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MARILIIS VAHT

Genes and alcohol use: effects of common genetic polymorphisms in general population

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Genes and alcohol use: effects of common genetic polymorphisms in general population

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Institute of Psychology, University of Tartu, Estonia.

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LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on the following original publications, further referred to by respective Roman numerals:

I. **Vaht M,** Merenäkk L, Mäestu J, Veidebaum T, Harro J (2014) Serotonin transporter gene promoter polymorphism (5-HTTLPR) and alcohol use in general population: interaction effect with birth cohort. *Psychopharmacology*, 231(13):2587–2594. doi:10.1007/s00213-013-3427-8

II. **Vaht M,** Kiive E, Veidebaum T, Harro J (2016) A functional vesicular monoamine transporter 1 (*VMAT1*) gene variant is associated with affect and the prevalence of anxiety, affective, and alcohol use disorders in a longitudinal population-representative birth cohort study. *International Journal of Neuropsychopharmacology,* 19(7):1–9. doi:10.1093/ijnp/pyw013

III. **Vaht M,** Laas K, Kiive E, Veidebaum T, Harro J A functional neuregulin-1 gene variant and stressful life events: effect on drug use in a longitudinal population-representative cohort study. *Journal of Psychopharmacology* (in press) doi:10.1177/0269881116655979

IV. **Vaht M,** Kurrikoff T, Laas K, Veidebaum T, Harro J (2016) Oxytocin receptor gene variation rs53576 and alcohol abuse in a longitudinal population representative study. *Psychoneuroendocrinology*, 74:333–341. doi:10.1016/j.psyneuen.2016.09.018

Contribution of the author

- For all the papers, the author of the dissertation formulated research hypotheses, conducted DNA extraction and genotyping of the polymorphisms, conducted the data analysis, wrote the first draft of the manuscript and was responsible for the final form.

- The author of the dissertation also participated in the ECPBHS data collection waves in 2007, 2008 and 2014, especially in data collection in the laboratory and the processing of biological samples.

ABBREVIATIONS

5-HT	serotonin
5-HTT	serotonin transporter
5-HTTLPR	serotonin transporter gene linked polymorphic region
AUD	alcohol use disorder
BDI	Beck Depression Inventory
CEE	Central and Eastern European
DNA	deoxyribonucleic acid
DALY	disability adjusted life year
G x E	gene-by-environment interaction
GABA	γ-aminobutyric acid
GWAS	genome-wide association study
EBBFI	Estonian Brief Big Five Inventory
ECPBHS	Estonian Children Personality Behaviour and Health Study
EPIP-NEO	Estonian Personality Item Pool NEO
MÅDRS	Montgomery-Åsberg Depression Rating Scale
MCMC	Markov chain Monte Carlo
M.I.N.I.	Mini-International Neuropsychiatric Interview
NEO-PI-R	Revised NEO Personality Inventory
NRG1	neuregulin-1
OXTR	oxytocin receptor gene
PCR	polymerase chain reaction
ppp	posterior predictive *p* values
SD	standard deviation
SLE	stressful life event
SNP	single nucleotide polymorphism
STAI	State-Trait Anxiety Inventory
VMAT1	vesicular monoamine transporter 1
VMAT2	vesicular monoamine transporter 2
VNTR	variable number of tandem repeats

1. INTRODUCTION AND REVIEW OF LITERATURE

1.1. Genetic vulnerability and environmental factors in alcohol use disorders

1.1.1. Harm of alcohol use disorders

Problematic use of alcohol is one of the leading causes of preventable deaths and disability. According to the Global Burden of Disease Study 2013, alcohol use continues to be a major contributor to disease burden worldwide (Degenhardt et al. 2016). Alcohol consumption is a causal factor in more than 200 disease and injury conditions, causing death and disability relatively early in life: In the age group 20–39 years approximately 25% of the total deaths are alcohol-attributable (WHO 2014). Based on the harm to the individual and others, alcohol was considered to be the most harmful drug in UK (Nutt et al. 2010). For men in Eastern Europe, the highest loss of disability adjusted life years (DALYs) due to mental disorders has been attributed to alcohol abuse (Wittchen et al. 2011). According to the data presented by the Organisation for Economic Co-operation and Development (OECD) in their 2015 economics and public health policy report, Estonia is one of the largest consumers of pure alcohol of all its member states (OECD 2015). The chronic and relapsing nature of alcoholism is the reason why the search for predictive biomarkers to help clinicians select and monitor a therapeutic course of action and to help researchers evaluate new therapeutic interventions is so urgent (Volkow and Baler 2013).

1.1.2. Vulnerability factors for alcohol use disorders

1.1.2.1. Genetic vulnerability

The fundamental goal of neuroscience is to understand the neurobiological mechanisms that shape the behaviour. Genetic factors mediate sensitivity to the pathogenic effects of environmental risk factors through control of, sensitivity to, and exposure to the environment (Kendler and Prescott 2006). The inborn differences in the activity of several neurotransmitter systems are one important reason why alcohol consumption differs between individuals (Kimura and Higuchi 2011). Heaviness of alcohol consumption and dependence symptoms have a high degree of genetic overlap, implying that genetic influences on dependence risk in the general population are acting to a considerable degree through heaviness of use, and that quantitative measures of consumption will likely have a useful role in the identification of gene variants contributing to alcohol dependence (Grant et al. 2009). Evidence from family, adoption, and twin studies converges on the relevance of heritability on substance use and addiction with estimates ranging from 0.39 to 0.72 (Goldman et al. 2005).

Identification of specific genes conveying increased risks of drug use has promise not only for understanding the causes and potential treatments for disease, but also for increasing our knowledge of how genetic and environmental risks interact to shape liability to addiction (Agrawal and Lynskey 2008).

However, according to evidence from genome-wide association studies (GWAS), the contribution of individual genetic variants to the risk for alcohol-related disorders is small, probably due to gene-environment interactions (G × E) (Samochowiec et al. 2014). G × E are defined as different expression of a gene across environments or equivalently, the effect of the environment varying on the observed phenotype by genotype (Gunzerath and Goldman 2003). Some of the signals that emerge from GWAS may turn out to reflect the action of modifiable (*e.g.*, environmental or behavioural) exposures, rather than more direct biological effects (Gage et al. 2016).

1.1.2.2. Environmental stressors

Exposure to stress has been described as an important component in high risk for drug use (Balk et al. 2009; Keyes et al. 2011a; Stogner and Gibson 2013). Since Caspi and colleagues' seminal study (2002), research on individual differences in genetic susceptibility to stressful experiences have gained momentum as an important factor that amplifies a host of negative behavioural and psychological outcomes for youth (Stogner and Gibson 2013).

Common stressful life events (SLEs) significantly influence substance use and mental health symptoms (Balk et al. 2009; Booker et al. 2008; Nation and Heflinger 2006). SLEs are described as discrete quantifiable circumstances that can have severe negative impact (Low et al. 2012). SLEs have been linked to the level of substance use and increases in use over time (Wills et al. 2001). Specifically, cumulative exposure to adversities such as domestic violence, physical, emotional and sexual abuse, neglect, and parental dysfunction due to alcohol or drug use increases propensity for drug use disorders across life span (reviewed by Myers et al. 2014). High emotional stress that can result from such events has been associated with the loss of control over impulses and an inability to inhibit inappropriate behaviours and to delay gratification (Mischel et al. 1989).

1.1.2.3. The role of gender

Women generally drink less alcohol and have fewer alcohol-related problems than men (World Health Organization 2014). Yet some studies suggest that, in recent decades, the gender difference in drinking- and alcohol-related problems has decreased (MacArthur et al. 2012; Wagoner et al. 2012; Wilsnack et al. 2009). It is acknowledged that alcohol use problems are more likely in people who start drinking at an early age and that drinking problems are on the rise, in particular in girls, and especially in Northern and Eastern Europe and in the

USA (Keyes et al. 2008; Wilsnack and Wilsnack 2013). A cohort-specific increase in female drinking would signal the need for better targeted prevention and intervention efforts to address drinking problems (Keyes et al. 2011a).

1.1.2.4. Birth cohort effects

Alcohol consumption has been shown to be subject to birth cohort effects (Johnson and Gerstein 1998; Pabst et al. 2010; Rice et al. 2003). Birth cohort effects reflect the socioeconomic environment experienced by different generations. Economic fluctuation, political instability, policies and laws, social norms and awareness are group-level exposures that can vary between time periods and countries (Keyes et al. 2011a), potentially impacting particular birth cohorts in ways that affect their risk for earlier or more frequent drug use and substance use disorders. Restrictions, prices, and advertisements related to alcohol directly influence drinking behaviour at younger ages (Paschall et al. 2009), which tends to predict patterns of alcohol use over the life course (Pitkanen et al. 2005; Eliasen et al. 2009). Recent studies on alcohol consumption trends have found that birth cohorts are significant explanatory factors (Meng et al. 20014; Kraus et al. 2015). Using data from several European countries, the inclusion of the birth cohort dimension has been shown to improve the understanding of alcohol-attributable health problems in both males and females (Trias-Llimós et al. 2016). This dimension has however not been included in G × E studies so far.

Countries of Central and Eastern Europe (CEE) are very often referred to as transition societies. Here, transition societies are former socialist countries undergoing a process of democratisation. The term refers to a situation in which the political structure is changing from a single-party rule to a parliamentary system, administrative institutions are being reorganized, central planning is changing to a capitalist free market economy and a society of shortages is changing to a consumer society (Allaste and Bennet 2013; Nugin et al. 2016). Estonia is a representative CEE transition society that moved away from socialism in the late 1980s and became an independent and highly liberal economy since 1991. The Estonian economy was one of the fastest growing in the world until 2007 (World Bank 2015), bringing about rapid but multifaceted social changes. As described by Allaste and Bennet (2013), Soviet-type regimes strived for homogenisation of their populations in regard of lifestyle; post-socialist transformation turned them into participants of the global lifestyle market. The idea of citizens as autonomous contract-making individuals did not emerge from capitalism of its own accord, it has been a subject of struggles over ways of life and the distribution of freedoms over two and a half centuries of capitalist development (Sulkunen 2009). A distinguishing characteristic of a transition society is the discrepancy between the speed of institutional reform and the slowness of cultural changes and liminality – the sense of being in between the two social orders (Allaste and Bennet 2013). However, the former socialist countries – including Estonia – missed the process of the most relevant develop-

ments of the twentieth century (*e.g.,* orientation towards consumerism and leisure, privatization and free market economy, diversity of lifestyles and visibility of alternative subcultures), starting from the 1960s, and have been adopting the Western lifestyle only in the last two and a half decades with vigor at enhanced speed. Therefore, if present, the cohort effects could be observable within a relatively brief time span.

1.2. Candidate genes for alcohol use disorders

1.2.1. Serotonin transporter gene and its role in alcohol use disorders

The level of 5-HT in the synaptic clefts throughout the brain is mainly regulated by the 5-HT transporter (5-HTT) (Voineskos et al. 2007). 5-HTT carries out the reuptake of serotonin from the synaptic cleft, terminating neurotransmission and restoring serotonin reserves in presynaptic terminals. The human 5-HTT gene contains a polymorphic region (5-HTTLPR) with two functional variants: a short (s) allele consisting of 14 tandem repeats and having low transcriptional efficiency, and a long (l) allele consisting of 16 tandem repeats and having high transcriptional efficiency (Lesch et al. 1996). In addition, an A/G nucleotide substitution in the l allele (rs25531) renders the 5-HTTLPR tri-allelic, with the lg allele functionally equivalent to the s allele in vitro (Hu et al. 2006; Jasinska and Perkins 2009).

Carriers of the s allele display increased reactivity of the amygdala to fearful stimuli (Hariri et al. 2002), reduced amygdalar (Pezawas et al. 2005) and hippocampal volume (Everaerd et al. 2012), and enhanced functional coupling between the amygdala and the ventromedial prefrontal cortex (Heinz et al. 2005). Low-expressing allele carriers who experience stressful life events have been found to have a higher risk of depression (Caspi et al. 2003). On the other hand, low-expressing allele carriers often respond more positively to environmental enrichment than high-expressing allele homozygotes (Belsky et al. 2009). Such "hypervigilance" may be moderately harmful in the day-to-day, but highly beneficial for survival under circumstances that have major impacts on fitness, such as when life-threatening situations arise (Dobson and Brent 2013; Homberg and Lesch 2011).

The role of serotonin in alcohol consumption has been studied in animal models. Serotonergic system has been found to have only a minor role in mediating sensitivity to high doses of alcohol (reviewed by Vengeliene et al. 2008), but to be crucial for the development of alcohol reinforcement. It has been shown that alcohol potentiates the action of 5-HT (Lovinger and Zhou 1994), and it has been suggested that inborn serotonergic dysfunction might be of importance for the initial alcohol preference. Low levels of 5-HT in limbic structures have been identified in several alcohol-preferring rat lines (McBride and Li 1998). In humans, lower activity of platelet monoamine oxidase (MAO),

a marker for the central serotonergic system (Oreland 2004), has been demonstrated in chronic alcoholic patients and associated with alcohol-related problem behaviour (Eensoo et al. 2004; Nilsson et al. 2008; Pivac et al. 2004; Schmidt et al. 1997).

A number of studies have investigated the role of 5-HTTLPR in alcohol consumption, with contradictory results (reviewed by Dick and Foroud 2003; McHugh et al. 2010). In some studies, an association between the short allele of 5-HTTLPR and higher alcohol consumption (Covault et al. 2007; van der Zwaluw et al. 2010; Merenäkk et al., 2011), 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 detected. 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 and 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. Several 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). This is not surprising, given that alcohol consumption is influenced by social, economic, and political conditions that vary between countries and can vary within a country between time periods. Therefore, analyzing the effects of 5-HTTLPR genotype on alcohol use in the context of birth cohorts may shed light on the background of these varied results.

1.2.2. Vesicular monoamine transporter 1 gene and its role in mental health disorders

Two structurally related but pharmacologically distinct human vesicular monoamine transporters have been identified, encoded by separate genes, *VMAT1* (*SLC18A1*) located on chromosome 8p21 and *VMAT2* (*SLC18A2*) on chromosome 10q25 (Peter et al. 1993). It was initially reported that only VMAT2 is expressed in the brain (Peter et al. 1995; Erickson et al. 1996). However, it was later found that VMAT1 is also widely expressed in human brain at the mRNA and protein level (Lohoff et al. 2006). VMATs carry monoamines such as serotonin, dopamine, adrenaline and noradrenaline from the cytoplasm into storage vesicles (Edwards 1992). The transporters share common substrates with the exception of histamine, which is believed to be preferentially packaged by VMAT2 (for review, see Bernstein et al. 2014). They also differ in affinities: VMAT1 shows higher affinity for serotonin (Brunk et al. 2006).

