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Susanna Apter

The Effect of Alcohol on Testosterone and Corticosterone Levels in Alcohol-Preferring and Non-Preferring Rat Lines

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Department of Mental Health and Alcohol Research
National Public Health Institute
and
Faculty of Biosciences
University of Helsinki

Helsinki, Finland 2008

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We find no real satisfaction or happiness in life without obstacles to conquer and goals to achieve.

Maxwell Maltz, 2004

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ABSTRACT

The large individual variation in the disposition of alcohol dependence has been explained by a combination of a range of both environmental and genetic factors. One possible genetic factor is the steroid hormone testosterone, or more precisely, differences in the concentrations of this hormone. A subgroup of alcoholics has been described to possess characteristics, which also have been associated with high testosterone levels. Furthermore, this subgroup generally begins drinking in early adolescence, which is when testosterone levels increase in boys. Briefly, this could explain why alcoholism is more common among men than women.

Male rats of AA and ANA rat lines, developed for high and low alcohol preference respectively, were used in this study. The AA rats represent an animal model of human alcoholics and large-scale consumers, while the ANA rats are comparable to abstainers and individuals consuming very small amounts of alcohol. The development of these rat lines was based solemnly on the animals' free will to drink alcohol, and therefore, any differences between the lines ought to be connected to alcohol drinking.

The aims of this study were to investigate if the AA and ANA rat lines differ in control and/or alcohol-induced levels of testosterone and corticosterone, and if a mildly stressful situation affects the alcohol-induced changes in testosterone. Stress has been reported to induce alcohol drinking although alcohol drinking *per se* has been shown to increase stress hormone levels.

Half of the experimental animals of each rat line were housed in groups of four and half were housed individually. All animals were subjected to all treatment groups, which varied by alcohol dose (control, 0.75g/kg, 1.5g/kg alcohol) and time of day (morning and afternoon). Blood samples

were collected before as well as 1, 2 and 3 hours post injection of the control or alcohol dose. Testosterone and corticosterone levels were measured from serum samples using radioimmunological techniques.

The AA rats were shown to have higher testosterone levels than the ANA rats.

The high alcohol dose reduced testosterone levels of group-housed rats of both rat lines, while the low dose only had a reducing effect in the ANA rats. Social isolation attenuated the effects of alcohol on testosterone. In isolated rats, the only testosterone-reducing effect of alcohol was following the high dose in the ANA rats in the afternoon session.

The results of this study support a role of testosterone in alcohol drinking, although this role seems to be complex. The role of testosterone in alcohol drinking may be enhanced in individuals with a genetic disposition for becoming dependent on alcohol and modulated by stress.

Keywords: alcohol, alcohol dependence, testosterone, corticosterone, social isolation.

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ABBREVIATIONS

AA rat	Alcohol-preferring rat of the Alko, Alcohol rat line
ANA rat	Alcohol-avoiding rat of the Alko, Non-Alcohol rat line
ADH	Alcohol dehydrogenase
ALDH	Aldehyde dehydrogenase
DHT	5-α-Dihydrotestosterone
CV	Coefficient of variation
GnRH	Gonadotropin-releasing hormone
HPA-axis	Hypothalamic-pituitary-gonadal-axis
HPG-axis	Hypothalamic-pituitary-gonadal-axis
LH	Luteinizing hormone
NAD^+	Nicotinamide adenine dinucleotide
w/v	Weight/volume

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles referred to in the text by their Roman numerals:

I Apter, SJ. and Eriksson, CJP. (2003) The Effect of Alcohol on Testosterone Concentrations in Alcohol-Preferring and Non-Preferring Rat Lines. *Alcoholism Clin Exp Res* **27**:1190-1193.

II Apter, SJ. and Eriksson, CJP. (2006) The Role of Social Isolation in the Effects of Alcohol on Corticosterone and Testosterone Levels of Alcohol-Preferring and Non-Preferring Rats. *Alcohol Alcohol* **41**:33-38.

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1 INTRODUCTION

Alcohol drinking behaviors have throughout history been molded by culturally-bound attributes, such as religion, moral standards, and legislation. These attributes exert their affects by influencing the availability of alcoholic beverages and the general opinion about drinking. Although different environmental factors have an important effect on both alcohol abuse and the development of alcoholism, environmental influences alone cannot explain the large individual variation in the disposition for alcohol dependence. The variation can only be accounted for by a combination of genetic and environmental factors: both "nature" and "nurture". Gender and other genetically-determined differences act as modulators in the development of alcohol addiction (Devor and Cloninger, 1989).

Alcohol today constitutes one of the substances causing the greatest number of cases of dependence in our society. Alcoholics are categorized by some researchers into two subtypes: type 1 and type 2 (Devor and Cloninger, 1989). The male-limited type 2 alcoholism is highly heritable from father to son regardless of the environmental background. The type 2 alcoholics have been described as displaying low harm avoidance, but high sensation seeking and impulsive, aggressive, and antisocial behavior. Simultaneously, these characteristics have also been associated with high concentrations of testosterone. Furthermore, type 2 alcoholics generally begin drinking in early adolescence, which is when testosterone levels increase in boys (Devor and Cloninger, 1989). A positive relation between the male sex hormone, testosterone, and alcohol drinking could represent a general mechanism that perhaps partly could explain why men typically drink more than women and why alcoholism is more common in men than in women.

Heavy alcohol abuse is known to cause a broad variety of symptoms and diseases. Clinically, the most important endocrine consequences of long term alcohol use are its effects on the gonads (Välimäki and Ylikahri, 1985). A connection between alcohol abuse and testicular atrophy with resulting gynecomastia was reported more than 100 years ago. Male alcoholics and heavy consumers also frequently report problems such as erectile dysfunction and lowered fertility (Välimäki and Ylikahri, 1985; Bannister and Lowosky, 1987), effects that have been explained by an alcohol-induced reduction in testosterone (Bannister and Lowosky, 1987).

Although acute alcohol has generally been shown to decrease testosterone levels in both men and in male experimental animals (Cicero and Badger, 1977; Cicero et al., 1978; Cicero et al., 1979; Chiao and Van Thiel, 1983; Eriksson et al., 1983; Eriksson et al., 1984; Bannister and Lowosky, 1987; Emanuele et al., 1995; Steiner et al., 1996), alcohol-induced elevations of testosterone have also been reported (Cicero and Badger, 1977; Eriksson et al., 1994; Alomary et al., 2003; Sarkola and Eriksson, 2003). These diverging results may be explained by variations in genetic components and/or situational factors.

It is well-established that the activation of opioid receptors in the brain is usually involved in the development of alcohol addiction (Herz, 1997; Sinclair, 2001). Alcohol has been known to cause a release of endorphins, but the mechanism producing this effect has not been indentified. Recently, a possible role for the opiate system on continued alcohol drinking through an alcohol-induced effect on testosterone levels was proposed on the basis of results in a rat line with a disposition for becoming addicted to alcohol (Apter and Eriksson, 2003). An alcohol-induced increase in testosterone could through the negative feed-back regulation of testosterone increase the release of β-endorphin in the hypothalamus. This could, in turn, reinforce alcohol drinking and thus lead to continued alcohol intake.

Alcohol-induced elevations of testosterone levels might only occur in interaction with or be more prominent in the presence of a situational factor such as stress; if so, this could shed light on the paradoxical relation of stress and drinking, that is, the "alcohol paradox theory" (Pohorecky, 1991; Stritzke et al., 1996). Stress is reported by some individuals to increase their alcohol intake, although alcohol *per se* has been shown to elevate stress hormone levels in both humans and experimental animals. The opiate system of the central nervous system is known to regulate both stress and sex hormone metabolism.

The use of experimental animals has become an important tool in alcohol research. Due to ethical concerns and experimental difficulties, research concerning alcohol use, abuse, and dependence is often impossible to perform on human subjects. Therefore, the use of experimental animals has enabled the research on questions that could not otherwise be investigated. Furthermore, several animal models have been developed specifically for studies on alcohol-seeking behavior, alcohol-related organ damage, tolerance to alcohol as well as dependence on alcohol. And, as it is possible to genetically manipulate animal models, they have also become valuable in

research on the genetic determinants of alcoholism. The validity of using animal models is often predictive, that is, results gained through studies with an animal model are predictive of for example the actions of medications on alcohol drinking in humans (Sinclair, 1980).

