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Correlations between mRNA expression of neurotransmitter receptors in the brain and alcohol self-administration in rats

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Title (English)

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Title (Swedish)

Abstract

In this thesis it was investigated whether there was a correlation between the mRNA expression of neurotransmitter receptors in the brain and self-administration of ethanol in rats. Forty naïve Wistar rats were trained to orally self-administer ethanol during a nine day training period. A high divergence in alcohol consumption was observed over the population. mRNA expression levels of a number of receptors was determined using real-time PCR. Prefrontal cortex (PFC), hippocampus and amygdala were studied, three brain structures that interact with the core regions of the reward pathway and are involved in learning and memory. Several correlations between mRNA expression and number of alcohol deliveries were found, especially in the PFC. In particular, we found correlations between three adrenergic receptor subtypes and alcohol deliveries which could indicate an involvement of stress in the choice to self-administer.

Keywords

Ethanol, self-administration, Neurotransmitter receptors, Real-time RT-PCR

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Sammanfattning

Alkohol är den vanligaste drogen i det svenska samhället och den är accepterad i många sociala sammanhang. De flesta människor klarar att kontrollera sitt användande av alkohol men en liten andel utvecklar beroende. Etanol påverkar användaren på många sätt, troligen genom många mekanismer som fortfarande är okända. I den här studien var vi intresserade av att försöka förstå vilka medfödda biologiska egenskaper som ligger bakom en ökad känslighet för att utveckla beroende av alkohol, något som i förlängningen kan användas för att utveckla läkemedel mot beroende. Undersökningen bygger på en beteendestudie där råttor lär sig att de får en vätska som innehåller alkohol (etanol) då de trycker på en pedal. Därefter användes real-tids PCR, en metod som anger mRNA nivåer i en vävnad, för att upptäcka skillnader mellan individerna. Vi undersökte mängden mRNA av ett antal receptorer i tre strukturer i hjärnan som enligt tidigare forskning kan vara inblandade i etanols effekt på centrala nervsystemet. Vi fann ett antal samband mellan mRNA uttryck och etanolintag i speciellt prefrontala cortex, en region i hjärnan som är inblandad i bl.a. bearbetning av nya intryck, minne och kontroll av beteende. Vi fann också indikationer på att stress kan vara en viktig faktor vid etanolintag.

Examensarbete 20 p i Molekylär bioteknikprogrammet

Uppsala Universitet juni 2005

1 Introduction

Alcohol has been used by humans throughout history. There is evidence of production and consumption of alcoholic beverages from the Stone Age about 10 000 years ago (Haglund, 2005). The *Centre for Social Research on Alcohol and Drugs* (SoRAD) surveys the alcohol consumption in Sweden today. The average yearly consumption for 2004 was 10.5 l of pure (100%) alcohol, which is an increase of 2.1 l since 1996. Men drink more than twice as much as women. 27 % of the total consumption consists of spirits, 37% is wine and 37% is beer. Almost half of the total amount is purchased at Systembolaget, one fourth is brought back from traveling abroad, one tenth is from restaurant visits and rest is from supermarkets (light beer), smuggling, and home brewing (SoRAD, 2005). According to *Centralförbundet för alcohol and narkotikaupplysning* (CAN), between 5 000 and 7 000 people die in Sweden every year from injuries related to alcohol consumption (CAN, 2005).

Why are some individuals more vulnerable to develop addiction to alcohol than others? There is convincing evidence that genetics are involved to a great extent but it is not fully clear how. Many experiments are performed to study how alcohol affects expression of genes but the clinical relevance of this can be questioned as high doses of ethanol are injected, often to the point of unconsciousness and the animal has no control over the situation. This study has the opposite approach, how does mRNA expression already present in the individual correlate to their voluntary use of alcohol? The common way to study this so far has been by using animal strains that are selectively bred as alcohol preferring or nonpreferring. However, behavior is a lot of times not examined in these studies. We are interested in differences in a normal population that is representative for the population at large. The use of cDNA microarrays is a popular way to study expression patterns but we wanted to use a more sensitive method that gives more quantitative information about a selected number of genes. Real-time PCR has those qualities and because extensive studies have been made in the alcohol field we can make a qualified hypothesis about which genes are involved in alcohol-seeking behavior. We have previously shown that expression of receptors is consistent and do not change due to ethanol intake over the 9-day training period (Pickering *et al.*, 2005). So, therefore this study is focused primarily on the expression of these receptors. The brain regions that are immediately involved in the reward pathway, ventral tegmental area (VTA) and nucleus accumbens (NAc), have already been extensively studied in alcohol research. It is obvious, however, that other regions also play a role so we decided to apply this novel approach to investigate the involvement of prefrontal cortex, hippocampus and amygdala in alcohol self-administration. We also wanted to show that the method is potentially useful to generate new targets for further research or development of pharmacological treatment, in alcoholism.

2 Background

2.1 Alcohol and addiction

Addiction is defined as "a compulsive drug use that becomes the main goal-directed activity of the subject" (Piazza & Le Moal, 1998). There are of course many different views about how alcoholism should be diagnosed. American Psychiatry Association (APA) has set up criteria for both alcohol abuse and dependence. There are four DSM-IV criteria for alcohol abuse and a person is considered to abuse alcohol if they fulfill one or more of these criteria for more than one year.

1. Role impairment (failed work or home obligation)
2. Hazardous use (driving under the influence)
3. Legal problems related to alcohol use
4. Social or interpersonal problems due to alcohol.

According to the DSM-IV criteria for alcohol dependence, a person would fulfill three or more of the following seven criteria for over a year if they were diagnosed as an alcoholic.

1. Tolerance
2. Alcohol withdrawal sign or symptoms
3. Drinking more than intended
4. Unsuccessful attempts to cut down on use
5. Excessive time related to alcohol (obtaining, hangover)
6. Impaired social or work activities due to alcohol
7. Use despite physical or psychological consequences

According to APA, the prevalence for alcohol dependence during lifetime is 8-14% and the symptoms usually first show up in the age 15-19 years.

2.2 Self administration

To understand alcohol consumption in humans and possibly develop new anticraving drugs, it is necessary to use animal models. The majority of all preclinical studies are performed on rats or mice. There are a large number of models for studying different parts of consumption like intoxication, withdrawal, abstinence etc (McGregor & Gallate, 2004). The animal can, for example have a choice between two bottles (with and without alcohol) to drink from or have to perform a task (press a lever) to obtain alcohol. There is a basic problem with alcohol studies in rodents since they are reluctant to consume ethanol to the point of intoxication. This could reflect the fact that 10% ethanol in water (used in most research) does not taste good. There are basically four ways to try and overcome this problem. One is the use of alcohol-preferring strains of rats and mice. Forced consumption of different forms is also used, ethanol may be the only available fluid or the animal may be exposed to ethanol in vapor chambers. Addition of different kinds of sugars is also a way to overcome the taste problem. Studies have also been made where rats were allowed to drink beer and it was shown that rats, like humans, had more appetite for beer than for ethanol in water. Rats seemed to be intoxicated and also experience hangover and dependence from the beer (McGregor & Gallate, 2004). In our

study, rats were offered a solution with ethanol and saccharin. Saccharin has a sweet taste but does not contain any calories so it therefore does not affect absorption and metabolism of ethanol (Matthews *et al.*, 2001).
Since the 1960's, several lines of alcohol-preferring and alcohol-nonpreferring strains of rats have been developed. Examples are the Alcohol-Preferring (P) and Alcohol-Nonpreferring (NP) lines, the High Alcohol Drinking (HAD1 and HAD2) and Low Alcohol Drinking (LAD1 and LAD1) lines and the Alko-alcohol (AA) and Alko Non-alcohol (ANA) lines. In a comparative study, rats were allowed to choose between a 10% Ethanol + water solution and only water. There was a big difference in preference between preferring strains that chose to drink 70-75% of the total from the ethanol bottle, and nonpreferring strains that only drink 8-16% from the ethanol containing liquid (Table 1). It was clear that genetic factors are important for ethanol consumption (Samson *et al.*, 1998).

Table 1. Comparison of ethanol preference between different alcohol-preferring and –nonpreferring rat strains. With "% ethanol preference" means the percentage liquid the rats drink from the ethanol containing bottle when they have a choice between a 10% ethanol solution and only water.

Rat strain	P	NP	HAD1	LAD1	HAD2	LAD2	AA	ANA
% ethanol preference	70	20	71	8	75	16	73	14

There are, however, also considerable differences in behavior between the different preferring and nonpreferring strains, respectively. The AA line, for example, consumed less alcohol than other preferring strains, under an operant experiment (Files *et al.*, 1998) but consumed more when alcohol was available in bottles in the homecage (Samson *et al.*, 1998). It has been suggested that genes related to ethanol preference when access is free may be different to genes related to ethanol intake under operant conditions (Files *et al.*, 1998). Because of the involvement of multiple genes, these strains may have important differences in genotype. But since the behavior between strains is inconsistent and these animals are difficult to obtain, outbred Wistar rats were used for this experiment. Earlier studies have shown that the alcohol self-administration behavior in these rats varies greatly over a given population.

2.3 Individual vulnerability

Alcohol is an accepted part of our society and is used by a large part of the population. Many people also try other drugs but only a few develop addiction. There are two main theories that try to explain why some individuals develop addiction while others do not. The drug-centered vision suggests that the drug induces changes in the subject that result in addiction. It also proposes that the individuals that develop addiction are those that are in an environment that give them many opportunities to use the drug (Piazza & Le Moal, 1998). The individual-centered vision suggests that the tendency to develop addiction is a preexisting condition. The organism's biological characteristics affect the response to the drug and make some individuals more likely to develop addiction (Piazza & Le Moal, 1998). The general view is that both genetic and environmental factors play a role in addiction and recent evidence from animal experiments also suggests this. The degree of exposure to a drug and the degree of vulnerability in the individual seems to interact (Deroche-Gamonet *et al.*, 2004). Sixty percent heritability has been reported for

alcoholism but there is certainly not only one gene responsible. Multiple and interacting genes seem to be involved but they are yet impossible to distinguish (Mayer & Höllt, 2005).

