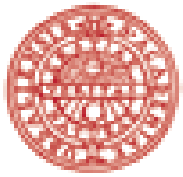

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Identification and characterization of genes that are involved in reward mechanisms in ethanol self-administration

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Identifiering och karakterisering av gener som är inblandade i belöningsmekanismer vid självadministrering av etanol

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Abstract

Alcohol addiction is one of the most common drug addictions amongst societies. Alcohol abuse, can lead serious injuries, severe diseases or even death. Despite intensive research, complete knowledge about alcohol addiction and a specific “ethanol receptor” has not been found yet. However, persistent researchers discovered that ethanol consumption and abuse is associated to the brain reward pathway and acts on the receptors of the system to elicit addiction. This project aimed to identify and characterize genes involved in the vulnerability for developing alcohol addiction. mRNA expression levels of neurotransmitter receptors were correlated to ethanol self administration in 11 rat brains. Measurement of the neurotransmitter receptor’s mRNA expression was carried out by using quantitative real-time PCR. The tested brain areas were the ventral tegmental area (VTA), nucleus accumbens (NAc), substantial nigra (Sn) and the caudate putamen (CPu). These areas have important roles in the reward pathway and contain the mesocorticolimbic dopamine pathway, which is believed to be behind the reward mechanisms. We found that the iontropic glutamate AMPA receptor subtype 3 (GluR3) in VTA correlated with ethanol self-administration. Two additional correlations were found in the NAc; the glutamate AMPA receptor subtype 3 (GluR3) and the dopamine receptor subtype 2 (DR2). Glutamatergic and dopaminergic receptors in VTA and NAc have been pointed out to be important in alcohol abuse, addiction behavior and reward. The correlated results showed a collaboration between the glutamatergic and the dopaminergic system in the VTA and NAc, which actuate alcohol addiction and behavior.

Sammanfattning

Alkoholberoende är ett av de vanligaste drogberoendena hos olika samhällen. Alkoholmissbruk kan leda till skador, allvarliga sjukdomar och till och med dödlig utgång. Trots intensiv forskning, är kunskapen om alkoholmissbruk ofullständig och en särskild "etanol receptor" har ännu inte hittas. Men ihärdiga forskare har funnit att etanol konsumtion och missbruk är kopplade till hjärnans belöningssystem och verkar på systemets receptorer för att framkalla beroende.
Med detta projekt försökte vi att identifiera och karaktärisera gener som är involverade i benägenheten för att utveckla alkoholism. Genuttrycket av de neurotransmittoriska receptorerna korrelerades med etanol självadministrering hos 11 råttor. Uttrycket av receptorerna mättes kvantitativt med realtids-PCR. De testades hjärnområden var det ventrala tegmentområdet (VTA), accumbenskärnan (NAc), substantia nigra (Sn) och caudate putamen (CPu). Dessa områden har viktiga roller i belöningssystemet och där ligger även den mesokortikolimbiska dopamin systemet som anses vara själva belöningsmekanismen. Vi fann att nivå av den ionotrop glutamat AMPA receptor subtyp 3 (GluR3) i VTA korrelerade till etanol självadministrering. I NAc fann vi även korrelation med GluR3 och dopamin receptor subtyp 2 (DR2). Glutamat och dopamin receptorer i VTA och NAc är utpekade som viktiga i alkoholmissbruk, beroende och i belöningssystemet. De korrelerande resultaten indikerar en samverkan mellan glutamat och dopamin systemet, som i sin tur påverkar alkoholberoendet och beteende.

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Introduction

Alcohol consumption and addiction

Alcohol is a substance that is defined as a drug, which can lead to abuse and addiction. Drug addiction can be defined as a disease of the brain reward system (Vetulani 2001). The brain reward pathway is affected by several different groups of substances which can produce addiction, and in humans addiction is considered as a chronic disease, which is characterized by the absolute dominance of drug seeking behavior. Other behaviors are inhibited by the urgency to receive the drugs (Vetulani 2001).

Two theories have been developed to explain the transition from drug use to addiction and these are the drug centered vision and the individual vision (Piazza and Le Moal 1998). The drug centered vision theory explains drug addiction as a disease resulting from the changes induced in brain function arising from repeated drug use. Chronic drug use induces mechanisms like tolerance and sensitization, which are defined as a dependent state. This is responsible for the change from drug use to abuse.
The other theory concerning the development of an addiction is the individual centered vision, which explains the cause of drug addiction as a preexisting pathological condition triggered by drug use. Certain individuals with a vulnerable biological background are easily generating a pathological response to drugs, which render them at greater risk to develop addiction. Stress is one of the biological factors that play an important role in the development of drug addiction (Piazza and Le Moal 1998).

The World Health Organization has set up a classification system for different diagnosis which is an accepted method to classify diseases. The definition of syndromes of dependency can be found in the 10th revision of the International Classification of Diseases and Health Problems (ICD-10). The ICD-10 definition is applicable for a specific psychoactive substance like alcohol or tobacco or for a wider range of pharmacologically different psychoactive substances. To identify the psychoactive substance a wide range of sources of information should be used as much as possible. This information is including self-report data, blood and body fluid analysis, different physical and psychological symptoms, clinical signs and behavior and evidence of patient's possession of drug, or information from a third part. To diagnose a dependency of alcohol three or more of the criteria have to be fulfilled at the same time during the previous year. Repeated alcohol using develops these behavioral, cognitive and psychological phenomena (WHO 1992):

- Strong or compulsive desire to consume alcohol
- Difficulties in controlling its use
- Obstinate using the drugs despite physical or psychological consequences
- Neglect activities and obligations due to the alcohol use
- Tolerance
- Physical withdrawal state

Animal models of alcohol addiction and self administration

One of the most common disorders among humans is alcoholism. Alcoholism is a complex disorder, which makes it difficult to study. Studies on alcoholism in humans face many experimental and ethical difficulties. Therefore, scientists have developed different animal models, which mimic human conditions for investigating the disorder, where they can break down the complex disorder of alcoholism into its single phases and reveal the mechanisms behind the alcoholism (Tabakoff and Hoffman 2000).
In the early 1960s, scientists who studied alcohol consumption and addiction were using nonhuman primates in preclinical studies and found out that these nonhuman primates were willing to consume alcohol over longer periods. Based on these results alcohol addiction researchers got a method to investigate biological impacts of alcohol consumption (Clark and Polish 1960).Use of nonhuman primates as models turned out to be expensive and unethical. Therefore, these animals were discarded from such studies and the majority of studies instead began to using rodents, mostly mice or rats.
A good model should have the ability to simulate human alcohol dependence, reinforcement, and tolerance, reveal the mechanism of craving, and relapse. These simulations provide information about alcohol consumption, binge, intoxication, withdrawal, abstinence and relapse cycle on physiological, biochemical and molecular levels (Tabakoff and Hoffman 2000; McGregor and Gallate 2004).

One of the simplest animal models to investigate human alcohol consumption is the alcohol preference model, in which the animals are provided with a free choice between water or alcohol solutions. Food is also freely available. Studies made with the alcohol preference model found out that the animals prefer alcohol solutions at lower concentration (>6 % weight/volume), which have a sweeter taste, to alcohol solutions at higher concentration which rodents averse due to the taste. The investigators suggested according to these studies that animals prefer alcohol due to the taste rather than due to the stimulating effects on the central nervous system. Alcohol preference-models are not necessarily revealing addictive behaviors but show controlled alcohol consumption. The major factor that define alcohol dependence is the uncontrolled drinking. Therefore, alcohol preference models are not the best models to study alcohol dependence (Spanagel 2000). To get a deeper understanding, varieties of models have been developed and are commonly make use of rodents. Some of these models were compiled and discussed in the article “Rats on the grog: Novel pharmacotherapies for alcohol craving” by Iain McGregor and Jason Gallate from University of Sydney. The following table is an excerpt from this article, which illustrates the different animal models that are valuable in alcohol addiction research.

Table 1. Animal models used in alcohol addiction studies. The table is derived from MacGregor and Gallate (2004)

Model	Simulates	Description	Example reference
Forced consumption	Consumption	Alcohol as only solution: forced intake	(Blokland, Prickaerts et al. 1992)
Ethanol vapour chamber	Intoxication	Animal forcibly exposed to ethanol vapour to induce intoxication and dependence	(Rimondini, Arlinde et al. 2002)
Withdrawal induced anxiety	Withdrawal	Dependent animals withdrawn from alcohol and signs of anxiety monitored in specific models such as the elevated plus maze	(Gallate, Morley et al. 2003)
Free access	Consumption	Alcohol consumption under conditions of free access with choice of alcohol vs. water	(McGregor, Saharov et al. 1999)
Limited access	Consumption	Alcohol consumption under conditions where alcohol is only available for a limited time each day	(McGregor, Saharov et al. 1999)
Operant (fixed ratio)	Craving / Consumption	Alcohol consumption under conditions where alcohol is obtained by performing operant response (e.g., lever press)	(Thorsell, Rimondini et al. 2002)
Operant (progressive ratio)	Craving / Consumption	Alcohol consumption under conditions where alcohol is obtained by performing operant response where response requirement is more demanding over time	(Gallate and McGregor 1999)
Extinction	Craving	After a period of alcohol consumption under operant conditions, alcohol is no longer available, the persistence of responding in the face of extinction is measure	(Topple, Hunt et al. 1998)
Reinstatement	Relapse	After a period of operant access, responding extinguished. The ability of various stimuli (cues) or interventions (stress) to reinstate responding for alcohol is measured	(Le and Shaham 2002)
Alcohol deprivation effect	Relapse / Consumption	After a period of abstinence, renewed access to alcohol brings out over-consumption	(Spanagel 2003)

Physiological aspects based on alcohol consumption has also been investigated and models such as continuous intragastric administration of alcohol and nutritionally, with defined low fat liquid diet produce severe liver damage. Alcohol-induced cardiomyopathy model has been shown to induce disorders of the heart muscle (Lieber and DeCarli 1974; Patel, Why et al. 1997). Other approaches to animal models in the field of alcohol research are to use genetically modified animals to reveal if the genetic background has any impacts. Different lines of alcohol preferring and alcohol non-preferring strains for examples alcohol preferring (P), alcohol-non preferring (NP), high alcohol drinking (HAD1&HAD2), low alcohol drinking (LAD1&LAD2), Alko-alcohol (AA) and Alko non-alcohol (ANA) lines has been selected for behavioral studies. The out coming results showed that the genetic factors are important for alcohol consumption (Samson, Files et al. 1998).

Alcohol is reinforcing in humans and the best procedure for studying reinforcement is the self-administration model. The self-administration paradigm shows the amount of work the animal has performed to gain access to a given amount of drugs, which indicate the strength of reinforcement induced by drugs. Self-administration is a valid method, as it reflects the human condition since the animals have a voluntary consumption. The self-administration model can be performed in different ways; intravenous self-administration, intragastric self-administration or oral self-administration. Intravenous- and intragastric self-administration is performed by a insertion of a cannula into the vein,

respectively implanting a tube into the stomach, and in both cases alcohol can be delivered directly (K.A 1990).
This project used the operant oral self-administration method; the animals have to perform a task (lever press) to obtain alcohol solution within an operant chamber. A common problem is that the rodents do not directly self administrate alcohol due to the taste. To overcome this problem the taste of the alcohol solutions were masked with a sweetener. Once the self-administration is established, the effect of reinforcement is taking over.

