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Early life experiences and alcohol use in youth

An emerging role of the Vesicular Glutamate Transporters

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Abstract
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Initiation of alcohol consumption usually takes place during adolescence, a period characterized by a plethora of physical and emotional changes. Towards early adulthood, hazardous drinking patterns can emerge and potentially lead to the development of Alcohol Use Disorder (AUD). Both positive and negative experiences during early life can shape brain development and, through interactions with the genetic make-up, can contribute to the vulnerability of an individual to develop AUD. Epigenetic mechanisms, such as DNA methylation, potentially mediate the effect of environmental influences on gene expression, thus serving as the missing link between gene, environment and phenotype. Among various neuroadaptive changes seen in AUD, those within the glutamatergic system appear particularly prominent, mainly in withdrawal and relapse states, but also in stress-related outcomes. The glutamatergic phenotype can be determined by the expression of the Vesicular Glutamate Transporters 1-3 (VGLUT1-3). To date, the relationship between early life experiences, alcohol consumption, and *Vgluts/VGLUTs* genes (rodents/humans) in the initial stage of alcohol consumption and during the sensitive period of late adolescence/young adulthood has not been investigated.

The present thesis, based on three studies on rodents and one on humans, aimed to examine *Vglut/VGLUT1-3* correlates of early life experiences and alcohol drinking during youth. The effect of co-exposure to nicotine, because of its high comorbidity with alcohol use, as well as the role of key DNA methylation-regulating genes was also investigated. The main finding showed that individuals exposed to early life stress were more sensitive to the effect of alcohol on *Vglut1-3* mRNA expression and DNA methylation, as well as expression of the DNA methylation-regulating genes, in limbic and striatal brain regions, as compared with controls. In an independent sample, prolonged nicotine co-exposure with alcohol during adolescence was associated with higher *Vglut2* expression in the ventral tegmental area of young adult rats. Lastly, the single nucleotide polymorphism *rs2290045* in *VGLUT2* was found to moderate the environmental sensitivity to alcohol-related problems in humans. Carriers of the minor allele (T) displayed differential susceptibility to the environment; increasing quality of parenting was associated with higher and lower alcohol-related problems in the absence and presence of previous maltreatment, respectively.

In conclusion, the findings highlight for the first time the role of *Vgluts/VGLUTs* in early stress-mediated sensitivity towards alcohol consumption and alcohol-related problems during adolescence and young adulthood, and especially a potential *Vglut2/VGLUT2*-mediated molecular signature behind the interactive mechanisms of these two aversive environmental factors, as well as of nicotine co-exposure.

Keywords: alcohol, early-life stress, brain, early adulthood, expression, DNA methylation, glutamate, Vesicular Glutamate Transporters

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To my family

"Πάντων δε κρηπίς και θεμέλιος η ενάργεια"
"Clearness is the foundation of everything"

Epicurus

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I. Vrettou M, Granholm L, Todkar A, Nilsson KW, Wallén-Mackenzie Å, Nylander I, Comasco E. "Ethanol affects limbic and striatal presynaptic glutamatergic and DNA methylation gene expression in outbred rats exposed to early-life stress." Addict Biol. 2017. 22(2): p. 369-380. doi: 10.1111/adb.12331.

II. Vrettou M, Yan L, Nilsson KW, Wallén-Mackenzie Å, Nylander I, Comasco E. "DNA methylation of *Vesicular Glutamate Transporters* in the mesocorticolimbic brain following early life stress and ethanol exposure in adulthood." Manuscript

III. Vrettou M, Thalhammer S, Svensson A-L, Nilsson KW, Wallén-Mackenzie Å, Fredriksson R, Nylander I, Comasco E. "*Vglut2* gene expression in limbic brain areas of outbred rats following exposure to ethanol and nicotine." Manuscript.

IV. Vrettou M, Nilsson KW, Tuvblad C, Rehn M, Åslund C, Andershed AK, Wallén-Mackenzie Å, Andershed H, Hodgins S, Nylander I, Comasco E. "VGLUT2 rs2290045 genotype moderates environmental sensitivity to alcohol-related problems in three samples of youths." Eur Child Adolesc Psychiatry. 2019 Oct;28(10):1329-1340. doi: 10.1007/s00787-019-01293-w.

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VI. Nylander I, Todkar A, Granholm L, Vrettou M, Bendre M, Boon W, Andershed H, Tuvblad C, Nilsson KW, Comasco E. "Evidence for a Link Between Fkbp5/FKBP5, Early Life Social Relations and Alcohol Drinking in Young Adult Rats and Humans." Mol Neurobiol. 2017 Oct;54(8):6225-6234. doi: 10.1007/s12035-016-0157-z.

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Abbreviations

Acb	Nucleus Accumbens
AFR	Animal Facility Reared
AUD	Alcohol Use Disorder
AUDIT	Alcohol Use Disorder Identification Test
AUDIT-C	Alcohol Use Disorder Identification Test-Consumption
BSCT	Biological Sensitivity to Context Theory
cDNA	Complementary DNA
CEPBD	CCAAT/enhancer binding protein delta
CISH	Chromogenic *In Situ* Hybridization
CpG	5′-cytosine-phosphate-guanosine-3′
CREB	cAMP Responsive Element-Binding Protein
CS	Clinical Sample
DNMT1	DNA-Methyltransferase 1
DST	Differential Susceptibility Theory
dStr	Dorsal Striatum
ELS	Early Life Stress
ESPAD	European School Survey Project on Alcohol and Other Drugs
EWAS	Epigenome Wide Association Studies
FISH	Fluorescent *In Situ* Hybridization
gDNA	Genomic DNA
GxE	Gene-by-Environment
GLM	Generalized Linear Models
GP	General Population
GWAS	Genome Wide Association Studies
HPA	Hypothalamic-pituitary-adrenal
MeA	Medial Amygdala
MECP2	Methyl CpG Binding Protein 2
mPFC	Medial Prefrontal Cortex
MS	Maternal Separation
PASCQ	Parents as Social Context Questionnaire
PBP	Parabrachial Pigmented Nucleus
PND	Postnatal Day
PNW	Postnatal Week
PVT	Paraventricular Nucleus of Thalamus
RLi	Rostral Linear Nucleus
ROI	Region of Interest
qPCR	Quantitative real-time Polymerase Chain Reaction

SLC	Solute Carriers
SNP	Single Nucleotide Polymorphism
TH	Tyrosine Hydroxylase
TF	Transcription Factor
TFBS	Transcription Factor Binding Site
USF	Upstream Transcription Factor
UTR	Untranslated Region
VGLUT	Vesicular Glutamate Transporter
VMH	Ventromedial Hypothalamus
VTA	Ventral Tegmental Area
VTAR	Ventral Tegmental Area Rostral Nucleus

Introduction

Alcohol consumption and alcohol use disorder

Alcohol is a psychoactive substance with euphoric and stress-alleviating effects, suggested to be even beneficial when consumed in low to moderate doses, but potentially detrimental and addictive in higher levels (Spanagel, 2009). Evolutionary theories have proposed alcohol consumption as a common behavior in nature, shaped over the course of million years, and not just a novel human behavioral feature (Spanagel, 2009). Nonetheless, alcohol misuse can lead to significant health (e.g. neuropsychiatric disorders, oncological, gastrointestinal, cardiovascular and infectious diseases) and socioeconomic (e.g. unemployment, accidents, violence) problems. Approximately 5.9% of deaths worldwide and 5.1% of the global burden of disease and injury are attributable to alcohol use and misuse, with the latter being 7.1% for males and 2.2% for females (WHO, 2018).

Alcohol Use Disorder (AUD) is the major neuropsychiatric alcohol-attributable condition. AUD is a polygenetic, multi-factorial and highly heterogeneous psychiatric disorder, resulting from a complex interplay between genetic and environmental factors (Spanagel, 2009). The 5th edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM–5) states eleven criteria to diagnose AUD (e.g., "alcohol is often taken in larger amounts or over a longer period than was intended" or feeling of "craving, or a strong desire or urge to use alcohol"), any two of which are sufficient for a clinical diagnosis. The sub-classification of AUD to mild, moderate or severe depends on the number of criteria met (Dawson et al., 2013).

Alcohol consumption and nicotine use among adolescents and young adults

Adolescence, defined as a "gradual period of transition from childhood to adulthood", is characterized by dramatic physical and emotional changes as well as increased peer-social interactions, novelty-seeking and risk-taking behavior (Spear, 2000). As a result of these factors, along with alcohol availability, initiation of alcohol consumption commonly occurs during this period, while during middle to late adolescence more hazardous drinking patterns emerge (Brown et al., 2008). Adolescent alcohol use is a major public health

concern in many European and North American countries (i.e. the "western world"). In Sweden, though alcohol use among adolescents has decreased in the last decade and lifetime alcohol use rates are relatively low (less than 60%) (ESPAD, 2020), alcohol consumption follows a binge-drinking pattern. Binge drinking is defined as consuming enough alcohol to raise blood alcohol concentration (BAC) to ≥0.8g% within a two-hour period (NIAAA, 2004). In 2019, according to European School Survey Project on Alcohol and Other Drugs (ESPAD), though frequency (or occasions) of alcohol use in the last month was reported to be within the lowest range, alcohol intake on the last drinking session was among the highest in Europe, along with other Scandinavian countries, while the preferred alcohol beverage was spirits for more than half of the students (ESPAD, 2020). Similar findings were demonstrated by the report of the European Commission, with binge-drinking being more common in western and northern-European countries, including Sweden (Steketee, 2013). Binge alcohol use in adolescents is associated with alterations in verbal learning, memory and attention, visual–spatial processing, but also with deficits in development and integrity of grey and white matter (Spear, 2018). Along with binge-alcohol consumption, early onset of drinking is also an alerting phenomenon and according to ESPAD 2019, one third (33%) of adolescents report having drunk alcohol before the age of thirteen (ESPAD, 2020).

During adolescence, initiation of nicotine use, and in fact in an intermittent way similar to binge-drinking pattern, typically takes place as well (Harrison et al., 2008). Nicotine use is highly comorbid with alcohol use (Goldman et al., 2005), displaying cross-reinforcement (potentiation of each other's rewarding effects) and cross-tolerance (reduction of each other's negative effects) (Adams, 2017). Among adolescents, 93% of those who use cigarettes have also used alcohol, while 54% of those who consumed alcohol have also used cigarettes (Kraus, 2015). In Sweden, moist oral snuff (snus) is widely used, even among adolescents and has been suggested to predict or facilitate future smoking (Joffer et al., 2014). Furthermore, according to ESPAD 2019, the lifetime nicotine use prevalence is 53% for both cigarettes (41% alone) and electronic (e-)-cigarettes (40% alone); whereas almost one fifth (18%), and more than one tenth (11%) of adolescents report having smoked cigarettes, or e-cigarettes, respectively, before the age of thirteen (ESPAD, 2020). Early onset and binge pattern of drug use and misuse can influence the course of normative brain development (Spear, 2013) and consequently increase the risk for psychopathology, including AUD (Dawson et al., 2008, Brown et al., 2008, Witt, 2010) and nicotine dependence (Camenga and Klein, 2016), thus calling for studies on this sensitive period.

Early life stress and plasticity in the developing brain

Studies of twins have demonstrated that genes account for 40-60% of AUD heritability (Goldman et al., 2005). Therefore, environmental influences are equally important for the development of this disorder, while DNA remains unchanged throughout life, these influences can change over time (Kendler et al., 2008, Heinrich et al., 2016). Early in life, brain development and maturation are ongoing processes that continue during early adulthood (Semple et al., 2013). Throughout this period, the brain is characterized by heightened neuroplasticity and is markedly sensitive to external cues that can shape it, leading to adaptive, but also maladaptive programming (Maccari et al., 2014).

Stress is defined as the body's response to any demand of change (Selye, 1936). Early life stress (ELS) has been shown to modulate brain development, by inducing stable changes in gene expression, neural circuit function and behavior, thus in turn exerting long-lasting effects on adult health (Maccari et al., 2014, Peña et al., 2014, Watt et al., 2017). ELS in humans, as a form of physical, sexual, psychological (or emotional) maltreatment or neglect during childhood and adolescence (Gilbert et al., 2009), can contribute to adult psychopathology (Teicher et al., 2016), including AUD (Enoch, 2011), independently of family history of alcoholism (Anda et al., 2002).

ELS in rodents can be exerted postnatally by various types of environmental manipulations. Postnatal stress paradigms implicate disturbance of mother-pup interaction during the initial weeks after birth either through separation of the pups from the mother (maternal separation) (Nylander and Roman, 2013) or by limited bedding and nesting material in the home cage that stresses the mother and alters her behavior and maternal care (Molet et al., 2014). Maternal separation (MS) in rodents is a model with high construct validity for studying alcohol-related outcomes, such as propensity to higher alcohol intake and preference (Nylander and Roman, 2013).

Models of MS are suggested to exert their effect via alteration of the maternal behavior and care towards the offspring (Meaney, 2001). Of high relevance is also the timing and duration of MS, considering the stress hypo-responsive period of hypothalamic-pituitary-adrenal (HPA)- axis in pups, during postnatal day 2-14, when the HPA axis response to stress is attenuated, a phenomenon that serves a protective mechanism on the developing brain (Sapolsky and Meaney, 1986). In humans, a similar state has been reported up until about 12 months of age, but not extending throughout childhood when the majority of reported maltreatment occurs (Gunnar and Donzella, 2002). Yet, during this period, the response to stress is mediated by the sensitivity, responsiveness, and attention of the caregivers that appears critical in maintaining low cortisol activity (Gunnar and Donzella, 2002). Indeed, highly heterogeneous maternal care can impact the infant's brain programming, and consequently affect individual's mental health (Baram et al., 2012). Furthermore, contrary to the one-year window of stress hyporesponsive period in humans, various brain regions show specific periods of sensitivity to the effects

of ELS throughout childhood and adolescence (Teicher et al., 2016). Furthermore, individuals exposed to ELS show dysregulation (both blunted and hyperactive) of HPA-axis response to stress and a higher risk towards vulnerability to addiction (Kirsch and Lippard, 2020). Hence, preclinical models of ELS (i.e. MS) are crucial for investigating this variability, allowing to overcome ethical limitations of studying maltreatment in humans, and offer an excellent tool for translational studies of causality (Molet et al., 2014, Teicher et al., 2006, Teicher et al., 2016).

Epigenetic mechanisms

During the last decades, a large body of studies has focused on how early life experience can impact phenotype. The suggested mechanism through which ELS exert long-lasting effects on phenotype is epigenetic programming (Maccari et al., 2014, Peña et al., 2014, Szyf et al., 2016), proposing epigenetics as the missing link between genetics, environment and phenotype (Petronis, 2010). Epigenetics refers to heritable, but potentially reversible, changes in gene expression without changes in the DNA sequence (Henikoff and Matzke, 1997, Szyf et al., 2016). The concept of "phenotypic plasticity" (Feinberg, 2007) states that cells have the ability to alter their behavior responding to internal and external environmental stimuli. Disruption of phenotypic plasticity can result in disease state through epigenetic mechanisms.

One of these mechanisms is DNA methylation (Moore et al., 2013, Szyf and Bick, 2013), referring to the addition of a methyl group to the fifth residue of a cytosine resulting in 5-methylcytosine (5mC) formation, a reaction catalyzed by DNA methyltransferases (DNMTs). In mammals, the majority of DNA methylation occurs on cytosines that precede a guanine nucleotide or CpG (5′-cytosine-phosphate-guanosine-3′) sites (Moore et al., 2013). DNMT1, a member of the DNMT family, acts on hemi-methylated sites maintaining the DNA methylation pattern during replication (Moore et al., 2013) along with DNMT3A and DNMT3B (Jones, 2012). DNA methylation is a normally occurring procedure during embryonic development and genomic imprinting (Henikoff and Matzke, 1997) and affects transcription in a genomic-context way (i.e. depending on whether it is on a transcriptional start site (TSS)/promoter, gene body, regulatory or repeated regions). DNA methylation in the TSS of the promoter region is usually associated with transcriptional silencing, while DNA methylation of the gene body is associated with higher transcription (Jones, 2012). The gene body regards the region of the gene after the first exon, as methylation of exon 1, similar to promoter methylation, is associated with gene silencing (Brenet et al., 2011). Research is still inconclusive on whether DNA methylation precedes or follows transcriptional

silencing (Jones, 2012). Much evidence shows that methylation actually ensures silencing, and that genes that are highly expressed are less likely to be *de novo* methylated and silenced (Jones, 2012).

DNA methylation-mediated alteration in gene expression can occur by modulation of the tethering of transcription factors (TFs) as a result of the methylation on its own or by recruiting specific repression proteins on the methylated site (Szyf et al., 2016, Moore et al., 2013). In regards to the first mechanism, differential methylation levels of specific CpG sites have the potential to block or induce TF binding and further regulate the transcriptional landscape (Jones, 2012). As far as the second mechanism is concerned, one of these proteins is Methyl-CpG binding protein 2 (MECP2), which can act as a suppressor of gene activity (Adkins and Georgel, 2011), but also as a recruiter of DNMT1 to hemi-methylated DNA, thus posing a unique role in the maintenance of DNA methylation as well (Moore et al., 2013). Both DNMT1 and MECP2 are highly expressed in the brain and are crucially involved in neuronal development (Goto et al., 1994, Gonzales and LaSalle, 2010). Altered expression of these genes in the brain has been observed in rodents exposed to ELS (Boku et al., 2015, Blaze and Roth, 2013, Lewis et al., 2013) and upon alcohol consumption (Warnault et al., 2013, Repunte-Canonigo et al., 2013) or other drugs of abuse (Lewis et al., 2013, Deng et al., 2010). A recent epigenome-wide association study (EWAS) found an association between alcohol intake and three CpGs in the body of *DNMT1* and the promoter and body of *MECP2* (Dugué et al., 2019). Collectively, these findings suggest *Dnmt1* and *Mecp2* as interesting candidates to study ELS-mediated impact on alcohol-related phenotypes in adulthood.

Gene-by-environment (GxE) interaction studies

Both genetic and environmental factors contribute to the etiology of AUD (Spanagel, 2009), with studies suggesting the importance of social and familial factors (Kendler et al., 2008) as well as of personality traits of adolescents (Heinrich et al., 2016) to alcohol use initiation, while the genetic component is more implicated in later stages of alcohol consumption (Kendler et al., 2008). Genetic studies have demonstrated that not only different factors are implicated in the emergence of alcohol-related phenotypes across development, but also distinct genes and neurocircuitries are involved in this progression (Dick, 2011, Dick et al., 2014). Candidate gene association studies have been performed since long to identify risk markers for AUD, with little replicability success (Buckland, 2001) except for the involvement of the acetaldehyde and alcohol dehydrogenase genes (Quertemont, 2004).

Genome-Wide Association Studies (GWAS), which have been a promising approach in identifying common risk variants in many complex disorders (TheWellcomeTrustCaseControlConsortium, 2007), typically focus on the

most common type of genetic variation, the single nucleotide polymorphisms (SNPs), which refer to variations in a single nucleotide of the DNA sequence. GWAS have been also performed on AUD (Treutlein and Rietschel, 2011), with findings suggesting different genes or loci, i.e. implication of a gene cluster in the chromosome 11 (Edenberg et al., 2010) or polymorphisms in the chromosome 2q35 (Treutlein et al., 2009) or in the glutamate receptor subunit *NR2A* (Schumann et al., 2008). Furthermore, towards a developmental approach, a genome-wide meta-analysis deduced that genes involved in the glutamatergic neurotransmission pathways mediated adolescent alcohol consumption (Adkins et al., 2015). Nonetheless, findings from GWAS also show inconsistency and low replicability, while very large samples are necessary for the identification of risk loci other than those encoding for the alcohol-metabolizing enzymes (Hart and Kranzler, 2015). A possible explanation behind the low explained variance in GWAS could be the lack of consideration of interactions between the genetic makeup and environmental factors (GxE).

