Allopregnanolone effects on food intake and weight gain

Ellinor Holmberg
Cover illustrations by Gianna Ragagnin, Umeå, Sweden

Front and back cover illustrations are inspired by the Mad Tea-Party from Alice’s Adventures in Wonderland by Lewis Carroll.

Cover synopsis
In a deep, still unexplored and sometimes dark wood of firing neurons we performed our experiments. We wanted to know what our nice rats like to eat, and why. They are our small Lords and Ladies, therefore we love to treat them well, offering them the most delicious cookies in the tidiest environment (but not looking so much because Blinded studies are the rule). Maybe some lipophilic cream of Allopregnanolone will increase their appetite? And how much? Will they even gain weight? And which neurons might fire? I wrote the doses on my hat, but I had to change them sometimes, you know how it works. You will discover it by reading this book: you can well imagine that, after so many years at my Rats service, I know some of their secrets. At the end, on the Path of Knowledge, we understood beyond any doubt the impact of the steroid web of Allopregnanolone, and it may have endless and still unknown connections to this intricate forest of neurons within the brain.

Interpretations of Quotes from Alice’s Adventures in Wonderland (next page)

The first quote represents a reminder to keep an open mind in research and if necessary change roads... The second quote was coined by Lewis Carroll and has been interpreted as “increasingly strange”. However, as the word curious may mean both strange and inquisitive the quote may just as well mean “increasingly inquisitive”.... Both interpretations may apply during different periods when writing this thesis.

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If you don´t know where you are going, any road can take you there.

.... curiouser and curiouser....

/ from Alice's Adventures in Wonderland by Lewis Carroll
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Abstract

Background

Obesity is currently one of the major causes of ill health and it is clear that consumption of more calories than needed, i.e. overeating, is the cause of obesity. However, the actions of many endogenous factors that contribute to overeating are still not well understood. Gamma-aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the brain and GABA-ergic transmission has in several studies been shown to be of great importance for food intake regulation. The progesterone metabolite allopregnanolone is a positive GABA<sub>A</sub> receptor modulating steroid (GAMS) and has a potent positive effect on the GABA<sub>A</sub> receptor. In humans, elevated allopregnanolone levels have been suggested to be involved in increased food intake, eating disorders and also in overweight and obesity. Allopregnanolone shares many properties of action with benzodiazepines at the GABA<sub>A</sub> receptor and studies on benzodiazepine induced food intake have indicated that the effect is primarily mediated by GABA<sub>A</sub> receptors that express the α2 and α3 subunits. Therefore, the aim of the work upon which this thesis is based was to further investigate the effect of allopregnanolone on food intake, feeding behavior, possible effects on weight gain and also to characterize a possible antagonist at α2β3γ2 and α3β3γ2 GABA<sub>A</sub> receptors.

Methods

Allopregnanolone effects on food intake of different food items were recorded in male Wistar rats. In addition, feeding patterns of allopregnanolone treated rats were analyzed. Food preference tests were also conducted to investigate if the animals had a preference for any specific food item when treated with allopregnanolone. To investigate if repeated allopregnanolone exposure influences the weight gain, rats were exposed to allopregnanolone during 5 consecutive days. Repeated exposure to allopregnanolone was also applied to schedule fed rats and rats schedule fed a high fat diet. Rats that were schedule fed a high fat diet, were separated into obesity prone and obesity resistant groups and analyzed separately. To deeper investigate GABA<sub>A</sub> receptor subtypes suggested to be involved in food intake regulation, a compound with previously shown antagonistic properties to GAMS-induced potentiation, UC1020, was selected and electrophysiological whole-cell patch-clamp recordings were performed to identify the specificity of this compound. For that purpose we produced HEK293-cells permanently expressing human GABA<sub>A</sub> receptors suggested to be involved in food intake regulation, human α2β3γ2 GABA<sub>A</sub> receptors and
human α3β3γ2 GABA_A receptors, which then were subjected to tests with UC1020.

**Results**

Allopregnanolone increased the intake of standard chow, cookies and a high fat diet in male Wistar rats. Preferentially, allopregnanolone increased the rats’ intake of the more calorie dense food type. Allopregnanolone reduced feeding latency and prolonged feeding duration of the next coming meal after injection, which indicates an increased meal size. The increased chow intake induced by allopregnanolone was more pronounced at the beginning of the rats’ active/dark period compared to the inactive/light period. Repeated allopregnanolone administration during 5 consecutive days led to an increased body weight gain, more evident in schedule fed rats on a high fat diet. In addition both obesity prone and obesity resistant rats gained significantly more weight with repeated allopregnanolone exposure and the increased body weight gain correlated with increased food intake. The compound UC1020 was a potent antagonist of GAMS-enhanced GABA evoked currents at human α3β3γ2 GABA_A receptors, whereas it had no effect at α2β3γ2 GABA_A receptors.

**Conclusions**

Allopregnanolone seemed to act at several levels of feeding and may lead to an increased meal size. This is important since in both rats and humans, meal size rather than meal numbers has been associated with obesity. In addition, allopregnanolone had a more prominent effect on food intake during the active/dark period of the rats, which could indicate that its effect might depend on GABA-ergic AgRP neurons that are important in food intake regulation and display a diurnal variation in their activity. As allopregnanolone preferentially increased the consumption of the more calorie dense food offered in food preference tests, it could be speculated that allopregnanolone favors an obesogenic diet. In addition an increased weight gain was recorded for rats repeatedly exposed to allopregnanolone when schedule fed a high fat diet. Thus, our findings indicate that allopregnanolone may be one of the endogenous factors involved in weight gain, especially when the diet is rich in fat. The compound UC1020 may prove useful for investigating the involvement of the α2 and α3 GABA_A receptor subtype in GAMS-induced hyperphagia.
Sammanfattning på svenska

Bakgrund


Mål

Eftersom det finns indikationer på kopplingar mellan allopregnanolon och påverkan på både födointag och vikt var målet med denna avhandling att vidare undersöka dessa samband. Mer specifika mål var att i en råttmodell undersöka effekter av allopregnanolon på intag av och preferens för olika typer av födoämnen, hur allopregnanolon påverkar matbeteendet samt viktuppgång hos råttorna och avslutningsvis granska en möjlig blockerare för allopregnanolons effekter på matintag.

Resultat

I artikel I undersökt dygnsskillnader i allopregnanolons effekter på intag av vanlig råttmat samt matintagsbeteende. Resultatet visade att allopregnanolon hade en större effekt under råttornas aktiva/mörka period jämfört med under deras inaktiva/ljusa period. Allopregnanolone påverkade även ätbeteendet så att råttorna påbörjade sin måltid tidigare samt åt under en längre tid jämfört med råttor som fick placebobehandling. Sammantaget indikerar detta en ökad måltidsstorlek.


I artikel III undersökt om dagligen upprepade allopregnanolon-behandlingar kunde öka råttornas kroppsvikt samt om råttor som är motståndskraftiga mot fetma skulle reagera på annat vis än råttor med en benägenhet för fetma när de behandlades med allopregnanolon. Det visade sig att 5 dagars upprepade allopregnanolon-behandlingar gav en ökad kroppsvikt för råttor som hade tillgång till mat med högt fettinnehåll 4 timmar per dag. Dessutom var vikttätheten korrelerad till ett ökat matintag. Både råttor som var motståndskraftiga mot fetma och råttor med en benägenhet för fetma gick upp i vikt mer med allopregnanolon än med placebobehandling när de fick en hög fettdiet 4 timmar per dag.
I artikel IV undersöktes om och hur en möjlig blockerare för allopregnanolons effekt på födointaget, UC1020, påverkade GABAₐ receptorer innehållande subenheter som anses vara av vikt för reglering av födointag. UC1020 visade sig vara effektiv för att blockera allopregnanolons effekt på α₃-innehållande GABAₐ receptorer, men den visade ingen effekt på α₂-innehållande GABAₐ receptorer.

**Slutsats**

# Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>ACC</td>
<td>American College of Cardiology</td>
</tr>
<tr>
<td>AFE</td>
<td>Atwater fuel energy</td>
</tr>
<tr>
<td>AgRP</td>
<td>Agouti-related protein</td>
</tr>
<tr>
<td>AHA</td>
<td>American Heart Association</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ARC</td>
<td>Arcuate nucleus</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>CCK</td>
<td>Cholecystokinin</td>
</tr>
<tr>
<td>Cpm</td>
<td>Counts per minute</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CRH</td>
<td>Corticotropin-releasing hormone</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular diseases</td>
</tr>
<tr>
<td>DHEAS</td>
<td>Dehydroepiandrosteronesulfate</td>
</tr>
<tr>
<td>DIO</td>
<td>Diet induced obesity</td>
</tr>
<tr>
<td>EC</td>
<td>Extracellular</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
</tr>
<tr>
<td>GABA&lt;sub&gt;A&lt;/sub&gt; receptor</td>
<td>γ-aminobutyric acid receptor type A</td>
</tr>
<tr>
<td>GAD</td>
<td>Glutamic acid decarboxylase</td>
</tr>
<tr>
<td>GAMS</td>
<td>GABA&lt;sub&gt;A&lt;/sub&gt; receptor modulating steroids</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal</td>
</tr>
<tr>
<td>GLP-1</td>
<td>Glucagon like peptide-1</td>
</tr>
<tr>
<td>HFD</td>
<td>High fat diet</td>
</tr>
<tr>
<td>IC</td>
<td>Intracellular</td>
</tr>
<tr>
<td>LH</td>
<td>Lateral hypothalamus</td>
</tr>
<tr>
<td>LPL</td>
<td>Lipoprotein lipase</td>
</tr>
<tr>
<td>MCH</td>
<td>Melanin-concentrating hormone</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>NAc</td>
<td>Nucleus accumbens</td>
</tr>
<tr>
<td>NES</td>
<td>Night eating syndrome</td>
</tr>
<tr>
<td>NHI</td>
<td>National Institutes of Health</td>
</tr>
</tbody>
</table>
NHLBI  National Heart, Lung, and Blood Institute
NPY  Neuropeptide Y
NTS  Nucleus of the solitary tract
OP  Obesity prone
OR  Obesity resistant
PBN  Parabrachial nucleus
PCOS  Polycystic ovary syndrome
POMC  Proopiomelanocortin
PMS  Premenstrual syndrome
PVN  Paraventricular nucleus
PYY  Peptide YY
RIA  Radio-labelled immunoassay
s.c.  Subcutaneously
SEM  Standard error of mean
sIPSC  Spontaneous inhibitory postsynaptic current
THDOC  Tetra-hydro-deoxy-corticosterone
TOS  Obesity Society
Vgat  Vesicular GABA transporter gene
VMH  Ventromedial hypothalamus
VTA  Ventral tegmental area
WC  Waist circumference
WHO  World Health Organisation
WHR  Waist-to-hip ratio
Original papers

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IV. J. Strömberg, E. Holmberg, E. Malinina, D. Haage, M. Johansson and T. Bäckström. Subunit specific antagonism of allopregnanolone potentiation at GABA<sub>A</sub> receptor types suggested being involved in feeding. In manuscript
Introduction

Obesity and overweight

Health issues associated with obesity and overweight

It is not a problem per se to have a high body weight. The main issue is that obesity is linked to a large number of serious health conditions, such as cardiovascular diseases (CVD), hypertension, coronary heart disease, stroke, insulin resistance, type 2 diabetes, sleep apnea, obesity related cancer and dementia (Poirier et al., 2006, Eckel and Krauss, 1998, Qatanani and Lazar, 2007, Ford et al., 1997, Bearpark et al., 1995, Gorospe and Dave, 2007, Ning et al., 2010, Park et al., 2014). Most researchers agree that obesity (BMI ≥ 30) is associated with an increased mortality, but the risks associated with overweight (BMI ≥ 25 and <30) are still under debate (Flegal et al., 2013).

The prevalence of obesity and overweight in adults

There has been a global increase in the prevalence of obesity since the 1970s, also known as “the obesity epidemic” (James et al., 2001) or even “the obesity pandemic” (Swinburn et al., 2011). Worldwide, from 1980 to 2013, the combined prevalence of overweight and obesity in adults rose from 28.8% to 36.9% in men, and from 29.8% to 38.0% in women, giving a total of 2.1 billion overweight and obese persons in the world in 2013. However, there is much variation between countries regarding the rates of overweight and obesity and trends over the years (Ng et al., 2014). The US in particular has been highly affected by the initial escalating increase in obesity. In the US in 2013, 32% of men and 34% of women were obese and 71% of men and 62% of women were either overweight or obese (Ng et al., 2014)(Fig 1). However, recent studies have suggested that the rapid increase in obesity prevalence seen in 1980-1999 appears to generally be slowing down in the US, as well as in Sweden. Even if the increase is levelling off, the current rates of obesity are still alarmingly high (Flegal et al., 2012, Norberg et al., 2010, Sundquist et al., 2010). In addition, there has been a dramatic increase in obesity in developing countries recently (Popkin and Gordon-Larsen, 2004, Ng et al., 2014).
**Figure 1.** Age-adjusted prevalence of overweight, obesity in US adults aged 20-74. The figure was constructed from online data, Source: CDC/NCHS, National Health Examination Survey and National Health and Nutrition Examination Survey. Overweight; BMI ≥25.0 kg/m$^2$ and < 30.0 kg/m$^2$. Obese; BMI ≥ 30.0 kg/m$^2$.

**Obesity and overweight in adults**

Body mass index (BMI) is commonly used to define overweight and obesity and is calculated as body weight (kg) divided by height squared (m$^2$). For adults the World Health Organisation (WHO) and the National Institutes of Health (NHI) have defined a BMI ≥ 25 as overweight and BMI ≥ 30 as obesity (WHO., 2013, Clinical Guidelines, 1998). This split is widely used in scientific research, although sometimes other definitions are used, such as waist circumference (WC) or waist-to-hip ratio (WHR) which can give additional information on who is at risk for obesity related disorders (Oliveros et al., 2014, Caballero, 2007).