Monoamine systems undergo extensive and interdependent functional reorganization as affective disorders develop (*e.g.*, Harro and Oreland 2001). Several functional polymorphisms in the monoaminergic (dopaminergic, serotonergic,

and noradrenergic) systems have been reported to moderate anxiety and affective disorders (for review, see Lacerda-Pinheiro et al. 2014) and alcohol use (Guo et al. 2007). Although deviations in monoaminergic function probably vary between disorders, a common source of vulnerability could lie in the vesicular function that controls monoamine storage and homeostasis. Studies in vitro show that lithium and valproate, effective pharmacotherapies for bipolar disorder, increase the expression of VMAT1, suggesting that the VMAT1 might be a target for therapeutic drug action (Lohoff 2010; Wimalasena 2011). Several recent genetic case-control studies have documented an association between common missense variations in the VMAT1 gene and susceptibility to bipolar disorder (Lohoff et al. 2006) and schizophrenia (Bly 2005; Chen et al. 2007; Lohoff et al. 2008a).

A common single nucleotide polymorphism in the VMAT1 gene (rs1390938 G/A) that results in threonine or isoleucine at amino acid 136 (Thr136Ile) has recently been shown to be functional in vitro, with the 136Ile variant leading to increased monoamine transport into presynaptic vesicles (Khalifa et al. 2012). Thr136Ile polymorphism is located in the intravesicular loop 1, and the frequency of the hyperfunction allele (A; 136Ile) is ~0.25 in European and Caucasian samples and <0.1 in African samples.

Carriers of the 136Ile (A) variant show diminished hemodynamic responses to negative emotional words in the medial prefrontal cortex and pregenual anterior cingulate cortex when compared with Thr136 homozygotes, suggesting that the *VMAT1* hyperfunction allele may predispose certain individuals to a diminished cortical response to negative stimuli (Lohoff et al. 2014). An association of the 136Thr variant with bipolar disorder (Lohoff et al. 2006) and higher self-report State-Trait Anxiety Inventory (STAI) scores in Thr/Ile heterozygous females (Lohoff et al. 2008b) has also been described. Considering that symptoms of anxiety and depressiveness have been associated with problematic alcohol use (de Abreu Costa et al. 2013; Edwards et al. 2014), the possibility that the rs1390938 polymorphism has a role in alcohol consumption should be considered.

1.2.3. Neuregulin-1 gene and its role in mental health disorders

NRG1 is a signaling protein that affects neuronal survival and development, synaptic plasticity and glial functioning, and has been described as being critical for how an organism responds and adapts to the environment (Stefansson et al. 2004). The gene encoding NRG1 is located on 8p12–21, and about 15 known NRG1 isoforms are generated through alternative promoter usage and splicing (Buonanno and Fischbach 2001; Falls 2003). These isoforms participate in neuronal migration and specification, oligodendrocyte differentiation and myelination, and regulation of cholinergic neurotransmission and expression of glutamate and γ-aminobutyric acid (GABA) receptors (Mei and Xiong 2008). NRG1 type IV has only been detected in brain and a putative type IV NRG1 protein of 66 kDa is similarly brain-specific (Tan et al. 2007). A functional polymorphism in

the promoter region of the brain-specific type IV neuregulin-1 (*NRG1*) gene – SNP8NRG243177/rs6994992; (C/T) has recently become of target in mental health research.

Frequency of the minor (T) allele of the rs6994992 polymorphism has been found to be ~0.35 in European samples (Barnes et al. 2012; Kéri 2009; McIntosh 2008). The rs6994992 polymorphism affects *NRG1* transcription rates: hippocampal mRNA expression of type IV NRG1 has been found to be higher in individuals carrying the T allele (Law et al. 2006). It has been confirmed by single point mutagenesis that promoter activity of the construct rs6994992-T is ~65% higher than that of the construct rs6994992-C (Tan et al. 2007). This genetic variant has also been demonstrated to affect brain structure as the T allele was associated with decreased grey matter volume and white matter density (Barnes et al. 2012; McIntosh et al. 2008), and also with reduced white matter integrity (Sprooten et al. 2009) in several brain regions.

NRG1 has originally been investigated as a candidate susceptibility gene for schizophrenia; the presence of the minor T allele has been associated with mental health problems. *NRG1* was first associated with schizophrenia in the Icelandic population (Stefansson et al. 2002). The majority of subsequent association studies, including two in Scottish populations (Stefansson et al. 2003; Thomson et al. 2007) have shown association of *NRG1* with both schizophrenia and bipolar disorder (Green et al. 2005), while the results have not been uniform (Crowley et al. 2008). In a genome-wide association study of alcohol dependence, the novel associations have suggested direct involvement of, or interaction with, genes previously identified as schizophrenia risk loci (Gelernter et al. 2014). In a genome-wide linkage and association study on an African-American sample it was found that *NRG1* is a likely susceptibility gene for cannabis dependence (Han et al. 2012).

Experimentally, it has been demonstrated that manipulation at the neuregulin-1 gene alters the sensitivity to the behavioural effects of cannabinoids: compared to wild type-like animals, heterozygous *Nrg1* transmembrane-domain knockout mice were more sensitive to the sedative action of cannabinoids (Boucher et al. 2007a) and also to the effects of Δ^9-tetrahydrocannabinol (THC) on stress response (Boucher et al. 2007b). Interestingly, partial deletion of *Nrg1* interacts with stress to promote neurobehavioural deficits, as *Nrg1* heterozygous mice displayed greater acute stress-induced anxiety-related behaviour than wild-type mice (Chohan et al. 2014).

Results from an animal model and a genome-wide linkage and association study in humans suggest that *NRG1* is a likely susceptibility gene for drug use. Given that individuals with schizophrenia and their non-psychotic siblings report higher rates of alcohol, cannabis, and nicotine use and that *NRG1* rs6994992 polymorphism has been associated with schizophrenia risk, the NRG1 genotype could also affect alcohol use. The rs6994992 polymorphism has been found to interact with psychosocial stress (Kéri et al. 2009b).

1.2.4. Oxytocin receptor gene and its role in alcohol use disorders

Oxytocin, a nine amino acid neuropeptide (nonapeptide), is synthesized primarily in the magnocellular neurosecretory cells of the paraventricular and supraoptic nuclei of the hypothalamus, and stored in the posterior pituitary gland, but extensive pathways containing oxytocin are present already in teleosts and highly developed mesolimbic tracts exist in mammalians (Grinevich et al. 2016). High density of oxytocin receptors has been found in brain regions involved in regulating mood, social behaviour and addictive processes, such as the central nucleus of amygdala, nucleus accumbens and ventral pallidum (Gimpl and Fahrenholz 2001). Oxytocin is a potent modulator of a variety of brain functions including learning, memory, emotions, mood, sexual behaviour, and adapting to social environment (reviewed by Sarnyai 2011).

There is accumulating evidence of an interaction between the neural substrates of affiliative behaviour and those of drug reward (reviewed by McGregor and Bowen 2012), with a role for brain oxytocin systems in modulating acute and long-term drug effects (McGregor et al. 2008). Intranasal administration of oxytocin has been found to elicit a variety of physiological and behavioural effects in humans, including reduction of anxiety (de Oliveira et al. 2011), and plasma levels of oxytocin-reactive autoantibodies correlate with mood states (Garcia et al. 2011). However, these results should be interpreted with caution: it has recently been brought up that studies analyzing the effects of intranasally administered oxytocin are generally underpowered (Walum et al. 2016) and it is unclear what percentage of peripherally administered oxytocin reaches oxytocin receptors in the brain (Leng and Ludwig 2016).

It has been suggested that anxiety disorders increase the risk for developing alcohol use disorders (Boschloo et al. 2013; Kessler et al. 1997). Oxytocin has been found to enhance functional connectivity between the amygdala and the bilateral insula and middle cingulate/dorsal anterior cingulate gyrus during the processing of fearful stimuli, suggesting that oxytocin may have broad prosocial implications such as enhancing the integration and modulation of social responses especially in anxiogenic contexts (Kirsch et al. 2005; Gorka et al. 2015). By reducing anxiety, increasing the ability to cope with stress, and possibly reversing established alcohol tolerance, oxytocin treatment may diminish craving and facilitate sobriety. Indeed, oxytocin treatment not only blocks alcohol withdrawal in human subjects (Pedersen et al. 2013) but has also been shown to decrease alcohol preference in animals. Recent preclinical studies in rodents have reported a remarkable ability of exogenously delivered oxytocin to inhibit stimulant and alcohol self-administration, to alter associated drug-induced changes in dopamine, glutamate and Fos expression in cortical and basal ganglia sites, and to prevent stress- and priming-induced relapse to drug seeking (reviewed by McGregor and Bowen, 2012). Based on previous findings it can be hypothesized that susceptibility to alcohol abuse is affected by individual differences in the oxytocinergic system.

The human oxytocin receptor gene (*OXTR*) is located on chromosome 3p25, spans about 17 kb, consists of three introns and four exons (Inoue et al. 1994), and encodes a 389-amino acid polypeptide with seven transmembrane domains belonging to the class I of the G-protein-coupled receptor family (Gimpl and Fahrenholz 2001). One of the common polymorphisms (rs53576) in the oxytocin receptor gene has recently been found to modulate the effect of oxytocin administration (Feng et al. 2015): oxytocin increased the reward or salience of positive social interactions for male major allele (G) homozygotes, while decreasing those processes for female major allele (G) homozygotes. This single nucleotide polymorphism (SNP) of an adenine (A, f≈0.4) or guanine (G, f≈0.6) within the third intron (rs53576) appears as a particularly promising marker of inter-individual differences in oxytocinergic function (Tost et al. 2010; Wu et al. 2005). Although the molecular functionality of this SNP is still unknown (Feng et al. 2015), the A allele has been suggested to be associated with less efficient oxytocinergic functioning in experimental settings (Marsh et al. 2012), and this would be theoretically consistent with association studies: the A allele carriers have lower levels of optimism, mastery, and self-esteem (Saphire-Bernstein et al. 2011), lower general sociality (Li et al. 2015), empathy, and higher levels of stress reactivity (Rodrigues et al. 2009). Different *OXTR* polymorphisms have been found to moderate the effects of alcohol use on aggressive behaviour in males, suggesting that alcohol has a larger effect on aggressive behaviour for those who, due to altered oxytocin signaling, already in a sober state have more difficulties with social abilities (Johansson et al. 2012a, 2012b; LoParo et al. 2016).

2. AIMS OF THE STUDY

This dissertation comprises of analyses aimed to explore the effects of common neurotransmission-related genetic polymorphisms (involved in different psychiatric disorders and mediating the adaptation to the environment) in relation to alcohol use in a population-representative sample.

More specifically,

1. Is the association of alcohol use and 5-HTTLPR genotype also present in the older birth cohort of the ECPBHS, and subject to cohort effects? (**Paper I**)
2. Does the *VMAT1* rs1390938 polymorphism affect the rates of alcohol use and whether any eventual associations depend on gender, birth cohort and environmental factors? (**Paper II**)
3. Is the *NRG1* rs6994992 polymorphism associated with rates of alcohol use and whether any eventual associations depend on gender, birth cohort and environmental factors? (**Paper III**)
4. Does the *OXTR* rs53576 polymorphism affect the rates of alcohol use and whether any of the associations depend on gender, birth cohort and environmental factors? (**Paper IV**)

3. MATERIALS AND METHODS

3.1. Study population

Research in this dissertation is based on the ECPBHS sample. This is the original Estonian sample of the European Youth Heart Study (1998/99) which was subsequently incorporated into the longitudinal Estonian Children Personality Behaviour and Health Study (ECPBHS). All the subjects are of Caucasian descent. The selection of the original sample and procedure of first data collection has been described in detail elsewhere (Harro et al. 2001). In brief, this is a representative sample of the Tartu city and county with a school as the sampling unit. All schools of Tartu County, Estonia, that agreed to participate (54 of the total of 56) were included into the sampling using the probability proportional to the number of students of the respective age groups in the school, and 25 schools were selected. All children from grades 3 (younger birth cohort) and 9 (older birth cohort) were invited to participate.

The total number of subjects in the first wave in 1998/99 was 1176 (45.7% males); 583 in the younger cohort (mean age=9.5, SD=0.5) and 593 in the older cohort (mean age=15.4, SD=0.6). The follow-up studies for the younger cohort took place in 2004 (n=483, mean age=15.3, SD=0.7), 2007 (n=454, mean age=18.3, SD=0.5) and 2014 (n=440, mean age=25.3, SD=0.5). For older cohort, the follow-ups were in 2001 (n=479, including 62 additional subjects, mean age=18.4, SD=0.9) and 2008 (n=541, mean age=24.7, SD=0.7). ECPBHS is population representative, while 79.1% of subjects of the randomized regional sample participated in the original sampling. The study was approved by the Ethics Review Committee on Human Research of the University of Tartu, and written informed consent was obtained from all the participants, and in case of minors, also from their parents.

3.2. Measurements

3.2.1. Estimation of alcohol consumption

Data collection was performed in uniform conditions of the laboratory at each wave. The measures in questionnaires varied by study waves. Subjects reported the age when they first consumed half a unit of alcohol during all follow-up studies (**Papers I, II and III**). One unit 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.). In all data collection waves the participants reported how often they had consumed different types of alcoholic beverages (**Papers II, III and IV**). According to the most frequently consumed type of alcoholic beverage, a 5-point total alcohol use scale was constructed: 1=almost never, 2=less than once a month, 3= at least once a month, 4= at least once a week, 5=every day, as previously described in Merenäkk et al. (2003). In **Paper**

I, data from the older birth cohort regarding the frequency of alcohol consumption during the previous 30 days at ages 18 and 25 was utilized, using a seven-point scale: 1=not at all, 2=once, 3=all together two to three times, 4=once or twice a week, 5=three to four times a week, 6=five to six times a week, and 7=every day (Merenäkk et al. 2011). In **Paper III**, data from the older birth cohort regarding the frequency of consuming more than 5 units of alcohol at a time (i.e., binge drinking) during the previous 12 months at the age of 25 was analyzed, using a five-point scale similar to the one used in the case of the most frequently consumed type of alcoholic beverage.

3.2.2. Use of tobacco products

Smoking habits were also assessed in all waves (**Paper III**). The proportion of smokers at age 18 in both cohorts and at age 25 in the older birth cohort was analyzed. In addition, the frequency of consuming tobacco products during the previous 30 days in the older birth cohort at the age of 25 was included in the analysis. A six-point scale was used: 1=never, 2=once or twice a month, 3=once or twice a week, 4=almost every day, 5=every day, 6=several times a day.

3.2.3. Illicit drug use

Nine years old children were not asked about illicit drugs. In all other data collection waves, subjects of both birth cohorts reported whether they had ever used illicit drugs (**Paper III**).