GxE studies provide the basis of research into possible mechanisms behind individuals' differences in the risk of developing complex disorders, including AUD. AUD indeed results from a complex cross-talk between genes and environment (Spanagel, 2009, Enoch, 2006). A theoretical schematic representation of this interplay is depicted in Figure 1. Only some individuals, but not all, will continue escalating their alcohol consumption during the transition from adolescence towards early adulthood (Brown et al., 2008) (from 18 to 25 years of age), a period with the highest risk for AUD onset (Brown et al., 2008, Sussman and Arnett, 2014). The first GxE study that demonstrated the moderating effect of a genotype on environmental risk (maltreatment) for antisocial behavior was conducted by Caspi et al. (Caspi et al., 2002). Since then, numerous GxE studies confirmed the presence of GxE in psychiatric disorders (Caspi and Moffitt, 2006), including AUD (Dick and Kendler, 2012); however the low replicability observed also among these studies led to criticisms regarding their actual contribution in understanding the etiology of psychiatric disorders (Munafo et al., 2014). Nonetheless, appropriate statistics including proper control for potential confounders (Keller, 2014), consideration of different developmental stages (Dick and Kendler, 2012), but also of both negative and positive environmental experiences (Pluess and Belsky, 2012) are suggested to improve replicability of GxE studies (Nilsson et al., 2018), and thus to contribute to our understanding of whether and/or under which circumstances the genetic and environmental influences come together to determine vulnerability to develop AUD.

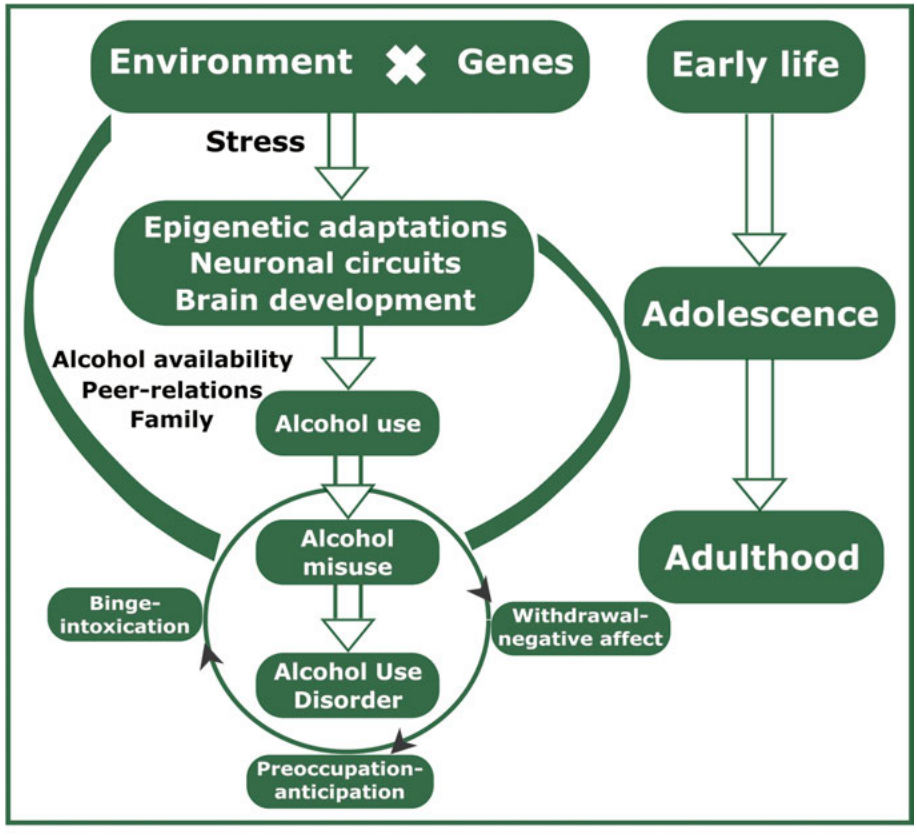

Figure 1. A theoretical schematic representation of the complex interplay between environmental and genetic factors involved in the development of Alcohol Use Disorder (AUD). Early life and adolescence are crucial periods for brain development. Stress during early life (ELS) can modulate brain development, by inducing stable changes in gene expression, neural circuit function and behavior, through epigenetic adaptations. Together with other factors (i.e. alcohol availability, peer-relations, familial factors), these long-lasting effects can contribute to alcohol use/misuse later in life (i.e. late adolescence/adulthood), which in turn, upon further cross-talk with the environment and the epigenome, can lead to the addictive cycle of AUD.

Developmental stress hypotheses

Development of AUD is the result of a complex interplay between genes, environment and the proposed missing link between those, that is epigenetics (Figure 1). AUD is indeed a multi-factorial disorder; several biopsychological models attempted to explain individual's susceptibility or resilience to alcohol use, misuse and AUD. For instance, the "diathesis-stress" (Monroe and Simons, 1991, Zuckerman, 1999) and the "resilience" model (Fergus and Zimmerman, 2005) could be considered as two sides of the same coin called

individual's susceptibility. The former proposes that risk-allele carriers have a greater vulnerability to negative environmental factors, while the latter highlights that individual's sensitivity to negative environment can be reduced by inherent factors. Further, the "vantage sensitivity" (Pluess and Belsky, 2013) hypothesis suggests that inherent characteristics of an individual drive their response to positive environmental factors.

Nonetheless, these models seem rather unilateral, in terms of the nature of environmental factors they are focused on (i.e. only negative or only positive) and the level of focus (i.e. the individual rather than the population). To answer the question of "*why early life experience has a lasting impact on phenotype?"* (Chaby, 2016), including AUD, various developmental stress hypotheses employing a population-level/evolutionary approach have been proposed. Examples of these integrative models, which also consider both positive and negative environmental experiences, comprise the "environmental match/mismatch hypothesis" (Schmidt, 2011), the "differential susceptibility" (DST) (Belsky et al., 2007) and "biological sensitivity to context" (BSCT) (Boyce and Ellis, 2005) theories. Though the environmental mismatch hypothesis can explain why early environment has lasting effects on phenotype, through a mismatch between maternal and offspring qualities/environments that would lead to a decreased fitness, it does not account for the genetic component (Schmidt, 2011). The more recent DST and BSCT theories, considering genetic factors and integrating the abovementioned 'unilateral' models, state that individuals systematically differ in their susceptibility to environmental factors of both negative and positive nature in a bidirectional manner (Ellis et al., 2011). The two theories define "susceptibility" differently; i.e. "sensitivity" in DST, relative to behavioral level, and "reactivity" in BSCT relative to stress response systems. The difference also lies on the origins of both theories, i.e. bet-hedging/heritable variation in susceptibility (DST) compared to conditional adaptation model (BSCT); and on consequences, i.e. plastic/malleable individuals that achieve fitness in wide range on niches (DST) compared to highly reactive individuals that perform poorly in adversity but well upon support (BSCT). Nonetheless, the core conceptual framework of "environmental sensitivity" remains the same, that is considering both stressful and enriching environmental experiences during development; these perspectives should be integrated in GxE studies (Pluess and Belsky, 2012, Pluess, 2015, Nilsson et al., 2018).

Glutamate in the neurobiology of addiction and stress

Several neurotransmitters have been implicated in alcohol consumption and AUD, due to the complex nature of these phenotypes (Spanagel, 2009). Indeed, according to *Koob and Volkow* (Koob and Volkow, 2010), the cycle of

addiction consists of three stages: 1) binge/intoxication, 2) withdrawal/negative effect, and 3) preoccupation/craving, in each of which distinct brain neurocircuitries are involved (Koob and Volkow, 2016).

The mesocorticolimbic dopamine system, consisting of the ventral tegmental area (VTA) with projections to the nucleus accumbens (Acb) and the prefrontal cortex (PFC), plays a central role in the binge/intoxication stage, mediating aspects of acute alcohol reward and reinforcement (Koob and Volkow, 2010, Koob and Volkow, 2016). All drugs of abuse, including alcohol and nicotine, lead to a mesolimbic dopamine increase (Di Chiara and Imperato, 1988), especially in the Acb shell. During the transition from voluntary to more habitual and compulsive alcohol-drinking patterns (withdrawal/negative effect), there is a shift in activation from the ventral (Acb) to the dorsal striatum (dStr) (Everitt and Robbins, 2013). In the later stage of the addiction cycle, during preoccupation/craving, the major neurotransmitter implicated is glutamate through activation of pathways involving projections from the medial PFC (mPFC) to the amygdala and the Acb (Koob and Volkow, 2010, Koob and Volkow, 2016). Another major source of glutamatergic projections to the Acb is the thalamus (Kirouac, 2015). The paraventricular nucleus of thalamus (PVT) receives dopaminergic projections from the mPFC and the hypothalamus, which are found to be activated upon drug-reward signalling cues (Hamlin et al., 2009, Dayas et al., 2008), and sends mainly glutamatergic projections to the PFC along with the Acb and extended amygdala (Kirouac, 2015). The amygdala receives dopaminergic but also glutamatergic projections from the VTA, and projects further to the Acb. A schematic representation of the dopaminergic and glutamatergic projections between all these regions is depicted in Figure 2. The dopaminergic connections between these structures further trigger drug-seeking and intake (Koob and Volkow, 2010, Jones and Bonci, 2005, Koob and Volkow, 2016). Though a dysfunctional glutamatergic system has been mainly linked to withdrawal (Tsai and Coyle, 1998) and craving/relapse stages of the addiction cycle (Koob and Volkow, 2016) potentially via a glutamate homeostasis imbalance (Kalivas, 2009), glutamate as the major excitatory neurotransmitter in the brain, is involved in several stages from initial and voluntary to chronic and compulsory alcohol use (Holmes et al., 2013, Kalivas et al., 2009).

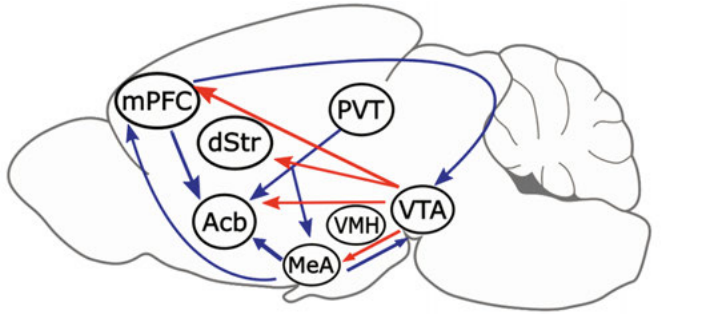

Figure 2. Schematic representation of the glutamatergic (blue) and dopaminergic (red) projections in the regions involved in the reward system. Acb: nucleus accumbens; dStr: dorsal striatum; MeA: medial amygdala; mPFC: medial prefrontal cortex; PVT: paraventricular nucleus of thalamus; VMH: ventromedial hypothalamus; VTA: ventral tegmental area

Stress plays an important role in addiction; a dysregulation of the HPA-axis, the core system regulating the stress response (Tsigos and Chrousos, 2002), has been demonstrated in animal models of alcohol consumption and in AUD (Ciccocioppo et al., 2009, Pucci et al., 2019, Becker et al., 2011). The physiological response to stress is mediated by release of glucocorticoid hormones, mainly cortisol in humans and corticosterone in rats, following activation of the HPA-axis (Tsigos and Chrousos, 2002). Glucocorticoids exert their effects by binding to glucocorticoid receptors, expressed throughout the brain, which upon activation interact with glucocorticoid responsive elements on targeted genes, leading to various endocrine, metabolic, behavioral and neurochemical changes (Tsigos and Chrousos, 2002, Arnsten, 2009). All these stress-induced changes are achieved through, apart from the HPA-axis, the involvement of other brain regions, such as the hippocampus, the PFC and amygdala (Teicher et al., 2016), and neurotransmitter systems, such as the dopamine and the glutamate systems (Ulrich-Lai and Herman, 2009, Arnsten, 2009, McEwen et al., 2016). Within amygdala, it has been shown that the medial amygdala (MeA) is highly involved in HPA-axis response to an emotional stressor (Dayas et al., 1999) and recently in alcohol-related behaviors among alcohol-preferring rats via potential interaction with the stress system (Ayanwuyi et al., 2015). Lastly, the ventromedial hypothalamus (VMH), a highly glutamatergic region, has also been shown to regulate HPA-axis activity (Suemaru et al., 1995). Both acute and chronic stress alters glutamatergic transmission in the PFC and midbrain dopamine neurons (Yuan and Hou, 2015). Repeated stress during childhood and adolescence (i.e. ELS) leads to aberrant reward processing and drug-seeking behaviors, likely mediated by disturbances in dopaminergic and glutamatergic neurotransmission in the PFC and the Acb (Watt et al., 2017). Overall, the role of glutamate alone or with its interplay with dopamine in stress or addiction phenotypes, has been highlighted. Yet, almost until 2000, the glutamatergic system was not that extensively investigated in relation to these factors, and in contrast to the dopaminergic neurocircuitry, partly due to

lack of valid molecular markers (Bimpisidis and Wallén-Mackenzie, 2019). During the last two decades, the identification of the vesicular glutamate transporters (VGLUTs), the best markers of the glutamatergic system, opened a new road of glutamate-focused investigation within the field of addiction (Bimpisidis and Wallén-Mackenzie, 2019).

The vesicular glutamate transporters

Glutamate is the major excitatory neurotransmitter in the brain, implicated in a wide array of physiologic actions, but also in numerous neurological and psychiatric disorders, including AUD (Tsai and Coyle, 1998, Holmes et al., 2013). Glutamatergic transmission involves a cycle of glutamate synthesis from glutamine in the presynaptic neuron, packaging in synaptic vesicles, release into synaptic cleft through exocytosis, binding to postsynaptic glutamate receptors that mediate synaptic plasticity (Figure 3A), and eventually extracellular glutamate clearance by transporters located on glia (Kalivas et al., 2009, Martinez-Lozada and Ortega, 2015). The glutamatergic phenotype can be identified by the expression of the VGLUTs 1-3, which are encoded by the solute carrier superfamily genes *Slc17a7, Slc17a6* and *Slc17a8*, respectively (Anne and Gasnier, 2014). VGLUTs actively package glutamate into presynaptic vesicles in neurons through an electrochemical proton-dependent gradient (Anne and Gasnier, 2014), and every neuron that expresses a *Vglut* gene has the ability to release glutamate (Takamori et al., 2000, Takamori et al., 2001). *Vgluts* can thus be considered the best markers of glutamatergic phenotype (Figure 3A). VGLUTs show different temporal expression in the brain. VGLUT2 has an early onset of expression, already present at birth (Miyazaki et al., 2003, Fortin et al., 2012, Gras et al., 2005), whereas neonatal VGLUT1 expression is very low, increasing from postnatal day (PND) 6 to PND 10 and reaching adult levels after PND 15 (Minelli et al., 2003, Gras et al., 2005). VGLUT3 temporal pattern of expression differs from VGLUT1 and 2; more than 60% of adult VGLUT3 levels are present at birth, with a peak around PND10, a small decrease until PND 15 and a steady increase until adulthood (Gras et al., 2005). Along with different temporal expression, changes in VGLUTs' spatial distribution throughout development have also been observed, suggesting an intriguing level of plasticity in the glutamatergic system (Gras et al., 2005, Boulland et al., 2004).In the adult rodent and human brain, VGLUTs have a complementary distribution (Vigneault et al., 2015, El Mestikawy et al., 2011) (Figure 3B). VGLUT1 is mainly found in the PFC, hippocampus and cortical cerebellum (Bellocchio et al., 1998, Ni et al., 1995), while VGLUT2 is located in the thalamus, brainstem and subcortical cerebellum, but also in dopaminergic neurons of the VTA (Fremeau et al., 2001, Herzog et al., 2001), and all of its sub-nuclei, namely parabrachial pigmented nucleus (PBP), VTA rostral nucleus (VTAR) and the rostral linear nucleus

(RLi) (Birgner et al., 2010). Yet, in some developing and adult glutamatergic neurons VGLUT1 and 2 co-localize (Fremeau et al., 2004, Herzog et al., 2006). VGLUT3 is sparsely found in the brain, in subgroups of primarily glutamatergic neurons in the raphe, hypothalamus and habenula (El Mestikawy et al., 2011, Vigneault et al., 2015), but also in non-glutamatergic neurons, such as cholinergic interneurons in the Acb, a subset of gamma-aminobutyric acid- (GABA)-ergic interneurons in the cortex and hippocampus, and in serotonergic neurons of the raphe nuclei (Herzog et al., 2004). Developmentally regulated expression of VGLUT1 and 2 in subpopulations of cholinergic, dopamine or GABA neurons, has also been observed (Trudeau and El Mestikawy, 2018).

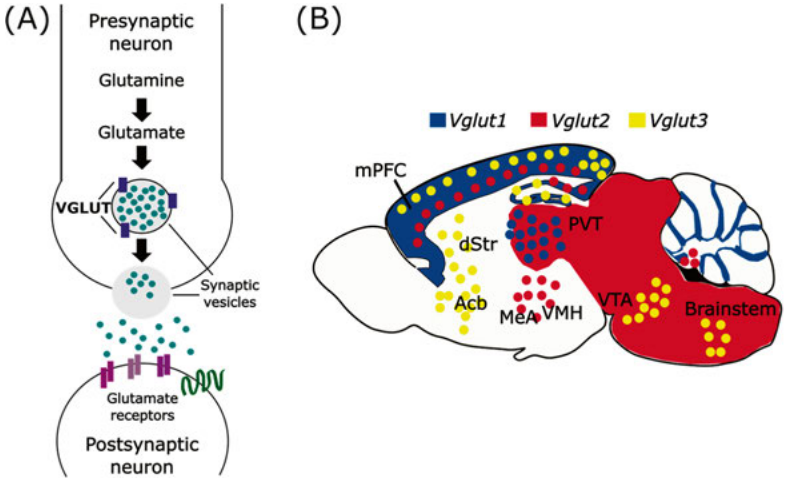

Figure 3. Schematic representation of A) The glutamatergic synapse. VGLUTs are located in the membrane of the synaptic vesicles and actively package glutamate into presynaptic vesicles. B) Vglut1-*3* mRNA expression in the rat brain, sagittal section; adapted from El Mestikawy et al., 2011. Acb: nucleus accumbens; dStr: dorsal striatum; MeA: medial amygdala; mPFC: medial prefrontal cortex; PVT: paraventricular nucleus of thalamus; VGLUT: Vesicular Glutamate Transporter; VMH: ventromedial hypothalamus; VTA: ventral tegmental areaa

VGLUT co-expression has been suggested to play a role, not only in glutamate co-release by these non-glutamatergic neurons, but also in enhanced release of the 'primary' neurotransmitter (i.e. dopamine or acetylcholine) along with glutamate, a phenomenon termed as "vesicular synergy" (El Mestikawy et al., 2011). This discovery has further strengthened the hypothesis of an interplay between different neurotransmitter systems that govern various aspects of neurophysiological processes (Gras et al., 2008, Trudeau and El Mestikawy, 2018), including reward (El Mestikawy et al., 2011) and addiction (Frahm et al., 2015, Bimpisidis and Wallén-Mackenzie, 2019, Papathanou et al., 2018).

The existence of a mesocorticolimbic glutamatergic pathway has been proven (Morales and Root, 2014, Yamaguchi et al., 2011); neurons originating

from the VTA innervate the mPFC and Acb. Approximately half of these mesocortical neurons are VGLUT2-only, while one fourth of those are co-expressing Tyrosine Hydroxylase (TH, the rate-limiting enzyme of dopamine synthesis and a marker for dopaminergic neurons) (Yamaguchi et al., 2011). Drug-induced activation of dopaminergic neurons in the VTA alters glutamatergic transmission in the VTA and Acb further supporting the existence of such pathway and of dopaminergic/glutamatergic interplay of relevance to addiction (Mameli et al., 2009, Rodriguez Parkitna and Engblom, 2012). Further evidence for this interplay emerges from studies of mice lacking the *Vglut2* gene in the midbrain neurons. These *Vglut2*-conditional knockout mice show addiction-related phenotypes, such as blunted response, assessed as attenuated locomotor activity, to acute injections of the psychostimulants amphetamine (Birgner et al., 2010) and cocaine (Hnasko et al., 2010), and higher cocaine self-administration and cue-induced drug seeking (Alsiö et al., 2011). *Vglut2* has also been implicated in alcohol-related phenotypes in rodents; altered *Vglut2* expression (Zhou et al., 2006, McBride et al., 2013, McClintick et al., 2015), but also promoter DNA methylation (Zhang et al., 2015) has been demonstrated in relation to alcohol exposure. Additionally, *Vglut1* and *Vglut3* have been linked to reward and addiction; altered gene expression has been found upon alcohol and/or nicotine co-administration (Truitt et al., 2014, McClintick et al., 2015). Furthermore, *Vglut3* KO mice were more responsive to cocaine as compared to their wild-type counterparts (Gras et al., 2008, Sakae et al., 2015). The involvement of *VGLUTs* in addiction has been also highlighted in human studies. Among common variants in the *VGLUT1*-3 genes, *VGLUT2 rs2290045* SNP was over-represented in women with alcohol dependence (Comasco et al., 2014), while frequency of rare variations of *VGLUT3* was higher in a cohort of severe cocaine or opiate abusers (Sakae et al., 2015). Moreover, *VGLUT1-2* expression in the VTA of human post mortem brains was robustly induced among smokers compared to controls, but the effect was reduced upon alcohol co-exposure (Flatscher-Bader et al., 2008). Lastly, an association between alcohol intake and two CpGs within the body of *VGLUT1* and the promoter of *VGLUT2* in humans was demonstrated by a recent EWAS study (Dugué et al., 2019).