In the present research, the acute effects of alcohol on testosterone concentrations in rat lines developed for diverging alcohol preference are investigated. The possible modulating effect of stress on the alcohol-induced testosterone effects is also assessed. The main aim is to explore whether the rat lines respond differently to alcohol and if this difference is associated with their inherited preference to alcohol.

Altogether, an alcohol-induced testosterone increase could theoretically be a new marker of an elevated risk of becoming addicted to alcohol. If so, a novel mechanism for the development of an alcohol addiction would be revealed. In time, this knowledge could be used for both preventive measures and the treatment of alcoholism. The following experimental animal study is the first in a series of studies from our laboratory concerning the possible role of testosterone in the development of an alcohol addiction.

2 REVIEW OF THE LITERATURE

2.1 Alcoholism

Alcoholism is a disease, which includes the development of an alcohol addiction, a state of increased alcohol tolerance, and the presence of withdrawal symptoms during abstinence. As stated previously, alcoholism has been classed into two subtypes (Cloninger et al., 1981). Type 1 alcoholics make up about 80% of all alcoholics. This type of alcoholism is promoted by environmental factors and occurs in both women and men. Type 2 alcoholism, on the other hand, is hereditary and male-limited. Type 2 alcoholics generally begin drinking in early adolescence, which is concurrent with the rise of testosterone levels in boys, and also typically suffer from recurrent social and legal problems. Type 2 alcoholics have been described as possessing personality traits such as low harm avoidance, high sensation seeking, impulsiveness, as well as aggressive and antisocial behavior.

2.2 Alcohol Metabolism

Once consumed, the small ethanol molecules (molecular formula: CH_3CH_2OH; molecular weight: 46.07 atomic mass units) are quickly absorbed from the gastrointestinal tract by passive diffusion. They readily pass through membranes and enter tissues, exerting their effects on the body. Rat studies have shown that significant ethanol concentrations can be measured in the brain already 1 to 2 minutes following intraperitoneal administration (Nurmi et al., 1994).

Due to its low lipid solubility, ethanol is primarily distributed to the water-soluble parts of the body. The lower water content in women compared to men (on average 0.59L/kg versus 0.73L/kg in men) explains the higher blood alcohol levels found in women following intake of a standard amount of alcohol per body weight (Marshall et al., 1983).

2.2.1 Ethanol Oxidation

In mammals, ethanol is mainly metabolized in the liver. However, almost all tissues are capable of oxidizing some ethanol and small amounts of alcohol are also eliminated through respiration, sweat, and urine (Salaspuro et al., 2003).

The oxidation of ethanol is primarily carried out by alcohol dehydrogenase, whose catalytic properties rely on the presence of the oxidized form of nicotinamide adenine dinucleotide (NAD^+). In essence, alcohol dehydrogenase oxidizes ethanol to acetaldehyde, and, simultaneously, NAD^+ is reduced to NADH (Salaspuro et al., 2003).

Two other ethanol metabolizing pathways are known: the catalase pathway and the microsomal ethanol oxidizing system (MEOS). The role of catalase is small: the pathway accounts for less than 2% of the total ethanol metabolism. It is generally accepted that the rise in alcohol elimination rate seen during long-term drinking is due to an increase in the activity of hepatic CYP2E1, which is the major component in the microsomal ethanol oxidizing system (Salaspuro et al., 2003).

The intermediate product of ethanol metabolism, acetaldehyde, is far more toxic than ethanol itself. A dose of 0.75g acetaldehyde/kg of body weight is lethal in mice. In contrast, an alcohol dose of 6.5g/kg of body weight is fatal only in seven out of ten cases in mice. The toxicity of acetaldehyde lies in its inhibitory effect on the sodium-potassium pump, protein synthesis of the cell, and cell respiration (Tabakoff et al., 1989). In addition, acetaldehyde is carcinogenic (Salaspuro et al., 2003).

The concentration of acetaldehyde is usually kept low due to the high activity of aldehyde dehydrogenase, ALDH. In individuals with reduced ALDH activity, high acetaldehyde levels after alcohol intake cause symptoms such as tachycardia, hypotension, nausea, and facial flushing. Hence, ALDH inhibitors such as disulfiram are used to promote abstinence in alcoholics. The end product of the catalytic action of aldehyde dehydrogenase, acetate, is further modified in the body to carbon dioxide and water (Salaspuro et al., 2003).

2.3 Testosterone

Testosterone is the most important androgen in both men and women. Testosterone has androgenic, anabolic, and psychological effects. The presence of testosterone is crucial for the development of reproductive tissues and secondary male characteristics. It is essential for normal spermatogenesis, and it contributes to general growth and protein synthesis. Testosterone also effects libido, sexual

potency, and behaviors such as aggressive and sexual behavior (Nieschlag and Behre, 2004).

Testosterone and behavior share a bidirectional interaction; testosterone can influence behavior while behavior can affect testosterone levels. During fetal and neonatal life, testosterone affects brain development through involvement in the organization of certain brain regions. At puberty, it is believed that these brain structures and the behavioral correlates that follow are activated.

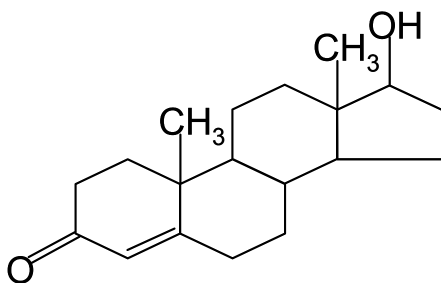

Figure 1. ***The testosterone molecule.***

During puberty in boys, androgen production is increased in the testes, which, in turn, increases testosterone levels. The physiological range of testosterone in men is large, between 10-35nmol/L (Välimäki et al., 2000; Nieschlag and Behre, 2004). The upper portion of this range is much higher than is required for normal sexual functions. The critical level has been shown to be around 10nmol/L (Nieschlag, 1979). The testosterone concentration in women is around 5-10% of that in man (Nieschlag and Behre, 2004).

There are large diurnal variations in levels of testosterone in men, with 25-50% higher concentrations in the mornings than evenings, due primarily to changes in production. With increasing age, testosterone levels gradually decrease and the sexual interest, and potency of men usually declines (Välimäki et al., 2000).

2.4 Testosterone Metabolism and Action

All bioactive steroid hormones, testosterone included, are produced from cholesterol. In men, testosterone is mainly produced by the testes, while in women, testosterone is produced by the ovaries, by the adrenals, and in peripheral tissue. In women, testosterone concentrations are decreased following menopause as the production of testosterone by the ovaries is reduced. The main source of androgens in postmenopausal women is the adrenal cortex (Välimäki et al., 2000).

The production of testosterone is regulated by negative feedback. Hypothalamic gonadotropin-releasing hormone (GnRH) stimulates the release of luteinizing hormone (LH) from the pituitary. In the testes, LH stimulates testosterone production. Testosterone itself suppresses the release of LH by preventing GnRH release from the hypothalamus. The catabolism of testosterone, as well as that of other androgens, takes place mainly in the liver, although testosterone is metabolized into other active hormones in several places of the body. Secretion products are excreted from the body via urine and the skin (Välimäki et al., 2000).

