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The effect of des-acyl ghrelin on alcohol consumption in male and female rats

- in an intermittent access to alcohol model

Degree Project in Medicine

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CONTENT

ABSTRACT

Since the new millenium, multiple studies have investigated the appetite-regulating peptide *ghrelin's* effect on alcohol intake and reward in rodents. Particularly, *ghrelin receptor* (growth hormone secretagogue receptor 1a) *antagonists* have been of interest as they decrease alcohol intake, reward and conditioned place preference in rodents. Recently, attention has been drawn to one of ghrelin's metabolites – *des-acyl ghrelin* (DAG) – a peptide previously thought to be inert. Preclinical studies have reported DAG to have multiple effects, some of which are opposing to ghrelin. As the role of DAG in relation to reward and addiction is unknown, we wanted to study DAGs effect on alcohol intake in rats. We therefore chose to (1) examine the effects of an acute injection with DAG on alcohol intake in male and female rats and (2) investigate how an acute injection with DAG influences locomotor activity in alcohol-naïve male and female rats.

After 12 weeks of voluntary alcohol consumption, 24 male and 24 femalre rats were injected with multiple different doses of DAG. When analysed with one-way ANOVA, no significant difference was noted in alcohol intake. A t-test was performed for the highest dose of DAG (1 mg/kg) and revealed a significant decrease in alcohol intake and preference for both male and female rats. No differences were observed in the locomotor activity experiment at any given dose when analysed with one-way ANOVA.

These findings suggest that DAG decreases alcohol intake in male and female rats and that this decrease is not due to changes in locomotor behaviour. We suggest that DAG should be considered a candidate for further preclinical studies in search of novel treatments for alcohol use disorders.

Keywords: alcohol, AUD, rats, appetite-regulating hormones, ghrelin, des-acyl ghrelin

ABBREVIATIONS

5-HT3-R	5-hydroxytryptamine receptor 3
AUD	Alcohol use disorder
DAG	des-acyl ghrelin
GABAA	γ-aminobutyric acid A receptor
Ghsr gene	growth-hormone secretagogue receptor gene
GHSR-1a	growth hormone secretagogue receptor 1a
GLP-1	glucagone-like peptide-1
GlyR	glycine receptor
GOAT	ghrelin O-acyltransferase
IAASD	intermittent alcohol access study design
LDTg	laterodorsal tegmental area
NaAc	nucleus accumbens
nAChR	nicotinic acetylcholine receptor
NMDAR	N-methyl-D-aspartate-receptors
ob/ob	obese mice
PFC	prefrontal cortex
pLGICs	pentameric ligand-gated ion channels
VTA	ventral tegmental area

DEFINITIONS IN SHORT

Acute treatment	Short-term treatment
Allosteric modulator	A substance which enhances the function of a receptor without binding to the activation site
Anorexigenic	Decreases appetite and food intake
Aversion conditioning	A process when an undesirable behaviour is paired with an unpleaseant stimulus to
Conditioned place preference	An animal may choose between two connected chambers which have been previously paired with two states, eg non-drug and drug state. The preference for a chamber is measured by time freely spent in the chamber.
Face validity	The degree to which an experiment measures what it is supposed to measure
Locomotor activity box	A box with photobeams that measure locomotor activity of a subject by detecting beams broken by the subject
Mesocorticolimbic dopamine system	The neurocircuits which modulate reward
Orexigenic	Increases appetite and food intake
Post-translational modification	Modification of proteins following protein synthesis

INTRODUCTION

Alcohol use disorder (AUD) is one of the most common psychiatric diseases in the world, causing substantial suffering worldwide. Furthermore, alcohol is among the leading risk factors for disability and mortality. According to the Global Burden of Disease report 2016 around 2.8 million deaths were attributed to alcohol use globally.[1] Addiction along with AUD has been widely researched but being a complex and heterogenous disease, uncertainty still exists surrounding the complex neurobiological and psychiatric processes that lead to addiction. Alcohol use disorder (AUD) is a disease characterised by symptoms such as recurrent use of alcohol, loss of control, increased tolerance and withdrawal in absence of the drug.[2] Furthermore, it is not uncommon for the alcohol dependent individuals to consume alcohol in larger amounts or over a longer period than was intended.[2]

As of now, four pharmacological therapies are currently on the market for treatment of AUD. However, studies show these pharmacotherapies have limited or varied efficacy.[3] The demand for novel neurochemical targets is considerable – in order to allow further development of pharmacotherapies. This thesis will focus on one of the promising targets, *des-acyl ghrelin (DAG)*.

Throughout this paper the terms ethanol and alcohol will be used interchangeably and will both refer to the chemical substance ethanol.

Background

The reward system and addiction

The reward system is a term for the parts of the brain that process the experience of reward and the associated learning processes with reward (conditioning and positive reinforcement).[4] Natural rewards include behaviours that may be necessary for survival such as food, water, exercise and sex while extrinsic rewards are often learnt and not necessary for survival, eg alcohol, drugs and gambling.[4] A few major circuits in the brain are important for the processing of reward, namely the mesocorticolimbic dopamine system. [4]

Dopamine is suggested to be one of the main neurotransmitters involved in the mesocorticolimbic dopamine system.[5] The *ventral tegmental area (VTA)* is one of the principal areas in the reward system. It contains the highest levels of dopaminergic neurons in the brain[6] and has projections to the *prefrontal cortex (PFC)*, *nucleus accumbens (NAc)*, *amygdala* and *hippocampus.*[7] The cortical projections (VTA to PFC) are often related to the motivational aspects of reward, while the limbic projections (VTA to amygdala and hippocampus) are related to learning processes and the mesoaccumbal projections (VTA to NAc) to an experience of pleasure.[7] Another important area of the reward system is the *laterodorsal tegmental area (LDTg)* which regulates the dopamine release in the VTA by cholinergic projections.[7,8]

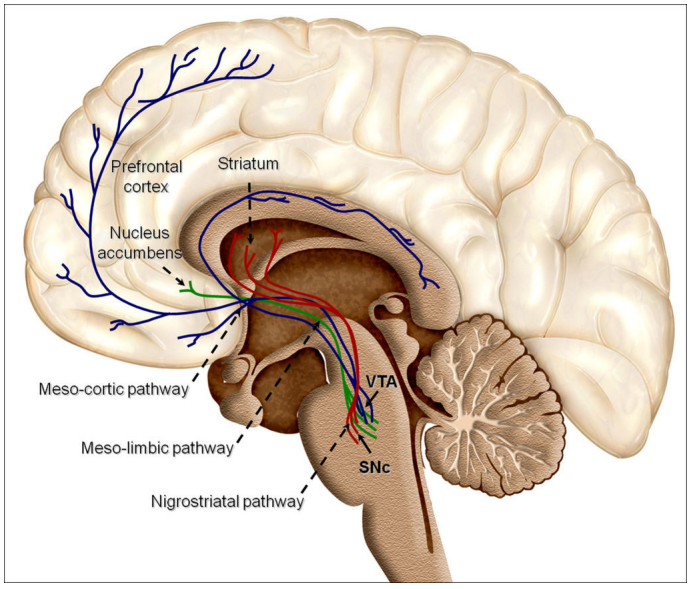

Figure 1: Overview of reward related areas in the brain. From: Oscar Arias-Carrión1, Maria Stamelou, Eric Murillo-Rodríguez, Manuel Menéndez-González and Ernst Pöppel. / CC BY (https://creativecommons.org/licenses/by/3.0)

Alcohol (i.e. ethanol) has been shown to affect reward processes in the VTA, NAc and LDTg.[9,10] Acute alcohol intake increases dopamine release in the NAc[11] while chronic alcohol intake has been shown to decrease

dopamine release[12] as well as the expression of dopamine receptor[12] in NAc in rats. Similar findings have been seen in humans, where long-term alcohol intake has shown to be correlated with lower D2-receptors in dorsal striatum.[13] This is believed to be one of the reasons for withdrawal, craving and relapse.[5,14]

Alcohol

Ethanol is the biochemical name for what is often referred to simply as "alcohol". Ethanol crosses the blood-brain-barrier[15,16] and studies suggest that ethanol blood concentrations are comparable to concentrations in the central nervous system.[17] Among other physiological effects, alcohol has stimulating as well as sedating properties. The stimulation leads to behaviours such as increased mood, increased sociability and increased confidence whereas the depression causes effects such as decreased anxiety, decreased cognitive, sensory and motor function as well as generalized depression of the nervous system with higher intake (loss of consciousness, respiratory dysfunction or arrest and death).[18,19] Chronic effects of ethanol include compensatory effects to maintain the body's homeostasis.[20]

The reason for this broad spectrum of symptoms/effects is ethanol's lack of specificity for a single target.[20] Rather, ethanol has multiple targets of action all throughout the brain. Among others, ethanol binds to a group of receptors called *pentameric ligand-gated ion channels (pLGICs)*, a group which includes receptors such as *γ-aminobutyric acid A receptor ($GABA_A$)*, *nicotinic acetylcholine receptor (nAChR)*, *5-hydroxytryptamine receptor 3 (5-HT3-R)* and *glycine receptor (GlyR)*.[21] However, ethanol also has ethanol-sensitive binding sites on other types of receptors, e.g. on *N-metyl-D-aspartate-receptors (NMDAR)* or *L-type Ca^{2+}-channels*.[21] Caused by this, the numerous targets give a diverse range of symptoms induced by alcohol consumption. However, ethanol does not function as a direct agonist/antagonist on any

receptor. Most commonly it is an allosteric modulator to the different receptors meaning that the receptors function is enhanced in the presence of ethanol, without ethanol binding to the receptors binding site, thus activating it on their own.[22] Ethanol does this either by increasing the probability of an ion channel opening, or by increasing other agonists affinity to the receptor.[21]

Pharmacotherapies against AUD

It is almost 70 years since *Disulfiram* was approved as the first pharmacological treatment against alcohol dependence.[23] Early treatment options for AUD had the aim of helping the affected to complete abstinence from alcohol [24] and Disulfiram had a role in that by aversion conditioning, though it is now prescribed for abstinence support rather than aversion.[24] Disulfiram works by impeding *aldehyde dehydrogenase 2* – an enzyme necessary for the degradation of alcohol. [25] Intake of alcohol and Disulfiram simultaneously leads to an accumulation of acetaldehyde in the body, [26] which gives rise to multiple symptoms such as flushing, sweating, dyspnoea, tachycardia, palpitations, dizziness, headache and nausea. A large intake of alcohol may also lead to more severe symptoms such as hypotension, arrhythmia, seizures and a risk of circular collapse.[27] This is one of the reasons why Disulfiram is not an ideal drug. The risk of severe adverse reactions, which might possibly lead to death, cannot be disregarded, even though the dose today is quite lower than the original treatment regimen[27] Another reason why Disulfiram is not an efficacious treatment for a large population of patients is that Disulfiram does not *per se* decrease craving or reward from alcohol.[24] More practical obstacles of Disulfiram treatment include a requirement of complete abstinence before initiation of treatment and the disadvantage that liver disease is a contraindication.[28]