Brain reward pathway and brain areas

Natural stimulants such as food, sex and social interactions produces reward and reinforcement in humans and in animals through the brain reward pathway which is essential for surviving. Rewards are experiences that have left a pleasure impression in the brain system that are appreciated and desired, hence a pursuance. When rewards like food, sex or drugs are utilized a state of pleasure is achieved, which initiates a learning process to enhance an affection to the reward, recognize cues of rewards and estimate the value and motivational status to the reward so the organism can determine how much efforts it is willing to perform in order to reach a specific goal or reward (Kelley and Berridge 2002; Berridge and Robinson 2003; Hyman, Malenka et al. 2006).
Beside natural stimulants, pharmacological agents like cocaine, amphetamine, opiates, nicotine, cannabinoids and ethyl alcohol are also potent activators of the brain reward pathway (Hyman, Malenka et al. 2006). When these stimulants like alcohol targets the brain reward pathway and activate it, dopamine is released from the dopaminergic neurons in the ventral tegmental area (VTA) of the midbrain into the limbic brain structures, especially to nucleus accumbens (NAc) (Nestler 2005).The dopamine release into NAc has an important role in the self-administration of ethanol and other drugs.
The ventral tegmental area and the NAc are the key areas in the brain reward pathway. Caudate nucleus and putamen parts of the striatum, amygdala, hippocampus, substantia nigra and prefrontal cortex are other brain areas that are involved in the brain reward pathway (Hyman, Malenka et al. 2006).

Areas that are in focus/focused on this project are the NAc, VTA, the substantia nigra (Sn) and the caudate putamen (CPu). The NAc is located both in the basal ganglia and the limbic system of the brain and can be divided into two major subunits, the shell and the core. The shell is considered being a part of the extended amygdala, which is a region of the limbic system. On the basal ganglia region, more specifically on the striatal complex site, lays the core of the NAc (Bowirrat and Oscar-Berman 2005). The NAc is participating in various functions such as the brain reward pathway, which includes sexual behavior, stress, reward and drug self-administration. Other functions of the NAc are the feeding mechanism, sensory motor gating and motivation. The two subunits of the NAc have different functional roles. The shell has functions such as processing response to emotions, involving in stimulus-reward associations whereas consumption of drugs of abuse triggers a dopamine release in the shell. The core has roles in drug-associated behaviors and in the integration of motor responses. Of the two subunits of the NAc, the

shell is suggested to have a more important role in the development of alcohol addiction because of a higher sensitivity to alcohol than the core (Bowirrat and Oscar-Berman 2005). The CPu is linked to the NAc because of the location in the striatum on the basal ganglia. The striatum is consisting of the ventral striatum whereas the NAc core is located and the dorsal striatum, where the CPu is located. Roles of the CPu are in reward-related motor learning, decision making, response selection, sensory processes and memory. The area is receiving dopamine inputs from the midbrain and glutamate inputs from the cortex and projects outputs to the cortex via the substantia nigra pars reticular (Wickens, Reynolds et al. 2003; Wickens, Budd et al. 2007). The VTA is located in the midbrain, medial to the Sn, ventral to the red nucleus and between the caudal hypothalamus and the brainstem reticular formation. The VTA consists of dopaminergic and GABAergic cells and is involved in processes of reinforcing of natural rewards, memory and motivation (Fields, Hjelmstad et al. 2007). One main function of VTA is the dopamine projection to amygdala, hippocampus, PFC and NAc, which together compose the mesocorticolimbic dopamine system. The VTA receive inputs from areas like the hypothalamic area and the pre frontal cortex (Korotkova, Sergeeva et al. 2003).

The origin of dopaminergic neurons is, besides the VTA, also the Sn. The Sn is located in the midbrain and containing a major part of the subcortical nuclei division in the basal ganglia, which are involved in functions like movement, memory and cognition.
The Sn is also connected to the striatum both functionally and anatomically. The Sn is divided into two units, the substantia nigra pars compacta that contains the dopaminergic neurons and the substantia nigra pars reticular, which is composed of GABAergic neurons. The dopaminergic containing part of Sn is also contributing to the mesocorticolimbic system by projecting dopamine to the striatum and NAc and getting inputs from GABAergic neurons from the forebrain (Bowirrat and Oscar-Berman 2005).

Neurotransmitter systems

When drugs of abuse are consumed and the active substances are entering the brain, circulation will transport the active substances to all regions in the brain. Different drugs have different specific receptors to act on, but ethanol has not any specific receptor to act on and seems to be interacting with all the different neurotransmitter systems. The mechanisms of ethanol on the neurotransmitter systems are still unrevealed.

Dopamine

Dopamine belongs to the catecholamine neurotransmitters family and is derived from a multi-step processing of tyrosine. First L-tyrosine is converted to L-dopa by the enzyme tyrosine hydroxylase and then the L-dopa is converted to dopamine by dopadecarboxylase. Dopamine is a neurotransmitter in the brain system and a precursor to the noradrenaline. It is produced by a small number of nuclei in the brain; the VTA, substantia nigra pars compacta and the arcuate nucleus of the mediobasal hypothalamus. Dopamine projections from the VTA to NAc, PFC and cingulate cortex constitute the mesocorticolimbic dopamine system and have functions in the reward pathway, cognitive

and emotions functions. Projections of dopamine from the substantia nigra pars compacta to CPU represent the nigrostriatal dopamine system, which is involved in the roles of the basal ganglia and voluntary movement. The tuberoinfundibular dopamine system is projections of dopamine from the hypothalamic arcuate nucleus to pituitary gland. The release of dopamine in this area regulates the secretion of prolaction (Nestler, Hyman et al. 2001; Nestler and Carlezon 2006). Dopaminergic receptors belong to the G-protein coupled receptor super family and can be divided into two groups, D1-like and D2-like receptors. The D1-like family is consisting of dopamine receptor 1 (D1) and dopamine receptor 5 (D5). D1 can be found in brain areas like the cerebral cortex, olfactory tubercle while NAc and D5 occur in hippocampus and hypothalamus. The second group of dopaminergic receptors is consisting of dopamine receptors 2, 3 and 4. D2 is presents in olfactory tubercle and NAc, D3 in NAc and the island of Calleja and D4 in the midbrain and amygdala. Studies have implicated the D2 receptor plays an important role in alcohol and drug addiction (Nestler, Hyman et al. 2001; Thanos, Katana et al. 2005).

Glutamate

The neurotransmitter glutamate is a non essential amino acid which has not the ability to cross the blood-brain barrier. Therefore, the neurotransmitter cannot be delivered to the brain by systemic circulation and has to be synthesized in the brain from glucose and other precursors. Glutamate plays a key role in most of the excitatory neurotransmission actions in the vertebrate brain and is involved in pathological conditions such as ischemia, epilepsy, brain damage and addiction (Nestler, Hyman et al. 2001). Ethanol has an effect on the glutamate transmission in the brain and this involvement in turn may affect other brain functions, such as memory and the interference can be an explanation to the “alcoholic blackout”, a condition associated with alcohol consumption. In brain areas like pre frontal cortex, ventral striatum and NAc, a relationship between glutamatergic and dopaminergic afferents have been observed and indicates that excitatory amino acids enhance the release of dopamine (Bowirrat and Oscar-Berman 2005).

The glutamate receptors family can be divided into two large families; the iontropic receptors family and the metabotropic receptors family. Iontropic receptors are ligand-gated ion channels and the macromolecular structure is consisting of a glutamate binding site and an associated ion channel. The iontropic receptors family is consisting of N-methyl-D-aspartate (NMDA) receptors, α-amino-3-hydroy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and kainate receptors. NMDA receptors are consisting of subunits, NR1 which has nine splice variants, NR2A-D and NR3. NMDA receptors are believed to have an important role in ethanol actions. The ethanol inhibits neuronal NMDA receptors in human and non-human primates. From behavioral and pharmacological studies the indication is that inhibition of NMDA receptors has a key role in acute intoxication. Chronic alcohol use upregulates NMDA receptor expression, and in brain reward areas chronic alcohol exposure is enhancing NMDA sensitivity (Zhou, Anthony et al. 2007).

AMPA receptors are consisting of four subunits, GluR 1-4 (the subunits could also be named as GluR A-D). The AMPA channels are permeable to Na^{2+}, K^{+}, Co^{+} and Ca^{2+}. However, influx of Ca^{2+} occurs only in striatum, hippocampus and the cerebellum. The permeability to different ions is decided by the composition of the receptors. AMPA receptors seem to have a role in alcohol seeking and relapse behavior, this assumption arrives from studies with AMPA receptor antagonists, like the antagonist GYKI52466, which reduces the ethanol-seeking behavior (Sanchis-Segura, Borchardt et al. 2006). Other studies have also shown that AMPA receptor increase in DA cells, especially the subunit GluR1 is increasing which enhances rewarding and motivational effects of drugs of abuse (Choi, Clements et al. 2005). Kainate receptors are consisting of subunits GluR5-6 and KA1-2 and kainate receptors have a high affinity to kainates.

The other large family of glutamate receptors is the metabotropic receptors family. This family has 8 receptors, mGluR1-8, which belong to the G-protein coupled receptor super family. The receptors can be further divided into three groups according to their sequence homology at the amino acids level, agonist pharmacology and which signal pathways the receptors are coupled to. The division is the following, group 1 (mGluR 1 and 5), group 2 (mGluR 2 and 3) and group 3 is consisting of mGluR 4, 5, 6, 7 and 8 (Nestler, Hyman et al. 2001; Choi, Clements et al. 2005).

GABA

The brain neurotransmitter γ–aminobutyric acid, GABA, is a major inhibitory neurotransmitter and this neurotransmitter is derived from the precursor glucose. Many neuronal disorders such as epilepsy, Huntington disease, tardive dyskinesia, sleep disorders and alcoholism have an altered GABA function and a degeneration of GABAergic neurons which reveal the involvement and the important of GABA in the brain. The type A of GABA receptors is believed to connect to the effects of ethanol in CNS (Nestler, Hyman et al. 2001; Davies 2003).

GABA receptors are divided into two main functional receptor-groups; the iontropic GABAA receptors and the G-protein coupled GABAB receptors. Accumulated evidence show that GABAA have central roles in the effects of ethanol in CNS. GABAA receptors belong to the iontropic family, which also includes the nicotinic acetylcholine, glycine and 5-HT3 receptors. GABAA receptors are mostly located in postsynaptic membranes and the receptors are pentameric with a GABA binding site coupled with a Cl^{-} ion channel. Once the postsynaptic cell is activated, chloride ions influx through the ion channel and moves the postsynaptic membrane potential away from the firing threshold, which is the mechanism that makes GABA classified as an inhibitory neurotransmitter (Nestler, Hyman et al. 2001). The receptors have 19 subunits and are further divided into 7 functionality divisions containing 6α, 4β, 3ρ, 2γ, 1δ, 1ε, 1θ and 1π subunits. Receptor subtype consisting of α1, β2 and γ2 subunits is most common in mammalian tissue and the most common subunit combination in CNS is 2α-2β-1γ. The GABAA subunits can by combinations from 2000 distinct GABAA receptors. These combinations of subunits resulting in different distinct receptors and the expression of subunits are interesting and important in understanding alcoholism whereas it is known that GABAA expression is

altered in alcoholism. Studies on GABAergic systems in ethanol consumption have shown that the systems are strongly connected with self-administration of ethanol and probably affecting the mesolimbic system by stimulating the reward pathway. Different compounds that activate or potentate GABAergic systems together with GABA have been correlated to increased ethanol intake, while compounds that inhibit or block the GABAergic systems decreased the ethanol intake. These compounds are benzodiazepines, barbiturates, neurosteroids and anesthetics. Benzodiazepines enhance the receptors inhibitory synaptic transmission by binding to the allosteric modulatory site on the GABAA, whereas the influx of chloride ions increase and hyperpolarizes the neuron. Ethanol is believed to have the same effects on GABAA receptors as benzodiazepines (Nestler, Hyman et al. 2001; Davies 2003). GABAA receptors with the subunit α5 have been shown in the study of identification of neurotransmitter receptors involved in self-administration of ethanol in rat PFC, hippocampus and amygdala to be strongly correlated with ethanol consumption. Pharmacological studies have also shown that the α5 subunit is correlated with alcohol consumption by using the α5-selective inverse agonist RY024 with results of decreased self-administration and blockage of alcohol-induced sedation and motor impairment (McKay, Foster et al. 2004). Further evidence that connect GABAergic system with alcohol disorders is long term ethanol exposure change GABAA receptor subunits mRNA and protein level (Davies 2003).

