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**EFFECTS OF SEROTONIN TRANSPORTER PROMOTER  
POLYMORPHISM (5-HTTLPR) AND NEUROPEPTIDE Y GENE  
VARIANTS ON ALCOHOL USE IN YOUNG ADULTS**

Master's thesis

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Tartu 2012

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## **Abstract**

**Objective:** To elucidate the possible main effects and interactions of 5-HT transporter gene-linked polymorphic region (5-HTTLPR) and two functional polymorphisms in the neuropeptide Y (NPY) gene – rs16139, rs16147 – on alcohol use in young adults.

**Methods:** The sample of the longitudinal Estonian Children Personality Behaviour and Health Study (initially cohorts of 9 and 15 year old children) was initially examined in 1998. In the given study, data collected at the time when the younger cohort (n=580) was 18 and the older (n=654) 25 years old was used. Subjects filled self-report questionnaires about their alcohol consumption; in addition, subjects from the older cohort were interviewed by a psychologist in order to determine the occurrence of alcohol use disorder.

**Results:** In the given sample, the subjects from the younger cohort started consuming alcohol at an earlier age than the subjects from the older cohort. Alcohol consumption was more problematic among male subjects, both in the older and younger cohort. In interaction with subjects' gender and cohort, 5-HTTLPR had significant effects on the age when subjects first consumed alcohol. Subjects who were NPY rs16139 C allele carriers and also had NPY rs16147 T/T genotype were found to have more problematic alcohol use. Some interactive effects on alcohol use between 5-HTTLPR and NPY polymorphisms were also identified in the course of exploratory data analysis.

**Conclusions:** Serotonergic and NPY-ergic systems have a role in youth's alcohol consumption, but the effects are dependent on gender and cohort.

Keywords: serotonin, 5-HTTLPR, neuropeptide Y, alcohol, gene-gene interaction

## Kokkuvõte

**Eesmärk:** selgitada serotoniini transporteri geeni promootorpiirkonna polümorfismi (5-HTTLPR) ja neuropeptiid Y (NPY) geeni kahe funktsionaalse polümorfismi – rs16139, rs16147 – võimalikku tähendust noorte täiskasvanute alkoholitarbimisele.

**Meetod:** Eesti Laste Isiksuse, Käitumise ja Tervise Uuringu (ELIKTU) näol on tegemist 1998. aastal alguse saanud longituuduuringuga. Antud valim koostati kahest kohordist – 9- ja 15-aastastest. Antud uuringus kasutatakse alkoholi tarvitamist puudutavaid andmeid, mis koguti ajal, kui noorema kohordi uuritavad (n=580) olid keskmiselt 18 ja vanema kohordi uuritavad (n=654) 25 aastat vanad. Valdavalt on tegemist enesekohastest küsimustikest pärinevate andmetega; vanema kohordi uuritavate puhul ka psühhiaatrilise intervjuu tulemused alkoholi kuritarvitamise ja sõltuvuse kohta.

**Tulemused:** antud valimis tarbisid noorema kohordi uuritavad alkoholi esmakordselt nooremas eas kui vanema kohordi uuritavad. Nii nooremas kui vanemas kohordis oli alkoholi tarbimine meessoost uuritavate seas problemaatilisem. 5-HTTLPR genotüübil oli interaktsioonis soo ja kohordiga mõju vanusele, mil uuritavad esmakordselt alkoholi tarvitasid. Uuritavate puhul, kes olid NPY rs16139 C-alleeli kandjad ning ühtlasi ka NPY rs16147 heterosügootid, oli tegemist probleemsema alkoholitarvitamisega. Eksploratiivse andmeanalüüsi käigus tuvastati alkoholitarbimisele ka interaktiivseid efekte 5-HTTLPR-i ja NPY polümorfismide vahel.

**Järeldus:** nii serotonergiline kui ka NPY-eriline süsteem omavad rolli noorte alkoholitarbimises, kuid avalduvad efektid sõltuvad soost ja kohordist.

Märksõnad: serotoniin, 5-HTTLPR, neuropeptiid Y, alkohol, geenidevaheline interaktsioon

## Alcohol Use Disorder

Alcohol consumption is an integral part of daily life in many societies, yet the benefits associated with the production, sale, and use of alcoholic beverages come at an enormous cost. The harmful use of alcohol is a global problem which compromises both individual and social development.

### Health Consequences of Excessive Alcohol Consumption

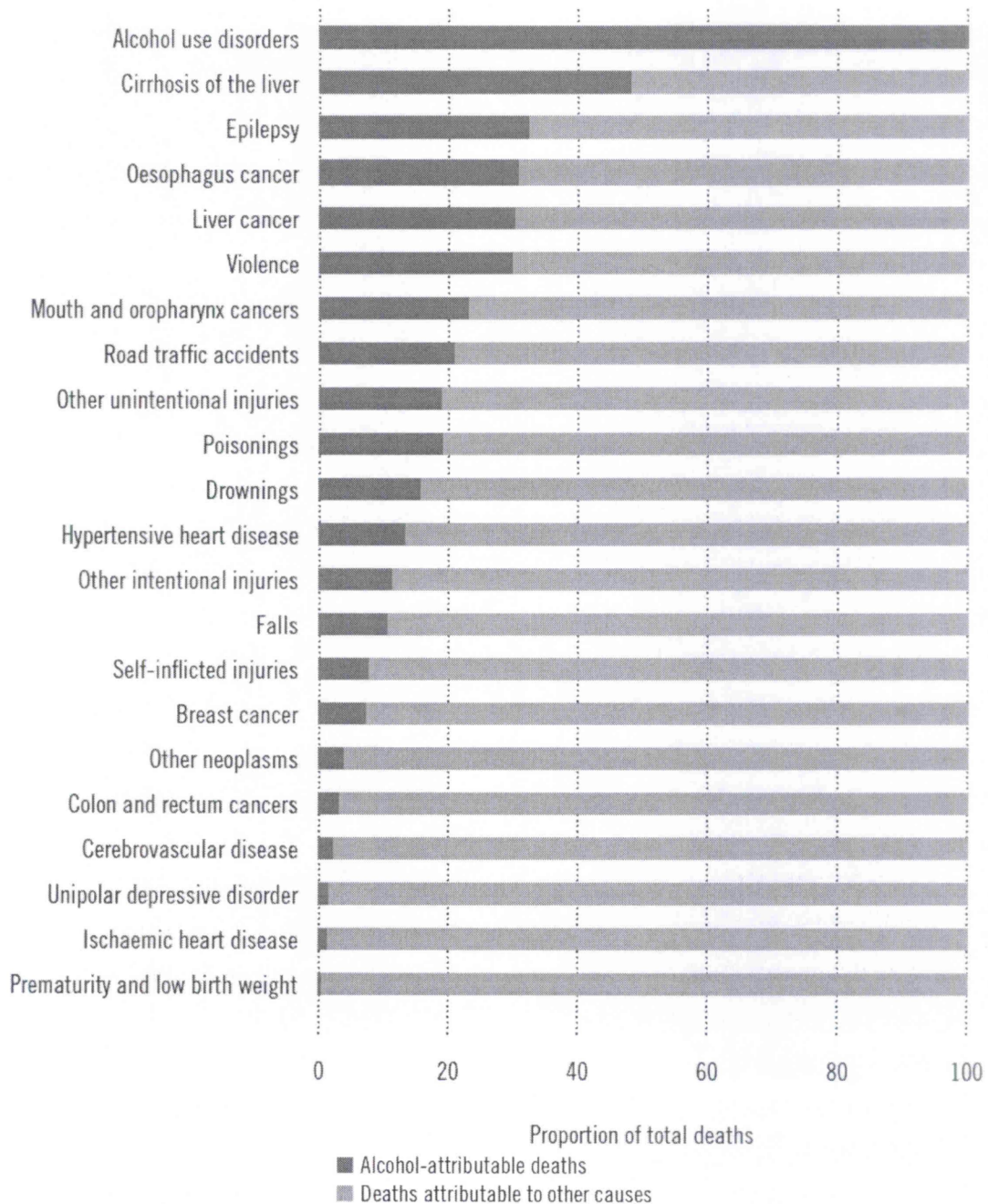


Fig. 1. Global distribution of all alcohol-attributable deaths by disease or injury, 2004 (WHO, 2011b).

According to the World Health Organization (WHO), about 2.5 million deaths a year worldwide are attributed to alcohol — more than the number of deaths caused by HIV/AIDS, tuberculosis or malaria. Alcohol is the world's third largest risk factor for disease burden; it is the leading risk factor in the Western Pacific and the Americas and the second largest in Europe (WHO, 2011a). Global distribution of all alcohol-attributable deaths by disease or injury is depicted in Fig. 1.

Alcohol is linked both to the incidence of disease and the course of disease. The impact of alcohol consumption on disease and injury is associated with two separate but related dimensions of drinking by individuals: the volume of alcohol consumed and the pattern of drinking. More than 30 International Classification of Diseases (ICD)-10 codes include alcohol in their name or definition, indicating that alcohol consumption is a necessary cause. Of these, alcohol use disorders (AUDs) are the most significant. In addition, alcohol has been identified as a component cause for over 200 ICD-10 disease codes. A component cause may be one among a number of components, none of which alone is sufficient to cause the disease. When a number of the components are present, the sufficient cause is formed (WHO, 2011b).

According to WHO, major disease and injury categories causally linked to alcohol are neuropsychiatric disorders, cardiovascular and gastrointestinal diseases, cancer, intentional and unintentional injuries, fetal alcohol syndrome and pre-term birth complications, and diabetes mellitus.

In the category of neuropsychiatric disorders, AUDs are the most important disorders caused by alcohol consumption in this category. Epilepsy is another disease causally impacted by alcohol, over and above withdrawal-induced seizures (Samokhvalov *et al*, 2010). Many other neuropsychiatric disorders are associated with alcohol, but whether they are caused or the extent to which they are caused by alcohol consumption is not clear.

The relationship between alcohol consumption and cardiovascular diseases is rather complex. Light to moderate drinking can have a beneficial impact on morbidity and mortality for ischaemic heart disease and ischaemic stroke. However, the beneficial cardioprotective effect of drinking disappears with heavy drinking occasions. Roerecke and Rehm (2011) have shown, based on meta-analyses, that, on average, light to moderate drinkers experienced no protective effect if they reported at least one heavy drinking occasion per month. Moreover,

excessive alcohol consumption has detrimental effects on hypertension, cardiac dysrhythmias and haemorrhagic stroke, regardless of the drinking pattern (Rehm *et al*, 2010).

In the case of gastrointestinal diseases, liver cirrhosis and pancreatitis (both acute and chronic) can be caused by alcohol consumption. Higher levels of alcohol consumption create an exponential risk increase. The impact of alcohol is so large for both disease categories that there are subcategories that are labelled as “alcoholic” or “alcohol-induced”.

Alcohol consumption has been identified as carcinogenic for the following cancer categories (Baan *et al*, 2007): cancers of the colorectum, female breast, larynx, liver, oesophagus, oral cavity and pharynx. The higher the consumption of alcohol, the greater the risk for these cancers: even the consumption of two drinks per day causes an increased risk for some cancers, such as breast cancer (Hamajima *et al*, 2002).

Heavy drinking has been linked to suicide and violence and almost all categories of unintentional injury are impacted by alcohol consumption. The effect is strongly linked to the level of alcohol concentration in the blood and the resulting effects on psychomotor abilities. Higher levels of alcohol consumption create an exponential risk increase. This kind of unintentional injuries include road traffic accidents, falls, drowning, poisoning and others (WHO, 2011b).

In addition to the disease and injury categories mentioned, new evidence points to a causal link between alcohol and infectious diseases. Namely, alcohol consumption weakens the immune system, thus enabling infections by pathogens, which cause pneumonia and tuberculosis. This effect is markedly more pronounced with heavy drinking and there may be a threshold effect (Lönnroth *et al*, 2008). A strong association exists between alcohol consumption and HIV infection and sexually transmitted diseases (Baliunas *et al*, 2009). It may be that a common third cause, such as having particular personality traits, impacts on both alcohol consumption and risky sexual behaviour leading to infectious diseases (Shuper *et al*, 2010). However, there is a clear causal effect of alcohol consumption on HIV/AIDS patients' adherence to antiretroviral treatment (Hendershot *et al*, 2009).

## **Alcohol Abuse and Dependence**

According to Diagnostic and Statistical Manual of Mental Disorders, fourth edition, DSM-IV, (American Psychiatric Association, 1994), criteria for alcohol abuse are:

1. A maladaptive pattern of alcohol abuse leading to clinically significant impairment or distress, as manifested by one or more of the following, occurring within a 12-month period:
  - Recurrent alcohol use resulting in failure to fulfil major role obligations at work, school, or home (e.g., repeated absences or poor work performance related to substance use; substance-related absences, suspensions or expulsions from school; or neglect of children or household).
  - Recurrent alcohol use in situations in which it is physically hazardous (e.g., driving an automobile or operating a machine).
  - Recurrent alcohol-related legal problems (e.g., arrests for alcohol-related disorderly conduct).
  - Continued alcohol use despite persistent or recurrent social or interpersonal problems caused or exacerbated by the effects of the alcohol (e.g., arguments with spouse about consequences of intoxication or physical fights).
2. These symptoms must never have met the criteria for alcohol dependence.

Alcohol dependence, according to DSM-IV, is characterized by a maladaptive pattern of alcohol use, leading to clinically significant impairment or distress, as manifested by three or more of the following seven criteria, occurring at any time in the same 12-month period:

1. Tolerance, as defined by either of the following:
  - A need for markedly increased amounts of alcohol to achieve intoxication or desired effect.
  - Markedly diminished effect with continued use of the same amount of alcohol.
2. Withdrawal, as defined by either of the following:
  - The characteristic withdrawal syndrome for alcohol (refer to DSM-IV for further details).
  - Alcohol is taken to relieve or avoid withdrawal symptoms.
3. Alcohol is often taken in larger amounts or over a longer period than was intended.

4. There is a persistent desire or there are unsuccessful efforts to cut down or control alcohol use.
5. A great deal of time is spent in activities necessary to obtain alcohol, use alcohol or recover from its effects.
6. Important social, occupational, or recreational activities are given up or reduced because of alcohol use.
7. Alcohol use is continued despite knowledge of having a persistent or recurrent physical or psychological problem that is likely to have been caused or exacerbated by the alcohol (e.g., continued drinking despite recognition that an ulcer was made worse by alcohol consumption).