The newer treatment options for AUD have the advantage to allow administration even with risk of relapse or with an intake of alcohol. [24]

Naltrexone is an opioid antagonist with minimal agonist activity. [24] Opioid receptors are present throughout the mesolimbic dopamine system and opioid receptors can be found on GABA inhibitory neurons that inhibit dopaminergic neuron. [29] The opioid receptors inhibit the GABA inhibitory neurons and thereby increase the dopamine release. [29] By modulating this endogenous opioid system by supplying an opioid antagonist the alcohol-induced reward decreases as the inhibition from the GABAergic neurons increases. [30] The most prominent effect of naltrexone against AUD is by lowering the risk of relapse as well as reducing alcohol consumption [31,32]

Acamprosate has a structure which is very similar to the structure of the neurotransmitters taurine and glutamate.[33] This makes it possible for Acamprosate to work as a modulator for the NMDA-receptor.[34] In chronic alcohol exposure Acamprosate can antagonise the hyperexcitation of the NMDA-receptor which helps with restoring the balance between the excitating glutamate and inhibiting GABA.[34] This has the effect of lowering craving and withdrawal symptoms and thereby preventing relapse. [35]

Nalmefene is the newest of the pharmacological treatments for AUD, being approved in 2013 by the European medicines agency, though it was first developed in the early 1970s. [24] Nalmefene is similar to Naltrexone in its effect on the opioid system, being an antagonist for *mu* and *delta* opioid receptors and a partial agonist for the *kappa* opioid receptor. [36] The most prominent effect of Nalmefene is the lowering of heavy drinking days in a population with AUD and should thus be administered if a patient continues a heavy intake of alcohol after an

initial assessment. [37] The efficacy of Nalmefene is still a controversial subject as studies have showed varied effectiveness. [38-42]

What is common for these four drugs, is the varied efficacy and often their low effect. [3] The low efficacy and limited treatment options have led researchers to investigating new targets of treatments. One of these potential targets, that is currently being researched, are the gut-brain peptides.

Gut-brain peptides

Between the years 1995-2000, several studies reported a significant correlation between the life-time comorbidity of bulimia nervosa and addiction.[43-45]. This fact, as well as the finding of appetite-regulatory peptide receptors in reward-related areas of the brain,[46,47] has led to research on the possibility of overlapping reward regulation between feeding and addictive substances.

To date, several gut-brain peptides have been studied in relation to the brains reward system, such as *ghrelin*, *glucagone-like peptide-1 (GLP-1)*, *amylin* and *neuromedin U* [46,48]. In particular, ghrelin and GLP-1 have been extensively studied, with ghrelin increasing, – and GLP-1 decreasing - drug intake and reward. However, there is lack of studies on desacyl ghrelin, (DAG), which recently was believed to be an inactive peptide.

Ghrelin and DAG

Both ghrelin and DAG are derived from the *Growth hormone secretagogue receptor gene (Ghsr gene)*. The Ghrs gene is transcribed into three different mRNAs and one of the pathways is the subject of interest in the present paper. The Ghsr gene is first transcribed to ghrelin mRNA which then is translated into preproghrelin. Preproghrelin is subsequently cleaved into proghrelin. Early evidence pointed towards proghrelin being cleaved again to

DAG or C-ghrelin and then being subjected to post-translational modification by the *ghrelin O-acyltransferase* (GOAT) which acylates the hydroxyl group at *Ser3*, forming ghrelin.[49,50] However, recent studies suggest that proteolytic cleavage is not necessary for the post-translational modification by GOAT. *Zhu et al.* reported finding des-acyl proghrelin but not deacyl-ghrelin during the biosynthesis of ghrelin which suggests that DAG is not an intermediate in this process.[51,52] It has been established that DAG is formed with the deacylation of ghrelin in plasma but the exact synthesis of DAG in tissue has not been discovered. Nonetheless, it is certain that DAG is present in various tissues, such as throughout the GI-tract and in parts of the brain.

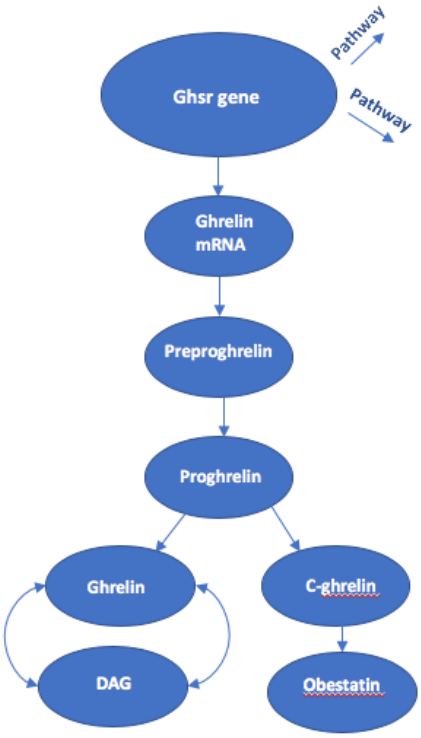

Figure 2: Overview of DAG/ghrelin-synthesis.

Ghrelin, DAG and C-ghrelin have all been found in human plasma, while studies regarding the detection of obestatin in plasma differ[53-55]. It has been established that the main production site of ghrelin is the gastrointestinal tract, with the largest synthesis *in X/A-like cells* in the gastric fundus [56]. Besides the GI-tissues, peripheral ghrelin production has also been identified in tissues such as the pancreatic islets, adrenal glands, placenta, lungs and liver [57,58].

As ghrelin has a wide distribution in the body, it also has a wide range of functions. One of ghrelin's most prominent functions is the regulation of energy homeostasis, particularly its orexigenic effect, with increase of appetite and increase of food intake.[59] Ghrelin also has a role in growth-hormone release, glucose regulation (decreases insulin secretion and increases blood glucose), lipid metabolism, gastric acid secretion, gastric movement and bone metabolism.[59,60]

The elimination of ghrelin and DAG is closely linked. After entering the circulation, a considerable amount of ghrelin is converted back to its deacylated form [49], an effect which has also been noted in collected blood samples [61]. The DAG/ghrelin-ratio has been found to be around 2:1 in the stomach compared to 9:1 in plasma and it is believed this is not only due to deacylation but also to ghrelin binding systemic GHSR-1a receptors. Thus, the elimination of DAG seems not to be insignificant for the elimination of ghrelin. A study by *Yoshimoto et al.*[62] reports that DAG-levels in plasma have a significant correlation with the serum-creatinin levels in patients with mild to severe renal failure. Furthermore, DAG-levels rise considerably after a bilateral nephrectomy in mice, without significant changes in gastric production of ghrelin. Few studies have investigated the subject, but this finding suggests the kidney's involvement in the elimination of DAG, though further studies will be needed for clarification.

Ghrelin's best studied receptor is the *growth hormone secretagogue receptor 1a* (GHSR-1a), which is found both centrally and peripherally. In the brain, GHSR-1a is found primarily in the pituitary and hypothalamus, but is also found in lower amounts in other parts of the brain, such as reward-related areas. [58] Sometimes called the "ghrelin receptor", the GHSR-1a seems to be necessary for ghrelin's effect on reward. [63] Additionally, the acylation of ghrelin is essential for ghrelin binding to GHSR-1a´s activation site. Therefore, DAG is not considered an agonist for GHSR-1a, even though studies have found that DAG might have a certain effect at supra-physiological concentrations. [50] At this time, no specific DAG-receptor has been molecularly identified, despite the fact that many studies indicate one may exist. [49,50,64]

Ghrelin's impact on reward and dependence

Several preclinical studies have focused on ghrelin's effect on reward. A study in mice, reported increased dopamine levels in nucleus accumbens and enhanced locomotor activity when given central injections of ghrelin. This is explained as the activation of the reward-associated mesoaccumbal dopamine system.[65] Interestingly, the effect of the ghrelin administration was prevented when the mice were simultaneously given peripheral injection of an unselective nicotinic antagonist. This implies that ghrelin's effect is mediated via involvement of cholinergic afferents to the ventral tegmental area.[65] In further support for a modulatory role in reward are the findings revealing that alcohol-induced locomotor stimulation, accumbal dopamine release and conditioned place preference was nullified in GHSR-1a knock out mice, as well as mice treated with either of two different GHSR-1a antagonists[66]. Additionally, a study examining ghrelin knockout mice reported decreased alcohol-induced locomotor activity and accumbal dopamine release, although the effect was not as strong as in GHSR-1a knockout mice.[67] This can be explained by that the GHSR-1a *per se* having significant constitutive activity without activation being required. [67] Similarly, other studies examining GHSR-1a antagonists have found a decrease in alcohol-induced locomotor stimulation and alcohol intake in different subjects.[68-71]

Physiological properties of DAG

Few studies have investigated DAG's relation to addiction and reward. Only in the last few years, studies have directly addressed the function and physiology of DAG, as DAG was previously believed to be solely an inactive form of ghrelin. Even if research on DAG is limited, and the results are not conclusive, a diverse range of effects have been attributed to DAG, from opposing to mimicking ghrelin's effects and even have an effect on cells where ghrelin does not.[72]

The results of a number of studies that have investigated DAG's effects and receptors are inconsistent and contradictory. It is probable that DAGs effect is not mediated by GHSR-1a,

that is, does not work as an agonist at physiological levels. [50,73] It is, however, probable that DAG affects cells through other receptors than GHSR-1a. Recent evidence shows DAG and ghrelin having effect on tissues without GHSR-1a being involved. One study by *Thompson et al.* on rats, studied bone marrow adiposity after treatment with either ghrelin, DAG or a GHSR-1a agonist (L-163,255), reporting an increase with both ghrelin and DAG treatment, but not with the GHSR-1a agonist. This, together with the fact that the dose (720 ng/day) injected into the tibial marrow cavity was the same for both ghrelin and DAG, suggests that the increase in bone marrow adiposity was not mediated via GHSR-1a.[74] *Togliatti et al.* published in 2002 a study from where systemic administration of an infusion of DAG (3 μg/kg/h) but not ghrelin (1 μg/kg/hour) attenuated diabetes-induced circulating *endothelial progenitor cell* damage in humans with diabetes type II and *ob/ob* mice, increasing the potential of vascular regenerative processes.[75] Therefore, it seems like there is a receptor or receptors which are stimulated by DAG or by both DAG and ghrelin at similar concentrations.[72]

There is considerable discrepancy in research on DAG, on one hand, and ghrelin on the other hand, when it comes to these agents' effect on appetite and food intake. Research on ghrelin indicates that it has clear effect on both.[59] In the case of DAG, the results are inconclusive. A study by *Asakawa et al.* and a study by *Chen et al.* showed decreased food intake after an intraperitoneal injection of DAG in fasted rodents (mice and rats)[64,76], as well as intracisternal injections [77]. Other studies however, in rodents and goldfish, have found no difference in food intake with or without fasting,[78-81] though studies have found that the administration of DAG together with ghrelin did not increase food intake compared to only administration of ghrelin.[79,80] A study of transgenic mice overexpressing DAG was conducted, showing that they had 10-44-fold higher levels of DAG in plasma than control mice, but nonetheless the

same levels of ghrelin. Compared to controls, these transgenic mice were shorter, measured by nose-to-anus, had a lower body weight and food intake. No difference could be seen in BMI or food intake per unit body weight. To be noted, the transgenic mice also had lower serum-IGF-1 levels seeming that the smaller phenotype is a result of lower food intake cannot be confirmed. [82]

Thus, it is not possible to draw certain conclusions, but there appears to be an inclination towards DAG having an anorexigenic effect.[49,83] If that is the fact, DAG may also have an effect on the reward system related to drugs.