The metabotropic GABAB receptors are belonging to the G-protein coupled receptors family. The receptors are insensitive to GABAA receptors ligands, such as bicuculline. Instead baclofen is a potent agonist and the receptors are inhibited by the competitive antagonist phaclofen. Two major subunits have been cloned for GABAB receptors and these are the GABABR1A and GABABR1B. Another identified GABAB receptor is the novel GABABR2. GABAB receptors are expressed both on presynaptic and postsynaptic membranes and the receptors can open potassium ion channels, decrease $Ca2^{+}$ influx and inhibit adenylyl cyclase (Nestler, Hyman et al. 2001). GABAB receptors are believed to have a role in immoderate alcohol consumption. Studies with the GABAB agonist baclofen show that the compound has an anti craving effect and moderate alcohol abstinence (McGregor and Gallate 2004).

Nicotinic acetylcholine

One of the first identified neurotransmitters is acetylcholine (Ach), which is functioning at the neuromuscular junction and in the CNS. Ach acts both on the peripheral nervous system and the CNS on the muscarinic subtypes of acetylcholine receptor (mAChR). The mAChRs are G protein-coupled receptors, with five subtypes and are located in the peripheral tissues, the CNS and in the autonomic nervous system. These receptors have roles in mediating neuromuscular and autonomic transmission. The nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels and have 8 α-subunits (α2-α9) and 3 β-subunits (β2-β4). The receptor has a pentameric structure with nicotine as ligand whereas activation results in influx of Na^{+} and Ca^{2+} and the ability of Ca^{2+} influx is setting by the composition of the different subunits (Nestler, Hyman et al. 2001). Much of the nAChRs is activated by nicotine, but some of the subset of the receptors can

interact with other drugs. In vertebrates nAChRs are located in CNS, the autonomic nervous system, neuromuscular junctions and the adrenal medulla (Powledge 2004). The different subunits are expressed in different amounts in different brain areas; the α4 subunit is expressed in higher level in thalamus, in the deeper layer of cerebral cortex, VTA and Sn pars compacta, while the α7 subunit is more expressed in the hippocampal formation. nAChRs located in CNS activated by nicotine, produces effects on cognitive performance, vigilance, memory, learning processes, respiration, cortical blood flow and pain perception. These effects may also be explained by the fact that nAChRs activation by nicotine is modulating other neurotransmitter systems that in turn produces those effects, including increased emission of Ach, DA, NE, 5-HT, GABA and glutamate (Lloyd and Williams 2000). Different studies included electrophysiological, pharmacological and neurochemical methods have suggested that ethanol is interacting with nAChRs and these studies indicate ethanol and nicotine addiction is to be coupled, since 80-90% of alcoholics are also nicotine addicted (Larsson and Engel 2004). The hippocampus is known to be a brain area that is vulnerable to ethanol exposure and nAChRs in this area are involved in cognitive performance, memory and learning. These hippocampal functions are alternated during chronic alcohol consumption, and it is believed that these alterations are coupled with the interaction between ethanol and nAChRs. A study with moderate chronic ethanol consumption in rats during 10 weeks showed that the hippocampal subtypes of nAChRs, α7 and α4β2 decreased (Robles and Sabria 2008).

Real-time PCR analysis

The real time PCR technique was developed in the early 1990s by Higuchi et al., and makes it possible to monitor PCR products in real time during the amplification by measuring the fluorescence signal which increases in proportion to the produced amount of DNA in the reaction (Kubista, Andrade et al. 2006). The method is sensitive, has high sample throughput and high accuracy, and is more preferred over conventional end-point PCR methods. Real time PCR is an important tool in neuroscience, development biology and medical diagnostics, with approaches like confirmation of the gene expression pattern and comparison of mRNA levels in different samples (Bustin 2002; Ramakers, Ruijter et al. 2003).

Molecular biology has many different methods for detection and quantification of nucleic acids sequences. Methods beside the real time PCR for that purpose are Northern and Southern hybridization, RNAase protection assay, In situ hybridization and RNA microarray assays. In comparison to the real time PCR, these methods are more or less time consuming, costly, have lower sensitivity, specificity, dynamic range and less quantitative. These issues can be overcome by using the real time PCR. Disadvantages with the real time PCR are that the technique is vulnerable to contamination, which can derive from the different preparation steps before the quantitative real time PCR step. RNA used in this technique, which is far less stable than DNA, and the real time PCR results are affected by the quantity and quality of the RNA in the samples. The real time PCR is only quantifying the mRNA expression or rather transcript level, but does not

reflect the gene expression product without special design of primers (Valasek and Repa 2005).

Background of gene correlation of self-administration of alcohol consumption in the reward pathway.

Alcohol is consumed everywhere in the world at different occasions and mostly people can handle alcohol consumption at a moderate level. However, some times this moderate consumption of alcohol is transmitting into abuse. Many studies have been done on alcohol consumption and abuse, but the mechanism behind alcohol addiction is still unrevealed. In the study "Identification of neurotransmitter receptor genes involved in alcohol self-administration in the rat prefrontal cortex, hippocampus and amygdale", by Doctor Christopher Pickering et al., gene expression of neurotransmitter receptors from self-administrating rats in prefrontal cortex, hippocampus and amygdala which all are essential elements of the brain reward pathway, was analyzed. GABAAα5, GABAB1, GluR1, NEα1A, NEα1B, NEα2A, nAChα7 and 5-HT3A receptors in prefrontal cortex were found to correlate to self-administration of ethanol. For the hippocampus NEα1A and GR receptors were correlated and in amygdala only NR2A was correlated to ethanol self-administration. The method used in the study was using quantitative real-time PCR for quantification of mRNA expression of target receptors in brain areas that are believed to be involved in mechanisms of alcohol impact in human. The study indicates that it is possible to investigate different genes in different brain areas and that the genetic background of the individual is important in the beginning of addiction. It also shows that prefrontal cortex has an important role in that process. The results show that ethanol is acting on the brain reward pathway, since the brain areas investigated in this study are all involved in the reward pathway and especially the prefrontal cortex (Pickering, Avesson et al. 2007). This project is based on these correlations found, using the same method applied to other brain areas that have a role in the brain reward pathway.

Aim of this study

The general aim of this project was to identify genes or molecular substrates involved in the individual vulnerability for developing alcohol addiction. Focus will be on the neuronal pathways that are involved in the brain reward system and how the interaction of these regions mediates addictive behaviors.
This approach could identify novel targets beneficial for drug design to treat alcohol addiction.

Brain areas used in this project are the NAc, VTA, Sn and CPu taken from rats in self-administration of ethanol models. The aim was to investigate gene transcripts of genes associated with drug addiction in these areas in order identify which gene or genes that is or are activated in self-administration of ethanol individuals and therefore could reveal how alcohol addiction develops.

Another approach in this project was to apply the same method to investigate the expression of the G-coupled protein receptor 125 in ethanol self-administration rats and in particularly the prefrontal cortex.

Materials and Methods

Animal training

The animal training was performed by Dr Chris Pickering at Karolinska Institutet . The Ethnical Committee for Use of Animal Subject at Karolinska Institutet approved the animal experiments. The guidelines of Swedish legislation on animal experimentation (Animal welfare Act SFS1998:56) and EU legislation (Convention ETS123 and Directive 86/609/EEC) were the setup for the course of actions of the animal care. The animals used in the experiments were 40 male Winstar rats with a weight of 250 g in the beginning of the experiment. The animals were obtained from Scanbur/B&K (Sollentuna, Sweden). After delivery from Scanbur/B&K the animals were housed with 4 rats per cage during one week to allow acclimatizion to the new environment. The animals were kept in a climate-controlled animal facility with a temperature set to 22^{o}C, 50% humidity and 12h light and dark cycle with lights turned on at 07.00. All self-administration sessions were performed between 09.00 and 12.00. That time period is the time of day when the rats normally are less active.
MED-PC operant chambers (Med Associates Inc., VT, USA) were used for the self-administration training. The chamber consisted of a receptacle located centrally on one of the chamber's walls with two levers placed on each side. The receptacle contains two small cups, one left to collect the fluid when the left lever was pressed and one right to collect the fluid when the right lever was pressed. The fluids were delivered by a pump located on top of the chamber, through tubes connected to the receptacle. Above each lever a cue light was placed and when respective lever was pressed, the corresponding cue light was activated. In this animal experiment, the left lever was settled to be the

active lever and the right lever was to be the inactive one. When the active lever was pressed the pump was activated for 3 seconds and 0.1 ml liquid delivered to the left cup. If the inactive lever was pressed, no fluid was delivered. The cue light was activated either when the left or the right lever was pressed. The cue light was not a help for the rats to learn which lever was the active one. The only help the rats had too know which lever is the active one was at the beginning of each session when a small amount of fluid was present in the left cup. The animal training was performed in 9 days and to optimize the animals' motivation of drinking, water deprivation was performed with the rats from 17:00 one day before the onset of training. On training day 1, the rats were delivered free 0.2 % saccharin every minute to get the animals more eager to approach the receptacle. Both levers were also active for delivering fluid during the session within one hour. After the training session, the animals were returned to the home cage with access to water, but the water was removed again at 17:00 like before. For day 2 the rats were not provided free saccharin, the only saccharin delivery was through pressing the levers. The water deprivation was performed as before for day 3 and on that day the saccharin delivery was only occurring by pressing the active one of the levers, the left lever. For training day 4 and 6 the session went on as the same as before but without the water deprivation. From day 7 to day 9, the fluid consisted of 5 % (w/v) ethanol/0.2% saccharin instead of only 0.2% saccharin. The animal training stopped after day 9 and the animals were kept for 20 additional days in their home cage. After those 20 days, the rats were beheaded and the brain dissected.

Quantitative real time PCR

Quantitative real time PCR is rapid method with high; sensitivity, accuracy, specificity and throughput and is also less labor-intensive than conventional quantitative PCR (Ong and Irvine 2002). The method is based on the conventional PCR with the additional ability to detect and monitor the PCR amplification in real time and PCR product analysis without further laboratory-analysis; instead computer software can be used to calculate the transcript level.

The technique is based on a fluorescent detection reporter that somehow correlates with the amount of produced PCR product and gives a recordable detection signal based on fluorescence. Three different fluorescent reporter systems are available for this technique; hydrolysis probes, hybridization probes and DNA-binding agents. Hydrolysis probes are sequence specific oligonucleotides labeled with a fluorescent dye and a quencher. As long as the probe is intact the fluorescence emission from the donor molecule is absorbed by the quencher. However, during the amplification process the probe will be hydrolyzed by Taq-polymerase, which separates the donor and acceptor fluorochrome resulting in emission of fluorescent light. The hybridization probe technique is using two probes, one labeled with a donor fluorophore at the 3'-end and an acceptor in the other end. During the annealing phase and the first part of the extension phase the two probes will be within 1-5 nucleotides from each other and a fluorescence resonance energy transfer can occur from the donor to the acceptor fluorophore and a fluorescence emission occurs (van der Velden, Hochhaus et al. 2003).

DNA-binding agents include ethidium bromide, BEBO and SYBR Green I fluorescence. The fluorescence detector used in this project was the asymmetric cyanine dye, SYBR Green I. The dye is incorporated in a non sequence specific manner into the minor grooves of the double stranded DNA and fluorescence increase when intercalated in the DNA helix (van der Velden, Hochhaus et al. 2003; Kubista, Andrade et al. 2006).