**The multifactorial etiology of obesity**

When energy intake is larger than the body’s energy expenditure, the term overeating can be used (Prentice, 2001). Excess body weight and, in the long run, obesity occurs when energy intake is greater than energy expenditure over a prolonged time. Excess energy is then stored as fat (Konturek et al., 2005).
However, the underlying causes of obesity are multifactorial. In general they can be divided into environmental and genetic causes, which both affect physiology and behavior (Speakman, 2004). The increased prevalence of obesity is most likely due to environmental and behavioral circumstances. The WHO has identified increasing access to high energy diets, fat and sugar together with a decreased energy expenditure as the most likely causes of the increasing obesity prevalence (WHO, 2000, Swinburn et al., 2011). However, genetic factors are also important. As Dr G.A. Bray has stated “the genetic background loads the gun, but the environment pulls the trigger” (Bray, 2004). There are some rare cases of obesity caused by single gene defects in genes coding for, e.g. leptin or the leptin receptor (Clement et al., 1998, Montague et al., 1997) and also rare obesity syndromes that display single gene defects, such as the Prader-Willi syndrome (Atkinson, 2005, Cassidy et al., 2012). However, the main genetic influence is caused by a combination of several genes. One review suggested that genetics may explain as much as 50-90% of the variation in BMI (Maes et al., 1997). Even though living in the same environment, the majority of the population is not obese. Thus, there seems to be an innate susceptibility for obesity or leanness. The concept that there are individuals that are obesity prone (OP) and those that are obesity resistant (OR) has been used for both humans and rodents (Thomas et al., 2013, Bessesen et al., 2008, Pagliassotti et al., 1997, Dourmashkin et al., 2006).

**Treatment today**

Today, there are three basic methods for the treatment of obesity: a) lifestyle changes, like diet and exercise, b) surgery, and c) pharmacotherapy. For an evaluation of treatments see “Guidelines for the management of overweight and obesity in adults 2013” (Jensen et al., 2014). Even though lifestyle changes such as diet and exercise may have positive health impacts, overall it seems as if this treatment strategy is insufficient to stop the increasing prevalence of obesity. According to the guidelines, bariatric surgery is a promising approach, but there is not today enough evidence of the benefits of bariatric surgery when BMI is < 35. However, adults with a BMI ≥ 40 or ≥35 with obesity related conditions (e.g. type 2 diabetes and CVD) who have not responded to behavioral treatment or pharmacology with enough weight loss to improve health may benefit from surgery. Thus, even if surgery can be effective for some patients, it is still an invasive procedure and the use of surgery on a large scale is impractical. Pharmacotherapy was not evaluated by the guidelines, but many anti-obesity drugs are hampered with poor
efficiency and undesirable side effects, such as negative cardiovascular effects and gastrointestinal problems (Walter et al., 2014).

The weight reducing effects of bariatric surgery besides decreasing the energy uptake from the intestine also produce reductions of orexigenic hormones, like ghrelin. Appetite regulating hormones in the stomach communicate with the brain and the possibilities of targeting appetite regulating hormones or their mechanism within the brain by pharmacotherapy could be one beneficial treatment strategy for obesity.

Regulation of food intake

Food intake is critical for our survival and is therefore under strict control. The control system is complex and contains both central and peripheral regulators that create an interaction between the gut, the adipose tissue and the brain (Sobrino Crespo et al., 2014). Regulation of food intake can also roughly be divided into the homeostatic and hedonistic system. The homeostatic system is primarily involved in regulation based on energy needs, whereas the hedonistic system regulates the reward values of feeding (Morton et al., 2006, Lutter and Nestler, 2009, Berthoud, 2011).

The homeostatic system

Central regulation

The homeostatic system can sense several signals and uses this information to maintain body weight homeostasis. Both short-term signals (immediate changes in nutritional status caused by, e.g. a meal) and long-term signals (correlated to amount of stored energy in adipose tissue) are sensed (Jobst et al., 2004). In the brain, the hypothalamus plays a major role in feeding control (Schwartz et al., 2000, Meister, 2007). In particular, the arcuate nucleus (ARC) within the hypothalamus has been proposed as a key node of feeding regulation. Since the ARC is located close to the median eminence where the blood brain barrier is weak, it can easily receive input of the body’s metabolic situation mediated by blood borne hormones (Peruzzo et al., 2000). In the ARC, there are at least two important cell populations for feeding regulation. One group of neurons, the orexigenic agouti-related protein (AgRP)/neuropeptide Y (NPY) neurons, stimulate food intake (Hahn
et al., 1998). The other group of neurons, the anorexigenic proopiomelanocortin (POMC) / cocaine- and amphetamine-regulated transcript (CART) neurons, on the other hand inhibit food intake (Coll et al., 2004, Elias et al., 1998). The typically satiety promoting leptin inhibits AgRP/NPY neurons, whereas they stimulate POMC/CART neurons (Cowley et al., 2001, Schwartz et al., 2000, Elias et al., 1998). The hunger hormone ghrelin has the opposite effect. It promotes hunger by stimulating AgRP/NPY neurons and indirectly inhibits POMC neurons (Nakazato et al., 2001, Riediger et al., 2003). ARC AgRP neurons project to several brain areas important for food intake regulation, e.g. POMC neurons in close proximity in the ARC (Cowley et al., 2001, Gropp et al., 2005), the paraventricular nucleus (PVN) (Atasoy et al., 2012), the parabrachial nucleus (PBN) (Wu et al., 2009) and the ventromedial hypothalamus (VMH) even though these projections are less dense (King, 2006, Li et al., 2002). Several other brain areas and neurons have been suggested an important role in food intake regulation, but this will not be discussed in this thesis.

The importance of GABA in food intake regulation has been highlighted in several studies. Increased local GABA levels in either the hypothalamus or nucleus accumbens (NAc) has been shown to stimulate food intake (Stratford and Kelley, 1997, Meister, 2007). The GABA_A receptors agonist muscimol increases food intake in satiated pigs, an effect that was completely blocked by the GABA_A receptor antagonist bicuculline (Baldwin et al., 1990). Additional studies further stress the importance of GABA-ergic transmission for normal regulation of food intake and/or energy balance. Mice lacking NPY, AgRP or both showed normal food intake and body weight (Erickson et al., 1996, Qian et al., 2002), but when AgRP expressing neurons were destroyed, mice decreased their food intake and lost weight, indicating the importance of these neurons for the regulation of energy balance (Phillips and Palmiter, 2008, Gropp et al., 2005). Moreover, in addition to NPY and AgRP, these neurons express GABA (Horvath et al., 1997, Cowley et al., 2001). Indeed, ablation of AgRP expressing neurons in mice led to starvation and body weight decline. Chronic treatment with the GABA_A receptor partial agonist bretazenil restored the feeding and weight to normal. Also, the effect was blocked by treatment with the GABA_A receptor antagonist flumazenil. This indicates that GABA and the GABA_A receptors may play an important role (Wu et al., 2009). This idea was further strengthened when Tong et al inactivated the vesicular GABA transporter gene (Vgat) in AgRP neurons of mice. With an inactivated Vgat, which is required for presynaptic GABA release (McIntire et al., 1997), the animals became lean and resistant to diet-induced obesity, again pointing towards the importance of GABA. However, no effect on food intake was detected in this study (Tong et al., 2008). Indeed, GABA neurons are widespread in the
brain but important areas implicated to be involved in the orexigenic effects mediated by GABA are: areas expressing POMC neurons in the ARC (Cowley et al., 2001, Gropp et al., 2005, Jobst et al., 2004), PVN (Atasoy et al., 2012, Tsujii and Bray, 1991), PBN (Wu et al., 2009) and VMH (Tsujii and Bray, 1991).

Peripheral regulation

Some of the important peripheral peptides/hormones involved in homeostatic food intake regulation are leptin, insulin, ghrelin, glucagon like peptide-1 (GLP-1), cholecystokinin (CCK) and peptide YY (PYY). Leptin and insulin are both involved in long-term signals for energy regulation, as they are secreted in proportion to the amount of adipose tissue in the body (Considine et al., 1996, Bagdade et al., 1967). However, insulin levels also increase rapidly immediately after food intake (Polonsky et al., 1988) and it has been suggested that also leptin may have a role in short-term food intake (Chapelot et al., 2000). Leptin is produced mainly by adipocytes and promotes satiety (Campfield et al., 1995). Insulin is a pancreatic hormone that is essential for stimulating glucose uptake and metabolism, and it also reduces energy intake (Woods et al., 1998). Ghrelin, GLP-1, CCK and PYY are examples of other short-term signals as their concentrations fluctuate in relation to food intake/meals (Woods and D’Alessio, 2008). Ghrelin is derived predominantly from the stomach and is the major circulating hunger promoting hormone (Wang et al., 2002a). Ghrelin-levels are elevated just before the onset of a meal (Cummings et al., 2001). GLP-1, CCK and PYY are all secreted from the gastrointestinal (GI) tract in response to food intake and are satiety promoting (Woods and D’Alessio, 2008). Their secretion also depends on the macronutrients (carbohydrates, fat and protein) present in the ingested food (Holst, 2007, Moran and Kinzig, 2004, Batterham et al., 2006). The GI peptides GLP-1, CCK and PYY primarily activate the vagal nerve, which projects to the nucleus of the solitary tract (NTS) in the brainstem. Gastric distention also affects the vagal nerve and signal to the NTS (Woods and D’Alessio, 2008, Schwartz et al., 2000, Sobrino Crespo et al., 2014).

The hedonistic system

We do not eat to merely meet an energy deficit. Other factors, such as the rewarding nature of feeding, also contribute to what, when and how much food is consumed. Berthoud concluded that the hedonistic system could also be termed the “non-homeostatic” system since it has no metabolic feedback
Neuronal circuits involved in the hedonistic regulation of food intake are mainly based in the cortico-limbic system. Several signaling systems are involved and interact with each other, e.g. the dopaminergic, opioid and cannabinoid system (Stanley et al., 2005). Dopamine is released from neurons in the ventral tegmental area (VTA) and project to several other brain areas. Dopamine has been suggested to primarily be involved in the “wanting” aspect (the willingness/motivation to work to obtain a reward, exemplified by cravings) of hedonistic feeding (Kelley and Berridge, 2002). However, it has also been suggested that it is rather the anticipation of especially highly palatable food that dopamine is involved in (Barbano and Cador, 2007). In rats, dopamine is released in the nucleus accumbens (NAc) as a response to intake of a palatable sucrose solution (Hajnal and Norgren, 2001). Dopamine is also released in response to consumption of favorite meals in human test subjects and it also increases the more pleasurable the food is perceived (Small et al., 2003). Several studies have indicated the involvement of the opioid system in the “liking” aspect (hedonic reaction to the pleasure of a reward) of hedonistic feeding. Hedonic hotspots for liking have also been identified in the NAc and ventral palladium (Pecina and Berridge, 2005, Kelley et al., 1996, Barbano and Cador, 2007). Much like opioids, endocannabinoids have been linked to the “liking” aspect of hedonistic feeding (Mahler et al., 2007).

A few studies have shown that GABA might be indirectly related to hedonistic feeding involving, e.g. the opioid and dopamine system. It has been shown that the food intake increase by the GABA_A receptor agonist muscimol is affected by different opioid receptor antagonists administered in the VTA and NAc (Khaimova et al., 2004). GABAergic inhibition of neurons in the VTA can indirectly lead to increased activity of dopamine neurons (Luscher and Malenka, 2011). In addition, allopregnanolone (a potent positive modulator of the GABA_A receptor (Majewska et al., 1986) that will be discussed more in detail later) has been shown to increase dopamine release in the NAc of rats (Rouge-Pont et al., 2002), and thus both GABA
and allopregnanolone might to some extent influence dopamine induced food intake.

**Meal size and frequency**

An increased meal size has been shown to be associated with obesity in both humans and rats (Berg et al., 2009, Furnes et al., 2009, Farley et al., 2003). In humans, skipping one meal a day has also been correlated to increased adiposity and obesity (Chapelot et al., 2006, Berg et al., 2009, Ma et al., 2003). In rats fed a high fat diet, an altered feeding pattern was detected in addition to weight gain, with fewer meals and increased meal size (Melhorn et al., 2010). Also, consumption of several smaller meals during the day has been suggested to be associated with a lower percentage of overweight compared to consumption of the same calories in fewer meals (Fabry and Tepperman, 1970). Generally, an increased meal size has been considered as more important than ingestive frequency for determination of the total energy intake, but the opposite has also recently been suggested (Mattes, 2014).

**Scheduled feeding**

Rats in the laboratory usually have ad libitum access to food, whereas humans consume their food in meals at fixed time points (Schwartz, 2004). One way to impose meal feeding on rats is to give them access to food only during certain times, i.e. scheduled feeding. Scheduled feeding affects the circuits of feeding regulation. In schedule fed rats or meal fed humans, increased neuronal activity has been reported before feeding in brain areas suggested to be involved in food intake regulation (Cummings et al., 2001, Verbaeys et al., 2011). An advantage with scheduled feeding is that it allows human meal related fluctuations of, e.g. ghrelin to be mimicked (Cummings et al., 2004, Cummings et al., 2001, Verbaeys et al., 2011). The feeding status also has other effects on substances involved in food intake regulation, such as, e.g. AgRP and dopamine. Plasma AgRP levels seem to correlate with whether the subjects are fasted or fed, with decreased AgRP levels after feeding and increased AgRP levels following a fast in both rats and humans (Shen et al., 2002). Similarly, dopamine levels in the VMH also appear to be decreased after feeding and increased following a fast, which suggest that the dopamine system is involved in increasing the rewarding properties of food after fasting (Vucetic and Reyes, 2010).
It has been suggested that the frequency of feeding can affect body composition. An increased fat storage has been detected in rats consuming their entire daily food ration (similar caloric intake) at a few occasions (Cohn, 1963, Verbaeys et al., 2011). However studies on Tunisian women who refrain from eating from dawn to dusk (more than 12 hour fast) during one month, Ramadan, report no effect of fasting on body weight or body composition. However, an increased percentage of energy derived from fat was reported (el Ati et al., 1995). Also, in rats, 48 h of food deprivation has been reported to alter macronutrient selection favoring fat intake (Bernardini et al., 1993).