Up to date, no research has been conducted on the connection between DAG and drugs, including alcohol. Therefore, it is of large interest to study a potential effect of DAG on drug-induced reward. With alcohol clearly being connected to appetite-regulating hormones, it is important to contribute to this growing area of research, exploring DAGs effect on alcohol intake and reward in rats.[82]

AIMS

The aims of the present thesis are:

1. To examine the effects of an acute injection with DAG on alcohol intake in male and female rats.
2. To investigate how an acute injection with DAG influences locomotor activity in alcohol-naïve male and female rats

MATERIALS AND METHODS

Study samples

We conducted two different, but related experiments; a locomotor activity experiment and an alcohol intake experiment. Both of these are based on animal models. The study and identification of potential novel pharmacological targets for treatment for addiction widely depend on animal models, and as rats respond similarly to alcohol and gut-brain peptides as humans,[84,85] they provide a useful tool in the study of addiction. The rats were males and females of the type Rcc Han Wistar. Since the physiological properties of DAG are not fully known and no similar studies of DAG have yet been conducted, the experiments were performed on both sexes and analysed separately to be able to detect any not yet known differences.

The rats were single-caged during 12 weeks, and as rats are social animals, being single-caged during a long time is considered a substantial stress factor. Therefore the severity of this experiment was classified as severe according to Directive 2010/63/EU on the protection of animals used for scientific purposes.[86] Nevertheless, as measuring alcohol intake with multiple rats in one cage is practically impossible, the single-cage model was chosen. All experiments were performed using as few animals as possible with the intention of minimising the suffering the rats were subjected to. Additionally, humane and experimental endpoints were continuously taken into consideration during the experiments.

The Locomotor Activity Study

For a first assessment of DAGs effect on the mesolimbic dopamine system, we performed a locomotor activity experiment. This is relevant to examine the effect of various doses on behaviour and thereby limit the possibility to use a dose of DAG to alcohol drinking rats, with an effect *per se* on behaviour. Alcohol has been known to increase locomotor activity, as a reflection of its effect on the mesolimbic dopamine system,[87] and in case of further studies it is essential to know the effect of DAG on locomotion. The locomotor experiment was conducted in six locomotor activity boxes (Open Field Activity System, Med Associates Inc; Georgia, Vermont, USA) on 12 male and 12 femalt alcohol-naïve Rcc-Han Wistar-rats.

In order to examine the effect of the different doses, the rats were divided into four groups based on the dose given: vehicle, 0.5mg/kg DAG, 1 mg/kg DAG or 2 mg/kg DAG. The total time recorded was 150 minutes, which consisted of a 60-minute habituation time following an injection of vehicle or DAG, after that 30 minutes of between injection time and finally, 60 minutes of recorded session time. The parameters analysed are shown in Table 1.

Table 1: Measured variables

Locomotor experiment, measured variables
Ambulatory distance*
Ambulatory episodes
Stereotypic counts
Jump counts
Zone entries
Zone session time
Verical counts
Average velocity

*** measured in cm distances/5min.**

Ambulatory distance was chosen as it is an indirect measurement of activation of the mesolimbic dopamine system. To investigate whether DAG affects anxiety-related behaviours such as thigmotaxis – we divided the locomotor field in an inner and outer zone where the outer zone was closer to the walls. In rodents, increased thigmotaxis – the inclination to remain close to the walls – correlates to increased anxiety.[88] The parameters of zone session time (inner zone and outer zone) and zone entries may therefore reflect differences in anxiety-related behaviour between the groups. Ambulatory episodes, stereotypic counts, jump counts, vertical counts and average velocity are parameters

that reflect stereotypy – that is abnormally repetitive behaviour related to neurological dysfunction.[89]

Intermittent Alcohol Access Study

An alcohol intake-experiment according to the intermittent alcohol access paradigm, using Rcc Han Wistar-rats– 24 males and 24 females – performed over a period of 12 weeks was also conducted. The intermittent alcohol access study design (IAASD) is a validated method to ensure adequate blood-alcohol concentration levels.[90] As shown by Simms et al. intermittent alcohol access leads to Wistar rats consuming high amounts of alcohol voluntarily, without the use of initiation procedures regarding continuous alcohol access. Consequently, this is the optimal method for the experiment.[90]

In this study, the rats were kept single-housed under stable temperature and humidity conditions (20°C and 50%) and were allowed to habituate for one week after arrival to the animal facilities. Water and food were provided *ad libitum*. During two 24-hour periods every week (Monday, Wednesday) and one 7-hour period (Friday), the subjects had unlimited access to a bottle of 20% ethanol-water solution, as well as a bottle of tap water. The remaining days the subjects had unlimited access to two bottles of tap water only. Water-, alcohol and food intake were measured after each period of alcohol/water-exchange and the subjects were weighed once a week. This was conducted over a 10-week period. Rats were the divided into treatment groups based on their average alcohol intake (Monday, Wednesday and Friday), ensuring that rats in future treatment groups had consumed similar levels of alcohol during the initial 10 weeks. The male and female rats were then on one occasion administered either vehicle or various doses of DAG (0.25 mg/kg, 0.5 mg/kg and 1 mg/kg).

Water- alcohol- and food intake was measured after 1, 4 and 24 hours after the administration of DAG.

Statistics

The results on alcohol intake at the different points of time were analysed using one-way ANOVA. Further analysis was performed with unpaired t-test to evaluate the effect of the separate doses. *Prism 8 GraphPad (GraphPad Software, San Diego) was* used for all statistical analyses. The results were considered statistically significant with a *p-value* <0,05.

Ethical permission

The experiments reported in this master thesis were approved by the *Regional Ethical Committee on Animal Research in Gothenburg* (registration number 1556/18 and 1457/18) and were conducted according to the *Swedish Animal Welfare act*.

RESULTS

Locomotor Activity Study

There were no significant differences in habituation behaviour in male nor female rats treated with DAG (**Figure 3; Figure 4**). As further shown in **Figure 3 and Figure 4**, DAG treatment did not influence ambulatory distance, stereotypic counts, jump counts, zone entries, time in outer zone, time in inner zone, the percentage of time in inner zone through total time, vertical counts or average velocity during running time of the experiment. A one-way ANOVA was used to calculate variance between the groups. For details, see Complimentary data, tables and figure on page 39-40.

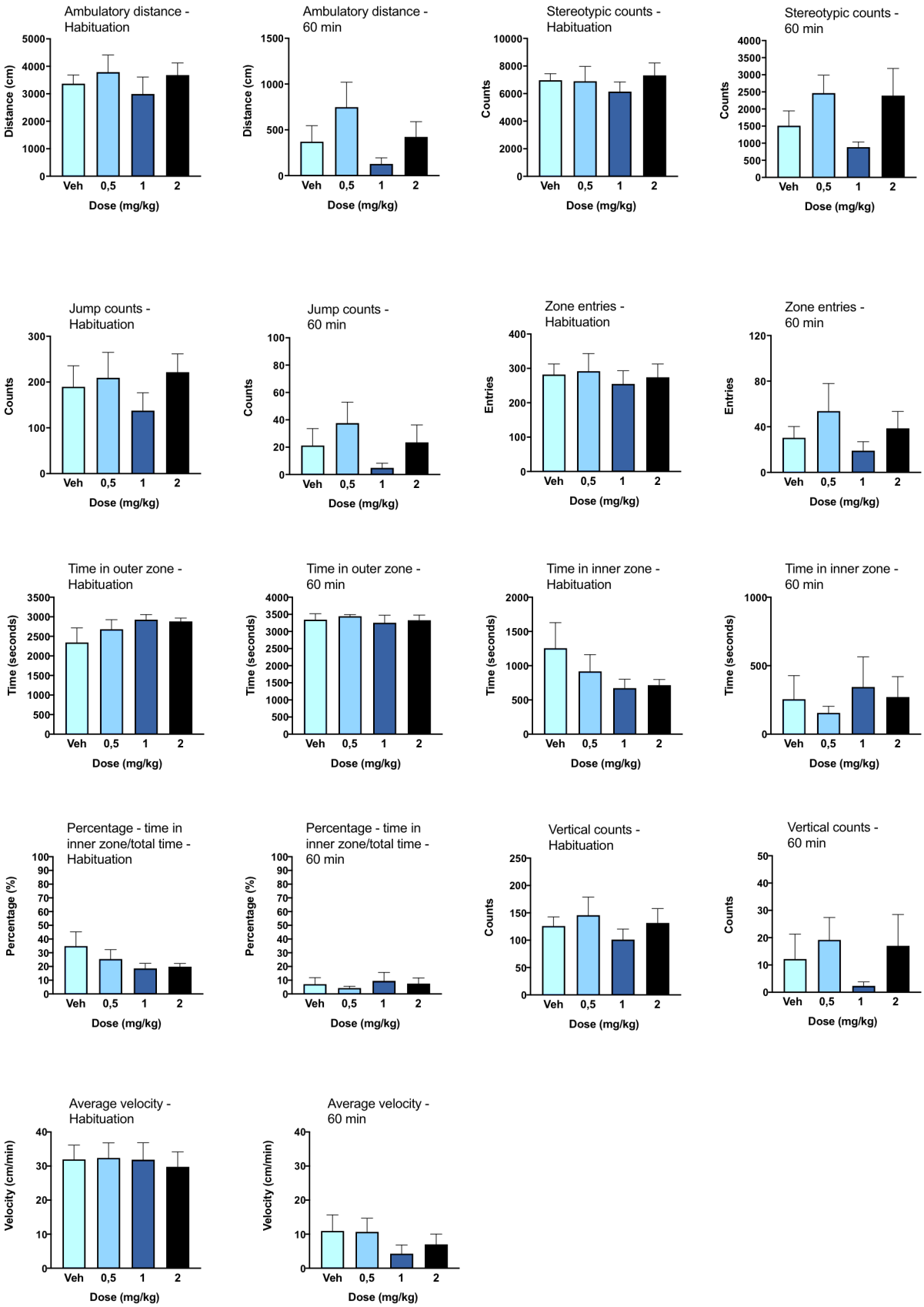

Figure 3: Effects of different doses of DAG-administration (0,5 mg/kg, 1 mg/kg, 2 mg/kg) or vehicle on locomotor activity measured by various parameters at time of habituation and 60 minutes, starting 30 minutes after administration of DAG, in female rats, analysed with one-way ANOVA. No significant differences between the various doses or vehicle was seen at any time.