2.4 Physiological effects

When ethanol is consumed it is quickly absorbed into the bloodstream and distributed to the whole body. Ethanol is metabolized in the liver to acetaldehyde by alcohol dehydrogenase and then to acetic acid by acetaldehyde dehydrogenase (Haddad, 2004). Acetaldehyde is poisonous and its accumulation cause flushing, nausea, headaches and other symptoms related to alcohol intoxification. Acetaldehyde can also cross the blood-brain barrier and affect neurotransmitter systems in the brain. Acetic acid can be used by the body as a source of energy (Haddad, 2004). The main effect of ethanol is as a CNS depressant. It has been shown to be reinforcing and addictive in humans and these effects can also be seen in many animal studies (Nestler *et al.*, 2001). Impaired coordination, slurred speech, increased self-confidence and euphoria are all examples of behavioral effects caused by ethanol. Ethanol also affects other systems in the body since it triggers vasodilation which causes a feeling of warmth but it actually makes the body lose heat. Among other things, ethanol also increases salivary and gastric secretion and also affects the endocrine system by stimulating the anterior pituitary gland (Rang *et al.*, 1999).

2.5 Neurotransmitter systems

There is no evidence for the existence of a receptor for ethanol. Instead, ethanol seems to have an effect on virtually all neurotransmitter systems by interactions with receptor or effector proteins or changes in plasma membrane fluidity (Dodd *et al.*, 2000).

2.5.1 GABA

Gamma-aminobutyric acid (GABA) is the major inhibitory neurotransmitter and is present throughout the brain. There are two types of GABA receptors. GABAB receptors are members of the G Protein Coupled Receptor (GPCR) superfamily and two major subunits have been cloned (Nestler *et al.,* 2001). GABAA receptors are members of the ligand-gated ion-channel family and at least 18 subunits divided into 7 groups have been cloned (Wafford *et al.*, 2004). Each channel is a pentameric complex that forms a water filled pore where Cl^- ions can pass when ligand is bound to the receptor. In the CNS, GABAA receptors are probably made up of $n\alpha + n\beta + n\gamma$ subunits (n = 1-3). The most common combination seems to be $2\alpha + 2\beta + 1\gamma$ but experiments suggest that several combinations exist in vivo (Nestler *et al.,* 2001). The GABAA receptor especially has been shown to play an important role in ethanol's effect on the CNS. Activation of GABAA receptors allows Cl^- ions to pass down its electrochemical gradient and the membrane potential is moved away from action potential threshold (hyperpolarization). There are several modulators that enhance the inhibitory effect of GABA, for example benzodiazepines and barbiturates. These activating modulators have a sedative or hypnotic effect. Ethanol also has this enhancing effect on at least some of the GABAA receptors. Ethanol makes the channels open longer and more frequently (Davies, 2003).

Blockade of GABAA receptor function with a GABAA antagonist can inhibit motivation to self-administer ethanol (Koob, 2004). The GABAB receptor agonist baclofen also appears to have an anticraving effect and reduce cravings for many drugs, including alcohol, cocaine and nicotine (McGregor & Gallate, 2004).

2.5.2 Glutamate

Glutamate is the major excitatory neurotransmitter in the brain. It is a non-essential amino acid that is synthesized in the brain from glucose and other precursors. The glutamate receptors include two families. N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate are all ion channels receptors while the 8 metabotropic glutamate receptors (mGluR) belong to the GPCR superfamily (Nestler *et al.*, 2001). Four AMPA receptor subunits have been cloned (GluR1-4) and the composition of the receptor determines its permeability to Na^+ and Ca^{2+}. AMPA receptors are involved in rapid excitation in the brain. The metabotropic receptors have been shown to be involved in both excitatory and inhibitory transmission. Seven subunits have been cloned (mGluR1-7) (Dodd *et al.*, 2000). NMDA receptors are often associated with ethanol effects. The receptors are probably comprised of 4 subunits, 2 NR1 (A & B) and 2 NR2 (A-D), which make a cation channel. Under normal conditions, the channel is blocked by Mg^{2+} but this blockade is removed when the membrane is depolarized. After depolarization, glutamate *and* glycine have to bind to the receptor for it to be activated. Activation increases the permeability to several cations but the influx of Ca^{2+} is the most important result (Krystal *et al.*, 2003). It is not clear how ethanol exert its effects on the NMDA receptor but there is evidence that it acts as an NMDA antagonist, thus inhibiting Ca^{2+} influx and making the neuron less likely to fire (Davis & Wu, 2001). The NMDA receptors have been proposed to be involved in long-term potentiation (LTP), which is the strengthening of the connection between two neurons and this is considered to be the basis for memory formation. This might be the explanation for ethanol's harmful effect on learning and memory (Nevo & Hamon, 1996).

2.5.3 Noradrenaline

Noradrenaline (NA) is a monoamine transmitter that belongs to the catecholamine family and is derived from tyrosine. There are relatively few NA neurons in the brain but they project to almost all areas of the CNS and, as such, the NA system is very important for many critical brain functions. Examples are the sleep-wake cycle, arousal, attention, learning and memory (Nestler *et al.*, 2001). The adrenergic receptors that NA activate are GPCR´s, nine different subtypes have been found and they are divided into two groups, α and β (Gibbs & Summers, 2002). The different receptor classes are associated with the differences in second messenger coupling. α1-receptors activate phospholipase C and adenylate cyclase is inhibited by α2-receptors and activated by β-receptors. Therefore, activation of these receptors has a wide range of effects on the organism. β1 receptors are found mainly in the heart and are important for heart rate and force (Rang *et al.*, 1999). Several α-receptors have been suggested to be involved in drug withdrawal and other responses to drugs. Clonidine is a α2-receptor agonist which has been used in withdrawal treatment. There are also studies that suggest that alcohol dependent individuals have a subsensitive α2-receptor (Fahlke *et al.*, 2000). Another group has shown the involvement

of α1b-receptors in locomotor response and dopamine release in response to morphine (Auclair *et al.*, 2004). The noradrenergic system in the brainstem regulates expression of corticotrophin releasing factor (CRF) and thus may have an important involvement in the HPA axis activity during stress (see section 2.6) Dysregulation of noradrenergic transmission has been connected to depression, stress and anxiety disorders (Forray & Gysling, 2004).

2.5.4 Dopamine

Dopamine (DA) is also derived from tyrosine and is both a precursor for noradrenaline and a neurotransmitter. DA mostly occurs in a few restricted areas in the brain, about 75% is found in the nigrostriatal pathway from substantia nigra to striatum. This pathway has an important role in voluntary movement. Deficiency of dopaminergic neurons in this system is associated with Parkinson's disease (Rang *et al., 1999*). The other major DA system is the mesolimbocortical pathway that originates in the ventral tegmental area (VTA) and projects to nucleus accumbens (NAc) and PFC. This system is called the reward pathway as this probably mediates the rewarding and reinforcing effects of drugs (Nestler *et al.*, 2001). DA receptors are GPCR's and can be divided into two families, D1 like (D1 and D5) and D2 like receptors (D2-D4). Ethanol enhances activation of the mesolimbocortical DA pathway and manipulations of the DA system have been shown to affect intake of ethanol in animal studies. It has also been suggested that D2 receptors may play a role in addiction; low levels have been observed among cocaine, heroin and metamphetamine abusers (Tupala & Tiihonen, 2004).

2.5.5 Serotonin

Serotonin or 5-hydroxytryptamine (5-HT) is a monoamine and synthesized from tryptophan. Serotonergic neurons are common in the brain and they project to all regions. Because the serotonergic system is so extensive it is hard to determine all the functions it is involved in, but mood, sensation seeking and sleep-wake cycle are usually mentioned (Nestler *et al.*, 2001). Seven families of mammalian serotonin receptors (5-HT1-7) with 14 subtypes have been found in the CNS. All families are GPCR's except 5-HT3 which is a ligand-gated ion-channel (Barnes & Sharp, 1999). A connection between serotonin and alcohol intake and dependence has been established in many studies. Reduced 5-HT concentrations in the brain increase intake of ethanol in animal studies, and increased levels reduce the intake (Nevo & Hamon, 1995). Fluxetine is a selective serotonin reuptake inhibitor (SSRI) and has a strong reducing effect on alcohol consumption, but unfortunately it has the same effect on intake of any kind of food or drinks (McGregor & Gallate, 2004). The 5-HT3 receptor has recently received attention as a possible therapeutic target for treatment of alcohol abuse. It has been shown that ethanol alters the function of this receptor. 5-HT3 antagonists reduce the increase in extracellular dopamine produced by ethanol and this decreases ethanol self-administration in rats (Hodge *et al.*, 2004). Administration of the 5-HT1B agonist RU24969 in rats significantly decreases self-administration of ethanol (Tomkins & O'Neill, 2000). A reduction in ethanol preference to various degrees has also been shown after treatment with 5-HT2A agonist or 5-HT2C/1B agonists (Maurel *et al.*, 1999).