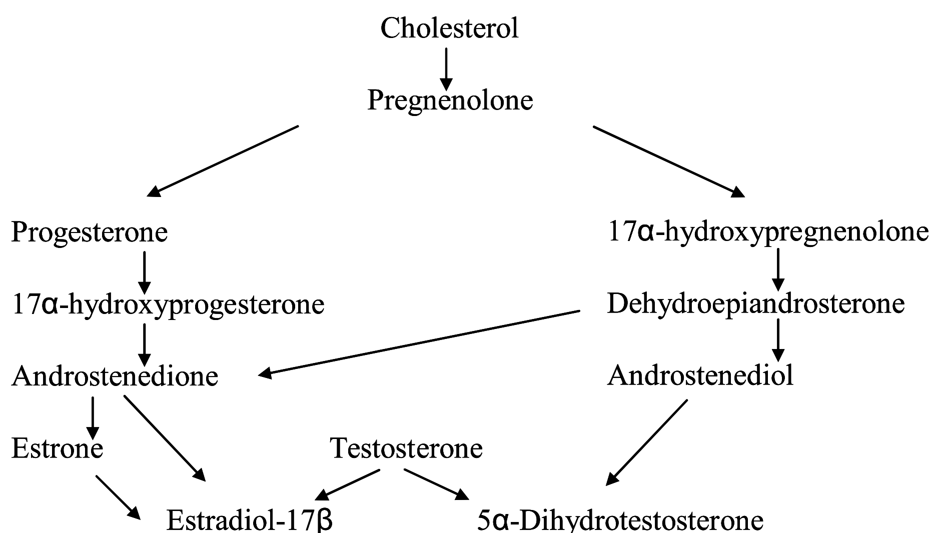

Figure 2. ***Synthesis of testosterone and its metabolites.***

Testosterone can rapidly pass through the membranes of the testicular Leydig cells, where it is produced. Within the body testosterone is transported mainly bound to different transport proteins. In men 44% and in women 66% of the total testosterone is bound to sex hormone binding globulins, SHBGs. Most of the remaining testosterone molecules, that is 50% of the total in men and 30% in women, are non-specifically bound to albumin (Nieschlag and Behre, 2004). SHBGs bind testosterone about 1000 times stronger than albumin does, but albumin is present at a much higher concentration. Due to the much higher affinity of testosterone to SHBG, it is only the unbound and albumin-bound testosterone that is biologically active. The SHBG-bound testosterone molecules serve as a reservoir (O'Malley and Strott, 1999). In contrast to humans, rats lack sex-hormone binding globulins, and testosterone is transported freely in the blood circulation (Välimäki and Ylikahri, 1985).

The testicular concentration of testosterone is about 100 times that in the periphery; the high testicular level is required for efficient spermatogenesis. The effect of testosterone on spermatogenesis is one of the few paracrine control mechanisms in the testis with shown physiological significance (Toppari and Huhtaniemi, 1999). As with other hormones, it is essential that testosterone levels are kept at appropriate levels. The overall half-life of free testosterone is only 12 minutes, so continuous production must be upheld (Nieschlag and Behre, 2004).

Testosterone exerts its effects both directly and via a conversion to either 5-α-dihydrotestosterone (DHT) or estradiol. The reduced form of testosterone, DHT, is a more active metabolite than testosterone itself, meaning that it binds to the same androgen receptors as testosterone itself but with a stronger affinity. Spermatogenesis is believed to be controlled by testosterone, while the effects of DHT are mainly seen outside the testes (Välimäki et al., 2000). The aromatization of testosterone to estradiol in the testes is small (around 20%) compared to that in the peripheral circulation (Nieschlag and Behre, 2004).

Similarly to testosterone and DHT, the actions of estradiol are initiated by the activation of certain steroid hormone receptors. Once inside a target cell, the steroid hormone molecules bind to either an androgen (testosterone and DHT) or estrogen receptor (estradiol). This hormone-receptor complex then undergoes a structural conformation and binds to specific DNA-sequences of the host cell, which influences the transcription of specific target genes within the cell (O'Malley and Strott, 1999).

2.5 Alcohol and Testosterone

During the last half century, researchers have become very interested in the effects of alcohol, and even more specifically, in the effects of alcohol on the reproductive system. Chronic consumption of alcoholic beverages is known to for example cause liver and brain damage, mental problems, cancer. Yet, one of the most important clinical effects of alcohol is its effects on the gonads.

The first reports concerning the effects of alcohol on testosterone were based on mouse (Badr and Bartke, 1974; Badr et al., 1977; Badr et al., 1979) and rat studies (Cicero and Badger, 1977; Cicero et al., 1978; Cicero et al., 1979; Eriksson et al., 1983; Eriksson et al., 1984). These investigations were later supplemented by *in vitro* protocols (Widenius et al., 1987; Akane et al., 1988; Orpana et al., 1989; Orpana et al., 1990a; Orpana et al., 1990b; Orpana et al., 1990c). Acute, short term, and long term alcohol use have all generally been shown to reduce testosterone in male rats, mice, monkeys, and humans (Chiao and Van Thiel, 1983). However, recent human studies have shown alcohol-mediated elevations in testosterone following a low dose in both women (Eriksson et al., 1994) and men (Sarkola and Eriksson, 2003).

In male rats, most publications have illustrated alcohol-induced reductions in testosterone following administration of a high dose (Cicero and Badger, 1977; Cicero et al., 1978; Cicero et al., 1979; Eriksson et al., 1983; Bannister and Lowosky, 1987; Emanuele et al., 1995). Only one recent publication has described alcohol-mediated peripheral testosterone elevations and elevations in the brain following administration of a high (3g/kg) alcohol dose (Alomary et al., 2003). However, the effects of a low dose have been rather contradictory. Cicero and Badger (1977) have reported an elevation in testosterone following administration of 0.75g alcohol per kg of body weight. Eriksson and coworkers (1983), on the other hand, did not find significant effects of doses between 0.3 and 0.9g/kg.

In the early experiments of Cicero and Badger (1977), testosterone concentrations were reduced 3 hours after ethanol administration (dose used 2.5g/kg). In a study by Eriksson et al. (1984), testosterone levels were reduced significantly already 2 hours after a 1.5g/kg dose. Steiner and his colleagues (1996) have reported a 20% decrease

in testosterone levels at the first time point of measure, which was 1.5 hours after ethanol administration of a 3g/kg dose. In this study testosterone levels remained reduced for 96 hours (Steiner et al., 1996).

Alcohol-induced reductions in testosterone can in theory be caused by a stimulatory effect on testosterone catabolism and/or inhibition of its synthesis. Research on the effect of alcohol on testosterone catabolism is limited in depth, while the effects of ethanol on testosterone synthesis have been widely investigated.

The reported alcohol-induced testosterone reductions have been relatively larger in male rats than in human males. This difference may at least partly be due to the absence of sex binding globulins in rats. The acute changes in testosterone may be greater and faster in rats since they lack the additional supply of testosterone in their blood (Välimäki and Ylikahri, 1985). Furthermore, since the biosynthetic processes may slightly differ between men and male rats, the diverging reactions may be unequally sensitive to the actions of alcohol (Välimäki and Ylikahri, 1985). However, as with all animal studies, we should expect at best only qualitative predictive validity and should not be surprised when a less than perfect match to human conditions is obtained.

2.5.1 Mechanisms of Action

Besides the ethanol molecule *per se*, the causes behind the alcohol-induced effects on testosterone have been attributed also to the metabolism of ethanol and to an intermediate product of ethanol metabolism, acetaldehyde. Acetaldehyde has been reported being 1000-4000 times more effective than ethanol in inhibiting testosterone synthesis *in vitro* (Cicero et al., 1980). Even so, Eriksson and his coworkers (1983) have demonstrated that acetaldehyde does not have an important role in the inhibition of testosterone synthesis *in vivo*. The authors assessed the role of acetaldehyde as a product of ethanol metabolism and concluded that increased acetaldehyde concentrations in blood and testicles did not inhibit testosterone synthesis.

The oxidation of ethanol requires nicotinamide adenine dinucleotide (NAD^+). The competition for this cofactor between the alcohol oxidizing enzymes and the enzymes involved in steroid synthesis can cause a reduction in testosterone synthesis. Addition of NAD^+ has been reported by some researchers (Eriksson et al., 1983), but not by all (Akane et al., 1988), to be able to restrain the ethanol-induced

inhibition of testosterone synthesis *in vitro*. In fact, the inhibitory mechanisms appear to differ between *in vivo* and *in vitro* conditions. Ethanol metabolism and its product acetaldehyde seem to have significant roles *in vitro*, while the oxidation process does not have such a clear role *in vivo* (Orpana et al., 1990c) since the testosterone-reducing effects of alcohol appear even when the ethanol metabolism is inhibited with the use of 4-methylpyrazole (Eriksson et al., 1983).

Male rats whose hormone synthesis has been stimulated by human chorioinic gonadotropin (hCG) have testosterone concentrations that are several times higher than normal levels. hCG stimulates steroid synthesis similarly to LH, but is more effective due to its higher affinity to the LH-receptor. The more stimulated an individual's steroid synthesis is, according to one study, the more central is the role of ethanol metabolism in producing the testosterone-reducing effects of alcohol. The ethanol-induced effects on testosterone in non-stimulated animals can be seen even when ethanol metabolism is inhibited (Eriksson et al., 1983; Orpana et al., 1990d).

2.5.2 Direct Effects on the Catabolism of Testosterone

Long-term alcohol use in men has been shown to lead to increased enzyme activities of 5-α-reductase and aromatase in the liver. This, in turn, leads to an increased testosterone catabolism and turnover to estrogens (Chiao and Van Thiel, 1983).