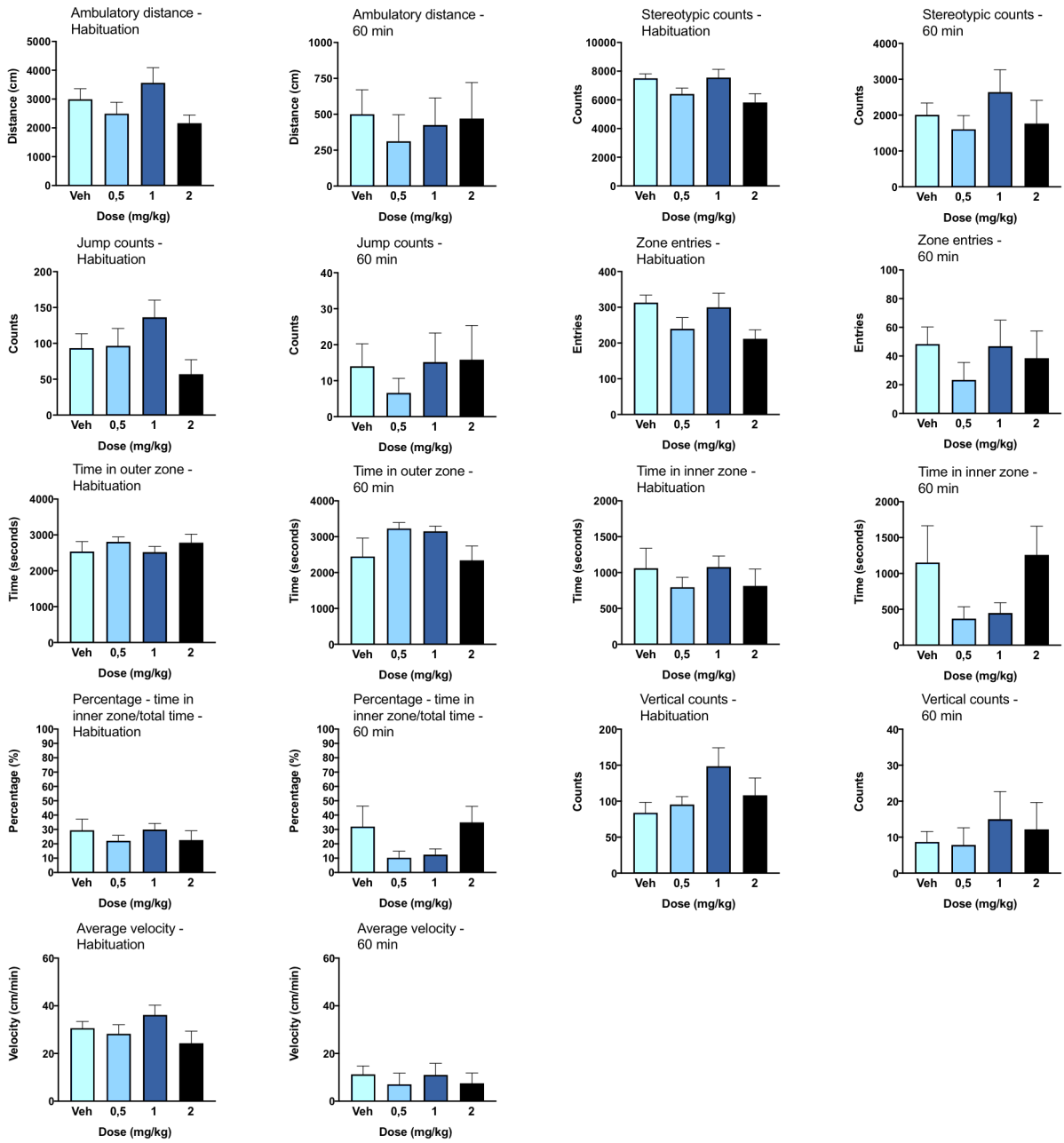

Figure 4: Effects of different doses of DAG-administration (0,5 mg/kg, 1 mg/kg, 2 mg/kg) or vehicle on locomotor activity measured by various parameters at time of habituation and 60 minutes, starting 30 minutes after administration of DAG, in male rats, analysed with one-way ANOVA. No significant differences between the various doses or vehicle was seen at any time.

Intermittent Alcohol Access Study

No difference in baseline ethanol intake (F(3,18)=0.34, P=0.7995) was detected between the rats assigned to different treatment groups (male: vehicle n=6, 0.25mg/kg n=60.5 mg/kg n=5, 1mg/kg n= 5; female: vehicle n=6, 0.25mg/kg n= 6, 0.5mg/kg n=6, 1mg/kg n=5).

In male rats, no significant effect of the various doses of DAG or vehicle was seen on ethanol intake, neither between 0-1 hr (F(3,18)=2.69, P=0.0768; **Figure 5A**), 1-4 hrs (F(3,18)=0.16, P=0.9221;**Figure 5B**) nor 4-24 hrs (F(3,18)=2.54, P=0.0885; **Figure 5C**). However, it was noted that the analysis revealed an inclination to overall effect of treatment, particularly with the highest dose of DAG (1mg/kg).

In ethanol intake of female rats, no significant treatment effect was seen at 0-1 hr (F(3,19)=0.39, P=0.7632; **Figure 6A**), at 1-4 hrs (F(3,19)=1.69, P=0.2025; **Figure 6B**) nor at 4-24 hrs (F(3,19)=0.28, P=0.8424; **Figure 6C**). Comparable to the male rats, an inclination to overall effect of treatment was seen, mostly so with the highest dose at 1 mg/kg at the 4-24 hrs interval.

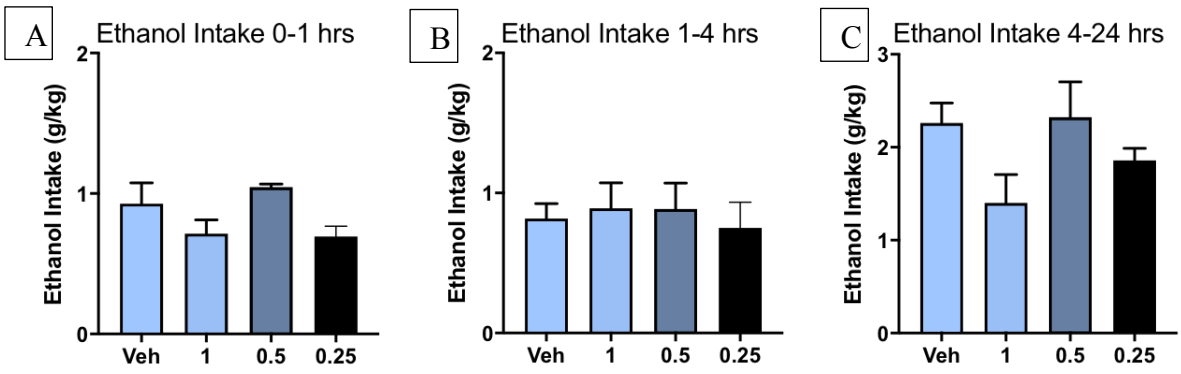

Figure 5A-C: Effects of various doses of DAG (0.25 mg/kg, 0.5 mg/kg, 1 mg/kg) or vehicle on ethanol intake at (A) 0-1 hr, (B) 1-4 hr and (C) 4-24 hr, male rats, analysed with one-way ANOVA. None of the doses were significant at any time. However, at 4-24 hr (xC) the analysis revealed a tendency to overall effect of treatment.

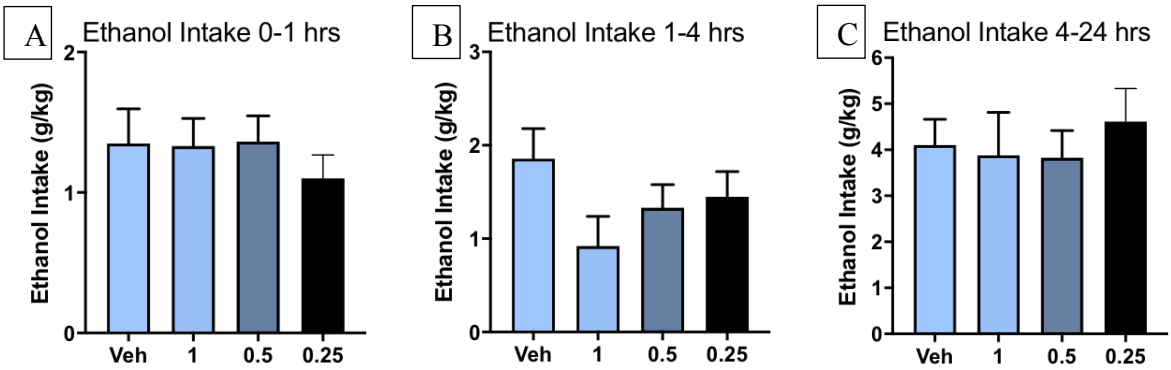

Figure 6 A-C: Effects of various doses of DAG (0.25 mg/kg, 0.5 mg/kg, 1 mg/kg) or vehicle on ethanol intake at (A) 0-1 hr, (B) 1-4 hr and (C) 4-24 hr, female rats, analysed with one-way ANOVA. None of the doses were significant at any time. However, at 1-4 hr (xC) the analysis revealed a tendency to overall effect of treatment.

As the same trend of effect was revealed in both male and female rats, further unpaired t-tests were performed, analysing the differences between the most promising dose of DAG (1mg/kg) and vehicle. The parameters analysed with the unpaired t-test were as follows: ethanol intake, ethanol preference, water intake, total fluid intake, food intake, rat weight and rat weight differences.

No significant difference in ethanol intake was seen between the groups at 0-1 hrs ($t(9)=1.134$, $P=0.1431$; **Figure 7A**) or at 1-4 hrs ($t(9)=0.361$, $P=0.3632$; **Figure 7B**). A significant decrease of ethanol intake was seen in male rats treated with 1mg/kg DAG at 4-24 hrs ($t(9)=2.352$, P0.0216; **Figure 7C**). Corresponding, no difference was seen at 0-1 hr ($t(9)=1.585$, $P=0.0737$; **Figure 5D**), an increase in alcohol preference was found at 1-4 hrs ($t(9)=1.844$, P0.0492; **Figure 5E**) while a decrease in alcohol preference was found at 4-24 hrs ($t(9)=3.001$, $P=0.0075$; **Figure 5F**) for the DAG-treated rats. An increase in water intake in the group treated with DAG 1 mg/kg was also found at 1-4 hrs ($t(9)=1.902$, $P=0.0448$; **Figure 5H**) while there were no differences in intake at 0-1 hr ($t(9)=0.824$, $P=0.2155$; **Figure 5G**) or at 4-24 hrs ($t(9)=1.712$, $P=0.0606$; **Figure 5I**). As shown in **Figures 5J-L**) no differences in total fluid intake was seen at any time. Food intake was similar between the two groups at 0-1 hr ($t(9)=0.098$, $P=0.4620$; **Figure 5M**) and 1-4 hrs ($t(9)=1.509$, $P=0.0828$; **Figure 5N**) while an

increase was noted at 4-24 hours (t(9)2.637, P=0.0135; **Figure 5O**) for the group treated with DAG.