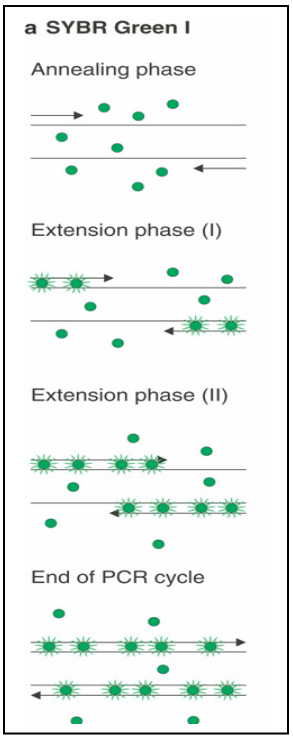

Figure 1. The DNA-binding agent SYBR Green I. Unbound SYBR Green I molecules have a week fluorescence emission, which is enhanced strongly when bound to the double stranded DNA.

During the first cycles of the amplification, the fluorescence signal is weak and cannot be distinguished from the background. When the amplification reaches the extension phase, the PCR products accumulates and increases exponentially, makes more double stranded DNA available for SYBR Green I to intercalate and the fluorescent signal increases exponentially with the produced PCR products. The following phase is the plateau phase where the critical components (i.e. primers, SYBR Green I and dNTPs) are limiting and the fluorescent signal level out.
The initial amount of the PCR product in the different samples can be calculated by measuring the number of PCR cycles required to reach a defined level of fluorescence, the threshold fluorescence. The more initial amount of DNA in the samples, the fewer cycles are required to reach the threshold relative to samples with lower initial amounts of DNA. The threshold cycle (Ct) is the cycle where the PCR product reaches the threshold level and the Ct is direct proportional to the amount of produced PCR product. Visualization of the amplification of the PCR product is done in an amplification plot where the fluorescent signal is plotted as relative fluorescence units (RFU) versus cycle number and in the plot the threshold line can be set on the point where the signal is just above the background and increase exponentially. By creating a standard curve containing samples with known concentrations the initial amount of PCR product can be quantified (Bustin 2002; Bustin and Mueller 2005).

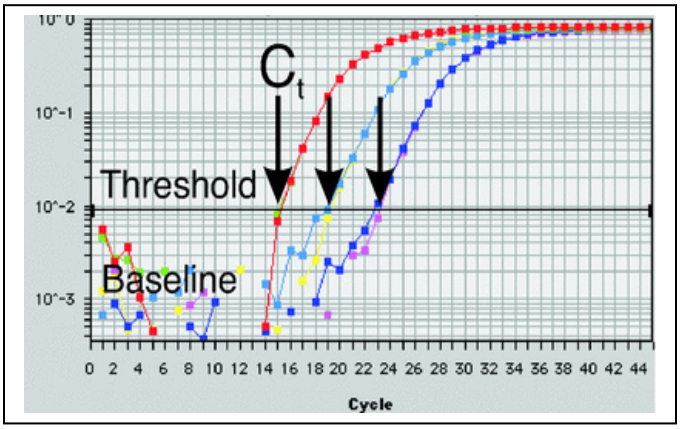

Figure 2. A real time PCR amplification plot. A threshold line is positioned above the background and in the exponential phase. The Ct value represent cycle where the amplicon reaches the threshold level (Bustin and Mueller 2005).

Relative quantification of the PCR product was estimated using the LinRegPCR method, which calculates the corrected Ct values and use assumption-free analysis to obtain the PCR efficiency.

The DNA intercalating SYBR Green I dye bind unspecifically to any double stranded DNA, like primer dimer products and undesired amplified PCR products. To ensure that a specific and accurate PCR product has been formed, a melting curve analysis can be performed after completed PCR amplification. PCR products of different length have different melting temperature; longer product length result in higher melting temperatures and shorter products, such as primer dimers, in lower melting temperatures. The melting curve is plotted with the product as negative derivate of the relative fluorescence units (-d(RFU)/dt) against temperature (^{o}C). Only one distinct peak will appear in the melting peak profile if the specific PCR product has been correctly amplified (van der Velden, Hochhaus et al. 2003).

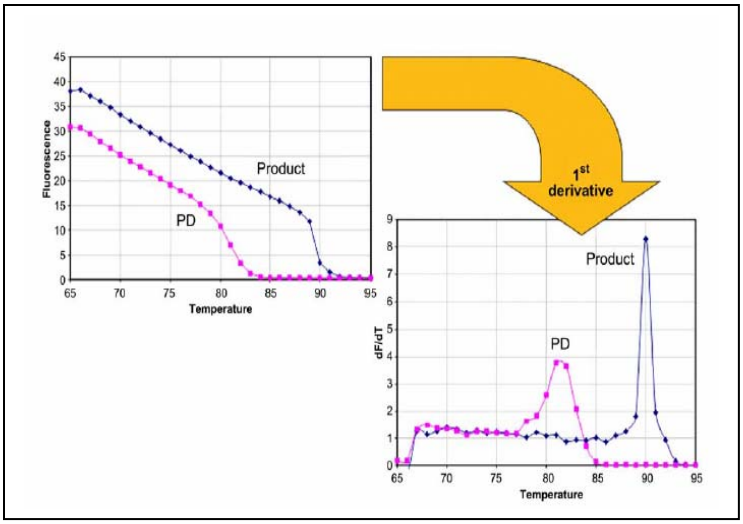

Figure 3. Melting curve analysis. The fluorescence intensiy as relative units is plotted as negative derivate versus temperature. Only one peak should appear in the melting curve if the specific PCR product has been correctly amplified. Here is an example of a primer dimer formation, the first curve since the melting temperature is lower. The second curve is a specific product since only one peak appears in the curve at the expected melting temperature. (Kubista, Andrade et al. 2006).

Normalization and housekeeping genes

The RNA material in the tested samples can differ in amounts of volume or concentration and the variation can cause misinterpretation of the PCR product levels. Therefore, it is necessary to normalize the sample to reference genes to compensate for any such variations. Internal controls referred to as housekeeping genes are used for normalization and the expression level of these genes should ideally be constant and independent of cells or tissues used and of development stage and should not change in response to treatment. Genes with that ability are often structural and metabolic genes (Bustin 2002). Since no genes are fulfilling these criteria for each and every experimental condition, it is necessary to validate the reference genes before use for normalization. Six housekeeping genes were used in this project and those were validated by using the software GeNorm. The program is a visual basic application for Microsoft Excel which calculates the stability of expression in the reference genes. A gene expression ratio between two housekeeping genes should be identical in all samples. The variation between all six

housekeeping used in this project were calculated to obtain the gene expression stability measure M for an internal control gene as the average pair wise variation for the gene of interest with all other control genes. High M value means low gene expression stability and by stepwise exclusion of these with new M values calculated after each time, the most stable control genes can be obtained and the normalization factor can be calculated. A normalization factor is a geometric mean of the expression levels of the most stable control genes. The recommended number of control genes for a normalization factor is three at a minimal (Vandesompele, De Preter et al. 2002).

RNA isolation

The dissected tissues were fixed in RNAlater and stored at -80^{o}C. Extraction for RNA from the RNAlater fixed tissues was based on the TRIzol (Invitrogen) method. Tissue was transferred to tubes containing 500-1000 µl reagent, depending on tissue size and kept on ice. Tissue was mechanically homogenized with ultra sound sonication. The equipment was cleaned with *RNase Away* (Invitrogen) and autoclaved water between each sample. Samples were then incubating at room temperature for 5 minutes. 100 µl of chloroform per 500 µl TRIzol were then added to the samples in order to separate the organic phase from the aqueous phase containing RNA. The samples were carefully inverted for 15 seconds and incubated at room temperature for approximately 3 minutes. The samples were then centrifuged at 13000 rpm for 20 minutes at 4^{o}C. The aqueous phase was then transferred to clean autoclaved tubes and the RNA was precipitated with 250 µl isopropanol per 500 µl added TRIzol at -20^{o}C for at least 2 hours. After the precipitation, the samples were centrifuged for 20 minutes at 4^{o}C in order to collect the precipitated RNA in the pellet. The supernatant was carefully removed from the centrifuged tubes and the pellet washed in 500 µl 75% room temperate ethanol per 500 µl TRIzol. The samples were then centrifuged at 13000 rpm for 10 minutes at 4^{o}C and the ethanol was removed. The washing process was repeated with 80% ice cold ethanol instead for 70% room temperate ethanol. After washing, the pellet was air dried at room temperature for a maximum of 30 minutes. The pellet was dissolved in 12-40 µl 1xDNAse buffer, depending on pellet size. The samples were then stored at -80^{o}C.

DNAse treatment and DNA contamination control

The extracted RNA samples were treated with DNase in order to remove all genomic DNA to avoid amplification in the cDNA synthesis. 1 µl DNase (Roche Diagnostics) was added to each sample and incubated for 1.5 hours for small size pellet and 2.5 hours for big size pellet at 37^{o}C. The DNase activity was inactivated by incubating the sample at 75^{o}C for 15 minutes. Condensation in the tubes originating from the inactivation step was brought down by centrifugation and samples were then stored at -80^{o}C. PCR were used to confirm that the DNase treatment was successful and the samples were completely free from DNA. For the PCR reaction reagents from Biotools B&M Labs were used. The mastermix for the PCR reaction was prepared within a total volume of 9.5 µl for each sample containing 1 µl 10x buffer, 0.3 µl 50 mM $MgCl_2$, 0.25 µl Tween, 0.1 µl 20mM dNTP solution, 1 µl of primer mix containing 10 pmol of each RNO 18s rRNA forward

and reverse primers for 490 bp, 0.5 units Tag polymerase enzyme (Biotools B&M Labs) and 6.75 µl autoclaved MilliQ (MQ) water. 0.5 µl of RNA sample was then added to a final reaction volume of 10 µl. The verification step with PCR included a positive control which was 5 ng/µl genomic DNA and a negative control, which only contained reaction solution. The setup for the PCR program was used was the following: 95^{o}C for 3 minutes, 35 cycles with 95^{o}C for 30 seconds, 58^{o}C for 30 seconds, 72^{o}C for 45 seconds and after 35 cycles, 72^{o}C for 5 minutes and finally a hold step at 4^{o}C. Analysis of the PCR product was done by gel electrophoresis using a 2 % agarose gel containing ethidium bromide stain. 1 µl 6x loading dye solution was added to the PCR product sample and loaded on the gel. The gel was analyzed with UV light and documented with a camera.

RNA concentration determination

The RNA concentrations of samples were determined by using Nanodrop 3000 (Nanodrop Technologies). The nanodrop measures the RNA concentration and the sample purity. The measurement was blanked with MQ water and 1 µl of the RNA sample was used in the measurement.

cDNA synthesis

The cDNA synthesis reaction buffer was composed of 12 µl template and 8 µl mastermix of reaction reagents. The required amount of RNA in the reaction buffer of 20 µl was 5 µg. Different volume of the extracted RNA were used and diluted to get the final concentration of 5 µg for the synthesis. When the RNA concentration was too low in the extracted samples, the whole volume was taken for the synthesis. The mastermix was contained the following reagents; 4 µl 5xFS buffer, 2 µl 0.1 M DTT, 0.5 µl 20mM dNTP, 0.5 µl random haxamers (N6) as primer and 200 units M-MLV Reverse transcriptase (Amersham Bioscience). The synthesis reaction buffer for each sample was mixed and incubated for 1 hour at 37^{o}C and the synthesis was inactivated at 95^{o}C for 15 minutes. Samples were kept on ice and the condensation was brought down by centrifugation. To verify if the cDNA synthesis was successful and all samples were containing cDNA, a control step using PCR and gel electrophoresis was performed. After verifying the presence of cDNA each sample was diluted with autoclaved MQ water to a final concentration of 5 ng/µl.