However, in women, the acute effects of alcohol include elevations in testosterone. Since alcohol causes a shift in the ratio between androstenedione and testosterone, it has been suggested that alcohol-mediated redox changes in the liver may cause an inhibition of testosterone catabolism (Sarkola et al., 2001).

2.5.3 Direct Effects on the Synthesis of Testosterone

Ethanol has been shown to influence testosterone synthesis through a direct effect on the gonads. This effect has been investigated by searching for possible shifts in concentrations, which could reveal inhibited reactions of the synthesis pathway. Significant reductions of progesterone, 17α-hydroxyprogesterone, and testosterone following an alcohol dose of 1.5g/kg have been reported (Eriksson et al., 1984). In the same study, a difference in the relation of substrate to product was found in the reaction from pregnenolone to progesterone and from progesterone to 17α-hydroxy-progesterone. These *in vivo* results suggest an effect of alcohol on testosterone production before the steps 17α-hydroxy-progesterone-androstenedione-

testosterone, but do not exclude effects before pregnenolone production (for overview of the production chain of testosterone, see figure 2 on page 18).

The effect that alcohol has on testosterone metabolism seems to be age-dependent. In contrast to the previously mentioned studies reporting mainly alcohol-induced reductions in testosterone, ethanol has been shown to stimulate testosterone synthesis in prepubescent rats (25-30days old) *in vivo* (Little et al., 1992).

2.5.4 Effects through a Stress-Induced Mechanism

Alcohol has repeatedly been shown to increase stress hormone levels; that is corticosterone levels in experimental animals and cortisol levels in humans. Nevertheless, some people tend to increase their alcohol intake when they are stressed. This has been called the "alcohol paradox"; although alcohol increases stress hormone concentrations, people appear to use alcohol as a means of self-medication to cope with stress (Pohorecky, 1991; Stritzke et al., 1996).

In women, it has been shown that alcohol increases testosterone levels, with the effect being more pronounced in women on the contraceptive pill (Eriksson et al., 1994). The use of contraceptive pills has also been shown to lead to increased cortisol levels (Meulenberg et al., 1987). Some studies have shown that alcohol, under certain circumstances, can increase testosterone levels also in men (Sarkola and Eriksson, 2003) and male experimental animals (Apter and Eriksson, 2003). The alcohol-induced increase in testosterone levels may, in accordance with the situation in women using contraceptive pills, be associated with elevated stress hormone levels. High concentrations of corticosterone have been shown to inhibit testosterone synthesis (Välimäki et al., 1984). Testicular interstitial cells express stress hormone (glucocorticoid) receptors on their cell membranes. The binding of receptor agonists to these receptors causes a stress response in the cells. It has been shown that glucocorticoids (corticosterone, cortisol) can reduce interstitial testosterone synthesis. They decrease the LH-induced cyclic adenosine monophosphate-production and the activity of 17α-hydroxylase *in vitro*, that is, the enzyme catalyzing the reactions from progesterone to 17α-hydroxyprogesterone and androstenedione.

2.5.5 Alcohol-Induced Effects on the Pituitary and the Hypothalamus

The alcohol-induced effects on testosterone may also be secondary, caused by a reduction in luteinizing hormone or GnRH that stimulate testosterone production. In

male rats, it has been shown that alcohol decreases LH concentrations. Furthermore, the association between the alcohol dose and the concentration drop in LH seems to be dose-related, as discussed in Cicero's review article (Cicero, 1982). However, in men, acute alcohol has been shown to increase, decrease as well as not affect LH concentrations (Widenius et al., 1989). Despite these contradictive results, researchers are in agreement that alcohol affects the hypothalamic-pituitary axis, since according to the negative feedback mechanism of testosterone production, lowered testosterone levels ought to be compensated for by a rise in LH via a rise in GnRH.

Alcohol has been shown to be able to decrease the number of gonadotropin receptors on the cell membranes of testicular interstitial cells (Bhalla et al., 1983). Normally LH regulates the number of receptors, and hence, the cell's sensitivity for stimulation of steroid synthesis. The more numerous the receptors on the cell membrane, the larger the probability is that steroid synthesis is induced, and, hence, the more product that is synthesized. The alcohol-induced down-regulation of receptors seems to require a high dose of alcohol (more than 10% volume/volume ethanol) *in vitro*, while *in vivo* it requires a prolonged alcohol use (5ml 20% volume/volume ethanol in saline/injection, two injections per day for 7 days) (Bhalla et al., 1983).

Nevertheless, the presence of alcohol does not alter the biological activity of luteinizing hormone (Välimäki et al., 1990), and LH is able stimulate testosterone synthesis also in intoxicated animals (Välimäki et al., 1984). However, since it has been reported that alcohol can inhibit the synthesis and secretion of glycoproteins in the liver, a similar mechanism may exist in the pituitary and affect LH, which also is a glycoprotein.

Rat studies have shown that the primary effect of ethanol on the hypothalamus-pituitary axis is located in the hypothalamus. When rats are injected with GnRH, the alcohol-induced reduction in luteinizing hormone disappears (Cicero et al., 1978; Cicero et al., 1979). Intoxicated rats can, in fact, respond in a normal fashion to stimulation by GnRH (Cicero et al., 1978; Cicero, 1982), even following a high alcohol dose (2.5g/kg). The responsiveness of the human male pituitary to GnRH under alcohol intoxication is similarly not affected by alcohol (Cicero, 1982). The mechanism or mechanisms by which alcohol inhibits the production and/or secretion of GnRH in the hypothalamus have not yet been determined.

Concentrations of β-endorphin have been shown to be increased in the interstitial fluid in testis, hypothalamus, pituitary gland, and serum following alcohol intake (Adams and Cicero, 1991). Increased levels of β-endorphin have been shown to reduce the production of GnRH and LH as well as to inhibit testicular steroid synthesis (Rivest and Rivier, 1993). Alcohol may inhibit GnRH production by activating opiate mechanisms, which are responsible for the inhibition of GnRH (Adams and Cicero, 1991). The opiate antagonist naloxone has been shown to block alcohol's effects on GnRH and LH secretion (Cicero et al., 1982) as well as hindering the alcohol-induced inhibition of testosterone synthesis (Cicero et al., 1990).

Endogenic opiate peptides produced in the gonads of alcohol-influenced animals probably have an autocrine or paracrine effect on testosterone synthesis. However, these peptides are not known to have a dominant role in the effects of alcohol on steroid synthesis.

2.6 Animal Research and the AA and ANA Rat Lines

The ultimate aim in alcohol research is to find a cure for alcoholism and other problems caused by alcohol drinking. However, due to ethical as well as practical reasons, human studies are sometimes difficult or even impossible to perform. Thus, the use of experimental animals offers a wide range of investigational possibilities. With the use of experimental animals, many factors, such as food and water intake as well as previous exposure to different drugs, can be controlled. Furthermore, the use of selectively bred animal models has granted researchers an exceptional opportunity: to study a genetically fairly homogenous population. In fact, several animal models of alcohol drinking have been developed, of which the Finnish Alcohol-preferring AA (Alko, Alcohol) and alcohol non-preferring ANA (Alko, Non-Alcohol) rat lines are two examples. Rats of the AA line voluntarily consume large quantities of alcohol while rats of the ANA line in a free-choice situation avoid practically all alcohol (Eriksson, 1968). The AA rats even learn to lever press for alcohol in the presence of food and water while the ANA rats in general will not work for alcohol (Sinclair, 1974).

The AA and ANA rat lines were developed during the 1960s in the Finnish Research Laboratories of the State Alcohol Monopoly (Alko, Helsinki, Finland) (Eriksson,

1968). The aim was to create animal models of alcohol drinking, which could be used for studying the heritability of voluntary alcohol consumption. The lines were developed with procedures intended to minimize an accidental divergence of the lines, thus increasing confidence that all observed differences between the two rat lines are related to the segregation of different genes contributing to their differential alcohol preferences.

Since the development of the AA and ANA rat lines, they have been studied extensively by researchers worldwide. A vast number of studies have been conducted, reaching from behavioral tests to physiological responses to different types of stimuli, and several line differences have been published. For instance, compared to AA rats, ANA rats have a lower alcohol oxidation rate, a higher acetaldehyde concentration after alcohol intake (Eriksson, 1973; Koivula et al., 1975; Eriksson, 1981), a higher ADH activity (Koivula et al., 1975; Eriksson, 1981), and a lower ALDH activity (Eriksson, 1973; Koivula et al., 1975; Koivisto and Eriksson, 1994). The alcohol intake of the AA rats can be manipulated by transplantation of livers from the ANA line (Eriksson et al., 1997). This suggests a key role of acetaldehyde metabolism in the differential alcohol intake of AA and ANA rats.