2.5.6 Acetylcholine

Acetylcholine (ACh) is synthesized from choline and acetyl coenzyme A (CoA). Cholinergic neurons project from the basal forebrain and the upper brain stem. Cerebral cortex and hippocampus are among the areas that receive these projections and this system is therefore involved in emotional state and the response to sensory input as well as learning and memory (Nestler *et al.*, 2001). ACh acts on two different types of receptors which are named according to their natural agonists. Muscarinic ACh receptors (mAChR) are GPCR's and five subtypes have been cloned. There is growing evidence that ethanol interacts with the other receptor type, nicotinic ACh receptors (nAChR), so only this type is discussed further. nACh receptors are ligand-gated ion channels and activation leads to rapid influx of Na^+ and Ca^{2+}. The receptors are pentameric and divided into α- (2-10) and β- (2-4) subunits. Ca^{2+} permeability of the receptor is influenced by its composition of the different subunits (Dajas-Bailador & Wonnascott, 2004). Several studies suggest that ethanol interacts with nACh receptors and both potentiating and inhibiting effect have been seen. It is also well demonstrated that there is a correlation between alcohol and nicotine addiction. According to several studies, 80-90% of alcoholics also smoke (Larsson & Engel, 2004).

2.6 Hypothalamic-Pituitary-Adrenal Axis

The hypothalamic-pituitary-adrenal (HPA) axis is a part of the neuroendocrine system and releases hormones in response to stress (Haddad, 2004). Corticotropin releasing factor (CRF) is released by the hypothalamus and transported to the pituitary where the release of adrenocorticotropic hormone (ACTH) is stimulated. ACTH is then transported via the bloodstream to the adrenal cortex where it causes synthesis and release of glucocorticoids (cortisol in humans and corticosterone in rodents). The release of CRF is influenced by the sleep/wake cycle but also by different stressors, both psychological and physical (Nestler *et al.*, 2001). The locus coeruleus (LC)-noradrenaline (NA) system is also an important "control station" for stress. Release of NA in the brain is an immediate response to stress and prepares the body for the "fight or flight" response. It activates the HPA axis and also the amygdala, hippocampus and striatum. There are neural connections between the CRF and LC/NA system as CRF and NA stimulate each other via α1-receptor involvement (Tsigos & Chrousos, 2002).

There are two types of receptors that glucocorticoids (released in response to HPA axis activation) can bind to. Mineralocorticoid receptors (MR) have high affinity for the hormone and are saturated under normal conditions. Glucocorticoid receptors (GR) have low affinity and are only activated by high levels of the hormone, for example after stress. A decrease in self-administration of ethanol and reduction in dopamine levels in the NAc has been observed after removal of the adrenal gland in animal studies. The reduction is corticosterone-dependent because the effect can be reversed by replacement of corticosterone (Marinelli & Piazza, 2002). The involvement of GR has also been shown by administration of a GR antagonist which decreases dopamine levels in the NAc. It seems like glucocorticoids, via GRs, regulates dopamine levels in the NAc. The hormone is released by stress, and the mechanism may be a way to compensate for the aversive effects of stress (Marinelli & Piazza, 2002). A connection between different kinds of stress and self-administration of drugs has been shown in several experiments

and administration of glucocorticoids increases self-administration of amphetamine. It has also been observed that the density of GRs in the hippocampus decreases after long-lasting high levels of corticosterone. This seems to disrupt the negative feedback loop that controls secretion of glucocorticoids. Altogether, chronic stress appears to sensitize the reward system which may make the individual more responsive to drugs and thus more vulnerable to develop addiction (Piazza & Le Moal, 1998).

2.7 Brain Areas

Alcohol and other drugs are thought to stimulate the brain reward pathway through activation of the dopaminergic system. Brain areas like nucleus accumbens (NAc) (in ventral striatum), VTA and substantia nigra (in midbrain) are central in reward and have been extensively studied in the alcohol and drug dependence field. Dopaminergic neurons originate from VTA and substantia nigra and terminate in the ventral striatum, especially NAc (Figure 1), known as the reward pathway (Bowirrat & Oscar-Berman, 2005). This study is focused on three areas that connect to and interact with the core regions of the reward pathway. The hippocampus plays a key role in memory, learning and processing of information from novel environments. The amygdala is a part of the limbic system, so emotions and learning are among the functions it is involved in. The prefrontal cortex (PFC) is very important in motivation, working memory and has an executive regulatory role over behavior. All these areas also project extensively to the striatum through glutamatergic transmission (Kelley, 2004). The PFC and hippocampus can together be considered as a memory system that combines information and enables working memory. Amygdala is also important in memory but has its main function in storage of emotional memories. It has been shown that different kinds of stress impair memory formation by the HC-PFC "system" but enhance processing in amygdala (Diamond *et al* 2004).

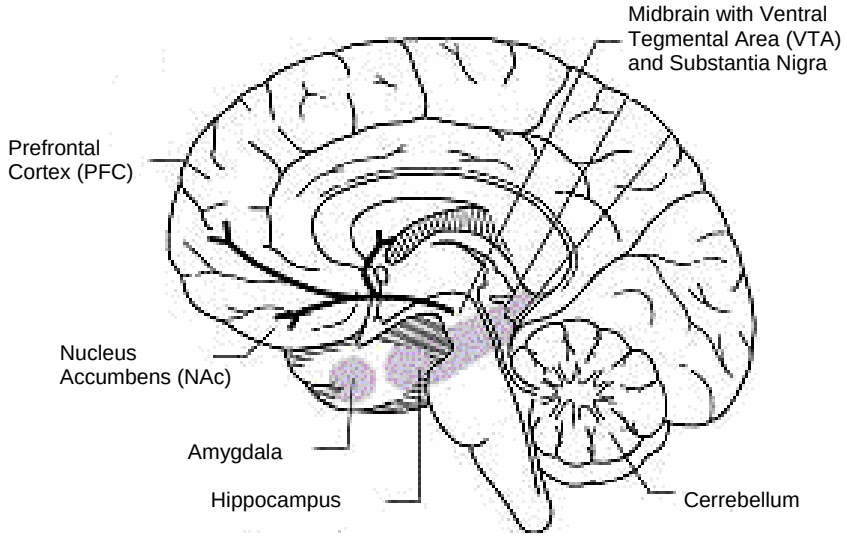

Figure 1. The figure shows the major brain areas involved in reward. In this study mRNA from PFC, Amygdala and Hippocampus was isolated and quantified. *Picture from NIDA notes (NIDA, 2005).*

The PFC is found in the cortical regions of the frontal lobe. It is difficult to understand the exact functional role of the PFC because it is not directly connected to any sensory or

motor neurons. But by studying the information that reach and leave the PFC some conclusions can be drawn. Cognitive and motivational/emotional processes that cause complex behavior (like decision making) seem to be highly influenced by the PFC (Groenewegen & Uylings, 2000). The PFC also interacts with the autonomic nervous system and is involved in regulation of heart rate, blood pressure, respiration, gastric motility and secretion and neuroendocrine response (Van Eden, 2000). There are incoming and outgoing projections to and from the PFC so it is a region that can integrate information from many sensory modalities and carry out executive control over striatal brain systems involved in motor response, behavioral initiation, cognitive and autonomic functions (Groenwegen & Uylings, 2000).

2.8 Real-time RT-PCR

Real-time reverse transcriptase polymerase chain reaction (RT-PCR) was developed in the 1990s and has become an important tool in nucleic acid quantification (Wilhelm & Pingoud, 2003). The principle of this technique is to detect PCR product as they accumulate at every cycle. This is done by measuring of fluorescence signals that are proportional to the amount of PCR product in the sample. There are two main categories of fluorescence reporters; fluorescent dyes that binds to double stranded DNA and sequence-specific fluorescent probes (Bustin, 2000). SYBR-green 1 is the most commonly used dye. It binds to the minor groove of dsDNA independent of sequence. The fluorescence increases over 1000 times when SYBR-green 1 is bound to dsDNA compared to free dye. During annealing and polymerization, more and more dye can bind to the newly synthesized DNA which results in light emission. The fluorescence is measured after each cycle and the increase can be monitored in real-time. The advantage of dsDNA binding dyes is that they can be used to detect any PCR product. But the problem is that non-specific products such as primer dimers also result in signals. It is therefore very important to design good primers. There are several different categories of fluorescence probes but they will not be discussed here.

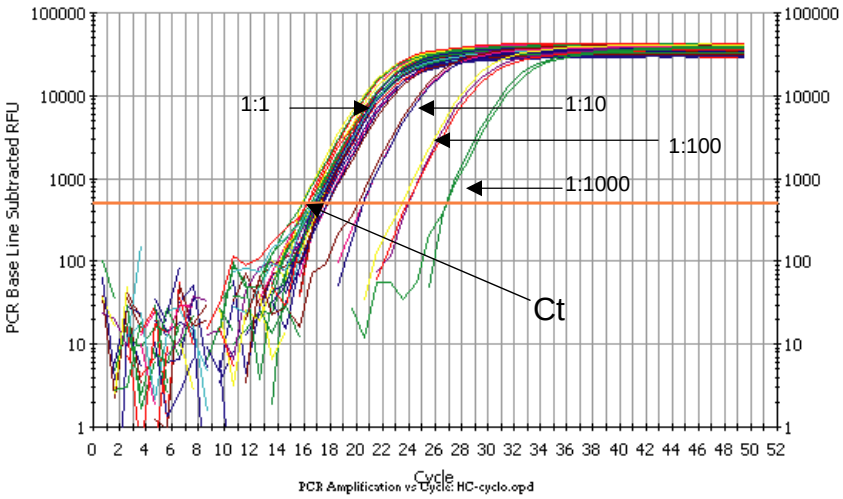

Figure 2. Example of amplification curves. The lower concentrations of the standard curve (1:10, 1:100, 1:1000) can easily be distinguished from the other samples. The 1:1 standard is hidden among the samples. Threshold is set to RFU=500 in all experiments in this study. This is cyclophilin run in the hippocampus.