Alcohol has pleiotropic effects on multiple systems, which may explain the diverse neuropsychiatric and medical pathology in alcohol dependence and abuse. Alcohol dependence is a chronic relapsing disorder with a heritability rate of more than 0.5 (Goldman *et al*, 2005; Heath *et al*, 1997), the heritability being higher for more severe forms of alcohol problems (Pickens *et al*, 1995).

The inborn differences in the activity of several neurotransmitter systems are one important reason why some individuals initiate alcohol drinking and others do not. This might as well explain the initiation of alcohol use early in life. The presence/absence of one or several alternative alleles of particular genes therefore results in an alcohol-sensitive phenotype that in the appropriate environment may lead to the initiation of alcohol drinking because the response of these individuals to appetitive properties of this drug will be much greater. Maintenance of alcohol consumption once an alcohol drinking behaviour is established, further chronic alcohol consumption influences brain function by altering the balance between inhibitory and excitatory neurotransmission through different neurotransmitter and neuropeptide systems (Vengeliene *et al*, 2008).

The first adoption-study evidence for an important genetic contribution to alcoholism risk was produced in Scandinavia. In Copenhagen, Denmark, Goodwin *et al* (1973, 1974, 1977a,b) used official registries to identify biological parents who had histories of alcoholism and who had given up a child for early adoption by nonrelatives. According to these findings, rates of alcoholism are significantly elevated in both the adopted and nonadopted sons of alcoholics, results which are consistent with a genetic influence on alcoholism risk in men. Results for women were found to be nonsignificant.

Despite earlier suggestions that alcohol dependence may be more heritable in men than in women, it has established that a similar proportion of variation in liability to alcohol dependence in men and women can be explained by additive genetic factors (Agrawal & Lynskey, 2008; Heath, 1995; Magnusson *et al*, 2010; Sartor *et al*, 2008). In a study by Ehlers *et al* (2010), among adults participating in the University of California San Francisco family study of alcoholism, women were even found to have a higher heritability estimate for alcohol dependence than men.

### **Alcohol Consumption in Estonia**

According to WHO's latest report on alcohol and health (WHO, 2011b), average adult and late teens (15+ years) per capita pure alcohol consumption (recorded and unrecorded) in the world in 2006 was 6.2 litres. Comparing different regions in the world, it was highest in Europe: 12.2 litres. In Estonia it was 15.6 litres. Prevalence estimates of alcohol use disorders among adults and late teens (15+ years) in 2006 among Estonians were 11.1% in male population and 1.6 % in female population which are one of the highest in Europe.

The consumption of alcoholic beverages by Estonian inhabitants had decreased every year since 2008 probably due to recession and amounted 9,7 liters per capita in pure alcohol in 2010. Yet, the indicator is still high. In 2010, there were more than 8,200 people in 2010 consulting specialists with direct alcohol-related diseases and the treatment costs of alcohol-related diseases together with drug compensations amounted to over 2.3 million EUR (Estonian Institute of Economic Research, 2011).

## **Overview of Serotonin (5-HT) and Its Effects on Behavior**

### **The Structure, Synthesis, and Inactivation of 5-HT**

Serotonin or 5-hydroxytryptamine (5-HT) is an amine known for about 60 years. It is a monoamine neurotransmitter - it contains one amino group that is connected to an aromatic ring by a two-carbon chain (-CH<sub>2</sub>-CH<sub>2</sub>-). Along with melatonin, it belongs to a subgroup of tryptamines. All monoamines are derived from aromatic amino acids by the action of aromatic amino acid decarboxylase enzymes. In animals including humans, 5-HT is

synthesized from the amino acid L-tryptophan by a short metabolic pathway consisting of two enzymes (Fig. 2): tryptophan hydroxylase and amino acid decarboxylase (Neckameyer *et al*, 2007).

In the brain, 5-HT biosynthesis depends on the quantity of tryptophan which crosses the blood-brain barrier. Only free plasma tryptophan, i.e. unbound to albumin, penetrates into the brain; decrease of its free ratio reduces its penetration. Moreover, other amino acids are in competition with free tryptophan and limit its entry in the brain. Plasma cortisol, whose level is increased in depressed patients, decreases free L-tyrosine and free L-tryptophan concentrations in plasma, i.e. the forms which penetrate into the brain. Insulin, of which secretion is increased by carbohydrates, has an opposite effect and decreases the concentration of the amino acids other than tryptophan (Pharmacorama, n.d.).

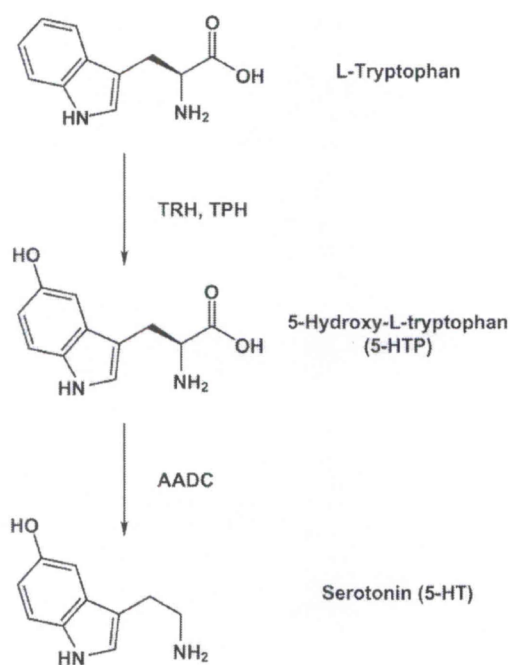


Fig. 2. 5-HT synthesis pathway (Watanabe *et al*, 2011).

As other transmitters, serotonin released in the synaptic cleft is mainly reuptaken by the presynaptic terminations by active transport with a specific carrier. 5-HT is converted into inactive molecules by oxydative deamination of the lateral amino chain by monoamine oxidase, leading to 5-hydroxy-indol-acetaldehyde which is then oxidized into 5-hydroxy-indol-acetic acid (5-HIAA) (Popova *et al*, 2001). The drugs which inhibit the inactivation of serotonin have antidepressant effects.

## 5-HT Receptors

Molecular biological methods have now been used to identify and clone 14 structurally and pharmacologically distinct mammalian 5-HT receptor subtypes (Barnes & Sharp, 1999; Popova & Naumenko, 2012). 5-HT receptors are classified into 7 types: 5-HT<sub>1</sub>, 5-HT<sub>2</sub>, 5-HT<sub>3</sub>, 5-HT<sub>4</sub>, 5-HT<sub>5</sub>, 5-HT<sub>6</sub> and 5-HT<sub>7</sub>. Each type can have subtypes A, B and so on. The evolutionary relationship between known human 5-HT receptor protein sequences can be seen in Fig. 3.

5-HT receptors are coupled to G-proteins except 5-HT<sub>3</sub> which are ionotropic - in the activated state, they are open and permeable to sodium and potassium cations (Barnes & Sharp, 1999). G-protein coupled receptors consist of a polypeptide chain containing 7 transmembrane domains forming a family with extracellular loops and N-terminal region transmitter binding sites. The third cytoplasmic loop and the C-terminal are responsible for the linkage with G-protein. Binding of transmitter leads to a change in the conformation of the receptor, resulting in dissociation of the G-protein into its subunits. These subunits have catalytic activity, such that they can alter intracellular processes (Popova & Naumenko, 2012).

The majority of 5-HT receptors are postsynaptic but receptors such as 5-HT<sub>1A</sub> and 5-HT<sub>1B</sub> are mainly presynaptic and modulate serotonin release. The signalling pathways to which these receptors are coupled are known but it is hardly possible to systematize clinical effects corresponding to their stimulation (Pharmacorama, n.d.).

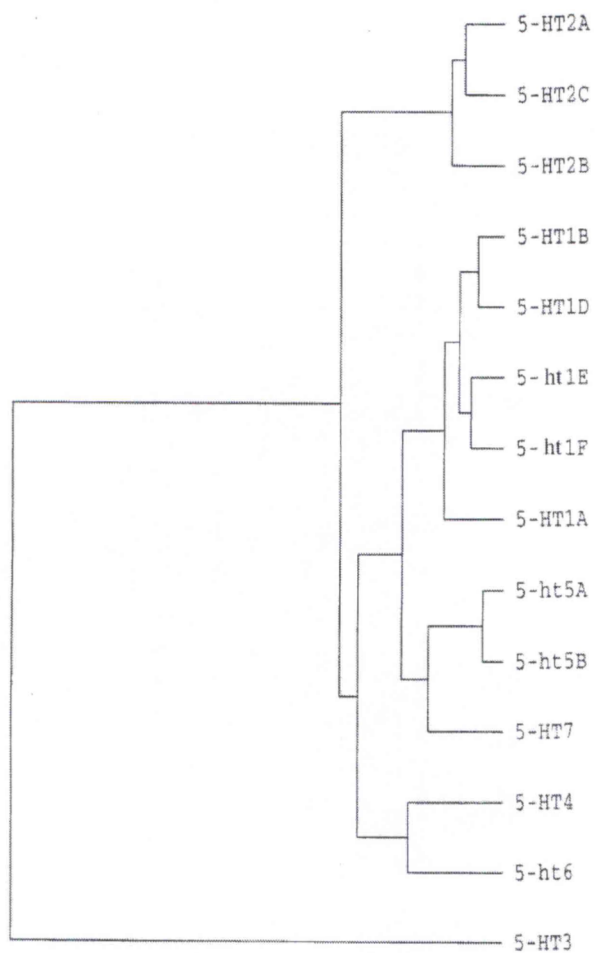


Fig. 3. Dendrogram showing the evolutionary relationship between various human 5-HT receptor protein sequences (Barnes & Sharp, 1999).

## 5-HT in Central Nervous System and Periphery

5-HT receptors have been found in virtually all organs (Hoyer *et al*, 2002; Siddiqui *et al*, 2005). Digestive tract contains about 95% of the total amount of 5-HT in the body, localized in enterochromaffin cells (Berger *et al*, 2009). In the central nervous system (CNS) of all species, higher concentrations are found in brainstem than in cortex. Less than one in a million CNS neurons produce 5-HT (Mengod *et al*, 2007). However, brainstem 5-HT neurons send ascending projections that terminate in a defined and organized manner in cortical, limbic, midbrain, and hindbrain regions (Fig. 4). All brain regions express multiple 5-HT receptors in a receptor subtype-specific fashion (Mengod *et al*, 2007). Additionally, individual neurons may express multiple 5-HT receptors.

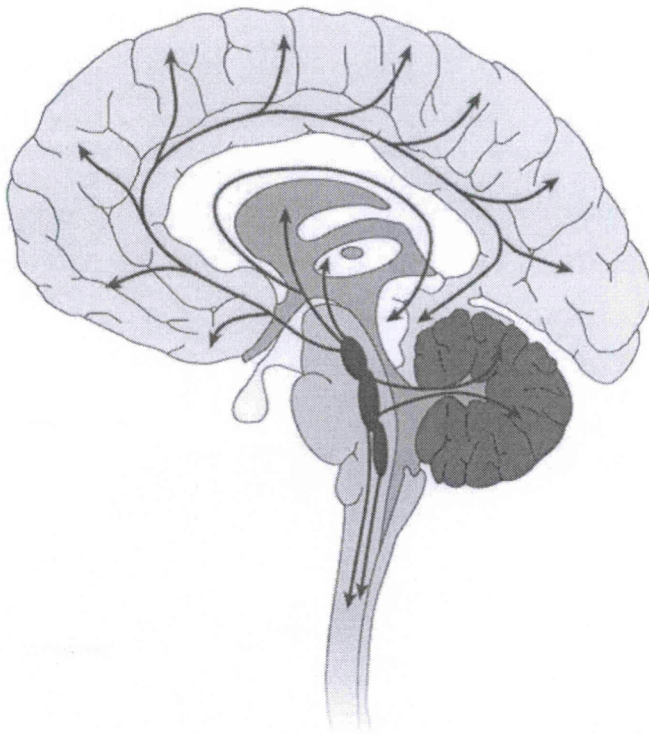


Fig. 4. Central serotonergic pathways (Berger *et al*, 2009).

Practically all blood 5-HT (concentration going from 100 to 200 micrograms per liter) is found in platelets which do not synthesize it, but take it from plasma where it is released by enterochromaffin cells. 5-HT released from platelets in plasma has a relatively localized effect on the vessels where it is released, for example during migraine (Pharmacorama, n.d.). When the platelets bind to a clot, they disgorge serotonin, where it serves as avasoconstrictor and helps to regulate hemostasis and blood clotting.

## Functions of 5-HT

Both within the CNS and throughout the body, 5-HT plays a number of roles in vascular biology, ranging from the control of vascular resistance and blood pressure to the control of hemostasis and platelet function. 5-HT also is a growth factor for some types of cells, which may give it a role in wound healing (Berger *et al*, 2009). 5-HT causes vasoconstriction or vasodilation in different vascular beds depending on the particular receptors that are expressed in each vessel wall and surrounding smooth muscle tissue (Kaumann & Levy, 2006). It regulates several different aspects of cardiac function, ranging from electrical conduction to valvular closure to post myocardial infarction remodeling. It also helps control breathing and respiratory drive through effects on brainstem respiratory control centers as well as on the pulmonary vasculature. 5-HT regulates digestion at multiple levels within the human gastrointestinal system and throughout the phylogenetic spectrum (Tecott, 2007).

5-HT and its receptors are important in the regulation of virtually all brain functions, and dysregulation of the serotonergic system has been implicated in the pathogenesis of many psychiatric and neurological disorders (Roth & Xia, 2004). As described by Berger *et al* (2009), 5-HT modulates virtually all human behavioral processes. The behavioral and neuropsychological processes modulated by 5-HT include mood, perception, reward, anger, aggression, appetite, memory, sexuality, and attention, among others. Indeed, it is difficult to find a human behavior that is not regulated by 5-HT.

A primary mechanism of the stress response is the activation of the hypothalamus–pituitary–adrenal (HPA) axis resulting in an enhanced secretion of cortisol. The serotonergic system influences HPA axis regulation by either stimulating or inhibiting its activity. Dense serotonergic projections, arising primarily from the dorsal and median raphé nuclei, innervate limbic structures including hippocampus and amygdala as well as the anterior hypothalamus (Hensler, 2006). The serotonergic system has been identified as a primary system involved in pre- and early postnatal ‘programming’ of the developing HPA axis with presumably long-term consequences for disease susceptibility in later life (Andrews & Matthews, 2004). Common psychiatric diseases, including major depression, have repeatedly been found to be associated with disturbances of both the HPA axis as well as the brain serotonergic system (Wüst *et al*, 2009).