**Diurnal variation in food intake**

In mammals, the circadian rhythm (ca. 24h fluctuations in physiology and behavior), is centrally controlled by a pacemaker or biological clock located in the suprachiasmatic nucleus (SCN) of the hypothalamus. There are also peripheral “time-givers” or “cues” which affect the circadian rhythm and one of the most important cues is light (Ralph et al., 1990, Berson, 2003, Bailey et al., 2014).

Circadian variations in eating have been documented in many species. In humans, eating at night has been linked to obesity (Berg et al., 2009). As the day progresses, the intermeal interval decreases. Thus, it appears that food intake during the late night is less satiating and also seems to be more calorie dense than food eaten earlier during the day (de Castro, 2004, de Castro, 2001). Night eating syndrome (NES), which is characterized by a delayed circadian pattern of food intake with frequent nocturnal feeding, has also been associated with obesity (Gallant et al., 2012, Milano et al., 2012).

The circadian system is also involved in homeostatic energy regulation. Several hormones (e.g. ghrelin, insulin and leptin) involved in homeostatic energy regulation are affected by the circadian rhythm and vice versa (Froy, 2010). In addition, interactions between the SCN and specific areas involved in the regulation of food intake, e.g. ARC have been shown by injections of neuronal tracers (Yi et al., 2006). The circadian clock affects metabolism, but metabolism also affects the circadian clock. Feeding at other time points than normal, as well as changing macronutrient composition, can result in circadian dyssynchrony, which has also been associated with metabolic disturbances (Bailey et al., 2014).
Rats are night active animals, in contrast to humans, who are active during the day. Even though this difference exists, the basic function of the circadian system seems to be conserved between day-active and night-active species (Smale et al., 2003). Some circadian fluctuations are opposed when comparing day-active and night-active animals, e.g. body temperature, but the effect of the important light cue, as well as GABA effects on the circadian rhythm, shows several similarities between night-active and day-active animals (Challet, 2007). As rats are night-active, they ingest most of their food during the dark period rather than during the light/inactive period (Farley et al., 2003, Bassil et al., 2007).

Expression of AgRP mRNA in rats also exhibits diurnal variations, with peaked expression at the beginning of the dark/active period and a nadir at the beginning of the light/inactive period. This diurnal fluctuation has also been shown to coincide with more food intake in ad libitum fed animals (Lu et al., 2002).

The aforementioned plasma ghrelin is affected by food intake, but ghrelin is also regulated by the diurnal rhythm. In humans, the diurnal variation in ghrelin levels follows a similar pattern to that of leptin levels, which increase throughout the day and reach a nadir just after breakfast the following morning (Cummings et al., 2001). Ghrelin levels in rats peak at the end of both the light/inactive and the dark/active periods (Murakami et al., 2002).

Steroid hormones in relation to food intake and weight gain

Steroid hormones

Steroid hormones are synthesized from cholesterol (Fig 2) and can be divided into progestagens, androgens, estrogens and corticosteroids. Progesterone is the primary progestagen, testosterone the primary androgen and estradiol the primary estrogen (Asarian and Geary, 2013). Steroid hormones are relatively small and lipid-soluble (lipophilic) and can therefore penetrate plasma membranes directly. Their classic effect is mediated through a genomic mechanism, in which they bind to specific intracellular receptors (e.g. progesterone to progesterone receptors, testosterone to androgen receptors, estrogen to estrogen receptors and cortisol to glucocorticoid or mineralocorticoid receptors) and regulate gene expression
(Kawata, 1995). The effect via these pathways may take hours and even days (Yamamoto, 1985, Paul and Purdy, 1992, Baulieu and Robel, 1995, McEwen, 1991). However, steroid hormones can also generate much faster effects (within a second) by a non-genomic mechanism, in which they bind to membrane bound receptors such as the GABA<sub>A</sub> receptor (Frye et al., 1992, McEwen, 1994, Moore and Evans, 1999). Steroid hormones and their receptors are present in both sexes, and both sexes are affected by exogenous administration of steroid hormones (Sodersten, 1984). The major differences between the sexes is that the steroid concentrations differ and the fact that the concentrations fluctuate with the menstrual cycle in fertile women (O’Malley and Strott, 1999).

**Figure 2.** Main pathways of steroid hormone synthesis subdivided into major classes. DHEA: dehydroepiandrosterone.
Steroid hormones, food intake and body weight

Steroid hormones like progestagens, estrogens and androgens are involved in regulating food intake and energy balance (Asarian and Geary, 2006).

In both rats and humans, food intake fluctuates during the estrus cycle/menstrual cycle, with larger food intake during the post-ovulatory luteal phase than during the pre-ovulatory follicular phase in humans (Asarian and Geary, 2006). There are some major differences between the estrus cycle of rats and menstrual cycle of humans. The cycle time is 4-5 days in rats compared to 28 days in humans. Also, no corpus luteum is formed during the estrus cycle of rats as it is in humans. As a consequence, humans experience a longer time with elevated progesterone levels (Becker et al., 2005). These differences aside, changes in food intake have been associated with fluctuating levels of primarily estradiol and progesterone. Estradiol levels are increased during the follicular phase when food intake is at its lowest, and it has been proposed that estradiol has a suppressing effect on food intake. A peak in food intake occurs during the luteal phase when progesterone levels are increased, which was initially attributed to progesterone alone, but later also to progesterone inhibition of estradiol (Buffenstein et al., 1995, Dye and Blundell, 1997). It has been discussed that only pharmacological concentrations of progesterone induce increased food intake (Butera, 2010). However, the importance of progesterone in increasing energy intake during the luteal phase is highlighted by the fact that the increase is only seen in ovulatory cycles (Barr et al., 1995). During ovulatory cycles a corpus luteum which secretes progesterone is formed. In anovulatory cycles on the contrary no corpus luteum is formed and thus progesterone levels are much lower. Testosterone stimulates food intake and seems to increase the number of meals rather than the size of the meal (Chai et al., 1999).

Steroid hormones are also involved in accumulation and distribution of body fat (Mayes and Watson, 2004). There are gender differences in the distribution of body fat. Men usually have more visceral (intra-abdominal) fat storage, resulting in an android obesity, whereas women usually have more gluteal/femoral fat storage, resulting in a gynoid obesity (Tchernof and Despres, 2000, Bjorntorp, 1996). Visceral fat storage has also been more strongly related to metabolic disorders (Tchernof and Despres, 2013). At menopause, women experience changes in steroid hormone concentrations. The cyclic changes typical of the menstrual cycle level off and concentrations of estrogens, progestagens and androgens decrease, but there are also reports that showed no changes in androgen levels (Mesch et al., 2008). Weight gain has also been associated with menopause (Wing et al., 1991) and
postmenopausal women tend to display a more visceral fat storage (Lovejoy et al., 2008). It has been speculated that the decline in steroid hormones at menopause could be related to changes in adipose tissue metabolism (Tchernof et al., 2004). Some studies show that hormone replacement therapy (HRT) may even counteract the postmenopausal fat increase, as well as increased visceral fat storage (Genazzani and Gambacciani, 2006, Haarbo et al., 1991). However, it has also been suggested that other factors than the hormonal changes of menopause influence mid-life weight gain, such as age and decline in energy expenditure (Al-Safi and Polotsky, 2014).

**Neuroactive steroids in relation to food intake and weight gain**

**Neuroactive steroids**

Steroids that regulate physiological functions of the central nervous system (CNS) have been named neuroactive steroids (Melcangi and Panzica, 2006). Like other steroid hormones, they can be synthesized from cholesterol (Mellon and Griffin, 2002, Melcangi et al., 2011) (Fig 3). Steroids that are both synthesized and have their action within the CNS are sometimes called neurosteroids (Baulieu, 1997, Corpechot et al., 1981). Enzymes required for the synthesis of neurosteroids from cholesterol are present in the brain (Do Rego et al., 2009). As aforementioned, steroids are lipophilic molecules, which mean that both precursor steroids and neuroactive steroids can easily cross the blood brain barrier from peripheral sources.

Several neuroactive steroids and neurosteroids are active at the GABA\textsubscript{A} receptor. To date, two different binding sites for some neuroactive steroids have been identified at the GABA\textsubscript{A} receptor and by binding to these sites the neuroactive steroids exert different effects. One site is localized in the transmembrane region of the α-subunit and by binding to this site, neuroactive steroids potentiate the effect of GABA. The other site is localized between the α and β-subunit and by binding to this site neuroactive steroids can directly activate the receptor (Hosie et al., 2006) (Fig 4). Typically, low concentrations of neuroactive steroids potentiate the GABA effect whereas higher concentrations activate the receptor directly (Belelli and Lambert, 2005, Hosie et al., 2006).

Two neuroactive steroids that are well studied and highly potent at the GABA\textsubscript{A} receptor are allopregnanolone and tetra-hydro-deoxy-corticosterone (THDOC). Allopregnanolone is synthesized from progesterone and THDOC
from deoxycorticosterone in two steps involving the enzymes 5α-reductase and 3α-hydroxysteroid dehydrogenase (Majewska et al., 1986, Paul and Purdy, 1992, Celotti et al., 1992) (Fig 3.).

*Figure 3.* Steroid synthesis. Neuroactive steroids like allopregnanolone and THDOC are synthesized from cholesterol.
Allopregnanolone

The neuroactive steroid and progesterone metabolite allopregnanolone (3α-hydroxy-5α-pregn-20-one) has been associated with feeding in several clinical studies. Allopregnanolone levels fluctuate during the menstrual cycle and follow levels of progesterone (Genazzani et al., 1998, Wang et al., 1996). They are therefore higher during the luteal phase of the menstrual cycle, which coincides with increased energy intake (Nyberg et al., 2007, Genazzani et al., 1998, Barr et al., 1995, Johnson et al., 1994, Bancroft et al., 1988). In women, circulating levels of allopregnanolone during the menstrual cycle are 1-4 nmol/l in the luteal phase compared to 0.2-0.8 nmol/l during the follicular phase (Nyberg et al., 2007, Kancheva et al., 2007, Genazzani et al., 1998, Havlikova et al., 2006, Pearson Murphy and Allison, 2000). Allopregnanolone levels in men, as well as in postmenopausal women are similar to those of fertile women during the follicular phase (Genazzani et al., 1998). The putative role of progesterone and its metabolites in feeding is supported by the fact that binge eating attacks are more frequent in the luteal phase, which coincides with elevated progesterone levels (Edler et al., 2007, Klump et al., 2008). There are also reports of increased energy intake during the luteal phase, and some women even experience food cravings (Barr et al., 1995, Johnson et al., 1994, Bancroft et al., 1988). Allopregnanolone and other neuroactive steroids have also been suggested to be involved in several eating disorders. Increased plasma levels of allopregnanolone have been detected in women with binge eating disorders and bulimia nervosa (Monteleone et al., 2001, Monteleone et al., 2003).

Allopregnanolone has also been associated with obesity and overweight in humans. Higher levels of allopregnanolone have been detected among obese individuals compared to individuals of normal weight, both women and men (Menozzi et al., 2002). In addition, overweight pubertal girls have been shown to have higher basal concentrations of allopregnanolone compared to girls of normal weight (Predieri et al., 2007). Higher allopregnanolone levels were also detected in obese prepubertal girls compared to lean prepubertal girls (Grosso et al., 2011). During pregnancy, allopregnanolone levels increase and the maximal levels measured can be as high as 100-250 nmol/l (Kancheva et al., 2007, Parizek et al., 2005, Luisi et al., 2000). As expected, pregnancy is associated with maternal weight gain. However, the maternal weight gain is often more than expected based on the weight of the fetus and fluids alone. In addition, women who gain more weight during pregnancy often experience difficulties reducing the weight to baseline post-partum (Mannan et al., 2013). Preliminary results in a study by A. Lundqvist indicate that women that gained more weight during pregnancy also had higher allopregnanolone levels (A. Lundqvist, personal communication).
allopregnanolone levels have also been detected following stress exposure in both humans and rats (Purdy et al., 1991, Droogleever Fortuyn et al., 2004).

In rodents, allopregnanolone has been linked to increased food intake in some studies (Chen et al., 1996, Reddy and Kulkarni, 1998, Reddy and Kulkarni, 1999), whereas others infer that GABA<sub>A</sub> receptor modulating steroids (GAMS) such as allopregnanolone merely reduce neophobia for new types of food, and thereby influence the food intake (Fudge et al., 2006, Higgs and Cooper, 1998). Recently, it was reported that rats treated with allopregnanolone during several days showed sustained elevated food intake, which resulted in increased weight gain (Nakhate et al., 2013). Allopregnanolone plasma levels in male rats have been measured as approximately 2 nmol/l (Barbaccia et al., 1998), whereas plasma levels in female rats fluctuate between approximately 6-30 nmol/l with the estrous cycle (Frye et al., 2000). However, levels in rat brain have been found to be higher than in circulation (Corpechot et al., 1993) and circulating allopregnanolone levels in rats are also increased during pregnancy (Concas et al., 1998).

Allopregnanolone can be synthesized within the brain (Purdy et al., 1991), as well as in the human ovary from the corpus luteum (Ottander et al., 2005), the adrenal glands (Corpechot et al., 1993) and the placenta (Dombroski et al., 1997).