The amplification of PCR product is visualized as a curve for each sample where fluorescence signal (Relative fluorescence units (RFU)) is plotted versus cycle number (Figure 2). Each curve can be divided into three phases. The initial lag phase is also called baseline and no change in signal can be detected. Eventually, enough products are present to generate a signal and an exponential phase follows. When reaction components become limiting, the amplification rate decreases and a plateau in fluorescence is reached. The threshold cycle (Ct) is defined as the cycle number when fluorescence from a sample passes a fixed threshold. This threshold should be set above baseline and where the curves are in the exponential phase. The more target DNA present in the sample at the start, the sooner Ct will be reached. By creating a standard curve from known concentrations, it is possible to determine starting copy number in a sample from Ct (Figure 3).

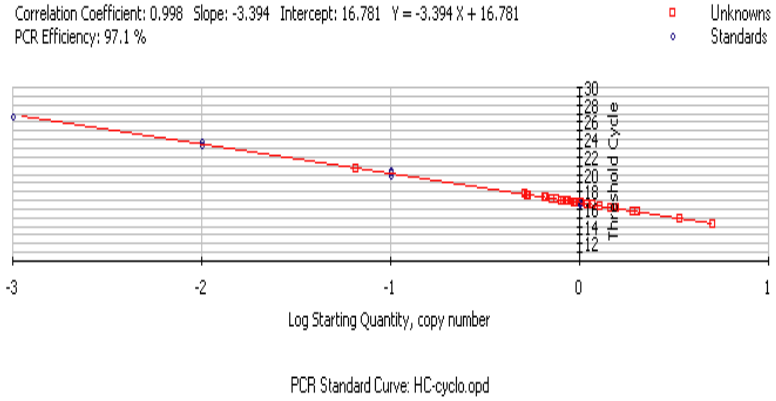

Figure 3. The standard curve is created from samples with known concentrations (1:1, 1:10, 1:100, 1:1000)

A melting curve analysis is also performed to verify that only the desired product is present (Figure 4). Products of different length and GC content melt at different temperatures. After the last PCR cycle, the temperature is raised and the change in fluorescence signal due to melting of product is plotted against temperature. More than one peak indicates multiple products. The most commonly used instruments for real-time PCR are based on a 96-well blockcycler with a fluorimeter device (Wilhelm & Pingoud, 2003).

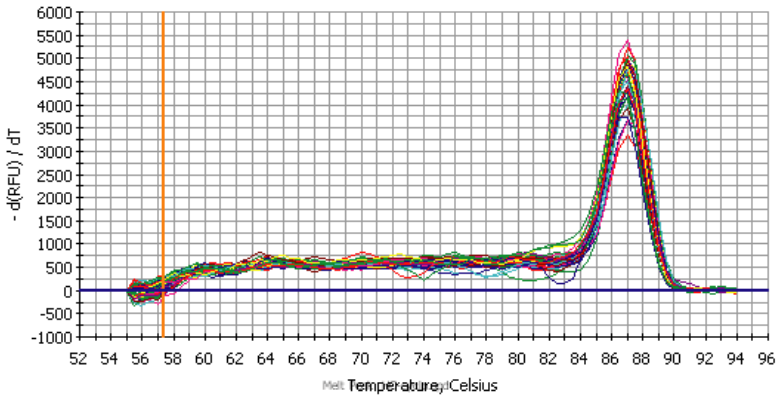

Figure 4. Example of melting curve. Only the desired product is present. Primer dimers or other secondary products usually melt at a lower temperature.

There are several other techniques that are used for detection and quantification of mRNA. Northern blotting is a method that gives information about the size of the mRNA, splicing and processing (Bustin, 2000). RNase protection assays are more sensitive than

northern blots and up to 10-12 different mRNA can be detected in the same sample. But RT-PCR can detect as few as 50-100 copies of mRNA, while northern and RNAse protection assay require 10^6 and 10^5 copies, respectively. (Rottman, 2002). The major advantage of *In Situ* hybridization is that the method gives the localization of the mRNA in a tissue, but it does not say much about quantity. cDNA microarrays is another, rather new, technique used to study the expression of a large number of genes in a tissue. Microarray data is often very large-scale which can make it difficult to sort out what is relevant. The method also has limitations in sensitivity, especially in studies of the nervous system, where tissues are complex with many different types of cells (Karsten & Geschwind, 2002). RT-PCR is superior in sensitivity to all other methods and with real-time monitoring the problems with quantification can be solved (Bustin, 2000). But there are problems with this technique that need to be considered, normalization being the most important one. First, it is important to make sure that the size of different samples matches, RNA is of good quality and the same amount of RNA is used for reverse transcription. More difficult is the internal normalization where the choice and validation of housekeeping genes are crucial (Hugget *et al.*, 2005).

2.9 Housekeeping genes

Expression of internal control genes, also called housekeeping genes, was used to normalize the expression of our genes of interest. A normalization factor (NF) is the geometric mean of the expression levels of the housekeeping genes in a sample. Expression of housekeeping genes should not vary in the tissue and should not change in response to treatment. This is, of course, not true for any genes. It is therefore important to evaluate the housekeeping genes for every region to obtain reliable normalization factors. This was done by using a method based on the principle that the expression ratio between two housekeeping genes should be the same in all samples. The pairwise variation in expression ratio was calculated between all internal control genes. The gene-stability measure (M) is the average pairwise variation between the gene and all other control genes. Seven housekeeping genes were used for every brain area in this study and then validated using GeNorm. GeNorm is a Visual Basic Application for Microsoft Excel that calculates M for all housekeeping genes. The gene with the highest M (least stable expression) is then removed and M for the remaining genes is recalculated. This procedure is then repeated and the most unstable control genes are stepwise excluded. The minimal use of the three most stable control genes are recommended for calculating NF. One control gene at the time is then included and new NF's are calculated. To determine if one more housekeeping gene should be included, the pair wise variation (V) between NF with and without that gene is calculated. A Low V value means that inclusion of that gene has no significant effect. It is then possible to determine how many housekeeping genes are necessary to obtain reliable normalization factors and which genes to use (Vandesompele *et al.*, 2002).

3 Method

3.1 Training

All training and collection of behavioral data was performed by Chris Pickering without participation by the author. It is still described here as it is essential for interpretation of the results.
All experiments were approved by the Ethical Committee for Use of Animal Subjects at Karolinska Institutet. Animal care procedures followed the guidelines of Swedish legislation on animal experimentation (Animal Welfare Act SFS1998:56) and EU legislation (Convention ETS123 and Directive 86/609/EEC). 40 male Wistar rats were used in the study and their weight at the start of the experiment was 250 g. The animals came from Scanbur/B&K (Sollentuna, Sweden) and the experiments were performed at Karolinska Institutet. Four rats were housed in every cage and they were allowed to acclimatize for one week after transport from Scanbur/B&K. The temperature in the animal room was controlled to 22°C and the humidity to 50%, and rats received as much standard lab chow as they wished. The light cycle was 12 hours and lights were turned on at 07.00. The self-administration sessions were performed between 09.00 and 12.00. This means that rats were trained during the light period, the time of the day when they normally are less active.
The self-administration experiments were performed in MED-PC operant chambers (Med Associates Inc., VT, USA) connected to a computer that recorded lever pressing and number of deliveries. The operant chamber is placed in closed and soundproof box during the experiments. This is to ensure that rats are not disturbed by the surrounding noise during training. On top of the chamber is a syringe in which the liquid was placed. It is connected to a syringe pump that delivers the fluid through a tube to a receptacle that is placed central on one of the walls in the chamber. The receptacle holds two cups, one on each side and two levers are placed to the left and right of the receptacle (Figure 5).

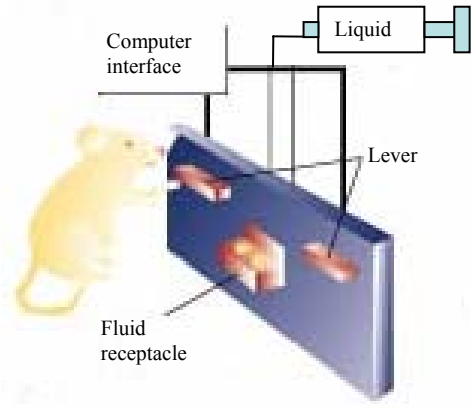

Figure 5. A picture describing the operant chambers. There are two levers that the rat can press. Pressing of the active lever (left) resulted in delivery of fluid into the left cup of the receptacle. Pressing of the inactive lever result in no delivery. The liquid that is delivered contain saccharin at the beginning of training and then saccharin and ethanol.

A cue light is placed above each lever and there is a house light on the opposite wall. If the active (left) lever is pressed, the pump is activated for 3 seconds and 0.1 ml liquid is delivered to the cup. If the inactive lever is pressed, no fluid is delivered. Every active lever press results in one delivery, except if the response is up to 10 seconds after pump activation, in that case no additional delivery is given but the response still recorded. The cue light over the lever is also activated upon lever press. Pressing of the inactive lever

also activates the cue light, so this does not help the rat localize the alcohol. The cue lights are there to remind the rat, especially early training, that something happens when a lever is pressed. Rats do receive one clue to which lever is the active one, a small amount of liquid is present in the left cup at the beginning of each session. This study is based on oral self-administration experiments with a total training period of 9 days. From 17.00 the day before the first day of training, rats were deprived of water to stimulate a motivation to drink. Rats were placed in the operant box for one hour on Day 1. To encourage rats to approach the delivery cup, free 0.2% saccharin deliveries were received every minute. Rats could also receive additional saccharin by pressing the active lever. Rats were then placed in home cage and approximately one hour after training water was returned. Rats were deprived of water in the same manner before Day 2 and 3 as before Day 1. From Day 2, rats were placed in the operant box for 30 minutes and no free deliveries were given. On Day 2 and 3, rats could now earn the same saccharin solution as earlier but only by pressing the active lever. The training continued in the same way on Day 4 to 6, but rats were no longer deprived of water prior to the session. On Day 7 to 9, the solution delivered upon lever pressing was exchanged for 5%(w/v) ethanol/0.2% saccharin. No more training was performed after Day 9. Rats were kept in their home cage for 20 more days and after that they were decapitated. The brains were dissected and regions of interest were stored in RNAlater solution (Ambion) at -20°C until RNA isolation.