Real-time PCR

Quantitative PCR was performed with a Bio-Rad iCycler real-time detection instrument (Bio-Rad laboratories). The RT-PCR was run in 96-well plates with a total volume of 20 µl in each well. On the plate three candidate genes with a positive control (genomic DNA) and a negative control (MQ-water) for each gene was settled up. All samples including positive and negative controls were run in duplicate. A mastermix for the reaction was made containing the following components: 9.52 µl MQ water, 2 µl 10x PCR buffer, 0.20 µl 20mM dNTP, 1.60 µl 50mM $MgCl_2$, 0.005 µl forward primer (100 pmol/µl), 0.05 µl reverse primer (100 pmol/µl), 1 µl DMSO, 0.5 µl SYBR Green I, 0.08

µl (5 units/µl) Taq polymerase (Biotools B&M Labs). A sample amount of 5 µl with a concentration of 5 ng/µl of cDNA was used in each well.

	Candidate gene 1				Candidate gene 2				Candidate gene 3			
Well	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	1	1	3	3	1	1	3	3	1	1	3	3
B	5	5	8	8	5	5	8	8	5	5	8	8
C	9	9	14	14	9	9	14	14	9	9	14	14
D	23	23	24	24	23	23	24	24	23	23	24	24
E	26	26	28	28	26	26	28	28	26	26	28	28
F	32	32	37	37	32	32	37	37	32	32	37	37
G	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
H	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos

Figure 4. Plate setup for real time PCR analysis. 12 samples were run for each candidate gene in duplicate. Three candidate genes were run on each plate and for each candidate gene positive and negative controls were included. The samples were labelled with the same number as the rat the tissue was taken from.

Primers

Sequences of primers used for this project are listed in table 1. All primers used in this project were provided by Dr Chris Pickering, Uppsala University. The primers used in the real time PCR were designed using the Beacon Designer 2.1 software (Premier Biosoft). With this software optimal primers based on SYBR Green I detection were designed and primers properties such as melting temperature, probability of primer dimer and hairpin formation were estimated. The primers used in this project had a length of 18-22 nucleotides and the melting points were between 55-60^{o}C. The primers were designed to produce PCR products of 70-100 bp.

Table 1. Primers sequences used for real time PCR analysis.

Primer	Forward sequence	Reverses sequence
B-AKT	cactgccgcatcctcttcct	aaccgctcattgccgatagtg
B-TUB	cggaaggaggcggagagc	agggtgcccatgccagagc
RPL19	tcgccaatgccaactctcgtc	agcccgggaatggacagtcac
H3b	attcgcaagctccccttttcag	tggaagcgcaggtctgttttg
SDCA	gggagtgccgtggtgtcattg	ttcgcccatagcccccagtag
GAPDH	acatgccgcctggagaaacct	gcccaggatgcccttttagtgg
DR1	cgggctgccagcggagag	tgcccaggagagtggacagg
DR2	agacgatgagccgcagaaag	gcagccagcagatgatgaac
$GABA_A\alpha1$	tgccagaaattccctcccaaag	cagagccgagaacacgaagg
$GABA_A\alpha3$	tgctgagaccaagacctacaac	tggcaaagagcacagggaag
GluR1	caaccaccgaggaaggatacc	ttcacagtcaaccaccaccag
GluR2	ttgtgaggactaccgcagaag	ggactccagcaagtaggcatac
GluR3	atggtgtccgtgtggcttatc	tgactgtttcccgcttctctg
nAChRα4	ctcctgtcctccacccaag	atgccatcttctgctgcttc
nAChRα7	ctgctctacattggcttcc	aggtgctcatcatgtgttg
NR2A	cagcagcaagccacagttatg	agtctcggtagccagggaag
NR2B	caagaacatggccaacctgt	ggtacacattgctgtccttc

The sequences are presented from 5' to 3' direction. The transcript for β-AKT primer is β-actin, for β-TUB is β-tubulin, for RPL19 is ribosomal protein L 19, for H3b is histone H3b, for SDCA is SDCA, for GAPDH is glyceraldehydes-3-phosphate dehydrogenase, for DR1 and DR2 are dopamine receptor 1 and 2, for GAGAAα1 and GAGAAα3 are gamma-aminobutyric acid A α receptor 1 and 3, for GLuR1, GLuR2 and GLuR3 are α-amino-3-hydroxy-5-methyl-4-4isoxazolepropionic acid receptor 1,2 and 3, for nAChRα4 and 7 are nicotine acetylcholine receptor α4 and 7, for NR2A and B are N-methyl-D-aspartate receptor 2A and 2B .

Data analysis

Results of the real-time quantitative PCR were analyzed using the software iCycle 3.0 which registers the PCR amplicons. Ct values for each sample were collected and a melting point analysis performed to ensure the specificity of the products.
The software GeNorm VBA applet for Microsoft Excel was used to determine the most stable expressed genes for those six housekeeping genes used in this experiment. The Ct values of the housekeeping genes were analyzed with GeNorm to determine the most stable genes. Assuming those most stable genes, the Normalization Factor (NF) was determined specific for each sample in that brain region the sample originated from.
The raw real-time PCR data were analyzed with LinRegPCR program for each sample to determine the primer efficiency. With the linear regression analysis, the primer efficiency for each primer was determined and the values were then analyzed with GRUBBS' outliers test to excluding all outliers and receive an average efficiency value with a standard deviation. Using data received from LinRegPCR and GRUBBS' outliers' test the corrected Ct values could be estimated and further calculations with data from GeNorm the normalized Ct values were determineded. The normalized Ct values for each sample were then statistically analyzed with MiniTab 14 and Graph Pad Prism 4 and all figures were made using Graph Pad Prism v4 (San Diego, CA, USA).

Results

Behavioral data

The active lever was pressed to various extents by each individual among those 12 trained animals. Some individuals did not press the active lever at all, while others were more active and pressed the active lever up to 176 times during the session .
For day 5 and day 6 the delivered fluid contained only 0.2% saccharin. The average number of deliveries was 68.42 for day 5 and 74.67 for day 6. Day 7 was the first day of alcohol delivery and the average number of deliveries was 42.33. For day 8 the deliveries increased significantly, but at day 9 the deliveries decreased. An average number of the deliveries on day 8 and day 9 were used to approximately estimate the amount that the animals would drink if the alcohol consumption was stable. The average number of deliveries for day 8/9 was 72.08. A correlation between the average number of deliveries for day 8/9 of the alcohol consumption and the average of deliveries for day 5/6 of the saccharin consumption was made but there was no correlation between those time points. The non parametric P value for the correlation was 0.301 and the Spearman correlation r value was 0.341.

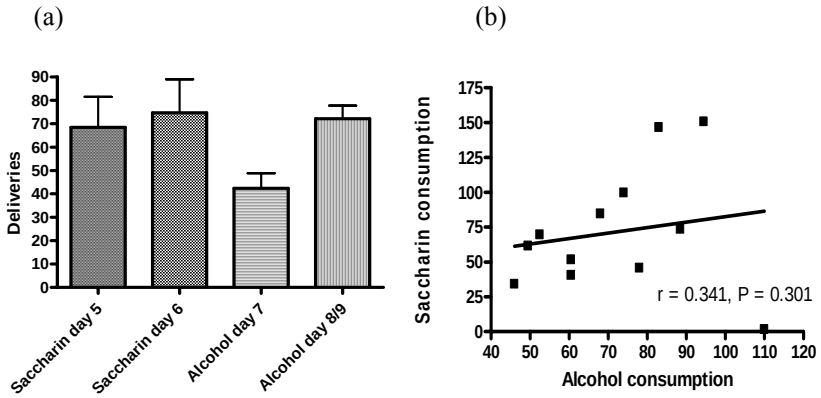

Figure 5. Number of fluid deliveries on day 5-9 for the high consumers in the study of correlation between self-administration alcohol consumption and gene expression. Correlation between the alcohol consumption and the saccharin consumption. a) On day 5 and day 6 only saccharin was delivered. Day 7 was the first day the alcohol was delivered and day 8 and 9 are present as average. b) Correlation between the average consumption of alcohol for day8/9 and the average consumption of saccharin for day 5/6. No significant correlation was found between these two consumptions, (r = 0.341, P = 0.301).

Reference gene validation

Six housekeeping genes were chosen for each brain region analysis and the validation was done with GeNorm to decide how many and which of these genes should be used for calculation of normalization factors. For the validation a cutoff value was set to 0.15, but this 0.15 value was not taken as a strict cutoff (Vandesompele, De Preter et al. 2002).

Caudate Putamen

Validation of the six housekeeping genes in CPu revealed that the optimal number of housekeeping genes to use in the calculation of the normalization factor was six. Of the six selected housekeeping genes β-tub and SDCA were the most stable ones and RPL19 was the least stable gene.

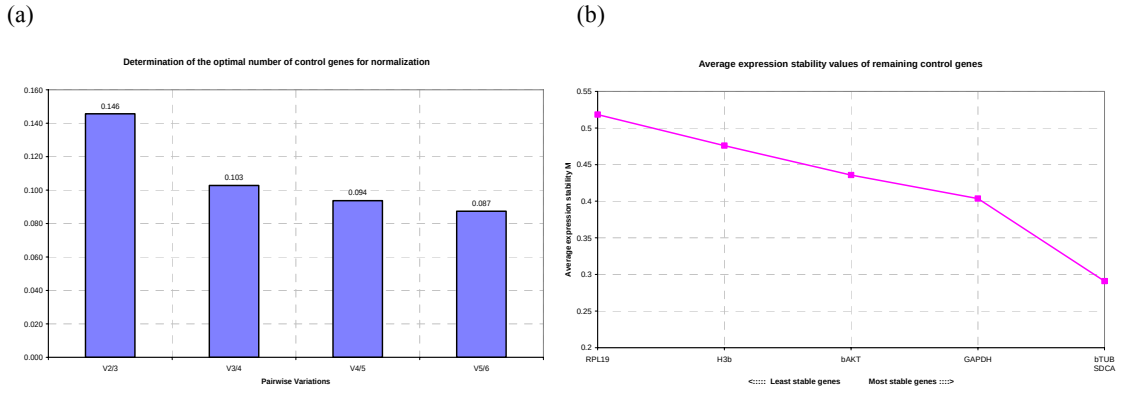

Figure 6. Determination of optimal number of housekeeping genes for normalization in CPu. a) The pairwise variation (V). b) Stability of the different housekeeping genes.

Nucleus accumbens

The optimal number of housekeeping genes for calculation of a normalization factor was five genes. The V value was lowest with four housekeeping genes and further inclusion increased the variation. The five most stable housekeeping genes used for normalization were βAKT, SDCA, GAPDH, H3b and RPL19.

(a) (b)

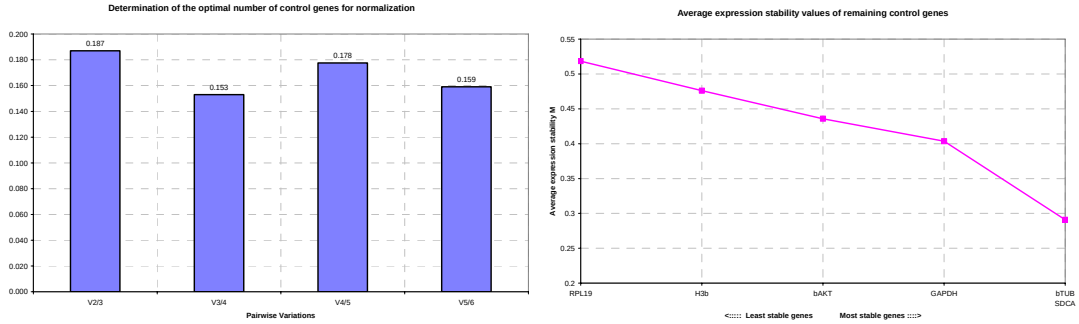

Figure 7. Determination of optimal number of housekeeping genes for normalization in NAc. a) The pairwise variation (V). b) Stability of the different housekeeping genes.

Substantia nigra

In Sn four housekeeping genes were selected as reference genes for normalization. The V value was lowest with inclusion of four housekeeping genes and further inclusion of one more gene will increase the variation. The four most stable housekeeping genes were β-TUB, GAPDH, H3b and RPL19.