## The Effect of 5-HT on Ethanol Consumption in Animal Models

The role of 5-HT in alcohol consumption have been vastly studied on animal models. The combination of gene deletion studies and pharmacological experiments in alcohol-preferring rat lines has produced a consistent set of results, and has provided a picture of the causes underlying high chronic alcohol consumption. 5-HT has been found to have only a minor role in mediating sensitivity to high doses of alcohol (reviewed by Vengeliene *et al*, 2008). Yet, in contrast, serotonergic system has been found to be crucial in the initiation of alcohol reinforcement. It has been shown that alcohol potentiates the action of 5-HT via the 5-HT<sub>3</sub> receptor (Lovinger & Zhou, 1994), and it is suggested that inborn serotonergic dysfunction might be of importance for the initial alcohol preference. For instance, low levels of 5-HT in several limbic structures have been identified in different alcohol-preferring rat lines as compared to their alcohol non-preferring counterpart lines (McBride & Li, 1998).

In addition, 5-HT<sub>3</sub> receptor antagonists have been shown to suppress the acquisition of voluntary alcohol consumption in alcohol-preferring rats (Rodd-Henricks *et al*, 2000). Administration of a 5-HT<sub>1A</sub> agonist, known to decrease 5-HT release, into median raphe nucleus was found to reinstate alcohol seeking (Lê & Funk, 2005), whereas systemic injections of 5-HT uptake blockers as well as 5-HT<sub>3</sub> antagonists attenuated intermittent foot-shock stress-induced reinstatement of alcohol seeking in rats (Lê *et al*, 1999, 2006; Lê & Funk, 2005). Free-choice alcohol drinking by alcohol-preferring rats was determined to alter 5-HT neurotransmission in the *nucleus accumbens* during a deprivation phase (reviewed by Vengeliene *et al*, 2008).

In mice, deletion of 5-HT<sub>1B</sub> receptors has been shown to increase alcohol intake (Crabbe *et al*, 1996). Deletion of 5-HT transporters (Kelaiï *et al*, 2003) or overexpression of 5-HT<sub>3</sub> receptors (Engel *et al*, 1998) has been determined to lead to a reduction in alcohol self-administration as compared with control mice. Pharmacological manipulations of 5-HT system activity revealed that administration of serotonin reuptake inhibitors, 5-HT<sub>1</sub>, 5-HT<sub>2</sub> and 5-HT<sub>3</sub> agonists as well as antagonists were capable of reducing alcohol consumption in common stock as well as alcohol-preferring animals (reviewed by Vengeliene *et al*, 2008).

## **A Functional Ins/Del Polymorphism in the 5-HT Transporter Gene - 5-HTTLPR**

The level of 5-HT in the synaptic cleft is mainly regulated by the 5-HT transporter (5-HTT) (Voineskos *et al*, 2007). The human 5-HTT gene (SLC6A4), located on chromosome 17q11.1–q12 (Lesch *et al*, 1994), contains a 5-HTT-linked polymorphic region (5-HTTLPR) with two functional variants: the short (S) form involving 14 copies of a 20-23 bp imperfect repeated sequence and the long (L) form involving 16 copies (Frik & Markus, 2009; Lesch *et al*, 1996). Due to a functional A→G substitution within the L allele, two functional variants exist within the L allele – L<sub>A</sub> and L<sub>G</sub>. The latter one – L<sub>G</sub> allele – which is the L allele with a common G substitution, creates a functional AP2 transcription-factor binding site and has a rate of 5-HTT expression comparable to the S (Hu *et al*, 2006). The short form of the 5-HTTLPR gene has been found to be less active than the L<sub>A</sub> allele, resulting in reduced transcriptional efficiency of the 5-HTT gene, decreased 5-HTT expression, and reduced 5-HT uptake relative to the long form (Greenberg *et al*, 1999; Lesch *et al*, 1996).

The allele frequencies between different populations vary: the short allele frequency among Caucasians has been determined to be about 0.4, among Asians 0.8 and among Africans 0.4 (Gelernter *et al*, 1997; Kunugi *et al*, 1997; Mannelli *et al*, 2006; Ng *et al*, 2006; Serretti *et al*, 2006).

Carriers of the short allele display increased amygdala reactivity to fearful stimuli (Hariri *et al*, 2002), reduced amygdala volume (Pezawas *et al*, 2005) and enhanced functional coupling between the amygdala and the ventromedial prefrontal cortex (Heinz *et al*, 2005). The short allele has been associated with anxiety (Goldman *et al*, 2005), increased risk of depression, poorer responses to the antidepressant effects of SSRIs, increased vulnerability to tryptophan depletion, and abnormal emotional processing (Frik & Markus, 2009). Carriers of the low-expressing 5-HTTLPR allele report a more negative attributional style, endorse more negative thoughts following a mood induction, display biased attention for emotional stimuli, recall more negative self-referent words, and report higher levels of rumination than long allele homozygotes (reviewed by Beevers *et al*, 2009).

A number of studies have investigated the role of 5-HTTLPR in alcohol consumption, with contradictory results (reviewed by Dick & Foroud, 2003; McHugh *et al*, 2010). In some studies, an association between the short allele of 5-HTTLPR and excess alcohol consumption (Covault *et al*, 2007; van der Zwaluw *et al*, 2010) alcohol dependence (Feinn *et al*, 2005; Hallikainen *et al*, 1999; Hammoumi *et al*, 1999; Lichtermann *et al*, 2000; McHugh *et al*,

2010; Sander *et al*, 1997) or binge drinking (Matsushita *et al*, 2001) has been determined. In others, the long 5-HTTLPR allele has been found to be associated with earlier onset of alcohol use (Twitchell *et al*, 2001) and dependence (Ishiguro *et al*, 1999), alcoholism (Parsian & Cloninger, 2001; Philibert *et al*, 2008; Schuckit *et al*, 1999), and compulsive craving in alcohol dependence (Bleich *et al*, 2007). In a study by Kaufman *et al* (2007), heterozygous children (L/S) were shown to have the greatest vulnerability to early alcohol use. Other studies have found no evidence of association between 5-HTTLPR and alcohol use (Edenberg *et al*, 1998; Köhnke *et al*, 2006; Preuss *et al*, 2000; Rasmussen *et al*, 2009; Shin *et al*, 2010; Thompson *et al*, 2010).

## **Overview of Neuropeptide Y (NPY) and Its Effects on Behavior**

### **NPY and Related Peptides**

Neuropeptide Y is the most abundant neuropeptide in the brain and is a widely diffused system that is involved in the regulation of multiple biological functions. NPY was originally isolated from porcine brain (Tatemoto, Carlquist, & Mutt, 1982). Lundberg and Tatemoto were the first to find the biological activity of NPY in 1982, demonstrating that NPY induced potent vasoconstriction (Tatemoto, 2004).

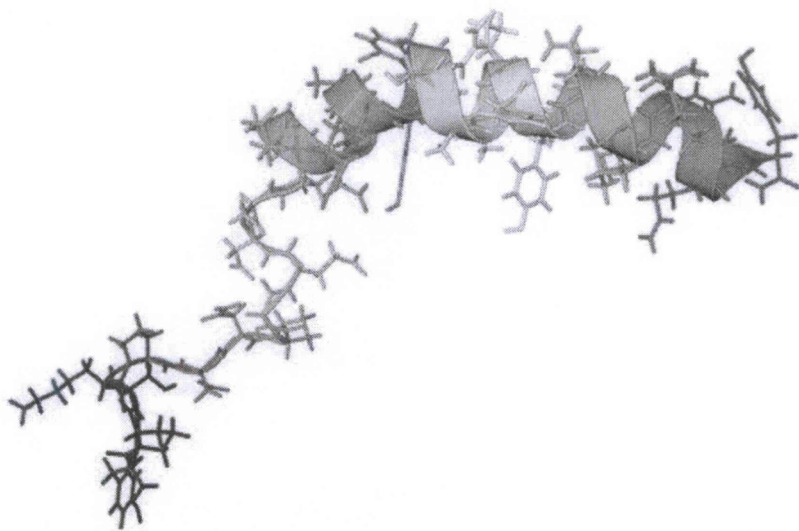
Neuropeptide Y (NPY), along with pancreatic peptide Y (PPY) and pancreatic polypeptide (PP) are often said to belong to the "pancreatic polypeptide family". A comparison of the primary structures of NPY, PYY and PP reveals a high degree of sequence homology between NPY and PYY, with a lesser degree of homology between NPY and PP, as shown in Fig. 5. However, because of the fact that NPY has been much more conserved during evolution and also exhibits much greater biological activity when compared to PP, this peptide family should more appropriately be called the NPY family (Larhammar *et al*, 1993).

		homology
NPY	<u>Y</u> <u>P</u> <u>S</u> <u>K</u> <u>P</u> <u>D</u> <u>N</u> <u>P</u> <u>G</u> <u>E</u> <u>D</u> <u>A</u> <u>P</u> <u>A</u> <u>E</u> <u>D</u> <u>L</u> <u>A</u> <u>R</u> <u>Y</u> <u>Y</u> <u>S</u> <u>A</u> <u>L</u> <u>R</u> <u>H</u> <u>Y</u> <u>I</u> <u>N</u> <u>L</u> <u>I</u> <u>T</u> <u>R</u> <u>Q</u> <u>R</u> <u>Y</u> *	100%
PYY	<u>Y</u> <u>P</u> <u>A</u> <u>K</u> <u>P</u> <u>E</u> <u>A</u> <u>P</u> <u>G</u> <u>E</u> <u>D</u> <u>A</u> <u>S</u> <u>P</u> <u>E</u> <u>E</u> <u>L</u> <u>S</u> <u>R</u> <u>Y</u> <u>Y</u> <u>A</u> <u>S</u> <u>L</u> <u>R</u> <u>H</u> <u>Y</u> <u>L</u> <u>N</u> <u>L</u> <u>V</u> <u>T</u> <u>R</u> <u>Q</u> <u>R</u> <u>Y</u> *	69%
PP	<u>A</u> <u>P</u> <u>L</u> <u>E</u> <u>P</u> <u>V</u> <u>Y</u> <u>P</u> <u>G</u> <u>D</u> <u>D</u> <u>A</u> <u>T</u> <u>P</u> <u>E</u> <u>Q</u> <u>M</u> <u>A</u> <u>Q</u> <u>Y</u> <u>A</u> <u>A</u> <u>E</u> <u>L</u> <u>R</u> <u>R</u> <u>Y</u> <u>I</u> <u>N</u> <u>M</u> <u>L</u> <u>T</u> <u>R</u> <u>P</u> <u>R</u> <u>Y</u> *	50%

Fig. 5. Comparison of the amino acid sequences of neuropeptide Y (NPY), peptide YY (PYY), and pancreatic polypeptide (PP) (porcine peptides). An asterisk indicates the amidated C-terminus. Identities are underlined (Tatemoto, 2004).

### The Structure of NPY

NPY is a linear 36-amino-acid polypeptide that consists of an alpha-helix folded underneath a proline helix with a tyrosine residue at the carboxy terminus (Bhaskar *et al*, 2007). Since NPY contains many tyrosine (Y) residues in its structure, it was named neuropeptide Y to distinguish it from PYY that possesses a very similar structure to NPY (Tatemoto, 2004). A characteristic tertiary structure is observed in NPY (as well as in PYY and PP) which consists of an N-terminal polyproline helix (residues 1–8) and an amphiphilic alpha-helix (residues 15–30), connected with a beta-turn, creating a hairpin-like loop (Fig. 6). This domain has been identified from the crystal structure of avian PP, and nuclear magnetic resonance studies also agree well with this three-dimensional configuration. The helices are kept together by hydrophobic interactions. The amidated C-terminal end (residues 30–36) projects away from the hairpin loop (Wahlestedt & Heilig, 1995; Bhaskar *et al*, 2007).



SCIENCEPHOTOLIBRARY

Fig. 6. The structure of NPY (Science Photo Library Ltd., 2011).

### NPY Receptors

Five NPY receptor subtypes have been identified in humans (Y1, Y2, Y4, Y5, Y6) and at least one other (Y3) has been suggested on the basis of pharmacological evidence (Protas *et al*, 2003). NPY receptors are a family of seven transmembrane G-protein coupled receptors (7t-GPCR), designated collectively as Y receptors that are expressed throughout the central and peripheral nervous system (Misra *et al*, 2005). These receptors are metabotropic, causing metabolic changes in the target cell rather than directly opening ion channels. The nomenclature reflects the large number of tyrosine (Y in the single letter code) present in their endogenous ligands (Berglund *et al*, 2003). These receptor subtypes have been found to share only modest sequence homologies (30–50%). NPY receptors have variable affinity to different NPY fragments as well as to PYY and PP (Blomqvist & Herzog, 1997). Some NPY receptors are structurally more related to G protein-coupled receptors outside of the NPY receptor family (Patel & Patel, 2010). Each of the receptor subtypes seems to be characterized by a distinct tissue localization and unique pharmacological profile (Tatemoto, 2004). The structural differences among NPY receptors are beneficial to drug discovery efforts since compounds with high affinity for a particular NPY receptor are less likely to interact with other NPY receptors (Patel & Patel, 2010).

## NPY in Peripheral and Central Nervous System

NPY-like immunoreactivity is widely and unevenly distributed in the human brain, and it is the most abundant neuropeptide known (Tatemoto, 2004). Similar to "classical" neurotransmitters, NPY seems to have a wide range of effects on peripheral (blood vessels, heart, airways, gastrointestinal tract, kidney, pancreas, thyroid gland, platelet, mast cells, and sympathetic, parasympathetic and sensory nerves) and central (effects on pituitary hormone release, behavior, central autonomic control, and other neurotransmitter mechanisms) targets. A number of these actions appear to be exerted by NPY per se, whereas others occur as a result of modulatory interactions with other agents (Wahlestedt & Heilig, 1995).

NPY is released from sympathetic neurons and exerts short-term (acute) effects on prejunctional nerve terminals and postjunctional cardiac ion channels. However, NPY also exerts long-term (trophic) effects on angiogenesis, cardiac hypertrophy, autonomic signaling, and cardiac ion channels, including effects on L-type Ca<sup>2+</sup> and pacemaker channels (Protas *et al*, 2003).