3.2 RNA isolation

Tissue fixed in RNAlater was transferred to tubes containing 500-1000 µl TRIzol (Invitrogen), depending on tissue size, and kept on ice. Ultrasound was used to homogenize the tissue. The equipment was cleaned with 70% EtOH and RNase free water between every sample. Large pieces of tissue were shred with a syringe prior to homogenizing with ultrasound. Samples were then kept in room temperature for five minutes before 100 µl chloroform was added per 500 µl TRIzol to separate the organic phase from the aqueous. The tubes were manually inverted for 15 seconds and then centrifuged at 12 000 rpm for 15 minutes at 4°C. The aqueous phase was transferred to clean tubes and the RNA was precipitated with 250 µl isopropanol per 500 µl of previously added TRIzol. Samples were vortexed and kept on ice for at least 10 minutes and then centrifuged at 12 000 rpm for 10 minutes at 4°C. The supernatant was removed and the pellet washed in 500-1000 µl of 75% EtOH. Samples were centrifuged at 7 500 rpm for 5 minutes at 4°C and the EtOH was removed. The wash step was repeated and after the second wash the pellet was air dried at room temperature. The pellet was dissolved in 20-50 µl 1×DNase buffer depending on pellet size. Samples were stored at -80°C.

3.3 DNAse treatment and DNA contamination control

RNA samples were treated with DNase to remove all genomic DNA and avoid amplification in the cDNA synthesis. 1 µl DNase (Roche Diagnostics) was added to each sample and tubes were incubated at 37°C for 3 hours. For RNA samples from large tissue

pieces, an additional µl of DNase was added after 1.5 hours. The reaction was stopped by inactivation of the enzyme at 75°C for 15 minutes.
PCR was then used to confirm that the DNAse treatment was successful and DNA was absent from the samples. Reagents from Taq DNA Polymerase kit (Invitrogen) were used to prepare a mastermix with final concentrations of 1×PCR buffer, 1.5mM $MgCl_2$, 0.025 V% Tween, 0.2 mM dNTP mixture, 1µM primermix and 0.5 units *Taq* DNA polymerase. 5 V% RNA sample, positive or negative control was added last, the final reaction volume was 10 µl. The following PCR program was used, 94°C for 5 min, then 40 cycles with 94°C for 30 s, 62°C for 30 s and 72°C for 30 s. Then 72°C for 7 min. PCR products were then analyzed by electrophoresis on a 1.5% agarose gel. Samples were mixed and loaded with 6×DNA loading buffer and the gel was stained with ethidium bromide. The nucleic acid was visualized under UV light and photographed for documentation.

3.4 cDNA-synthesis

RNA concentrations were determined with Nanodrop 3000 (Nanodrop Technologies). 5-10 µl RNA was diluted to 12 µl in MQ water. A mastermix was prepared with the following final concentrations, 1×M-MLV RT reaction buffer, 0.5 mM dNTP, Random Hexamers as primer and 10 units/µl M-MLV Reverse Transcriptase (Amersham Biosciences). The reaction volume was 20 µl. Each sample was mixed and incubated at 37°C for 1 h followed by denaturation of the enzyme for 15 min at 95°C. A control PCR to assure that the cDNA synthesis was successful was performed in the same way as after DNase treatment.

3.5 Real-time RT PCR

Real-time PCR was run in 96-well plates. Two genes of interest were analyzed on each plate and the 16 samples were run in duplicates. For every gene a standard curve was created from a mixture of cDNA from four random samples. The standard curve was run in triplicates with the cDNA concentrations 1 ng/µl, 0.1 ng/µl, 0.01 ng/µl and 0.001 ng/µl. Each plate also included negative controls without cDNA (Figure 6). A mastermix was prepared with the final concentration: 1×PCR buffer, 4mM $MgCl_2$, 0.2 mM dNTP, 0.8 µM primer mix, 1ng/µl cDNA, SYBR-green and 0.02 units/µl *Taq* polymerase. Water was used as solvent and the reaction volume was 25 µl. An iCycler real-time detection instrument (Bio-Rad Laboratories) was used.

1	1	2	2	3	3	4	4	5	5	6	6
7	7	8	8	9	9	10	10	12	12	14	14
15	15	16	16	17	17	18	18	19	19	20	20
21	21	22	22	23	23	24	24	25	25	26	26
27	27	28	28	29	29	30	30	31	31	32	32
33	33	-	-	-	-	34	34	-	-	-	-
1	1	1	0.1	0.1	0.1	0.01	0.01	0.01	0.001	0.001	0.001
1	1	1	0.1	0.1	0.1	0.01	0.01	0.01	0.001	0.001	0.001

Figure 6. Plate setup. Two genes are analysed on each plate. The sexteen samples were run in duplicates for every gene. Standard curves and negative controls are also included.

3.6 Primers

Primers were designed with Beacon Designer 2.1 software (Premier Biosoft). This is a program that rates possible primer pairs according to product length, melting temperature and other characteristics such as primer dimer and hairpin formation. Primers used in this study are 18-22 nucleotides in length and have a melting point between 55°-60°C. The product length is from 70-100 bp. The primers were further analyzed with BLAST searches to confirm that they were unique to the gene of interest. All forward and reverse primers are listed in Table 2.

Table 2. Primers used in the study. The DNA sequences are in the 5' to 3' direction.

Transcript	Primer	Forward	Reverse
β-tubulin	β-tub	cggaaggaggcggagagc	agggtgcccatgccagagc
SDCA	SDCA	gggagtgccgtggtgtcattg	ttcgcccatagcccccagtag
Histone H3b	H3b	attcgcaagctccccttttcag	tggaagcgcaggtctgttttg
Ribosomal protein L19	RPL19	tcgccaatgccaactctcatc	agcccgggaatggacagtcac
Cyclophilin	Cyclo	gagcgttttgggtccaggaat	aatgcccgcaagtcaaacaaa
β-actin	β-act	cactgccgcatcctcttcct	aacgctcattgccgatagtg
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	acatgccgcctggagaaacct	gcccaggatgccctttagtgg
Gamma-aminobutyric acid A α receptor 1	GABAAα1	tgccagaaattccctcccaaag	cagagccgagaacacgaagg
Gamma-aminobutyric acid A α receptor 3	GABAAα3	tgctgagaccaagacctacaac	tggcaaagagcacagggaag
Gamma-aminobutyric acid A α receptor 5	GABAAα5	caagtctgtggtggtggc	tgctggtgctgatgttctc
Gamma-aminobutyric acid B α receptor 1	GABAB1	tgggctatggctctatgttcac	ggtcttcctccactccttcttc
N-methyl-D-aspartate receptor 2A	NR2A	cagcagcaagccacagttatg	agtctcggtagccagggaag
N-methyl-D-aspartate receptor 2B	NR2B	tgactggctacggctacac	ctctcactctggcaggaagg
α-amino-3-hydroxy-5-methyl-4-	GluR1	caaccaccgaggaaggatacc	ttcacagtcaaccaccaccag
isoxazolepropionic acid receptor 1,2,3	GluR2	ttgtgaggactaccgcagaag	ggactccagcaagtaggcatac
	GluR3	gttacaaatcacgggcagagtc	tggcaggagcaggcttaaag
Metabotropic glutamate receptor 1	mGluR1	gccaccacaccacctctg	tgacggaatcagccaggaac
Metabotropic glutamate receptor 2	mGluR2	ctcacgccacatctgtcctg	atcactgtaggagccaccaatg
Metabotropic glutamate receptor 3	mGluR3	atggtgtccgtgtggcttatc	tgactgtttcccgcttctctg
Metabotropic glutamate receptor 5	mGluR5	tgtccaccaccaaccaactg	gcctccactctctgaatccc
Noradrenaline receptor α1A	NEα1A	cagaaggcggcggagtca	gcgtcttggcagctttcttct
Noradrenaline receptor α1B	NEα1B	gcgcccgggcactttta	ctcccgcccctacgatggt
Noradrenaline receptor α2A	NEα2A	tggccctcgacgtgctcttt	cgatggcctgcgtgatgga