Furthermore, the AA rats are less impaired by alcohol than the ANA rats (Nikander and Pekkanen, 1977). In fact, the ANA rats are intoxicated to a higher degree from a variety of drugs when comparing them to the AA rats (Eriksson, 1981). On the other hand, the AA rats have a higher general metabolic rate and a higher food intake as well as a lower body and brain weight (Eriksson, 1981).

Some individuals in Asian populations develop an accumulation of acetaldehyde following alcohol consumption causing symptoms such as tachycardia, hypotension, nausea, and facial flushing. This is caused by a mutation in the gene coding for ALDH, which reduces the activity of the enzyme, that is, the oxidation of acetaldehyde to acetate. Due to the unpleasant side-effects, these individuals usually consume less alcohol than the normal genotype carriers. Similarly, the impaired acetaldehyde metabolism in ANA rats may be one important factor controlling alcohol intake in these animals. In fact, the ANA rat line may operate as an animal model for ALDH deficient individuals. Likewise, the AA rat line may act as an animal model for the factors promoting alcohol drinking, perhaps partly through a testosterone-related model. In conclusion, the AA and ANA rat lines are used as animal models for studying factors that influence (human) alcohol drinking.

2.6.1 Social Isolation as a Stress Model in AA and ANA Rats

Stress is usually used as a term to describe a subjective feeling of pressure or tension. In a scientific meaning stress is according to Selye's theory depicted as a non-specific stereotyped response of the body to any demand upon it (Selye, 1936). The brain receives stimuli from various stressors and reacts by stress responses, that is, by actions on the nervous, endocrine, and immune systems.

The effects of stress on behaviors and physiological responses have been examined using various types of stress models. Commonly used stressors in rat studies include immobilization, electric foot shocks, fasting, and immersion into cold water (Tanaka, 1998). However, in addition to these physical stressors, also structural and social aspects, such as housing conditions, can generate stress responses (Rivier and Rivest, 1991; Brown and Grunberg, 1995; Serra et al., 2003; Esquifino et al., 2004). The non-physical nature of social isolation minimizes discomfort yet generally causes stress responses in animals. Therefore, it is suggested that social isolation could with perhaps even greater validity than physical, potentially painful stressors, be compared with social and psychological stressors, which naturally are of particular interest in humans. Furthermore, social isolation is a suitable test condition for measuring the combined effect of stress and alcohol on the AA and ANA rats since the development of these rat lines was carried out by measuring the alcohol preference by isolated individuals (Eriksson, 1971).

2.7 Alcohol and the Opioidergic System

The brain's opioidergic system is involved in the reinforcing actions of alcohol as well as in excessive alcohol intake (Herz, 1997; Sinclair, 2001). Alcohol increases the release of endogenous opioid peptides and alters the expression and function of opioid receptors in the brain. Several opioid antagonists, such as naltrexone and naloxone, have been shown to decrease alcohol consumption in both experimental animals and humans. Opioid receptors are a part of the regulation of alcohol intake and stimulation of these receptors reinforces alcohol consumption.

One factor causing some individuals to drink more alcohol than other people do is that they receive more reinforcement from alcohol. Higher responses from the reward systems in the brain may be a factor predisposing an individual to drug

abuse. It has been suggested that the alcohol-preferring AA rats are hypersensitive to the reinforcing effects of opioids (Honkanen et al., 1999). Genetically determined differences in the brain β-endorphin system of the AA and ANA rat lines have been reported (Gianoulakis et al., 1992). The receptor densities of the μ-, δ-, κ-receptors are greater in certain brain regions of the AA rats than in the ANA rats (Soini et al., 1998; Soini et al., 1999). Furthermore, the concentrations of opioid propeptide mRNA, opioid peptide mRNA, and peptide contents differ between the two rat lines (Gianoulakis et al., 1992; Nylander et al., 1994; Marinelli et al., 2000). The increased density of opioid receptors for β-endorphin (de Waele et al., 1995) may partly explain the stronger motivational force for continued drinking in the AA rats than in the ANA rats.

Furthermore, the line difference between the AA and ANA rats in the alcohol-induced testosterone effects may be related to these opioidergic differences and coupled to alcohol preference at the hypothalamic level. An alcohol-induced increase in testosterone levels could, via the negative feed-back regulation of testosterone synthesis, increase β-endorphin concentrations in the hypothalamus. An elevation of β-endorphins could enhance the reinforcement produced by alcohol administration and, hence, lead to additional alcohol intake (Apter and Eriksson, 2003).

3 AIMS OF THE STUDY

The main aim of the present work was to investigate effects of alcohol on testosterone and corticosterone in rat lines differing in their alcohol preference. All differences between the two animal models are viewed as possible explanations, at least in part, for the line difference in their alcohol preference. The following two subsidiary aims were also addressed:

I. To characterize control testosterone and corticosterone levels in AA and ANA rats.

II. To investigate if a mildly stressful situation affects the alcohol-induced changes in testosterone.

4 MATERIALS AND METHODS

4.1 Animals

Male rats of two rat lines differing in their alcohol preference were used in the study (Eriksson, 1971). Alcohol-preferring AA and alcohol non-preferring ANA rats belonging to the F80 generation were housed in either single cages (N=12 for AA and N=10 for ANA) or in groups of four individuals (N=12 for both rat lines). At the beginning of the experiment the AA rats weighed on average less than the ANA rats ($p<0.001$ for difference between rat lines), with the AA rats weighing 348 ± 5g and the ANA rats 398± 8g. The rats were about two months old when the experiment was begun. During the experiment the rats were given water and a standard laboratory chow (SDS RM1, Witham, Essex, England) ad libitum. In the animal facilities, air temperature was set at 20-21°C, humidity kept at 47.6% ± 2.1 and a 12h light 12 h dark cycle maintained (lights on at 6 a.m.). At the beginning of the study, the rats had had no previous contact with alcohol.

4.2 Experimental Procedures

The study consisted of six treatment conditions, differing by the alcohol dose (0, 0.75, and 1.50g ethanol/kg of body weight) and the time of day (morning and afternoon sessions). The alcohol dose was administered as a 10% ethanol intraperitoneal injection (w/v, diluted in 0.9% NaCl, i.e. final volumes 7.5ml/kg and 15ml/kg). The control dose consisted of 0.9% NaCl (i.e. 10ml/kg). Morning sessions were begun at about 8 a.m. and afternoon sessions at about 3 p.m. All rats were subjected to all treatments in a random order, with at least one week of rest between treatments. Data are missing for one ANA rat on the effects of the treatments with the low alcohol dose.

During experimental sessions, four tail blood samples of 200µL were collected, from which alcohol, testosterone, and corticosterone concentrations were determined. Blood samples were taken prior to the alcohol/saline injection and 1, 2, and 3 hours post-injection. The blood samples were immediately diluted with 500µL saline and then centrifuged. Serum samples were frozen and kept at -70°C until the analyses were made. The study was approved by the County Administrative

Board of Southern Finland and the ethical committee of the Finnish National Public Health Institute.

4.3 Analytical Methods

Hormone concentrations were measured from serum using commercially available radioimmunoassay kits. The quantifications of the radioimmunoassays were performed by a Wallac Wizard 1470 automatic gamma counter. Testosterone concentrations were determined with Orion Diagnostica's (Espoo, Finland) Spectria testosterone Radioimmunoassay kit. The inter-assay coefficient of variation (CV) was 7% at a testosterone concentration of 1.2 nmol/L, the intra-assay CV was 7.5% at a testosterone concentration of 1.6 nmol/L, and the minimum detectable concentration was 0.1nmol/L.

Corticosterone concentrations were measured using the ImmuChem Double Antibody Corticosterone RIA Kit by ICN Biomedicals (Costa Mesa, CA, USA). The inter-assay CV was 7.2%, and the intra-assay CV was 4.9% at corticosterone levels of 100-200 ng/ml.

Serum ethanol concentrations were determined using head-space gas chromatography (Perkin Elmer Sigma 2000) (Eriksson et al., 1977).