In the peripheral nervous system, NPY is located in postganglionic sympathetic neurons, adrenal medulla, enteric neurons, cardiac nonsympathetic neurons, certain noradrenergic perivascular neurons and parasympathetic neurons (Patel & Patel, 2010). There are dense plexuses of NPY-like immunoreactivity found in vascular beds throughout the body. In addition, NPY also occurs in nonadrenergic perivascular, enteric, and cardiac nonsympathetic and parasympathetic nerves (Wahlestedt & Heilig, 1995).

Neurons displaying NPY-like immunoreactivity are abundant in the central nervous system. NPY containing neuronal cell bodies are found primarily in the locus coeruleus, the nucleus of the solitary tract and the arcuate nucleus of the hypothalamus. In addition, these NPY containing neuronal cell bodies often contain other neurotransmitters, such as noradrenaline, and send projections throughout the brain; hence, NPY can be found in most brain regions, particularly in the cortex, hippocampus, thalamus, hypothalamus and brainstem (Laura *et al*, 2004). The highest concentrations of NPY have been found in the paraventricular hypothalamic nucleus, hypothalamic arcuate nucleus, suprachiasmatic nucleus, median eminence, dorsomedial hypothalamic nucleus, and paraventricular thalamic nucleus (Chronwall *et al*, 1985; Tatemoto, 2004). Coexistence with somatostatin and NADPH-

diaphorase/nitric oxide synthase is common in cortex and striatum. Although NPY neurons in the latter brain regions receive few inputs, they make numerous contacts with dendrites, including GABAergic neurons. NPY is also extensively colocalized with GABA in the cortex, but not in the striatum (Wahlestedt & Heilig, 1995).

The orexigenic effects of NPY are mediated through the hypothalamus, while the anxiolytic effects of NPY appear to be mediated through the amygdala (Patel & Patel, 2010). The amygdala receives dense NPY innervations from the nucleus of the solitary tract, arcuate nucleus, and lateral septum (Chronwall *et al*, 1985) and contains a moderate amount of NPY Y1 receptors, which are important for the anxiolytic effects of NPY (Primeaux *et al*, 2005).

It has also been demonstrated that the NPY occurs in a variety of brainstem monoaminergic neurons — coexisting with norepinephrine (the A1 group in the ventrolateral medulla, the A2 group of the dorsal medulla and the locus coeruleus), epinephrine (C1 and C2 groups and solitary nucleus), or serotonin (nucleus raphe pallidus) (Wahlestedt & Heilig, 1995).

## **Functions of NPY**

NPY has a role in the regulation of a variety of physiological processes, for example vasoconstriction, nasal congestion, blood pressure, intestinal motility, anxiety, depression, pain, feeding, reproductive endocrinology, neuronal excitability and memory retention (reviewed by Patel & Patel, 2010); energy metabolism, insulin release, stimulating lipoprotein lipase activity in white adipose tissue, inhibiting norepinephrine release and potentiating norepinephrine response, stimulating proliferation of human vascular smooth muscle cells, and promoting the overall capillary tube and vessel development (Jia *et al*, 2005). NPY and its expression has also been extensively studied in the CNS where it serves as a neurotransmitter and/or a modulator of the neuronal function.

NPY has an important role in the feeding behaviour. The “axis of hunger” is from arcuate nucleus of the hypothalamus to the paraventricular nucleus of the hypothalamus (de Quidt & Emson, 1986). Feeding behaviour has mainly been studied in animals (Kaipio, 2009). For example, injection of NPY into the hypothalamus of rats potently stimulates food intake and decreases energy expenditure while it simultaneously induces lipogenic enzymes in liver

and white adipose tissue; continuous or repeated central administration of NPY leads to obesity (Schwartz *et al*, 2000).

NPY signaling system has also been implicated in body weight regulation in humans (Ding *et al*, 2005). Cerebrospinal fluid NPY concentrations have been found to be significantly elevated in anorectic and bulimic patients (Kaye *et al*, 1990). NPY and its receptors have been of interest in the development of anti-obesity drugs. Data from the antisense oligodeoxynucleotides or blockade of NPY action by intraventricular infusion of NPY antibody has provided rather convincing evidence that appropriate antagonism of NPY action could lead to useful therapies for treating obesity (Kaipio, 2009). Numerous studies concerning Y1, Y2, Y4 and Y5 receptors and the putative anti-obesity drugs have already been conducted (Kaipio, 2009). Nevertheless, further investigations involving simultaneous activation and inhibition of NPY receptors are still required (Kaipio, 2009; Kamiji & Inui, 2007; MacNeil, 2007; Patel & Patel, 2010).

As NPY promotes hunger, it simultaneously shuts off the sexual drive and reproduction (reviewed by Kalra & Kalra, 2004, and Kaipio, 2009). NPY has also an important role in the neuroendocrine regulation as it influences the secretion and release of many hormones such as corticotrophin releasing hormone, thyrotropin releasing hormone, luteinizing hormone, gonadotropin releasing hormone, and growth hormone releasing hormone (reviewed by Tatemoto, 2004, and Kaipio, 2009). The activity of the neuroendocrine system is closely associated with the energy homeostasis (Pedrazzini *et al*, 2003).

Numerous studies have shown that NPY helps to restore calm after stressful events (Mickey *et al*, 2011). Fuxe *et al* (1983) demonstrated that central administration of NPY induced EEG synchronization. It is therefore suggested by Tatemoto (2004) that NPY produces behavioral signs of sedation. NPY-like immunoreactivity has been found to be significantly lower in cerebrospinal fluid from patients with a major depressive disorder compared with healthy controls (Widerlov *et al*, 1988). Also, Widerlöv *et al* (1988) and Heilig *et al* (2004) found decreased plasma levels of NPY from depressive and suicidal patients. It was also found that antidepressant drugs increased the concentrations of NPY-like immunoreactivity in the brain (Heilig *et al*, 1988). In case of depression treated with repeated electroconvulsive shock treatments, NPY levels in the depressed patients have been observed to be elevated (reviewed by Thorsell, 2007). Subjects with lower levels of NPY have been found to be more responsive to negative stimuli in key brain circuits related to emotion - and

are therefore less resilient in the face of stress and may be at higher risk for developing a major depressive disorder (Mickey *et al*, 2011). These observations support the hypothesis that NPY is involved in the pathophysiology of depressive illness.

### **The Role of NPY in Ethanol Consumption**

The effects of NPY and alcohol drinking have been examined mostly in animal studies. Thiele *et al* (1998) first reported that NPY-deficient mice showed increased ethanol consumption, while transgenic mice with NPY overexpression had a lower preference for ethanol. In Indiana alcohol preferring rats the amount of NPY in amygdala, frontal cortex and hippocampus was significantly lower in comparison to Indiana alcohol non-preferring rats (Ehlers *et al*, 1998). Furthermore, infusion of NPY has been shown to reduce ethanol intake in alcohol preferring rats (Gilpin *et al*, 2003). In a study by Spence *et al* (2005), in the case of alcohol preferring rats, NPY mRNA expression was observed at significantly decreased levels in the nucleus accumbens, frontal cortex, amygdala, hippocampus, caudate putamen, and hypothalamus. These data suggest that alcohol consumption and resistance are inversely related to the NPY levels in the brain (Tatemoto, 2004). Although, in the study by Ehlers *et al* (1998), in the case of Indiana alcohol preferring rats the amount of NPY in hypothalamus was higher in comparison to Indiana alcohol non-preferring rats.

In 2002, Thiele *et al* reported that knockout mice lacking the Y1 receptor showed increased ethanol consumption. It is suggested that the Y1 receptor regulates voluntary ethanol consumption and some of the intoxicating effects caused by administration of ethanol. It was shown by Thorsell *et al* (2002) that blockade of central Y2 receptors by a Y2 receptor antagonist, BIIE0246, reduced ethanol self-administration in rats. It was therefore suggested that the Y2 receptor would be a candidate target for developing pharmacological treatments for alcoholism (Tatemoto, 2004).

As mentioned before, NPY is anxiolytic and its release is induced by stress (Zhou *et al*, 2008). NPY possesses anxiolytic properties when infused into the area of amygdala (reviewed by Kaipio, 2009). Clinical studies of alcohol dependence have shown an association between initial anxiety and alcohol abuse possibly due to the anxiolytic action of alcohol (Pandey *et al*, 2003a; Pandey *et al*, 2003b). The hippocampal NPY system has found to be downregulated during ethanol withdrawal anxiety states (Olling *et al*, 2007). The brain

tissue from alcoholics has been found to have significantly lower NPY expression than brain tissue from controls (Mayfield *et al*, 2002). However, it is not clear if the decreased NPY levels observed were present before alcoholism began or are the consequence of alcohol consumption (Francès *et al*, 2011).

### **The Gene Encoding NPY**

The NPY gene is located on chromosome 7q15.1 and is about 8 kb in length with four exons interrupted by three introns of 965, 4300 and 2300 bp in length (Baker *et al*, 1995). NPY gene produces a precursor protein that includes a signal peptide, mature NPY and a carboxyl terminal flanking peptide (Minth *et al*, 1984).

The gene is highly conserved with 92% amino acid sequence identity between the cartilaginous fish *Torpedo marmorata* and mammals, which are separated by an evolutionary distance of more than 400 million years (Larhammar, 1996). This high level of conservation indicates that NPY presumably has a critical physiological function and that interindividual variation at this locus is likely to be minimal (Bhaskar *et al*, 2007).

The NPY gene is expressed in cells derived from the neural crest, and several factors are involved in its regulation. Consensus sequences for a number of DNA-binding proteins that could act as regulatory factors are contained within the NPY gene. These include five potential GC-rich SP-1 binding sites: two CCCCTC sites, a partial CAAT box, and one AP-1 binding site. Additional factors regulating NPY gene expression include activators of cyclic AMP and calcium- or phospholipid-dependent protein kinases nerves (Wahlestedt & Heilig, 1995).

### **A Functional Polymorphism Affecting the Signal Peptide of the Prepro-NPY: rs16139**

Studies on humans investigating the effect of NPY genetic variations on alcohol consumption have mainly focused on rs16139 polymorphism - a thymidine-to-cytosine polymorphism at position 1128 (-1128T/C) in the NPY gene resulting in leucine-to-proline substitution at position 7 (Leu7Pro) in the signal peptide of the NPY, originally found in 1998 by Karvonen *et al* (Francès *et al*, 2011). This polymorphism is located in the signal peptide of the prepro-NPY (Karvonen *et al*, 1998). This change in the signal sequence has been

attributed to altered packaging of the hormone in granules of endocrine cells, resulting in higher levels of peptide secretion (Mitchell *et al*, 2008) without affecting the peptide's binding to its receptors (Ding *et al*, 2005).

The NPY signal sequence 7Pro allele frequency among different populations varies widely. In Caucasian populations the frequency of the Leu7Pro polymorphism varies from 6% to 15% (Pesonen, 2008) and for example in the Asian population this polymorphism is extremely rare (Jia *et al*, 2005). The most studied population regarding the preproNPY Leu7Pro polymorphism is the Finnish population, among whom the NPY 7Pro allele variant frequency is approximately 12% (Kaipio, 2009).

The effect of rs16139 on plasmatic NPY levels is little known and evidence is contradictory (Francès *et al*, 2011). Kallio *et al* (2001) found that individuals with the Leu7/Pro7 genotype had an average of 42% higher maximal increases of plasma NPY in response to physiological stress compared with Leu7/Leu7 individuals. In 2003, Kallio *et al* demonstrated that carriers of the 7Pro allele have lower plasma NPY levels. Leu7Pro has also been found to alter the secretion and packaging of NPY and increase the peptide synthesis and secretion (Mitchell *et al*, 2008; Pesonen, 2006). Yet, study by Jaakkola *et al* (2007) suggests that this polymorphism does not affect plasmatic NPY levels.

Association studies investigating the effect of this polymorphism on the risk of alcoholism are also contradictory (Francès *et al*, 2011). Some studies associate the presence of the 7Pro variant with an increased alcoholism risk (Köhnke *et al*, 2002; Lappalainen *et al*, 2002; Mottagui-Tabar *et al*, 2005) and increasing ethanol consumption (Kauhanen *et al*, 2000), others found a lower frequency of the 7Pro allele in alcoholics (Ilveskoski *et al*, 2001). Furthermore, Zhu *et al* (2003) did not find significant differences in genotype frequencies between alcoholics and nonalcoholics from Finland and Sweden, and nor did Zill *et al* (2008) and Hu *et al* (2005) in German population.

In addition, the given polymorphism has been studied in association with depression. In Swedish samples, the 7Pro allele was found to protect against depression (Heilig *et al*, 2004; Sjöholm *et al*, 2009). Yet, the 7Pro variation has later been found to be significantly more frequent in patients with major depression in a Danish sample (Koefoed *et al*, 2012).

## **A Functional Polymorphism in the Promoter Region of the NPY Gene: rs16147**

An NPY gene promoter region SNP rs16147 - a thymidine-to-cytosine polymorphism at position 485 (-485T/C), analogous to cytosine-to-thymidine polymorphism at position 399 (-399C/T), has been shown to significantly reduce transcriptional activity of the NPY gene (Itokawa *et al*, 2003).

According to National Center for Biotechnology Information (NCBI) database, allelic distribution of rs16147 T/C is about 0.5:0.5 in European samples („Reference SNP Cluster Report: rs16147“, n.d.). In African American and Sub-Saharan African samples, T allele has been found to be more common. In Asian samples, it is quite the opposite – the C allele is more frequent.

The given polymorphism has been shown to predict levels of NPY messenger RNA in post-mortem brain and lymphoblasts, and levels of plasma NPY (Zhou *et al*, 2008). Tissue culture experiments with cells of neuronal and non-neuronal origin found strongly increased reporter gene expression under the control of C allele carrying promoters (Buckland *et al*, 2005; Itokawa *et al*, 2003). The C allele of the rs16147 has also been associated with higher NPY expression levels in the anterior cingulate cortex, a brain area of critical importance for affective processing (Drevets *et al*, 2008). In the study by Zhou *et al* (2008), the C allele was associated with lower NPY mRNA expression in postmortem brain and lymphoblasts.

However, the direction of the effect (plasma levels of NPY predicted by rs16147) seems to depend on the environmental conditions under which the sample was drawn (Sommer *et al*, 2010). When blood samples were obtained under resting conditions reduced NPY plasma levels were found in rs16147 C allele carriers (Zhou *et al*, 2008), while increased NPY plasma levels in rs16147 C allele carriers were found in a large sample obtained under highly stressful, preoperative conditions (Shah *et al*, 2009).