Noradrenaline receptor α2B	NEα2B	gacggcgcaacttccctcta	ggtgcccagcgtccctaca
Nicotinic Acetylcholine receptor α3	nAChRα3	gtctccctccctgtctatcg	cagcagcatcagcaccag
Nicotinic Acetylcholine receptor α4	nAChRα4	ctcctgtcctccacccaag	atgccatcttctgctgcttc
Nicotinic Acetylcholine receptor α6	nAChRα6	aggacacagggagcaacc	gcaagaatcagaccacgaaag
Nicotinic Acetylcholine receptor α7	nAChRα7	ctgctctacattggcttcc	aggtgctcatcatgtgttg
5-hydroxytryptamine receptor 1A	5-HT1A	ccgcacgcttccgaatcc	tgtccgttcaggctcttcttg
5-hydroxytryptamine receptor 1B	5-HT1B	caccttcttctggcgtcaag	accgtggagtagaccgttag
5-hydroxytryptamine receptor 2A	5-HT2A	aacggtccatccacagag	aacaggaagaacacgatgc
5-hydroxytryptamine receptor 2C	5-HT2C	ttggactgagggacgaagc	ggatgaagaatgccacgaagg
5-hydroxytryptamine receptor 3A	5-HT3A	caaggaagggtcaggatg	aaggaacagtgtggctctc
5-hydroxytryptamine receptor 6	5-HT6	gccgcatccactca	cctaccacctcctagtctcag
Dopamine receptor 1	DR1	cgggctgccagcggagag	tgcccaggagagtggacagg
Dopamine receptor 2	DR2	agacgatgagccgcagaaag	gcagccagcagatgatgaac
Glucocorticoid receptor	GR	accaacggaggcagtgtgaaa	ggggacccagcggaaaac
Mineralocorticoid receptor	MR	gacaattccaagcccgacacc	cttggcccacttcacgacctg
Corticotropin releasing factor receptor 1	CRF1	acttcgccagagcatctcag	gacacccaggcccactcacc
Corticotropin releasing factor receptor 2	CRF2	ccctccgagtgcctgtgg	gctgtctgcttgatgctgtgg

4 Results

4.1 Behavioral data

A great divergence in ethanol consumption was observed across the population. There were individuals that did not press the lever at all (no response), while others pressed up to 100 times or more during the 30 minute session (Figure 7). Eight high-responders and eight low responders were randomly selected for inclusion in the study.

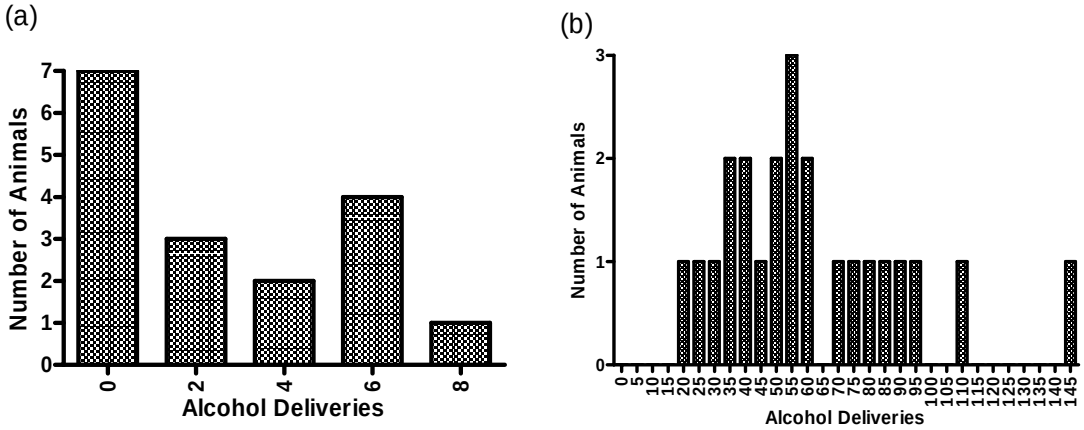

Figure 7. Alcohol consumption over the whole population. (a) Low or no responders with 0-8 deliveries. (b) High responders with 20-145 deliveries on average Day 8/9.

The numbers of liquid deliveries in the selected high consuming group over Day 5-9 are illustrated in Figure 8a. Figure 8b-d describes how rats responded (number of lever presses) during the 30 minute session on different days. Every point corresponds to the running total at that time while the point at 30 minutes is the total number of lever presses

during the session. The curves are nearly linear during the first 20 minutes which demonstrates that rats pressed the lever at a fairly constant rate. From 20 to 30 minutes the curves plateau or, in other words, rats stopped responding.

During training, rats had reached a fairly stable number of saccharin deliveries by Day 6. Figure 8b shows the difference in number of active lever presses between Day 6 (saccharin) and Day 7 (first day of alcohol). There was a significant decrease in active lever presses between the two days using 2-way repeated measure ANOVA (Main effect $F(1,15) = 5.26$; $p = 0.038$) and the difference was dependent on time (Interaction $F(13,195) = 4.99$; $p = 0.0002$). In other words, rats seemed to acknowledge that the solution contained something different and responded less. The difference between the two days was time dependent which means that the difference changed (increased) with time and rats stopped responding earlier for alcohol Day 7 compared to saccharin.

On Day 8, the second day of alcohol, rats earned significantly more alcohol deliveries than the day before (*) (Figure 8a). The response was back to the same level as before addition of alcohol.

There was a similarity between the response to saccharin and ethanol (Figure 8c). There was no significant difference in lever pressing for saccharin or alcohol using 2-way repeated measures ANOVA (Main effect $F(1,15) = 0.44$; $p = 0.52$). On Day 9, the third day of alcohol, there was a significant decrease in deliveries (#) (Figure 8c). An average of deliveries on Day 8 and Day 9 was used to approximate the amount rats would drink if they would have stable alcohol consumption and this was used in correlation analyses. There was a significant difference between number of active lever presses on the first day of alcohol and the average on Day 8/9 (Figure 8d) using 2-way repeated measures ANOVA (Main effect $F(1,15) = 7.11$; $p = 0.018$). The difference was time dependent (Interaction $F(13,195) = 8.57$; $p < 0.0001$)).

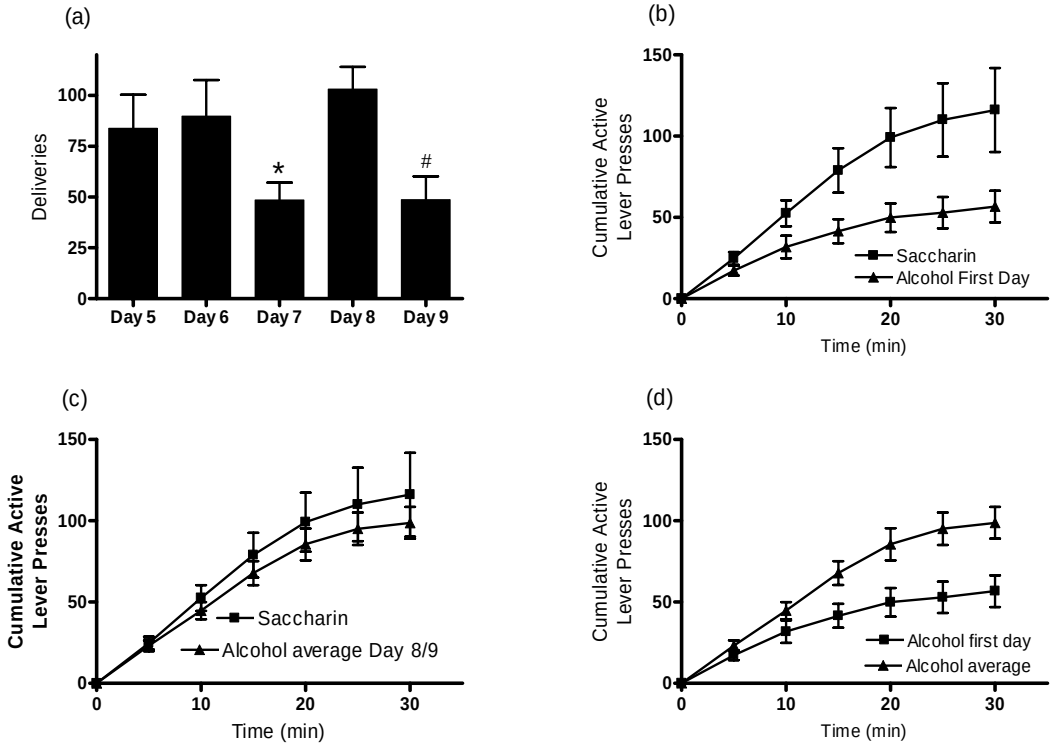

Figure 8
Illustration of behavioral data. Figure 8a shows the number of deliveries on day 5-9 in the selected high consuming group. On day 5 and 6 liquid contains saccharin and on day 7-8 the liquid contains saccharin and ethanol. Figure 8b-d compare lever pressing patterns between different days. 8b shows the decrease in lever pressing from day 6 (saccharin) and day 7 (first day with alcohol). 8c illustrate the similarity in lever pressing in response to saccharin and alcohol. Figure 8d shows the difference in lever pressing between the first day of alcohol and the average of day 8 and 9.

4.2 Validation of housekeeping genes

Seven housekeeping genes were run for the three regions studied. GeNorm was used to validate them and decide how many and which ones to use for calculation of normalization factors. We chose a combination of criteria when deciding which genes to use. A cutoff value for adding housekeeping genes was set to approximately 0.1 for the pairwise variation V. But more genes could be added if an even lower variation could be obtained.

4.2.1 Prefrontal Cortex

In the PFC, the inclusion of more than three housekeeping genes did not have a significant effect on the NF. We decided to use six housekeeping genes for calculations of expression levels in PFC (Figure 9).