4.4 Statistical Analysis

Data were analyzed using the SPSS (version 11.5, Inc., Chicago, IL). Non-parametric tests were used as the data did not fulfill the requirements of parametric tests even after logarithmic transformations. Differences within lines (related samples) were analyzed using the Wilcoxon test and differences between lines (independent samples) using the Mann-Whitney test. Basal differences in hormone levels were analyzed comparing the averages of the four measuring points within sessions, while the effects of alcohol (alcohol-control values) were measured only at relevant time points.

P-values ≤ 0.05 were considered statistically significant. Data are presented in the text as mean values ± standard error of mean (SE) and in the figures (GraphPad Prism version 4.0, GraphPad Software, Inc., San Diego, CA) as medians ± interquartile deviation (=interquartile range/2).

5 RESULTS

5.1 Control Testosterone Concentrations

The AA rats had significantly higher mean testosterone levels than the ANA rats during both morning (Figure 3, 152% higher, p=0.028) and afternoon sessions (75% higher, p=0.035). In both rat lines the average levels of testosterone were higher in mornings than in afternoons (AA rats 100% higher, p=0.039; ANA rats 39% higher, p=0.021). A large variation in testosterone concentrations was repeatedly found within the AA rats.

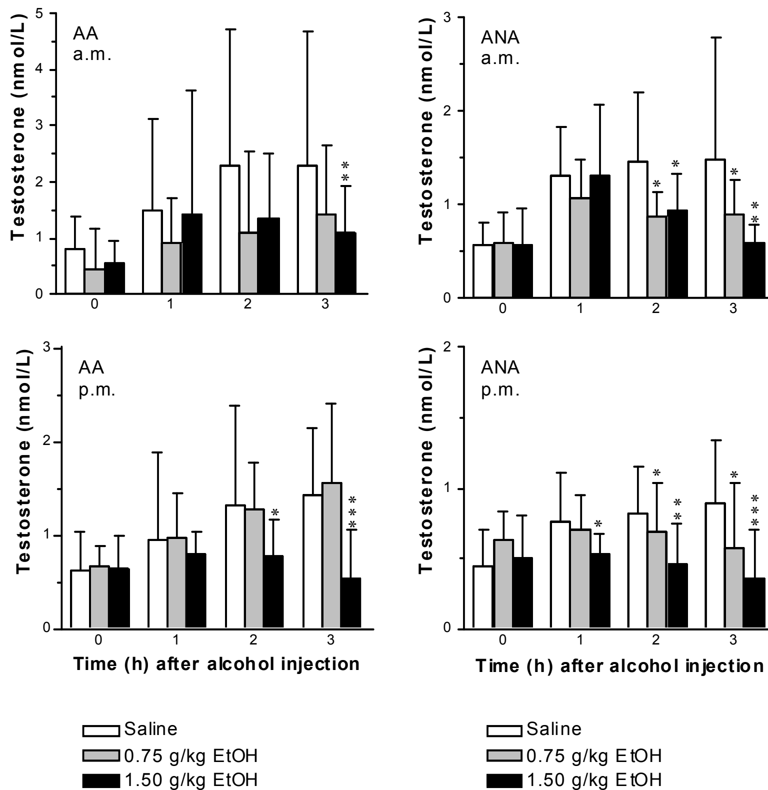

Figure 3. ***The effect of alcohol on testosterone concentrations in AA and ANA rats during morning and afternoon sessions. The medians of the testosterone concentrations during the different treatment conditions ± interquartile deviations are displayed. Significant differences between control and alcohol treatments are also shown; *p<0.05, **p<0.01, and ***p<0.001. Please note the use of different scales.***

5.2 Control Corticosterone Concentrations

Without alcohol, the group-caged AA rats had significantly lower corticosterone concentrations than group-caged ANA rats during morning sessions (Figure 4, the average corticosterone levels were 36±6ng/ml and 98±19ng/ml respectively, p=0.007). In the afternoon sessions, however, no significant differences were found. The concentrations of corticosterone during the afternoons were much higher than during morning sessions in both rat lines (a.m. levels mentioned above, p.m. levels for AA rats were 143±18ng/ml and for ANA 170±15ng/ml; p-values for the difference between morning and afternoon sessions, 0.003 for AA rats and 0.008 for ANA rats, respectively).

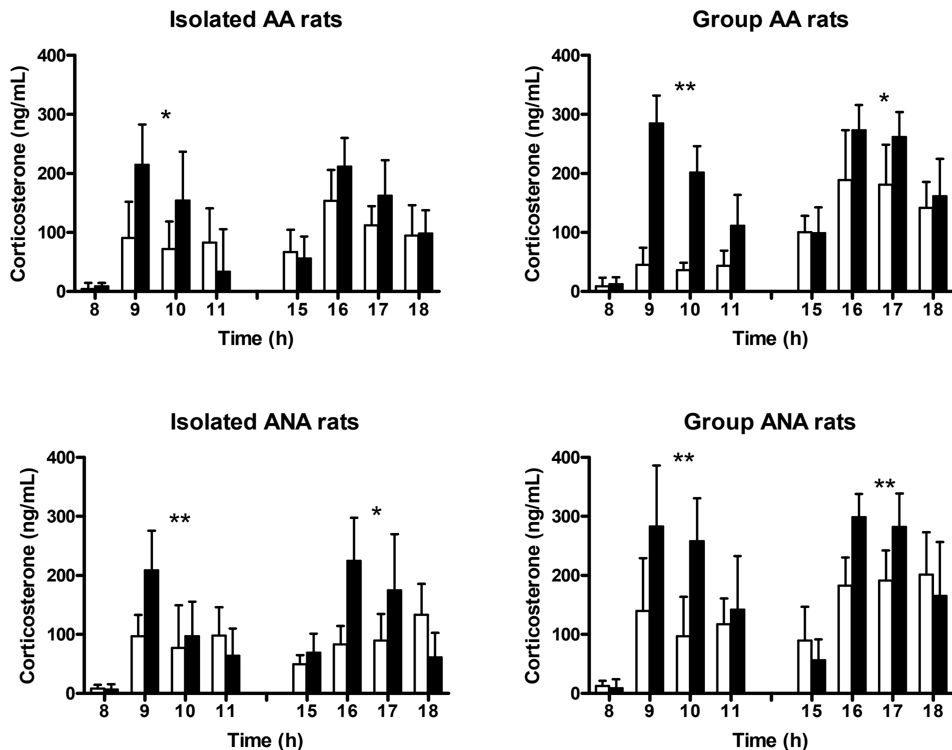

Figure 4. ***Corticosterone concentrations during morning and afternoon sessions. The white bars represent control concentrations and the black bars treatment with 1.5g/kg alcohol, median ± interquartile deviation. Significant increases during alcohol treatment are displayed (average of concentration at 1-2h post injection during alcohol treatment compared with the control session), *p<0.05 and **p<0.01.)***

5.3 Social Isolation and Testosterone

Splitting the animals into subgroups according to housing conditions did not have an effect on the reported line difference. Social isolation did not significantly affect basal testosterone levels within either rat line.

5.4 Social Isolation and Corticosterone

Social isolation in general did not significantly increase corticosterone levels (Figure 4). The only case in which isolation produced a significant increase was in the AA in the morning sessions (mean a.m. increase = 66±15ng/ml, that is an increase of 83%, $p=0.047$. Isolation had the opposite effect in ANA rats in the afternoon, significantly reducing corticosterone levels (mean decrease = 89±18ng/ml, that is a decrease of 48%, $p=0.016$) compared with the control group-caged animals.

In general, corticosterone levels were higher during afternoon than morning sessions ($p<0.010$). The effect was significant in all subgroups except in the isolated ANA rats ($p=0.173$).

It should be noted that no significant line or housing difference in corticosterone levels was found at the first time point of the morning and afternoon sessions, that is, the only time excluding a potential effect of the handling of the animals.

5.5 The Effects of Alcohol on Testosterone

Both alcohol doses reduced testosterone levels in ANA rats significantly during both morning and afternoon measurements (Figure 3). In the AA rats, an overall reduction in testosterone was only induced by the larger dose.

5.6 The Effects of Alcohol on Corticosterone

The lower alcohol dose did not affect corticosterone levels in either rat line ($p>0.05$), while the higher dose increased corticosterone levels in both (Figure 4).