(a) (b)

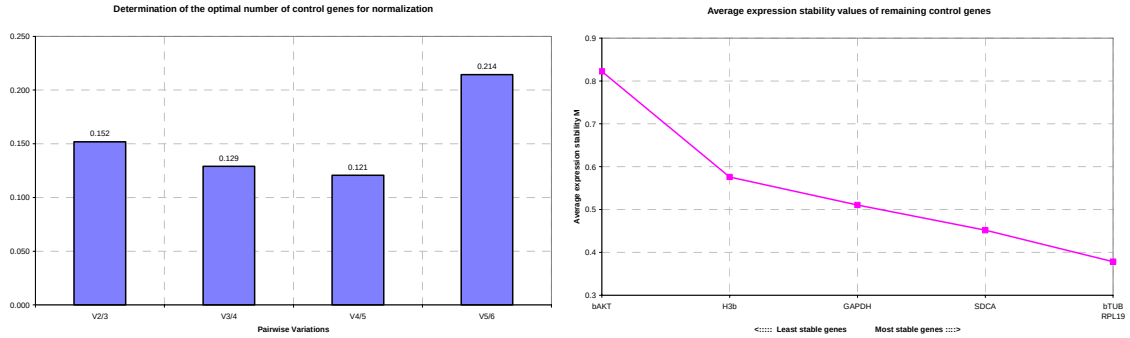

Figure 8. Determination of optimal number of housekeeping genes for normalization in Sn. a) Pairwise variation (V). b) Stability of the different housekeeping genes.

Ventral tegmental area

Considering the low total amount of RNA, only three housekeeping were analyzed for normalization in VTA. The optimal number of reference genes for VTA was to use two genes of the three housekeeping genes. With all three genes, the V value was significant over the cut-off value and by excluding the least stable one the variation decreased significantly. βTUB and Cyclo were the two most stable genes of the housekeeping genes.

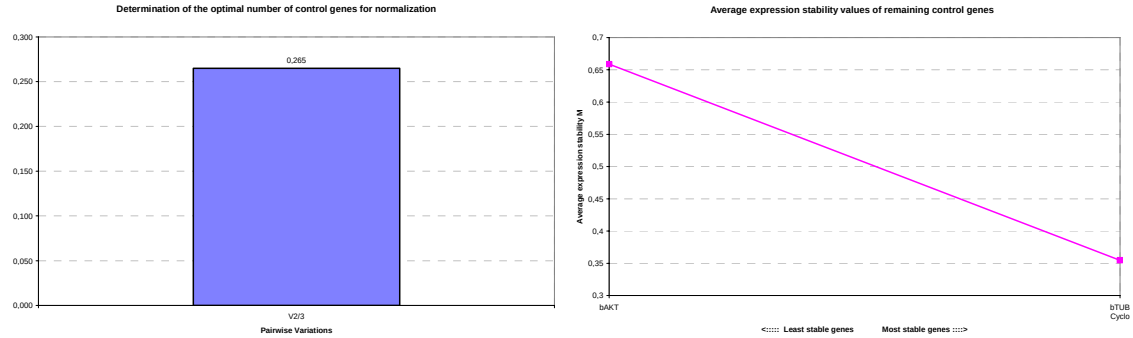

Figure 9. Determination of optimal number of housekeeping genes for normalization in VTA. a) Pairwise variation (V). b) Stability of the different housekeeping genes.

Correlation of the individual alcohol consumption to gene expression

The correlation between self-administrate alcohol consumption and gene expression was done by correlating the average number of alcohol deliveries on day 8 and 9 to the relative receptor transcript expression level. The average number of alcohol deliveries on day 8 and 9 was set to be the most representative estimation of long term individual ethanol consumption. The non parametric Spearman correlation was used to identify correlations between mRNA expression and behavior. The range of Spearman correlation r is between +1 to -1. A correlation of +1 indicates a perfect correlation and values above +0.5 indicates a strong correlation. The significance level for the p value was set to 0.05.

Caudate Putamen

In CPu no strong significant hits of correlation between gene expression and alcohol and saccharin consumption were found. Expression of dopamine receptor subunits was not correlated to alcohol consumption. Correlation analysis between mRNA expression and saccharin consumption showed that the dopamine receptor subunits 1 had a P value of 0.085 and an r value of -0.517, which indicated a trend of significant correlation. Glutamate and nicotine acetylcholine receptors expressions were not significantly correlated to alcohol and saccharin consumption.

Table 2. Correlation between mRNA expression and alcohol consumption and saccharin consumption in CPu.

Neurotransmitter receptor		Spearman correlation	P value
Correlation between mRNA expression and alcohol consumption			
Dopamine receptor	DR1	0.028	0.931
	DR2	-0.025	0.940
	DR3	-0.028	0.931
Glutamate ampa receptor	AMPA GluR1	-0.151	0.640
	AMPA GluR2	-0.091	0.778
	AMPA GluR3	-0.224	0.484
	NMDA NR2A	-0.018	0.958
Nicotine acetylcholine receptor	nAChRα4	-0.067	0.837
	nAChRα7	-0.284	0.372
Correlation between mRNA expression and saccharin consumption			
Dopamine receptor	**DR1**	**-0.517**	**0.085**
	DR2	0.280	0.379
	DR3	0.112	0.729
Glutamate receptor	AMPA GluR1	0.287	0.366
	AMPA GluR2	0.134	0.678
	AMPA GluR3	0.028	0.931
	NMDA NR2A	-0.226	0.404
Nicotine acetylcholine receptor	nAChRα4	-0.112	0.729
	nAChRα7	0.064	0.846

Nucleus accumbens

Two trends of significant correlation of mRNA expression to alcohol consumption were found within dopamine receptor subunit 2 and glutamate AMPA receptor subunit 1. The dopamine receptor subunit 1 had a P value of 0.087 and a r value of 0.515 indicating a trend of a strong significant correlation, which also was the case for the glutamate AMPA receptor subunit 1 with a P value of 0.060 and a r of 0.557.
In the correlation of mRNA expression to saccharin consumption the glutamate AMPA receptor subunit 2 was significantly correlated to the saccharin consumption. The P value for the correlated glutamate AMPA subunit 2 was 0.026 and the r value was 0.636.

Table 3. Correlation between mRNA expression and alcohol consumption and saccharin consumption in NAc.

Neurotransmitter receptor		Spearman correlation	P value
Correlation between mRNA expression and alcohol consumption			
Dopamine receptor	DR1	0.252	0.429
	DR2	**0.515**	**0.087**
GABA receptor	A α1	0.137	0.672
	A α3	0.382	0.221
Glutamate receptor	AMPA GluR1	0.189	0.556
	AMPA GluR2	0.340	0.280
	AMPA GluR3	**0.557**	**0.060**
Nicotine acetylcholine receptor	nAChRα4	-0.077	0.812
	nAChRα7	-0.103	0.778
Correlation between mRNA expression and saccharin consumption			
Dopamine receptor	DR1	0.315	0.319
	DR2	0.455	0.138
GABA receptor	A α1	0.042	0.897
	A α3	-0.133	0.681
Glutamate receptor	AMPA GluR1	-0.063	0.846
	AMPA GluR2	**0.636**	**0.026**
	AMPA GluR3	0.434	0.159
Nicotine acetylcholine receptor	nAChRα4	-0.420	0.175
	nAChRα7	-0.176	0.627

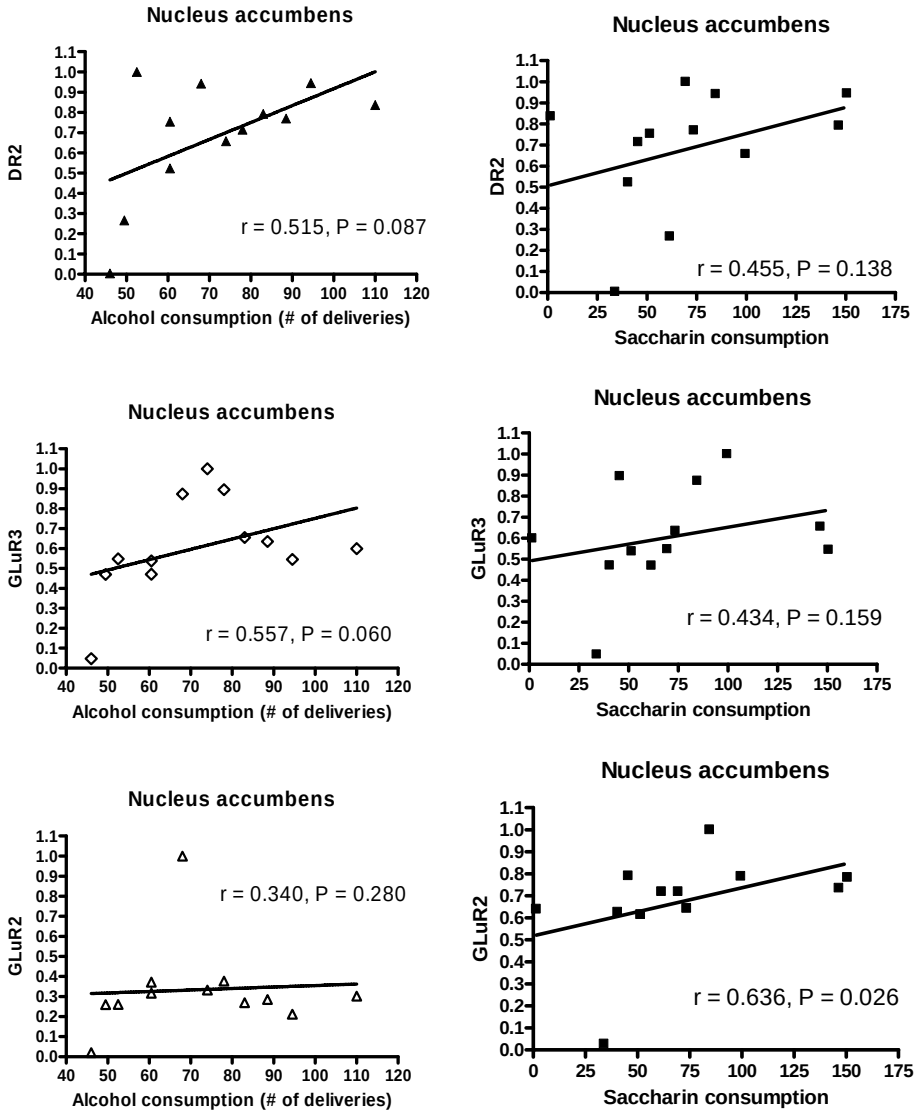

Figure 10. mRNA expression correlations within the NAc. Dopamine receptor subunit 2 (r = 0.515, P = 0.087) and glutamate AMPA receptor subunit 3 (r = 0.557, P = 0.060) were found to have a trend of significant correlation to the alcohol consumption. The two receptor subunits were not correlated to the saccharin consumption. Glutamate AMPA subunit 2 were found to correlated with saccharin consumption with r value of 0.636 and P value of 0.026.

Substantia nigra

No significant correlation between mRNA expression and alcohol consumption or saccharin consumption was found in Sn.

Table 4. Correlation between mRNA expression and alcohol consumption and saccharin consumption in Sn.

Neurotransmitter receptor		Spearman correlation	P value
Correlation between mRNA expression and alcohol consumption			
Dopamine receptor	DR2	0.329	0.296
Glutamate receptor	AMPA GluR1	0.053	0.871
	AMAP GluR2	0.088	0.787
	NMDA NR2A	0.112	0.729
Nicotine acetylcholine receptor	nAChRα4	-0.301	0.341
Correlation between mRNA expression and saccharin consumption			
Dopamine receptor	DR2	0.336	0.286
Glutamate receptor	AMPA GluR1	0.049	0.880
	AMPA GluR2	0.070	0.829
	NMDA NR2A	-0.196	0.542
Nicotine acetylcholine receptor	nAChRα4	0.070	0.829

Ventral tegmental area

In the VTA a significant correlation between mRNA expression of glutamate AMPA receptor subunit 3 and alcohol consumption was found. The P value was 0.038, which reach below the cut-off value for significance and an r value of 0.602, which indicated a strong correlation. In the correlation of mRNA expression to saccharin consumption, a trend of correlation was found with nicotine acetylcholine receptor subunit α4. The P value was 0.085 and the r value was 0.517.