Allopregnanolone has been suggested to be one of the most potent positive modulators of the GABA<sub>A</sub> receptor. Allopregnanolone binds with high affinity to the receptor (Majewska et al., 1986, Paul and Purdy, 1992) and increases the effect of GABA by affecting the frequency and duration of the Cl<sup>-</sup> channel opening (Lambert et al., 1995). The primary target for allopregnanolone is the GABA<sub>A</sub> receptor, although other possible targets have been suggested (Bali and Jaggi, 2014). In fact, Chen et al. were unable to block the allopregnanolone induced hyperphagia with picrotoxin (Chen et al., 1996), a known blocker of the ion channel in the GABA<sub>A</sub> receptor (Akaike et al., 1985). However, the hyperphagic effect of allopregnanolone in mice was effectively blocked by picrotoxin (Reddy and Kulkarni, 1998). Also, progesterone effects on food intake were also investigated by the same group and because the hyperphagic effect of progesterone was similar to the hyperphagic effect of allopregnanolone, they speculated that the progesterone effect could be mediated by its metabolite allopregnanolone (Reddy and Kulkarni, 1998). The dosage of allopregnanolone used by Chen et al. was higher than the dosage used by Reddy and Kulkarni while the dosage of picrotoxin was similar between the studies. This could be the reason for the discrepancy. Hence, there is evidence in the literature
supporting the GABA<sub>A</sub> receptor as the main mediator of the allopregnanolone induced hyperphagia.

**THDOC**

The neuroactive steroid THDOC (3α,21-dihydroxy-5α-pregnan-20-one) shares many properties with allopregnanolone. They are both increased in response to acute stress (Barbaccia et al., 1998, Purdy et al., 1991). They are both neuroactive steroids and are as such potent positive modulators of the GABA<sub>A</sub> receptor and share binding sites at the receptor (Majewska et al., 1986, Hosie et al., 2006). Regarding food intake and obesity, information on THDOC effects is scarce, but it has been shown in rats as well as humans that THDOCs precursor corticosteroids can stimulate food intake (Dallman et al., 1995, Tataranni et al., 1996). Increased cortisol levels have also been associated with obesity, especially abdominal/visceral obesity in humans (Peeke and Chrousos, 1995, Hollifield, 1968). In addition, several obese rodent models are hypercorticosteronemic (Guillaume-Gentil et al., 1990, Cunningham et al., 1986, McGinnis et al., 1992).

**The GABA neurotransmitter system**

**GABA**

γ-amino butyric acid (GABA) is the main inhibitory neurotransmitter in the CNS and approximately 20-30% of CNS neurons are GABAergic. GABA is synthesized from glutamate (the main excitatory neurotransmitter in the CNS) by the enzyme glutamic acid decarboxylase (GAD) (Petroff, 2002, Carver and Reddy, 2013) and is then released from GABAergic neurons. GABA can activate three types of receptors in the brain: GABA<sub>A</sub>, GABA<sub>B</sub> and GABA<sub>C</sub> receptors. The GABA<sub>A</sub> and GABA<sub>C</sub> receptors are fast responding ionotrophic ligand gated Cl<sup>-</sup> channels, whereas GABA<sub>B</sub> receptors are metabotropic G protein-coupled and slower in their response. Although GABA<sub>C</sub> receptors show a fast response, they are unaffected by typical GABA<sub>A</sub> receptor modulators, such as barbiturates, benzodiazepines and allopregnanolone (Barnard et al., 1998, Majewska et al., 1986).

GABA-ergic transmission and GABA<sub>A</sub> receptors are also important in the regulation of food intake. Food intake decreases after a reduction of GABAergic transmission from AgRP-expressing neurons and application of
GABA_A receptor agonists in hypothalamic key areas of food intake regulation result in hyperphagia (Tong et al., 2008, Wu et al., 2009, Pu et al., 1999, Tsujii and Bray, 1991).

**GABA_A receptors**

GABA_A receptors are, as aforementioned, chloride channels and they can be opened by the binding of GABA, which then allow negatively charged Cl⁻ to flow across the cell membrane. This alters the electrical potential, thereby affecting neuronal excitability. Generally, the concentration of Cl⁻ inside the cell is low and the GABA_A receptor mediated effect is inhibitory (Sieghart, 1995, Berne and Levy, 1993).

GABA_A receptors consist of five subunits forming a chloride channel (Barnard et al., 1998, Whiting et al., 1999). Several different subunits have been identified: α1-6, β1-3, γ1-3, δ, ε, θ, π. The composition of subunits can vary, but most GABA_A receptors are composed of two α, two β and one γ, δ or ε-subunit (Olsen and Sieghart, 2008) (Fig 4.). Each of the subunits has a distinct regional and cellular distribution in the brain (Pirker et al., 2000, Wisden et al., 1992). Different GABA_A receptor subunits or subunit combinations may mediate different physiological and pharmacological properties and may be associated to specific conditions, such as anxiety, feeding and drinking behavior, circadian rhythm, cognition, learning and memory (D'Hulst et al., 2009, Sieghart and Sperk, 2002).

GABA_A receptors containing the α1-subunit are the most abundant in the rodent brain. In the hypothalamus, which is highly important for regulation of food intake (Schwartz et al., 2000, Smith and Ferguson, 2008), the α2-subunit is the most common of the subunits, but α1, α3 and α5 are also found in this area (Wisden et al., 1992). β-subunits 1, 2 and 3 and γ-subunits 2 and 3 are also detected in the hypothalamus (Pirker et al., 2000). In the ARC of the hypothalamus, which is considered the key node for feeding regulation, it has been demonstrated that GABA-ergic AgRP neurons contain the α3-subunit, whereas POMC/CART neurons contain the α1-, α2- and α3-subunits (Backberg et al., 2004). Studies on benzodiazepine induced food intake suggest that the GABA_A receptor α-subunits involved are α2 and α3 (Morris et al., 2009, Cooper, 2005).
Several other substances than GABA can also modulate the GABA$_A$ receptor, e.g. benzodiazepines, barbiturates, ethanol and endogenous neurosteroids, which increases the GABA$_A$ receptor function (Sieghart, 1995, Korpi et al., 2002). These substances have many properties in common, typically sedative, anticonvulsant and anxiolytic (Majewska et al., 1986).

**Figure 4.** Schematic illustration of the GABA$_A$ receptor and its different subunits forming a pentameric ion-channel through the cell membrane. Binding sites for GABA, as well as neuroactive steroids like allopregnanolone and THDOC, are indicated in the picture.
**5β-pregnane-3β,20(R)-diol (UC1020)**

The 3α-hydroxy group within the A-ring of neuroactive steroids like allopregnanolone and THDOC has been suggested to be the essential for their positive modulatory effect on the GABA_A receptor (Harrison et al., 1987). Steroids with a 3β-hydroxy group within the A-ring (3β-hydroxy-steroids) on the other hand have previously been found to be antagonists to 3α-hydroxy-steroids, like allopregnanolone, in both rats and humans (Stromberg et al., 2009, Backstrom et al., 2005, Lundgren et al., 2003, Bengtsson et al., 2015, Turkmen et al., 2004). In this thesis work, the 3β-hydroxy-steroid 5β-pregnane-3β,20(R)-diol (UC1020), was characterized further in relation to food intake and GABA_A receptors suggested to be involved in food intake regulation. UC1020 has previously been tested in vitro in isolated cells from the medial preoptic nucleus and was found to significantly reduce allopregnanolone-induced prolongation of decay time for spontaneous inhibitory postsynaptic currents (sIPSCs) (Stromberg et al., 2009).

![Chemical structure of 5β-pregnane-3β,20(R)-diol (UC1020).](image)

**Figure 5.** Chemical structure of 5β-pregnane-3β,20(R)-diol (UC1020).
Aims

To elucidate acute effects of allopregnanolone on food intake in rats.

- To investigate feeding behavior in terms of latency to and duration of the next meal.
- To study intake of standard chow at two different time points during the day.
- To investigate if allopregnanolone affects rats’ preference for energy rich or palatable food.

To elucidate effects of repeated allopregnanolone exposure on food intake and body weight in rats.

- To investigate food intake and body weight gain under different feeding conditions.
- To investigate if obesity prone and obesity resistant rats are affected differently.

To characterize the endogenous compound UC1020.

- To characterize in vitro effects of UC1020 on allopregnanolone and THDOC-enhanced potentiation of GABA-evoked currents at GABA_A receptors expressing subunits suggested to play a role in food intake regulation, namely α2 and α3.
Materials and methods

The rat model – male Wistar rats

Male Wistar rats (Taconic, Denmark or Harlan, the Netherlands) were used in all the different experiments included in this thesis.

Since rats are nocturnal animals, a reversed dark-light-cycle was applied in all experiments to facilitate testing of the animals during their naturally active period. As diurnal variations have also been observed in the food intake of rodents (Farley et al., 2003, Bassil et al., 2007), care was taken to record food intake at exactly the same time point every day. In Paper I, allopregnanolone effects on food intake during the active period were also compared to food intake during the inactive period.

The rats were group housed (three rats per cage) in Paper I and II as well as initially in experiment 3 in Paper III (four rats per cage). Then, in some of the experiments (Paper I and II), they were transferred to single cages in which food and liquid intake was measured during one hour. Before onset of the experiments all rats were repeatedly habituated to spend one hour alone in the single cages. In the study detailed in Paper III, after the initial group housing, rats were housed in individual cages. Group housing of rats is preferred over single housing because it better resembles the natural environment of the species. Indeed, it has been shown that single housing can induce anxiety and depression-like behavior in rats (Djordjevic et al., 2012). However, in the study of Paper III, as the primary goal was to investigate the weight gain of individual rats and correlate this to food intake, rats were housed in single cages. To minimize possible stress due to housing, the cages were both larger and equipped with a higher roof so that the rats at all times could stand up, smell and hear the other rats. Also, additional paper to play with was introduced as environmental enrichment. Rats were also continuously handled in all experiments to reduce possible stress.

Male rats were chosen in these studies. As mentioned in the introduction, sex steroids are present in both sexes. Thus the progesterone metabolite allopregnanolone is also present in male rats but in more stable concentrations than in females (comparable to concentrations during diestrus) (Frye et al., 2000). The advantages of using male rats are to avoid interference from the natural hormonal fluctuations present in female rats.
Furthermore, in female rats, allopregnanolones effect on energy intake fluctuates with the estrous cycle (Reddy and Kulkarni, 1999). Therefore, if females are to be used, careful consideration must be taken to monitor the estrous cycle of every rat. With male rats, the time consuming and possibly confounding procedure of ovariectomy and hormonal and back therapies can be avoided. All the experimental protocols were approved by the Ethical Committees for Animal Studies, Umeå, Sweden.

**Diet induced obesity in rats**

Diet induced obesity (DIO) in animals is commonly used to study the susceptibility to and/or consequences of obesity. Diets used to induce obesity in animals are commonly high fat diets (HFD) with a fat content of > 30% or cafeteria diets with an selection of fat- and sugar-rich supermarket foods (Hariri and Thibault, 2010). Other common animal models for studying obesity are genetically obese Zucker rats, Wistar Kyoto fatty rat, and the obese ob/ob mouse (Lutz and Woods, 2012).

Both Wistar and Sprague Dawley rats display individual differences in weight gain when fed HFD (Dourmashkin et al., 2006, Pagliassotti et al., 1997, Buettner et al., 2007). This makes it possible to classify rats as obesity resistant (OR) or obesity prone (OP). In the work presented in this thesis, we used a classification based on weight gain during the first five days on a 45% HFD as previously used by Dourmashkin et al (Dourmashkin et al., 2006). It has been shown that there is a high and highly significant correlation between the initial weight gain (after one week) on a HFD and the weight gain after 5 weeks (Pagliassotti et al., 1997). In our experiment, out of total 48 rats, the highest tertile in weight gain was classified as OP, whereas the lowest tertile was classified as OR. The main advantage of using this type of model is it allows both environmental (diet) and genetic (individual differences) effects to be studied, as well as how they may interact.

**Weighing of foods, liquids and rats**

Rats were weighed on a laboratory scale in the same room where they were kept. The same scale was used to weigh different foods and liquids and these
weights were reported to one decimal place. To investigate if the room humidity affected the weights, some samples of chow pellets, cookies and HFD pellets were weighed separately over 2 days, but no differences were found.

All liquids (water and sucrose solution) and solid foods were presented to the rats in both the single and home cages prior to the onset of the experiments so that the rats became accustomed to the different types of diets.

**Filming and analysis of meal patterns**

In Paper I, rats in single cages were filmed using a digital camera after injection of allopregnanolone to document their feeding behavior. The light was set to the minimum level required to enable filming and rats were not disturbed during the filming. The films were then analyzed by an observer clocking the time of food consumption. All films were blinded for the observer. To assure accuracy, some films were scored separately by two observers and the results compared. This revealed no difference in scores. A meal was defined as terminated when more than 10 minutes had passed without further feeding activity (Demaria-Pesce and Nicolaidis, 1998).

**Different food types**

Different food types used in experiments included in this thesis is presented in Table 1 and 2. Nutrient percentages for the diets used in Paper II are presented in Table 1. The diets used in Paper III are presented in Table 2, which show nutrient percentages for standard chow diet and 45% HFD (both obtained from Special Diets Services) as (AFE) Atwater fuel energy, which is similar to metabolisable energy and takes into account energy loss, e.g. through urine and faeces (Merrill and Watt, 1973). Buettner et al reviewed studies on DIO by HFD during a period of 10 years and concluded that the most efficient way to induce obesity was to use diets high in animal fats (Buettner et al., 2007), therefore we used a diet with fat mostly from lard.
**Table 1.** Nutrient percentages (w/w %) of the different food types used in Paper II.

