare år har forskning inom ett flertal medicinska fält studerat kopplingen mellan mag-tarmkanalen och hjärnan. Hormonet glucagon-like peptide 1 (GLP-1) produceras i tunntarmen och i hjärnan och en av dess viktiga roller är reglering av blodsocker, varför läkemedel som efterliknar hormonet används som läkemedel mot diabetes typ 2. Senare upptäcktes att denna grupp av läkemedel också kan minska överdrivet matintag och orsaka viktnedgång och fick därför även godkännande för behandling av fetma och övervikt. Mat, liksom droger, aktiverar hjärnans belöningssystem och det är därför inte långt bort att tänka att läkemedel som sänker matintag också skulle kunna påverka droganvändning. Flera studier på möss och råttor har visat att läkemedel som efterliknar GLP-1 kan minska intaget av alkohol och dämpa beteenden relaterade till alkohol, såsom sökandet efter drogen. Liknande försök har gjorts med andra beroendeframkallande droger och gett liknande resultat. Försöken har även visat en direkt biokemisk effekt på belöningssystemet av dessa läkemedel, då de testade drogernas (inkl. alkohol) förmåga att öka nivåerna av dopamin (en signalsubstans viktig för belöning) försvagas. Hur läkemedlen utövar denna effekter är sedan tidigare okänt

och den här studien syftade till att undersöka mekanismerna bakom. Råttor fick därför en injektion av GLP-1-läkemedlet exendin-4 och nivåer av ett antal signalsubstanser mättes i nucleus accumbens (ett viktigt område i hjärnans belöningssystem). Nivåerna efter injektion jämfördes med nivåerna före injektion för att se om läkemedlet påverkade signalsubstanserna.

Studien visade att exendin-4 signifikant sänker koncentrationerna av signalsubstanserna serin, glycin och taurin i nucleus accumbens, medan andra signalsubstanser förblev opåverkade (glutamat) eller inte kunde detekteras (GABA). Tidigare studier har visat att om man blockerar den cellkomponent som serin, glycin och taurin binder in till kan alkohol inte längre öka nivåerna av dopamin. Vi har därför dragit den hypotetiska slutsatsen att även minskade nivåer av dessa signalsubstanser kan förhindra alkoholens dopamin-höjande effekt. Försöken gjordes på råttor som aldrig konsumerat alkohol och endast fick en dos av läkemedlet. Vidare studier bör undersöka vad som händer i alkoholkonsumerande djur och efter en längre tids behandling. En klinisk studie som testar exendin-4 på alkoholberoende patienter är redan igång, men för att bättre kunna ge rätt läkemedel till rätt patient och kunna kombinera olika läkemedel är det av stor nytta att känna till verkningsmekanismerna. Därför ger den här studien ett bidrag till fortsatt läkemedelsutveckling inom ett högt prioriterat område.

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