3.2.4. Psychiatric diagnosis

Psychiatric assessment based on DSM-IV was carried out in the older cohort at age 25 by experienced clinical psychologists using the Mini-International Neuropsychiatric Interview (M.I.N.I.5.0.0; Sheehan et al. 1998; Estonian version: Shlik et al. 1999) at age 25. In the analysis we used lifetime incidence of affective and anxiety disorders (**Paper II**), and substance use disorders (**Papers II, III and IV**) which in our sample almost exclusively consisted of alcohol use disorder (AUD) (Laas et al. 2015).

3.2.5. Stressful life events

History of stressful life events (SLEs) was self-reported in all follow-up studies (**Paper III**). Subjects were divided into low (0–2 events) and high (3 or more events) SLE exposure groups (Laas et al. 2015). The list of adverse life events varied across measurement times and consisted of 10–17 (dependent on the study wave) stressful experiences including parental death and divorce/separation, unemployed parent, parental alcoholism, poverty, poor living conditions, poor

health, accidents and traumas, physical abuse, emotional abuse, severe burden/ serious concerns, suicidal attempts, leaving home for several days without telling anyone, depression of a close relative, suicide attempt or committed suicide of a close relative. The events were recorded as dichotomous variables (present or not present) and were then counted to form the number of adverse life events experienced.

3.2.6. Social interactions

Relationship with teachers, classmates and family members were self-reported at ages 15 and 18 (**Paper IV**). The ECPBHS Child Questionnaire included eleven items on perceived school atmosphere and principal component analysis revealed three independent factors, reflecting relationships with teachers, relationships with schoolmates, and bullying/rejection. The score for adverse relations with teachers was obtained by adding the scores of the following three questions: "I'm not one of the teachers' favourites", "Teachers often treat me unfairly", "Teachers treat me well" (reversed score). The score for relations with classmates was obtained by adding the scores of the following three questions: "Classmates reckon with me", "Our class is like-minded", "I have a lot of friends at school" and "I feel secure at school". The score for bullying/rejection was obtained by adding the scores of the following three questions: "I am bullied by my classmates", "I feel lonely at school", "I'm afraid of going to school". Items were presented in terms of 4-point Likert scale. To obtain an overall score of school adversity the relationships with classmates subscale was reversed.

Social interactions in the family were measured by Tartu Family Relationships Scale (Paaver et al. 2008) which is a child-report scale. It was composed for ECPBHS and has four subscales: Closeness (15 items, *e.g.*, "Our family is dedicated to each other", "The marriage of my parents is happy"), Support (7 items, *e.g.*, "My family supports me", "Someone in the family helps (has helped) me to feel important and special"), Misprize (10 items, *e.g.*, "I can make no decision on my own", "I am depreciated at home"), and Abuse (emotional and physical, 7 items, *e.g.*, "Were you ever hit by someone in your family or have you experienced physical violence in your family?"). Items were presented in terms of 4- or 5-point Likert scale. At age 15, only questions later forming the Misprize subscale was administered to the older cohort. At age 18, all the four subscales were used.

3.2.7. Anxiety

The Spielberger State Trait Anxiety Inventory (STAI, Spielberger et al. 1983) was used to measure anxiety (**Paper II**). In the younger birth cohort, the Spielberger State Anxiety Inventory (STAI-S) was used at ages 15 and 18 and the Spielberger Trait Anxiety Inventory (STAI-T) at age 18. In the older birth cohort, STAI-S was used only at age 25, and STAI-T at ages 18 and 25.

3.2.8. Depressiveness

Depressiveness was measured using the self-report version of the Montgomery-Åsberg Depression Rating Scale (MÅDRS; Montgomery and Åsberg 1979) or Beck Depression Inventory (BDI; Beck et al. 1961) (**Paper II**). BDI was used to measure depressiveness in the younger birth cohort at age 15. MÅDRS was used in the younger cohort at age 18 and in the older birth cohort at ages 18 and 25.

3.2.9. Impulsivity

Self-reports for different facets of impulsivity were completed at ages 15 and 18 for the younger cohort and at ages 18 and 25 for the older cohort (**Paper II**). The Adaptive and Maladaptive Impulsivity Scale, which follows the concept of functional and dysfunctional impulsivity (Dickman 1990) and comprises subscales measuring fast decision-making and excitement seeking (functional or adaptive impulsivity) and disinhibition and thoughtlessness (dysfunctional or maladaptive impulsivity), was used (Laas et al. 2010).

3.2.10. Big Five Personality

Personality traits of the 5-factor model (Costa and McCrae 1989) (**Paper II**) were measured by self-reports with the Estonian version of Revised NEO Personality Inventory (NEO-PI-R) (Kallasmaa et al. 2000), EE.PIP-NEO (Mõttus et al. 2006), which is a semantically simplified 240-item version of the Inernational Personality Item Pool (IPIP), which emulates the NEO-PI-R, Short Five (S5) (Konstabel et al. 2011), which is a short inventory of five-factor personality, or Estonian Brief Big Five Inventory (EBBFI), which is a short and semantically simplified questionnaire (Harro et al. 2009, Laidra et al. 2006). All scales measure each of the FFM personality dimensions (Neuroticism, Extraversion, Openness to experience, Agreeableness and Conscientiousness), and their six subscales with the exception of EBBFI. All four instruments have been found to provide realistic and convergent assessment of personality according to the five-factor model (Konstabel et al. 2011, Laidra et al. 2006, Mõttus et al. 2006). Personality data were collected at age 15 (younger cohort: EE.PIP-NEO; older cohort: EBBFI), 18 (younger cohort: S5; older cohort: NEO-PI-R) and 25 (older cohort: EE.PIP-NEO).

3.2.11. Genotyping

Genomic DNA was extracted from venous blood samples using Qiagen QIAamp® DNA Blood Midi Kit.

3.2.11.1. 5-HTTLPR VNTR

Genotyping for triallelic classification (**Paper I**) was performed according to Anchordoquy et al. (2003). Genotyping was done in two stages. First all subjects were genotyped for the 5-HTTLPR VNTR polymorphism, then SNP rs25531 (A/G). The polymorphic region was amplified using the primers 5-HTTLPR-F: 5′-6FAM-ATG CCA GCA CCT AAC CCC TAA TGT-3′ and 5-HTTLPR-R: 5′-GGA CCG CAA GGT GGG CGG GA-3′. PCR reaction components and final concentration were as follows: 1 x of 5x HOT FIREPol BLEND with BSA 2.5 mM $MgCl_2$ (Solis Biodyne); 5% of DMSO; 1 x of 10x Solution S (Solis Biodyne); 380 μM each of the forward and reverse primers; 10–50 ng of template DNA. The amplification was conducted in a total volume of 20 μl. The touchdown PCR cycles were used as by Anchordoquy et al. (2003). The electrophoresis was made on ABI PRISM 3130XL genetic analyser and the components used were: 1 μl PCR product, 10 μl Hi-Di formamide, 0.25 μl LIZ 500 size standard. For genotyping of SNP rs25531 (la/lg) the MspI restriction analysis was conducted in a total volume of 10 μl (2 μl of PCR product and 8 μl of restriction master mix). The reaction components and final concentrations of the restriction master mix were as follows: 1 x Buffer Tango; 4 units of MspI restriction enzyme (Fermentas). Samples were then incubated on 37 °C for 3 h and on 65 °C for 20 min. MspI digest electrophoresis was conducted using ABI PRISM 3130XL genetic analyser and the components used were: 1 μl digest product; 10 μl Hi-Di formamide; 0.25 μl LIZ 500 size standard. Genotypes were generated using ABI Gene-Mapper V 4.0 software. Genotyping was performed blind to all phenotypic data. All DNA samples were successfully genotyped. In **Paper I**, the subjects were classified by genotype according to the transcriptional activity of the 5-HTT gene (Hu et al. 2005). Due to lg allele being functionally equivalent to the s allele, lg alleles were grouped together with s alleles for the statistical analysis. 5-HTTLPR genotype frequencies were in Hardy–Weinberg equilibrium and shown in Table 1.

Table 1. 5-HTTLPR genotype and allele frequencies in the ECPBHS sample.

	5-HTTLPR VNTR			SNP rs25531/SLC6A4		
	l/l	l/s	s/s	la	lg	s
Older cohort (*n*=654)	211 (32%)	302 (46%)	141 (22%)	0.55	0.08	0.37
Males (*n*=290)	91 (31%)	138 (48%)	61 (21%)			
Females (*n*=364)	120 (33%)	164 (45%)	80 (22%)			
Younger cohort (*n*=580)	194 (33%)	290 (50%)	96 (17%)	0.58	0.08	0.34
Males (*n*=277)	92 (33%)	132 (48%)	53 (19%)			
Females (*n*=303)	102 (34%)	158 (52%)	43 (14%)			

3.2.11.2. SNP polymorphisms: *VMAT1* rs1390938, *NRG1* rs6994992, *OXTR* rs53576

The real-time polymerase chain reaction (RT-PCR) for genotyping the three SNP polymorphisms was performed using TaqMan Pre-Designed SNP Genotyping Assays (Applied Biosystems; Foster City, CA, USA) containing primers and fluorescent probes. For *VMAT1* rs1390938 (**Paper II**), the Assay C___8804621_1_ was utilized; for *NRG1* rs6994992 (**Paper III**) and *OXTR* rs53576 (**Paper IV**) polymorphisms, the Assays C_____22019_10 and C___3290335_10 were used, respectively. Genotyping reactions were performed in a total volume of 10 ml with ~25 ng of template DNA. RT-PCR reaction components and final concentrations were as follows: 1:5 5 x HOT FIREPol® Probe qPCR Mix Plus (ROX) (Solis BioDyne) and 1:20 80 x TaqMan Primers Probe.

Context sequences [VIC/FAM] were as follows:
VMAT1 rs1390938 – AGCAAACAGAACCCCGACCCGGGTA[A/G]TCTCTT CCTCCAAGAAACCTGTGCC,
NRG1 rs6994992 – AAGCACCATGCAGGGTTCAAGTGAA[C/T]GTATACT GGAGGCCAGACCTGCCCA,
OXTR rs53576 – AAAGGTGTACGGGACATGCCCGAGG[A/G]TCCTCAGT CCCACAGAAACAGGGAG.

Reactions were performed on the Applied Biosystems ViiA™ 7 Real-Time PCR System. The amplification procedure consisted of an initial denaturation step at 95 °C for 12 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Positive and negative controls were added to each reaction plate. No inconsistencies occurred. Genotyping was performed blind to all phenotypic data. Allele frequencies agreed with National Center for Biotechnology Information database and published reports. All DNA samples but one (younger cohort, males, *VMAT1* rs1390938) were successfully genotyped. In **Paper III**, *NRG1* rs6994992 CT heterozygotes and TT homozygotes were grouped together for data analysis as T allele carriers due to functional similarity (Barnes et al. 2012). Other genotypes (*VMAT1* rs1390938, *OXTR* rs53576) were grouped based on their allelic variances. All genotype frequencies were in Hardy–Weinberg equilibrium and are shown in Table 2.

Table 2 *VMAT1* rs1390938, *NRG1* rs6994992 and *OXTR* rs53576 genotype frequencies in the ECPBHS sample.

	VMAT1 rs1390938			*NRG1* rs6994992			*OXTR* rs53576		
	AA	AG	GG	CC	CT	TT	AA	AG	GG
Older cohort (*n*=654)	76 (12%)	279 (43%)	299 (45%)	256 (39%)	320 (49%)	78 (12%)	85 (13%)	317 (48%)	252 (39%)
Males (*n*=290)	40 (14%)	129 (44%)	121 (42%)	105 (36%)	148 (51%)	37 (13%)	33 (11%)	141 (49%)	116 (40%)
Females (*n*=364)	36 (10%)	150 (41%)	178 (49%)	151 (42%)	172 (47%)	41 (11%)	52 (14%)	176 (49%)	136 (37%)
Younger cohort (*n*=580)	52 (9%)	265 (46%)	262 (45%)	231 (40%)	264 (45%)	85 (15%)	66 (11%)	286 (50%)	228 (39%)
Males (*n*=277)	28 (10%)	128 (46%)	120 (43%)	114 (41%)	122 (44%)	41 (15%)	30 (11%)	138 (50%)	109 (39%)
Females (*n*=303)	24 (8%)	137 (45%)	142 (47%)	117 (39%)	142 (47%)	44 (14%)	36 (12%)	148 (49%)	119 (39%)

3.3. Statistical analysis

Three-way analysis of variance (ANOVA) considering all interactions was utilized in the statistical analysis of age of first alcohol consumption (**Papers I, II and III**). Preplanned comparisons of cohorts by genotype and gender, genders by genotype and cohort, and genotypes by gender and cohort were performed using properly defined contrasts. In the same way, average values of genders by cohort and cohorts by genders were compared. To test the statistical significance of genotype × gender interactions by cohort for both cohorts, two-way ANOVA was performed; to test the overall genotype effect by cohort and gender, one-way ANOVA was used. The same methodology was applied when analyzing substance consumption frequency (**Papers I–IV**) and anxiety, depressiveness, impulsivity and personality scores (**Paper II**). Additionally, the repeated measurements of the same subjects were considered in three-way ANOVA. Results have been presented as F-statistic and raw *p* value. Fisher's least significance difference method was used in *post hoc* comparisons.

In the case of a dichotomous dependent variable (*e.g.,* alcohol use disorder), Pearson's chi-square ($\chi 2$) test or binary logistic regression with Wald chi-square ascertaining whether a variable is a significant predictor of the outcome was utilized, and results are reported in the form of odds ratios (OR) with confidence intervals (CI) (**Papers II–IV**). Binary logistic regression with Wald chi-square was also utilized when assessing the interaction of the number of stressful life events (**Paper III**) or unfavourable social interactions (**Paper IV**) and genotype on the prevalence of AUD or drug use.

In the study on the *OXTR* genotype (**Paper IV**), we tested a series of models with Bayesian estimation using Markov chain Monte Carlo (MCMC) sampling with SPSS AMOS in order to test the mediation and interaction effects of the genotype, alcohol use frequency and unfavourable relations with teachers on the occurrence of AUD. All the continuous variables were centered, and interaction terms were residual-centered to avoid statistical interference errors (Little et al. 2006). Both the direct and indirect effects were reported as standardised regression weights with 95% confidence intervals (CI), the association was regarded significant if the 95% CI did not cross zero. Models were evaluated by posterior predictive *p* values (ppp; the closer the ppp-value to 0.5 the better the model); posterior shapes, traces and autocorrelations; checked for impossible estimand values and non-normality; and compared with preliminary analyses presented in results.

As the personality data have been collected with different instruments (**Paper II**), all scores were transformed into Z-scores for statistical analysis. In addition, self-reported alcohol use frequencies were transformed into Z-scores in **Paper IV** due to the variations in the number of choice items between different time points. Linear mixed model (LMM) was used to assess the effect of the genotype on changes in alcohol consumption over the three measurement times, and presented as numerator degrees of freedom (df) and denominator df in parentheses, F-statistic, and raw *p*-value.