<table>
<thead>
<tr>
<th>Nutrients (%)</th>
<th>Standard chow</th>
<th>10% sucrose solution</th>
<th>Cookies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbohydrate</td>
<td>57.6</td>
<td>9.7</td>
<td>65.3</td>
</tr>
<tr>
<td>Fat</td>
<td>3.4</td>
<td>-</td>
<td>25.0</td>
</tr>
<tr>
<td>Protein</td>
<td>18.6</td>
<td>-</td>
<td>6.3</td>
</tr>
<tr>
<td>Kcal/g</td>
<td>3.5</td>
<td>0.4</td>
<td>5.2</td>
</tr>
</tbody>
</table>

**Table 2.** Nutrient percentages (AFE %) of the diets used in Paper III.

<table>
<thead>
<tr>
<th>Nutrients (AFE%)</th>
<th>Standard chow</th>
<th>High fat diet 45%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbohydrate</td>
<td>68</td>
<td>35</td>
</tr>
<tr>
<td>Fat</td>
<td>9</td>
<td>45</td>
</tr>
<tr>
<td>Protein</td>
<td>22</td>
<td>20</td>
</tr>
<tr>
<td>Kcal/g</td>
<td>3.5</td>
<td>4.6</td>
</tr>
</tbody>
</table>

**Food preference tests**

In Paper II, we used food preference tests to determine which type of food allopregnanolone treated rats prefer in a choice situation. The tests were similar to previously used food preference tests (Giraudo et al., 1999, Olszewski et al., 2003, Bomberg et al., 2007). These tests make it possible to distinguish if a treatment affects ingestion of a certain food type more exclusively. In the first food preference test, two food types were offered, i.e. standard chow and Maryland chocolate chip cookies. In this experiment the cookies were more calorie dense (5.2 compared to 3.5 Kcal/g) but also more palatable. The aim of this test was to investigate whether the more energy dense as well as palatable food type would be preferred by allopregnanolone treated rats in comparison to standard chow. In the second food preference test, two food types were again offered but this time they were standard chow and a 10% sucrose solution. The chow was more calorie dense than the sucrose solution (3.5 compared to 0.4 kcal/g), but the sucrose solution was more palatable than chow. In this test, the aim was to investigate whether at first hand the palatability or energy content was affected by allopregnanolone.
Ad libitum and scheduled feeding

Ad libitum feeding was used in the majority of the animal experiments. However, in Experiment 2 and 3 in Paper III, scheduled feeding was used. This scheduled feeding allowed rats access to food (chow or HFD) during 4 hours at the beginning of the dark/active period. Scheduled feeding influences different parameters important for feeding regulation, as discussed further in the Introduction part of this thesis. One may speculate that the 20 hour fast would stress the rats, but as we habituated the rats to this feeding regime before the experiments, this effect was likely to be insignificant. In addition, it has been shown that a 24 hour fast does not induce chronic stress or increase levels of stress markers in rats (De Boer et al., 1989).

Acute allopregnanolone s.c. injections

The vehicle used for acute allopregnanolone injections in this thesis work was a 2-hydroxypropyl-β-cyclodextrine (β-cd) solution (Sigma Chemical Co., St. Louis, MO, USA). Allopregnanolone (3α-hydroxy-5α-pregn-20-one) was obtained from Umecrine AB (Umeå, Sweden) and dissolved in β-cd by ultra-sonication. An initial dose response test was performed (Paper I), in which vehicle and different doses of allopregnanolone (1.25, 2.5, 5.0, 10.0 mg/kg) were subcutaneously (s.c.) injected into rats and the effects on intake of standard chow were measured. For the subsequent acute s.c injections used in the experiments of Paper I and II, the two lower doses were chosen (1.25, 2.5 mg/kg).

Repeated allopregnanolone s.c. injections

The vehicle used for repeated allopregnanolone injections in this thesis work was sesame oil (Sigma-Aldrich, St. Louis, MO, USA). A suspension of allopregnanolone in sesame oil was prepared by 48 hours’ stirring with a magnetic stirrer at room temperature. Sesame oil was used as the vehicle because we strived to achieve slow sustained drug release, resulting in prolonged drug duration. Therefore, the concentration used (20 mg/kg) in Paper III was also higher than in Paper I and II.
Allopregnanolone quantification

The plasma concentration of allopregnanolone obtained after acute s.c. injections of 1.25 and 2.5 mg/kg allopregnanolone was quantified at different time points after injection (at 10, 30, 60, 120, and 240 minutes) (Paper I, Fig 5)

The allopregnanolone assay has been described in detail elsewhere (Timby et al., 2006). Briefly, blood samples were drawn from the rats’ tail vein, then, after centrifugation, plasma was collected and frozen at -20°C.

**Diethyl ether extraction**

For quantification of allopregnanolone in the plasma samples, diethyl ether was added to the plasma to perform liquid-liquid extraction. The ether phase, which contained allopregnanolone, was decanted and evaporated by nitrogen gas.

**Celite chromatography purification**

The extractions were further purified and separated with celite chromatography to separate allopregnanolone from steroids that cross-react with the polyclonal antibody used in the radioimmune assay (RIA). The evaporated sample was first dissolved in isooctane saturated with ethylene glycol and then applied to a celite column. After washing with isooctane, the fraction containing allopregnanolone was collected and evaporated. The recovery rate of allopregnanolone was calculated by adding a control sample containing a known amount of tritium-labelled allopregnanolone. All results were compensated for the recovery. Two control samples with known amounts of allopregnanolone were also included in each assay.

**RIA**

The evaporated samples were quantified by employing a RIA. The antibody used was a polyclonal rabbit antiserum made against 3α-hydroxy-20-oxo-5α-pregnan-11-yl carboxymethyl ether coupled to bovine serum albumin (generous gift from Professor R.H. Purdy, The Scripps Research Institute, CA, USA) (Purdy et al., 1990). The radioactivity of the unbound tritium-labeled allopregnanolone in the samples was counted in a RackBeta (Wallac) scintillation counter in counts per minute (cpm). A standard curve was constructed showing the level of radioactivity (cpm) as a function of amount.
of allopregnanolone (ng). The inter-assay coefficient of variation for the allopregnanolone assay was 8.5%.

**Whole-cell voltage-clamp electrophysiology**

Electrophysiological techniques can be used to measure changes in ion flow over membranes e.g. through the GABA<sub>A</sub> receptor channel. In Paper IV, electrophysiological recordings from HEK-293 cells expressing different GABA<sub>A</sub> receptors were performed by the whole-cell patch-clamp technique under voltage-clamp conditions at a holding potential of -16 mV. By this technique recordings were made from single cells attached to a glass pipette containing an electrode and filled with intracellular solution. After attachment of a cell to the pipette, the small patch of membrane in the pipette tip was ruptured by gentle suction. This allowed good electrical control over the cell membrane (Fig 6). The recordings were made under voltage-clamp conditions, i.e. the membrane potential was kept stable by setting a reference value. This so-called holding potential was maintained by applying a compensatory current over the membrane. This current was recorded to provide information on the ion-flow over the membrane. In this case, the current response of the GABA<sub>A</sub> receptor to different steroids and compounds was measured. The Dynaflow™ system (Dynaflow Pro II Platform Zeiss Axiovert 25; Cellectronic AB, Sweden) with Resolve chips was used for all patch-clamp experiments.

**Figure 6.** Simplified schematic illustration of the whole-cell patch-clamp setup.
Transfection and expression of GABA\textsubscript{A} subunits in HEK-293 cells

The transfection procedure is described in detail in Paper IV. Briefly, vectors, including the human CMV promoter for constitutive expression of the human $\alpha_1$, $\beta_2$, and $\gamma_2$ GABA\textsubscript{A} receptor subunits ($\alpha_1\beta_2\gamma_2$), human $\alpha_2$, $\beta_3$, and $\gamma_2$ GABA\textsubscript{A} receptor subunits ($\alpha_2\beta_3\gamma_2$) or human $\alpha_3$, $\beta_3$, and $\gamma_2$ GABA\textsubscript{A} receptor subunits ($\alpha_3\beta_3\gamma_2$) were used to permanently transfect wild-type HEK-293 cells (originating from the Pasteur Institute, Paris, France). HEK-293 cells that expressed the GABA\textsubscript{A} receptors of interest were then used for electrophysiological recordings.

Electrophysiological recordings

Execution of the electrophysiological recordings is described in detail in Paper IV. Briefly, borosilicate patch pipettes were pulled, polished and filled with intracellular-like solution (IC; resistance 2-5 M$\Omega$). The chip was filled with extracellular-like solution (EC) and HEK-293 cells that expressed GABA\textsubscript{A} receptors were added to the bath and patched (Fig 6). The maximum series resistance allowed between the pipette and cell membrane was 20 M$\Omega$. The experimental setup was designed to mimic the conditions of phasic (synaptic) activation of GABA\textsubscript{A} receptors with brief high concentrations of GABA. Allopregnanolone/THDOC used alone or together with UC1020 was applied 20 s prior to and during GABA application. At least 2 min of wash out with control solution was used between trials. In addition, all cells tested were challenged with GABA alone, GABA and allopregnanolone/THDOC and GABA and allopregnanolone/THDOC and UC1020. Therefore, each cell served as its own control. The recordings were compared to the control value, e.g. GABA or GABA and GAMS, and reported as relative values.

Statistics

Statistical analyses were performed using SPSS version 18, 20 and 22 (SPSS Inc., Chicago, IL, USA) Software, GraphPad Prism (GraphPad Software, Inc. La Jolla, CA, USA) computer programs.

Cross-over design

In Paper I, Experiment 2, Paper II, Experiment 1 and 2, a cross-over design was used. The advantage of this design is that every rat receives all treatments. Therefore, every rat can serve as their own control, which decreases the risk of confounders. One disadvantage of this design is that
the preceding treatments might influence the effect of the following treatments, i.e. carry-over effect. To overcome this potential problem, a wash out period of at least 3 days was used between trials.

**Stratified randomization**

In Paper III, stratified randomization based on the rats´ body weights and baseline food intake was used to allocate rats into comparable treatment groups. In the last experiment of Paper III, rats were first subdivided into OP and OR groups and rats in each group were then randomized as described above.

**Tests used**

The non-parametric **Kruskall-Wallis test** was used to compare differences between several independent groups with a different set of rats in each group. This test was used because the data were not normally distributed, and therefore violated the assumption of one-way independent ANOVA. Kruskall-Wallis test was used in Paper I to compare allopregnanolone induced effects on food intake in the dose-response test, as well as for different time points (Experiment 1). In Paper I the Kruskall-Wallis test was also used to investigate differences in chow intake among rats that ate, i.e. “eaters”, (Experiment 2) as well as to compare the duration of the first meal within the different treatments (Experiment 3). In Paper III, the Kruskall Wallis test was used to compare HFD intake for OR and OP rats treated with allopregnanolone and vehicle (Experiment 3).

The non-parametric **Mann-Whitney U test** was used to compare two conditions with a different set of rats in each group. In Paper II, the Mann-Whitney U test was used to compare total liquid and total kcal intake between Experiment 1 and 2. The test was also used in Paper III to compare food intake between allopregnanolone and vehicle treated rats and also to compare the area under the curve (AUC)s for body weight gain (day vs. weight gain). The Mann-Whitney U test was also used as a post-hoc test to the Kruskall-Wallis test in Paper I and III.

**Friedman’s analysis of variance (ANOVA)** is the non-parametric version of repeated measures ANOVA. Friedman´s test was used in Paper I and II to compare effects of allopregnanolone on food intake in the cross-over designs. The test was also used to compare duration of the first meal between treatments. Since Friedman´s test is not available as a 2-way ANOVA, the AUC was calculated for each animal when comparing intake during the active and inactive period in Paper I. AUCs were also used in
Paper II when comparing intake of different food types in the food preference tests. To compare AUCs, the paired sample Wilcoxon signed rank test was used.

The non-parametric **paired sample Wilcoxon signed rank test** was used to compare two conditions where the same rats took part in both conditions. This test was used because the data violated the assumption of the dependent t-test, i.e. the data were not normally distributed. In Paper I, the test was used to compare AUCs as mentioned above. It was also used to compare the increase in chow intake induced by the highest allopregnanolone dose between the active and inactive period. Additionally, the paired sample Wilcoxon signed rank test was used as a post-hoc test to Friedman’s test in Paper I and II. In the in vitro study in Paper IV, the test was used to compare AUCs for the relative effect of UC1020 on GAMS potentiation to basic GAMS potentiation, as well as AUCs for the relative effect of UC1020 on GABA potentiation to basic GABA potentiation.

**Fisher’s exact test** was used in Paper I to examine whether the number of animals that initiated a meal differed between vehicle and allopregnanolone treated rats.

The **Kaplan-Meier analysis** was used in Paper I to compare how long it took for the rats to initiate their first meal after they had been treated with vehicle or allopregnanolone. This analysis was used because it could account for the fact that not all rats had initiated their first meal within the studied period (1 hour).

**Correlations by Spearman’s rho ($r_s$).** The non-parametric Spearman’s correlation coefficient, $r_s$, was used in Paper III to investigate correlations between food intake and weight gain. It was used because the data violated the assumption of a normal distribution required for using Pearson’s correlation coefficient ($r$).

Post hoc tests were adjusted by **Bonferroni corrections**. Each statistical test that is run on the same data increases the probability of obtaining at least one significant result by chance, and thereby increases the risks of obtaining false-positive results (type I error). The Bonferroni correction reduces the risk of this kind of error (Bland and Altman, 1995). In the work included in this thesis Bonferroni corrections were calculated by multiplying the obtained $p$-values by the number of tests performed, yielding a new corrected $p$-value. A disadvantage of the Bonferroni correction is that it is rigid, which increases the risks of obtaining false-negative results (type II error).
Results

Allopregnanolone increased food intake in male Wistar rats

Allopregnanolone dose dependently increased the intake of standard chow in male Wistar rats. Allopregnanolone also significantly increased the intake of chocolate chip cookies, as well as the 45% HFD. However, no effect was seen in the intake of sucrose solution when presented simultaneously with chow.

The experiments described in Paper I were repeated several times with the same animals but the effect of allopregnanolone on food intake did not decline. Thus, it seems unlikely that the allopregnanolone induced hyperphagia was merely caused by reduced neophobia as has been suggested (Higgs and Cooper, 1998, Fudge et al., 2006).