In the study by Zhou *et al* (2008), lower NPY expression predicted higher emotion-induced activation of the amygdala, as well as diminished resiliency as assessed by pain/stress-induced activations of endogenous opioid neurotransmission in various brain regions; rs16147 was found to alter NPY expression in vitro and account for more than half of the variation in expression in vivo. The C allele was associated with lower NPY mRNA expression and therefore higher emotion-induced activation of the amygdala. A later study failed to replicate the association with anxious personality traits, but used a different measure,

neuroticism, which makes comparison difficult (Cotton *et al*, 2009). However, there was a trend toward significance in this study when diplotype-predicted NPY mRNA levels were used in the analysis. Domschke *et al* (2010) found the rs16147 C allele to be associated with stronger bilateral amygdala activation in response to threatening faces in an allele-dose fashion which points towards a possible influence of functional NPY gene variation on antidepressant treatment response in anxious depression, potentially conveyed by altered emotional processing.

Rs16147 T allele has been associated with schizophrenia (Itokawa *et al*, 2003), and C allele with depressive illness (Dannlowski, 2010; Heilig *et al*, 2004; Sommer *et al*, 2010) in some studies, but not in others. For example, in a Danish population study no association was found between rs16147 and schizophrenia and depression or panic disorder (Lindberg *et al*, 2006). Rs16147 had no effect on panic disorder also in a study by Domschke *et al* (2008).

In a study by Vergne *et al* (2010), it was reported that alcoholics with two copies of the T allele had significantly less heavy drinking days. The T/T genotype was also associated with lower alcoholism severity score, and those with T/T genotype showed less of an association between anxiety and alcohol symptoms compared to the C/C and C/T genotypes where the association was greater.

## **Neurobiological Interaction between 5-HT and NPY in Animal Models**

Evidence from animal models suggests that 5-HT and NPY neurons in the hypothalamus, which respectively inhibit and stimulate food intake, interact to control energy homeostasis (Dryden *et al*, 1996; Gehlert *et al*, 2008). The given agents have also been shown to interact in the suprachiasmatic nuclei in controlling circadian clock phase (Guy *et al*, 1987; Lall & Harrington, 2006; Marchant *et al*, 1997; Prosser, 1998, 2000; Ueda *et al*, 1995).

Structural non-synaptic appositions between 5-HT nerve endings and NPY-containing neurons have been demonstrated in the rat arcuate nucleus; such cellular relationships are proposed to constitute a morphological substrate for putative 5-HT/NPY interactions in neuroendocrine hypothalamus (Guy *et al*, 1988). It has also been shown that in the brainstem, NPY occurs in a variety of brainstem monoaminergic neurons, coexisting also with or

serotonin in nucleus raphe pallidus (Hendry, 1993). NPY has also been determined to compensate for the impaired function of the serotonergic systems in rats' central nervous system (Kondo *et al*, 1993).

## **Materials and Methods**

### **Subjects**

The data were derived from the Estonian sample of the European Youth Heart Study (EYHS); an international study addressing cardiovascular disease risk factors in children and adolescents. The first stage of data collection took place in 1998/1999. During the following decade, it continued longitudinally in the form of Estonian Children Personality Behavior and Health Study.

The sampling frame was a complete list of public schools in the Tartu County. Of the 56 schools approached, 54 agreed to participate. A random sample of 25 schools was selected using cluster sampling (urban and rural schools with younger and older children from Estonian and Russian language schools) and probability proportional to school size. Of each school sampled, all children in the age of 9 and 15 years were asked to participate in the study. Of all subjects invited to participate, 76% of children and their parents agreed. The agreement rate was the highest in urban Estonian girls and the lowest in younger Russian children (Harro *et al*, 2001). 83% of the subjects were Estonian and 17% Russian. 28% of the children lived in rural areas. Mean age of younger children was  $9,5 \pm 0,5$  and of older children  $15,6 \pm 0,6$  years. All parents gave written informed consent for their child to participate, and in turn, all children gave their written consent. The study was approved by the ethical committee of the University of Tartu (protocol no 49/30-1997). In total, 1176 subjects participated; 638 (54%) of them female and 538 (46%) male, 583 in the younger cohort and 593 in the older one.

In 2001, a follow-up study was performed with 462 adolescents from the older cohort: 400 (68%) of those who participated in the first wave, plus 62 adolescents who did not participate in 1998. The mean age of adolescents in the second wave was  $18,4 \pm 0,9$  years. Because of the high attrition rate, an additional 62 students who had not participated in 1998 were recruited from among participants' classmates to participate in 2001. The next follow-up

with the same cohort was performed in 2008, when 541 subjects in the mean age of  $24.7 \pm 0.7$  agreed to take part in the study again.

In 2004, 483 (83%) children from the original younger cohort participated again. The mean age of the subjects studied in 2004 was  $15.3 \pm 0.5$  years. The next follow-up with the same cohort took place in 2007, when 453 (78%) subjects from the original sample in the mean age of  $17.8 \pm 0.5$  took part in the study.

In the current study, data from both of the cohorts is used, but analyzed as separate samples. Biological samples were obtained from all but one male subject from the older cohort, and all but three from the younger cohort, making the final number of subjects in the older cohort 654 and in the younger cohort 580.

### **Estimation of Alcohol Consumption**

In 2008, when the subjects from the older cohort were on the average 25 years old, they filled self-report questionnaires that included questions about the age when they first consumed half a dosage of alcohol, how often they had consumed at least 5 or more dosages of alcohol during the previous 12 months (1="never", 2="less than once a month", 3="at least once a month", 4="at least once a week", 5="every day"; considered reflecting binge drinking), and whether they had deliberately been in an intoxicated state. One dosage of alcohol was defined as a glass of light wine or champagne (12 cl), a shot of vodka (4 cl), or a bottle (33 cl) of light alcohol (beer, long drink, cider, etc). The question regarding the age of the initial drink was also asked in 2001 and when the subject had not answered that question or not participated in 2008, the data from 2001 was used.

In addition, during the follow-up with the older cohort in 2008 the subjects were interviewed by an experienced psychologist in order to determine the occurrence of mental health disorders. All diagnoses were made according to Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV) criteria using Mini International Neuropsychiatric Interview (M.I.N.I.). It is a short, structured psychiatric interview mainly used for psychiatric evaluation and outcome tracking in clinical psychopharmacology trials and epidemiological studies. The Structured Clinical Interview for DSM-IV Axis I Disorders (SCID-I), a semi-structured interview for making and specifying the major DSM-IV Axis I diagnoses was also utilized when a past or ongoing episode of mental health disorder had been identified using

M.I.N.I. Among other disorders, the occurrence of alcohol use disorder (alcohol abuse or dependence) was identified.

Data regarding the younger cohort's alcohol use is derived from the self-report questionnaire filled by the participants in 2007 when they were on the average 18 years old. Questions about the age when they first consumed half a dosage of alcohol, how often they had consumed at least 5 or more dosages of alcohol during the previous 6 months (1="never", 2="once", 3="twice", 4="3-5 times", 5="5-10 times", 6="10-20 times", 7="more than 20 times"; considered reflecting binge drinking), and how often during the previous 6 months had they consumed such a large quantity of alcohol that they regarded themselves as having been very drunk (1="never", 2="once", 3="2-3 times", 4="4-5 times", 5="5-10 times", 6="11-20 times", 7="more than 20 times") were used in the current study. The question regarding the age of the initial drink was also asked in 2004 and when the subject had not answered that question or not participated in 2007, the data from 2004 was used. One dosage of alcohol was defined as a glass of light wine or champagne (12 cl), a shot of vodka (4 cl), or a bottle (33 cl) of light alcohol (beer, long drink, cider, etc) as in the older cohort's questionnaire.

## **Genotyping**

Genomic DNA was extracted from whole blood samples using QIAamp Midi kit (Qiagen) according to the manufacturer's instructions (Qiagen).

Samples were genotyped on Applied Biosystems® ViiA7™ Real-Time PCR (polymerase chain reaction) System using TaqMan® Pre Designed SNP Genotyping Assays and 384 plates.

A Genotyping assay detects variants of a single nucleic acid sequence, without quantifying the target. The presence of two probes in each reaction allows genotyping of the two possible variants at the single nucleotide polymorphism (SNP) site in a target sequence. Each TaqMan® SNP Genotyping Assay consists of a single, ready-to-use tube containing two sequence-specific primers for amplifying the polymorphism of interest and two allele-specific TaqMan® minor groove binder (MGB) probes for detecting the alleles for the specific polymorphism of interest. Each allele-specific TaqMan® MGB probe has a reporter dye at its

5' end: VIC® dye is linked to the 5' end of the Allele 1 probe and FAM™ dye linked to the 5' end of the Allele 2 probe.

The PCR reactions contain primers designed to amplify the sequence containing the SNP and reagents to detect two different alleles. During PCR, each TaqMan® MGB probe anneals specifically to its complementary sequence between the forward and reverse primer sites. When the oligonucleotide probe is intact, the proximity of the quencher dye to the reporter dye quenches the reporter signal.

One can collect the results of a genotyping experiment in two different ways: At the end of the experiment, or continuously during the experiment. Data collection at the end of the experiment is called end-point data collection. Data collection during the experiment run is considered real-time PCR. The real-time data helps further data analysis.

PCR reaction components per sample and final concentration were as follows: 0,5 x 5x HOT FIREPol® Probe qPCR Mix Plus (Solis Biodyne), 0,125 µl TaqMan® Pre Designed SNP Genotyping Assays (Primer 1 conc.: 36 µM, Primer 2 conc.: 36 µM, Probe conc.: 8 µM, scale: 40x), 1 µl genomic DNA (conc.: 1-50 ng/ µl) and 6,875 µl H<sub>2</sub>O. The amplification was conducted in a total volume of 10 µl. The PCR thermal cycling was as follows: pre-read stage at 60°C for 30 seconds, hold stage at 95°C for 15 minutes (default: 10 minutes), PCR stage - 40 cycles at 95°C for 15 seconds and 60°C for 1 minute, post-read stage at 60°C for 30 seconds. Ramp rate was 1.6 °C/s.

## Statistical Analysis

Genotype frequencies were tested for Hardy–Weinberg equilibrium (HWE) using Chi-square tests with one degree of freedom using a calculator by Online Encyclopedia for Genetic Epidemiology studies (<http://www.oege.org/software/hwe-mr-calc.shtml>). In the given sample, allelic distribution of rs16147 was 0.5:0.5 in both cohorts. In the case of rs16139, minor allele frequency was 0.08 in the older cohort and 0.06 in the younger cohort. The frequencies were in HWE. Subjects were divided into groups by genotype for statistical analysis: 3 groups in the case of rs16147 (T/T, C/C, and C/T) and 2 groups in the case of rs16139 (C/C+C/T and T/T). Rs16139 C/C homozygotes were analyzed together in the same

group with C/T heterozygotes because of the low number of subjects with the C/C genotype (n=7; 4 male, 3 female).

In the case of 5HTTLPR, the genotypes were divided into 3 groups according to the transcriptional activity of the 5-HTT gene. L/L denotes the high-activity group consisting only of L<sub>A</sub>/ L<sub>A</sub> heterozygotes, L/S group consists of L<sub>A</sub>/ L<sub>G</sub> and L<sub>A</sub>/S genotypes that have the medium activity, and the latter S/S group comprises L<sub>G</sub>/ L<sub>G</sub>, L<sub>G</sub>/S, and S/S genotypes that have the lowest transcriptional activity.

Genotype frequencies in the given cohorts as a whole and by sexes are shown in Tables 1 and 2.

	NPY rs16147			NPY rs16139		
	C/C	C/T	T/T	C/C	C/T	T/T
Entire older cohort (n=654)	159 (24.3%)	336 (51.4%)	159 (24.3%)	4 (0.6%)	92 (14.1%)	558 (85.3%)
	HWE Chi-square=0.5			HWE Chi-square=0.01		
Males (n=290)	73 (25.2%)	137 (47.2%)	80 (27.6%)	2 (0.7%)	40 (13.8%)	248 (85.5%)
Females (n=364)	86 (23.6%)	199 (54.7%)	79 (21.7%)	2 (0.5%)	52 (14.3%)	310 (85.2%)
	5-HTTLPR (grouped according to transcriptional activity)			5-HTTLPR allele frequencies		
	L/L	L/S	S/S	L <sub>A</sub>	L <sub>G</sub>	S
Entire older cohort (n=654)	211 (32.3%)	301 (46.0%)	142 (21.7%)	0.55	0.08	0.37
Males (n=290)	91 (31.4%)	137 (47.2%)	62 (21.4%)			
Females (n=364)	120 (33.0%)	164 (45.1%)	80 (22.0%)			

Table 1. Genotype and allele frequencies in the older cohort.

	NPY rs16147			NPY rs16139		
	C/C	C/T	T/T	C/C	C/T	T/T
Entire younger cohort (n=580)	149 (25.7%)	291 (50.2%)	140 (24.1%)	3 (0.5%)	66 (11.4%)	511 (88.1%)
	HWE Chi-square=0.01			HWE Chi-square=0.3		
Males (n=277)	72 (26.0%)	136 (49.1%)	69 (24.9%)	2 (0.7%)	26 (9.4%)	249 (89.9%)
Females (n=303)	77 (25.4%)	155 (51.2%)	71 (23.4%)	1 (0.3%)	40 (13.2%)	262 (86.5%)
	5-HTTLPR (grouped according to transcriptional activity)			5-HTTLPR allele frequencies		
	L/L	L/S	S/S	L <sub>A</sub>	L <sub>G</sub>	S
Entire younger cohort (n=580)	194 (33.4%)	290 (50.0%)	96 (16.6%)	0.58	0.08	0.34
Males (n=277)	92 (33.2%)	132 (47.7%)	53 (19.1%)			
Females (n=303)	102 (33.7%)	158 (52.1%)	43 (14.2%)			

Table 2. Genotype and allele frequencies in the younger cohort.

The numbers of subjects having answered the questions in interest regarding their alcohol consumption are shown in Table 3. Chi-square test and Univariate Analysis of Variance (General Linear Model) were utilized in the statistical analysis. Scheffe's post hoc tests were used for pairwise comparison. Cases with missing values were excluded. Statistical analysis was done using IBM® SPSS® Statistics, Version 19.0. Three-way interactions between genotypes were excluded due to the sparseness of the data in high dimensions. Results were considered significant at the  $p < 0.05$  level.