It is therefore plausible that VGLUTs play an important role in stress- or alcohol-induced separate effects contributing to the emergence of alcohol-related phenotypes. Nevertheless, the interactive effect of both factors (ELS and alcohol) has never been investigated. Furthermore, the synergistic effect of alcohol and nicotine on VGLUTs has been sparsely studied (Truitt et al., 2014, Flatscher-Bader et al., 2008). To date, the main focus of research on addiction has been on the glutamatergic system during the withdrawal, alcohol-seeking and relapse states (Koob and Volkow, 2010, Tsai and Coyle, 1998). Alcohol-induced effects have been mainly investigated on the glutamatergic receptors or transporters responsible for glutamate clearance from the synaptic cleft (Kalivas, 2009). Only during the last two decades, after the discovery of the VGLUTs (Bellocchio et al., 1998, Takamori et al., 2000, Takamori et al.,

2001, Herzog et al., 2001, Takamori et al., 2002, Ni et al., 1995), research shifted towards the investigation of presynaptic transporter mechanisms that could modulate glutamate release and downstream processes such as transmission and synaptic plasticity. How VGLUTs are affected by alcohol and/or nicotine co-exposure and whether these effects contribute to the progression of the addiction stages has been virtually unstudied. Over and above that, investigation of the effect of ELS on VGLUTs remains elusive. The present thesis focused on these excellent glutamatergic markers and contributed to unravel *Vglut*/*VGLUT*-mediated underpinnings behind the interactive relationship of early-life experiences with alcohol, as well as the combined effect of the two most highly consumed drugs, alcohol and nicotine.

Aims

The present thesis sought to investigate the relationship between early life experiences, alcohol and *Vgluts/VGLUTs* in the initial stage of transition from voluntary to habitual alcohol consumption and during the sensitive period of late adolescence/young adulthood in rats and humans. The effect of co-exposure to nicotine, as well as the role of *Dnmt1* and *Mecp2,* was also investigated.

Paper I: The expression of *Vglut1-3*, *Dnmt1* and *Mecp2* genes was investigated in the brains of young adult male outbred Wistar rats exposed to different rearing conditions during early life and episodic weekend-like alcohol or water drinking during early adulthood. The following research questions were addressed:

- What is the effect of ELS, alone and in interaction with alcohol drinking, on *Vglut1-3, Dnmt1* and *Mecp2* expression?
- Do ELS and alcohol consumption influence correlations between the expression of the investigated genes?
- Do levels of alcohol consumption correlate with gene expression?
- Is there an effect of housing on gene expression?

Paper II: The DNA methylation within potential regulatory regions of *Vglut1-3* genes was investigated in the mesocorticolimbic brain of the same rats used in Paper I. The following research questions were addressed:

- What is the effect of ELS, alone and in interaction with alcohol drinking, on *Vglut1-3* DNA methylation?
- Is there any correlation with the transcriptional *Vglut1-3* and *Dnmt1* differences previously reported in Paper I?
- Does CpG methylation moderate the interactive effect of ELS and alcohol drinking on *Vgluts* expression?
- Is there any correlation between *Vglut1-3* DNA methylation and corticosterone levels or alcohol consumption?
- Are there potential transcription factor binding sites in the investigated CpGs?

Paper III: The mRNA expression of the *Vglut2* gene was investigated in the mesolimbic brain of young adult male outbred Wistar rats exposed to prolonged episodic weekend-like alcohol and/or nicotine during adolescence. The following research questions were addressed:

- What is the effect of alcohol and nicotine, alone and in combination, on *Vglut2* mRNA expression?
- Is there an implication of midbrain neurons co-expressing *Vglut2* and *Th*?

Paper IV: The moderating effect of the candidate *rs2290045* SNP in the *VGLUT2* gene on the relationship of alcohol-related problems with both aversive (i.e., maltreatment) and supportive (i.e., supportive parenting) environmental factors was investigated in three independent cohorts of adolescents and young adults: two general population samples and one clinical sample. The following research questions were addressed:

- Does *rs2290045* genotype interact with maltreatment and parenting to moderate alcohol-related problems in a bidirectional manner according to the "environmental sensitivity" framework?
- Are there sex differences? Does nicotine affect the results?
- Are interaction effects present in the general population and in "at risk" individuals as well as across different periods of adolescence and young adulthood?

Materials and Methods

Samples

Rodents (Paper I, II and III)

Outbred Wistar rats were used to mirror the individual differences and heterogeneity of alcohol-drinking patterns (Palm et al., 2011) encountered within the human population. For each study, twenty-five time-mated dams (RccHan: WI, Harlan, the Netherlands, Europe) of gestation day 15 were received in the laboratory. The dams were housed individually in standard cages (59 x 38 x 20 cm) under normal light-dark cycle having water and food *ad libitum*. Immediately after birth, at postnatal day (PND) 0, sexing and cross-fostering of the pups was performed to ensure that each experimental group would not include biological littermates, and each litter would be of the same size (in total 10 pups) and sex composition (6 males, 4 females). Only male rats were used to minimize the potential confounding effect of hormonal fluctuations during estrous cycle in the females (ter Horst et al., 2012). Moreover, alcohol-intake in adult female rats was found not to be affected by the MS paradigm (Paper I, II) (Roman et al., 2004).

Humans (Paper IV)

Three independent samples were included; two general population (GP) samples and one clinical sample (CS), whose age spanned the period from adolescence to early adulthood. The GP sample of adolescents (GP-Adolescents) included 14-year-old adolescents (baseline), followed-up at the age of 17. The other GP sample included young adults of 20 to 24 years of age (GP-Adults), assessed only in one time-point. Lastly, the CS included 17-year-old adolescents (baseline), followed-up at the age of 22. The study design of each sample is depicted below (Figure 4-6).

General Population sample of adolescents (GP-Adolescents)

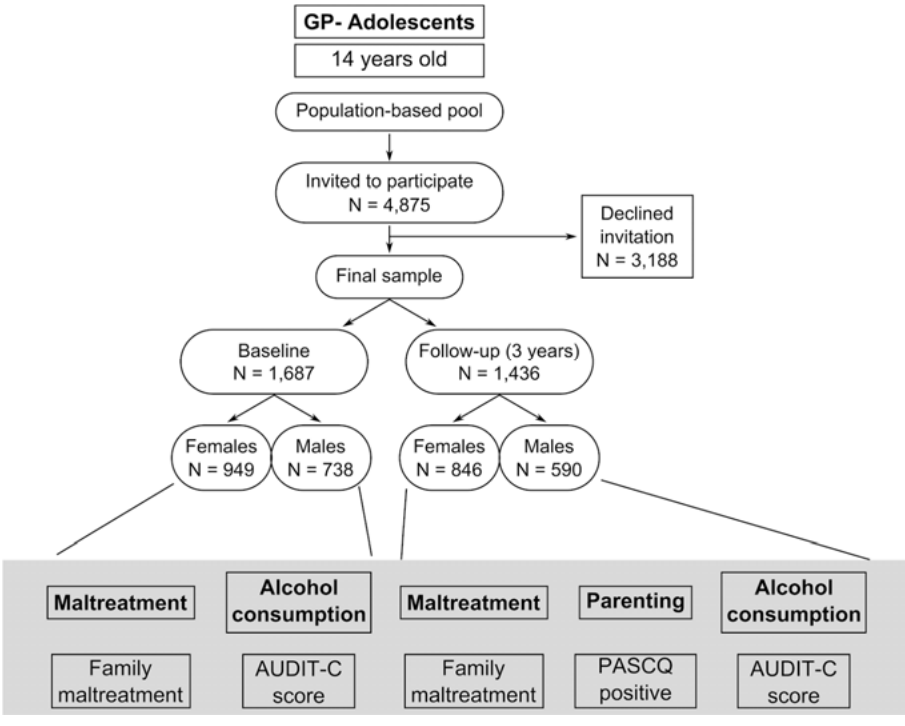

Figure 4. Design of the study on GP-Adolescents.
AUDIT-C: Alcohol Use Identification Test-Consumption, PASCQ: Parents as Social Context Questionnaire

General Population sample of young adults (GP-Adults)

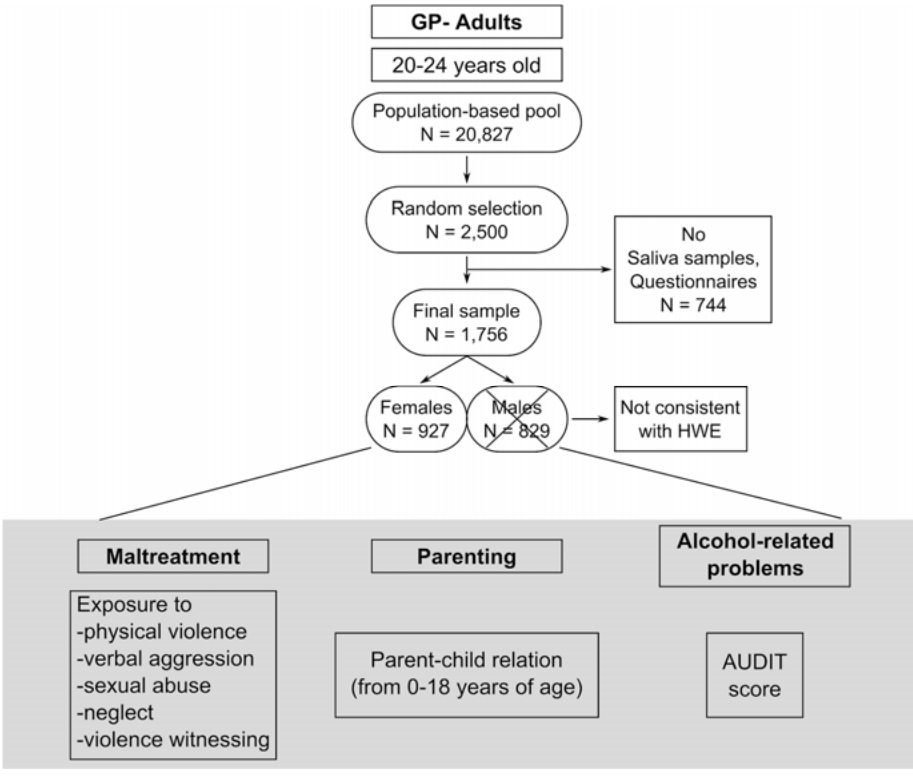

Figure 5. Design of the study on GP-Adults.
AUDIT: Alcohol Use Identification Test, HWE: Hardy-Weinberg Equilibrium

Clinical sample (CS)

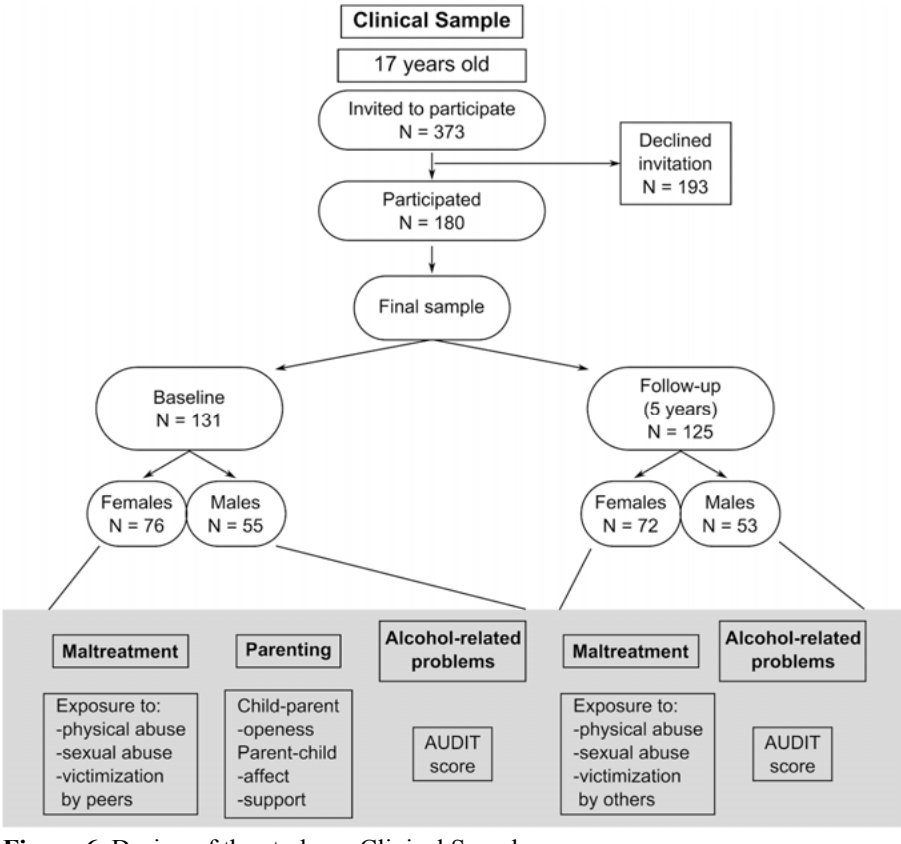

Figure 6. Design of the study on Clinical Sample.
AUDIT: Alcohol Use Identification Test

Environmental factors

The maternal separation (MS) model (Paper I, II)

In order to study ELS, a maternal separation (MS) paradigm was used to model different early-life rearing conditions during the first three postnatal weeks (PND 0 – 21) (Figure 7A). MS in rodents is a validated model with high construct validity to study alcohol-related outcomes, such as propensity to higher alcohol-intake and preference (Nylander and Roman, 2013). Two conditions were used herein: prolonged MS (MS360), in which the pups were daily separated from the dam for 360 minutes, and short MS (MS15), where the separations were done also daily but for 15 minutes only. MS360 was used to simulate a stressful and high-risk environment (ELS) and the MS15 was used as a control, simulating a naturalistic condition. Previous studies demonstrated higher propensity to alcohol consumption in the MS360 group, while a protective role of MS15 has been shown, with rats subjected to daily separations of short (0 to 15 min) duration (also called handling) displaying lower alcohol-intake over time (Lehmann and Feldon, 2000, Nylander and Roman, 2013).

The litters were randomly assigned to the different experimental groups (i.e. MS15, MS360). Separation from the dam was done litter-wise (the whole litter was kept together) during the light period, starting at 9.00 am, by the same experimenter and in the same room. Weighing of the litters took place every three days from PND 0 to 16 (i.e. PND 0, 3, 7, 10, 13 and 16) and cage change was done on PND 7 and 16. The pups were weaned on PND 22 and from postnatal week (PNW) 4 to 9 (adolescence, PND 22 – 69) they were group-housed, three per cage, with their littermates.

Animal facility-rearing condition (Paper I, III)

Rats reared according to conventional laboratory conditions (i.e. animal facility-reared (AFR) rats) were used in Paper III. On PND21, the pups were weaned and group-housed (2 – 3 per cage) under standard conditions (22◦C, 50 ± 10% humidity) in reversed light-dark cycle having access to tap water and pellet food *ad libitum* (Paper III). AFR rats were also included in Paper I as part of a parallel experiment to assess the effect of alcohol-drinking and single-housing in non-maternally separated animals (Figure 7A). The AFR rats were left undisturbed during the first three postnatal weeks, except for the weighing of the litters and the cage maintenance, which was performed on the same PNDs as in the MS rats (Paper I).

Maltreatment (Paper IV)

Stress in humans was conceptualized as maltreatment experienced by the participant until the time-point of the study. In GP-Adolescents family maltreatment was assessed at baseline and follow-up, using the summation index (ranging from 0 to 20) of four questions regarding presence of physical or verbal maltreatment between the parents, and from the parents towards the adolescent. Higher score indicated higher levels of maltreatment.

In GP-Adults, different types of lifetime maltreatment were assessed using self-reports; i.e. exposure to physical abuse, verbal aggression, sexual abuse, neglect and witnessing violence (Cater et al., 2014). For each type, maltreatment was defined if occurred twice or more (Cater et al., 2014) and then a combined maltreatment variable was created, which had six levels ranging from 0 (no/minor maltreatment) to 5 (five types of maltreatment).

In the CS, three different types of maltreatment were assessed at baseline and at follow-up; i.e. physical abuse by parents in childhood, sexual abuse, victimization by peers/others (Hodgins et al., 2014). Physical abuse was assessed using the Conflict Tactics Scale Parent-Child Version (Straus et al., 1998). Events of minor valence (e.g. slapped on the hand, pinched) were classified as minor abuse. Sexual abuse was assessed using the Sexual Experience Survey questionnaire (Karabatsos, 1997) at baseline. At follow-up it was assessed using the Sexual and Physical Abuse Questionnaire (Kooiman et al., 2002) or the McArthur Community Violence Instrument (Steadman et al., 1998). Victimization by others at baseline was assessed through a 7-item self-report questionnaire regarding victimization during the last semester, while at follow-up it was assessed using 8 items from the McArthur Community Violence Instrument (Steadman et al., 1998) regarding exposure to aggressive behavior. In each case, victimization was defined as an affirmative response to at least one item. Each type of maltreatment was dichotomized to 0 (no/minor maltreatment) and 1 (high maltreatment) and then a combined maltreatment variable was created, both at baseline and follow-up, with four levels ranging from 0 (no/minor maltreatment) to 3 (three types of maltreatment).

Parenting (Paper IV)

Parenting style in GP-Adolescents was assessed using the Parents as Social Context Questionnaire (PASCQ) including questions regarding three positive dimensions of parenting i.e. warmth, structure and autonomy (Skinner et al., 2005). A summation index was computed, with higher score indicating supportive parenting. In GP-Adults, parent-child relationship was assessed using two questions regarding perceived relationship of the participant with their mother or father. A summation index of these two questions was computed, with higher number indicating supportive parenting. In the CS, three different dimensions of parenting were assessed, i.e. child-parent openness, parent-

child affect, and parent-child support. A summation index for each dimension was computed with higher score indicating higher quality of parenting.

Alcohol and nicotine consumption

Alcohol-drinking model: two-bottle choice paradigm (Paper I, II)

From PNW 10 to 16, the light cycle was reversed and the rats were single-housed and subjected to a modified intermittent, two-bottle, free-choice paradigm between water and alcohol (Figure 7A). An additional group of AFR rats (n = 7) was kept group-housed with access only to water and it was used to assess the housing effect among the AFR rats. Single-housed MS and AFR rats were randomly assigned into water-drinking groups with access to water only (MS15W, n = 10; MS360W, n = 10; AFRW = 9), or alcohol-drinking groups with access to both water and alcohol (MS15A, n = 10; MS360A, n = 20; AFRA = 11). Due to the existence of MS360 "responders" and "non-responders" relative to alcohol intake (Nylander and Roman, 2013), twice as many animals were used in that group.

Alcohol-drinking rats had access to water or non-sweetened alcohol (made from 96% ethanol, Solveco AB, Rosersberg, Sweden) for three consecutive days per week, followed by four days with access to water only. This modified intermittent paradigm was used to mimic an episodic, weekend-like drinking in humans (Momeni and Roman, 2014, Palm and Nylander, 2014). The first drinking week (PNW 10), the rats had free (24 hours) access to 5% alcohol in order to initiate the alcohol consumption. The second drinking week (PNW 11), the access to 5% alcohol was limited to 2 hours to avoid variation in drinking bouts that is usually seen in 24-hours access paradigms. From the third to seventh drinking week (PNW 12 – 16), the rats had limited access to 20% alcohol to assess voluntary alcohol consumption due to the rewarding effects rather than the sweet, mild taste of lower alcohol concentrations (Sanchis-Segura and Spanagel, 2006) (Figure 7B).