During morning sessions, the alcohol-induced corticosterone elevations were significantly higher in the AA than in the ANA rats (AA rats +182 ng/ml and ANA rats +152 ng/ml, p=0.028). In the afternoons, the baselines were still elevated relative to those at 8 a.m., thus the increases were proportionally smaller, and no line difference was found.

5.7 The Modulation of Alcohol-Induced Effects on Testosterone by Social Isolation

The higher dose of ethanol significantly reduced testosterone concentrations in all subgroups of group-caged animals in both morning and afternoon sessions, but among isolated rats the only significant alcohol-induced reduction was seen in the ANA rats during afternoon sessions following the administration of the higher dose (Figure 5, p=0.009). Furthermore, during morning sessions, in fact, the median alcohol-induced effect in the AA rats tended to be an elevation of testosterone levels in contrast to median reductions in both ANA subgroups as well as group-caged AA rats. Although a trend towards a diverging effect of the high dose between the isolated and group-caged rats was found, the testosterone concentrations of the isolated AA rats were not at any time point significantly higher than during the control situation.

The lower dose did not significantly alter testosterone levels in neither AA nor ANA rats subjected to isolation.

5.8 The Modulation of Alcohol-Induced Effects on Corticosterone by Social Isolation

Similarly to the results in the group-caged ANA rats, the lower alcohol dose had no effect while the higher dose increased corticosterone levels in isolated ANA rats. However, in the isolated AA rats, neither alcohol dose affected corticosterone levels although a trend towards an alcohol-induced elevation of corticosterone was found (p=0.071).

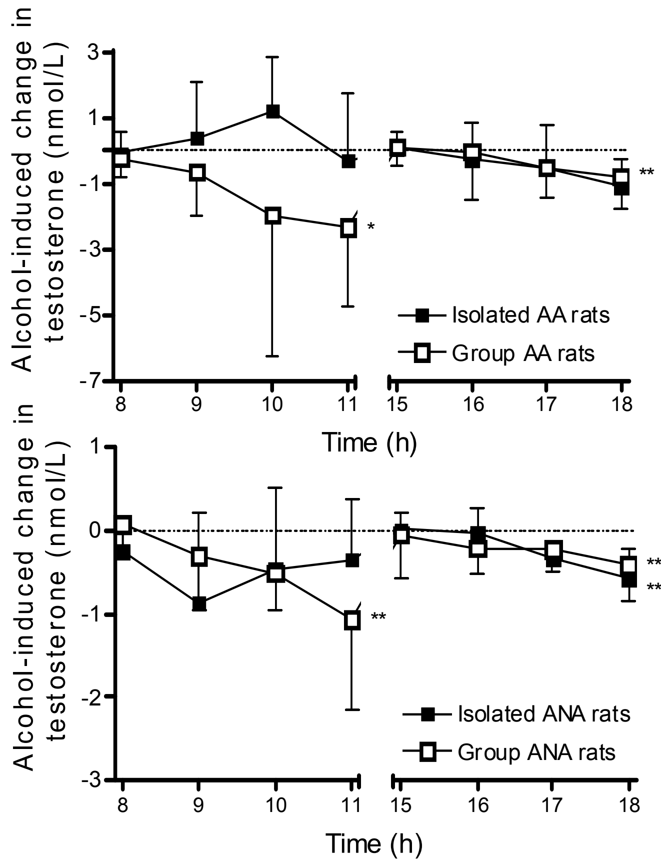

Figure 5. ***Alcohol-induced (1.5g/kg) changes in testosterone levels. Median values of alcohol-induced changes (alcohol-control values) ± interquartile deviation are displayed (deviations of single-caged animals are shown above and group-caged animals are shown below the median values). Significant changes have been calculated as the difference between the averages of the two last measuring points, *p<0.05 and **p<0.01.***

6 DISCUSSION

The present study focuses on three main issues. Do the AA and the ANA rat lines differ in basal testosterone or corticosterone levels? Do they differ in their hormonal responses to alcohol? And, third, does a mildly stressful situation affect the alcohol-induced changes in testosterone? All issues may be connected to why these rat lines, developed for diverging alcohol intake, actually differ in their alcohol preference.

6.1 Control Testosterone Levels

Our results showed a difference in testosterone levels between the AA and the ANA rats, with higher concentrations of testosterone found in the AA rats. To our knowledge only one study on animals has been published previously showing an association between high testosterone levels and alcohol drinking (Lakoza and Barkov, 1980). This is, however, also supported, indirectly, by behavioral associates of testosterone. Testosterone levels have been positively correlated with low harm avoidance, high sensation seeking, impulsiveness, as well as aggressive and antisocial behavior (Daitzman et al., 1978; Zuckerman et al., 1980; Dabbs and Morris, 1990; Archer, 1991; King et al., 1995). These characteristics have also been used to describe the type II alcoholics (Devor and Cloninger, 1989). Moreover, higher testosterone levels have been reported in sober alcoholics than in controls (King et al., 1995), and higher testosterone cerebrospinal fluid content has been found in alcoholic, impulsive offenders with antisocial personality disorder than in controls (Virkkunen et al., 1994). A positive relation between testosterone levels and alcohol drinking in men could characterize a general mechanism, explaining why men typically drink more than women and why alcoholism is more common in men than in women. It should be noted, however, that within the AA rat line, females drink more alcohol than do the males (Hilakivi et al., 1984).

6.2 Control Corticosterone Levels

The ANA rats had higher corticosterone concentrations than the AA rats during the morning control setting. A line difference was not, however, found in the afternoon session, during which the hormone levels were significantly higher than in the mornings. This difference, with lower stress hormone levels in the alcohol preferring

rat line, is contradictory to two earlier sets of results. First, previous reports have not found a line difference in corticosterone levels between AA and ANA rats (Gianoulakis et al., 1992; Fahlke et al., 1993). This divergence is likely to be caused at least partly by differences in handling and sampling. It should be noted that we also did not see differences at the initial 8 a.m. or 3 p.m. measure; the line differences seen later during the session can to some extent be seen as responses to the handling, injecting, and blood sampling at the first time point. Second, corticosterone and hypothalamic-pituitary-adrenal-activity have previously been related to increased alcohol drinking (Gianoulakis, 1998; Tanaka, 1998; Fahlke and Eriksson, 2000). This inconsistency can perhaps be explained by the reported line differences in AA and ANA rats concerning the relation between manipulation of the HPA-axis and alcohol drinking (see below).

Adrenalectomy has been shown to attenuate alcohol intake in rats (Morin and Forger, 1982; Fahlke et al., 1994; Fahlke et al., 1996; Lamblin and De Witte, 1996), and the effect has been reversed by corticosterone treatment (Fahlke et al., 1994; Fahlke et al., 1995). However, of the AA and ANA rat lines, only the AA rats have responded in this manner to the manipulation of the HPA-axis (Fahlke and Eriksson, 2000). No shift in alcohol intake was seen in the ANA rats after adrenalectomy or subsequent corticosterone replacement. Consequently, the authors concluded that corticosterone only stimulates alcohol drinking in animals with high alcohol preference. This theory is supported by reports of higher sensitivity to the reinforcing effects of glucocorticoids on alcohol drinking in individuals with a predisposition to develop drug abuse (Deroche et al., 1993; Piazza et al., 1993; Piazza and Le Moal, 1997; Marinelli and Piazza, 2002).

6.3 Social Isolation and Hormone Levels

Social isolation increased corticosterone levels in AA rats during morning sessions, while corticosterone levels were reduced in ANA rats during afternoon sessions. Due to technical reasons, the selective breeding of these two rat lines is based on alcohol intake during social isolation. Therefore, the relation between corticosterone and alcohol intake may be related to the increased corticosterone levels in isolated AA rats and reduced levels in isolated ANA rats, but this does not explain why the effects are only present part of the time. This difference may be fundamental in nature as the AA rats have previously been reported as less fearful, less sensitive to aversive stimuli, responding more calmly to stress and having a lower vulnerability

to stress gastric ulceration (Korpi et al., 1988; Sandbak et al., 1998). In the above mentioned studies measurements other than corticosterone levels were used, such as behavioral responses and epinephrine and dopamine levels.

Splitting the animals into subgroups according to housing conditions did not have an affect on the reported line difference nor did it significantly affect basal testosterone levels within either rat line.