	Self-reported age of first consuming alcohol	Binge drinking	Deliberate intoxication	Occurrence of alcohol use disorder	Subjective sensation of having been very drunk
Entire older cohort	584	510	532	501	
Males	255	213	225	221	
Females	329	297	307	280	
Entire younger cohort	492	373			383
Males	226	170			173
Females	266	203			210

Table 3. The number of subjects having answered the questions regarding their alcohol consumption.

## Results

### Effects on the Self-reported Age of First Consuming Alcohol

The male subjects from the older cohort were  $13.7 \pm 2.5$  and female subjects  $14.4 \pm 2.1$  years old when reportedly first consuming half a dosage of alcohol. In the older cohort, the difference between male and female subjects was significant ( $df=1$ ,  $F=10.0$ ,  $p=0.002$ ). The male subjects from the younger cohort were on the average the youngest when they reportedly first consumed half a dosage of alcohol:  $12.6 \pm 2.6$  years old. The female subjects from the younger cohort consumed their first half a dosage of alcohol when being on the average  $12.9 \pm 2.2$  years old. However, in the younger cohort, the difference between male and female subjects was not significant ( $df=1$ ,  $F=2.3$ ,  $p=0.127$ ). Both gender ( $df=1$ ,  $F=15.1$ ,  $p<0.001$ ) and cohort ( $df=1$ ,  $F=84.9$ ,  $p<0.001$ ) were valid predictors in the sample as a whole – male subjects started consuming alcohol at an earlier age than the females and subjects from the

younger cohort started consuming alcohol at an earlier age than the ones from the older cohort.

5-HTTLPR genotype had significant effect in interaction with gender and cohort ( $df=2$ ,  $F=8.4$ ,  $p<0.001$ ) on the self-reported age of first consuming alcohol. In the younger cohort, as mentioned above, the female subjects reportedly first consumed half a dosage of alcohol slightly later than their male counterparts. When the 5-HTTLPR genotype was taken into consideration, it occurred that among S/S homozygotes the relationship is reversed – the female subjects from the younger cohort were the ones who consumed their first half a dosage of alcohol at the youngest age ( $df=2$ ,  $F=6.0$ ,  $p=0.003$ ; Fig. 7). In the older cohort, the effect tended to be contrary – the age gap between male and female subjects with S/S genotype becomes even larger, making the female subjects from the older cohort the ones who consumed their first half a dosage of alcohol latest. However, when data from the older cohort was analyzed separately, the interaction between 5-HTTLPR genotype and subjects' gender turned out to be insignificant ( $df=2$ ,  $F=1.5$ ,  $p=0.213$ ).

In the younger cohort among males, the L/L homozygotes were the ones consuming a half a dosage of alcohol at the earliest: when being on the average 12.3 years old (Fig. 7). When these young male subjects were also NPY rs16139 C allele carriers, the average age of first consuming half a dosage of alcohol dropped to 10.1 years ( $df=2$ ,  $F=3.1$ ,  $p=0.045$ ; Fig. 8). Yet, due to a low number of C allele carriers, this interaction should be regarded with caution.

NPY rs16147 and NPY rs16139 genotypes had no significant main effect nor interaction on the age when the subjects reportedly first consumed half a dosage of alcohol in either cohort as a whole nor among male and female subjects analyzed separately (Tables 4 and 5).

	5-HTTLPR			NPY rs16147			NPY rs16139			rs16147*rs16139			5-HTTLPR* rs16147			5-HTTLPR* rs16139		
	df	F	p	df	F	p	df	F	p	df	F	p	df	F	p	df	F	p
Entire older cohort (N=581)	2	0.2	0.850	2	0.4	0.688	1	1.0	0.312	2	1.1	0.346	4	1.2	0.323	2	0.4	0.683
Males (N=253)	2	0.6	0.549	2	0.1	0.876	1	0.5	0.497	1	0.7	0.396	4	0.5	0.761	2	0.1	0.945
Females (N=328)	2	1.2	0.299	2	1.0	0.381	1	0.5	0.477	2	0.2	0.828	4	0.9	0.463	2	0.7	0.491

Table 4. Effects of 5-HTTLPR, NPY rs16147 and NPY rs16139 genotypes on the self-reported age when the subjects first consumed half a dosage of alcohol in the older cohort. Significance level:  $p < 0.05$ .

	5-HTTLPR			NPY rs16147			NPY rs16139			rs16147*rs16139			5-HTTLPR* rs16147			5-HTTLPR* rs16139		
	df	F	p	df	F	p	df	F	p	df	F	p	df	F	p	df	F	p
Entire younger cohort (N=491)	2	0.2	0.791	2	0	0.994	1	0.6	0.453	1	1.4	0.240	4	1.3	0.265	2	1.8	0.16
Males (N=225)	2	1.7	0.185	2	0.3	0.709	1	1.6	0.211	1	0.7	0.396	4	1.0	0.411	2	3.1	<b>0.045</b>
Females (N=266)	2	5.0	<b>0.008</b>	2	0.5	0.627	1	0	0.998	1	0.5	0.488	4	1.0	0.425	2	1.3	0.263

Table 5. Effects of 5-HTTLPR, NPY rs16147 and NPY rs16139 genotypes on the self-reported age when the subjects first consumed half a dosage of alcohol in the younger cohort. Significance level:  $p < 0.05$ .

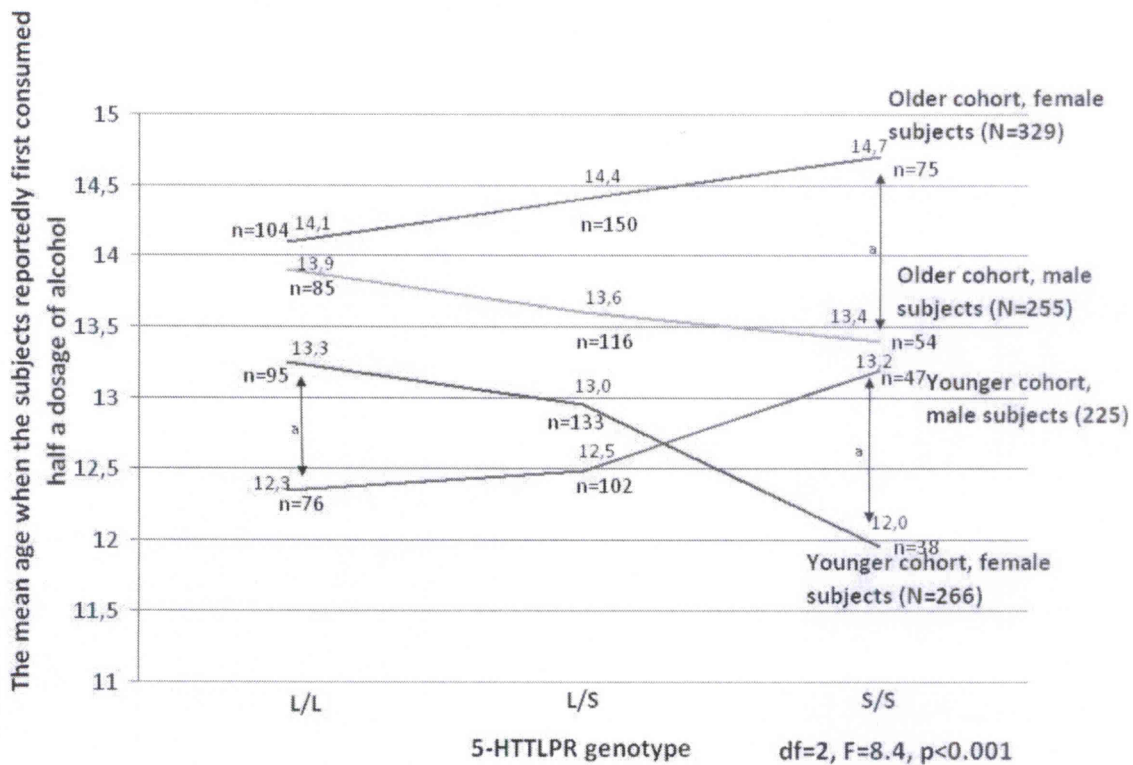


Fig. 7. The effect of 5-HTTLPR genotype in interaction with gender and cohort on the average age when the subjects first consumed half a dosage of alcohol. Scheffe's post hoc tests: <sup>a</sup>p<0.05.

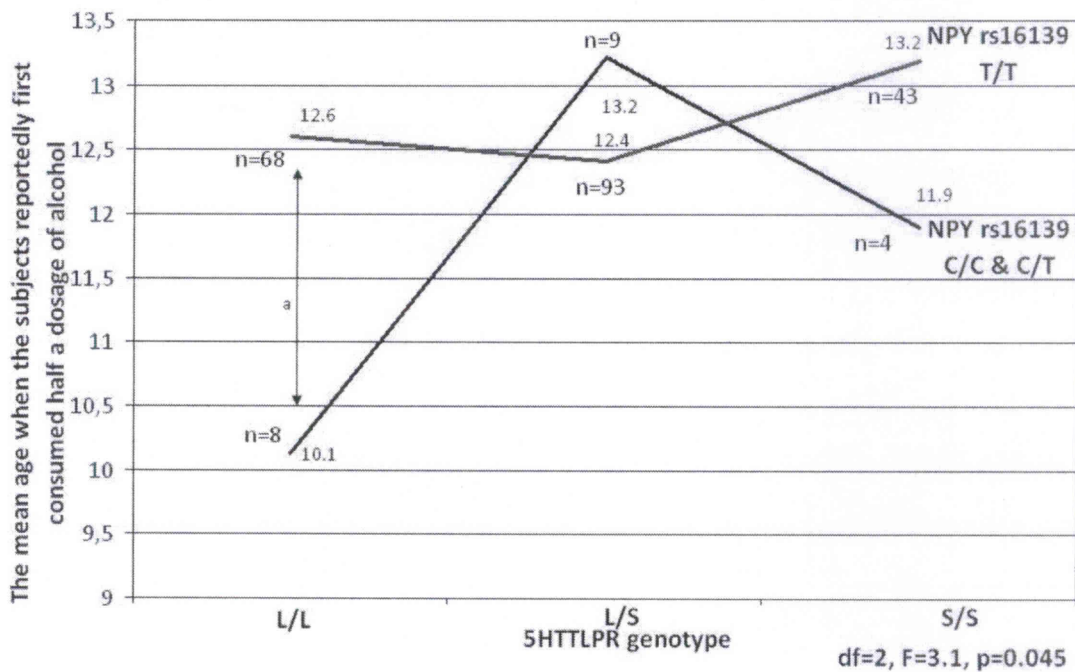


Fig. 8. The effect of 5-HTTLPR genotype in interaction with NPY rs16139 on the average age of the subjects when first consuming half a dosage of alcohol. Younger cohort, male subjects. Scheffe's post hoc tests: <sup>a</sup>p<0.05.

## Effects on Binge Drinking

The median and mode answer on how often the subjects had consumed at least 5 or more dosages of alcohol during the previous 12 months in the older cohort as a whole and separately among female subjects was “less than once a month“. Among male subjects in the older cohort, the median and mode answer was “at least once a month“. The gender of the subjects was found to significantly influence how often the subjects consumed at least 5 or more dosages of alcohol during the previous 12 months - male subjects consumed large amounts of alcohol more often than their female counterparts ( $df=1$ ,  $F=108.4$ ,  $p<0.001$ ). The subjects from the older cohort were on the average 25 years old when answering the question.

The median answer on how often the subjects had consumed at least 5 or more dosages of alcohol during the previous 6 months in the younger cohort as a whole and among female subjects “twice“. The mode answer in the younger cohort as a whole was “3-5 times“ and among female subjects “once“. Among male subjects in the younger cohort, the median and mode answer was “3-5 times“. The gender of the subjects was a valid predictor also in the younger cohort ( $df=1$ ,  $F=27.0$ ,  $p<0.001$ ) – male subjects consumed large amounts of alcohol more often than their female counterparts. The subjects from the younger cohort were on the average 18 years old when answering the given question.

5-HTTLPR, NPY rs16147 and NPY rs16139 genotypes had no significant main effect on how often the subjects had consumed large amounts of alcohol in both cohorts as a whole nor among male and female subjects analyzed separately (Tables 6 and 7). In the case of the older cohort, 5-HTTLPR S/S homozygotes who were also NPY rs16147 C allele carriers were the ones who tended to consume large amounts of alcohol slightly more frequently than others; on the other hand, 5-HTTLPR S/S homozygotes who were also NPY rs16147 T/T homozygotes were the ones who tended to consume large amounts of alcohol slightly less frequently than others ( $df=4$ ,  $F=2.7$ ,  $p=0.031$ ; Fig. 9). Although, according to Scheffe’s post hoc tests, the groups did not differ significantly. In the case of females from the younger cohort, there was a tendency ( $df=4$ ,  $F=4.0$ ,  $p=0.004$ ) among subjects with 5-HTTLPR L/L genotype to consume large amounts of alcohol less often when they were also NPY rs16147 C/T heterozygotes (Fig. 10). In the younger cohort as a whole, there was a tendency ( $df=1$ ,  $F=5.7$ ,  $p=0.018$ ) among subjects carrying the NPY rs16139 C allele to consume large amounts of alcohol more frequently when they were also NPY rs16147 T/T homozygotes (Fig. 11).

	5-HTTLPR			NPY rs16147			NPY rs16139			rs16147*rs16139			5-HTTLPR* rs16147			5-HTTLPR* rs16139		
	df	F	p	df	F	p	df	F	p	df	F	p	df	F	p	df	F	p
Entire older cohort (N=510)	2	0.6	0.566	2	0.1	0.947	1	0	0.853	2	0.4	0.704	4	2.7	<b>0.031</b>	2	0.3	0.706
Males (N=213)	2	2.8	0.062	2	0.1	0.933	1	0.3	0.582	1	0	0.927	4	1.1	0.349	2	0.3	0.727
Females (N=297)	2	0.8	0.461	2	0.4	0.695	1	0.4	0.504	2	0.8	0.449	4	0.9	0.485	2	0.5	0.606

Table 6. Effects of 5-HTTLPR, NPY rs16147 and NPY rs16139 genotypes on how often the subjects had reportedly consumed at least 5 or more dosages of alcohol during the previous 12 months. Data from the older cohort. Significance level:  $p < 0.05$ .