Table 5. Correlation between mRNA expression and alcohol consumption and saccharin consumption in VTA.

Neurotransmitter receptor		Spearman correlation	P value
Correlation between mRNA expression and alcohol consumption			
GABA receptor	A α1	-0.155	0.650
	A α3	-0.400	0.223
Glutamate receptor	AMPA GluR1	-0.074	0.820
	AMPA GluR2	-0.410	0.186
	AMPA GluR3	**0.602**	**0.038**
Nicotine acetylcholine receptor	nAChRα4	0.445	0.147
	nAChRα7	0.137	0.672
Correlation between mRNA expression and saccharin consumption			
GABA receptor	A α1	0.518	0.102
	A α3	-0.255	0.450
Glutamate receptor	AMPA GluR1	0.301	0.342
	AMPA GluR2	-0.385	0.371
	AMPA GluR3	0.000	1.000
Nicotine acetylcholine receptor	**nAChRα4**	**0.517**	**0.085**
	nAChRα7	-0.063	0.846

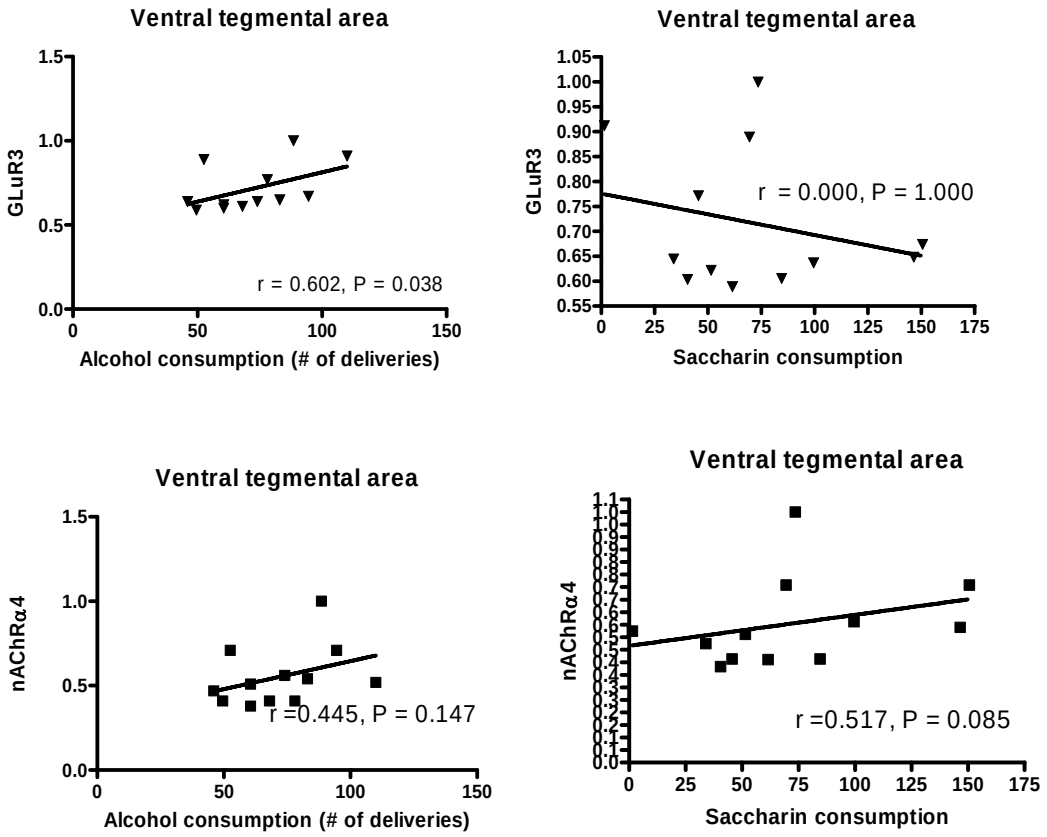

Figure 11. mRNA expression correlations within VTA. Glutamate AMPA subunit 3 was found to be significantly correlated to alcohol consumption (r = 0.602, P = 0.038), but not correlated to saccharin consumption. A trend of significant correlation was found for the nicotine acetylcholine receptor subunit α4 to the saccharin consumption (r = 0.517, P = 0.085) but not to the alcohol consumption.

Application to prefrontal cortex with G-protein coupled receptor GPR125

Behavioral data

The lever was pressed to varying degrees at different time points during the session by the trained animals. The average number of lever pressed responses for day 5/6 of saccharin consumption in the low consuming group was 2.3 and for the alcohol consumption in day8/9 as average was 7 times. In the high consuming group, the animals pressed the lever on an average of 85 times in day 5/6 for saccharin while the average of day 8/9 for alcohol was 76 times. Correlations analysis between saccharin and alcohol deliveries in both low consuming group and high consuming group revealed no significant correlations.

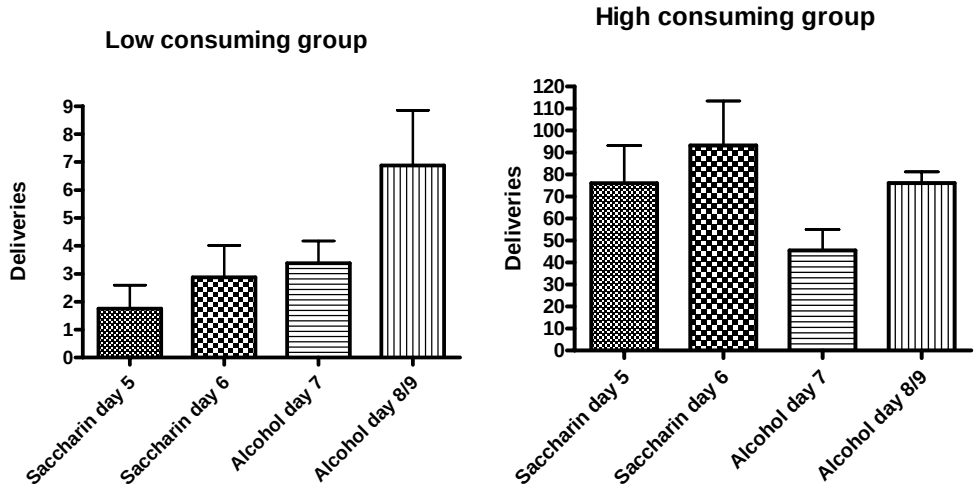

Figure 12. Number of fluid deliveries on day 5-9 for the high consuming group and low consuming group in the study of correlation between self-administrate alcohol consumption and gene expression. On day 5 and day 6 only saccharin was delivered. Day 7 was the first day the alcohol was delivered and day 8 and 9 are present as average.

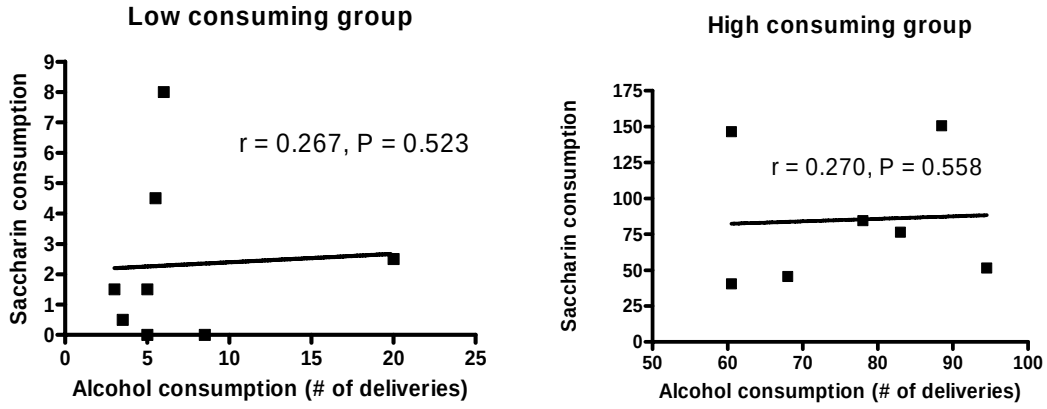

Figure 13. Correlation between average of day 8/9 alcohol consumption and average of day 5/6 saccharin consumption in the high consuming group and low consuming group. No correlation was found in those groups.

Reference genes validation

Prefrontal cortex

For the prefrontal cortex six housekeeping genes were used in the validation. Decision of how many and which one/ones to use was based on the validation software GeNorm VBA applet for Microsoft Excel. The optimal number of reference genes for normalization was five genes. With another inclusion of more than five housekeeping genes the variation increased. The V value for five housekeeping genes was 0.050, which is significantly below the cut-off value of 0.15. Those five housekeeping genes used for normalization in pre frontal cortex were βAKT, βTUB, GAPDH, H3b and RPL19.

(a) (b)

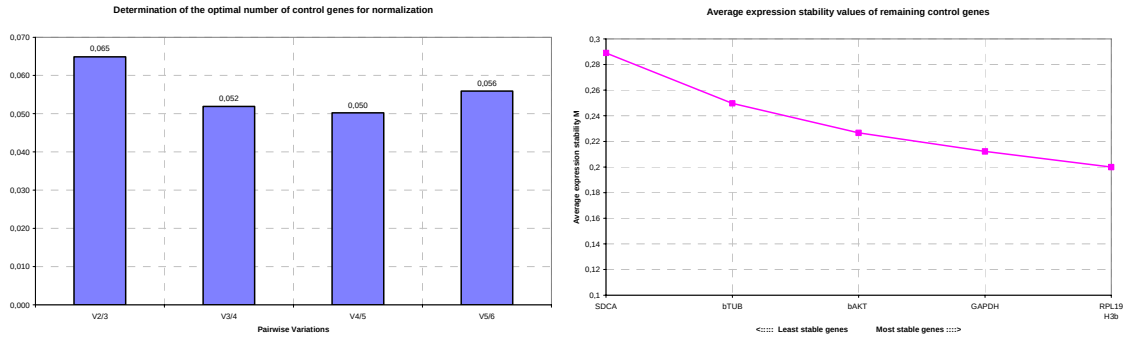

Figure 14. Determination of optimal number of housekeeping genes for normalization in pre frontal cortex. a) The pairwise variation (V). b) Stability of the different housekeeping genes.

Correlation of the alcohol consumption to G-protein receptor 125

The GPR125 expression was correlated to alcohol consumption in pre frontal cortex in a high consuming group and a low consumption group. In the high consuming group, the P value of 0.090 revealed a trend of significant correlation and the Spearman correlation r value of -0.685 revealed a strong negative correlation. For the low consuming group, there were not any correlations of GPR125 expression to the alcohol consumption. Both low consuming group and high consuming group of saccharin did not have any correlations for GPR125 to the saccharin consumption. An unpaired two-tailed t test was done with the GPR125 expressions in the pre frontal cortex between the high consumers and low consumers of alcohol. No significant correlation was found for the comparison between the groups. The P value was 0.7170. The GPR125 expression did not vary in the low consumers group while it was more variable in the high consumers group. This variation in the high consumers could explain the trend of GPR125 expression to correlate to the behavior.

Table 6. Correlation of GPR125 expression to alcohol consumption and saccharin consumption in prefrontal cortex.