Fresh alcohol and water was used in each session. To avoid position preference, the placement of the bottles was changed every day. Spillage was also minimized with the usage of bottles with nipples. Quantification of alcohol and water intake was done by weighing the bottles before and after each session. Alcohol preference was calculated as a fraction of alcohol to total volume of liquid consumed. On PNW 16, immediately after the last 2-hour drinking session, the rats were decapitated, the brain regions of interest (i.e. VTA, Acb, mPFC and dStr) were dissected on ice, snap-frozen on dry ice and stored at -80^{o}C until further analyses.

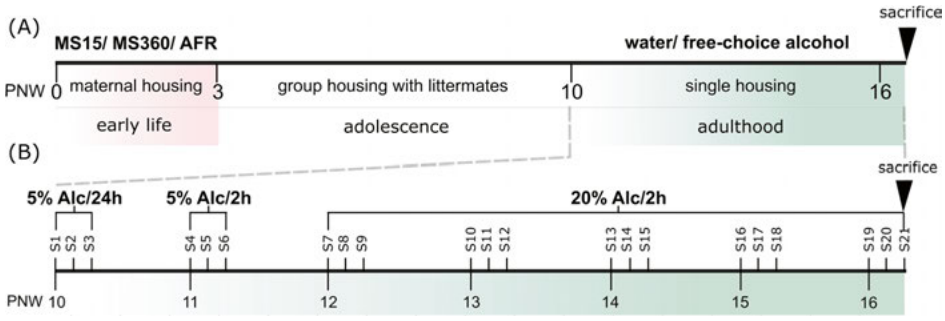

Figure 7. Experimental outline of the rodent studies I and II. **A)** Male Wistar rats were subjected to maternal separation for 15 min (MS15) or 360 min (MS360) or animal facility rearing (AFR) conditions during the first three postnatal weeks (PNW), group-housed during adolescence and single-housed during alcohol/water consumption. One additional water-drinking AFR group remained group-housed throughout the experiment. **B)** A schematic outline of the details of the intermittent alcohol-drinking model from PNW 10 to 16. AFR: Animal facility reared; Alc: Alcohol; h: hours; MS15: maternal separation for 15 min; MS360: maternal separation for 360 min; S: sessions, PNW: postnatal week

Alcohol and/or nicotine exposure (Paper III)

Between PNW 4 and 9, the animals were divided into four experimental groups (n = 10, per group) and exposed to i) alcohol-only; ii) nicotine-only; iii) combination of alcohol and nicotine; or iv) control for three consecutive days per week (Figure 8). Alcohol (made from Solveco Ethanol 96%, Solveco AB, Rosersberg, Sverige) was diluted in tap water and nicotine [(−)-Nicotine hydrogen tartrate salt, Sigma Aldrich] in saline. A vehicle solution was used, i.e. tap water for alcohol exposure and saline for nicotine, in order to control for the administration procedure. Alcohol and water were administrated via gavage whereas nicotine and the control saline solution were injected subcutaneously. Administration was done at 09:00 on PND 28-30, 36-38, 43-45, 50-52, 57-59. This exposure paradigm was chosen to mimic episodic drug binges commonly used among adolescents. The ethanol dose (2 g/kg 20%) was chosen to achieve blood alcohol levels > 0.08g/dl (Lundberg, 2020, Löf et al., 2007) (further supported by unpublished data) and the nicotine dose (0.35 mg/kg free base) was based on previous literature(Löf et al., 2007, Lundberg, 2020). At PNW 9, the animals were decapitated two hours after the last session of drug administration and the whole brain was collected, snap-frozen and stored in -80°C (Figure 8). Coronal cryosections of 14µm were collected on Superfrost slides (Menzel-Gläser, Braunschweig, Germany) from bregma -2.28 to -3.00 (targeting the PVT, MeA, VMH), and -4.92/-5.04 (targeting the anterior VTA, including PBP, VTAR and RLi), according to Paxinos and Watson, 6th edition (Paxinos G., 2007), using the Cryostar NX70 cryostat (Thermofisher Scientific, Waltham, MA, USA) and stored at -80°C until further analysis.

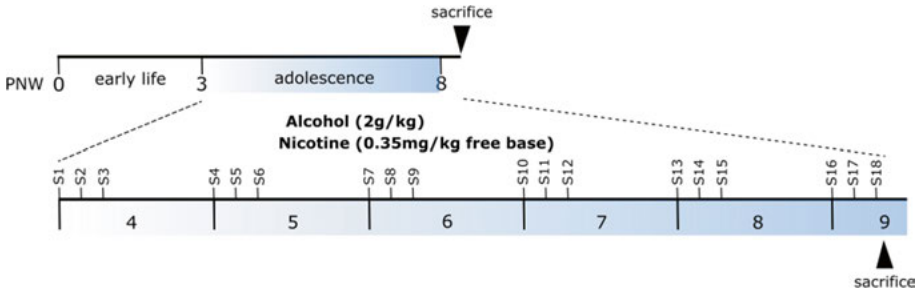

Figure 8. Experimental outline of the rodent study III. Male Wistar AFR rats were assigned to four experimental groups (control, alcohol, nicotine, and alcohol and nicotine; n = 10 animals per group). Administration of alcohol (2g/kg 20%) and nicotine (0.35 mg/kg free base) took place for three consecutive days per week for six weeks during adolescence. S: sessions, PNW: postnatal week

Alcohol consumption and alcohol-related problems (Paper IV)

The Alcohol Use Disorders Identification Test (AUDIT) is a 10-item screening tool for excessive drinking developed by the World Health Organization (Saunders et al., 1993). AUDIT comprises three conceptual domains regarding i) hazardous alcohol use (3 items); ii) alcohol dependence (3 items); and iii) harmful alcohol use (4 items). Each item is rated from 0 to 4 by the patient, thus AUDIT can range from 0 to 40, with higher score indicating higher levels of alcohol-related problems. AUDIT provides good discrimination and high internal consistency reliability and validity (Babor, 2001). The AUDIT-Consumption (AUDIT-C) (Bush et al., 1998) includes only the first three questions of AUDIT that are relevant to alcohol consumption (hazardous alcohol use domain), and its index ranges from 0 to 12.

In GP-Adolescents, due to their young age, the AUDIT-C was used to assess alcohol consumption. A slightly modified version of AUDIT-C was employed, appropriate for adolescents. In the first two questions the answer "monthly or less" was divided into "every other month or less" and "about once a month". In GP-Adults and in the CS individuals, the AUDIT was used to assess the whole spectrum of alcohol-related problems. Since high AUDIT-C score indicates alcohol misuse, a predictor of alcohol-related problems (Kelly et al., 2009), the same term was used also for GP-Adolescents.

Nicotine use (Paper IV)

Considering the high comorbidity and cross-transmission between nicotine use and alcohol misuse (Goldman et al., 2005), data for nicotine use were considered in the present study. Data were available for cigarette smoking and/or use of Swedish snus in GP-Adolescents and CS individuals, but only cigarette smoking in GP-Adults. Accordingly, nicotine use was defined as current cigarette smoking in GP-Adults and current cigarette smoking and/or snus in the

other two samples. In each sample, the variable was dichotomized into 0 (no use) vs. 1 (occasional/daily use).

Genetic analyses

Gene expression analyses (Paper I, II and III)

Quantitative real time polymerase chain reaction (Paper I and II)

Quantitative real time polymerase chain reaction (qPCR) was used to assess gene (mRNA) expression levels of *Vglut1-3*, *Dnmt1* and *Mecp2* in the rat VTA, Acb, mPFC and dStr. qPCR is one out of multiple methods (i.e. RNA protection assays, *in situ* hybridization, microarrays, Northern blot) for mRNA detection and it has been proven advantageous compare to the rest because of its high sensitivity, reproducibility and quantitative nature (Wong and Medrano, 2005).

A schematic representation of the experimental procedure is shown in Figure 9. Total RNA was extracted from the VTA, Acb, mPFC and dStr using the AllPrep DNA/RNA/miRNAUniversal kit (Qiagen AB Sollentuna, Sweden) and following the manufacturer's instructions. The homogenization of the tissue was performed by sonication in Cell Disruptor B15, Branson Sonifier (Kebo Lab). The procedure included on-column DNase treatment to avoid genomic DNA (gDNA) contamination. RNA concentration was measured using Nanodrop ND 1000 spectrometer and integrity was verified in 1% agarose gel electrophoresis for 10% of randomly selected samples.

Complementary DNA (cDNA) was synthesized by 350-700ng of total RNA with the QuantiTect Reverse Transcription Kit (Qiagen AB Sollentuna, Sweden) according to manufacturer's protocol with minor modifications regarding the incubation times: the gDNA wipeout reaction was prolonged to 5 minutes (instead of 2) and the final cDNA synthesis step was prolonged to 35 minutes (instead of 15) followed by a 5-minute inactivation (instead of 3) at 95^{o}C. The newly-synthesized cDNA was diluted 20-fold and stored at -20^{o}C until further analyses.

Primers for *Vglut1-3*, *Dnmt1* and *Mecp2* as well as for the three housekeeping genes *Actb* (actin beta), *Gapdh* (glyceraldehyde 3-phosphate dehydrogenase) and *Rpl19* (ribosomal protein L19), used as reference controls, were designed using Primer 3 (http://frodo.wi.mit.edu/) and cross-checked using Primer Map (http://www.bioinformatics.org/sms2/primer_map.html). A final control for gDNA contamination was applied at this step as well; primers were designed across two adjacent exons to avoid unspecific gDNA amplification.

The gene expression analyses were performed using CFX96 Touch Real-Time PCR Detection System (Applied Biosystems, Foster City, CA, USA). The final 20µl reaction contained 1x iQ SYBRGreen Supermix (Biorad Sweden), 0.15 µM of each primer and 3µl of diluted cDNA (20x) template. The

qPCR protocol included denaturation, annealing and elongation steps with optimal conditions for each primer, followed by melting curve analysis. Each PCR plate contained no-template controls as well as internal positive control samples. Unique PCR product for each gene was verified by gel electrophoresis.

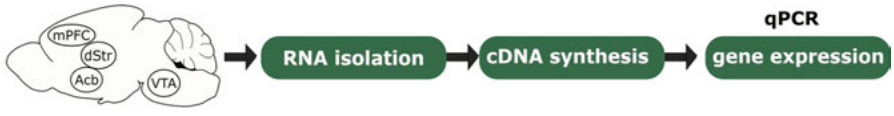

Figure 9. Experimental procedure in Paper I. RNA was isolated from the selected regions, converted to cDNA and the expression levels of the genes of interest were assessed by quantitative real time PCR.

The relative fluorescent units from each PCR plate were collected using Biorad CFX manager 3.1 software (Applied Biosystems) and were used to determine the baseline and calculate PCR efficiency and cycle (Cq) threshold in LinRegPCR software (Ruijter et al., 2009). Genes with PCR efficiency < 1.7 were excluded from the analyses. The positive controls from each plate were used to calculate correction factors that were applied for Cq value correction to achieve plate normalization within the same gene. The corrected Cq values were used to calculate relative gene expression to three housekeeping genes following the ΔCT method (Biorad real time PCR application guide, Bio-Rad, #170-9799). Each sample was run in triplicates; the standard deviation (SD) of their Cqs was calculated and those samples with SD > 0.5 were excluded from further analyses.

Data on expression of *Vglut3* in the VTA, *Vglut2* in the dStr, and *Mecp2* in both the VTA and the dStr were excluded from the analyses as they did not comply with the inclusion criteria (i.e. PCR efficiency > 1.7; no amplification in no-template control; unique PCR product for each gene; and SD < 0.5).

In situ hybridization (Paper III)

A schematic representation of the experimental procedure of Paper III is shown in Figure 10. Chromogenic *in situ* hybridization (CISH) with digoxigenin (DIG)-labelled RNA probes was performed to localize and quantify mRNA *Vglut2*-expressing cells. CISH is a semi-quantitative method, but it has high sensitivity, while its main advantage, compared to more quantitative techniques such as qPCR, is the anatomical and spatial information that provides about mRNA sequences *in situ,* as well as the assessment of neuronal co-phenotype. In the present study, the *Vglut2* riboprobe was generated from a plasmid construct (AI060126.1; Source Bioscience, UK Limited, Bingham, GB), following linearization with the restriction enzyme FokI (Fast Digest, Thermoscientific, Waltham, MA, USA). The DIG-antisense RNA probe (NM_053427.1, sequence: 3687-3982) was generated by T3 *in vitro* transcription [1µg linearized DNA template, 2µl RNA DIG-labeling mix (Roche,

Mannheim, Germany), 2µl transcription buffer (Roche, Mannheim, Germany), 2µl RNA T3 polymerase (Roche, Mannheim, Germany) and diethylpyrocarbonate (DEPC)-H2O up to 20µl, incubated for 10min at 37°C]. A DIG-sense RNA probe was also generated to control for unspecific staining signal. For the sense-probe generation, the T7 RNA polymerase was used, following linearization of the plasmid by the restriction enzyme TseI (New England Biolabs, Ipswich, MA, USA) overnight at 37°C.

The 14µm-cryosections were air-dried (10min), fixed (10min) with 4% formaldehyde (Histolab, Gothenburg, Sweden), treated with Proteinase K [1µg/ml; (Sigma, Saint Louis, MO, USA)] (10min) and re-fixed (5min). Acetylation treatment [(1.3% triethanolamine (Sigma, Saint Louis, MO, USA), 0.25% acetic anhydride (Fluka, Neu-Ulm, Germany), and 0.065% HCl diluted in water)] (10min) for background signal reduction and inactivation of RNases, and PBS-containing 1% Triton X-100 (Sigma, Saint Louis, MO, USA) incubation (10min) for tissue permeabilization was followed by prehybridization (2 h) for further reduction of background staining. The *Vglut2* probe was then diluted in a final concentration of 1µg/µl into hybridization solution and heated at 80°C for 5min. Hybridization (200µl per slide) was performed in a humidified chamber at 58°C for 16 hours. The following day, slides were washed at 58°C with Saline-Sodium Citrate (SSC) buffers of decreasing strength, immuno-blocked with 1% blocking solution in tris-buffered saline (TBS), and then incubated at 4°C with alkaline phosphatase-conjugated anti-DIG Fab fragments (Roche, Mannheim, Germany) diluted 1:5000 in TBS containing 1% blocking solution (2h). Thereafter, the slides were color-developed in BM-purple (Roche, Mannheim, Germany) for 20-24 hours at 37°C and mounted.

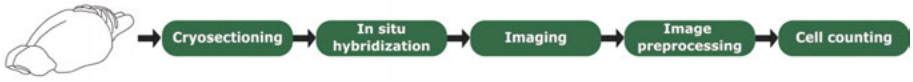

Figure 10. Experimental procedure in Paper III. Coronal cryosections of 14µm, including the PVT, MeA, VMH, and anterior VTA, were collected. Chromogenic and fluorescent i*n situ* hybridization with RNA probes was used to visualize and quantify *Vglut2* and *Th* mRNA. Image acquisition followed by image preprocessing and analysis for *Vglut2, Th* staining identification

Double Fluorescent ISH (d-FISH) was performed on adjacent sections of the previously-used slides using RNA probes to determine neurons co-expressing *Vglut2* (NM_080853.3, sequence 2315-3244) and the dopaminergic marker tyrosine hydroxylase (*Th*, NM_012740.3, sequence 456-1453). Riboprobes were synthesized with DIG- or fluorescein-labeled ribonucleoside tri-phosphate. dFISH was carried out on the 14µm-cryosections which were air-dried, fixed in 4% paraformaldehyde and acetylated in 0.25% acetic anhydride/100 mM triethanolamine (pH 8). Sections were hybridized for 18h at 65°C in 100µl of formamide-buffer containing 1µg/ml *Vglut2* DIG-labeled riboprobe

and 1µg/ml *Th* fluorescein-labeled, and they were then washed at 65°C with SSC buffers of decreasing strength, and blocked with 20% fetal bovine serum and 1% blocking solution. Fluorescein epitopes were detected with horseradish peroxidase (HRP) conjugated anti-fluorescein antibody at 1:5000 and revealed using Cy2-tyramide at 1:250. HRP-activity was stopped by incubation of sections in 0.1M glycine followed by a 3% H2O2 treatment. DIG epitopes were detected with HRP anti-DIG Fab fragments at 1:3000 and revealed using Cy3 tyramide at 1:100. Nuclear staining was performed with 4' 6-diamidino-2-phenylindole (DAPI).

Data from the VTA were excluded for one sample in nicotine group (dFISH) due to bad staining quality. Moreover, seven samples in alcohol group and six samples in nicotine group (CISH) were excluded because of inaccurate bregma estimation.

Genotype analyses (Paper IV)

Saliva samples (200µl), collected with the Oragene self-collection kit (DNA Genotek®, Canada), were used for DNA extraction using the silica-based Kleargene DNA extraction method. The *VGLUT2* SNP *rs2290045* was genotyped in all three study samples using the Kbioscience Allele-Specific Polymorphism assay based on competitive allele-specific PCR and bi-allelic scoring (LGC®, England). No-template control samples were included to enable the detection of contamination or non-specific amplification.

Deviation from Hardy-Weinberg Equilibrium (HWE) was tested using the online calculator developed by Michael Court (Court and Michael, 2012) to determine whether the observed genotypic frequencies of SNP *rs2290045* differed from the expected ones. In GP-Adult males, genotypic frequencies (CC: 72.5%; CT: 28.9%; TT: 3.6%) were not consistent with HWE ($p = 0.009$), thus these individuals were excluded from further analyses. In Paper IV, genotypes were grouped as homozygous and heterozygous of the *rs2290045* minor T allele vs. homozygous for the major allele (C) to address the statistical constraint imposed by the low frequency of the minor allele.

Image analyses (Paper III)

Cell imaging and counting

Visualization and imaging of the CISH stained tissue were carried out using the Zeiss AxioImager brightfield microscope (Zeiss, Oberkochen DE) and the AxioCam MRc camera (Zeiss, Oberkochen DE). A uniform light source intensity and the exposure time were chosen for every image. In order to obtain a high-resolution image of the whole brain slice, a 20x objective and a tile-setup was used. FISH stained slides were imaged using the Hamamatsu Nanozoomer 2.0-HT (Hamamatsu Photonics, Hamamatsu City, JPN). The whole slides were scanned as one batch with the same settings and a 40x objective, and the final images were saved in NDPI format. Using NDP.view 2, brightness, contrast and saturation of the individual channels were identically adjusted for all images.

A schematic representation of image preprocessing and analyses steps is depicted in Figure 11. Image-masking with the respective region of interest (ROI) of the corresponding bregma was performed by alignment of the figures of the rat brain atlas, Paxinos and Watson, 6th edition (Paxinos G., 2007) on Inkscape (version 0.92) as a template with specific anatomical reference points. The process included alignment of the image to match specific anatomical reference points in the template figure, e.g. ventricle system, optical tract (CISH) or the *Th* expression pattern (dFISH). To identify gene expression within the ROIs, the masked ROIs were assigned to a color (550000 Inkscape code), giving a specific intensity (0.333) in downstream analyses for ROI segmentation. The ROIs were the PVT (bregma -2.28 to -3.00), VMH (bregma -2.52 to -2.76), MeA including the basomedial amygdala (bregma -2.16 to -2.76) and anterior VTA (aVTA, bregma -4.92 to -5.04). Additionally, the sub-nuclei of the aVTA (PBP, VTAR and RLi) were analyzed separately.

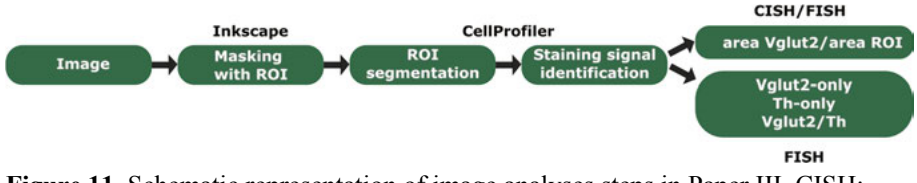

Figure 11. Schematic representation of image analyses steps in Paper III. CISH: Chromogenic *in situ* Hybridization (ISH); FISH: Fluorescent ISH; ROI: Region of Interest; Th: Tyrosine Hydroxylase; Vglut2: Vesicular Glutamate Transporter 2

A standardized staining signal identification was performed using CellProfiler (version 3.1.5) (Carpenter et al., 2006) through recognition of objects on the basis of size, shape, intensity, and texture of the signal. For CISH images a value for gene expression was generated by measurement of the area of the ROI occupied by *Vglut2*-specific expression signal (area occupied by *Vglut2*^{pos} signal within a ROI, divided by total area occupied by each ROI).