	5-HTTLPR			NPY rs16147			NPY rs16139			rs16147*rs16139			5-HTTLPR* rs16147			5-HTTLPR* rs16139		
	df	F	p	df	F	p	df	F	p	df	F	p	df	F	p	df	F	p
Entire younger cohort (N=371)	2	0.4	0.681	2	0.6	0.574	1	0.1	0.724	1	5.7	<b>0.018</b>	4	2.0	0.097	2	0.2	0.839
Males (N=168)	2	0.8	0.460	2	2.5	0.089	1	0	0.957	1	2.0	0.165	4	0.3	0.882	2	2.8	0.063
Females (N=203)	2	0.1	0.886	2	0.7	0.511	1	0	0.854	1	2.8	0.095	4	4.0	<b>0.004</b>	2	1.5	0.226

Table 7. Effects of 5-HTTLPR, NPY rs16147 and NPY rs16139 genotypes on how often the subjects had reportedly consumed at least 5 or more dosages of alcohol during the previous 6 months. Data from the younger cohort. Significance level:  $p < 0.05$ .

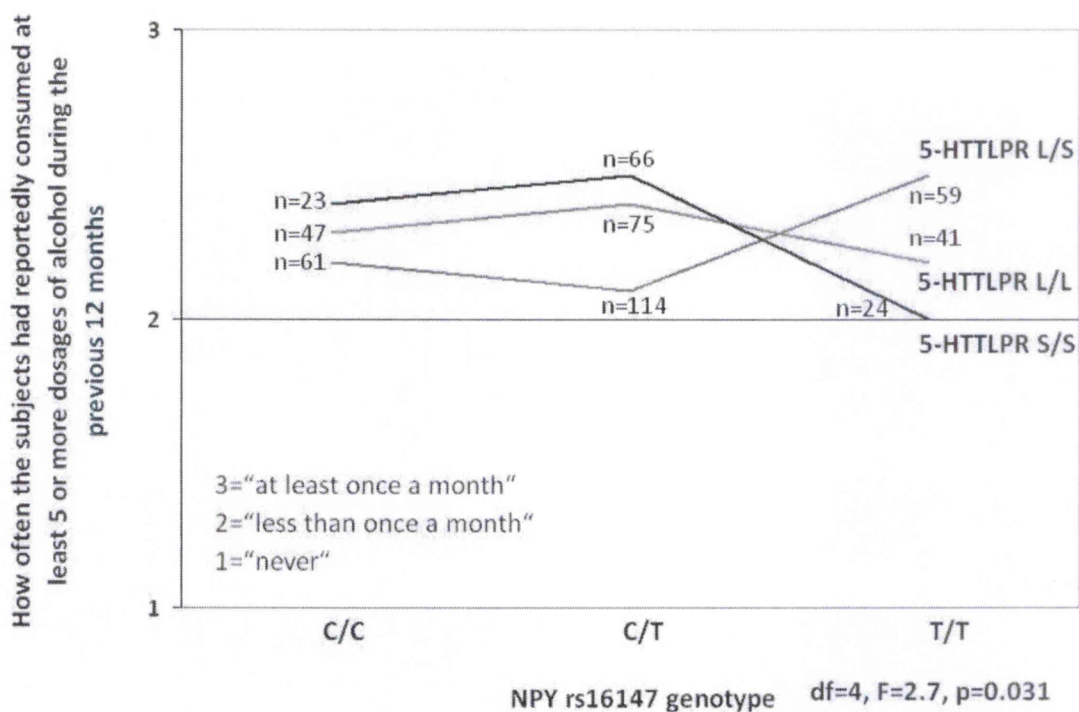


Fig. 9. The frequency of how often the subjects from the older cohort as a whole had consumed at least 5 or more dosages of alcohol during the previous 12 months influenced by 5-HTTLPR and NPY rs16147 genotypes.

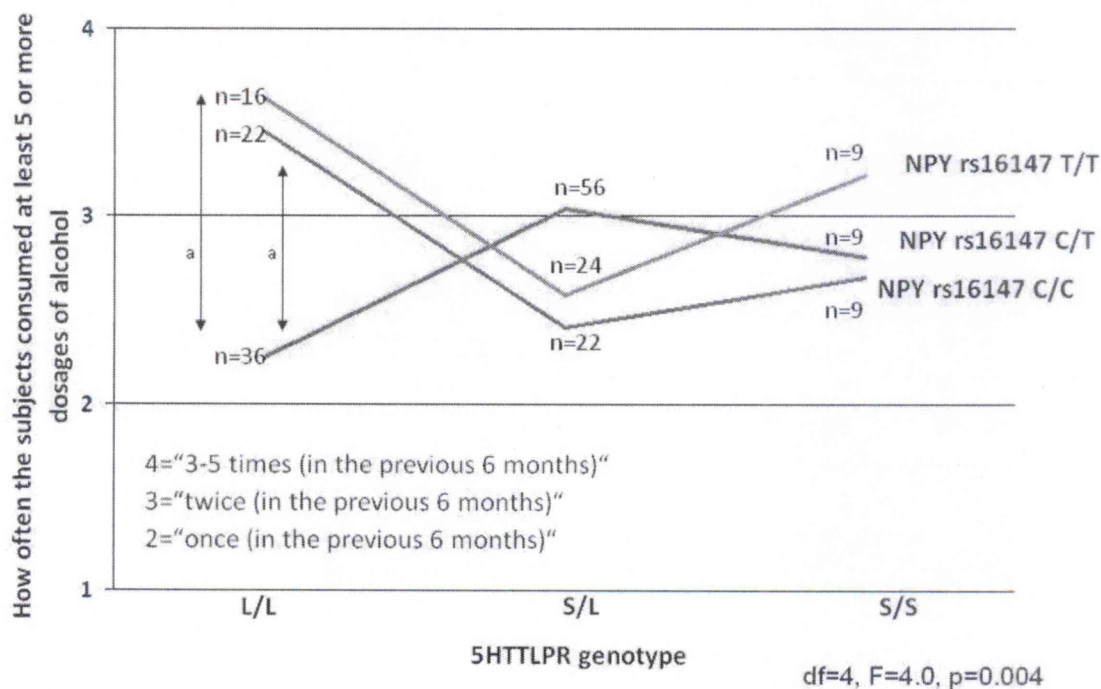


Fig. 10. The frequency of how often the female subjects from the younger cohort had consumed at least 5 or more dosages of alcohol during the previous 6 months influenced by 5-HTTLPR and NPY rs16147 genotypes. Scheffe's post hoc tests: <sup>a</sup>p<0.05.

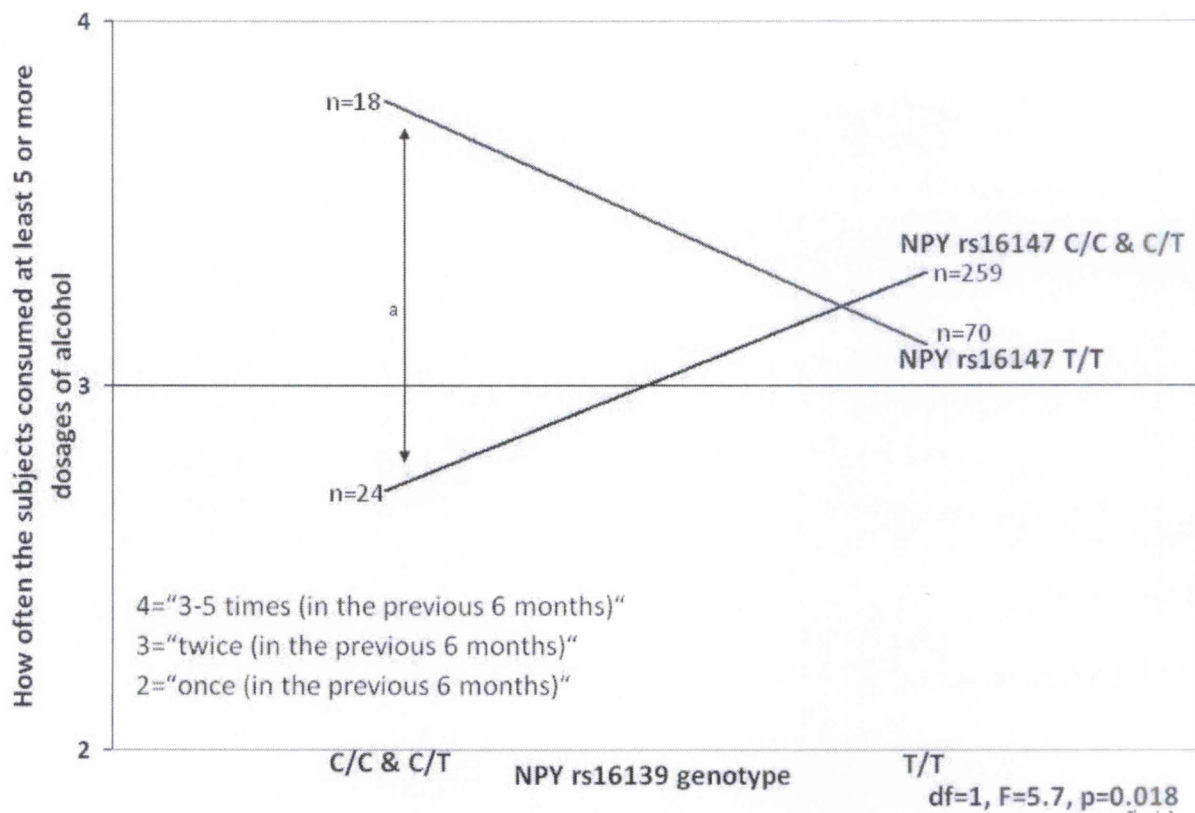


Fig. 11. The frequency of how often the subjects from the younger cohort as a whole had consumed at least 5 or more dosages of alcohol during the previous 6 months influenced by NPY rs16139 and NPY rs16147 genotypes. Scheffe's post hoc tests: <sup>a</sup>p<0.05.

### Effects on Deliberate Intoxication

The subjects from the older cohort answered the question about whether they had deliberately been in an intoxicated state. Male subjects reported deliberately been in an intoxicated state substantially more often than female subjects (df=1, Pearson  $\chi^2=9.1$ , p=0.003) – 68.0% of the male subjects and 55.0% of the female subjects answered affirmatively. 5-HTTLPR, NPY rs16147 and NPY rs16139 genotypes had no significant main effect on the subjects' answer (Table 8). However, there appeared to be a greater likelihood of having deliberately been in an intoxicated state among male subjects carrying the NPY rs16139 C allele when they were also NPY rs16147 T/T homozygotes (df=1, Pearson  $\chi^2=4.0$ , p=0.047; Fig. 12).

	5-HTTLPR			NPY rs16147			NPY rs16139			rs16147*rs16139			5-HTTLPR* rs16147			5-HTTLPR* rs16139		
	df	Pearson $\chi^2$	Asymp. Sig. (2- sided)	df	Pearson $\chi^2$	Asymp. Sig. (2- sided)	df	Pearson $\chi^2$	Asymp. Sig. (2- sided)	df	Pearson $\chi^2$	Asymp. Sig. (2- sided)	df	Pearson $\chi^2$	Asymp. Sig. (2- sided)	df	Pearson $\chi^2$	Asymp. Sig. (2- sided)
Entire older cohort (N=532)	2	0.56	0.756	2	0.76	0.684	1	0.321	0.571	1	2.17	0.14	4	2.57	0.632	2	0.575	0.75
Males (N=225)	2	0.499	0.779	2	1.477	0.478	1	0.397	0.529	1	3.95	<b>0.047</b>	4	9.21	0.056	2	2.13	0.345
Females (N=307)	2	1.914	0.384	2	0.801	0.67	1	0.021	0.885	1	1.24	0.265	4	3.02	0.554	2	1.65	0.438

Table 8. Effects of 5-HTTLPR, NPY rs16147 and NPY rs16139 genotypes on the subjects' perception of having deliberately been in an intoxicated state. Data from the older cohort. Significance level:  $p < 0.05$ .

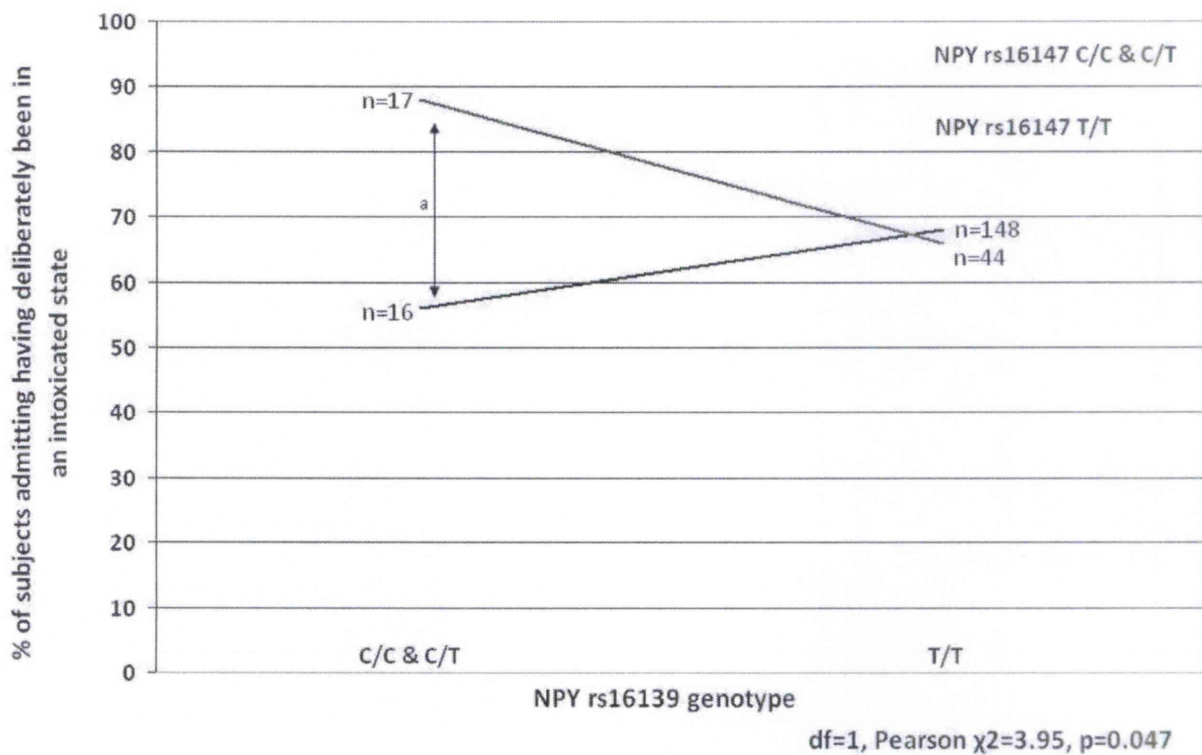


Fig. 12. The proportion of male subjects from the older cohort having deliberately been in an intoxicated state influenced by NPY NPY rs16139 and NPY rs16147 genotypes. Scheffe's post hoc tests: <sup>a</sup>p<0.05.