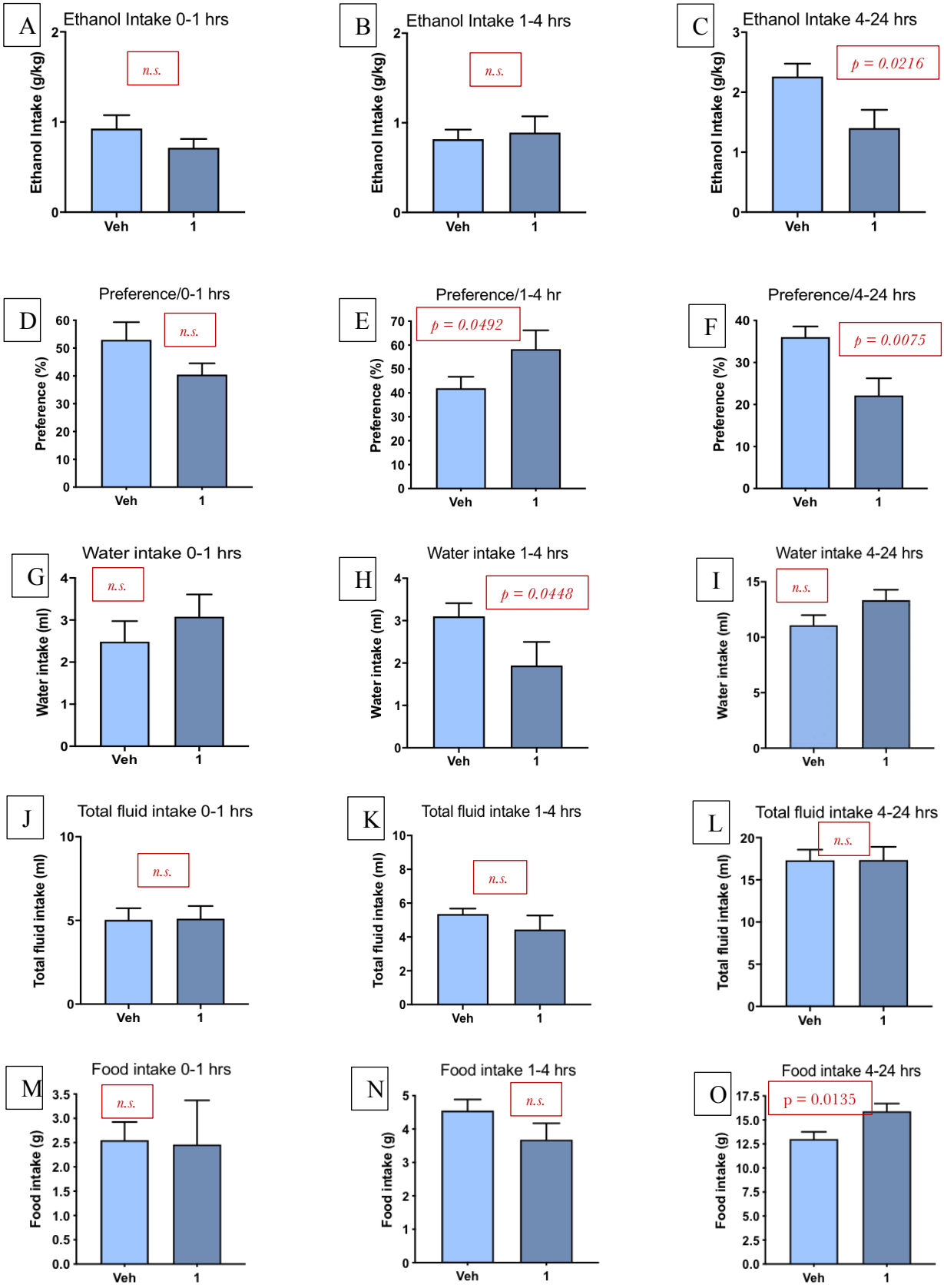

Figure 7: Effects of DAG-administration of 1 mg/kg or vehicle on ethanol intake, ethanol preference, water intake, total fluid intake and food intake at 0-1 hrs, 1-4 hrs and 4-24 hrs, in male rats, analysed with unpaired t-test. Significant decrease in ethanol intake as well as ethanol preference and increase in food intake was seen at 4-24 hrs. Significant decrease in water intake and increase in ethanol preference was seen at 1-4 hrs. N.s. = non-significant.

Concerning the results for the female rats, illustrated in **Figure 8A-O**, ethanol intake was significantly decreased at 1-4 hrs in the group treated with 1mg/kg DAG ($t(9)=2.054$, $P=0.0351$; **Figure 8B**). Correspondingly, ethanol preference was decreased in the DAG-treated group at the same time ($t(9)=0.216$, $P=0.0294$; **Figure 8E**). No other differences between the groups were seen at any time in ethanol intake (**Figure 8A-C**), ethanol preference (**Figure 8D-F**), water intake (**Figure 8G-I**), total fluid intake (**Figure 8J-L**) nor food intake **(Figure 8M-O**).

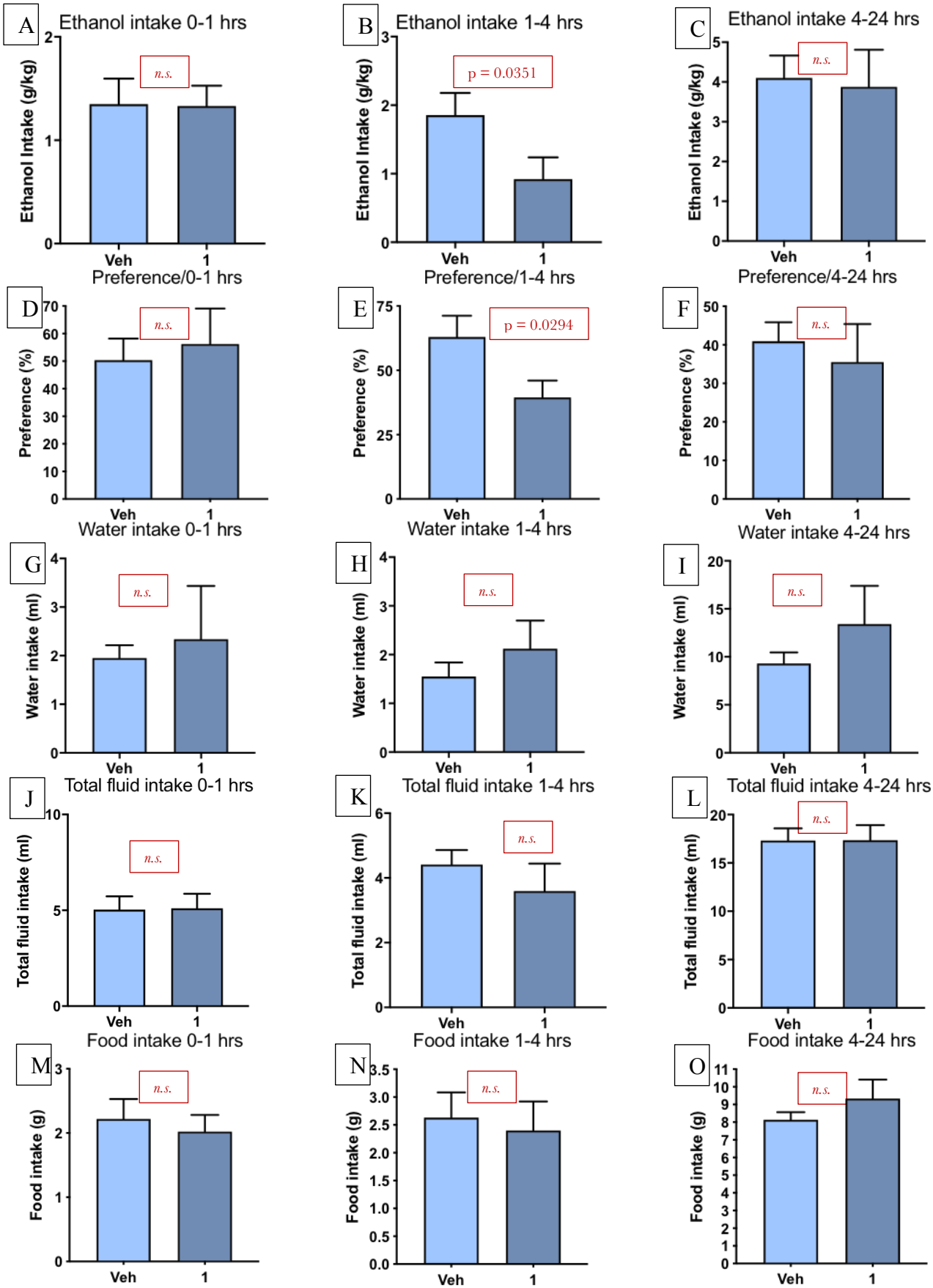

Figure 6 A-O: Effects of DAG-administration of 1 mg/kg or vehicle on ethanol intake, ethanol preference, water intake, total fluid intake and food intake at 0-1 hrs, 1-4 hrs and 4-24 hrs, in female rats, analysed with unpaired t-test. Significant decrease in ethanol intake as well as ethanol preference was seen at 1-4 hrs. N.s. = non-significant.

DISCUSSION

Main results

Intermittent Alcohol Access Study

The main findings of the present study shows no significant overall effect of the DAG treatment when analysed with one-way ANOVA. However, when analysed with t-test, in rats treated with 1mg/kg DAG, a significant decrease in both ethanol intake and preference was seen in male rats at 4 - 24 hrs, with a significant decrease in female rats at 1 - 4 hrs respectively. This suggests that there is an effect on ethanol intake with acute treatment of high doses of DAG, even if the decrease is not large enough to show an overall effect on the treatment. As a significant effect was only noted at the highest dose, it is possible the doses given were not large enough. Being the first study of this kind, there is neither experience nor evidence to depend on, predicting the optimal dose for an optimal effect. However, the results establish that DAG-treatment indeed, does affect alcohol intake in Wistar rats. It is also likely, that if the subjects had been more numerous, the statistical model would have been stronger.

As no other published studies have been performed on DAGs effect on alcohol intake, a comparison of the results of the present study is not possible to make. However, the decrease in alcohol intake following DAG-treatment was not unexpected. As previously stated, several studies indicate that DAG works as an anorexigenic hormone.[49,50] Seeing that studies suggest suggest several anorexigenic gut-brain hormones lower alcohol intake in rats,[48,46] it is reasonable that DAG does as well. Furthermore, in unpublished data we have seen that DAG blocks increased alcohol-induced locomotor activity, decreases alcohol-induced conditioned place preference and decreases alcohol-induced dopamine release in NAc. This indicates that DAG decreases the reward induced from alcohol and as rats largely drink alcohol for the experienced euphoria it is plausible the alcohol intake decreases when treated with DAG.