The same outcome variable was created for FISH images using a separate pipeline. Additionally, the count of *Vglut2*-positive/*Th*-negative (*Vglut2*^{pos}/*Th*^{neg}), *Vglut2*-negative/*Th*-positive (*Vglut2*^{neg}/*Th*^{pos}) and *Vglut2*-positive/*Th*-positive (*Vglut2*^{pos}/*Th*^{pos}) neurons was determined in dFISH images by use of a third pipeline, which related identified mRNA (*Vglut2*/*Th*) signal to the associated nuclei. The percentage of *Th* and *Vglut2* neurons vs. the total number of DAPI-stained nuclei within a ROI was also estimated. The range of the size of positive signal was set to 8 – 50 (CISH) or 5 – 50 (FISH) pixels. Positive signal was defined as staining above background [intensity threshold value: CISH: 0.05 (PVT) or 0.58 (MeA, VMH, VTA, PBP, RLi, VTAR); FISH: 0.15 for all ROIs] and surrounding a DAPI-stained nucleus (dFISH). Co-localization of *Vglut2* and *Th* was determined by the presence of the signal of both probes in the nucleus of the same cell (dFISH).

Epigenetic analyses (Paper II)

For Paper II, the log2 of the fold change in mean gene expression was calculated on a group- and individual-level for each region to assess the homogeneity of each group in terms of gene expression. Heterogeneity of *Vglut1-2* expression in MS360A in the VTA (that is, almost half of the samples had the opposite (than expected) direction of gene expression in MS360A) motivated an individual DNA methylation assessment of these genes. Expression of *Vglut1-3* in the dStr, mPFC and Acb, respectively, was largely homogeneous between the individuals within the same experimental group, with the exception of three different samples per gene. This homogeneity motivated DNA methylation analysis of all animals within a group after pooling of the samples. The threes samples that differed in their direction of expression were excluded before pooling of the samples to minimize potential noise in downstream DNA methylation analyses.

In silico design

All methylation analyses were performed by EpigenDx Inc (Hopkinton, MA, USA). *In silico* design of target CpG regions of interest (ROIs) for DNA methylation analysis across the promoter region and gene body of *Vglut1* (VTA, dStr), *Vglut2* (VTA, mPFC) and *Vglut3* (Acb) was performed. Each regulatory element of *Vglut1-3* was carefully evaluated using Ensembl and UCSC genome browser; gene sequences containing repetitive elements, low sequence complexity, high thymidine content and overall CpG density were excluded from the process of assay design.

A total of 41 assays were designed, 14 for *Vglut1*, 13 for *Vglut2* and 14 for *Vglut3*. The assays were grouped by gene and then based on GC %, amplicon size, and design score. A gradient PCR was run on each group of assays at

several different annealing temperatures using stock bisulfite-treated DNA (bsDNA). The most successful annealing temperature was chosen to be sequenced from each group. EpigenDx's custom library preparation method was performed on the chosen test samples, then the test samples were templated using the Ion Chef™ system (Thermo Fisher, CA, USA) and sequenced using the Ion S5™ sequencer (Thermo Fisher, CA, USA). Read counts from this sequencing run were used to regroup all the assays into final multiplex PCR conditions. Assays that failed PCR optimization were excluded from analyses. The final number of successful assays (>30 reads) was 33 (11 for *Vglut1*, 10 for *Vglut2* and 12 for *Vglut3*).

More specifically, for *Vglut1*, 61 CpG sites were targeted in 11 ROIs within 5'-upstream, exon 7, 9 and 12, intron 8-9, and 3'-UTR (untranslated region). For *Vglut2*, 53 CpG sites were targeted in 10 ROIs within 5'-upstream, 5'-UTR, exon1, and intron 1-3. Lastly, for *Vglut3*, 51 CpG sites were targeted in 12 ROIs within 5'-upstream, 5'-UTR, intron 1, 2 and 10, and exon 12 (Figure 12a-c).

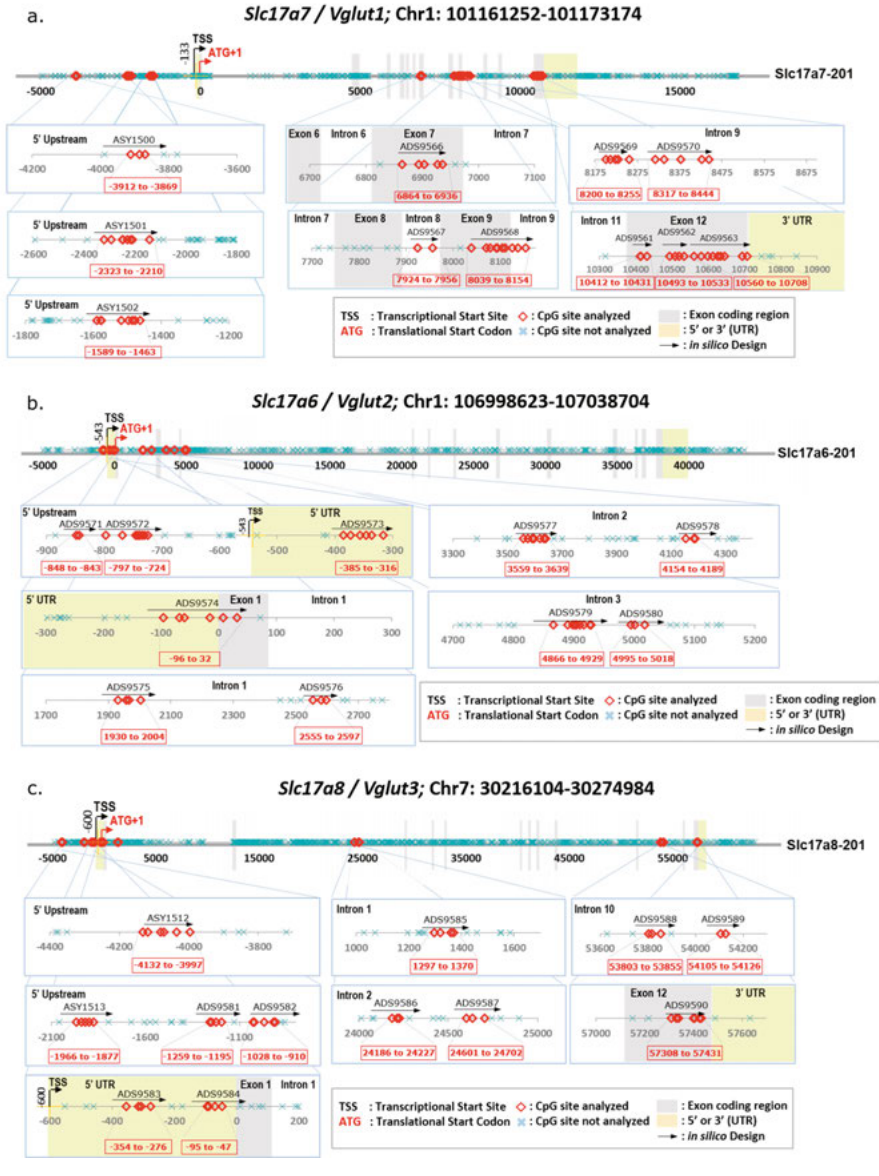

Figure 12. CpGs analyzed within target CpG regions for DNA methylation analysis across the promoter region and gene body of a) *Slc17a7/Vglut1*; b) *Slc17a6/Vglut2*; c) *Slc17a8/Vglut3*. *Slc:* Solute carriers; *Vglut:* Vesicular Glutamate Transporter

DNA methylation analyses

DNA was isolated from the rat VTA, Acb, mPFC and dStr, using AllPrep DNA/RNA/miRNA Universal Kit according to the manufacturer's protocol (Qiagen AB Sollentuna, Sweden), and quantified using a Nanodrop ND 1000 spectrometer. Targeted next generation bisulfite sequencing (tNGBS) was performed to identify differentially methylated CpG sites in the target CpG ROIs on 48 samples for *Vglut1* and *Vglut2* in the VTA, and 4 pooled samples (one for each experimental group i.e. MS15W, MS360W, MS15A and MS360A) for *Vglut1* in the dStr, *Vglut3* in the Acb and *Vglut2* in the mPFC (Figure 13).

A total of 300 ng of extracted DNA samples were bisulfite modified using Zymo EZ- 96 DNA Methylation™ Kit (Zymoresearch, CA, USA) according to manufacturer's protocol with minor modifications. Bisulfite treatment of DNA converts cytosine to uracil but leaves 5□mC intact. Thus, 5□mC patterns can be mapped by subsequent sequencing (Jones, 2012). The bsDNA samples were eluted using M-elution buffer in 46ul and amplified using 4 separate multiplex or simplex PCRs included 0.5 units of Qiagen HotStarTaq (Qiagen, MD, Catalogue number 203205), 0.2µM primers, and 2 µl of bsDNA in a 20 µl reaction. All PCR products were verified and quantified using the QIAxcel Advanced System (Qiagen, Germany). Prior to library preparation, PCR products from the same sample were pooled and purified using QIAquick PCR Purification Kit columns (Qiagen, MD, USA).

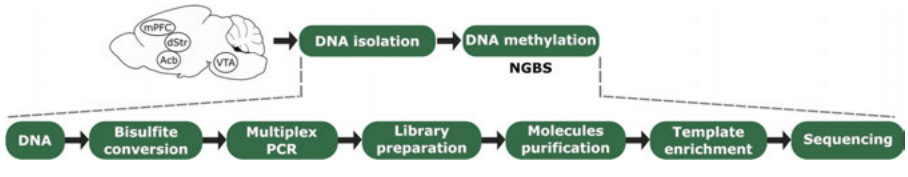

Figure 13. Schematic representation of DNA methylation analyses using next generation bisulfite sequencing (NGBS). Acb: nucleus accumbens; dStr: dorsal striatum; VTA: ventral tegmental area; mPFC: medial prefrontal cortex

Libraries were prepared using a custom Library Preparation method created by EpigenDx Inc (Hopkinton, MA, USA). Next, library molecules were purified using Agencourt AMPure XP beads (Beckman Coulter Inc., CA, USA) and quantified using the Qiagen QIAxcel Advanced System (Qiagen, Germany). Barcoded samples were then pooled in an equimolar fashion before template preparation and enrichment were performed on the Ion Chef™ system (Thermo Fisher Scientific Inc., MA, USA) using Ion 520™ & Ion 530™ ExT Chef reagents. Following this, enriched, template-positive library molecules were then sequenced on the Ion S5™ sequencer using an Ion 530™ sequencing chip (Thermo Fisher Scientific Inc., MA, USA).

FASTQ files from the Ion Torrent S5 server were aligned to the local reference database using open-source Bismark Bisulfite Read Mapper with the

Bowtie2 alignment algorithm (https://www.bioinformatics.babraham.ac.uk/projects/bismark/) (Krueger and Andrews, 2011). Methylation levels were calculated in Bismark by dividing the number of methylated reads by the total number of reads.

Lastly, potential transcription factor binding sites (TFBS) at each CpG were assessed using ALGGEN PROMO (Farré et al., 2003, Messeguer et al., 2002) which predicts TFBSs using TRANSFAC database version 8 (http://alggen.lsi.upc.es/cgi-in/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3)

Statistical analyses

Shapiro-Wilk (Paper I - IV)

The distribution of gene expression (Paper I, III), DNA methylation data (Paper II), and AUDIT/AUDIT-C score (Paper IV) was verified using the Shapiro-Wilk test, which computes a similarity percentage between the observed and the normal distribution and then the probability of finding this observed -or a smaller- similarity percentage. For not normally distributed data ($p < 0.05$), non-parametric tests were applied.

Modified Levene's test (Paper II)

The methylation levels of *Vglut1-2* CpGs (VTA) were assessed for homogeneity of variances using the modified Levene's test, which computes the absolute differences between all scores and the group median. The modified Levene's test is considered more robust for not normally distributed data as compared to the classic Levene's test, which is based on means and *F* statistics (Parra-Frutos, 2009).

Generalized Linear Models (Paper I, II and IV)

Generalized Linear Models are a large class of models that includes the Univariate General Linear Model (GLM), but expands it by allowing the outcome variable to have non-normal distribution and also to be linearly related to the factors and covariates via a specified link function. The GLM test, two-way ANOVA with type III sum of squares was used to study the interaction effects between ELS and alcohol drinking on gene expression (Paper I) and DNA methylation in MS rats (Paper II), and the interaction between *rs2290045*, maltreatment and parenting on AUDIT/AUDIT-C score (Paper IV), even though the data were not normally distributed, as it is considered robust to violations of normality (Glass et al., 1972). GLM implements both regression and analysis of variance on a dependent continuous variable, having as independent predictors one or more factors, of categorical nature (fixed factors) or

continuous (covariates). With this test (type III sum of squares) it is possible to investigate the interaction effect on the outcome variable, after adjusting for the main effect between the predictor factors. In Paper I, estimated marginal means were calculated (i.e. the mean response of the outcome variable (i.e. gene expression) for each factor, adjusted for any other variables in the model) and used to generate GLM plots for visualization of the interaction directions. In Paper IV, apart from the GLM, the Negative Binomial (NB) model with log link function was applied to compare the results obtained with GLM. The NB was chosen as the dependent variable (AUDIT/AUDIT-C) consisted of count data (i.e. a type of data in which the observations can take only the non-negative integer values, and where these integers arise from counting rather than ranking). The NB was chosen over the Poisson model (another model for count data), as it can handle more efficiently over-dispersion (i.e. greater variability in a dataset than would be expected based on a given statistical model). The Maximum Likelihood Estimation (MLE) was used to estimate the dispersion parameter. In Paper IV, a p value < 0.10 was considered significant for interaction effects (Fleiss, 1999). Fit regression lines were used to depict the interaction between SNP *rs2290045* and negative and positive environmental factors on alcohol-related problems in T carriers and CC group separately.

Moderation analysis (Paper II, IV)

Moderation analysis attempts to answer the question of *when* an effect exists or in other words an *interaction*. Thus, moderation analysis determines whether the size or sign of the effect of one or more predictor variables [i.e. ELS and/or alcohol (Paper II) or maltreatment and parenting (Paper IV)] on the outcome variable [i.e. *Vglut* expression (Paper II) or AUDIT/AUDIT-C scores (Paper IV)] depends on another, moderator variable [i.e. *Vglut* DNA methylation (Paper II) or *rs2290045* genotype (Paper IV)]. In Paper II, moderated moderation analysis was performed to assess whether the interaction between rearing [MS15 vs. MS360 (ELS)] and drinking (water vs. alcohol) on *Vgluts* expression was dependent on *Vgluts* methylation. In Paper IV, moderated moderation analysis was performed to assess whether *VGLUT2 rs2290045* genotype would moderate the interaction of maltreatment x parenting on alcohol-related problems (measured as AUDIT/AUDIT-C scores). Moderation analysis was performed using SPSS PROCESS macro v2.16 (Hayes and Matthes, 2009). The Johnson-Neyman technique was used to probe the interaction and determine the region of significance (ROS) in the distribution of the moderator where the interaction between the two predictors on outcome variable is significant (Hayes and Matthes, 2009).

Mann-Whitney U tests (Paper I - IV) and independent t-test (Paper III)

Between-group differences in gene expression (Paper I), DNA methylation (Paper II) and continuous variables (AUDIT/AUDIT-C, family maltreatment in GP-Adolescents, parenting; Paper IV) were assessed using the Mann-Whitney U test, which is the non-parametric equivalent to independent t-test. Differences on percentage of *Th* and *Vglut2* neurons (Paper III) were assessed using the independent t-test and Mann-Whitney for not normally distributed variables. Mann-Whitney is used to compare differences between two independent groups on a non-normally distributed outcome variable depending on the data's distribution. When the data of the independent groups do not have the same shape of distribution, as in the present studies, the Mann-Whitney U test can be used to compare mean ranks. Whisker boxplots indicating the median and data distribution were used to visualize gene expression in each group in Paper I.

Kruskal Wallis H test (Paper I, II and III) and one-way ANOVA (Paper III)

The Kruskal Wallis H test is the non-parametric equivalent to one-way ANOVA (Analysis of Variance) and an extension of Mann-Whitney U test, as it allows for comparison between three or more independent groups. In Paper I, Kruskal Wallis was used to test differences between the three AFR groups (group-housed water-drinking rats, single-housed water- and alcohol-drinking rats) to discriminate between a possible effect of housing and the effect of alcohol on gene expression levels. In Paper II, Kruskal-Wallis was used to test for differences on DNA methylation between low, moderate and high MS360 drinking rats, as well as between MS15A and low (< one fold-change) and high (> one fold-change) *Vglut1-2* expressers in the MS360A group. In Paper III, one-way ANOVA was used to compare the area occupied by $Vglut2^{pos}$ cells between the groups followed by Tukey *post-hoc* tests and Kruskal-Wallis was used for not normally distributed variables.

Pearson chi-square test (Paper IV)

Pearson chi-square test is designed to analyze group differences in categorical variables, assessing the probability of independence of data distribution. That is, it compares the observed data to a theoretical distribution that assumes independency of the variables. In Paper IV, group differences in categorical variables (i.e. genotype, sex, maltreatment in GP-Adults and CS individuals, nicotine use) were tested using the Pearson chi-square test.

Spearman rank-order correlation test (Paper I, II and IV)

Correlation analyses were performed using the Spearman rank-order correlation test, which is the nonparametric equivalent of the Pearson correlation test. Spearman's correlation coefficient (ρ) measures the strength and direction of association between two ranked variables and it can range from '-1' to '+1'. A value of ρ equal to '0' indicates absence of any correlation, while a value lower than '0' and towards '-1', or a value higher than '0' and towards '+1' signifies a negative and positive correlation, respectively, between the two variables assessed. In Paper I, scatter plots were used for visualization of correlations.

Correction for multiple comparisons (Paper I and II)

Correction for multiple testing was done following the Bonferroni method, considering the number of tests performed region-wise (Armstrong, 2014). Accordingly, in Paper I, the level of accepted *p*-value within Acb and mPFC was ≤ 0.05; and within VTA and dStr was ≤ 0.017. For correlations among different regions between *Vgluts* expression and between *Vgluts* and DNA methylation genes, $p \leq 0.0014$ and $p \leq 0.0038$ was considered, respectively. For the correlation analyses between gene expression and alcohol intake during PNW15, $p \leq 0.003$ was accepted. In Paper II, a Bonferroni-adjusted *p*-value was calculated considering the number of tests performed gene-wise in the VTA; i.e. $p < 0.0008$ for *Vglut1* and $p < 0.0009$ for *Vglut2*. Nevertheless, considering the exploratory nature of the study, nominal (i.e. not surviving Bonferroni correction) associations were reported.

Wilcoxon signed-rank test (Paper IV)

Changes in alcohol-related problems over time in GP-Adolescents and CS individuals were tested using the Wilcoxon signed-rank test, which is the nonparametric equivalent to paired t-test and it compares the mean score of two related variables. The test is called the signed rank as it pools all differences, ranks them and applies a negative sign to all the ranks where the difference between the two observations is negative. It is very robust to outliers.

Covariates (Paper IV)

Following *Keller et al.* (Keller, 2014), covariate-by-genotype and covariate-by-environment (two- and three-level interaction terms) terms were added in the model to appropriately control for confounding effects. Sex was used as a covariate in Paper IV, except for the GP-Adults because males had been excluded from the analyses. In case the three-way interaction of interest (*rs2290045* x maltreatment x parenting) was not statistically significant, sex

was included in the interaction term (4-way interaction term) to test for interactions modified by sex; when significant 4-way interactions were observed, the relationship was further investigated separately by sex. Considering the high comorbidity and cross-transmission between nicotine use and alcohol misuse (Goldman et al., 2005), the former was taken into account as potential confounder in separate analyses. In the CS, the analysis at follow-up was adjusted for maltreatment at baseline.

Other variables such as family history for AUD, co-morbidity with other disorders, or co-use of other drugs were not taken into account. That was partly because such information was not available for all the studied samples but also to minimize over-fitting of the model.