Contrasts were calculated for significant model effects. All p values are reported as 2-tailed, and results are considered significant at the $p<0.05$ level. Statistical analyses were performed using IBM® SPSS® Statistics (versions 19 and 20) and R (version 3.1.1., package "stats").

4. RESULTS AND DISCUSSION

4.1. Alcohol use in the ECPBHS sample (Papers I–IV)

In the current population-representative birth cohort study in a CEE country, the subjects of the younger cohort reportedly started consuming alcohol at an earlier age (F[1, 1063]=84.9, p<0.001) (Figure 1). Male subjects started consuming alcohol statistically significantly earlier than females in the older birth cohort (F[1, 579]=14.6, p<0.001), and also on a trend level in the younger cohort (F[1, 490]=2.0, p=0.158). This is similar to what has been described in the WHO Health Behaviour in School-Aged Children (HBSC) study reports that also include data from Estonia – the gender gap in alcohol use is narrowing (Currie et al. 2000, 2004, 2008; Pärna et al. 2012).

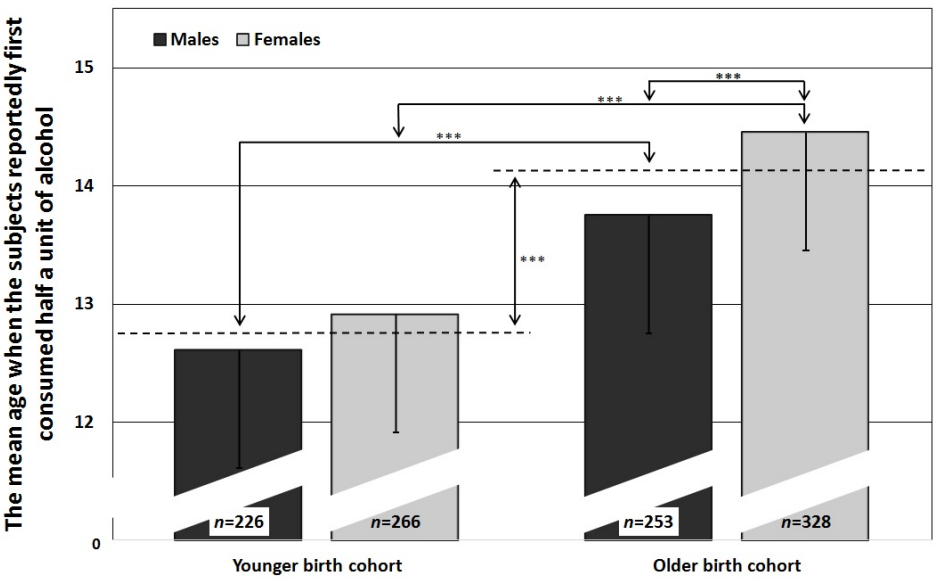

Figure 1 The effect of gender and birth cohort on the average age when the subjects first consumed half a unit of alcohol. The dashed lines indicate the mean age when the subjects from the respective cohort first consumed half a unit of alcohol. *N* represents the number of subjects. Vertical bars denote standard deviations. Significant differences between groups: ***p<0.001.

Higher alcohol consumption among individuals reporting earlier drinking onset has been shown in population-representative studies (Lee et al. 2012; York et al. 2004), so such a change in drinking behaviour of the population can bear public health consequences. Indeed, individuals who have reported an early age of drinking onset have much more likely been found to meet the criteria for life-time alcohol abuse and dependence (Grant and Dawson 1997). The lower mean age when the subjects reportedly first consumed half a unit of alcohol also

increased the likelihood of developing AUD in the ECPBHS sample (OR=1.15[1.08–1.23], $p<0.001$). There were significantly more subjects diagnosed with AUD by age 25 in the younger cohort (Figure 2), who, as mentioned previously, reportedly started consuming alcohol at an earlier age. The notion that the tendency of more recent birth cohorts to start consuming alcohol at an earlier age places them at a greater risk of developing alcohol use disorders was thus confirmed also in the present study.

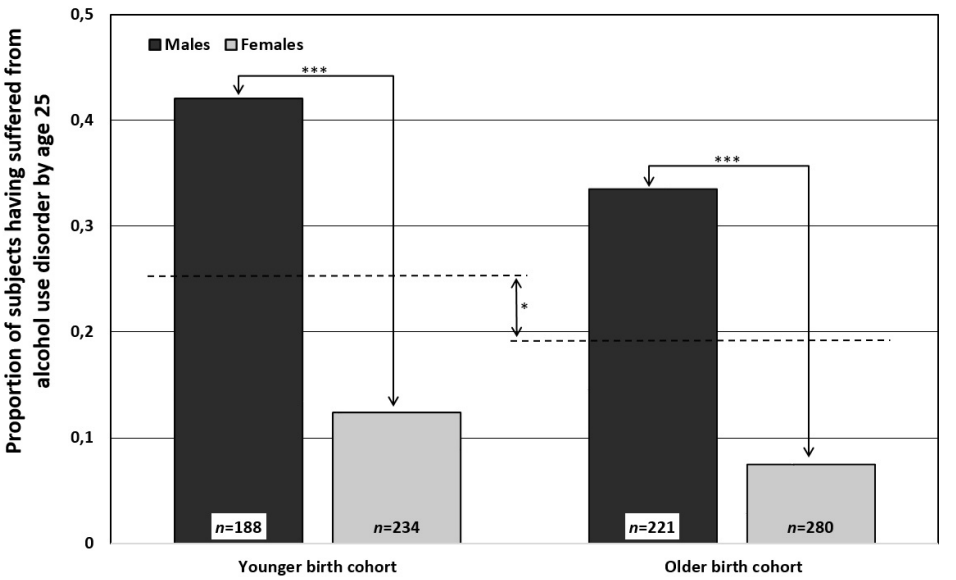

Figure 2 Lifetime Prevalence of AUD assessed at age 25 in the ECPBHS sample. The dashed lines indicate the mean proportions of subjects in the respective cohorts. *N* represents the number of subjects in the group (100%). Significant differences between groups: *$p<0.05$, ***$p<0.001$.

Male subjects from both cohorts were more frequent alcohol consumers both at the age of 18 and at the age of 25. At age 15, the younger cohort was using alcohol more frequently than the older cohort ($F[1, 1032]=59.1$, $p<0.001$). Self-reported alcohol use frequency was not, however, statistically significantly different between the cohorts at ages 18 ($F[1, 891]=0.23$, $p=0.63$) nor 25 ($F[1, 961]=0.87$, $p=0.35$). Higher frequency of consuming alcohol (according to the most frequently consumed type of alcohol) increased the likelihood of lifetime AUD by the age of 25 in both older (Figure 3) and younger birth cohort (Figure 4). Subjects who had had AUD by age 25 had reported higher frequency of consuming alcohol at the age of 15 (OR=1.45[1.16–1.81], $p=0.001$ and OR=1.60[1.21–2.10], $p=0.001$, for the older and younger cohort, respectively), 18 (OR=2.02[1.37–2.97], $p<0.001$ and OR=2.26[1.61–3.18], $p<0.001$, for the older and younger cohort, respectively) and 25 (OR=1.79[1.35–2.38], $p<0.001$ and OR=2.43[1.76–3.36], $p<0.001$, for the older and younger cohort, respectively).

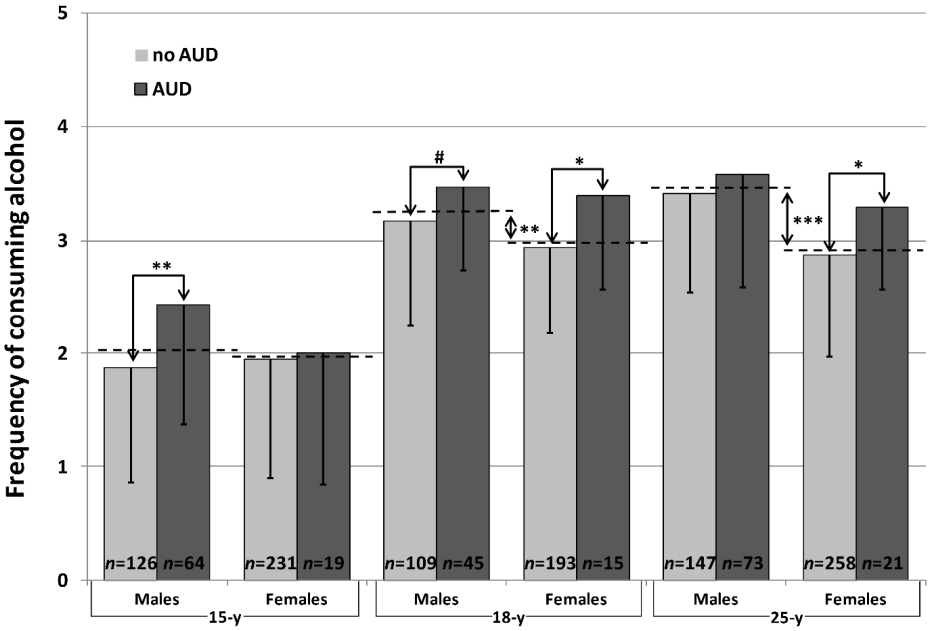

Figure 3. Frequency of consuming alcohol and the occurrence of lifetime AUD assessed at the age of 25 in the older birth cohort. Alcohol use frequency is according to the most frequently consumed type of alcohol. A five point scale was used at ages 18 and 25: *1*=never, *2*=less than once a month, *3*=at least once a month, *4*=at least once a week, *5*=every day. At age 15, a four point scale was used, excluding the option *5*=every day. *N* represents the number of subjects in the group. The dashed line indicates the mean frequency in the group. Vertical bars denote standard deviations. Significant differences between groups: **p*<0.05, ***p*<0.01, ****p*<0.001, #*p*=0.056.

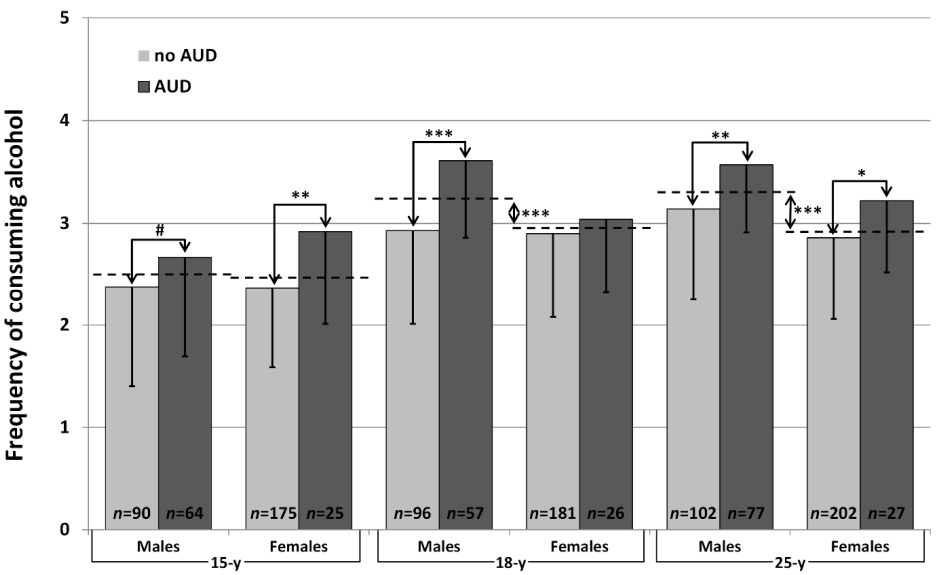

Figure 4. Frequency of consuming alcohol and the occurrence of lifetime AUD assessed at the age of 25 in the younger birth cohort. Alcohol use frequency is according to the most frequently consumed type of alcohol. A five point scale was used: *1*=never, *2*=less than once a month, *3*=at least once a month, *4*=at least once a week, *5*=every day. *N* represents the number of subjects in the group. The dashed line indicates the mean frequency in the group. Vertical bars denote standard deviations. Significant differences between groups: **p*<0.05, ***p*<0.01, ****p*<0.001, #*p*=0.07.

The number of SLEs reported at age 15 did not have any significant effect on the likelihood of developing AUD. SLEs reported at age 18 however had a significant effect on the likelihood of having been diagnosed with AUD by age 25 in both younger (OR=3.30[1.98–5.48], $p<0.001$) and older birth cohort (OR=2.77[1.57–4.88], $p<0.001$) (Figure 5). SLEs reported at age 25 affected the likelihood of developing AUD only in males of the older birth cohort. Therefore, in our sample of young adults, the life stress experienced during late teens was the most influential.

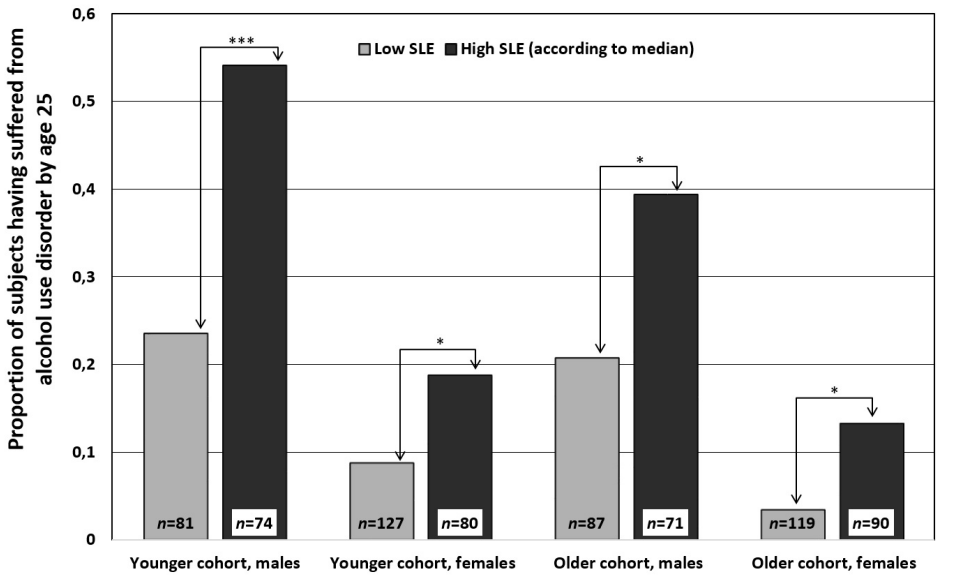

Figure 5 The effect of stressful life events (SLEs) reported at age 18 on the likelihood of having suffered from alcohol use disorder by age 25. *N* represents the number of subjects in the group. Significant differences between groups: **p*<0.05, ****p*<0.001.