### Effects on the Occurrence of Alcohol Use Disorder

Subjects from the older cohort were interviewed by a psychologist in order to determine the occurrence of mental health disorders. 33.5% of the male subjects and 7.5% of the female subjects were determined to have had or currently having an alcohol use disorder (AUD), making male subjects substantially more in risk of AUD (df=1, Pearson  $\chi^2=54.3$ , p<0.001). Again, 5-HTTLPR, NPY rs16147 and NPY rs16139 genotypes had no significant main effect (Table 9). There appeared to be 2 interactive effects in the case of female subjects (Table 9), but due to the extreme sparseness of the data in the given case – low number of female subjects having been diagnosed with alcohol use disorder (Tables 10 and 11) –, these interactions should not be taken into account.

	5-HTTLPR			NPY rs16147			NPY rs16139			rs16147*rs16139			5-HTTLPR* rs16147			5-HTTLPR* rs16139		
	df	Pearson $\chi^2$	Asymp. Sig. (2- sided)	df	Pearson $\chi^2$	Asymp. Sig. (2- sided)	df	Pearson $\chi^2$	Asymp. Sig. (2- sided)	df	Pearson $\chi^2$	Asymp. Sig. (2- sided)	df	Pearson $\chi^2$	Asymp. Sig. (2- sided)	df	Pearson $\chi^2$	Asymp. Sig. (2- sided)
Entire older cohort (N=501)	2	0.34	0.843	2	4.635	0.099	1	0.322	0.57	1	2.38	0.123	4	5.13	0.274	2	1.06	0.588
Males (N=221)	2	0.434	0.805	2	4.797	0.091	1	1.771	0.183	1	3.74	0.053	4	4.68	0.322	2	0.994	0.608
Females (N=280)	2	0.105	0.949	2	0.644	0.725	1	1.502	0.22	1	3.33	0.068	4	15.0	<b>0.005</b>	2	10.4	<b>0.005</b>

Table 9. Effects of 5-HTTLPR, NPY rs16147 and NPY rs16139 genotypes on the occurrence of alcohol use disorder. Data from the older cohort. Significance level:  $p < 0.05$ .

		5-HTTLPR genotype					
		L/L		L/S		S/S	
		1	0	1	0	1	0
NPY rs16139	C/C & C/T	0	11	1	16	0	10
	T/T	7	68	8	104	5	50

Table 10. The distribution of the number of female subjects in the older cohort by 5-HTTLPR and NPY rs16139 genotypes diagnosed with alcohol use disorder (1=affected, 0=unaffected).

		5-HTTLPR genotype					
		L/L		L/S		S/S	
		1	0	1	0	1	0
NPY rs16147	C/C	3	26	1	29	3	11
	C/T	3	37	6	66	1	31
	T/T	1	16	2	25	1	18

Table 11. The distribution of the number of female subjects in the older cohort by 5-HTTLPR and NPY rs16147 genotypes diagnosed with alcohol use disorder (1=affected, 0=unaffected).

### Effects on Subjective Sensation of Having Been Very Drunk

Subjects from the younger cohort answered the question about how often during the previous 6 months had they consumed such a large quantity of alcohol that they regarded themselves as having been very drunk. Subjects were on the average 18 years old when answering the given question.

The median answer in the younger cohort as a whole and separately among female subjects was “once (in the previous 6 months)“. The mode answer in the younger cohort as a whole and separately among female subjects was “never“. Among male subjects in the younger cohort, the median and mode answer was “2-3 times (in the previous 6 months)“. The gender of the subjects had a significant effect ( $df=1$ ,  $F=11.3$ ,  $p=0.001$ ) – male subjects regarded themselves as having been very drunk during the previous 6 months more often than their female counterparts.

	5-HTTLPR			NPY rs16147			NPY rs16139			rs16147*rs16139			5-HTTLPR* rs16147			5-HTTLPR* rs16139		
	df	F	p	df	F	p	df	F	p	df	F	p	df	F	p	df	F	p
Entire younger cohort (N=382)	2	0.2	0.833	2	0.4	0.690	1	0.8	0.386	1	4.9	<b>0.028</b>	4	1.9	0.110	2	0.2	0.810
Males (N=172)	2	0.5	0.631	2	1.5	0.219	1	0.1	0.700	1	2.4	0.125	4	0.8	0.530	2	1.3	0.274
Females (N=210)	2	1.1	0.323	2	0.3	0.739	1	1.6	0.202	1	2.0	0.161	4	2.1	0.085	2	1.2	0.290

Table 12. Effects of 5-HTTLPR, NPY rs16147 and NPY rs16139 genotypes on the subjects' perception of having consumed such a large quantity of alcohol that they regarded themselves as having been very drunk. Data from the younger cohort. Significance level:  $p < 0.05$ .

5-HTTLPR, NPY rs16147 and NPY rs16139 genotypes had no significant main effect on the subjects' answer (Table 12). However, in the younger cohort as a whole, there was a tendency ( $df=1$ ,  $F=4.9$ ,  $p=0.028$ ) among subjects carrying the NPY rs16139 C allele to perceive themselves as having been really drunk more often when they were also NPY rs16147 T/T homozygotes (Fig. 13). Although, according to Scheffe's post hoc tests, the groups did not differ significantly.

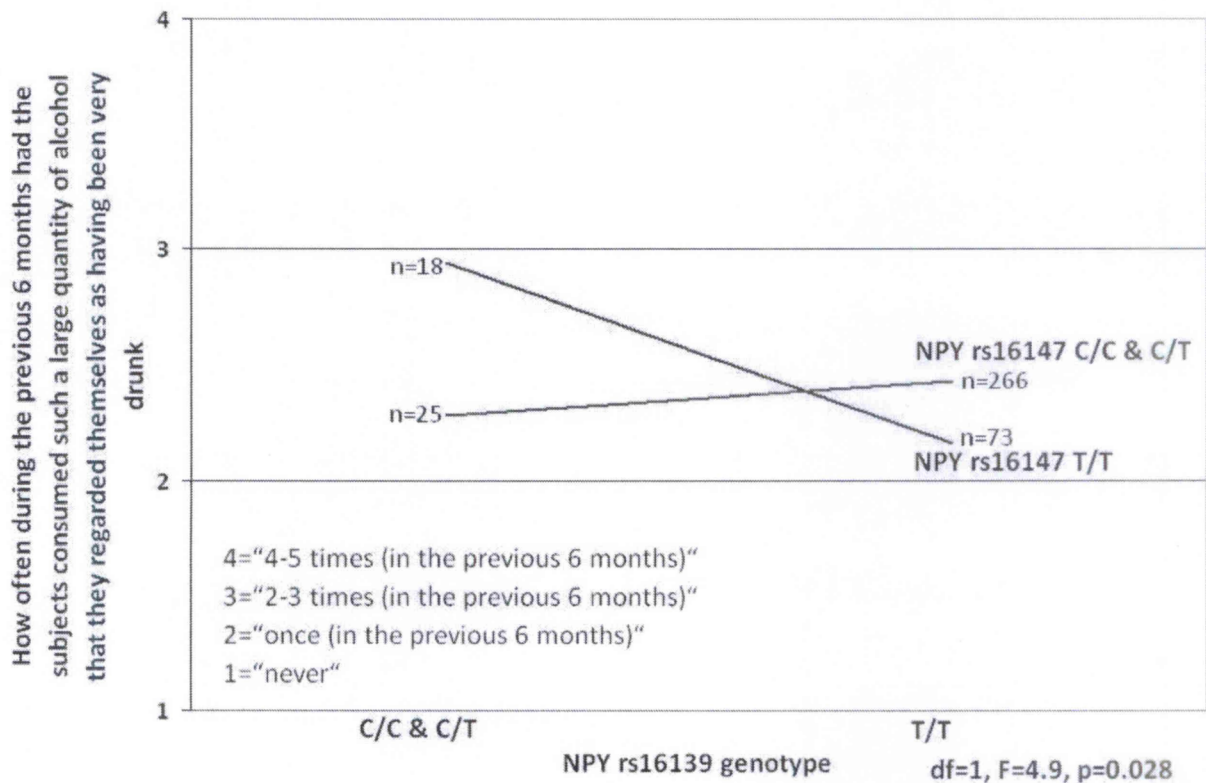


Fig. 13. The frequency of how often the subjects from the younger cohort as a whole regarded themselves as having consumed such a large quantity of alcohol that they perceived themselves as having been very drunk during the previous 6 months; influenced by NPY rs16139 and NPY rs16147 genotypes.

## Discussion

In the given sample, the subjects from the younger cohort reportedly started consuming alcohol at an earlier age than the subjects from the older cohort; similar trend is also visible in WHO's Health Behaviour in School-aged Children (HBSC) study reports about Estonia over time (Currie *et al*, 2000, 2004, 2008; Pärna *et al*, 2012). Alcohol consumption was more problematic among male subjects, both in the older (~25 years old when

participating; born in the early 1980's) and younger cohort (~18 years old when participating; born in the late 1980's). In the older cohort, male subjects started consuming alcohol at a younger age, consumed large amounts of alcohol more frequently, were more likely to be deliberately in an intoxicated state and also were diagnosed to have had or currently having an alcohol use disorder more often than their female counterparts. In the younger cohort, male subjects started consuming alcohol at about the same age as the females, but consumed large amounts of alcohol and regarded themselves as having been very drunk more often than female subjects. Given results are in accordance with previous data – in Estonia, excess alcohol consumption is indeed a larger concern among men compared to women and the problems are apparently visible already in early adulthood (Pärna *et al*, 2012).

The functional polymorphism in the 5-HT transporter gene – 5-HTTLPR – that was focused on in the current study had significant effects on subjects' alcohol consumption. As mentioned above, the female subjects from the older cohort started consuming alcohol later than male subjects and in the younger cohort, male subjects started consuming alcohol at about the same age as the females. When 5-HTTLPR genotype was taken into account, it became apparent that in the younger cohort, the female subjects also started consuming alcohol later except for the ones who were 5-HTTLPR S/S homozygotes (Fig. 7). As opposed to the female S/S homozygotes, the male S/S homozygotes from the younger cohort started consuming alcohol later than their female counterparts or other male subjects with 5-HTTLPR L/L or L/S genotypes. Given that the carriers of the short allele have been found to be more sensitive to the detection of socially relevant information (Lonsdorf *et al*, 2011) and more susceptible to environmental influences (Pluess *et al*, 2010), it might be that in the given age group, the girls were under more severe peer pressure to consume alcohol and the ones being more vulnerable conformed more easily. Indeed, peer pressure has been more strongly associated with drinking for girls than it has for boys (Donovan, 2002; Simons-Morton *et al*, 2001).

Environmental differences might be the reason why the S/S homozygotes from only the younger cohort behave differently. Although genes determine the features an organism may develop, the features that actually develop depend upon the complex interaction between genes and environment. Gene–environment interactions are important because genes produce their effects in an indirect way (through proteins and cellular activity), and, therefore, the ultimate outcome of gene action may vary in different circumstances. Although genes do not change over the life course (creating the impression of causal links), many traits in later life

demonstrate very high environmental plasticity; that is, they can be modified in response to an environmental change (Ryff & Singer, 2005). Given the rapid economical and political changes in Estonia during the time the subjects in the current study were growing up, it might be possible that the environment was different enough for the two cohorts to influence the genetic effects.

The functional polymorphism located in the signal peptide of the prepro-NPY – rs16139 – and the other one in the promoter region of the NPY gene – rs16147 – that were focused on in the current study had significant interactive effects on subjects' alcohol consumption. In the older cohort, male subjects who were NPY rs16139 C allele carriers and also had NPY rs16147 T/T genotype were more likely to be deliberately in an intoxicated state. The same kind of pattern emerged also in the younger cohort as a whole – subjects who were NPY rs16139 C allele carriers and also had NPY rs16147 T/T genotype consumed large amounts of alcohol and regarded themselves as having been very drunk more often. NPY rs16139 C allele carriers have been found to have higher levels of peptide secretion (Mitchell *et al*, 2008) without affecting the peptide's binding to its receptors (Ding *et al*, 2005) and NPY rs16147 T/T genotype has been associated with higher NPY mRNA expression and plasma NPY levels (Zhou *et al*, 2008). It could be argued that this kind of combination results in a high NPY release potential – due to NPY rs16147 T/T genotype, more NPY is produced and due to NPY rs16139 C allele, more is being released into the synaptic cleft. Therefore, based on the given results, high NPY release potential could be associated with problematic alcohol consumption.

Some interactive effects between 5-HTTLPR and NPY polymorphisms were also identified in the course of exploratory data analysis. This supports the notion that serotonergic and NPY-ergic systems, having previously been determined to have interactions in animal models, also have interactive effects on human behavior. Given that problematic alcohol consumption is indeed a behavioral disorder in nature, it is more likely to involve several neurotransmitter systems than just one. Detecting interactions between loci elucidates the biological and biochemical pathways that underpin the given disease.

Although compelling epidemiological evidence indicates that ~50% of the risk for becoming alcoholic stems from genetic susceptibility (Kalsi *et al*, 2008), the importance of social factors should not be forgotten. Children do not start consuming alcohol in a vacuum. WHO has been called upon to impose its instruments to reduce alcohol supply (Sridhar,

2012), but the importance of educating should not be forgotten, either. In Estonia, further alcohol policy actions should include the reduction of the density of alcohol outlets, more comprehensive advertisement bans, clearer separation of alcoholic beverages from other goods in retail stores and full implementation of brief alcohol interventions in primary health care (Lai & Habicht, 2011), but there should also be an alcohol consumption prevention program targeted to adolescents and their parents (Pärna *et al*, 2012). Children need positive role models. If excessive alcohol use is accepted in children's home environment – kids see their parents drink and get no directions on how to consume alcohol moderately, drunk people are being considered funny, alcohol is an inseparable part of every social event, dad drinks beer in front of the TV on every single evening – then placing the responsibility on WHO for not making legislative moves to reduce the supply of alcohol is really not the sole answer.

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Käesolevaga kinnitan, et olen korrektselt viidanud kõigile oma töös kasutatud teiste autorite poolt loodud kirjalikele töödele, lausetele, mõtetele, ideedele või andmetele.

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Mariliis Vaht