For both chow and HFD, the increase in food intake induced by allopregnanolone was more pronounced during the first hours following administration. When allopregnanolone was s.c. injected as a solution increased food intake was mainly seen during the first hour, followed by three hours of lower but stable intake comparable to that of vehicle treated animals. However, when allopregnanolone was s.c injected as a suspension the main increase in food intake was seen during the first two hours. This could be due to slower drug release. No rebound effect was seen in the experiments. It could be speculated that the reason for the transient effect was that increased satiety signals lead to decreased activity of GABAergic orexigenic neurons, thereby limiting the effect of allopregnanolone, and thus influencing later food intake (Riediger et al., 2004, Valassi et al., 2008).

Rats are nocturnal animals, active during the dark period and commonly consume the major part of their food during this period (Farley et al., 2003, Bassil et al., 2007). In all food intake studies, except one where we showed that allopregnanolone had a much weaker effect at the beginning of the light period, allopregnanolone was given at the onset of darkness, i.e. the time point when ghrelin levels are high (Murakami et al., 2002) and rats usually consume a large meal. However, the effect of allopregnanolone at other time points was not investigated but would be an interesting aspect to investigate in future work.
Diurnal variation in allopregnanolone´s effect on food intake

Previous studies on allopregnanolone and feeding in rodents have produced diverse results (Chen et al., 1996, Reddy and Kulkarni, 1998, Reddy and Kulkarni, 1999, Fudge et al., 2006). Therefore, we investigated whether the discrepancies could be traced to a diurnal difference in the effect of allopregnanolone. When comparing allopregnanolone effects at the beginning of either the rats´ active/dark period or the beginning of their inactive/light period, we found that the allopregnanolone effect on food intake was more prominent during the beginning of the active/dark period (Fig 7). Allopregnanolone also had an effect during the inactive/light period but the increase induced by 2.5 mg/kg allopregnanolone was half as large as with the same dose during the active/dark period. In this aspect, the effect of allopregnanolone appears to differ from the effect of ghrelin because the latter is not dependent on the time of day (Finger et al., 2011). It was not within the scope of this thesis to study the mechanism by which allopregnanolone might act, but the diurnal differences detected are in line with the idea that allopregnanolone´s effect is mediated by neurons that show higher activity during the active/dark period, such as e.g. AgRP neurons in the ARC (Lu et al., 2002, Peterfi et al., 2004).

![Graph](image)

**Figure 7.** Allopregnanolone-induced increase in chow intake was more prominent at the beginning of the rats´ active period compared to at the beginning of the inactive period of the day. Chow intake per rat during 1 hour (mean ± SEM) (n= 24 per treatment, per time point). Significant differences compared with the corresponding vehicle are marked *** (p≤0.001) and ** (p≤0.01).
Allopregnanolone reduced feeding latency and increased duration of the first meal

In the feeding behavior study in Paper I, chow was introduced at different time points: immediately 10, 20, 30, or 60 minutes after treatment. Allopregnanolone significantly reduced the feeding latency compared to vehicle for all time points except for 10 min (for which $p=0.065$). The feeding latency of rats with immediate access to chow after injections is shown in Fig 8.

![Feeding latency graph](image)

**Figure 8.** Allopregnanolone reduced rats’ latency to the next coming meal after treatment compared to vehicle. Feeding latency was measured as the time to next meal for each animal after immediate access to standard chow and water following subcutaneous injections of vehicle or allopregnanolone (n=12 per treatment).

The duration of the first meal after treatment was also analyzed and 2.5 mg/kg allopregnanolone was found to significantly prolong the duration of the next coming meal compared to vehicle (Fig 9).

The time of food introduction did not seem to have any major influence on the duration of the following meal. However, the plasma concentration of allopregnanolone for the dosages used was elevated during all these time points. This makes it difficult to draw any conclusions on possible correlations between food intake and plasma concentration of allopregnanolone.
### Figure 9. Allopregnanolone increased the duration of the next coming meal after treatment. Data are presented as mean ± SEM. Significant difference compared with the corresponding vehicle is marked ** (p≤0.01).

Thus, from the study on feeding behavior in Paper I, we concluded that allopregnanolone reduced feeding latency as well as increased the duration of the next coming meal, thus indicating an increased meal size. Increased meal size has been associated with obesity in both humans and rats (Berg et al., 2009, Furnes et al., 2009, Farley et al., 2003). That allopregnanolone may influence the meal size emphasizes the importance of further investigations on allopregnanolone and food intake.

**Rats preferred a more calorie dense food type to a more calorie dilute but palatable food when treated with allopregnanolone**

In Paper II, rats were subjected to two different food preference tests to elucidate which type of food allopregnanolone treated animals prefer. When the rats had access to standard chow and cookies, which are more calorie dense and more palatable than chow, allopregnanolone treated rats significantly increased their intake of cookies but not of chow (Fig 10). On the other hand when the rats had access to standard chow and a 10% sucrose solution, which is less calorie dense but more palatable than chow, allopregnanolone treated rats significantly increased their intake of chow, but no effect was seen for the intake of sucrose solution (Fig 11). The 10%
sucrose solution had an energy density that was comparable to most sweetened beverages, i.e. 0.4 kcal/g (DiMeglio and Mattes, 2000).

**Figure 10.** Allopregnanolone increased the energy intake from cookies but not from chow in a food choice test. Consumption was measured for 1 h after subcutaneous injections of allopregnanolone or vehicle. Data are presented as means ± SEM (n= 24 per treatment per food type). Significant differences compared to the corresponding vehicle are marked * (p≤ 0.05) and ***(p≤ 0.001).

**Figure 11.** Allopregnanolone increased the energy intake from chow but not from 10% sucrose solution in a food choice test. Consumption was measured for 1 h after subcutaneous injections of allopregnanolone or vehicle. Data are presented as means ± SEM (n= 24 per treatment per food type). Significant differences compared to the corresponding vehicle are marked **(p≤ 0.01).
Previous studies have reported increased intake of both bland and more palatable food in animals treated with allopregnanolone (Holmberg et al., 2013, Chen et al., 1996, Reddy and Kulkarni, 1998, Reddy and Kulkarni, 1999). However, in those studies, there was no choice offered and all foods (except in Holmberg et al 2013) were palatability enhanced chow, which is relatively high in calories. Fudge et al. used a calorie dilute sucrose solution in their experiments. However, they only detected a hyperphagic effect of allopregnanolone during the initial experiments and suggested that this was due to reduced neophobia. Taken together, these studies support our view that allopregnanolone appears to at first hand increase the intake of energy rich food, whereas palatability is of secondary importance.

Cookies were used as an energy dense as well as highly palatable food source in Paper II. Cookies are examples of the kind of food often consumed during binge eating attacks (Hadigan et al., 1989, Guertin and Conger, 1999). In fact, in humans, allopregnanolone has been associated with binge eating attacks (Monteleone et al., 2003). In addition, elevated energy intakes in women have been reported during the luteal phase of the menstrual cycle (Barr et al., 1995, Johnson et al., 1994). Moreover, Barr et al compared energy intake during ovulatory and anovulatory cycles and concluded that the luteal increase in energy intake is only present during ovulatory cycles (Barr et al., 1995) where a corpus luteum producing progesterone/ allopregnanolone is formed (Ottander et al., 2005). Thus, our results showing that allopregnanolone primarily increases the intake of cookies compared to chow are in line with these observations and could suggest possible allopregnanolone mediated effects on food intake during the luteal phase of the menstrual cycle. It should be noted that the luteal phase has been associated with elevated symptoms of PMS (Backstrom et al., 1983), and uncontrolled ingestion of energy dense, highly palatable foods may represent a way to self-medicate mood disorders (Markus et al., 2000, Prasad and Prasad, 1996). Intriguingly, a paper examining this question reported no association between premenstrual food craving and mood change, either in timing or severity (Bancroft et al., 1988). This indicates that premenstrual food cravings could be a separate symptom, possibly induced by increased energy needs during the luteal phase that is first of all a preparatory phase for a forthcoming pregnancy (Butte and King, 2005). However, sex hormones like progesterone and estradiol also fluctuate with the menstrual cycle and there are reports of positive associations between binge eating attacks and progesterone, as well as negative association between binge eating attacks and estradiol (Klump et al., 2008, Edler et al., 2007). Thus, there may be other possible mediators for the increased energy intake reported during the menstrual cycle.
The allopregnanolone precursor progesterone has also been related to increased intake of fat in rats rather than carbohydrates and proteins (Leibowitz et al., 1998, Leibowitz et al., 2007). In humans, the data are more diverse and progesterone has been linked to both elevated intake of fat and carbohydrates (Tarasuk and Beaton, 1991, Johnson et al., 1994, Dalvit-McPhillips, 1983). Allopregnanolone levels during the estrous cycle in rats follow the fluctuations of progesterone (Ichikawa et al., 1974, Genazzani et al., 1998) and the results from our experiments suggest increased intake of the higher fat containing food item (25% fat for cookies compared to less than 5% fat for chow). One could hypothesize that allopregnanolone like progesterone might have an impact on macronutrient selection, favoring fat. However, at this stage, this is merely a hypothesis which needs to be further investigated.

Other researchers have used food preference tests offering a choice between standard chow and sucrose solution to study the effects of different food regulating peptides (ghrelin, NPY, AgRP, and μ-opioid agonist DAMGO in rats (Giraudo et al., 1999, Olszewski et al., 2003, Bomberg et al., 2007). Ghrelin, AgRP and NPY preferentially increased the intake of standard chow, the more energy dense food type. The authors suggested that the food intake induced by these peptides was thus primarily driven by energy needs rather than reward. In contrast, the μ-opioid agonist DAMGO preferentially increased the intake of the calorie dilute sucrose solution, suggesting that DAMGO affected the reward component of food intake. The food preference tests we used for the work in Paper II were based on the abovementioned studies. As allopregnanolone preferentially increased the intake of the energy dense standard chow and had no effect on the simultaneously presented sucrose solution, it could be speculated that the allopregnanolone induced hyperphagia might primarily be driven by energy needs.

In the first food preference test, the rats had access to both standard chow and cookies. Allopregnanolone increased intake of the more energy dense cookies. However, the cookies were also the more palatable food type present, so possible influences on food intake caused by palatability cannot be disregarded. However, the increase in cookie intake (120% increase compared to vehicle) induced by allopregnanolone in the choice situation with cookies and chow present was not larger than the increase of chow intake (155% increase compared to vehicle) in the choice situation with chow and sucrose solution. Thus, allopregnanolone appears to increase consumption of the most energy-rich food available regardless of palatability. Also, it has been reported that food-deprived rats tend to choose more energy dense food compared to ad libitum-fed rats, which tend to choose more rewarding food (Scheggi et al., 2013). In our study, rats had ad
libitum access to food and would thus be even more inclined to choose a more rewarding food type, the sucrose solution, but we still detected no effect of allopregnanolone on intake of sucrose solution.

Indeed there was a large difference in energy content between the two energy sources in the food preference test with sucrose and chow (0.4 kcal/g for sucrose and 3.5 kcal/g for chow) However, no significant effect of allopregnanolone on sucrose consumption was found regardless of whether the weight (1.8 g increase compared to vehicle) or the energy content (0.7 kcal increase compared to vehicle) was used for calculation. Also, no ceiling effect should have been present as rats have been reported to have a capacity for consuming more liquid than the highest recorded intake in our experiments (Giraudo et al., 1999, Kendig et al., 2013).

**A scheduled feeding paradigm and repeated exposure of allopregnanolone increased rats’ daily intake of standard chow**

In Paper III, rats were schedule fed, with chow access during 4 hours per day, at the same time as they were s.c. injected with allopregnanolone once a day during 5 consecutive days. During this scheduled feeding paradigm, allopregnanolone significantly increased the daily intake of standard chow in contrast to when chow was given ad libitum (Fig 12 A and 13 A). Schedule fed rats treated with allopregnanolone also increased their body weight gain compared to vehicle but only on day 4 and 5. This pattern differed from that of the ad libitum fed animals, for which no significant weight gain was recorded, even if a slightly increasing tendency was also detected for the ad libitum fed animals (Fig 12 B and 13 B).

Schedule feeding was used since it enabled us to study the effect of allopregnanolone under conditions that to a larger extent resemble the feeding patterns in humans with distinct meals. In humans, there is a pre-prandial rise of orexigenic ghrelin before the onset of a meal in parallel with increased hunger ratings (Cummings et al., 2004). Increased ghrelin levels have also been suggested to be involved in meal initiation (Cummings et al., 2001). In rats, a similar pattern of ghrelin has been recorded in schedule fed animals (Verbaeys et al., 2011). It is thus likely that our schedule fed animals were exposed to elevated ghrelin levels at the time point when allopregnanolone was administered.
Figure 12. Allopregnanolone had a minor effect on energy intake and weight gain in rats fed chow ad libitum. Rats were subcutaneously injected with allopregnanolone (n= 12) or control (n=18) once daily for five consecutive days. A) Intake of chow during the first 2 h and the daily total over 24 h (mean ± SEM). B) Body weight gain (mean ± SEM) during treatments. Significant differences compared to the corresponding vehicle are marked * (p≤ 0.05).

Figure 13. Allopregnanolone increased the energy intake as well as body weight gain in rats schedule fed chow. Rats were subcutaneously injected with allopregnanolone (n= 12) or control (n=18) once daily for five consecutive days. A) Intake of chow during the first 2 h and daily total over 4 h (mean ± SEM). B) Body weight gain (mean ± SEM) during treatments. Significant differences are marked * (p≤ 0.05).