Even though comparison to other studies investigating DAG cannot be made, there is the possibility to compare the results to studies that have investigated GHSR-1a antagonists, which is an agent, thought to operate in a similar way as DAG. A study by *Suchankova et al.* investigated the GHSR-1a antagonist *JMV2959.*[68] This study operated according to IAASD, where male Wistar rats consumed 20% alcohol for 2 and 5 months, following acute treatment with JMV2959 as well as repeated treatment after 8 months. Results reported significant decrease in alcohol intake, both in acute treatment, repeated treatment and after alcohol deprivation. The study design is similar to the experiment in this paper, where the rats followed the IAASD for 2 months as well. However, as this study does not study DAG, the results are not quite comparable. Nonetheless, some parallells might be drawn between the studies. Interestingly, the JMV2959-study saw increased significant effect on the intake of rats that had followed the IAASD for 5 months before treatment as well as with repeated administration. Even if DAG is not an antagonist to GHSR-1a at physiological levels, it is possible that DAGs effects behave in a similar way with increased effect after longer time of drinking alcohol before treatment or increased effect with repeated administration. However, by increasing the dose of DAG it is possible that it would have an effect of GHSR-1a but by instead activating it, and in that way counteract the effect that decreases the alcohol intake of Wistar rats.[50] Further studies, with focus on higher doses of DAG, longer alcohol treatment and/or repeated measures will be needed to further elucidate DAGs effect on alcohol intake.

Another finding in the present study are the differences between male and female rats concerning the time frame of the DAG effect. The decrease in ethanol intake and preference was seen at 4 - 24 hours in the male group, whereas it was seen at 1-4 hours in the female group respectively. There could be several reasons for this difference. DAG has previously been shown to cross the blood-brain barrier in an unsaturable manner in mice and rats[64,91] but with some difference in the rate between human and mice ghrelin as well as DAG. No studies

have investigated whether there is a difference in blood-brain crossing rate between male and female rats, but this can not be excluded as a factor for the observed differences.

It is also not impossible that oestrogen affects the effect of DAG or its metabolism. Studies examining ghrelin have reported oestrogen-modulated expression of ghrelin in rat stomach[92] as well as oestrogen-dependent decrease in the orexigenic effects of ghrelin[93]. No studies on this subject has been performed for DAG, and can be a subject for future studies.

Another unanticipated difference between the male and female groups was the decrease in food intake observed in males between 4-24 hours (same interval as the decrease in alcohol intake), whereas no difference in food intake was observed in females. A similarity can be found in a study, which revealed that ghrelins orexigenic potency decreased significantly in non-ovarectomised female rats, as well as when estradiol was administrated to ovarectomised female rats.[93] It is possible that DAGs anorexigenic effects are similarly affected by oestrogen.

Locomotor Activity Experiment

The main results of the locomotor activity experiment was that no dose of DAG affected any of the parameters examined compared to vehicle. This was expected and in line with previous studies done on DAG, even if none of them have studied locomotor activity in rodents. A study examining ghrelins and DAGs effect on locomotor activity in goldfish, saw increased locomotor activity with ghrelin-injection intraperitoneally and intracerebroventricularly, but not with a DAG-injection in the same locations.[94] Our results from the locomotor activity study suggest that peripheral injections of DAG do not affect the mesolimbic dopamine system on its own. Our locomotor activity experiment did not evaluate centrally administered DAG and can therefore not be compared directly to the goldfish studies.

The locomotor activity experiment was important to elucidate any changes in male or female rats' behaviour when treated with DAG. This is to make sure that any differences found in the intermittent alcohol access study is not driven by changes in behaviour from the DAG-treatment. As no such differences in behaviour were found, neither with male nor female rats, we can conclude that the differences in alcohol intake is not due to changes in behaviour, eg sedation.

Strengths and weaknesses

The present study is the first of its kind with the aim of examining whether DAG has an effect on alcohol intake in Wistar rats, and furthermore if there is a reason to investigate this peptide further. The study investigates acute treatment with DAG on alcohol intake, which gives a basic, if not complete, indication of its effect. The study is designed for this purpose. Preclinical animal studies have long been a crucial part of addiction research.[95] Addiction research with rats as subjects is generally considered the preferable choice, as rats display many of the behaviours correlated with dependence in humans.[85] Non-operant self-administration models such as the IAASD show high face validity[84] and have been proven an important tool in identifying drugs that are now on the market, such as Naltrexone and Acamprosate.[96]

AUD is a complex neuropsychiatric disorder. One study examining DAGs acute effect on alcohol intake in Wistar rats, can not give a complete answer if DAG can be used as a novel target for pharmacotherapies against AUD. Hopefully however, it can be one of more building blocks in the search for treatment of this common and challenging disorder.

CONCLUSIONS

Acute administration with 1 mg/kg DAG decreases alcohol intake in rats. Furthermore, DAG on its own does not affect behaviour in male nor female rats, as measured by locomotor activity. These promising results however are solely the starting point in research of DAG and addiction. Before a definitive answer on DAGs effect on alcohol intake and dependence can be concluded, more research will have to be conducted. We suggest further preclinical studies investigating repeated administration with DAG, its effect on the alcohol deprivation effect as well as microdialysis studies on DAG and DAG in combination with alcohol.

This thesis argues that DAG is a substance that is worth further investigation as a promising novel target for new treatments against AUD.

SVENSK POPULÄRVETENSKAPLIG SAMMANFATTNING

Desacyl-ghrelins påverkan på han- och honråttors alkoholkonsumtion

Alkoholberoende är en av de vanligaste psykiatriska sjukdomarna i världen. Det är en komplex neuropsykiatrisk sjukdom som orsakar mycket lidande, hög sjukdomsbörda samt betydande dödlighet. Trots detta finns det bara fyra läkemedel på marknaden och som ofta endast har en begränsad effekt. Sedan millennieskiftet har man börjat studera kopplingen mellan aptitreglerande hormoner och alkoholberoende. Detta eftersom att man bl.a. har hittat receptorer för aptit-reglerande hormoner i belöningscentra i hjärnan. Sammanfattningsvis tyder forskningen på att det finns ett överlapp mellan reglering av belöning från mat och alkohol.

Ett av hormonerna som har studerats är *ghrelin* – ett hormon som bl.a. ökar aptiten – och har setts öka alkoholintag och belöning. Nyligen har man börjat studera desacyl-ghrelin (DAG) – en av ghrelins nedbrytningsprodukter – som till viss del har effekter motsatta till ghrelin. Därför ville vi undersöka om DAG påverkar alkoholintag hos han- och honråttor. Dessutom ville vi undersöka om DAG påverkar den motoriska aktiviteten i han- och honråttor.

Vi gjorde två experiment. I ett av experimenten hade vi både han- och honråttor som fick tillgång till en alkohol-vattenlösning tre dagar i veckan under 12 veckor. Efter dessa 12 veckor delades de in i grupper där varje grupp fick en injektion av en av tre doser DAG eller saltlösning. Vi mätte därefter alkohol-, vatten- och matintag efter 1, 4 och 24 timmar.

I det andra experimentet hade vi han- och honråttor som fick en injektion av en av tre doser DAG eller saltlösning innan vi mätte deras motoriska aktivitet i aktivitetsboxar.

I det första experimentet såg vi först ingen skillnad på alkoholintag när vi tittade på alla doser. Däremot, när vi jämförde den högsta dosen med saltlösning såg vi en minskning i alkoholintag mellan 1:a och 4:e timmen hos honorna och mellan 4:e och 24:e timmen hos hanarna. I det andra experimentet såg vi ingen skillnad i den motorisk aktiviteten beroende på dos.

Detta säger oss att vi såg en minskning i alkoholintag och den uppmätta minskningen beror inte på skillnad i motorisk aktivitet, t.ex. sedering – som gör att råttorna blir trötta och då dricker mindre.

Denna studien är dock inte designad för att ge oss ett fullständigt svar på hur DAG påverkar alkoholintag i råttor - den ger oss en basal och relativt grov förståelse för DAGs effekter på alkoholintag. Sammanfattningsvis så har vi fått resultat som tyder på att DAG minskar alkoholintag i han- och honråttor. Av detta drar vi slutsatsen att DAG är en substans som är värd att studera vidare för en mer utförlig förståelse av dess effekter samt för att belysa om DAG är en potentiell farmakologisk behandling mot alkoholberoende.

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REFERENCES

1. Life expectancy and disease burden in the Nordic countries: results from the Global Burden of Diseases, Injuries, and Risk Factors Study 2017. Lancet Public Health 2019;4:e658-e69.

2. Association AP. Diagnostic and Statistical Manual of Mental Disorders. 5th ed. Washington, DC2013.

3. Farokhnia M, Browning BD, Leggio L. Prospects for pharmacotherapies to treat alcohol use disorder: an update on recent human studies. Curr Opin Psychiatry 2019;32:255-65.

4. Berridge Kent C, Kringelbach Morten L. Pleasure Systems in the Brain. Neuron 2015;86:646-64.

5. Everitt BJ, Dickinson A, Robbins TW. The neuropsychological basis of addictive behaviour. Brain Research Reviews 2001;36:129-38.

6. Kalivas PW. Neurotransmitter regulation of dopamine neurons in the ventral tegmental area. Brain Research Reviews 1993;18:75-113.

7. Russo SJ, Nestler EJ. The brain reward circuitry in mood disorders. Nature Reviews Neuroscience 2013;14:609-25.

8. Dobbs LK, Mark GP. Acetylcholine from the mesopontine tegmental nuclei differentially affects methamphetamine induced locomotor activity and neurotransmitter levels in the mesolimbic pathway. Behav Brain Res 2012;226:224-34.

9. Gessa GL, Muntoni F, Collu M, Vargiu L, Mereu G. Low doses of ethanol activate dopaminergic neurons in the ventral tegmental area. Brain Research 1985;348:201-3.

10. Larsson A, EdstrÖM L, Svensson L, SÖDerpalm BO, Engel JA. VOLUNTARY ETHANOL INTAKE INCREASES EXTRACELLULAR ACETYLCHOLINE LEVELS IN THE VENTRAL TEGMENTAL AREA IN THE RAT. Alcohol and Alcoholism 2005;40:349-58.

11. Ericson M, Löf E, Stomberg R, Chau P, Söderpalm B. Nicotinic Acetylcholine Receptors in the Anterior, but Not Posterior, Ventral Tegmental Area Mediate Ethanol-Induced Elevation of Accumbal Dopamine Levels. Journal of Pharmacology and Experimental Therapeutics 2008;326:76.

12. Feltmann K, Borroto-Escuela DO, Rüegg J, et al. Effects of Long-Term Alcohol Drinking on the Dopamine D2 Receptor: Gene Expression and Heteroreceptor Complexes in the Striatum in Rats. Alcoholism: Clinical and Experimental Research 2018;42:338-51.

13. Everitt BJ, Belin D, Economidou D, Pelloux Y, Dalley JW, Robbins TW. Review. Neural mechanisms underlying the vulnerability to develop compulsive drug-seeking habits and addiction. Philos Trans R Soc Lond B Biol Sci 2008;363:3125-35.

14. Everitt BJ, Robbins TW. Neural systems of reinforcement for drug addiction: from actions to habits to compulsion. Nature Neuroscience 2005;8:1481-9.