Results and Discussion

The effect of ELS and alcohol consumption on *Vglut1-3* expression (Paper I) and methylation (Paper II)

To date, the studies presented here are the first to investigate the relationship between ELS and alcohol on *Vgluts* expression and DNA methylation, while only a handful of studies has focused separately on ELS- or alcohol-related effects on VGLUTs. Rats were sacrificed at a time point when differences in drinking patterns just started to emerge to assess gene expression, and consequently DNA methylation levels, at this specific time-point of transition towards potential escalation of alcohol consumption. Indeed, the median (min, max) alcohol intake (g/kg/2h) in the different groups was similar: MS360: 1.32 (0.60, 2.05); MS15: 1.32 (0.39, 1.77); AFR: 1.18 (0.62, 1.66), likely because the duration of alcohol-drinking paradigm was too short to observe such differences (Nylander and Roman, 2013). Yet, in line with previous evidence of "responders" and "non-responders" to alcohol intake (Gustafsson and Nylander, 2006), three subgroups emerged within the MS360A group with low (< 1g/kg/2h), moderate (1 – 1.5g/kg/2h) and high (>1.5g/kg/2h) alcohol-drinking. These subgroups did not differ in their gene expression levels; nonetheless differences in single CpG-methylation of *Vglut1* (VTA) were observed among the subgroups as well as some correlations with *Vgluts* expression and alcohol intake.

Region-specific interaction effects between ELS and voluntary alcohol consumption were observed on *Vgluts, Dnmt1* and *Mecp2* expression. More specifically, expression of *Vglut2* was affected in the VTA, *Vglut3, Dnmt1* and *Mecp2* in the Acb, and *Vglut1* in the dStr. Separately, ELS or alcohol affected *Vglut1* expression in the VTA, *Vglut2* expression in the mPFC and *Dnmt1* expression in the VTA and dStr (Figure 14-15). The findings regarding *Vgluts'* expression were followed-up by assessing whether *Vglut1-3* DNA methylation at single CpGs was affected as well by ELS and/or alcohol and whether it correlated with the abovementioned transcriptional differences. *Vgluts* methylation varied in a gene- or region-dependent way, whereas there was not a clear association depending on the gene targeted regulatory region (i.e. promoter or gene body). The effects were also reflected in correlations between gene expression and methylation, but also between methylation with corticosterone levels and alcohol consumption.

Overall, we found altered *Vglut1-3* expression, concomitant with differential (< 10%) CpG-specific methylation in the VTA, Acb, dStr and mPFC (Paper II). Largely, ELS- or alcohol-only were associated with higher *Vglut1* CpG-specific methylation in the dStr, but with lower *Vglut2* CpG-specific methylation in the mPFC. Both factors however had bidirectional effects on *Vglut1-2* methylation in both regions. Different to those patterns, each factor alone (ELS- or alcohol-only) had mixed effects on *Vglut3* CpG methylation in the Acb. The same CpGs were affected (some were hyper- and others hypo-methylated) by either factor alone, while combination of the factors seemed to reverse these effects. Lastly, in the VTA, spanning almost all targeted regions, ELS-alone had a mixed effect on *Vglut2* CpG methylation, while alcohol-alone was associated with largely higher *Vglut2* CpG methylation. Furthermore, ELS- or alcohol-only were associated with both hypo- and hyper-methylation of *Vglut1* promoter and gene body methylation. However, in both *Vglut1* and *2,* ELS in alcohol-drinking rats (MS360A vs. MS15A) was associated with hypo-methylation of *Vglut1* gene body and *Vglut2* promoter and gene body (Figure 16).

The moderating effect of methylation on expression was also assessed to explore the potential regulatory role of methylation on gene expression in different early-life and drinking conditions. Although no significant moderating effect was observed for any CpG on neither *Vglut1* or *Vglut2* expression, for the latter a region-of-significance emerged. This had a specific range, for most of the sites around the mean methylation, where the effect of ELS and alcohol was opposite in MS15 and MS360 rats. Such interesting pattern was noted only for *Vglut2,* but not for *Vglut1*. It is highly probable that the three-way interaction did not reach significance due to the small sample size (i.e. 50 animals). It nevertheless hints towards an interesting pattern in the relationship between *Vglut2* methylation and expression in response to alcohol consumption in different early-life conditions in a small subgroup. These observations support the heterogeneity and inter-individual differences commonly seen in ELS- (Baram et al., 2012) and alcohol-drinking models (Palm et al., 2011). A more detailed discussion of the results by gene is presented below.

The *Vglut2* role in ELS and alcohol outcomes

The relevance of VGLUT2 to addiction has been highlighted, mainly by preclinical studies (El Mestikawy et al., 2011, Bimpisidis and Wallén-Mackenzie, 2019). Studies on alcohol (Zhou et al., 2006, McBride et al., 2013, McClintick et al., 2015, Comasco et al., 2014) or ELS (Martisova et al., 2012) separately have been scarce, and even less research has been done on epigenetic modifications in relation to those (Zhang et al., 2015). The present thesis investigated for the first time the effect of both ELS and alcohol on *Vglut2* expression and CpG-specific methylation in outbred, non-dependent male rats. In the VTA and mPFC, rats exposed to ELS (MS360W) displayed lower gene expression

compared to controls (MS15W) (Figure 14). This finding is in contrast with a previous study where MS180 rats displayed higher VGLUT2 hippocampal protein expression (Martisova et al., 2012). The discrepancy can reflect different effects of MS exposure (Nylander and Roman, 2013) or most likely region-dependent effects. ELS in the mPFC was associated with two- to three-fold lower *Vglut2* DNA methylation of intron 2 and 3. On the contrary, CpG59 methylation in intron 2 was higher in the same group not only in the mPFC, but also in the VTA. The Upstream Transcription Factor (USF)-1 is predicted to bind to CpG59; higher methylation of this site could lead to decreased TF binding and in turn resulting to lower *Vglut2* expression observed between MS360W and MS15W in both regions (Figure 14). Between the same groups in the VTA, higher methylation was observed also at CpG-36 (5'-upstream) and CpG112 (intron 3), but lower at CpG2 (exon 1) and CpG110 (intron 3) (Figure 15). Notably, the direction of the effects was not homogeneous in regards to genomic location (i.e. promoter or gene body). Although methylation in the promoter is largely associated with silencing whilst methylation in the gene body is associated with transactivation (Jones, 2012), herein, both hyper- and hypo-methylation of gene body (intron 2, 3) as well as around promoter region (5'-upstream, exon 1) was observed, highlighting specific CpG methylation signatures of ELS and hinting towards a dynamic epigenetic repertoire on the single CpG level. Subtle changes (less than 10% or even 1-5%) in methylation of single CpGs have been suggested to occur as a result of external environmental factors and being a hallmark of complex diseases, associated with their onset as well as play a role in a large diversity of phenotypes (Leenen et al., 2016).

Voluntary alcohol drinking was associated with lower *Vglut2* expression in the mPFC of MS15 rats (Figure 14). In line, alcohol consumption in adolescent alcohol-preferring rats resulted in lower *Vglut2* expression in the dorsal raphe nucleus (McClintick et al., 2015). On the contrary, higher *Vglut2* expression was found in the Acb shell of alcohol-preferring rats compared to the alcohol-non preferring counterparts (McBride et al., 2013), as well as higher number of VGLUT2-expressing terminals upon repeated alcohol deprivation (Zhou et al., 2006). Furthermore, prenatal alcohol exposure was associated with adult *Vglut2* up-regulation in the murine hippocampus of males accompanied by decreased DNA methylation of the promoter *Vglut2*, which was even more pronounced in mice with the highest *Vglut2* expression (Zhang et al., 2015). Corroborating these findings, in the present study, high *Vglut2* expressers in the MS360A group had also lower methylation of 3 CpGs in the promoter and exon 1 of *Vglut2,* but higher methylation of CpG113 within intron 3 as compared to low *Vglut2* expressers. Methylation of exon 1, similar to promoter methylation, is associated with gene silencing (Brenet et al., 2011), while methylation in gene body has been associated with higher gene expression (Jones, 2012). Indeed, voluntary alcohol drinking here was largely associated with lower CpG-specific methylation especially within intron 3 in

the mPFC. This observed hypomethylation could explain the lower *Vglut2* expression in MS15A. In the pituitary and hypothalamus of the same rats, alcohol drinking was also associated with lower CpG-specific methylation but in the promoter of the stress-related genes proopiomelanocortin (*Pomc*), arginine vasopressin (*Avp*) and FK506 Binding Protein 5 (*Fkbp5*) (Todkar et al., 2015). In line with these findings, although measured in blood, a recent EWAS in humans showed greater alcohol intake to be associated with lower global DNA methylation (Dugué et al., 2019). The same study found an association between alcohol intake and one CpG within the *VGLUT2* promoter. In contrast to that EWAS, in rodents chronic alcohol exposure has been associated with global DNA hyper-methylation in various brain regions, although acute alcohol intake might have the opposite effect leading to global DNA hypomethylation (Berkel and Pandey, 2017).

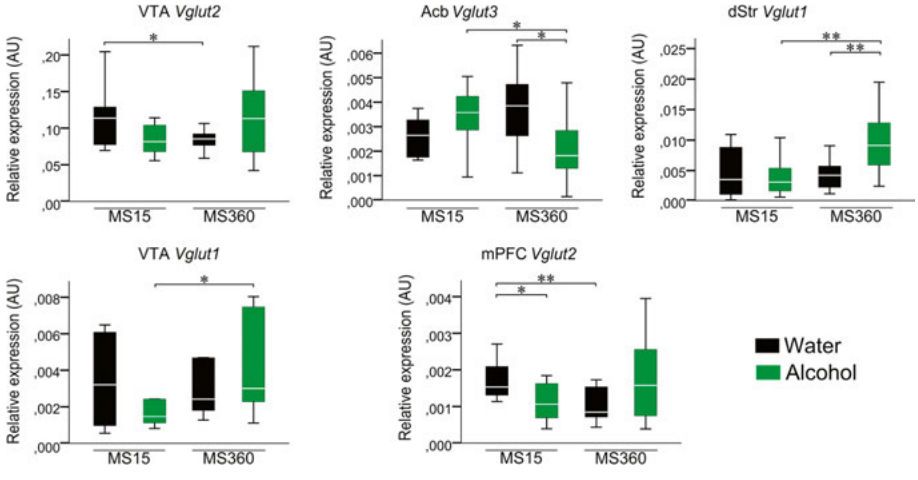

Figure 14. Between-group differences on relative *Vglut1-3* expression.
Acb: nucleus accumbens, AU: arbitrary units, dStr: dorsal striatum, mPFC: medial prefrontal cortex, MS: Maternal Separation, *Vglut*: vesicular glutamate transporter, VTA: ventral tegmental area. (*: $p \leq 0.05$; **: $p \leq 0.01$)

In regards to both ELS and alcohol, there was an interactive effect in the VTA where both factors were associated with higher *Vglut2* expression (Figure 14). *Vglut2* methylation was affected in a specular manner. Relative methylation at single CpGs was 19-50% lower within 5'-upstream, and intron 1-3 in the MS360A rats compared to MS15A or MS360W (Figure 15). The interactive effect of ELS and alcohol on *Vglut2* had never been assessed before. Evidence from the present study shows that *Vglut2* expression is sensitive to voluntary alcohol drinking depending on early-life rearing conditions. The effect emerged already in the initial stage of transition from voluntary to more ha-

bitual alcohol consumption and is accompanied by changes in DNA methylation as well, complementing previous research in the field (Szyf et al., 2016, Zhang and Gelernter, 2017).

The *Vglut1* role in ELS and alcohol outcomes

Vglut1 has also been linked to reward and addiction (Truitt et al., 2014, McClintick et al., 2015). An interactive effect of ELS with alcohol was observed on *Vglut1* expression in the dStr; rats exposed to both factors (MS360A) displayed higher gene expression compared to rats exposed to either stressor alone (MS15A or MS360W) (Figure 14). *Vglut1* methylation of intron 9 and exon 12 in the dStr was lower in 20% and higher in 13% of the analyzed CpGs in MS360A compared to the rest of the groups. In the VTA of the same group (MS360A), *Vglut1* expression was also higher compared to MS15A rats (Figure 14), accompanied by lower methylation of 3 CpGs within exon 9 and 12 (Figure 15). *In silico* analysis identified potential binding site for CCAAT/enhancer binding protein delta (CEBPD) at one of these sites, CpG198. Increased CEBPD in the brain has been associated with chronic ethanol consumption (Erickson et al., 2019) as well as higher corticosterone levels (MacDougald et al., 1994). It is plausible that lower methylation of CpG 198 facilitates CEBPD binding and in turn contributes to higher *Vglut1* expression. In line with this increase, in alcohol-preferring rats, adolescent alcohol consumption led to higher *Vglut1* expression in the dorsal raphe nucleus (McClintick et al., 2015), while acute alcohol and nicotine co-administration in the posterior VTA resulted in higher *Vglut1* expression in the Acb shell (Truitt et al., 2014). In the amygdala and the superior frontal cortex of human post-mortem brains of alcoholics, *VGLUT1* expression was also upregulated (Ponomarev et al., 2012).

Although an interaction effect between ELS and alcohol was not present on *Vglut1* expression in the VTA, nominal interaction effects were observed on *Vglut1* methylation within 5'-upstream, intron 9 and exon 12. ELS in alcohol-drinking rats was associated with 2 – 7% lower methylation of 3 CpGs in intron 9 and exon 12, but with 4 – 12% higher methylation in water-drinking counterparts. At 5'-upstream, ELS and alcohol affected in opposite direction two CpGs and the relative hyper- or hypo-methylation seemed pronounced due to their general un-methylated status. For instance, ELS in alcohol-drinking rats was associated with 90% lower methylation of one of these sites, but with 250% higher methylation in water-drinking counterparts. For CpG-59, ELS in water-drinking rats was associated with 34% lower methylation, while alcohol-drinking counterparts were not that affected. Although measured in blood, a recent EWAS in humans, found an association between alcohol intake and one CpG within the body of *VGLUT1* (Dugué et al., 2019), while a study on hippocampal post-mortem tissue of alcohol abusers revealed differences in

histone methylation and gene expression networks involving *VGLUT1* compared to healthy controls (Farris et al., 2015).

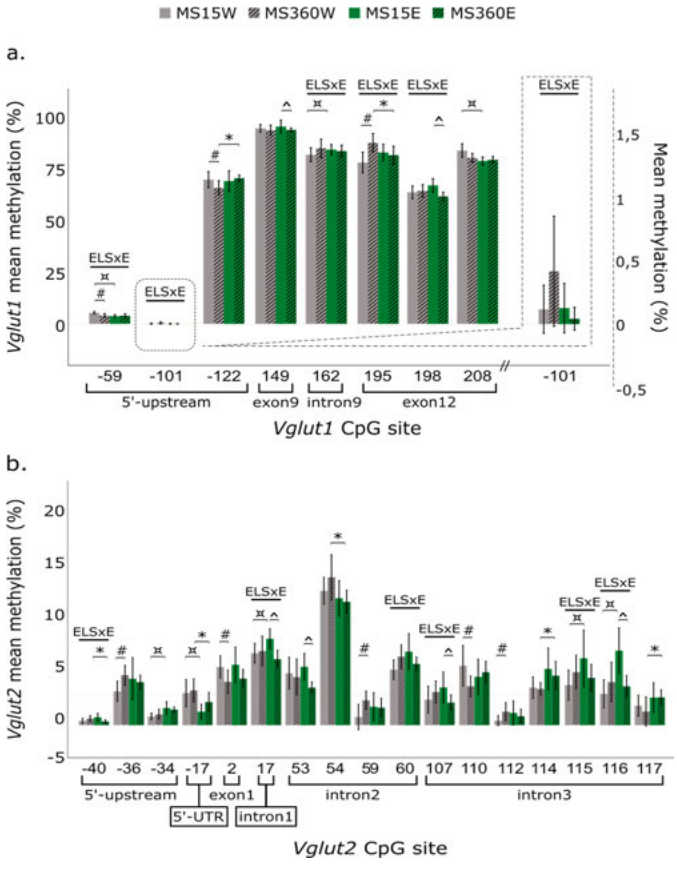

Figure 15. Interactive effects of ELS and/or alcohol and between-group differences on mean *Vglut1 and 2* CpG methylation (%) in the VTA; Error bars: 95% confidence intervals; ELS: early life stress; E: ethanol; MS: maternal separation; W: water; * MS360A vs. MS360W; # for MS360W vs. MS15W; ¤ MS15A vs. MS15W; ^ MS360A vs. MS15A; $p \leq 0.05$

The *Vglut3* role in ELS and alcohol outcomes

VGLUT3 has been associated to ELS and addiction-related phenotypes as well. Male rat pups lacking *Vglut3* and exposed to ELS showed lower stress reactivity and enhanced anxiety-like behavior (Balázsfi et al., 2018), adding up to previous research that had linked *Vglut3* deletion to anxiety-like phenotype in mice (Amilhon et al., 2010). Furthermore, *Vglut3* KO mice have been more responsive to cocaine compared to their wild type counterparts (Gras et al., 2008, Sakae et al., 2015) and frequency of rare variations of *VGLUT3* was higher in a cohort of severe cocaine or opiate abusers (Sakae et al., 2015). The

present study provides evidence for the first time of an interaction between ELS and alcohol in the Acb; rats exposed to both factors (MS360A) displayed lower *Vglut3* expression than rats exposed to either alcohol (MS15A) or ELS (MS360W) alone (Figure 14). Indeed, ELS was associated with higher accumbal *Vglut3* expression in water-drinking rats but with lower expression in alcohol-drinking counterparts (MS360A vs. MS15A). Furthermore, alcohol consumption in ELS animals resulted in lower *Vglut3* expression (MS360A vs. MS360W). In line, alcohol consumption in adolescent alcohol-preferring rats resulted in lower *Vglut3* expression in the dorsal raphe nucleus (McClintick et al., 2015). A dorsal raphe-VGLUT3 pathway to the VTA has been found with the majority of these neurons to be glutamatergic (VGLUT3-expressing) only. In the VTA, these neurons create synapses with mainly dopaminergic neurons, some of which innervate the Acb (Qi et al., 2014), linking this pathway to reward and motivation. Though the involvement of cholinergic neurotransmission could be hypothesized, as the majority of VGLUT3-expressing neurons in the Acb co-express acetylcholine (Gras et al., 2008), the aforementioned pathway suggests a more complex interplay among the different circuits relative to alcohol-rewarding effects.

The lower *Vglut3* expression in MS360A (compared to both MS15A and MS360W) was accompanied by 6-15% higher methylation within 5'-upstream, 5'-UTR, intron 1, 2 and 10 as well as 5-11% lower methylation within 5'-upstream and 5' UTR. Research on *Nr3c1* promoter has shown that small changes in methylation throughout the promoter and 5'-UTR can impact gene expression via mechanisms such as alternative splicing and transcriptional microvariability, which refers to the permissivity of transcriptional initiation based on differential use of available TSSs (Leenen et al., 2016). Furthermore, hyper-methylation of more than two-fold was observed at one of these sites (CpG316) in intron 2, in MS360A compared to MS15A. Potential binding of USF-2 and cAMP Responsive Element-Binding Protein (CREB) was also predicted at this site by *in silico* analysis. The CREB pathway has been implicated in addiction; especially in the Acb, chronic exposure to various drugs of abuse leads to activation of CREB which in turn decreases the reinforcing value of drug reward and may contribute to the emergence of withdrawal/negative effect state (Koob and Volkow, 2016). Upon chronic alcohol use CREB has been suggested to modulate connectivity and synaptic plasticity via regulation of brain-derived neurotrophic factor and activity-regulated cytoskeleton-associated protein expression (Berkel and Pandey, 2017). The present findings may add a novel small piece of knowledge in these neuro-adaptations, suggesting an accumbal *Vglut3*-mediated role.