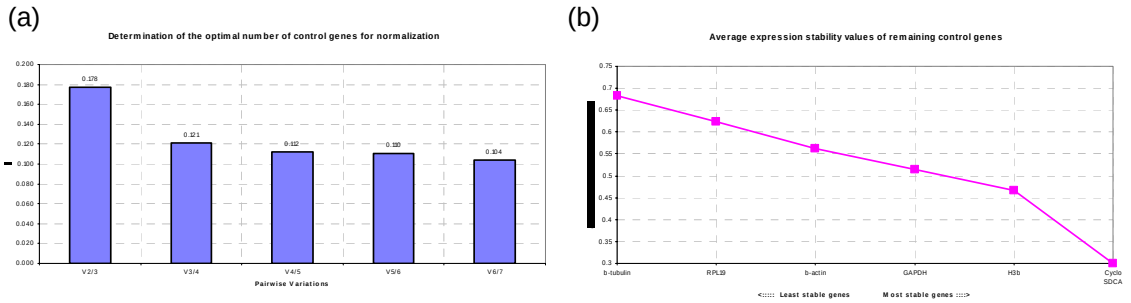

Figure 9. Determination of optimal number of housekeeping genes in PFC. (a) The pairwise variation V (b) Stability of the different housekeeping genes

4.2.2 Hippocampus

For the hippocampus, V is very close to 0.1 with only three control genes. Inclusion of a fourth brings V below cutoff while further inclusion increased variation (Figure 10).

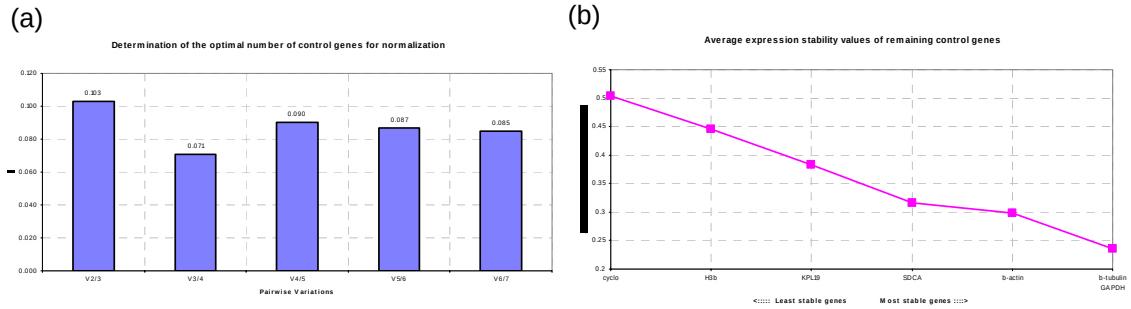

Figure 10. Determination of optimal number of housekeeping genes in hippocampus. (a) The pairwise variation V (b) Stability of the different housekeeping genes.

4.2.3 Amygdala

Three housekeeping genes are enough to get V below 0.1 in the amygdala. However, V decreases with the inclusion of up to six housekeeping genes so we decided to use six (Figure 11).

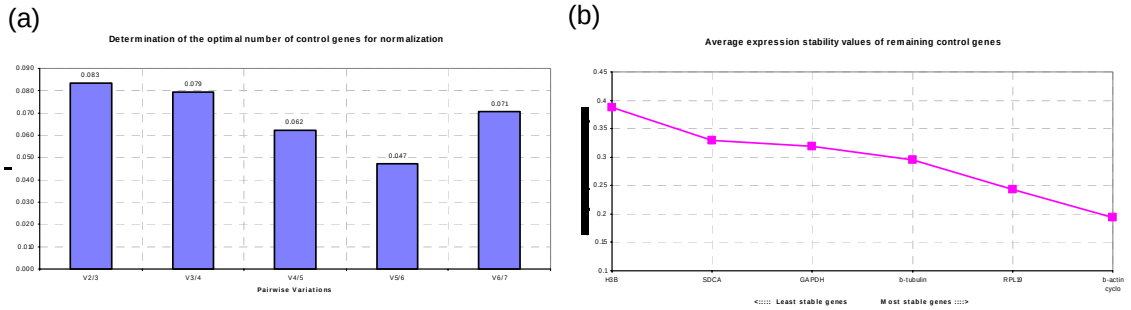

Figure 11. Determination of optimal number of housekeeping genes in the amygdala (a) The pairwise variation V (b) Stability of the different housekeeping genes.

4.3 Correlation analysis

Expression of receptor subunit RNA were correlated to the average number of alcohol deliveries on day 8 and 9 which was considered the most representative of how much alcohol a given individual would consume. Pearson correlation was used to compare RNA expression to behavior. The significance level was set to p=0.05.

4.3.1 Prefrontal Cortex

High consuming group

Expression of the GABAA α5 receptor subunit in PFC was found to be positively correlated to alcohol deliveries (Figure 12a) while α1 and α3 were not. GABAB1 was also significantly correlated (Figure 12b). GluR1 (Figure 12c) was correlated but no significant correlations were found among the other glutamate receptor subunits. All three adrenergic receptor subunits studied in PFC were significantly and positively correlated to the number of alcohol deliveries (Figure 12d-f). The nicotinic ACh receptor subunit α7 (Figure 12g) but not α4, was correlated in PFC. Three serotonin receptor subunits were studied. Expression of 5-HT3A was correlated to alcohol deliveries (Figure 12h) while 5-HT1A and 2A were not. Neither of the dopamine receptors D1 or D2 or the GR receptor was correlated to alcohol deliveries in PFC. Correlations for all genes studied in the PFC are collected in Table 3.

Table 3. Correlations between expression of receptor subunit mRNA and alcohol deliveries for all genes studied in the prefrontal cortex.

Primer	Pearson r	P value
GABAAα1	0.36	0.38
GABAAα3	0.32	0.44
GABAAα5	0.99***	<0.0001
GABAB1	0.90**	0.0059
NR2A	-0.50	0.21
NR2B	0.22	0.60
GluR1	0.86*	0.0128
GluR2	0.69	0.09
GluR3	0.64	0.12
NEα1A	0.89**	0.008
NEα1B	0.84*	0.018
NEα2A	0.96**	0.0007
nAChα4	0.17	0.68
nAChα7	0.77*	0.0042
5-HT1A	-0.11	0.79
5-HT2A	0.54	0.17
5-HT3A	0.88**	0.0086
DA-D1	0.31	0.46
DA-D2	0.33	0.42
GR	0.12	0.77

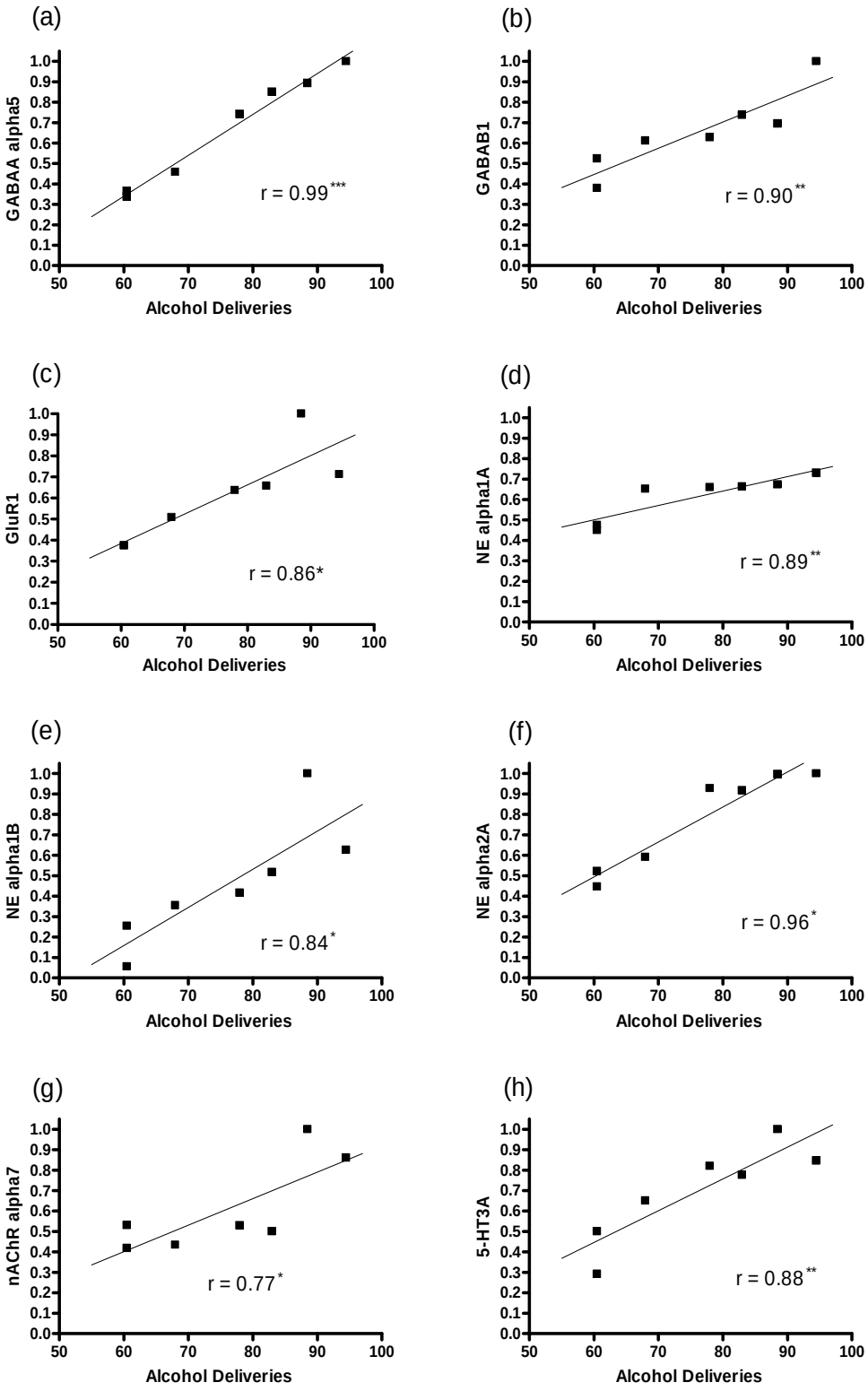

Figure 12. Significant correlations in the PFC. Relative expression levels of neurotransmitter receptors are normalized so that highest expression = 1 and plotted against number of alcohol deliveries.