G-protein receptor 125	Sperman correlation	P value
	Correlation of GPR125 to alcohol consumption	
High consuming group	**-0.685**	**0.090**
Low consuming group	0.096	0.821
	Correlation of GPR125 to saccharin consumption	
High consuming group	-0.423	0.337
Low consuming group	-0.313	0.450

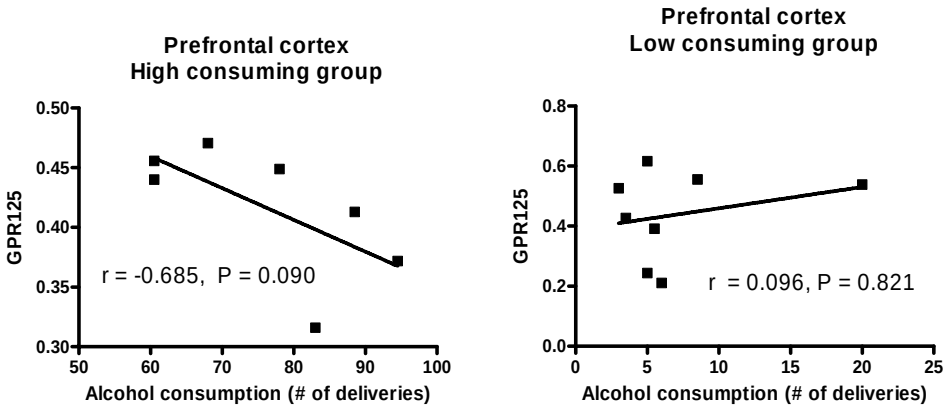

Figure 15. GPR125 expressions in the high and low consuming group correlated to the alcohol consumption. GPR125 had weak a trend of significant correlation to the alcohol consumption in the high consuming group. No correlation was found for the GPR125 expression in the low consuming group.

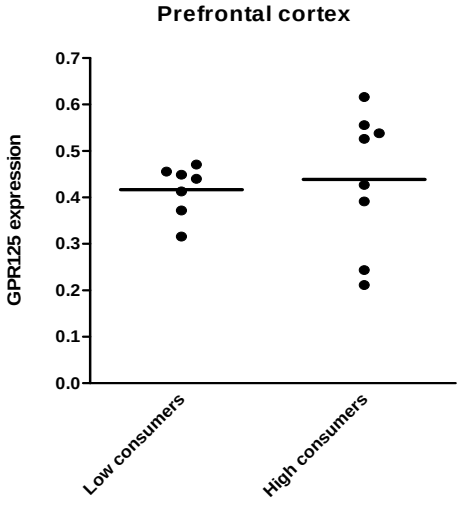

Figure 16. Gene expression comparison between the low consuming group and the high consuming group of alcohol. The low consuming group included 7 animals and the high consuming group included 8 animals. P value of the comparison was 0.7170, the mean of column A: 0.4165, the mean of column B: 0.4387 and difference between the means: -0.02213.

Discussion

This study was focused on mRNA expression of receptor types that are involved in drug consumption, especially thought to be involved in alcohol consumption and expressed in brain areas that have a role in the brain reward pathway. With the quantitative real-time PCR method, mRNA expression could be obtained and a correlation analysis to ethanol self-administration could be performed.

Three correlations between mRNA expression and ethanol self-administration were found, where two cases showed a trend of significant correlation and the third was significantly correlated. Two receptors were found in NAc correlated to ethanol self-administration, the iontropic glutamate AMPA receptor subtype 3; GluR3, and the dopamine receptor subtype 2; DR2. These two correlations had p-values (DR2: 0,087, GluR3: 0,060) just above the significance level, which indicates a strong trend of significance. The last correlation was found in the VTA, the GluR3 (p-value: 0,038) was significantly correlated to ethanol self-administration.
Many studies have yielded evidences that the brain reward pathway is a target for alcohol and that these actions are elicited through the mesolimbic dopaminergic system, which originates from the VTA (Samson, Hodge et al. 1993; Bowirrat and Oscar-Berman 2005). The VTA and NAc are known to have significant roles in brain reward and drug addiction mechanisms. The VTA projects dopamine to NAc, which is an important projection in the reward pathway and this particular dopamine signal way has been proven to promote ethanol self-administration. Additionally, when VTA is inhibited or dopamine transmission in NAc is decreased the ethanol self-administration and ethanol responses will also decrease (Rassnick, Stinus et al. 1993; Samson, Hodge et al. 1993). NAc has been suggested through behavioral studies to be an important brain area for the rewarding effects of drugs like opiate or ethanol (Nie, Madamba et al. 1994).

More correlations were actually expected from this investigation, since other studies have indicated the glutamate receptor subtype NMDA and GABAergic receptors to have roles in ethanol and drug addiction (Nie, Madamba et al. 1994). The NMDA and GABAA are believed to be directly modulated by ethanol, as in acute ethanol exposure, where the ethanol potentiates the GABAA receptors and inhibits NMDA receptors, which seem to have a role in acute intoxication (Hoffman, Rabe et al. 1989; Davies 2003).
The correlation with GluR3 in VTA was surprising; since the GluR1 and GluR2 are more common to be coupled with reward and drug abuse. The GluR1 has been proven to increase in VTA after chronic ethanol and administration of drugs of abuse (Ortiz, Fitzgerald et al. 1995; Fitzgerald, Ortiz et al. 1996).

Glutamatergic transmission is suggested to have connection with alcohol and this connection may affect several different brain areas and functions. The NAc is receiving glutamatergic projections from several surrounding areas like amygdala, PFC and hippocampus, and glutamatergic receptors in NAc is associated with addiction behavior. Relapse to drug seeking have been proven to be triggered by increased NAc glutamatergic transmission. Studies with cocaine addiction have revealed that accumbal glutamate transmission facilitates the effect of cocaine-associated cues on cocaine-

seeking behavior (Backstrom and Hyytia 2007). Another study that indicates the importance of glutamate transmission in drug craving and relapse was done by Sanchis-Segura, Borchardt et al. (2006), which found that the glutamate AMPA receptor subtype GluR3 has an important role in ethanol-seeking behavior and is involved in drug craving and relapse. The GluR3 was shown to be upregulated during ethanol abstinence and when the GluR3 receptors were inhibited with the AMPA receptor antagonist GYKI 52466, the ethanol-seeking behavior was reduced. Identical phenotype was also seen in the GluR3 knockout mice (Sanchis-Segura, Borchardt et al. 2006).
Studies on cocaine- and ethanol-seeking behavior, using the non-specific AMPA/kainate receptors antagonist CNQX, revealed a decreased drug-seeking behavior, which support the role of the AMPA subtype GluR3's involvement in drug craving and relapse (Cornish and Kalivas 2000; Backstrom and Hyytia 2004).

The AMPA receptors have also been indicated to have an important role in different studies on different mechanism underlying alcohol consumption and the effects of alcohol. One of those is that the AMPA receptors in combination with kainate receptors AMPA/kainate, are involved in rapid tolerance to alcohol. This result was obtained by investigation with the AMPA/kainate receptor agonist aniracetam and the antagonist DNQX on ethanol-induced motor impairment. The results were that aniracetam enhanced the development of rapid tolerance to ethanol and this rapid tolerance was blocked by the antagonist DNQX (Rial, Takahashi et al. 2008). Another postule that indicates a possibility of a relationship between sensitivity to ethanol and AMPA/kainate receptor was done by Lu and Yeh, (1999), where they showed ethanol to modulate AMPA receptor-mediated responses of neurons in the rat primary somatosensory cortex (Lu and Yeh 1999). Ethanol sensitivity differs among the various AMPA receptors and the sensitivity to modulation by ethanol depends on the subunit composition. In recombinant AMPA/kainate receptors, the homomeric GluR3, which mediate kainate currents, is more sensitive to ethanol than other GluRs. GluR3 has been shown to desensitize more quickly than GluR1 currents (Weight, Peoples et al. 1993; Dildy-Mayfield and Harris 1995; Lu and Yeh 1999).

Studies have been indicating that dopamine and glutamate are interacting with each other in the mesocorticolimbic pathway and this interaction may regulate motivation and reward and give to arise to schizophrenia and drug abuse (Clements and Greenshaw 2005). The correlations of GluR3 and DR2 in NAc suggest functional interaction with each other since NAc is receiving glutamate from PFC, hippocampus, amygdala and thalamus, and also dopamine from VTA. The acccumbal dopamine is believed to modulate the glutamate signals from these brain areas, which gates and regulates the glutamate signals into NAc (Cardinal, Parkinson et al. 2002).
The dopamine projection from VTA to NAc is one of the key parts of the reward pathway. Study on voluntary ethanol consumption by Stuber et al., 2008; have showed that ethanol self-administration increased in AMPA receptors functioning in VTA dopamine neurons. The increased AMPA receptor function may facilitate the drive to consume ethanol by regulating the VTA neuron firing, enhance the reinforcing and activating effects of drugs of abuse. Increase of AMPA function has been seen following

several weeks of ethanol self-administration, hence an assumption that AMPA receptors may promote ethanol seeking and consumption (Stuber, Hopf et al. 2008).
The dopamine projection from VTA has been proven to increase the activation of excitatory amino acid receptors, AMPA and NMDA subtypes in VTA. The study was carried out by intra-VTA infusion of the ionotropic excitatory amino acid receptor agonist AMPA and NMDA dose-dependently (10 and 100 μM) which increased the dopamine release from VTA to ventral striatum and surrounding areas like NAc. This increasing dopamine burst firing triggered by activated AMPA receptors induced by the agonist AMPA indicate that the AMPA receptors are involved in the regulation of dopamine release from VTA to target areas (Karreman, Westerink et al. 1996). Evidences have been found in acute ethanol administration that ethanol increase glutamate release at synapses on the VTA dopamine neurons and promotes dopamine firing (Xiao, Shao et al. 2008).

D2-like dopamine receptors in the NAc are believed to have a role in facilitating the reward or motivating effects of the VTA-stimulation (Schultz 2000). Genetic investigations have also found the TaqI A minor allele of the DR2 gene may be involved in alcohol addiction (Blum, Braverman et al. 2000; Noble 2000). Dopamine receptor agonist stimulation of DR2 resulted in a higher decrease in anxiety, craving behavior and increased ethanol consumption in two-bottle choice experiments (Lawford, Young et al. 1995; Sluyter, Hof et al. 2000). Ethanol-reinforced responding has been shown to decrease with decreasing dopamine transmission in the NAc and increase with increased dopamine transmission and the NAc is suggested as a site for ethanol reward associated with the DR2 receptor (Self and Nestler 1995; Myers and Robinson 1999).

In summary this project indicates a useful method by using the quantitative real-time PCR to study mRNA expression of receptor types that are involved in brain reward pathway and drug addiction.The study also provides information on appropriate housekeeping genes to use for normalisation of transcript levels in different tissues. We found in ethanol self-administering rats correlations between the ethanol self-administration and mRNA expression of receptor types. The correlations were found in brain areas important for the brain reward pathway, the VTA and NAc. In VTA, we found the glutamate AMPA receptor subtype GluR3 and in the NAc the dopamine receptor subtype DR2 and the glutamate receptor subtype GluR3. The correlated receptors and the brain areas indicate an interaction between the glutamatergic and the dopaminergic pathway in alcohol consumption and reward mechanisms. More specifically it seems to be interactions between iontropic glutamate AMPA receptors and dopamine receptors in both VTA and NAc that facilitate ethanol consumption and behaviors.

Since these receptors have been identified previously in different studies to be involved in different mechanisms of drug addiction and behavior, this indicates that our strategy can be useful to identify receptor types involved in drug consumption and addiction, such as ethanol. However, this method is only identifying receptors involved in the initial stages of alcohol self-administration and since ethanol self-administration is a complex

behavior, other methods, animal models and behavior study are needed to get a more complete understanding.

The application to prefrontal cortex with G-protein coupled receptor GPR125 was a novel investigation of the GPR125. The correlation between the GPR125 and the high consuming group of ethanol in prefrontal cortex indicates that GPR125 might have some role in alcohol consumption and behavior.
However, since this investigation with the GPR125 was only a novel search for correlations of the GPR125 mRNA expression within brain areas related to reward, the result indicates an opening for further more investigations.

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