Relationships at home and in school also significantly affected the likelihood of developing AUD. Subjects having suffered from AUD by age 25 reported higher scores of abuse in the family at age 18 in both younger (OR=1.09[1.02–1.17], $p=0.009$) and older birth cohort (OR=1.10[1.03–1.16], $p=0.003$) (Figure 6). In school, the quality of relationships with teachers was relevant – subjects having suffered from AUD by age 25 reported worse relationships with teachers at age 15 in both younger (OR=1.20[1.06–1.35], $p=0.005$) and older birth cohort (OR=1.26[1.12–1.42], $p<0.001$). In both birth cohorts, the effect was mostly attributable to male subjects (Figure 7).

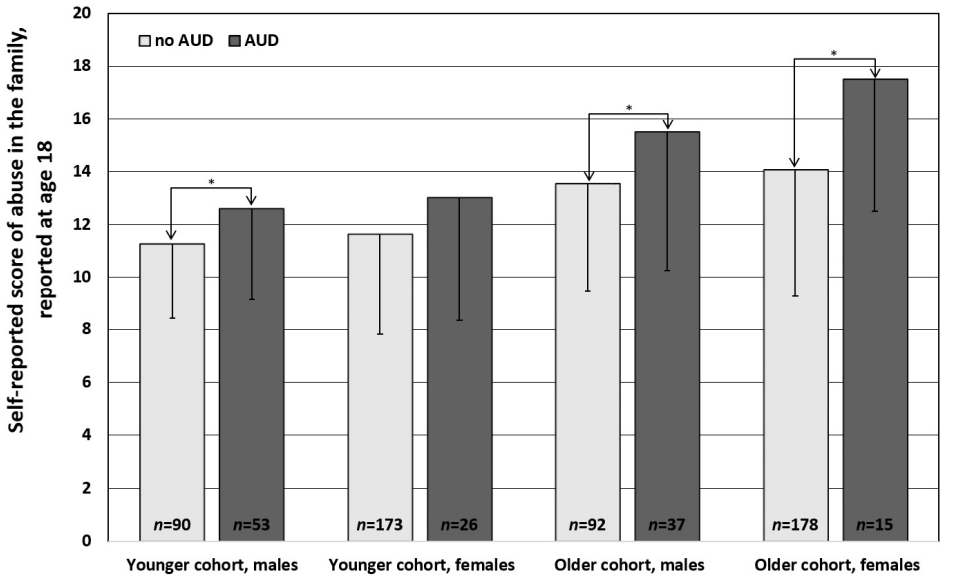

Figure 6. The effect of abuse in the family reported at age 18 on the likelihood of having suffered from alcohol use disorder (AUD) by age 25. *N* represents the number of subjects in the group. Vertical bars denote standard deviations. Significant differences between groups: **p*<0.05.

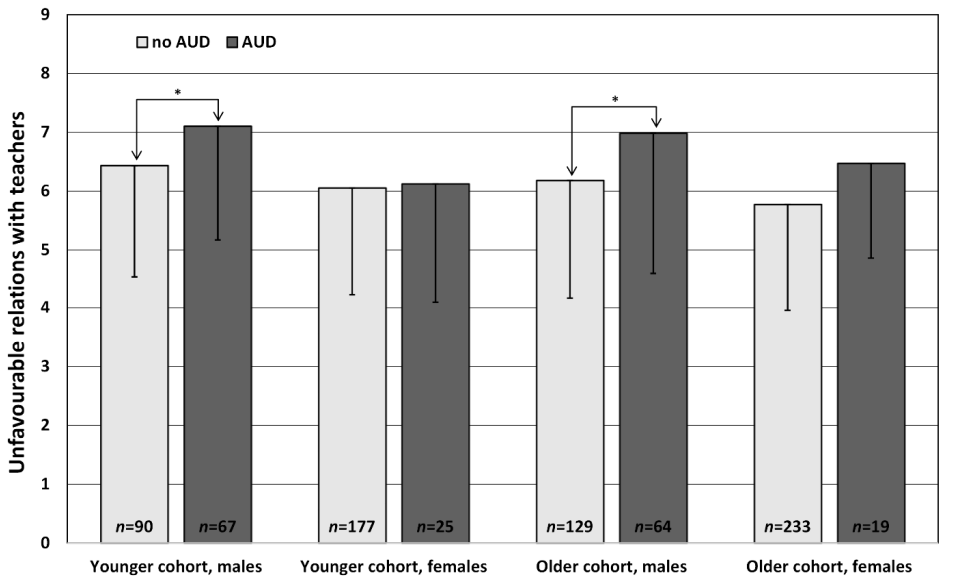

Figure 7. Unfavourable relations with teachers reported at age 15 and the likelihood of having suffered from alcohol use disorder (AUD) by age 25. *N* represents the number of subjects in the group. Vertical bars denote standard deviations. Significant differences between groups: **p*<0.05.

In sum, the subjects of the younger birth cohort (born in 1988/1989) reportedly started consuming alcohol at an earlier age than subjects of the older birth cohort (born in 1982/1983), and male subjects started consuming alcohol earlier and were more frequent alcohol consumers than females. There were significantly more subjects who had suffered from AUD by age 25 among males and in the younger birth cohort as a whole. Subjects having suffered from AUD by age 25 had reported higher frequency of consuming alcohol at the age of 15, 18 and 25. Environmental stressors (SLEs), relationships at home and in school also significantly affected the likelihood of developing AUD. Subjects having suffered from AUD by age 25 reported higher number of experienced stressful life events and higher scores of abuse in the family at age 18. In school, the subjects (especially boys) having suffered from AUD by age 25 had reported worse relationships with teachers at age 15.

4.2. The association of 5-HTTLPR and alcohol use (Paper I)

The 5-HTTLPR genotype had a significant effect on the initiation of alcohol consumption when it was considered in the interaction with the gender and birth cohort ($F_{5\text{-HTTLPR} \times \text{gender} \times \text{cohort}}$ [2, 1063]=7.2, p<0.001) on the self-reported age of first consumption of alcohol (Figure 8). Female subjects with the s/s genotype were the latest experimenters with alcohol if they belonged to the older cohort (born in 1982/1983), but the youngest if to the younger cohort (born in 1988/1989). In males, there was no significant cohort difference among the s/s homozygotes. Peer pressure has been more strongly associated with drinking for girls than it has for boys (Donovan 2002; Simons-Morton et al. 2001), and carriers of the short allele have been found to show increased social conformity (Homberg and Lesch 2011), to be more sensitive to the detection of socially relevant information (Lonsdorf et al. 2011), and to be more susceptible to environmental influences (Pluess et al. 2010). Environmental conditions and demands have been rather different for the two birth cohorts, and with regard to response with changes in alcohol use behaviour, this appears to be reflected most prominently in 5-HTTLPR s/s females.

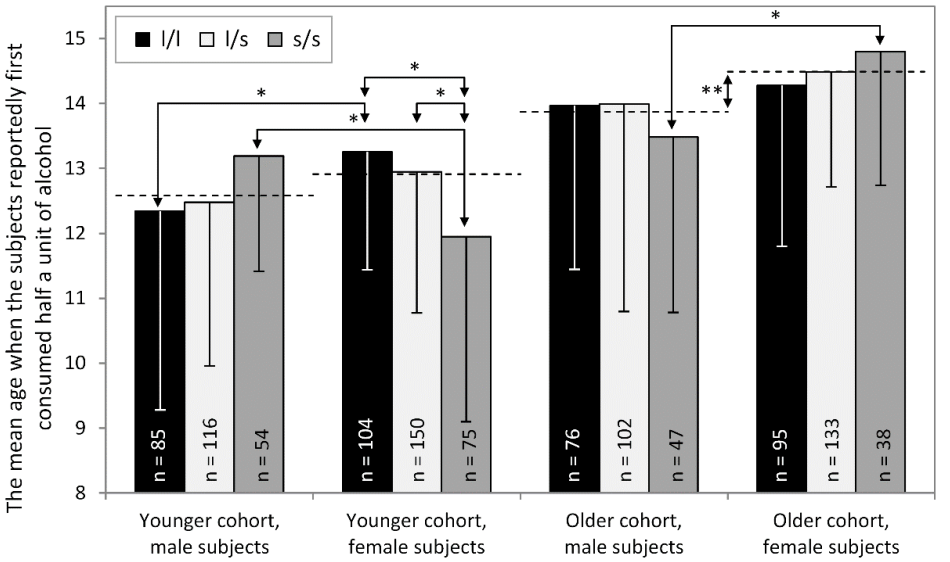

Figure 8 The effect of 5-HTTLPR genotype in interaction with gender and cohort on the average age when the subjects first consumed half a unit of alcohol. The dashed lines indicate the mean age when the subjects from the respective cohort and gender first consumed half a unit of alcohol. *N* represents the number of subjects. Vertical bars denote standard deviations. Differences between cohorts by genotype and gender were significant after Bonferroni–Holm correction for multiple testing except for the males with s/s genotype. Other significant differences between groups are denoted as follows: **p*<0.05, ***p*<0.01.

By the age of 25, the 5-HTTLPR genotype had an effect on the frequency of alcohol consumption in interaction with gender in the older birth cohort ($F_{\text{5-HTTLPR} \times \text{gender}}$ [5, 520]=3.6, p=0.028) (Figure 9). Male s/s homozygotes were the most frequent alcohol consumers; the effect was strongest in the case of male subjects with the s/s genotype (F[2, 221]=3.7, p=0.028). We have previously described a similar finding in the younger cohort where subjects with the s/s genotype were more active alcohol users by the age of 18 (Merenäkk et al. 2011); reanalysis of the data revealed that while there was no significant interaction with gender, this difference was also largely derived from the male subjects. So in both cohorts, larger alcohol use is significantly associated with the s allele and, in particular, with the s/s genotype in males, but this difference became significant in the older cohort at later age. Alcohol use was lower in females, and while the earlier onset of alcohol consumption is significantly associated with increased future use also in this sample, no genotype effect has become significant within the age constraints of the study population. Our results support the notion that subjects with the s/s genotype are most affected by environmental changes and that the genotype effect may differ between male and female subjects. For the 5-HTTLPR, it would seem more appropriate to consider it a "plasticity variant" rather than a "vulnerability genotype" (Belsky et al. 2009), and this

may explain why no clear negative effects of the 5-HTTLPR genotype on the prevalence of AUD have been detected in the ECPBHS sample by the age of 25.

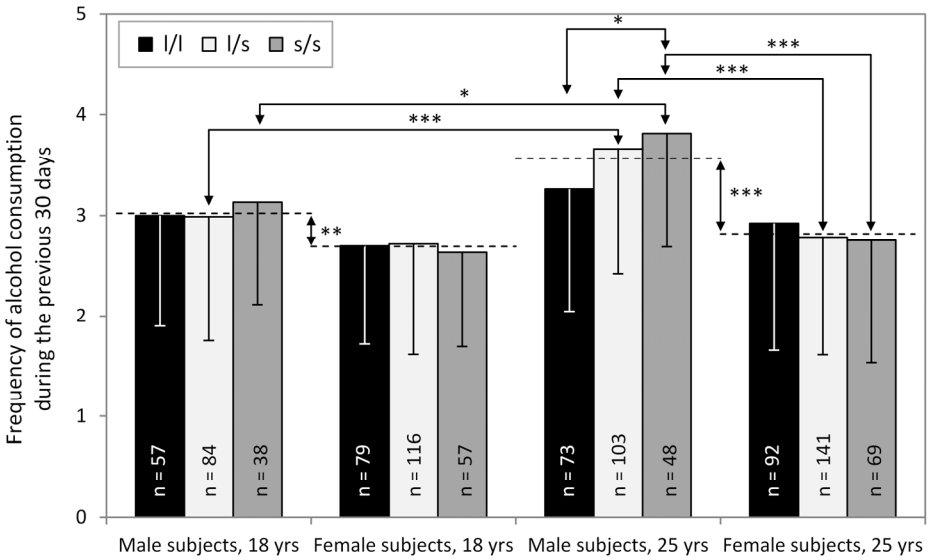

Figure 9 The effect of 5-HTTLPR genotype on the average frequency of alcohol consumption during the previous 30 days in the older cohort. A seven-point scale, described in 3.2.1, was used. The dashed lines indicate the mean frequency of alcohol consumption in the respective age and gender. Vertical bars denote standard deviations. Differences between groups after Bonferroni–Holm correction for multiple testing: *p<0.05, **p<0.01, ***p<0.001

4.3. *VMAT1* effects on alcohol use and mental health disorders (Paper II)

VMAT1 genotype was associated with the average age when the subjects reportedly first consumed half a unit of alcohol in both younger (F[2, 488]=3.0, p=0.053) and older (F[2, 578]=4.2, p=0.015) birth cohorts, but in opposite directions (Figure 10). In the younger birth cohort, GG homozygotes were the first and AA homozygotes the last to start experimenting; in the older cohort, it was the other way around. Unlike in the case of G allele carriers, there was no cohort difference in the mean age when AA homozygotes started experimenting with alcohol. Given that peer drinking serves as a model for alcohol use (Milgram 2001) and socially anxious youth can be motivated to use alcohol to manage their anxious arousal (Blumenthal et al. 2010), one possible explanation could be that AA homozygotes may be less sensitive to peer pressure in this regard. As described before, it was found by Lohoff et al. (2014) that carriers of the *VMAT1* hyperfunction allele (A) may be predisposed to a diminished cortical response to negative stimuli. Activity of prefrontal regions is a critical

component of regulating emotional arousal, particularly those triggered in response to environmental factors.

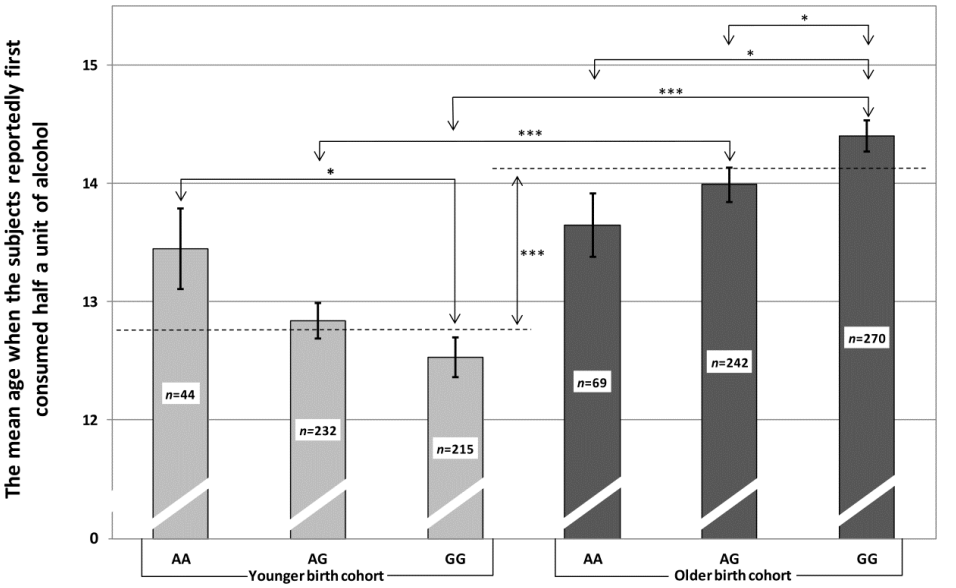

Figure 10. The effect of *VMAT1* rs1390938 polymorphism in interaction with birth cohort on the average age when the subjects reportedly first consumed one half a unit of alcohol ($F_{VMAT1\ rs1390938\ *\ cohort}$ [2, 1066]=6.9, p=0.001). The dashed line indicates the mean age when the subjects from the respective cohort first consumed half a unit of alcohol. *N* represents the number of subjects. Vertical bars denote standard errors of mean. Significant differences between groups denoted as follows: *p<0.05, **p<0.01.