The *Dnmt1* and *Mecp2* role in ELS and alcohol outcomes

DNA methylation potentially mediates the effect of ELS on later psychopathology (Maccari et al., 2014), including AUD (Pucci et al., 2019). In both

rodents and humans, alcohol exposure has been associated with differential DNA methylation in blood and various brain regions (Berkel and Pandey, 2017, Zhang and Gelernter, 2017, Zhang et al., 2015, Zhang et al., 2013, Dugué et al., 2019). Two regulatory genes of the DNA methylation machinery are *Dnmt1* and *Mecp2* (Moore et al., 2013) and have both been implicated in ELS and addiction-related behaviors. *Lewis et al.,* using an MS paradigm, found increased methamphetamine intake in ELS rats (i.e. exposed to MS180) that was accompanied by decreased MECP2-immunoreactivity in the Acb core compared to MS15 rats (Lewis et al., 2013). In the present study (Paper I), ELS alone did not have an impact on drug (i.e. alcohol) consumption, but similarly to *Lewis et al*, ELS in alcohol-drinking rats (MS360A) was associated with lower *Mecp2* expression in the Acb compared to the control (MS15A) rats. Furthermore, *Mecp2* expression was lower upon alcohol consumption in ELS rats (MS360A vs. MS360W) (Figure 16). Another study has shown that local knock-down of MECP2 in the Acb of adult alcohol-preferring mice enhanced amphetamine-induced reward, a finding that was inverted by MECP2 overexpression (Deng et al., 2010). Moreover, differentially expressed MECP2 has been observed in the murine PFC and Acb shell in an alcohol-dependence model during protracted withdrawal (Repunte-Canonigo et al., 2013). In the same study, MECP2 was found to contribute to alcohol sensitivity and intake via regulation of alcohol-related genes. Lastly, the effect of ELS-only on *Mecp2* expression was assessed in the mPFC of male rats; it was reduced in ELS rats in adolescence (PND 30), and adulthood (PND 90), but not right after exposure (PND 8) (Blaze and Roth, 2013). Notably, in that study, ELS was modeled by a different paradigm from ours. During PND 1 – 7, pups were exposed daily for 30 minutes to a non-biological, stressed (due to limited nesting material) dam. *Dnmt1* expression was also assessed in the mPFC; following this maltreatment condition, reduced *Dnmt1* expression was observed in ELS rats only in adulthood, and not in PND 8 or during adolescence (PND 30) (Blaze and Roth, 2013). Contrary to that study, herein, main or interaction effects on *Dnmt1* or *Mecp2* expression were not observed in the mPFC, nevertheless, the methodological differences in ELS paradigm should be taken into account.

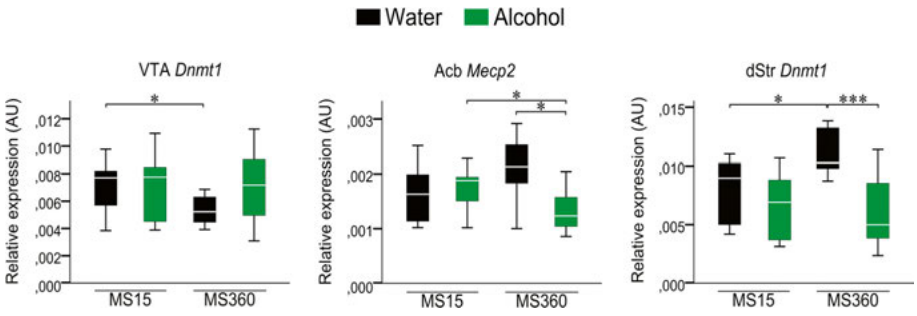

Figure 16. Between-group differences on relative *Dnmt1* and *Mecp2* expression. Acb: nucleus accumbens, AU: arbitrary units, dStr: dorsal striatum, Dnmt1: DNA methyltransferase 1, Mecp2: Methyl-CpG-binding protein 2, MS: Maternal Separation, VTA: ventral tegmental area. (*: $p \leq 0.05$; ***: $p \leq 0.001$)

Another study, focused on the effect of ELS-only, found instead higher adult *Dnmt1* expression in the murine hippocampus of MS180 rats compared to AFR (Boku et al., 2015). Region-dependent differences were observed in Paper I as well; rats exposed to ELS (MS360W) displayed lower *Dnmt1* expression in the VTA but higher in the dStr compared to control (MS15W) rats (Figure 16), suggesting long-term effects of ELS on the expression of this key regulator of DNA methylation maintenance (Moore et al., 2013), corroborating previous research, although substantial methodological differences between those and our studies should be considered, i.e. ELS model (Blaze and Roth, 2013) and selection of the control group (Boku et al., 2015).

To date, there are no studies investigating the interactive effect of ELS and alcohol on DNMT1. In the present study (Paper I), voluntary alcohol consumption in ELS rats was associated with lower *Dnmt1* expression in the Acb, and in the dStr of MS360 but not MS15 rats (Figure 16). *DNMT1* downregulation as a result of chronic alcohol consumption has been demonstrated in the amygdala and the superior frontal cortex of post-mortem brain of alcoholics (Ponomarev et al., 2012). Contrary to that, *Warnault et al.* found higher *Dnmt1* expression in the Acb of C57Bl/6J mice upon excessive alcohol drinking (Warnault et al., 2013). Yet, though both studies show an alcohol-induced effect on *Dnmt1* expression, they did not consider ELS, although one could expect that a portion of alcoholics included in the study of Ponomarev et al., 2012 would have experienced some form of ELS, as individuals with alcohol dependence typically experience more negative, and fewer positive, environmental factors, than their healthy peers (Dube et al., 2002).

The effect of ELS and alcohol consumption on correlations between gene expression, methylation, corticosterone and alcohol intake

Overall, correlations reflected the ELS x alcohol effects. In presence of both, they were largely disrupted; this was observed region-wise and gene-wise (i.e. correlations were disrupted in affected regions or between affected genes) or group-wise (correlations were prominent in all experimental groups, but sparser in the rats exposed to both ELS and alcohol), suggesting that the molecular mechanisms regulating gene expression and DNA methylation are potentially different in the presence of both aversive environmental factors as compared to only one or none.

ELS and alcohol consumption effect on correlations between gene expression and methylation

Correlational analyses by experimental group indicated a positive relationship between the investigated genes' expression in all regions except for the dStr, and reflected the observed interactive effects between alcohol and ELS (Paper I). Correlations between *Vgluts* (mostly between *Vglut1* and *Vglut2*) were observed mainly in the Acb, where *Vglut1* and *Vglut2* expression was not disturbed by ELS and/or alcohol. On the contrary, *Vglut3* expression was positively correlated with the expression of both *Dnmt1* and *Mecp2* only in the MS360A rats, where the expression of all these three genes was downregulated by ELS and alcohol interaction (Paper I). We hypothesized that lower *Dnmt1* could contribute to lower methylation of specific CpGs in the MS360A group. Indeed, in the Acb of MS360A rats, CpG-specific *Vglut3* methylation was lower in the majority of targeted CpGs within the 5'-upstream, but higher in most of analyzed CpGs within 5'-UTR, intron 1 and 2 (Paper II). The DNA methylation genes, *Dnmt1* and *Mecp2* were positively correlated in the mPFC in all groups. This relationship could not be explored in the VTA and the dStr, where *Mecp2* was not assessed, due to methodological reasons (Paper I). Positive correlations between the DNA methylation genes and the *Vgluts* were mainly noted in the VTA and the Acb (Paper I). In the VTA, lower *Dnmt1* in MS360W rats may have contributed to the hypo-methylation of *Vglut2* CpG -37, and 110, although methylation of these sites did not correlate with *Dnmt1* expression (Paper II). Surprisingly in the dStr, where the direction of the interactive effect of ELS and alcohol on *Dnmt1* was in the opposite direction than for *Vglut1*, no correlations were observed between the two genes (Paper I). Nevertheless, and similarly to the Acb, lower *Dnmt1* expression was hypothesized to contribute to hypo-methylation of *Vglut1* CpGs in the MS360A group. Indeed, almost 25% of the successfully analyzed CpGs, mainly within intron 9 and 12 of *Vglut1,* had lower (> 5%) methylation in MS360A rats, but another 17% of CpGs were hyper-methylated (Paper II).

Vglut1-2 expression was correlated with CpG-specific methylation of each gene in all groups (Paper II). The correlations were high and bidirectional in all groups, but not in the MS360A; in these rats, moderate unilateral correlations were observed instead. That is, *Vglut1* expression was positively correlated with CpG-methylation in the gene body, while *Vglut2* expression was negatively correlated with CpG-methylation in the promoter.

The effect of ELS and alcohol consumption on correlations between gene expression, methylation and corticosterone levels

Blood corticosterone levels were not correlated with gene expression, except for *Mecp2* in the Acb, for which a positive high correlation (r = 0.95) was noted in MS15A group only (Paper I). However, correlations between blood corticosterone levels and *Vglut1-2* DNA methylation in the VTA were present in all groups. Both negative and positive high correlations were observed, but in the MS360A group correlations were moderate (Paper II).

Blood corticosterone levels of water-drinking rats exposed to ELS (MS360W) in the present studies (Paper I, II) were higher compared to alcohol-drinking counterparts (MS360A), but not when compared to MS15W rats (Bendre et al., 2015). In line, expression of the glucocorticoid-receptor gene *(Nr3c1)*, was not affected by ELS in the animals used in the present studies (Paper I, II) in any brain region (Todkar et al., 2015). Interestingly, the lower corticosterone levels of MS360A rats compared to MS360W but also to MS15A possibly reflect stress-alleviating effects of alcohol, although *Nr3c1* expression in MS360A rats did not differ from the controls (Todkar et al., 2015). Yet, even more supporting for such effect, although not significantly different, MS360A high-drinkers had the lowest corticosterone levels (mean: 63.4 ng/ml), followed by moderate-drinkers (mean: 77 ng/ml), while corticosterone levels were highest in low-drinking MS360A rats (mean: 91.7 ng/ml). The seminal studies of *Weaver et al.* and *McGowan et al.* had shown already over a decade ago that differences in maternal care in rats and humans have the potential to alter HPA-axis response to stress via differential methylation of the *Nr3c1* promoter (Weaver et al., 2004, McGowan et al., 2009), although an effect on *Nr3c1* promoter methylation was not observed after a MS paradigm by another study (Daniels et al., 2009). Similarly, in the pituitary and hypothalamus of the present rats, ELS did not have any effect on *Nr3c1* promoter methylation, nor did alcohol (Todkar et al., 2015). Alcohol drinking was instead associated with lower CpG-specific methylation in the promoter of the stress-related genes *Pomc*, *Avp* and *Fkbp5* (Todkar et al., 2015).

The effect of ELS and alcohol consumption on correlations between gene expression, methylation and alcohol intake

Alcohol intake levels during PNW 15 were considered for correlation analyses with gene expression and DNA methylation levels. PNW 15, and not the last week before decapitation (PNW16), was selected to avoid a possible confounding effect on alcohol consumption due to higher stress levels during the last week. Negative nominal correlations were observed between alcohol intake and expression of *Dnmt1* and *Mecp2* in the Acb of high-drinking MS360 rats (the 'responder' subgroup of MS360A, which displayed escalated alcohol consumption over time; >1.5g/kg/2h during PNW 15) (Paper I). This finding could hint towards a *Dnmt1*- and *Mecp2*-involvement in escalating drinking patterns of 'responder' MS360 rats. This hypothesis could not be tested by examining correlations between alcohol intake and DNA methylation of that group in the Acb, as in that region, DNA methylation of pooled samples was assessed (Paper II). However, in the VTA, high alcohol intake was positively correlated with *Vglut2* promoter methylation in MS360A rats, whereas low alcohol intake was negatively correlated with *Vglut2* promoter and intron 1 CpG-methylation. Furthermore, *Vglut1* exon 12 methylation was positively correlated with alcohol consumption in low- and moderate-drinking MS360A, but not in the high MS360A drinkers, where this correlation was negative. Correlations of opposite nature between alcohol intake and methylation of the same CpGs in exons 9 and 12 were observed in MS15A (negative correlations) and high MS360A drinkers (positive correlations) (Paper II). These findings suggest DNA methylation markers of different alcohol doses in the VTA; yet whether these signatures precede or follow alcohol consumption remains to be clarified.

The effect of single-housing on gene expression

Single-housing of animals in voluntary alcohol-drinking paradigms is a common practice for individual liquid measurements, but can represent an extra stressful factor considering that rats are social animals (Palm and Nylander, 2014). Age-dependent differences in alcohol consumption have been shown between group- versus single-housed AFR rats; single-housed rats consume more alcohol compared to their group-housed counterparts when housing isolation is occurring during weaning, but no differences in alcohol intake were seen between the groups when the isolation is performed in adulthood (Schenk et al., 1990). In the Paper I, single-housing in adulthood did not confound alcohol-drinking effects on *Vglut1-3, Dnmt1* and *Mecp2* expression in AFR rats. However, the single-housing effect in adulthood was not assessed in the MS rats. Animals previously exposed to ELS can be more sensitive to stressors later in life (Murthy and Gould, 2018), so even though single-housing did not

affect AFR animals, this finding could not discard a potential effect in MS rats, thus further studies are needed to confirm or discard this hypothesis.

The effect of alcohol and/or nicotine exposure on *Vglut2* expression (Paper III)

Concurrent use of alcohol and nicotine is high (Goldman et al., 2005), even during the adolescent years (Kraus, 2015). Few studies have focused on the effect of both drugs on *Vglut2/VGLUT2* (Truitt et al., 2014, Flatscher-Bader et al., 2008). Only one of those found an effect; in post-mortem human brains *VGLUT2* expression in the VTA was higher among alcoholic smokers, and even higher among smokers (Flatscher-Bader et al., 2008). Following the same direction, it is demonstrated here for the first time, that young adult rats exposed to prolonged episodic adolescent alcohol and nicotine, have higher *Vglut2*-expression in the PBP of the VTA compared to controls (Figure 17A).

It has been suggested that only a small proportion of neurons within a brain region could be responsible for addiction-related behaviors (Leão et al., 2015) and that individual VTA *Vglut2* subpopulations could play a unique role in drug addiction (Morales and Root, 2014). Thus, we further investigated the contribution of the VTA *Vglut2*-subpolations (Figure 17C) to the abovementioned difference in *Vglut2* expression and found that, although not statistically significant, it was driven by higher ratio of $Vglut2^{pos}/Th^{neg}$ neurons. As shown in Figure 17B, *Vglut2* mRNA level, though not statistically significant, was higher in all drug treated groups (alcohol: mean = 2.13; SD = 0.92; nicotine: mean = 2.21; SD = 0.86; alcohol and nicotine: mean = 1.92; SD = 1.02) compared to the control group (mean = 1.55; SD = 0.70). On the contrary, the percentage of $Vglut2^{pos}/Th^{pos}$ neurons and $Vglut2^{neg}/Th^{pos}$ was lower in co-exposed group compared to the control. Moreover, in the alcohol group there was a strong trend towards higher percentage of $Vglut2^{pos}/Th^{neg}$ neurons in the PBP, and towards a lower percentage of $Vglut2^{pos}/Th^{pos}$ neurons in the VTAR. This effect might have been missed by CISH analysis, owing to the high amount of animals excluded from alcohol group due to prior mis-estimation of bregma co-ordinates. Although, not such an effect of alcohol alone was seen in the VTA of MS15 rats in Paper I, and in fact lower instead of higher *Vglut2* expression was observed in the mPFC upon alcohol only, one should consider the methodological differences in alcohol exposure between the two studies, as well as that the rats in Paper III were AFR. Differences in early experience could have long lasting effects even among the widely used standard control condition. Indeed, MS15 has been associated with reduced VGLUT2 expression in the paraventricular hypothalamus of rats (assessed in PND9), although this change did not persist into late adolescence (PND45)

(Korosi et al., 2010). Nevertheless, such findings further highlight the influence of early life environment on neuroplasticity and its implications for mental health (Baram et al., 2012, Levine, 1967, Meaney, 2001), the importance of the control group (Nylander and Roman, 2013), but also region-dependent differences, as well as effects of developmental stage and of alcohol consumption pattern (Sommer, 2013).

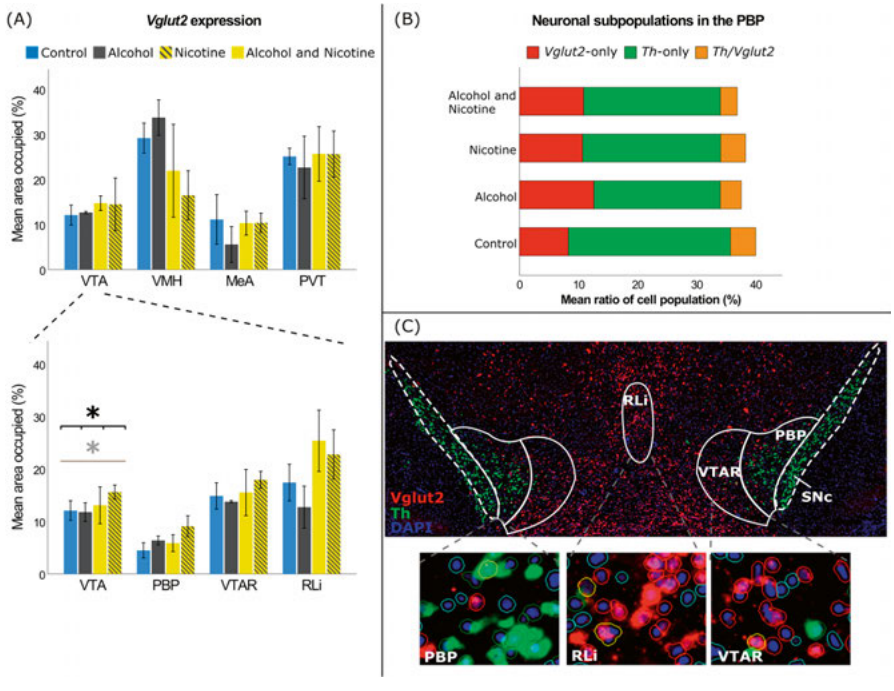

Figure 17. A) Area occupied by *Vglut2* expression, as assessed by CISH in each region of interest and aVTA subnuclei; **B)** Percentages of *Vglut2*-only, *Th*-only and *Th/Vglut2* neurons among all cells by experimental group in the PBP, as assessed by dFISH; **C)** Staining of *Vglut2* (red), *Th* (green) and DAPI (blue) in the aVTA (-4.92mm) and exemplary closeup images of CellProfiler output for each subnuclei showing $Vglut2^{neg}/Th^{neg}$ (blue outline), $Vglut2^{neg}/Th^{pos}$ (green outline), $Vglut2^{pos}/Th^{neg}$ (red outline) and $Vglut2^{pos}/Th^{pos}$ (yellow outline).
MeA: medial amygdala; PBP: parabrachial pigmented nucleus; PVT: paraventricular thalamic nucleus; RLi: rostral linear nucleus; SNc: substantia nigra pars compacta; Th: tyrosine hydroxylase; Vglut2*:* Vesicular Glutamate Transporter 2; VMH: ventromedial hypothalamus; VTA: ventral tegmental area; VTAR: VTA rostral nucleus

It has been long shown that alcohol and nicotine act synergistically on behavior via dopamine release in the Acb (Di Chiara and Imperato, 1988). Overexpression of VGLUT2 in dopaminergic neurons of the VTA is associated with increased glutamate and dopamine signaling in the Acb by co-release (El Mestikawy et al., 2011) and might be one of the underlying mechanisms leading to reinforcing effects of the drugs (Birgner et al., 2010, Flatscher-Bader et al.,

2008). The present results confirm only partly this hypothesis, as they suggest that higher *Vglut2* expression in the PBP of the group co-exposed to alcohol and nicotine could be triggered by an increase in *Vglut2*-only neurons. Many glutamatergic neurons express nAChRs (Jones and Wonnacott, 2004) and direct or indirect activation of nAChRs, especially in the aVTA, mediates alcohol-induced elevation of accumbal dopamine levels (Soderpalm and Ericson, 2013, Ericson et al., 2008, Larsson et al., 2005, Yan et al., 2018). Alcohol-mediated excitement of co-localized nAChRs within glutamate releasing synapses in the VTA (Yan et al., 2018) could lead to enhanced glutamatergic excitatory transmission within the VTA, and from there to the Acb, thus resulting in increased accumbal dopamine release and long-term effect of nicotine (Ikemoto, 2007). The strong trend towards higher percentage of *Vglut2*-only neurons in the PBP of the alcohol-only group, further supports this hypothesis. Altogether, the present findings describe a potential *Vglut2*-mediated molecular signature behind the cross-reinforcement mechanism of alcohol and nicotine co-exposure in the aVTA.

Another region involved in regulation of presynaptic accumbal dopamine release via activation of the ionotropic glutamate receptors in dopaminergic fibers is the PVT (Kirouac, 2015). In rats, nicotine has been shown to stimulate the thalamocortical projections to PFC (Gioanni et al., 1999), which are primarily *Vglut2*-positive. Repeated treatment with antipsychotic drugs was found to increase *Vglut2* mRNA expression in the murine PVT (Moutsimilli et al., 2008). The PVT also mediates context-induced reinstatement of extinguished reward seeking (Hamlin et al., 2009), and is activated by alcohol intake, especially the anterior part (rostral to -2.04mm) (Barson et al., 2015). Our study on the central part of the PVT (-2.28 to-3.00mm) found no evidence for an effect of alcohol and/or nicotine exposure on *Vglut2* expression (Figure 17A). The lack of an effect could be due to differences in bregma co-ordinates or time-points (herein, two hours after the last drug administration).