15. Wong AD, Ye M, Levy AF, Rothstein JD, Bergles DE, Searson PC. The blood-brain barrier: an engineering perspective. Front Neuroeng 2013;6:7-.

16. Harris RA. Ethanol Actions on Multiple Ion Channels: Which Are Important? Alcoholism: Clinical and Experimental Research 1999;23:1563-70.

17. Olsen RW, Hanchar HJ, Meera P, Wallner M. GABAA receptor subtypes: the “one glass of wine” receptors. Alcohol 2007;41:201-9.

18. Hendler RA, Ramchandani VA, Gilman J, Hommer DW. Stimulant and Sedative Effects of Alcohol. In: Sommer WH, Spanagel R, eds. Behavioral Neurobiology of Alcohol Addiction. Berlin, Heidelberg: Springer Berlin Heidelberg; 2013:489-509.

19. McIntosh C, Chick J. Alcohol and the nervous system. J Neurol Neurosurg Psychiatry 2004;75 Suppl 3:iii16-iii21.

20. Gilpin NW, Koob GF. Neurobiology of alcohol dependence: focus on motivational mechanisms. Alcohol Research & Health 2008;31:185-95.

21. Lovinger DM, Roberto M. Synaptic effects induced by alcohol. Curr Top Behav Neurosci 2013;13:31-86.

22. Söderpalm B, Lidö HH, Ericson M. The Glycine Receptor—A Functionally Important Primary Brain Target of Ethanol. Alcoholism: Clinical and Experimental Research 2017;41:1816-30.

23. Blanco-Gandía MC, Rodríguez-Arias M. Pharmacological treatments for opiate and alcohol addiction: A historical perspective of the last 50 years. Eur J Pharmacol 2018;836:89-101.

24. Blanco-Gandía MC, Rodríguez-Arias M. Pharmacological treatments for opiate and alcohol addiction: A historical perspective of the last 50 years. European Journal of Pharmacology 2018;836:89-101.

25. Vasiliou V, Malamas M, Marselos M. The Mechanism of Alcohol Intolerance Produced by Various Therapeutic Agents. Acta Pharmacologica et Toxicologica 1986;58:305-10.

26. Hald J, Jacobsen E. The Formation of Acetaldehyde in the Organism after Ingestion of Antabuse (Tetraethylthiuramdisulphide) and Alcohol. Acta Pharmacologica et Toxicologica 1948;4:305-10.

27. Suh JJ, Pettinati HM, Kampman KM, O'Brien CP. The Status of Disulfiram: A Half of a Century Later. Journal of Clinical Psychopharmacology 2006;26:290-302.

28. FULLER RK, GORDIS E. Does disulfiram have a role in alcoholism treatment today? Addiction 2004;99:21-4.

29. Nutt DJ. The role of the opioid system in alcohol dependence. Journal of Psychopharmacology 2014;28:8-22.

30. Benjamin D, Grant ER, Pohorecky LA. Naltrexone reverses ethanol-induced dopamine release in the nucleus accumbens in awake, freely moving rats. Brain Research 1993;621:137-40.

31. O'Malley SS, Jaffe AJ, Chang G, Schottenfeld RS, Meyer RE, Rounsaville B. Naltrexone and Coping Skills Therapy for Alcohol Dependence: A Controlled Study. Archives of General Psychiatry 1992;49:881-7.

32. Volpicelli JR, Alterman AI, Hayashida M, O'Brien CP. Naltrexone in the Treatment of Alcohol Dependence. Archives of General Psychiatry 1992;49:876-80.

33. Littleton J. Acamprosate in alcohol dependence: how does it work? Addiction 1995;90:1179-88.

34. Spanagel R, Zieglgänsberger W. Anti-craving compounds for ethanol: new pharmacological tools to study addictive processes. Trends in Pharmacological Sciences 1997;18:54-9.

35. Rösner S, Hackl-Herrwerth A, Leucht S, Lehert P, Vecchi S, Soyka M. Acamprosate for alcohol dependence. Cochrane Database of Systematic Reviews 2010.

36. Soyka M. Nalmefene for the treatment of alcohol use disorders: recent data and clinical potential. Expert Opinion on Pharmacotherapy 2016;17:619-26.

37. Keating GM. Nalmefene: A Review of Its Use in the Treatment of Alcohol Dependence. CNS Drugs 2013;27:761-72.

38. Karhuvaara S, Simojoki K, Virta A, et al. Targeted Nalmefene With Simple Medical Management in the Treatment of Heavy Drinkers: A Randomized Double-Blind Placebo-Controlled Multicenter Study. Alcoholism: Clinical and Experimental Research 2007;31:1179-87.

39. Anton RF, Pettinati H, Zweben A, et al. A Multi-site Dose Ranging Study of Nalmefene in the Treatment of Alcohol Dependence. Journal of Clinical Psychopharmacology 2004;24.

40. Gual A, He Y, Torup L, van den Brink W, Mann K. A randomised, double-blind, placebo-controlled, efficacy study of nalmefene, as-needed use, in patients with alcohol dependence. European Neuropsychopharmacology 2013;23:1432-42.

41. Fitzgerald N, Angus K, Elders A, et al. Weak evidence on nalmefene creates dilemmas for clinicians and poses questions for regulators and researchers. Addiction 2016;111:1477-87.

42. Mann K, Torup L, Sørensen P, et al. Nalmefene for the management of alcohol dependence: review on its pharmacology, mechanism of action and meta-analysis on its clinical efficacy. European Neuropsychopharmacology 2016;26:1941-9.

43. Bulik CM, Sullivan PF, Carter FA, Joyce PR. Lifetime comorbidity of alcohol dependence in women with bulimia nervosa. Addict Behav 1997;22:437-46.

44. Wolfe WL, Maisto SA. The relationship between eating disorders and substance use: moving beyond co-prevalence research. Clin Psychol Rev 2000;20:617-31.

45. Dansky BS, Brewerton TD, Kilpatrick DG. Comorbidity of bulimia nervosa and alcohol use disorders: results from the National Women's Study. Int J Eat Disord 2000;27:180-90.

46. Engel JA, Jerlhag E. Role of appetite-regulating peptides in the pathophysiology of addiction: implications for pharmacotherapy. CNS Drugs 2014;28:875-86.

47. Guan XM, Yu H, Palyha OC, et al. Distribution of mRNA encoding the growth hormone secretagogue receptor in brain and peripheral tissues. Brain Res Mol Brain Res 1997;48:23-9.

48. Jerlhag E. Gut-brain axis and addictive disorders: A review with focus on alcohol and drugs of abuse. Pharmacol Ther 2019;196:1-14.

49. Soares JB, Leite-Moreira AF. Ghrelin, des-acyl ghrelin and obestatin: three pieces of the same puzzle. Peptides 2008;29:1255-70.

50. Delhanty PJ, Neggers SJ, van der Lely AJ. Mechanisms in endocrinology: Ghrelin: the differences between acyl- and des-acyl ghrelin. Eur J Endocrinol 2012;167:601-8.

51. Yang J, Brown MS, Liang G, Grishin NV, Goldstein JL. Identification of the Acyltransferase that Octanoylates Ghrelin, an Appetite-Stimulating Peptide Hormone. Cell 2008;132:387-96.

52. Zhu X, Cao Y, Voodg K, Steiner DF. On the processing of proghrelin to ghrelin. Journal of Biological Chemistry 2006;281:38867-70.

53. Angela SB, Steven GS, Tim GY, Richards AM, Chris JP. Characterisation of proghrelin peptides in mammalian tissue and plasma. Journal of Endocrinology;192:313-23.

54. Harada T, Nakahara T, Yasuhara D, et al. Obestatin, acyl ghrelin, and des-acyl ghrelin responses to an oral glucose tolerance test in the restricting type of anorexia nervosa. Biol Psychiatry 2008;63:245-7.

55. Zizzari P, Longchamps R, Epelbaum J, Bluet-Pajot MT. Obestatin partially affects ghrelin stimulation of food intake and growth hormone secretion in rodents. Endocrinology 2007;148:1648-53.

56. Date Y, Kojima M, Hosoda H, et al. Ghrelin, a novel growth hormone-releasing acylated peptide, is synthesized in a distinct endocrine cell type in the gastrointestinal tracts of rats and humans. Endocrinology 2000;141:4255-61.

57. Zhao CM, Furnes MW, Stenström B, Kulseng B, Chen D. Characterization of obestatin- and ghrelin-producing cells in the gastrointestinal tract and pancreas of rats: an immunohistochemical and electron-microscopic study. Cell Tissue Res 2008;331:575-87.

58. Ghelardoni S, Carnicelli V, Frascarelli S, Ronca-Testoni S, Zucchi R. Ghrelin tissue distribution: comparison between gene and protein expression. J Endocrinol Invest 2006;29:115-21.

59. Nogueiras R, Williams LM, Dieguez C. Ghrelin: new molecular pathways modulating appetite and adiposity. Obes Facts 2010;3:285-92.

60. Sato T, Nakamura Y, Shiimura Y, Ohgusu H, Kangawa K, Kojima M. Structure, regulation and function of ghrelin. The Journal of Biochemistry 2011;151:119-28.

61. Hosoda H, Doi K, Nagaya N, et al. Optimum Collection and Storage Conditions for Ghrelin Measurements: Octanoyl Modification of Ghrelin Is Rapidly Hydrolyzed to Desacyl Ghrelin in Blood Samples. Clinical Chemistry 2004;50:1077-80.

62. Yoshimoto A, Mori K, Sugawara A, et al. Plasma Ghrelin and Desacyl Ghrelin Concentrations in Renal Failure. Journal of the American Society of Nephrology 2002;13:2748-52.

63. Jerlhag E, Egecioglu E, Landgren S, et al. Requirement of central ghrelin signaling for alcohol reward. Proceedings of the National Academy of Sciences of the United States of America 2009;106:11318-23.

64. Chen C-Y, Inui A, Asakawa A, et al. Des-acyl Ghrelin Acts by CRF Type 2 Receptors to Disrupt Fasted Stomach Motility in Conscious Rats. Gastroenterology 2005;129:8-25.

65. Jerlhag E, Egecioglu E, Dickson SL, Andersson M, Svensson L, Engel JA. Ghrelin stimulates locomotor activity and accumbal dopamine-overflow via central cholinergic systems in mice: implications for its involvement in brain reward. Addict Biol 2006;11:45-54.

66. Jerlhag E, Egecioglu E, Landgren S, et al. Requirement of central ghrelin signaling for alcohol reward. Proc Natl Acad Sci U S A 2009;106:11318-23.

67. Jerlhag E, Landgren S, Egecioglu E, Dickson SL, Engel JA. The alcohol-induced locomotor stimulation and accumbal dopamine release is suppressed in ghrelin knockout mice. Alcohol 2011;45:341-7.

68. Suchankova P, Steensland P, Fredriksson I, Engel JA, Jerlhag E. Ghrelin receptor (GHS-R1A) antagonism suppresses both alcohol consumption and the alcohol deprivation effect in rats following long-term voluntary alcohol consumption. PLoS One 2013;8:e71284.