AA homozygotes of the older birth cohort were less likely to have been diagnosed with an affective, anxiety, and/or alcohol use disorder (Table 3) and, in addition, reported lower state and trait anxiety, depressiveness, maladaptive impulsivity, and neuroticism by young adulthood compared with G allele carriers. All these associations were similar for men and women. Subjects homozygous for the hyperfunction allele (AA, Ile/Ile; predisposed to diminished cortical response to negative stimuli) appeared to have features supporting resiliency to negative emotionality and these disorders. These results are essentially consistent with previous research: The A allele has been found to be associated with reduced connectivity in networks that show a general increased connectivity in alcoholics, indicating a potential protective effect (Zhu et al. 2015). It has also been found that the gain-of-function A allele is protective against bipolar disorder (Lohoff et al. 2006), and the AA homozygotes have lower STAI state and trait scores (Lohoff et al. 2008b). Genetic variation in plasma membrane transporters (serotonin, noradrenaline, and dopamine transporters) can serve as a basis for inter-individual differences in brain circuits associated with affective behaviour (Bevilacqua and Goldman 2011). These transporters are mainly

involved in synaptic neurotransmitter reuptake, which contributes to the duration of signaling. In contrast, variation in the magnitude of signaling may be more closely related to mechanisms regulating synaptic neurotransmitter release (Lohoff et al. 2014). Efficient reuptake of the transmitter from the synaptic cleft through plasma membrane monoamine transporters followed by reaccumulation into synaptic vesicles through the VMATs constitute crucial interlinked steps of monoamine neurotransmission (Wimalasena 2011).

Table 3 *VMAT1* rs1390938 effects on the lifetime prevalence of affective, anxiety, and alcohol use disorders in the older birth cohort assessed at age 25.

Psychiatric disorders	Total (*n*=501)	Main statistics (Pearson's χ^2)	AA (*n*=62)	AG (*n*=208)	GG (*n*=231)
Affective disorders	114 (23%)	χ^2=(2, *N*=501)=4.86, *p*=0.088	10 (16%)	41 (20%)	63 (27%)
Anxiety disorders	84 (17%)	χ^2=(2, *N*=501)=3.85, *p*=0.146	5 (8%)	37 (18%)	42 (18%)
Affective or anxiety disorder or both	152 (30%)	χ^2=(2, *N*=501)=4.52, *p*=0.104	12 (19%)	63 (30 %)	77 (33%)
Alcohol use disorder (AUD)	95 (19%)	χ^2=(2, *N*=501)=2.15, *p*=0.341	8 (13%)	44 (21%)	43 (19%)
Affective, anxiety, and/or AUD	214 (43%)	**χ^2=(2, *N*=501)=6.78, *p*=0.034**	**17 (27%)**	**94 (45%)**	**103 (45%)**

Significant differences presented in bold.

The amino acid interchange produced by Thr136Ile polymorphism is located in the first luminal domain of the transporter. This region of the protein interacts with inhibitors and substrates (Sievert and Ruoho 1997). The first luminal loop of VMATs also represents a G-protein-coupled receptor that adapts vesicular filling (Brunk et al. 2006). 136Thr has been related to decreased monoamine transport in vitro. Reduced storage and release of monoamines in brain regions expressing VMAT1 and in adrenal medulla where VMAT1 is the major type of VMAT have been suggested to alter the balance of monoamine availability both peripherally and centrally (Khalifa et al. 2012). Such presynaptic components are likely part of a shared pathway of vulnerability to a range of neuropsychiatric phenotypes (Lohoff et al. 2014).

The association of *VMAT1* rs1390938/Thr136Ile polymorphism with psychological measures and prevalence of psychiatric disorders in the older birth cohort is straightforward and the findings provide a remarkably coherent picture. However, we cannot see a similar association in the younger birth cohort. Therefore, we have to consider that as environmental conditions and demands have

been different enough for the two birth cohorts, this might have brought about a change in the E component of the G × E formula; in turn, such a change in environment indeed should be reflected in how specific gene variants relate to the behaviour in question, in this case, psychological measures and prevalence of psychiatric disorders (Harro 2010).

4.4. *NRG1* and stressful life events: impact on drug use (Paper III)

The *NRG1* rs6994992 variation was associated with substance use. There was a cohort- and gender-specific effect of *NRG1* on AUD. In the older birth cohort, the C/C homozygous females were several times more likely to have had AUD than T allele carriers (OR=6.01[2.13–16.94], p=0.001). Also, SLEs reported at age 25 had an interaction effect with the *NRG1* genotype on the occurrence of AUD among women, as the C/C homozygous females with greater numbers of SLEs were more likely to have been diagnosed with AUD by the age of 25 (OR=2.08[1.43–3.02], p<0.001) (Figure 11). Among females carrying the T allele, SLEs did not affect the likelihood of developing AUD. In addition, the C/C homozygous females who reported exposure to SLEs were significantly more likely to be active smokers at age 18 (OR=0.76[0.60–0.96], p=0.019) and the most frequent consumers of tobacco products at age 25 ($F_{NRG1 \times \text{gender} \times \text{SLE25}}$ [1, 461]=7.3, p=0.007). The observed gender differences may be due to gender differences in stress resilience, as the psychological consequences of trauma exposure are worse for women (Kline et al. 2013; Tolin and Foa 2006). Regarding tobacco use, environmental adversities have a greater deleterious effect on continued abstinence and the ability to quit smoking in women (McKee et al. 2003).

When the older birth cohort (born in 1982/1983) was aged 15, illicit drugs were only just beginning to become available in most of Estonia (Paimre 2013). By the age of 18, the older birth cohort had caught up with the younger cohort in prevalence of trying illicit drugs; however, differences in use between genotypes were not significant. In the younger cohort, born six years later, drug use was much more prevalent by the age of 15, and male C/C homozygotes were the leaders in drug use, remaining so three years later. Male C/C homozygotes of the younger birth cohort were twice as likely to have tried illicit drugs compared with male T allele carriers at age 15 (OR=2.41[1.29–4.52], p=0.006) and age 18 (OR=2.16[1.20–3.88], p=0.011). In addition, the *NRG1* genotype had a significant interaction effect with SLEs on trying illicit substances among male subjects (of the entire sample) at the age of both 15 (OR=1.32[1.07–1.62], p=0.009) and 18 (OR=1.28[1.08–1.53], p=0.005), as SLEs had a larger effect in C/C homozygotes.

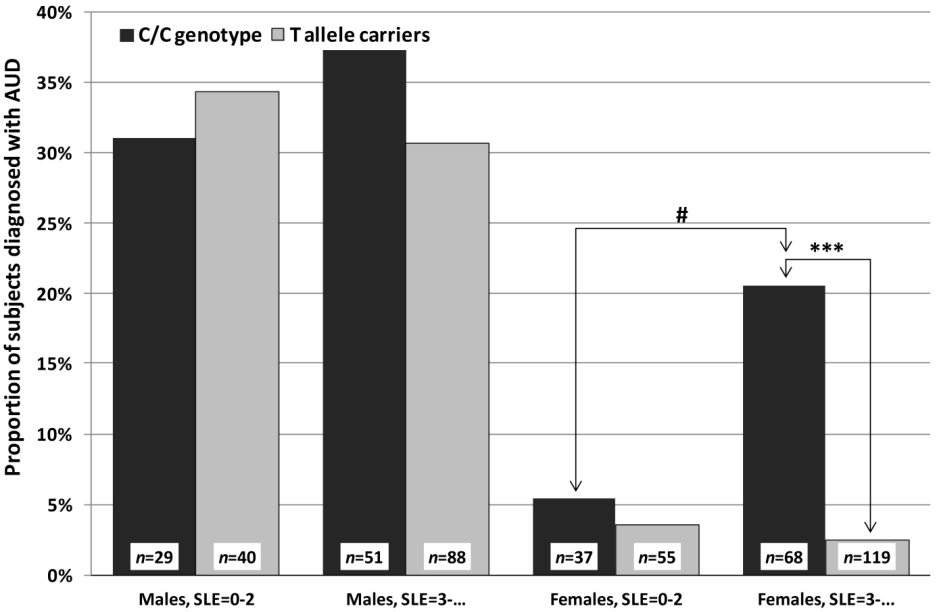

Figure 11 The effect of *NRG1* rs6994992 genotype and interaction with SLEs on the occurrence of AUD in the older birth cohort by the age of 25 (OR=1.49[1.23–1.81], $p<0.001$). *N* represents the number of subjects in the whole sample (100%). Significant differences between groups: ***$p<0.001$, #$p=0.05$.

Because originally the *NRG1* T allele was associated with mental health problems, the higher prevalence of all kinds of drug issues in the C/C homozygotes may at first appear surprising. Indeed, the C allele has been associated with higher working memory capacity (Stefanis et al. 2007), lower risk of developing psychotic illness, and higher premorbid IQ in samples at risk for psychosis (Hall et al. 2006; Keri et al. 2009a). Functional MRI studies have shown increases in frontal lobe activation associated with T homozygote status (Hall et al. 2006; Mechelli et al. 2009, 2010), with the interpretation that neural processing is more effective in C allele carriers (Barnes et al. 2012). One speculative interpretation is that the C/C individuals may be more open to environmental influences, both positive and negative. We found that higher exposure to SLEs brought about a further increase in the prevalence of substance use in C/C homozygotes. T allele carriers seem to be more resilient in this regard: adverse life events did not elevate the measures of substance use in this group. Indeed, T/T homozygosity has previously also been found advantageous in studies on community samples: children with the T/T genotype had better episodic memory performance in late adolescence (Douet et al. 2014), and among people with high intellectual and academic performance, the highest creative achievements and creative thinking scores were in T/T homozygotes (Keri 2009). Higher resilience to substance use in carriers of the gain-of-function T allele is consistent with animal experiments that have suggested higher sensitivity to cannabinoids in mice with lower expression of Nrg1 (Boucher et al. 2007a, 2007b).

4.5. Variation in the *OXTR* gene and alcohol abuse (Paper IV)

The *OXTR* rs53576 polymorphism was associated with alcohol use and prevalence of AUD in the older cohort of the ECPBHS sample and the effects varied by gender. The frequency of alcohol use measured at ages 15, 18, and 25 was significantly affected by the interaction of *OXTR rs53576* genotype and gender (LMM; $F_{OXTR*gender}$ [3, 694]=19.8, p<0.001). In males, the AA homozygotes (the genotype presumably with the least efficient oxytocinergic neurotransmission) were the most frequent alcohol consumers at ages 15 and 18. By the age of 25, the frequency of alcohol use was similar for all genotypes (Figure 12). In females and subjects of the younger birth cohort, the *OXTR* genotype did not affect the levels on alcohol use either over time.

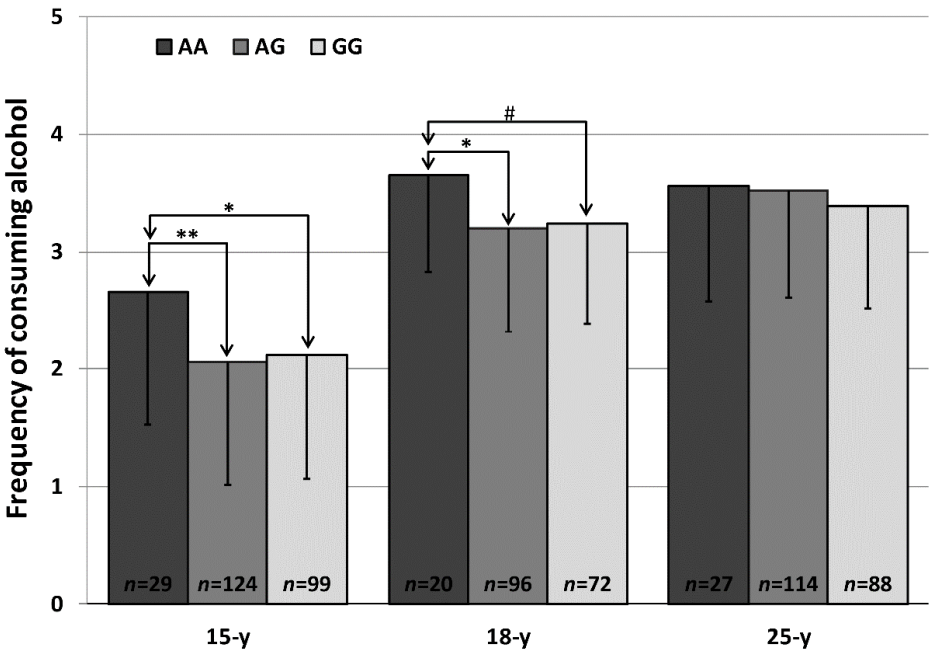

Figure 12 The effect of *OXTR* rs53576 genotype on the frequency of consuming alcohol (according to the most frequently consumed type of alcohol) in male subjects of the older birth cohort at ages 15, 18, and 25 (LMM results of three measurement times regarding *OXTR* effect on alcohol use: F_{OXTR}[2, 314]=3.9, p=0.021). A five point scale was used at ages 18 and 25: *1*=never, *2*=less than once a month, *3*=at least once a month, *4*=at least once a week, *5*=every day. At age 15, a four point scale was used, excluding the option *5*=every day. *N* represents the number of subjects in the group. Vertical bars denote standard deviations. *Post hoc*: Fisher's least significant difference (LSD). Significant differences between groups: *p<0.05, **p<0.01, #p=0.06.