Scheduled feeding also affects other feeding regulating substances. Increased levels of e.g. AgRP have been measured in fasting animals (Shen et al., 2002). AgRP neurons coexpress GABA, and GABAergic-transmission has been suggested to be crucial for maintaining normal feeding regulation (Wu et al., 2009, Cowley et al., 2001, Horvath et al., 1997). Allopregnanolone is a
potent positive modulator of the GABA_A receptor (Majewska et al., 1986). Thus, we hypothesized that the effect of allopregnanolone would be elevated during a schedule feeding paradigm as these animals should have a stronger hunger drive from the outset due to higher activity of AgRP neurons and concurrent GABA release. In Paper III, we showed that allopregnanolone had a slightly more explicit effect on food-intake with schedule fed animals compared to animals fed ad libitum. Similar results were also obtained for body weight gain. These results are in line with our hypothesis. However, it is important to note that the increased food intake in the schedule fed rats compared to ad libitum fed rats was of the same magnitude.

Weight gain due to repeated allopregnanolone administration has previously been reported in male Sprague Dawley rats (Nakhate et al., 2013). Nakhate et al s.c. injected rats once daily with allopregnanolone for 7 days and reported significantly increased intake of chow, as well as increased body weight gain. In that study, the rats had ad libitum access to chow and allopregnanolone was given in solution (1 and 2 mg/kg). In our experiments, the rats were s.c. injected with a suspension (20 mg/kg allopregnanolone in sesame oil), and contrary to Nakhate et al, we did not detect any significant increase in daily chow intake nor weight gain with daily repeated allopregnanolone treatment in ad libitum fed rats. One major difference between the studies is the type of vehicle used, namely that we used allopregnanolone in a suspension. In Paper I and II we used a vehicle and dosages more similar to that used by Nakhate et al and under such conditions we obtained results of similar magnitude (Holmberg et al., 2013, Holmberg et al., 2014). Also, Nakhate et al continued their experiment for 7 days. Therefore if we had repeated our injections two days longer to a total of 7 days, we might also have reached significance in our data since there was a tendency in that direction. We aimed to achieve a relatively slow and more sustained allopregnanolone release through the use of a sesame oil suspension. In fact, different results have been reported in animal experiments where allopregnanolone has been administered either in a repetitive way or by continuous delivery (Chen et al., 2011, Bengtsson et al., 2012). Much like our results in Paper I, Nakhate et al detected the main increase in chow intake during the first hours.

Nakhate et al also s.c. injected rats with dehydroepiandrosteronesulfate (DHEAS), which is a neurosteroid that modulates the GABA_A receptor negatively. In their experiment, DHEAS decreased both food intake and body weight gain, which indicates that these effects are mediated by the GABA_A receptor. Furthermore, they mapped expression of CART neurons, which mediate satiety, following allopregnanolone administration and detected a decrease in CART immunoreactivity in the ARC, PVN, lateral
hypothalamus (LH) and NAc (Nakhate et al., 2013). This supports our idea that allopregnanolone could act postsynaptically to inhibit satiety promoting neurons.

**Repetitive exposure to allopregnanolone during scheduled feeding of a high fat diet increased the rats weight gain**

In Paper III, allopregnanolone was found to significantly increase both the daily energy intake (+9.2 kcal/day) and body weight gain (+8 g/5 days) of rats schedule fed a 45% HFD compared to vehicle treated rats (Fig 14). Moreover, the body weight gain correlated with the overall energy intake. Although correlation does not prove causation, the results of our study suggest that the increased intake of HFD induced by allopregnanolone in schedule fed rats led to an increased body weight gain. In support of this conclusion, the amount of energy intake measured in our experiments was in line with the observed level of weight gain (Le Magnen and Devos, 1982). This makes it less likely that a decrease in activity due to the putative sedative effects of allopregnanolone would have influenced the weight gain (Wieland et al., 1995). Also, in earlier behavioral studies (data not shown), we documented no effect of allopregnanolone on overall activity of the rats.

**Figure 14.** Allopregnanolone increased energy intake as well as body weight gain in rats schedule fed a high fat diet. Rats were subcutaneously injected with allopregnanolone (n=16) or vehicle (n=16) once daily for five consecutive days. A) Intake of high fat diet during the first 2 h and daily total over 4 h (mean ± SEM). B) Body weight gain (mean ± SEM) during treatments. Significant differences compared to the corresponding vehicle are marked * (p ≤ 0.05) and ** (p ≤ 0.01).
Both energy intake and body weight gain were larger when rats were schedule fed a HFD compared to schedule fed standard chow. It has been described that progesterone can increase fat storage in adipocytes (Kim and Kalkhoff, 1975, Steingrimsdottir et al., 1980, Mayes and Watson, 2004). As allopregnanolone is a progesterone metabolite, it may have similar effects. This could be one explanation for why the body weight gain induced by allopregnanolone in schedule fed rats on HFD was more pronounced than the body weight gain induced by allopregnanolone in schedule fed rats on chow. However, this is merely a hypothesis that needs further investigation.

Schedule fed rats on HFD that were treated with allopregnanolone ate approximately 9 kcal more per day than vehicle treated rats. For comparison in humans, Raclette et al discussed that such a small surplus of energy intake as 10 calories per day results in a 0.45 kg weight gain each year, and if continued, this can be of importance for health (Racette et al., 2003). Thus, this indicates that the level of energy increase induced by allopregnanolone could be of importance in the discussion on unhealthy weight gain.

In our experiments, allopregnanolone treated rats had a surplus in body weight of 8 g/5 days. This might seem minor, but an increase of 8 g/5 days over longer time will result in substantial weight increase. In addition, it has been concluded that losing even a small amount of weight can be highly beneficial especially for overweight and obese persons. In 2013, on the National Heart, Lung, and Blood Institutes (NHLBI) initiative, the American College of Cardiology (ACC), the American Heart Association (AHA) and the Obesity Society (TOS) established guidelines for clinicians on the management of overweight and obesity in adults. They found strong evidence that overweight and obese adults with cardiovascular risk factors benefit from even a modest sustained weight loss of 3-5%. Of course, even greater health benefits are achieved by greater weight loss (Jensen et al., 2014).

**Both obesity resistant (OR) and obesity prone (OP) rats increased their weight gain with allopregnanolone treatment**

Allopregnanolone treatment significantly increased the body weight gain in both OR and OP rats that were schedule fed a 45% HFD (Fig 15). The body weight gain also significantly correlated with total energy intake. Compared
to vehicle treated animals, allopregnanolone treated OR rats gained on average 9.0 g more than vehicle treated OR rats and allopregnanolone treated OP rats gained on average 6.4 g more than vehicle treated OP rats. Thus, there was a tendency for OR rats to gain more in body weight compared to OP rats. However the difference was not significant.

Figure 15. Allopregnanolone increased the body weight gain in both obesity resistant and obesity prone rats schedule fed a high fat diet. Body weight gains (mean ± SEM) of A) obesity resistant rats (n= 16, n=8/treatment) and B) obesity prone rats (n= 16, n=8/treatment). Rats were subcutaneously injected with allopregnanolone or vehicle once daily for five consecutive days. Significant differences compared to the corresponding vehicle are marked ** (p≤ 0.05).

Allopregnanolone treated OR rats that were schedule fed a HFD increased their food intake and ate as much as vehicle treated OP rats. The body weight gains of allopregnanolone-treated OR rats were also in a similar range to that of vehicle treated OP treated rats.

The difference in average weight gain between the untreated OR and OP rats after 5 days of HFD in our experiments was comparable to weight changes measured by Dourmashkin et al (Dourmashkin et al., 2006). As both Pagliasotti et al and Dourmashkin et al have argued that the initial body weight gain on HFD is the strongest predictor of long term weight gain, as well as fat accumulation, we feel encouraged that our separation into OR and OP groups is relevant.

In our experiments, rats were fed a HFD for only 4 weeks. As a longer period on HFD is necessary for the rats to become obese per se, it is possible that OP rats would have responded differently to allopregnanolone exposure after an established obesity. This would have been very interesting to study.
further. However, already after 5 days access to a HFD, higher levels of, e.g. leptin, insulin and increased lipoprotein lipase (LPL) activity have been measured in OP Sprague Dawley rats (Dourmashkin et al., 2006), indicating early changes in important feeding regulatory hormones during a HFD similar to changes observed in markedly obese rats (Boivin and Deshaies, 2000, Levin et al., 1997, Lauterio et al., 1999).

Higher levels of allopregnanolone, as well as plasma AgRP levels, have been measured in obese men and women compared to controls of normal weight (Menozzi et al., 2002, Katsuki et al., 2001). It could therefore be speculated that this would be similar in OP rats and that this could influence their food intake and weight gain. Differences between OR and OP rats are described in the literature. OP rats are, e.g. known to be more likely to store fat in adipose tissue, whereas OR rats seem to be more efficient at oxidizing fat (Bessesen et al., 2008). However, in our experiments, allopregnanolone effects on food intake and weight gain were detected in both OP and OR rats.

**UC1020 effectively antagonized GAMS-enhanced activation of GABA at α3β3γ2 GABA\(_A\) receptors but had no antagonistic effect at α2β3γ2GABA\(_A\) receptors.**

Both GAMSs allopregnanolone and THDOC enhanced GABA evoked currents in the electrophysiological studies in Paper IV. This was expected since both allopregnanolone and THDOC are known positive modulators of the GABA\(_A\) receptor. UC1020 effectively antagonized the GAMS enhanced GABA evoked currents at the α3β3γ2 GABA\(_A\) receptor (Fig 16). However, UC1020 had no antagonistic effect at the α2β3γ2 receptor (Fig 16). A smaller antagonism of GAMS-enhanced GABA current was also detected at the α1β2γ2-receptor. Thus, the UC1020 effect seems to depend on the alpha subunit of the GABA\(_A\) receptor. This indicates that UC1020 could be useful to elucidate whether the α2 and α3 receptor subtype is of importance for allopregnanolone-induced hyperphagia.

Furthermore, UC1020 did not antagonize GABA directly, showing that the effect of UC1020 is specific to the GAMS enhancement of GABA evoked currents. This is important as inhibition of GABA directly can induce convulsions. This in turn would make UC1020 unsuitable for treatment of CNS disorders.
Figure 16. UC1020 effectively antagonized GAMS-enhanced activation of GABA at α3β3γ2 GABA_A receptors but had no antagonistic effect on GAMS-enhanced activation of GABA at α2β3γ2 GABA_A receptors. Data are presented as mean ± SEM. Significant inhibition at α3β3γ2 GABA_A receptors; Allopregnanolone (n=9); ** p < 0.01, THDOC (n=12); *** p < 0.001.

Orexigenic GABA effects have been implicated to be mediated by several brain areas involved in food intake. AgRP/GABA neurons project from the ventromedial part of the ARC to POMC neurons in the ventrolateral part of the ARC, PVN, PBN and VMH (Atasoy et al., 2012, Gropp et al., 2005, Jobst et al., 2004, Wu et al., 2009, Tsujii and Bray, 1991, King, 2006). Like allopregnanolone, benzodiazepines are positive modulators of GABA_A receptors that increase food intake (Reddy and Kulkarni, 1999). α3-containing GABA_A receptors (Morris et al., 2009) or α3- and α2-containing GABA_A receptors (Cooper, 2005) have been implicated in benzodiazepine-induced hyperphagia. POMC/CART neurons in the ARC show immunoreactivity for α1, α2 and α3 whereas AgRP neurons show immunoreactivity for α3. In the PVN and VMH, immunoreactivity has been found for several different α-subunits; α1, α2, α3 as well as α5. However, generally the staining was weaker for all subunits in the PVN as well as for α1 in the VMH (Backberg et al., 2004). To the best of our knowledge, there is currently no specific information available on GABA_A receptor α-subunits in the PBN. UC1020 in our experiments was found to antagonize GAMS-enhanced GABA activation at α3-containing GABA_A receptors but had no effect at α2-containing GABA_A receptors. Thus, all the above implicated action sites (uncertain for the PBN) contain the α3-subunit, which probably is a prerequisite for the antagonizing effect of UC1020. Future studies on whether or not the hyperphagic effect of allopregnanolone could be blocked
in vivo by UC1020 would be highly interesting to further elucidate possible involvement of α3-containing GABA<sub>A</sub> receptors.

Like allopregnanolone, THDOC is also a highly potent positive modulator of the GABA<sub>A</sub> receptor (Majewska et al., 1986). Furthermore, allopregnanolone and THDOC share binding sites at the GABA<sub>A</sub> receptor (Hosie et al., 2006). In addition, in our whole-cell patch-clamp experiments (see above) we found similar results regarding the effect of UC1020 on GAMS enhanced GABA evoked currents at the different GABA<sub>A</sub> receptor subtypes tested regardless of whether allopregnanolone or THDOC was used as GAMS. Therefore, to further study the mechanism of the UC1020’s antagonistic effect at α3β3γ2 GABA<sub>A</sub> receptors, THDOC was used in additional experiments. UC1020 seemed to act as a non-competitive antagonist to THDOC at the α3β3γ2 GABA<sub>A</sub> receptor (Fig 17). This is in line with previous data on 3β-hydroxy-steroids (Wang et al., 2002b). However, contrary to the findings of Wang et al for other 3β-hydroxy-steroids, our results indicate that the effect of UC1020 is not use-dependent as the antagonistic effect did not depend on the concentration of THDOC. The effect of UC1020 was also reversible as it was possible to wash out UC1020 between trials, and thus the α3β3γ2 response to THDOC-enhanced GABA evoked currents after UC1020 exposure was the same as before UC1020 exposure.