6.4 Acute Effects of Alcohol on Testosterone

All alcohol administration circumstances (both doses and time points) reduced concentrations of serum testosterone in group caged ANA rats. However, in the group caged AA rats, only the higher doses had this effect, while the lower doses had no significant effect. Also, a divergence in the alcohol-induced effects of the lower dose was found between the two rat lines, with an almost equal amount of AA rats showing increases and decreases and nearly all ANA rats showing decreases in testosterone following alcohol administration. However, based on this study it is impossible to state whether the observed line difference in the effects of alcohol are a result of a higher prevalence of alcohol-induced elevations or by a higher resistance to alcohol-mediated reductions in testosterone in the AA line.

As previously mentioned, both alcohol-mediated increases and decreases of testosterone have been reported. This discrepancy concerning the effect of a low alcohol dose with experimental animals is supported by human data in both women and men (Eriksson et al., 1994; Sarkola and Eriksson, 2003). However, the lower sensitivity of the AA than the ANA rats to the testosterone-reducing effects of alcohol may be associated with the higher alcohol drinking in the AA rats. Furthermore, this difference in sensitivity may be a sign of differential mechanisms of action by ethanol in the two rat lines. In rats, which have been subjected to hormonal stimulation to increase testosterone levels, ethanol metabolism has been reported to be important for the appearance of the testosterone-related effects. On the other hand, in non-stimulated animals ethanol-mediated effects were seen even when ethanol metabolism was inhibited (Eriksson et al., 1983; Orpana et al., 1990d). Since the AA rats were shown to have higher testosterone levels in the control sessions, differential mechanisms or a different balance of the possible mechanisms may act in the rat lines. The AA rats have been shown to metabolize ethanol more

effectively (Eriksson, 1973; Koivula et al., 1975; Eriksson, 1981; Koivisto and Eriksson, 1994), and this may influence the sum of the effects.

6.5 Acute Effects of Alcohol on Corticosterone

The observed alcohol-induced changes in corticosterone concentrations are in line with previous reports (Rivier, 1996) demonstrating that high doses increase and low doses do not affect corticosterone concentrations. The only situation in which the effect of a high alcohol dose failed to reach significance was during the afternoon session with the isolated AA rats. In the group-cage situation during the morning session, the alcohol-induced elevations were significantly higher in the AA rats than in the ANA rats. In the afternoon sessions, the levels at 15:00 were higher so the subsequent ethanol-induced increases were proportionally smaller, and no line differences were seen. It has been reported that the response of the hypothalamus-pituitary-adrenal axis depends not only on the type of stressor, but also on the time of day that the stressor is applied (Retana-Márquez et al., 2003), as is also the case here.

6.6 The Modulation of Alcohol-Induced Effects on Testosterone by Social Isolation

Although social isolation *per se* had no significant effects on control testosterone levels, it modified the effects of alcohol on testosterone. Among group caged animals, the high dose of alcohol significantly reduced testosterone in both rat lines during both morning and afternoon sessions, but among the isolated rats the reduction reached significance only in the ANA rats in the afternoon session. Moreover, the testosterone-reducing effect of the low dose in the group-caged ANA rats was absent in the isolated ANA rats.

During the morning sessions, the median alcohol-induced effect of the high dose in isolated AA rats seemed to be a minor elevation in testosterone levels. Although the alcohol-induced changes in testosterone concentrations tended to differ between isolated and group caged animals, increases were not found when comparing the alcohol-influenced values to the animals own control sessions.

7 CONCLUSIONS

This study is among the first in a line of studies in our laboratory focusing on the possible role of the steroid hormone testosterone in alcohol drinking and the development of an alcohol addiction. For decades investigators have been interested in the effects of alcohol on testosterone, while to our knowledge only one study has been carried out concerning the effects of testosterone on alcohol drinking (Lakoza and Barkov, 1980). The aim of our research team is to investigate the possibility of a connection between alcohol drinking and testosterone, which well may be two-way.

In this study, three main outcomes were found. First, higher testosterone levels were found in the alcohol-preferring AA than in the non-preferring ANA rats. Second, ethanol significantly reduced testosterone in both lines, with more significant reductions in the ANA rats, and, third, social isolation attenuated the alcohol-induced effects on testosterone in both rat lines.

Based on the results of this study, the role of testosterone in alcohol drinking is supported. The higher testosterone levels in the AA rats may promote alcohol drinking through behavioral correlates associated with the hormone. Moreover, the higher levels may be a result of lower β-endorphin levels, which by inducing hypersensitivity in opiate receptors through an increased density of receptors could lead to increased reinforcement by alcohol. Greater densities of opioid receptors have been measured in several brain areas in AA compared to ANA rats (Soini et al., 1998; Soini et al., 1999).

Although the influence testosterone levels on alcohol drinking is supported by the results, there is less evidence that alcohol-mediated testosterone elevations play the role hypothesized for them, although increases were seen in some AA rats after both alcohol doses. Moreover, β-endorphin levels were not measured in this study. Thus, the promotion of alcohol drinking through the feedback regulation of testosterone via an increase in β-endorphin levels cannot be confirmed here.

In the introductory part of this thesis, it was hypothesized, that alcohol-induced testosterone elevations may occur only in interaction with or be more prominent in the presence of a situational factor such as stress. However, social isolation only

increased corticosterone levels in one test group (AA, morning) and even a decrease was found (ANA, afternoon). This suggests that the relationship between individual housing and stress is more complicated than previously assumed. Housing conditions do influence behavior and physiologically reactions to ethanol, as we observed, but not necessarily through the intervening factor of stress as measured with corticosterone levels.

Furthermore, two issues need to be pointed out. First, in this experiment alcohol was administered to the animals by injection. The results might be different if the experiment was done as a free-choice paradigm. The injection and handling of the animals is likely to have caused some distress, as can be seen by the rise in corticosterone concentrations after the first time point within the sessions. Second, the sessions in this experiment were in the morning and in the afternoon, while it is known that rats are nocturnal and drink primarily during the night (Hyytiä and Sinclair, 1990) when the basal levels of the investigated hormones may be quite different. Corticosterone levels of rats have been reported to begin increasing before the beginning of the dark period with a peak at about one hour after lights have been turned off. The reports on the diurnal variation of testosterone in rats, on the other hand, have been more inconsistent, with peaks reported in the early mornings (Kinson and Liu, 1973), mornings (Heywood, 1980), afternoons (Leal and Moreira, 1997), and just before midnight (Keating and Tcholakian, 1979). This discrepancy can at least partly be explained by differences in experimental settings (light-dark cycle, sampling methods, timing of samples, housing), age and strain of experimental animals as well as seasonal variations (Keating and Tcholakian, 1979).

The limitations to the conclusions that can be made based on this data are acknowledged. If an association between alcohol, corticosterone, and testosterone exists, the experimental setting of this study may be the reason for it not being found here. The results of this study support the role of testosterone in alcohol drinking, although this role seems to be complex. Alcohol drinking can be reinforced by alcohol-induced increases in β-endorphin, caused either directly or indirectly. A direct alcohol-induced increase in β-endorphin could cause a reduction of testosterone through the feedback regulation of testosterone, while an indirect increase could be caused by an alcohol-induced testosterone increase, which again, through the feedback inhibition of testosterone would cause β-endorphin levels to increase. This study supports more the direct hypothesis. However, there might be an interspecies difference here, which could explain the testosterone increases observed in men (Sarkola and Eriksson, 2003). It is possible that endorphins provide

reinforcement in both rats and humans, but that the way in which these alcohol-induced endorphins are produced differs between the two species. It is possible that in rats, alcohol increases endorphins through a system(s) separate from testosterone, while in humans, the testosterone link could provide much of the endorphin. In essence this could explain for example why in rats females drink more than males (Hilakivi et al., 1984), while in humans men drink more than women.

Future studies, some of which already are under way, include both rat and human experiments. We will further investigate the effects of alcohol on hormone levels following voluntary alcohol drinking rather than injected doses of alcohol, to see if a connection between testosterone and continued alcohol drinking can be found. Moreover, we will try to find stressful situations were an unexpected rise in testosterone might be connected to continued alcohol drinking. The role of testosterone in alcohol drinking may be enhanced in individuals with a genetic disposition for becoming dependent on alcohol and modulated by stress (Apter and Eriksson, 2006). Our main aim is to discover testosterone-related mechanisms controlling and/or influencing alcohol consumption. In the future, we hope the new mechanisms can be of use in the treatment and prevention of alcoholism and alcohol abuse. And, considering the extensive effects of alcohol on our society today, tools for minimizing them are most certainly welcomed.

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