OXTR rs53576 genotype also had a gender-specific effect on lifetime AUD assessed at age 25 in the older birth cohort (Figure 13): AA homozygous males were significantly more likely to have had alcohol abuse or addiction than male G allele carriers (OR=2.89[1.24–6.72], p=0.014). Earlier findings have supported the notion that the disproportionate risk for dysfunction in males has a gender-related neural basis. There are distinct gender differences in alcohol use. As reviewed by Erol and Karpyak (2015), more women are lifetime abstainers, drink less, and are less likely to engage in problem drinking, develop alcohol-related disorders or alcohol withdrawal symptoms; however, women drinking excessively develop more medical problems. Biological factors, including differences in alcohol pharmacokinetics as well as its effect on brain function and the levels of sex hormones may contribute to some of those differences. The disproportionate risks are likely to have gender-related neural basis, and these could in part be mediated by *OXTR*. Using multimodal neuroimaging in a large sample of healthy human subjects, Tost et al. (2010) identified genotype-dependent differences in brain structure, brain function, and personality. Specifically, the A allele carriers showed a significant allele load-dependent decrease in gray matter volume in the oxytocinergic 'core' of the brain, the hypothalamus, a finding that predicted reduced reward dependence in males. The subsequent cross-correlation of voxel-based morphometry and personality questionnaire measures confirmed that lower hypothalamic volumes predicted lower sociality in male, but not in female subjects. Furthermore, a genetic effect on the structural connectivity of the hypothalamus was indicated, which showed a strong increase in the correlation of the gray matter volume of the hypothalamus and that of the dorsal anterior cingulate gyrus and amygdala in *OXTR* A allele carriers. Male carriers of the A allele were characterized by an increase in the right amygdala volume which was negatively correlated with pro-social temperament scores. Additionally, in a study by Wang et al. (2013) the AA homozygous individuals showed lower local functional connectivity density in the hypothalamus and weaker resting-state functional connectivity between the hypothalamus and left dorsolateral prefrontal cortex, and these differences were again only found in males.

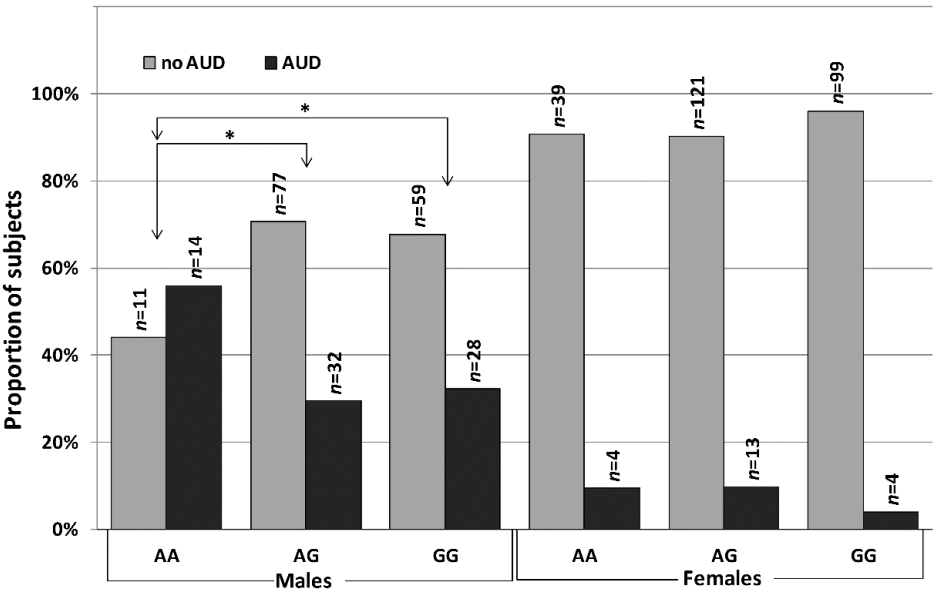

Figure 13 *OXTR* rs53576 genotype and occurrence of AUD by the age of 25 in the older birth cohort ($\chi^2_{OXTR*gender}$=[2, *N*=501]=32.09, p<0.001). *N* represents the number of subjects in the group. Significant differences between groups: *p<0.05.

Oxytocin has been suggested as a potential treatment for alcoholism: In a study of alcoholics in inpatient setting, oxytocin effectively decreased both alcohol craving and withdrawal symptoms (Pedersen et al. 2013). Efficacy of oxytocin for decreasing ethanol intake has also been shown in animal models (Kovacs et al. 1998; MacFadyen et al. 2016; Peters et al. 2013). Effective treatments promoting recovery from addictions appear to incorporate a factor that comprises a form of social rehabilitation or social reintegration. Oxytocin may amplify the reinforcing properties of social engagement, perhaps priming social reward rather than providing the reward itself (McGregor and Bowen 2012). In a study by Young et al. (2014), it was demonstrated that oxytocin administration restores social bonding in drug-exposed animals. It was further elaborated that the largely conserved role of oxytocin in social behaviour across species (Meyer-Lindenberg et al. 2011) and the evidence of altered oxytocinergic systems in human drug users (Light et al. 2004) support the potential of the oxytocinergic system as a neural target for the pharmacological treatment of social deficits in addiction.

Indeed, insufficient interpersonal skills appear as a potential contributory factor in developing AUD in the older cohort of the ECPBHS sample. The association was similar for men and women. AA homozygotes who had scored higher in the Unfavourable School Relationships Scale at age 15 were more likely to develop AUD by age 25 (χ^2=[2, *N*=438]=7.64, p=0.022). This effect was mainly attributable to unfavourable relations with teachers (Figure 14) even though there was a similar trend regarding bullying (χ^2=[2, *N*=448]=5.48, p=0.065). However, unfavourable relations with classmates did not have a

facilitative role in the association of the *OXTR* rs53576 polymorphism and alcohol use, nor did social interactions in the family. As AA homozygotes reportedly have lower general sociality (Li et al. 2015), empathy and self-esteem (Saphire-Bernstein et al. 2011), and higher levels of stress reactivity (Rodrigues et al. 2009), their probability to develop interpersonal problems or even get victimized at school may be higher (Crawford and Manassis 2011). The AA homozygotes have also been found to seek less emotional support during stress (Kim et al. 2010) and be less able to benefit from social support (Chen et al. 2011). Another study has found A allele carriers less likely to adopt problem-focused strategies in the face of unsupportive interactions (McInnis et al. 2015). Victimization at school consistently predicts higher alcohol consumption and problematic drinking (Rospenda et al., 2013), as victimized adolescents are likely to adopt a drinking style to cope (Archimi and Kuntsche 2014). In turn, drinking to cope is associated with a higher risk of alcohol-related problems (Kuntsche et al. 2005). Therefore, if AA homozygous subjects do not obtain adaptive coping methods or communications skills already in adolescence, they might be more prone to develop AUD.

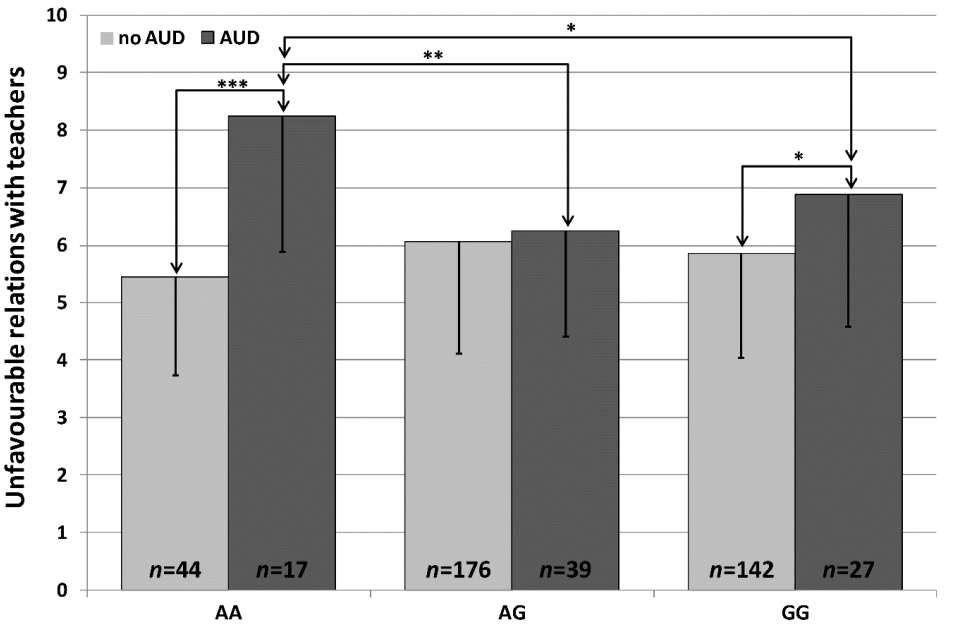

Figure 14 The relationship between *OXTR* rs53576 genotype, likelihood of AUD diagnosis by age 25 and unfavourable relations with teachers at age 15 (χ^2=[2, 445]=9.15, p<0.01). *N* represents the number of subjects. Vertical bars denote standard deviations. Significant differences between groups: *p<0.05; **p<0.01; ***p<0.001.

Based on the previous analyses, we tested both mediation and interaction models (Baron and Kenny 1986) with Bayesian MCMC sampling for predicting AUD with genotype, alcohol use frequency and unfavourable relations with teachers. All the tested models converged; there were no negative (impossible) variances, and posterior distributions were statistically and visually appropriate. We tested the models separately in males and females of the older birth cohort as previous results had indicated gender differences, starting from the models where both relations with teachers and alcohol use were modeled both as mediators and moderators of *OXTR* effects on AUD. As the model fit was poor in both genders (ppp<0.01), we continued with models where moderation and mediation effects were varied in addition to direct effects. Figure 15 presents the final reduced models (ppp-value for males 0.29 indicating a good fit, and for females, ppp=0.01 indicating modest fit). *OXTR* x Alcohol use interaction term and unfavourable relations with teachers at age 18 were omitted from this model as the interaction term was not a significant predictor and relations at age 18 was a weaker predictor compared to relations reported at age 15. So, in males of the older birth cohort, *OXTR* genotype exerted its effects on the developing of AUD both indirectly via alcohol use in adolescence and by interacting with unfavourable relations with teachers. The direct effect of *OXTR* on AUD in a similar model was not significant: males, 0.090 (–0.088; 0.264); females, 0.020 (–0.117; 0.137). In females, *OXTR* influenced AUD by interacting with unfavourable relations with teachers at age 15, not via alcohol use. In the older birth cohort as a whole, the only direct significant genotype-related predictor was Genotype × Unfavourable relations with teachers at age 15 [interaction term 0.198 (0.069; 0.333); ppp=0.01]. In all the models, alcohol use both at ages 15 and 18 contributed significantly to AUD. According to the summary model of developing AUD, the moderating effect of unfavourable relations was independent of the mediating effect of alcohol use in both male and female rs53576 AA homozygotes. Even though interpersonal relations and alcohol use behaviour in teens are thought to be interweaved, the association between drinking frequency and relations with teachers at the same time point was weak while statistically significant (at age 15 r=0.128, p=0.002; and at age 18 r=0.190, p<0.001) indicating that they do not greatly overlap in time. So, according to the final model, for the AA homozygotes, there could be two different pathways starting from adolescence that could lead to AUD: the first via more frequent alcohol use and the second by interacting with insufficient interpersonal skills, especially with teachers.

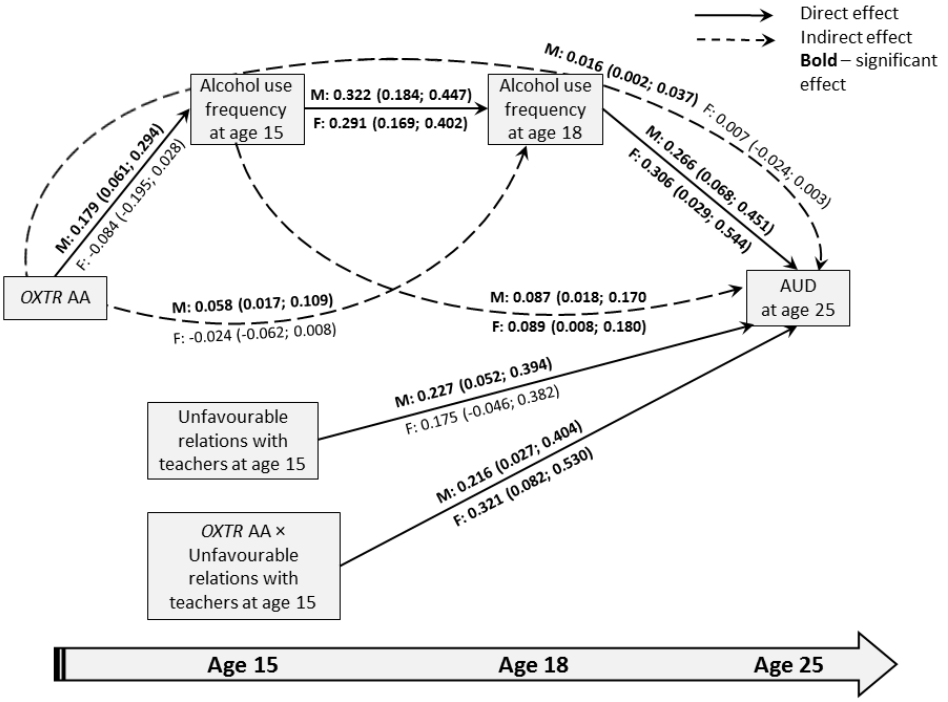

Figure 15 Predicting AUD with *OXTR*, alcohol use frequency and unfavourable relations with teachers in the final SEM model. Results are given separately for males (M) and females (F) as standardized direct (solid arrows) and indirect (dashed and curved arrows) effects with 95% CI. The direct effects from one variable straight to another show that male AA homozygotes use alcohol more frequently at age 15, subjects who are more frequent alcohol users at age 15 are more frequent alcohol users at age 18, and subjects who use alcohol more frequently at age 18 more likely develop AUD. Additionally, while relations with teachers has itself a direct effect, both sexes with the AA genotype who have unfavourable relations with teachers develop AUD more often (moderator Genotype x relations effect). Indirect effects of *OXTR* describe the pathways via where alcohol use variables are mediators: Male AA homozygotes use alcohol more frequently and the effect of *OXTR* on alcohol use at age 18 and AUD are exerted via alcohol use at earlier ages (mediation effects). Results come from SEM model with Bayesian estimation using MCMC sampling. Model ppp-value for males is 0.29 and for females 0.01.

The role that oxytocin plays in animal models in driving the formation of attachments between conspecifics has led to the hypothesis that oxytocin can act on the brain's reward circuits by facilitating the release of dopamine by ventral tegmental area (VTA) neurons into the nucleus accumbens (Mitchell et al. 2015). Indeed, it has been shown in rodents that oxytocin dose-dependently excites dopamine neurons in the VTA (Tang et al. 2014), and oxytocin infusion into the prelimbic cortex alters dopamine levels in accumbens (Young et al. 2014). Using an oxytocin analog carbetocin and viral-mediated overexpression of the oxytocin receptors in nucleus accumbens it was shown that both oxytocin

receptor stimulation and overexpression globally suppresses the rewarding properties of ethanol in mice (Bahi 2015). In a study on opiate tolerance and dependence by Sarnyai and Kovács (1994) it was demonstrated that chronic oxytocin treatment decreased the number of apparent binding sites of dopamine in the basal forebrain area and also inhibited a cocaine-induced increase in dopamine utilization in the nucleus accumbens. It was concluded that oxytocin may act as a neuromodulator on dopaminergic neurotransmission in limbic-basal forebrain structures to regulate adaptive CNS processes leading to drug addiction, at least in animal models.