![Graph showing the effect of THDOC and THDOC + 1µM UC1020 on GABA evoked currents](image)

**Figure 17.** A non-competitive UC1020 inhibition is suggested to THDOC at the α3β3γ2 GABA<sub>A</sub> receptor. THDOC 1-1000 nM enhanced the GABA evoked current at α3β3γ2-GABA<sub>A</sub> receptors to higher E<sub>max</sub> compared to 1-1000 nM THDOC in presence of 1 µM UC1020.
Allopregnanolone could be one endogenous factor involved in weight gain, especially with an energy-rich diet

In Paper III, we showed that rats schedule fed on a HFD increased their energy intake more than rats schedule fed standard chow. In Paper II, allopregnanolone treated rats offered a choice between more energy dense cookies and standard chow, preferentially chose the more energy dense and fat rich cookies. This is in line with our results regarding the HFD in Paper III, even though the rats in the latter study were deprived of an optional food item. However, in Paper II we showed that the allopregnanolone induced increase in energy intake from cookies (in a cookie/chow choice) was comparable to the allopregnanolone induced increase in energy intake from chow when chow was the more calorie dense option (in a chow/sucrose choice). Thus, it seems as if allopregnanolone might increase food intake to a predestined value.

Taken together, the result that allopregnanolone preferentially increased energy rich food intake in rats suggests that allopregnanolone can lead to overconsumption, which eventually may favor a body weight increase. Our results thus indicate that allopregnanolone might be one of the endogenous factors involved in overweight and obesity.
The GABA- hypothesis

As several studies have pointed out the importance of GABA in promoting food intake and that allopregnanolone is a potent positive modulator of the GABA\textsubscript{A} receptor, we propose the hypothesis that the hyperphagic effect of allopregnanolone is mediated through GABA-ergic transmission, which we refer to as “the GABA- hypothesis” (Fig 18).

GABA-ergic transmission from the AgRP neurons in the ARC of the hypothalamus seems to be of great importance for feeding regulation (Morton et al., 2006, Tong et al., 2008, Wu et al., 2009). The AgRP neurons project to nearby POMC/CART neurons in the ARC, as well as to neurons in the PVN, VMH and PBN (Atasoy et al., 2012, King, 2006, Gropp et al., 2005, Wu et al., 2009). Allopregnanolone could positively modulate GABA\textsubscript{A} receptors in postsynaptic neurons, and thus inhibit satiety promoting neurons, leading to increased hunger/hyperphagia.

Several pieces of evidence in this thesis are in line with these ideas. Firstly, AgRP neurons showed higher activity during the active/dark period of the rats and allopregnanolone induced a more prominent hyperphagia during this part of the day (Paper I). As AgRP neurons coexpress GABA and allopregnanolone is a potent positive modulator of the GABA\textsubscript{A} receptor (Majewska et al., 1986), it is plausible that allopregnanolone could act downstream of the AgRP neurons. As allopregnanolone did not induce a preference for sucrose (Paper II) the mechanism is likely mediated by more homeostatic neuronal circuits, such as the AgRP-POMC circuit. Also, as schedule feeding is known to increase plasma ghrelin levels before feeding (Verbaeys et al., 2011) and ghrelin promotes hunger by stimulating AgRP neurons (Nakazato et al., 2001), one would expect more explicit food intake in schedule fed rats during the time of food access as compared to ad libitum fed rats. This was seen in Paper II. Furthermore, the allopregnanolone-induced weight gain reported in Paper III correlated with increased food intake. This supports the view that allopregnanolone also in this case exerts its effect by increased hyperphagia.
Figure 18. Simplified schematic illustration of brain areas implicated in GABA-ergic regulation of food intake showing the proposed mechanism whereby allopregnanolone enhances the GABA-ergic orexigenic effect, the GABA hypothesis.

The arcuate nucleus (ARC) in the hypothalamus receives input from, e.g. the important hunger hormone ghrelin and the satiety hormone leptin. AgRP neurons in the ARC coexpress GABA and project to, e.g. nearby POMC/CART neurons in the ARC, the paraventricular nucleus (PVN) and the ventromedial hypothalamus (VMH) in the hypothalamus, as well as to the parabrachial nucleus (PBN) in the brainstem.

We hypothesize that allopregnanolone-induced hyperphagia is caused by allopregnanolone positive modulation of GABA_A receptors in postsynaptic satiety promoting neurons. Thereby, the satiety promoting neurons are inhibited and the orexigenic effect is enhanced.

Hunger-promoting AgRP/GABA neurons project from close proximity to satiety promoting POMC/CART neurons in the ARC and also to satiety promoting neurons within the PVN, VMH and PBN. AgRP/GABA neurons mediate hunger by inhibition of satiety promoting neurons. Allopregnanolone-enhancement of the GABA-ergic inhibition in these areas may further inhibit satiety, and thereby increase food intake.
Methodological considerations

The dosages used in Paper I and II were quite high. To fully assess the allopregnanolone effect on food intake and food preference, a follow up with more physiological doses would be advisable. However, we know that allopregnanolone is produced within the brain and can thus be locally high. Thus, this initial stage of investigation provides useful information for future research. Our results suggest that allopregnanolone effects on food intake are diurnally dependent, may increase the meal size have a larger impact on intake of more energy dense food and may have a larger impact on homeostatic neuronal circuits. In addition, allopregnanolone induced hyperphagia appears to lead to increased body weight.

Only male rats were used in this study for reasons previously stated. It is however important to follow up the results in this thesis in female rats as well as in humans, as it is not possible to rule out differences between sexes and species. The hyperphagic effect of allopregnanolone in female rats differs between the phases of the estrous cycle (Reddy and Kulkarni, 1999). Therefore, the importance of monitoring estrous phase/menstrual cycle phase when working with females and food intake cannot be understated. Possible effects on weight gain induced by allopregnanolone in female rodents or humans have to our knowledge not yet been investigated.

The cookies used in Experiment 1 in Paper II contained small chocolate chips. This particular food item was chosen as it contains higher energy density than chow and is also highly preferred by rats (Rothwell and Stock, 1979, Rolls et al., 1980). However, the fact that chocolate itself contain substances like caffeine and theobromine known to affect the autonomous nervous system as well as cannabinoid-like fatty acids (Bruinsma and Taren, 1999), might have influenced the results. It was obvious though that the rats did not pick out the chocolate chips in particular and their consumption in the presence of allopregnanolone did not show any preference for chocolate over the rest of the cookie.

In this thesis work, we classified rats as OP or OR based on their initial weight gain during the first week on a HFD as previously described (Dourmashkin et al., 2006, Pagliassotti et al., 1997). In contrast, Levin et al selectively bred Sprague Dawley rats to express either an OP (DIO) or OR (DR) phenotype (Levin and Dunn-Meynell, 2002, Levin et al., 1997). Therefore, results may differ depending on the method used. With a larger sample size in our experiments, we might have identified rats with more extreme differences in body weight gain. This in turn could have made the
differences in effect more pronounced. However, in this thesis work, further subdivision of the OP and OR rats to look at the highest and lowest within each group yielded similar results. Also, inclusion of a set of rats fed only standard chow would have been favorable but weight gain in rats fed chow was monitored in an earlier study. In that study, the rats were of comparable baseline weights compared to in the HFD study and rats fed chow gained less than OR rats, but were closer in weight gain than OR and OP rats were. It is difficult to precisely compare the two studies as the chow fed rats also were single housed, which could have contributed to the differences.

It has been suggested that sesame oil when used as a supplement can have estrogenic effects (Wu et al., 2006). However, in our experiments, we detected no difference between saline and vehicle (sesame oil) treated animals either in food intake or weight gain. Consequently, no saline control was included in Experiment 3 in Paper III. This would have been preferable, but as we detected no effect in the previous two experiments, we felt encouraged that any effect due to the use of sesame oil as vehicle would not be of major importance for the outcome of the study. In addition the sesame oil was not digested by the rats, but s.c injected.

Initially, we planned to also investigate body constitution by Magnetic resonance imaging (MRI) to reveal whether an increased body weight was due to increased body fat mass, and consequently whether allopregnanolone affects body fat distribution. However, it was not possible to investigate this within the time-frame of this PhD. Thus it would be an interesting topic for future study.

In addition, to be absolutely sure that allopregnanolone did not have any effect on the activity of the rats, it would have been preferable to also measure their ambulatory activity level. However, as the weight gain induced by allopregnanolone in Paper III correlated to the energy intake we feel encouraged that the weight gain primarily was caused by an increased energy intake.

In the whole-cell patch-clamp experiments, we used HEK cells in which GABA\textsubscript{A} receptors were expressed. This is a simplified system for investigating receptor activity. The properties of the GABA\textsubscript{A} receptor may differ depending on what type of cell it is expressed in. Furthermore, neurons also contain intrinsic factors that influence the properties of the membrane proteins. These factors may vary between different brain areas and types of neurons. However, the advantage of using HEK-cells is that it is possible to record from GABA\textsubscript{A} receptors of a specific subunit composition.
Clinical implications

The results in this thesis suggest that allopregnanolone increases the energy intake and promotes energy-rich food intake. Evolutionary, this would be beneficial during pregnancy when it is important for the growing fetus that the mother increases her food intake when food is available. In human history, the availability of food was often scarce, and thus increasing one’s energy intake when food was available was important for survival. Therefore, persons with weaker satiety signals had a survival advantage. It could be that allopregnanolone is one of the endogenous factors mediating this effect. However, in the modern society, this has become a disadvantage as food, especially energy dense food, is available always and everywhere.

As increased allopregnanolone levels have been reported in obese men and women (Menozzi et al., 2002), as well as in overweight young girls (Predieri et al., 2007, Grosso et al., 2011), it could be speculated that for some overweight or obese patients increased allopregnanolone levels might be associated with increased hunger maintaining the elevated food intake, which might contribute to the undesired weight gain. Also, as allopregnanolone seems to increase the homeostatic drive, one could speculate that in a situation where a person tries to lose weight by dieting, increased allopregnanolone levels may be one factor that impairs the chances of maintaining the diet.

There are reports of increased premenstrual energy intake (Barr et al., 1995, Johnson et al., 1994, Bancroft et al., 1988). As allopregnanolone levels are known to be higher during the premenstrual period (Nyberg et al., 2007) the presence of allopregnanolone could be related to this increase. In addition, binge eating attacks are more frequent during the premenstrual phase (Klump et al., 2008, Edler et al., 2007) and binge eaters have higher allopregnanolone levels (Monteleone et al., 2003).

Polycystic ovary syndrome (PCOS) affects 5-10% of women in child-bearing ages worldwide (Norman et al., 2004) and 50% of them are obese or overweight (Azziz et al., 2009, Norman et al., 2004, Gambineri et al., 2002). PCOS patients also have higher allopregnanolone levels (Genazzani et al., 2006). The testosterone metabolite androstanediol is another important GAMS which is elevated in this group of patients (Meczekalski et al., 2007). Not all women experience a premenstrual increase in food intake and not all PCOS patients are overweight or obese. One explanation may be an individual difference in sensitivity to allopregnanolone. However, the etiology of obesity is multifactorial and there are several other factors that may contribute to an increased weight gain.
Thus, why some people gain weight while other do not despite having high allopregnanolone levels is still an open question. However, the presented results suggest that allopregnanolone exposure may be one factor that promotes overeating and weight increase. This also indicates a potential for pharmacotherapy targeting mechanisms involved in allopregnanolone induced hyperphagia.

**Future implications**

More long-term studies on allopregnanolone effects on weight gain would be highly interesting in addition to investigations on body composition to clarify if allopregnanolone induced increased body weight gain would correlate to increased adipose tissue. Also the use of MRI could be advantageous to study adipose tissue distribution and disclose if allopregnanolone may have any impact on that parameter as well.

Also, use of an animal model with naturally elevated physiological allopregnanolone concentrations, such as pseudopregnant female rats, could be suitable for further studies investigating more long-term effects of allopregnanolone and potential antagonists.

In this thesis work, electrophysiological studies showed that UC1020 was an effective antagonist for GAMS-enhanced GABA evoked currents at α3β3γ2 GABA_A receptors, whereas it had no effect on α2β3γ2 GABA_A receptors. This indicates that UC1020 could be used to discriminate between action sites of these two receptor subtypes in the brain. This could be highly relevant in the future, particularly when disentangling which neuronal GABA circuits involved in food intake regulation allopregnanolone may affect. Also additional animal studies on the effect of UC1020 on allopregnanolone induced food intake are warranted to disclose possible antagonistic effects of UC1020 on food intake.

It has been discussed that obesity prone rats and humans differ from those that seem more resistant to weight gain. In the future, it would be interesting to further investigate whether allopregnanolone might have different effects in animal models with established obesity.

The results in this thesis are derived from a rat model and the data are not simply transferable to a human situation. It would thus be interesting to perform a follow-up with a study into the effects of physiological allopregnanolone concentrations on food intake and weight gain in humans, to elucidate the clinical relevance.
Main conclusions

Acute effects of allopregnanolone on food intake in rats

- Allopregnanolone dose-dependently increased intake of standard chow.

- Allopregnanolone injections reduced the feeding latency and prolonged feeding duration of the next coming meal, indicating an increased meal size.

- The increased chow intake induced by allopregnanolone was more pronounced at the beginning of the rats’ active/dark period compared to at the beginning of the inactive/light period.

- For both chow and HFD, the increase in food intake induced by allopregnanolone was more pronounced during the first hours following treatment.

- In a choice situation between standard chow and more palatable as well as more calorie dense cookies, allopregnanolone exclusively increased cookie intake.

- In a choice situation, allopregnanolone injections resulted in an increased intake of the calorie dense, less palatable standard chow over a calorie dilute more palatable sucrose solution.

Chronic effects of allopregnanolone on food intake and body weight in rats

- Repeated allopregnanolone administration during five consecutive days led to increased body weight gain, which was most clearly seen in schedule fed rats on a high fat diet, and the increased body weight gain correlated with increased food intake.

- Both obesity resistant and obesity prone rats that were schedule fed on a high fat diet increased their weight gain with repeated allopregnanolone administration.
Effects of UC1020 as an antagonist to allopregnanolone potentiation of GABA evoked currents

- The compound UC1020 effectively antagonized GAMS-enhanced GABA evoked currents at α3β3γ2 GABA_A receptors but had no antagonistic effect on GAMS-enhanced GABA evoked currents at α2β3γ2GABA_A receptors.
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