4.3.2 Hippocampus

High consuming group

None of the GABAA receptor subunits α1, α3 and α5 expression levels in the hippocampus were correlated to the number of alcohol deliveries. No correlation was found among the glutamate receptor subunits NR2A, NR2B and GluR2 either. Three adrenergic receptor subtypes were studied in the hippocampus; α1A was negatively correlated (Figure 13b) while α2A and α2B showed no correlation. The α4 and α7 subunits of the nicotinic Ach receptor did not correlate. Five serotonin receptor subunits were studied, 5-HT1A, 1B, 2A, 2C and 3A but no correlation was found. Expression of GR was negatively correlated to alcohol deliveries (Figure 13a) but no correlation was found for MR. Correlations for all genes in the hippocampus are collected in Table 4.

Table 4. Correlations between expression of receptor subunit mRNA and alcohol deliveries for all genes studied in the hippocampus.

Primer	Pearson r	P value
GABAAα1	0.06	0.88
GABAAα3	0.12	0.78
GABAAα5	-0.04	0.93
GABAB1	0.31	0.46
NR2A	-0.09	0.84
NR2B	0.40	0.33
GluR1	0.025	0.95
GluR2	-0.05	0.90
GluR3	-0.39	0.34
mGluR1	0.26	0.54
mGluR3	-0.14	0.74
mGluR5	0.22	0.60
NEα1A	-0.79*	0.03
NEα2A	-0.42	0.30
NEα2B	0.39	0.33
nAChα4	-0.27	0.51
nAChα7	0.06	0.89
GR	-0.85*	0.016
MR	0.09	0.83

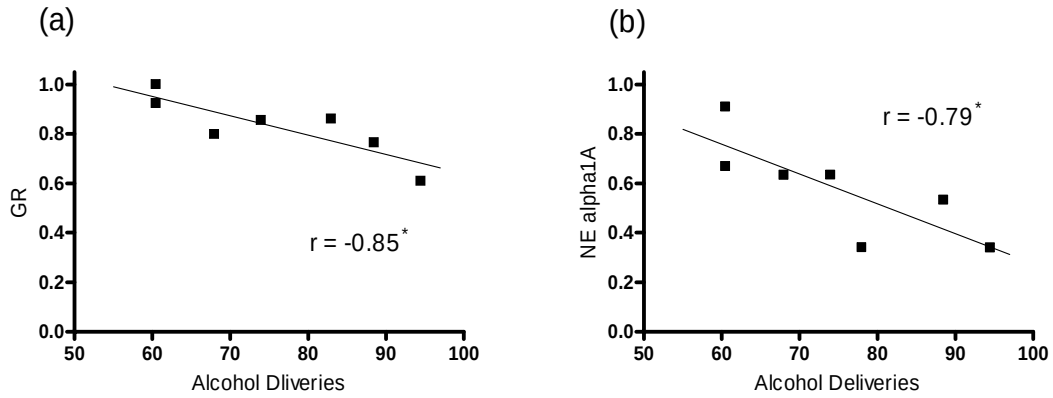

Figure13. Significant correlations in the hippocampus. Relative expression levels of neurotransmitter receptors are normalized so that highest expression = 1 and plotted against number of alcohol deliveries.

4.3.3 Amygdala

High consuming group

In the amygdala, one correlation was found among the glutamate receptor subunits. NR2A correlated negatively with number of alcohol deliveries (Figure 14) while NR2B, mGluR3 and mGluR5 did not correlate. The NA α1A subunit did not correlate in amygdala. No correlation was found among any of the three serotonin receptor subunits, 5-HT1A, 2C and 3A that were studied in this structure. Expression of GR, CRFR1 and CRFR2 was not correlated with alcohol deliveries. Correlations for all genes in amygdala are collected in Table 5.

Table 5. Correlations between expression of receptor subunit mRNA and alcohol deliveries for all genes studied in the amygdala.

Primer	Pearson r	P value
NR2A	-0.79*	0.035
NR2B	-0.20	0.64
GluR1	-0.42	0.30
GluR3	-0.28	0.30
mGluR1	0.42	0.31
mGluR3	-0.09	0.83
mGluR5	0.39	0.33
NEα1A	0.26	0.58
5-HT1A	0.43	0.29
5-HT2C	0.55	0.15
5-HT3A	0.11	0.80
GR	0.32	0.44
CRFR1	0.04	0.93
CRFR2	0.61	0.11

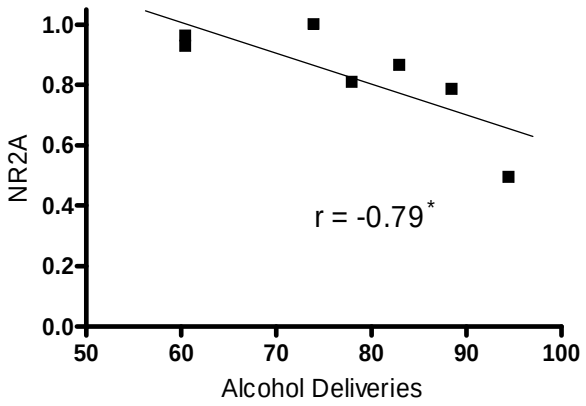

Figure 14. Significant correlations in the amygdala. Relative expression levels of neurotransmitter receptor mRNA are normalized so that highest expression = 1 and plotted against number of alcohol deliveries.

4.3.4 Low consuming group

mRNA expression of our genes of interest in this study were very diverge within the low consuming group (data not shown). The expression levels did not fit in with any of the correlations with alcohol deliveries found in the high consuming group. It is possible however, that other behavioral data collected during the experiments (not analyzed here), could be of interest when studying the low consuming group.

5 Discussion

We found that there was a high divergence with respect to ethanol consumption in a naive population of 40 outbred wistar rats. Rats self-administered ethanol over a range of 0-145 deliveries during the 30 minute session so it was clear that individual differences in behavior occur. A number of correlations were found between self-administration of ethanol and expression of mRNA, especially in the prefrontal cortex. In the PFC, we observed correlations in almost all neurotransmitter systems which follows the previous observation that ethanol affects many targets through many different mechanisms, rather than via action at a single receptor.

The correlation of gene expression in the PFC should not be so surprising considering its important role in the CNS. The PFC, for example, plays a role in learning and decision making and both should be involved when a rat chooses whether to press the lever again or not. It has been shown that the PFC is activated especially when a new task is learned (De Bruin, 2000) and our training method involves initial learning in an unfamiliar environment. Dysfunction of the PFC is observed in many psychiatric conditions like depression, schizophrenia (Groenewegen & Uylings, 2000) and ADHD (Sullivan & Brake, 2003). So it seems natural to include addiction in this list.

It is interesting that we found correlations between three out of four adrenergic receptor subtypes in PFC. NA is, among other things, involved in the body's immediate response to stress (Feenstra, 2000). An involvement of stress could also be suggested by the negative correlation we found between the glucocorticoid receptor and ethanol consumption in the hippocampus. Previous studies have proposed that increased levels of glucocorticoid hormones in stressed subjects enhance the drug-induced release of dopamine in NAc and thus increase the response to the drug. It is also observed that those individuals have a lower number of glucocorticoid receptors in hippocampus. The decrease in receptors could be due to a feedback loop that changes receptor number in response to glucocorticoid levels (Piazza & Le Moal, 1998).

The GABA system is classically regarded as one of the main targets of ethanol and the correlation between high expression of the GABAA α5 subunit and high alcohol consumption is consistent with data from other studies. For example has a recent study showed that administration of the α5 selective inverse agonist RY024 reduces self administration of ethanol in rats (McKay *et al.*, 2004). Thus, rats with a higher number of receptors with α5 subunits may respond more to ethanol.

The original goal of this experiment was to compare mRNA expression levels between eight high and eight low consumers as groups. But it soon became evident that the low consumers had very scattered expression levels while the high group was more homogenous. The low group had a very small range in the number of alcohol deliveries and, as such, correlation is not possible when one out of two variables does not vary. The great differences in expression levels in the low group despite their similar behavior (deliveries) can have several explanations. Low consuming rats may just have been slow learners and may not have learned to press the lever over such a short training period. They could also have been oversensitive to ethanol perhaps due to differences in liver enzymes. These rats that consume such low levels of ethanol probably do not feel any pharmacological effect so it is doubtful if any ethanol-related genotype can be

distinguished. It is also possible that mRNA expression within the low consuming group could be correlated to some other behavioral data that was collected during the study. Because we observed several significant correlations within the high group only, we decided to change our approach and only study those eight individuals.

The choice to use the average deliveries between the second and third day of alcohol is based on unpublished results from a previous study. Rats decreased their response on the first day of alcohol and this corresponds with the idea that alcohol is aversive to the rat at first (McGregor & Gallate, 2004). The response is then unstable for a couple of days so it is hard to determine what numbers are representative for the individual. In the previous study, rats were trained in the same manner as in this study but the self-administration sessions continued for 30 days. It was observed that the number of alcohol deliveries leveled out with time to approximately the average of the second and third day of ethanol. Since we wished to keep the ethanol effect on mRNA expression to a minimum, the three day ethanol training period was used and the consumption was approximated as best as possible.

The method is very promising because of its use of easily obtained outbred rats and its novel approach from the individual vulnerability view. But lessons can be learned from this study and things can be improved in the future. It is especially important to better distinguish response to the drug of interest and to reward in general as the number of saccharin deliveries correlated with the number of ethanol deliveries. In future studies more animals could also be included, since some of the correlations were rather strong but not significant (e.g. PFC NR2A in Table 3).

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