4.6. Conclusions

In the current population-representative birth cohort study in an CEE country, the subjects of the younger birth cohort (born in 1988/1989) reportedly started consuming alcohol at an earlier age than subjects of the older birth cohort (born in 1982/1983), and male subjects started consuming alcohol earlier and were more frequent alcohol consumers than females. There were significantly more subjects diagnosed with AUD at some point of their life by age 25 among males and in the younger birth cohort as a whole. Subjects who had had AUD by age 25 had reported higher frequency of consuming alcohol at the age of 15, 18 and 25.

Environmental stressors (SLEs), relationships at home and in school also significantly affected the likelihood of developing AUD. The number of experienced stressful life events and the abuse in the family reported at age 18 had a significant effect on the likelihood of having been diagnosed with AUD. In school, the quality of relationships with teachers was relevant, especially for boys – the worse relationships the students reported at age 15, the higher the likelihood of having been diagnosed with AUD.

5-HTTLPR genotype had a significant effect on alcohol consumption, but the association was different in the two birth cohorts, suggestive of the significance of gene–environment interactions. Female subjects with the s/s genotype were the latest experimenters with alcohol if they belonged to the older cohort, but the youngest if to the younger cohort. In the older birth cohort, male s/s homozygotes were the most frequent alcohol consumers by the age of 25; in the younger cohort, male subjects with the s/s genotype were more active alcohol users by the age of 18. Our results support the notion that subjects with the s/s genotype are most affected by possible environmental changes and that the genotype effect may differ between male and female subjects. Therefore, for 5-HTTLPR, it would seem more appropriate to consider it a "plasticity gene" variant rather than of a "vulnerability gene" (Belsky et al. 2009), and this may explain why no overt negative effects of early alcohol use have been detected in the ECPBHS sample by the age of 25.

We found the *VMAT1* rs1390938/Thr136Ile polymorphism to be associated with lifetime prevalence of affective, anxiety, and alcohol use disorders, but, again, in a cohort-specific manner. Subjects of the older birth cohort homozygous

for the hyperfunction allele (AA, Ile/Ile; predisposed to diminished cortical response to negative stimuli) appeared to have features supporting resiliency to negative emotionality and these disorders. Genetic variation in plasma membrane transporters (serotonin, noradrenaline, and dopamine transporters) can serve as a basis for inter-individual differences in brain circuits associated with affective behaviour (Bevilacqua and Goldman 2011). These transporters are mainly involved in synaptic neurotransmitter reuptake, which contributes to the duration of signaling. In contrast, variation in the magnitude of signaling may be more closely related to mechanisms regulating synaptic neurotransmitter release (Lohoff et al. 2014). Efficient reuptake of the transmitter from the synaptic cleft through plasma membrane monoamine transporters followed by reaccumulation into synaptic vesicles through the VMATs constitute crucial interlinked steps of monoamine neurotransmission (Wimalasena 2011).

NRG1 variant rs6994992 (SNP8NRG243177) affected substance use and the relationship was moderated by adverse life events. It appears that in a general population, the C/C homozygotes, especially those who had experienced a higher number of SLEs, had more often developed AUD by young adulthood and either were generally more active consumers of tobacco products (older birth cohort) or had more likely used illicit drugs (younger birth cohort). Based on previous studies, we have offered an interpretation that the C/C individuals may be more open to environmental influences, both positive and negative. T allele carriers seem to be more resilient in this regard: adverse life events did not elevate the measures of substance use in this group.

The *OXTR* rs53576 polymorphism was found to be associated with alcohol use and prevalence of alcohol use disorders in the older birth cohort; the effects varied by gender and quality of interpersonal relations. In males, the AA homozygotes (the genotype associated with the least efficient oxytocinergic neurotransmission, lower general sociality and higher levels of stress reactivity) were the most frequent alcohol consumers and more likely to have had alcohol abuse or addiction. Furthermore, the AA homozygotes who reported more un-favourable relations at school at age 15 were more likely to develop AUD by age 25. Therefore, if AA homozygous subjects do not obtain adaptive coping methods or communications skills already in adolescence, they might be more prone to develop AUD.

5. CLOSING REMARKS

With this dissertation we have contributed to substance use research by demonstrating in a population-representative sample the effects of specific common genetic polymorphisms on substance use and that genetic factors that influence liability to substance use are systematically dependent on birth cohorts and other environmental factors.

When analyzing the effects of common genetic polymorphisms (5-HTTLPR VNTR, *VMAT1* rs1390938, *NRG1* rs6994992, *OXTR* rs53576) in general population on alcohol use and abuse, the genotypes associated with higher levels of stress reactivity, openness to environmental influences and affectability by environmental changes seem to be the ones also linked to problematic alcohol use. However, the relations strongly depend on birth cohort as a proxy for the socioeconomic environment experienced by different generations. A study investigating societal-level disapproval of drug use (marijuana) defined by birth cohort or by time period documents that adolescents who mature in birth cohorts with low disapproval of drug use are at higher risk of using drugs during their teenage years, regardless of individual-level disapproval, perceived social norms, or perceived availability (Keyes et al. 2011b). It is deduced that social norms and attitudes regarding drug use cluster in birth cohorts, and this clustering has a direct effect on drug use even after controlling for individual attitudes and perceptions of norms. The finding that drug use is predicted by a cohort effect rather than a period effect is figured to suggest that adolescents are more influenced by individuals of similar age than by broad socio-cultural influences that affect all adolescents simultaneously (*e.g.*, policy and law changes).

Variation in substance abuse liability stems not only from genotypic differences and environmental circumstances but also from their interactions: it occurs when the expression of a gene varies in different environments, or at different ages, or when the influence of the environment varies by genotype (Gunzerath and Goldman 2003). As reviewed by Young-Wolff et al. (2011), there are two principal processes whereby environmental circumstances have been theorized to interact with genetic influences with respect to drinking behaviours. First, environmental restrictions, including social norms promoting abstinence and restricted availability of alcohol, are hypothesized to dampen the expression of genetic influences on drinking behaviours (Shanahan and Hofer 2005). In environments characterized by high levels of social control, a large proportion of individuals, irrespective of genotype, are expected to exhibit low levels of drinking. Conversely, in more permissive settings, people's alcohol consumption will reflect the full range of their genotypes. A second mechanism is that the social context can act as a stressor that potentiates the behavioural expression of genetic liability on risk for alcohol consumption and AUDs. In effect, this renders individuals with genetic risk even more sensitive to the pathogenic effects of environmental stressors (Rende and Plomin 1992). Interestingly, in a study by Brun et al. (2009), more genetically influenced traits were found to be the more

variable phenotypes and environmental influences to be greater for the later-developing brain regions (*e.g.*, the frontal lobes), suggesting that often environment acts to reduce the genetically produced variability (Harro 2010).

The rapid socioeconomic changes that have taken place in Estonia since the beginning of 1990s and are still ongoing affect the values, activities, relationships, leisure time choices and everyday functioning of the people living in this transition society. Intercultural transformation processes continuously shape the identities and lifestyles of individuals, and, as we have demonstrated with this dissertation, also may moderate the genetic effects on alcohol consumption. However, further analyses are needed to fully grasp and determine the exact mechanisms responsible for the different qualities observed in the two birth cohorts.

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SUMMARY IN ESTONIAN

Geenid ja alkoholitarvitamine: levinud geenipolümorfismide mõju rahvastikus

Käesolevas väitekirjas uurisime suurel rahvastiku suhtes esinduslikul valimil, kuidas mõjutavad geneetilised faktorid (4 levinud funktsionaalset geenipolümorfismi), keskkondlik stress ja sünnikohort alkoholi tarvitamist ning alkoholiprobleemide kujunemist. Leidsime, et noorema kohordi uuritavad (sündinud enamasti 1988/1989) alustasid alkoholi tarvitamisega varasemas eas kui vanema kohordi uuritavad (sündinud 1982/1983) ning meessoost uuritavad alustasid alkoholi tarvitamisega varem ning tarvitasid alkoholi sagedamini kui naissoost uuritavad. 25. eluaastaks oli alkoholiprobleeme kogenuid rohkem meeste seas ning nooremas kohordis tervikuna. Uuritavad, kes olid 25. eluaastaks alkoholiprobleeme kogenud, raporteerisid 15, 18 ja 25 aasta vanuselt sagedasemat alkoholitarvitamist. Alkoholiprobleemide kogemise tõenäosust tõstsid ka stressirikkad elusündmused ning halvad suhted kodus ja koolis.

Serotoniini (5-HT) hulk sünaptilistes piludes on reguleeritud serotoniini transporteri (5-HTT) poolt. 5-HTT geeni promootorpiirkond sisaldab polümorfismi (5-HTTLPR), millel on 2 funktsionaalset varianti – lühike ja pikk alleel. Lühikest alleeli on varasemalt seostatud mandelkeha suurema reaktiivsusega hirmutavatele stiimulitele. Oma uuringus leidsime, et 5-HTTLPR genotüüp mõjutab alkoholi tarvitamist, kuid antud seos erineb kohorditi, viidates geenikeskkonna interaktsioonile. Nimelt olid kahe lühikese alleeliga noorema kohordi tüdrukud kõige varasemad alkoholi proovijad, kahe lühikese alleeliga vanema kohordi tüdrukud aga kõige hilisemad alkoholi proovijad. Kahe lühikese alleeliga mehed olid kõige aktiivsemad alkoholi tarvitajad nooremas kohordis 18. eluaastaks ning vanemas kohordis 25. eluaastaks. Meie tulemused toetavad seisukohta, et kahe lühikese alleeliga inimesed on keskkondlikele mõjutustele kõige vastuvõtvamad ning efektid võivad sugude kaupa erineda. 5-HTTLPR näol on seega tegemist pigem närvisüsteemi ja käitumise plastilisuse, mitte haavatavusega seotud polümorfismiga, mis võib seletada ka fakti, et antud valimis ei mõjutanud 5-HTTLPR alkoholiprobleemide esinemise tõenäosust.

Monoamiine – serotoniini, dopamiini, adrenaliini, noradrenaliini jt – transpordivad tsütoplasmast säilituspõiekestesse vesikulaarsed monoamiini transporterid (VMAT). 1. tüüpi VMAT-i geenis leidub polümorfism rs1390938, mis tingib muudatuse valgu struktuuris. Antud polümorfismi A-alleel on seotud monoamiinide suurenenud transpordiga vesiikulitesse ning on seega n-ö hüperfunktsiooni alleel. Meie valimis on rs1390938 polümorfism seotud ärevus- ja meeleoluhäirete ning alkoholiprobleemide esinemissagedusega, kuid taas sõltuvad efektid sünnikohordist. Vanema kohordi uuritavad, kellel oli 2 hüperfunktsiooni alleeli, olid ärevus- ja meeleoluhäiretele ning alkoholiprobleemide eest rohkem kaitstud.

Neureguliin-1 on signaalvalk, mis mõjutab neuronite arengut ja surma, sünaptilist plastilisust ja gliia funtsioone ning sellele on omistatud kriitiline roll

organismi kohanemisvõimes. Neureguliin-1 geeni promootorpiirkonnas asub polümorfism rs6994992 (C/T), mille C-alleeli on seostatud efektiivsema infotöötluse, suurema töömälu mahu ja kõrgema vaimse võimekusega. Leidsime, et C-alleeli homosügoodid, kes olid kogenud rohkem stressirikkaid elusündmusi, olid 25. eluaastaks tõenäolisemalt kogenud alkoholiprobleeme, tarvitasid aktiivsemalt tubakatooteid ning olid suurema tõenäosusega proovinud keelatud uimasteid. Uuritavatel, kellel oli T-alleel, ei mõjutanud stressirikkad elusündmused alkoholi, tubakatoodete ja keelatud uimastite tarvitamist. Võttes arvesse varasemaid tulemusi, võib põhjuseks olla C-alleeli homosügootide suurem vastuvõtlikkus keskkondlikele mõjutustele, nii positiivsetele kui negatiivsetele.

Oksütotsiini – õppimise, mälu, meeleolu, paljunemise ja sotsiaalses kontekstis kohanemisega seotud hormooni – nasaalne manustamine on leitud avaldavat mõju mitmetele inimese füsioloogilistele ja psüühilistele reaktsioonidele, tuues teiste seas kaasa ka ärevuse ning alkoholi võõrutusnähtude vähenemise. Oksütotsiini nasaalse manustamise efektid on leitud olevat mõjutatud oksütotsiini retseptori geenis leiduva polümorfismi rs53576 (A/G) poolt. Selle A-alleeli on seostatud vähemefektiivse oksütotsiinergilise süsteemi, madalama sotsiaalsuse ja suurema reaktiivsusega stressile. Meie valimi meessoost A/A homosügoodid olid kõige sagedasemad alkoholitarvitajad ning nad olid nooreks täiskasvanueaks kõige tõenäolisemalt kogenud alkoholiprobleeme. Lisaks olid 25. eluaastaks tõenäolisemalt alkoholiprobleeme kogenud need uuritavad (nii mees- kui naissoost), kes raporteerisid 9. klassis halbu koolisuhteid. Sellest järeldub, et kui A/A homosügoodid ei omanda teismeeaks kohaseid suhtlemisoskusi ega käitumisviise, võivad nad suurema tõenäosusega kogeda alkoholiprobleeme. Antud seos esines aga üksnes vanemas kohordis, viidates geenide ja keskkonnatingimuste interaktsioonile.

Analüüsides levinud geenipolümorfismide mõju alkoholitarbimisele, leidsime, et kõrgema stressireaktiivsuse ja keskkondlike mõjude suhtes avatusega seonduvad polümorfismid olid ühtlasi ka need, mis seondusid probleemse alkoholitarbimisega. Seosed sõltusid suures osas aga sünnikohordist, mis väljendab erinevate generatsioonide poolt kogetud sotsiaalmajanduslikku keskkonda. Kiired ühiskondlikud muutused, mis said alguse 1990. aastatel ning toimuvad praegugi, mõjutavad meie siirdeühiskonnas väärtushinnanguid, vaba aja tegevusi, suhteid ja igapäevast toimetulekut. Keskkondlikud piirangud, sh sotsiaalsed normid ja kättesaadavuse piiramine vähendavad geneetilisi mõjusid alkoholitarbimisele. Vabamates tingimustes avalduvad geeniefektid aga ilmekamalt. Sotsiaalne kontekst võib toimida ka stressorina, mis võimendab geneetilisi eelsoodumusi. Kultuurisisesed ja ühiskondlikud muutused vormivad pidevalt inimeste identiteeti ja elustiili ning, nagu käesolevas väitekirjas demonstreeritud, võivad vahendada ka geeniefekte alkoholitarbimisele.

PUBLICATIONS

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