69. Stevenson JR, Francomacaro LM, Bohidar AE, et al. Ghrelin receptor (GHS-R1A) antagonism alters preference for ethanol and sucrose in a concentration-dependent manner in prairie voles. Physiol Behav 2016;155:231-6.

70. Stevenson JR, Buirkle JM, Buckley LE, Young KA, Albertini KM, Bohidar AE. GHS-R1A antagonism reduces alcohol but not sucrose preference in prairie voles. Physiol Behav 2015;147:23-9.

71. Gomez JL, Cunningham CL, Finn DA, et al. Differential effects of ghrelin antagonists on alcohol drinking and reinforcement in mouse and rat models of alcohol dependence. Neuropharmacology 2015;97:182-93.

72. Callaghan B, Furness JB. Novel and Conventional Receptors for Ghrelin, Desacyl-Ghrelin, and Pharmacologically Related Compounds. Pharmacological Reviews 2014;66:984.

73. Gauna C, van de Zande B, van Kerkwijk A, Themmen AP, van der Lely AJ, Delhanty PJ. Unacylated ghrelin is not a functional antagonist but a full agonist of the type 1a growth hormone secretagogue receptor (GHS-R). Mol Cell Endocrinol 2007;274:30-4.

74. Thompson NM, Gill DA, Davies R, et al. Ghrelin and des-octanoyl ghrelin promote adipogenesis directly in vivo by a mechanism independent of the type 1a growth hormone secretagogue receptor. Endocrinology 2004;145:234-42.

75. Togliatto G, Trombetta A, Dentelli P, et al. Unacylated Ghrelin Rescues Endothelial Progenitor Cell Function in Individuals With Type 2 Diabetes. Diabetes 2010;59:1016.

76. Asakawa A, Inui A, Fujimiya M, et al. Stomach regulates energy balance via acylated ghrelin and desacyl ghrelin. Gut 2005;54:18.

77. Chen CY, Chao Y, Chang FY, Chien EJ, Lee SD, Doong ML. Intracisternal des-acyl ghrelin inhibits food intake and non-nutrient gastric emptying in conscious rats. Int J Mol Med 2005;16:695-9.

78. Neary NM, Druce MR, Small CJ, Bloom SR. Acylated ghrelin stimulates food intake in the fed and fasted states but desacylated ghrelin has no effect. Gut 2006;55:135.

79. Matsuda K, Miura T, Kaiya H, et al. Regulation of food intake by acyl and des-acyl ghrelins in the goldfish. Peptides 2006;27:2321-5.

80. Inhoff T, Mönnikes H, Noetzel S, et al. Desacyl ghrelin inhibits the orexigenic effect of peripherally injected ghrelin in rats. Peptides 2008;29:2159-68.

81. Toshinai K, Yamaguchi H, Sun Y, et al. Des-Acyl Ghrelin Induces Food Intake by a Mechanism Independent of the Growth Hormone Secretagogue Receptor. Endocrinology 2006;147:2306-14.

82. Ariyasu H, Takaya K, Iwakura H, et al. Transgenic Mice Overexpressing Des-Acyl Ghrelin Show Small Phenotype. Endocrinology 2005;146:355-64.

83. Inhoff T, Wiedenmann B, Klapp BF, Mönnikes H, Kobelt P. Is desacyl ghrelin a modulator of food intake? Peptides 2009;30:991-4.

84. Sanchis-Segura C, Spanagel R. REVIEW: Behavioural assessment of drug reinforcement and addictive features in rodents: an overview. Addiction Biology 2006;11:2-38.

85. Spanagel R. Animal models of addiction. Dialogues Clin Neurosci 2017;19:247-58.

86. Smith D, Anderson D, Degryse A-D, et al. Classification and reporting of severity experienced by animals used in scientific procedures: FELASA/ECLAM/ESLAV Working Group report. Lab Anim 2018;52:5-57.

87. Liljequist S, Berggren U, Engel J. The effect of catecholamine receptor antagonists on ethanol-induced locomotor stimulation. Neuroscience Letters 1981;24:S376.

88. Simon P, Dupuis R, Costentin J. Thigmotaxis as an index of anxiety in mice. Influence of dopaminergic transmissions. Behavioural Brain Research 1994;61:59-64.

89. Fowler SC. Motor Activity and Stereotypy. In: Stolerman IP, ed. Encyclopedia of Psychopharmacology. Berlin, Heidelberg: Springer Berlin Heidelberg; 2010:799-805.

90. Simms JA, Steensland P, Medina B, et al. Intermittent access to 20% ethanol induces high ethanol consumption in Long-Evans and Wistar rats. Alcohol Clin Exp Res 2008;32:1816-23.

91. Banks WA, Tschöp M, Robinson SM, Heiman ML. Extent and Direction of Ghrelin Transport Across the Blood-Brain Barrier Is Determined by Its Unique Primary Structure. Journal of Pharmacology and Experimental Therapeutics 2002;302:822.

92. Matsubara M, Sakata I, Wada R, Yamazaki M, Inoue K, Sakai T. Estrogen modulates ghrelin expression in the female rat stomach. Peptides 2004;25:289-97.

93. Clegg DJ, Brown LM, Zigman JM, et al. Estradiol-Dependent Decrease in the Orexigenic Potency of Ghrelin in Female Rats. Diabetes 2007;56:1051.

94. Matsuda K, Miura T, Kaiya H, et al. Stimulatory effect of n-octanoylated ghrelin on locomotor activity in the goldfish, Carassius auratus. Peptides 2006;27:1335-40.

95. Lamontagne SJ, Olmstead MC. Animal models in addiction research: A dimensional approach. Neuroscience & Biobehavioral Reviews 2019;106:91-101.

96. Spanagel R, Zieglgänsberger W. Anti-craving compounds for ethanol: new pharmacological tools to study addictive processes. Trends Pharmacol Sci 1997;18:54-9.

COMPLIMENTARY DATA, TABLES AND FIGURES

Table 2: Summary of various parameters for male rats, analysed with unpaired t-test.

Parameter	Unpaired t-test		
	0-1 hrs	1-4 hrs	4-24 hrs
Ethanol intake	t(9)=1.134, p=0.1431	t(9)=0.361, p=0.3632	t(9)=2.352, p=0.0216*
Ethanol preference	t(9)=1.585, p=0.0737	t(9)=1.844, p=0.0492*	t(9)=3.001, p=0.0075*
Water intake	t(9)=0.824, p=0.2155	t(9)=1.902, p=0.0448*	t(9)=1.712, p=0.0606
Total fluid intake	t(9)=0.640, p=0.4752	t(9)=1.088, p=0.1524	t(9)=0.017, p=0.4935
Food intake	t(9)=0.098, p=0.4620	t(9)=1.509, p=0.0828	t(9)2.637, p=0.0135*

* statistically significant

Table 3: Summary of various parameters for female rats, analysed with unpaired t-test.

Parameter	Unpaired t-test		
	0-1 hrs	1-4 hrs	4-24 hrs
Ethanol intake	t(9)=0.560, p=0.4783	t(9)=2.054, p=0.0351*	t(9)=0.217, p=0.4165
Ethanol preference	t(9)=0.400, p=0.3493	t(9)=0.216, p=0.0294*	t(9)=0.516, p=0.3092
Water intake	t(9)=0.378, p=0.3569	t(9)=0.188, p=0.9309	t(9)=1.070, p=0.1563
Total fluid intake	t(9)=0.473, p=0.3236	t(9)=0.897, p=0.1965	t(9)=1.128, p=0.1442
Food intake	t(9)=0.473, p=0.3236	t(9)=0.340, p=0.3707	t(9)=1.122, p=0.1454

* statistically significant

Table 4: Summary of locomotor activity parameters in male rats. at time of habituation and 60 minutes, starting 30 minutes after DAG-administration. analysed with one-way ANOVA.

Parameter	One-Way ANOVA values	
	Habituation	60-Minute Value
Ambulatory Distance	F(3,19)=0.49, p=0.4895	F(3,19)=1.97, p=0.1521
Stereotypic Count	F(3,19)=0.40, p=0.7516	F(3,19)=2.02, p=0.1448
Jump Count	F(3,19)=0.70, p=0.5630	F(3,19)=1.29, p=0.3075
Zone Entries	F(3,19)=0.15, p=0.9259	F(3,19)=0.96, p=0.4298
Time in Outer Zone	F(3,19)=1.33, p=0.2953	F(3,19)=0.20, p=0.8921
Time in Inner Zone	F(3,19)=1.33, p=0.2953	F(3,19)=0.20, p=0.8921
Percentage - Time in Inner Zone - Total Time	F(3,19)=1.33, p=0.2953	(3,19)=0.20, p=0.8922
Vertical Count	F(3,19)=0.60, p=0.6242	F(3,19)=0.78, p=0.5214
Average Velocity	F(3,19)=0.07, p=0.9765	F(3,19)=0.77, p=0.5275

Table 5: Summary of locomotor activity parameters in female rats. at time of habituation and 60 minutes, starting 30 minutes after DAG-administration. analysed with one-way ANOVA.

Parameter	One-Way ANOVA	
	Habituation	60-Minute Value
Ambulatory Distance	F(3,19)=0.23, p=0.1066	F(3,19)=0.15, p=0.9255
Stereotypic Count	F(3,19)=2.982, p=0.0572	F(3,19)=0.75, p=0.5375
Jump Count	F(3,19)=0.25, p=0.1159	F(3,19)=0.29, p=0.8388
Zone Entries	F(3,19)=2.54, p=0.0792	F(3,19)=0.47, p=0.7055
Time in Outer Zone	F(3,19)=0.50, p=0.6873	F(3,19)=1.67 p=0.2077
Time in Inner Zone	F(3,19)=0.50, p=0.6873	F(3,19)=1.67 p=0.2077
Percentage - Time in Inner Zone - Total Time	F(3,19)=0.50, p=0.6873	F(3,19)=1.67 p=0.2077
Vertical Count	F(3,19)=1.93, p=0.1589	F(3,19)=0.29, p=0.8349
Average Velocity	F(3,19)=1.56, p=0.2332	F(3,19)=0.26, p=0.8506

Table 6: Summary of ethanol intake results for male rats, analysed with one-way ANOVA.

Parameter	One-way ANOVA		
	0-1 hr	1-4 hr	4-24 hr
Ethanol intake (g/kg/time)	F(3,18)=2.69, P=0.0768	F(3,18)=0.16, P=0.9221	F(3,18)=2.54, P=0,0885

Table 7: Summary of ethanol intake results for female rats, analysed with one-way ANOVA.

Parameter	One-way ANOVA		
	0-1 hr	1-4 hr	4-24 hr
Ethanol intake (g/kg/time)	F(3,19)=0.39, P=0.7632	F(3,19)=1.69, P=0.2025	F(3,19)=0.28, P=0.8424

Table 8: Summary of weight difference between 0-24 hrs, in male and female rats, analysed with unpaired t-test.

Parameter	Unpaired t-test	
	Male rats	Female rats
Weight - 24 hrs	t(9)=0.628, p=0.2729	t(9)=0.591, p=0.2844
Weight difference -24 hrs	t(9)=0.825, p=0.2154	t(9)=0.137, p=0.4469