Similarly, *Vglut2* expression in the VMH and MeA was insensitive to alcohol and/or nicotine exposure (Figure 17A). The role of these regions in addiction has not been explored, although a potential role of MeA in reward-related processes has been highlighted lately (Ayanwuyi et al., 2015, Campbell et al., 2017). Both regions though have been linked to stress-related experiences (Dayas et al., 2001, Dayas et al., 1999) and activity of the stress-response system (HPA-axis) (Suemaru et al., 1995), thus an involvement in later phases of the addiction cycle, i.e. the withdrawal phase, is plausible via interaction with stress systems (Koob and Volkow, 2016).

Interactive effects between *VGLUT2* genotype, maltreatment and parenting in humans (Paper IV)

Interactive effects between *VGLUT2* genotype and environmental experiences on alcohol-related problems of adolescents and young adults were assessed in humans as well, but here, both negative and positive environmental factors were considered, i.e. maltreatment and parenting. An association of *VGLUT2* genotype with alcohol dependence had been found by our previous exploratory, haplotype-tag Single Nucleotide Polymorphisms (SNPs) study of the three *VGLUT* genes, such that the minor allele of the SNP *rs2290045* in *VGLUT2* was overrepresented (OR = 1.660) in a sample of 191 women with alcohol dependence as compared to 184 healthy women (Comasco et al., 2014). Herein, two GP samples and one CS of adolescents and young adults were included. The mean age of GP-Adolescents was 14.4 ± 1.04 at baseline (56% females) and 17.3 ± 1.04 at follow-up (59% females); mean age of GP-Adults was 22.15 ± 1.4 (53% females), and of CS individuals 16.5 ± 1.9 at baseline (58% females) and 22.2 ± 1.8 at follow-up (58% females). Among GP-Adolescents, only 12% (N = 201) were consuming alcohol at baseline and alcohol consumption differed over time ($Z = -25.304$, $p < 0.0001$). Differences in alcohol consumption over time were not seen in the CS. Weak correlations were observed between AUDIT/AUDIT-C score and environmental variables, whereas no gene-environment correlation was found.

Interactive effects between *VGLUT2* genotype (i.e. *rs2290045*), maltreatment, and parenting on AUDIT/AUDIT-C score were observed in all three samples. T carriers seemed to respond to environmental influences of both negative and positive nature in a bidirectional manner according to the environmental sensitivity framework (Ellis et al., 2011). Upon exposure to high levels of maltreatment, T carriers reported less alcohol-related problems (lower AUDIT score) with increasing quality of parenting, while an opposite pattern was observed in low levels of maltreatment (Figure 18). By assessing the positive environment as well, the differential susceptibility of T carriers, who would otherwise be considered as the vulnerable individuals, could be highlighted. This differential susceptibility was not observed in the CC group, who reported similar or less alcohol-related problems with increasing quality of parenting (Figure 18), irrespective of maltreatment.

In line with both approaches of DST and BSCT, T carriers displayed greater conditional adaptation to the environment agreeing with the malleable-phenotype feature of DST (Belsky et al., 2007) making the "best" out of each situation (Ellis et al., 2011). Although higher AUDIT-C/AUDIT score in the presence of high-quality environment (low maltreatment, supportive parenting) cannot be considered as the "best" reaction, adolescence and early adulthood are the periods when high alcohol consumption often occurs as a social phenomenon (Comasco et al., 2010). These findings could reflect a good level

of social integration and peer-acceptance, as well as potential effect of alcohol-specific parental behaviors. There is some evidence suggesting that adolescents will use alcohol, imitating their parents' use, especially when there is a close parent–child relationship (Andrews et al., 1997). However, parental alcohol use was not considered in this study. On the contrary, higher AUDIT-C/AUDIT score in the group of individuals exposed to maltreatment and poor parenting, could indicate an unhealthy coping strategy to stress, and a compensation effort to the lack of supportive environment at home (Comasco et al., 2010). Indeed, individuals with a history of adverse childhood experiences (including maltreatment and family-related stress) are more likely to use alcohol to cope with problems than individuals without such a history (Rothman et al., 2008). Of relevance is the important role of peer contexts and relationships which drive the social motives or pressures during adolescence (Spear, 2000). It is plausible that adolescents exposed to maltreatment and family-related stress (i.e. poor parenting) give an especially strong emphasis on peer relationships. Such peer contexts and relationships may be characterized by minimal adult supervision and high social pressure to engage in high alcohol drinking (Kirsch and Lippard, 2020). On the other hand, T carriers exposed to high maltreatment displayed a tendency toward less alcohol-related problems, even less than their CC counterparts, when they perceive the relationship with their parents to be supportive, highlighting the important role of favorable environments during early life and adolescence especially in higher-risk groups (Kendler et al., 2016). Rules about adolescent alcohol use have been suggested to be more protective when established by parents in the context of a good parent–adolescent relationship (Yap et al., 2017), and this is likely more apparent in these high-risk adolescents. A study of twins has demonstrated that the influence of shared environmental factors including family and peer-relationships is heightened during early adolescence (14 years of age), decreasing in later stages (until 23 years of age), when genetic influences become more pronounced (Kendler et al., 2008). Further follow-ups would be helpful to determine whether these associations will persist later-on in life and result in reduction of alcohol consumption after these experimentation years for the "protected" individuals or in development of AUD for the "highly stressed" group.

The potential functional role of *VGLUT2 rs2290045*, which is located in an intronic region of *SLC17A6/VGLUT2* on chromosome 11p14, was investigated *in silico* in the present study. Previous GWAS had shown an implication of a gene cluster in the chromosome 11 (Edenberg et al., 2010). However, here *in silico* search indicated no evidence for a functional role for *rs2290045,* though other variations in high linkage disequilibrium with this could have it. Furthermore, the SNP was investigated as potential expression quantitative trait loci, but no evidence for that was found in any brain region either. Nonetheless, the number of available samples in the online databases was too small to draw robust conclusions.

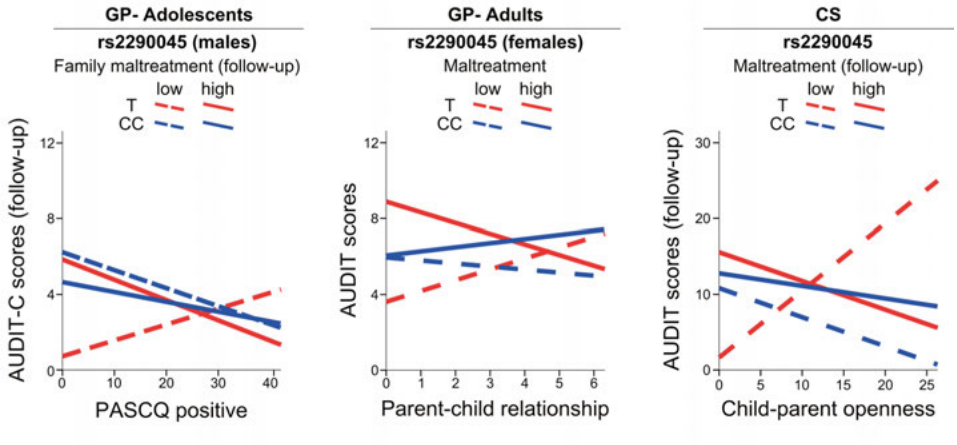

Figure 18. Fit regression lines depicting the interaction between negative and positive environmental factors, on alcohol-related problems in *rs2290045* CC and T carriers. GP-Adolescents: *rs2290045* x family maltreatment x PASCQ on AUDIT-C (follow-up): GLM: $F_{(1,581)} = 3.754$, $\eta^2 = 0.006$, $p = 0.053$, Adj. $R^2 = 0.009$; NB: Wald $\chi^2 = 4.485$, $p = 0.034$; Low family maltreatment: <1.1; high family maltreatment: ≥1.1; GP-Adults: *rs2290045* x maltreatment x parent-child relationship on AUDIT: GLM: $F_{(1,919)} = 9.409$, $\eta^2 = 0.01$, $p = 0.002$; Adj. $R^2 = 0.030$; NB: Wald $\chi^2 = 9.121$, $p = 0.003$; Low maltreatment: ≤1; high maltreatment: ≥2; CS: *rs2290045* x maltreatment x child-parent openness on AUDIT (follow-up): GLM: $F_{(1,96)} = 10.285$, $\eta^2 = 0.097$, $p = 0.002$; Adj. $R^2 = 0.210$; NB: Wald $\chi^2 = 18.683$, $p = 0.00002$; Low maltreatment: <1; high maltreatment: ≥1.
AUDIT: Alcohol Use Disorders Identification Test; AUDIT-C: AUDIT-Consumption; CS: Clinical Sample; GP: General Population; PASCQ: Parents as Social Context Questionnaire.

Sex, nicotine effects and replicability of GxE findings (Paper IV)

Both males and females were studied. In the literature, there is evidence of sex influences on ELS (Forster et al., 2018) or addiction outcomes (Becker et al., 2017), including AUD (Flores-Bonilla and Richardson, 2020). No sex-differences were observed in the present study, even though this could not be confirmed in GP-Adults, as males had been excluded from the analysis, due to methodological reasons. In GP-Adolescents, the direction of the interaction at follow-up was similar in both sexes, but in females it did not reach statistical significance. This relationship could not be assessed at baseline, as very few adolescents consumed alcohol at that time-point, and additionally, information about parenting was collected at follow-up. In the CS, the interaction was present in the whole population but only at follow-up. Nonetheless, a similar trend was observed at baseline. This observation, in line with previous research (Dick et al., 2014), supports the hypothesis that genetic influences

are not that pronounced during adolescence (Kendler et al., 2008, Heinrich et al., 2016), whereas heighten expression of genetic effects is seen later-on in environments with lower social control (i.e., at follow-up, when individuals are older) or higher alcohol availability (Dick and Kendler, 2012, Kendler et al., 2008). In a separate analysis, nicotine use was included to assess its possible confounding effect on the findings. Nicotine use did not confound the results, as the interactive effects of interest remained unchanged in all three samples. The effect became slightly weaker in the GP-Adolescents sample, stronger in GP-Adults and the same in CS individuals. In all samples, inclusion of nicotine use in the model increased the percentage of explained variance in predicting alcohol-related problems.

Replicability of GxE studies in the literature has been low and their actual contribution in understanding the etiology of psychiatric disorders has been thus criticized (Munafo et al., 2014). By employing proper control for potential confounders (Keller, 2014), as well as considering both negative and positive environmental experiences (Pluess and Belsky, 2012), the present study provided robust evidence of GxExE effects that were similar in all three samples, regardless of alcohol-drinking stage (initial vs. habitual and high-risk), and/or instruments used to assess maltreatment and parenting. Interestingly, in GP-Adolescents the amount of variance explained by the interaction term in prediction to alcohol-related problems was lower compared to GP-Adults and CS, which implies that genetic influences become stronger later in life, from early to middle adulthood (Kendler et al., 2008, Heinrich et al., 2016), although also expected due to the low quantity of alcohol consumed by this sample. The amount of variance explained was higher in the CS than in the two GP samples, as expected because the former is a "high-risk", more extreme sample. Despite all the aforementioned sample-related differences, the present findings support generalization of *rs2290045* x maltreatment x parenting interaction, suggesting that the T allele of SNP *rs2290045* confers increased sensitivity to negative and positive environmental factors. These individuals could be more responsive to prevention and treatment than CC carriers.

Contribution of the thesis

In the non-addicted brain, the activity of circuits involved in reward, motivation, memory, habituation and executive control is balanced, resulting in proper inhibitory control and decision making (Koob and Volkow, 2016). In the addicted brain, this balance is disturbed, as the enhanced expectation of the drug stimulates the reward (Acb, VTA), motivation (VTA, dStr), memory and habituation (dStr) circuits, overcoming the control circuit (PFC) (Volkow et al., 2011). Glutamate plays an important role in the neuroadaptations behind the transition between the two states. Repeated drug use results in a preference

shift from the consumed drug to the conditioned stimulus (Volkow et al., 2011). These conditioned responses are mediated by enhanced glutamatergic signaling from the PFC and amygdala to the VTA and Acb (Kalivas, 2009), which could be associated with changes in *Vglut/VGLUT* expression. The involvement of *VGLUT2* and *VGLUT3* in addiction has been highlighted in human studies (Comasco et al., 2014, Sakae et al., 2015) whereas studies in rodents have linked *Vglut1-3* to addiction-related phenotypes (Zhou et al., 2006, Truitt et al., 2014, Sakae et al., 2015, Alsiö et al., 2011, Birgner et al., 2010, Hnasko et al., 2010).

The present thesis adds a small piece to this big puzzle (Figure 19), considering for the first time early life environmental experiences as well, focusing on adolescence and young adulthood, and on the stage of potential transition from initial alcohol use to misuse. The results show higher *Vglut2* and *Vglut1* expression in the VTA and dStr, respectively, and lower *Vglut3* expression in the Acb upon interaction of ELS with alcohol. These changes are accompanied by altered single CpG-specific methylation for each *Vglut*, which is the first evidence of ELS x alcohol effects on *Vglut* DNA methylation. The studies also provide evidence for the first time that in non-dependent outbred male young adult rats, episodic concurrent exposure to alcohol and nicotine is associated with higher *Vglut2* expression in the VTA, but with no changes in MeA, VMH or PVT, suggesting a midbrain-specific molecular *Vglut2*-mediated mechanism already during the initial stage of drug use. Further, the findings of the study in humans demonstrated the moderating role of *rs2290045 VGLUT2* genotype on environmental sensitivity to alcohol-related problems during the transitional period of alcohol use to misuse.

Differences in methylation of single CpG sites in potential regulatory regions of *Vgluts/VGLUTs* could be a plausible mechanism of gene expression regulation. Even subtle differences (< 10% or even 1 – 5%) in methylation of single CpG sites potentially have functional relevance, not likely as on/off switch of gene expression, but rather in redistribution of the transcriptional landscape, affecting translational isoform production and the proteome (Leenen et al., 2016). *In silico* analysis identified potential TFBS for CEBPD, c-Fos, USF-1, and -2, and CREB at specific CpGs within all *Vgluts*, suggesting a potential mechanism between changes in methylation and expression. It has been indeed proposed that TFs control the levels of gene expression (McGowan et al., 2009) as well as that differential methylation levels of specific CpG sites have the potential to block or induce TF binding (Jones, 2012). Some of these TFs (i.e. CEBPD, CREB, c-Fos) have been consistently linked to alcohol-related phenotypes (Berkel and Pandey, 2017, Erickson et al., 2019) or ELS (Horii-Hayashi et al., 2013). Especially the CREB pathway, as CREB activation is observed upon chronic drug use in the Acb, contributing to a decrease in the reward value of the drug and likely to the emergence of withdrawal/negative affect state (Koob and Volkow, 2016).

Stress plays an important role in addiction (Koob and Volkow, 2016), including AUD (Ciccocioppo et al., 2009, Pucci et al., 2019). Acute stress and application of corticosterone *in vitro* increase the glutamate readily-releasable-pool of synaptic vesicles through glucocorticoid receptors-mediated mechanisms in the PFC (Treccani et al., 2014). Heightened plasma corticosterone levels have been found in adult mice previously exposed to ELS, as a result of increased excitatory glutamatergic neurotransmission in the paraventricular nucleus of the hypothalamus (Gunn et al., 2013), while maternal deprivation has been shown to result in disturbed homeostasis of glutamatergic synapses (Toya et al., 2014). Considering that synaptic plasticity is controlled pre-synaptically through the regulation of glutamate release (Malenka and Bear, 2004) and post-synaptically through the activation of ionotropic glutamate receptors (Bowers et al., 2010), alterations in *Vgluts* gene expression could lead to altered VGLUT protein expression in the synaptic vesicles regulating glutamate release and hence glutamatergic synaptic transmission and plasticity (Wojcik et al., 2004, Daniels et al., 2004, Herman et al., 2014). Ablation of VGLUT2 in mature dopamine neurons of adult mice was recently shown to contribute to decreased glutamatergic neurotransmission and synaptic strength in the Acb (Papathanou et al., 2018). *In vitro* administration of VGLUT inhibitors reduced glutamatergic synaptic transmission in murine PFC and hippocampal slices (Neale et al., 2014), while downregulation of VGLUT1 in mice led to altered glutamatergic synapses contributing to increased anxiety, depressive-like behavior and impaired memory (Tordera et al., 2007). Furthermore, *in vivo* inhibition of VGLUT activity attenuated methamphetamine-induced conditional place preference (He et al., 2014).

Relative to the findings of Paper IV, allelic variation in *VGLUT2* polymorphisms could influence various behaviors related to the glutamatergic system and addiction, for example in acute intoxication, reinforcement or craving (Kalivas et al., 2009). Because chronic excessive alcohol use ultimately results in a hyperglutamatergic state, characterized by elevated extracellular glutamate and altered glutamate receptors and transporters (Holmes et al., 2013, Heilig et al., 2016), *Vgluts/VGLUTs* (as important elements of glutamatergic neurotransmission) could serve as promising targets of pharmacological manipulation that could potentially alter a number of alcohol-related phenotypes, such as acute intoxication, alcohol-seeking and consumption, and withdrawal. Along with *Vgluts/VGLUTs,* inhibition of DNMTs could be another promising therapeutic option in AUD (Berkel and Pandey, 2017). Differences in expression of the two regulatory DNA methylation genes, *Dnmt1* and *Mecp2,* were observed herein.

Taken together, changes in the expression of *Vgluts/VGLUTs,* likely regulated by differential DNA methylation, during the initial stage of alcohol use to potential misuse may contribute later on to the enhanced glutamatergic transmission seen in the addicted brain, where impaired glutamate-induced

plasticity has been linked to drug-seeking behavior (Kalivas, 2009). Unravelling the genetic and epigenetic profile (i.e. DNA methylation patterns) of key neuronal markers such as *Vgluts/VGLUTs* and their relationship with regulators of the DNA methylation machinery (i.e. *Dnmt1* and *Mecp2*) in reward and stress-related brain regions will contribute in a better understanding of alcohol-related phenotypes and the AUD, possibly aiding towards the development of efficient treatments for this detrimental multifactorial disorder.

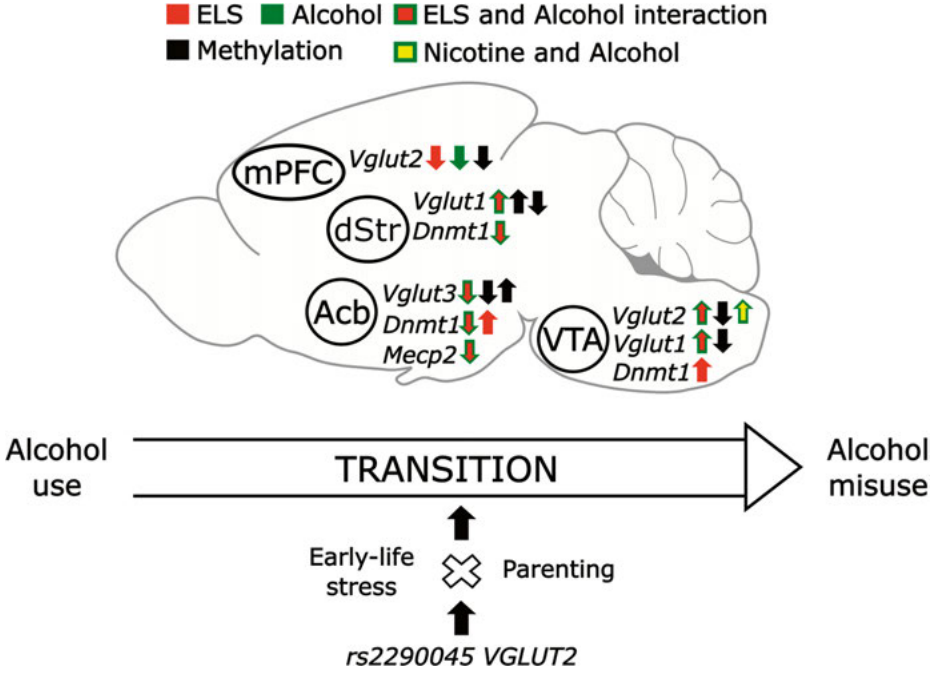

Figure 19. The contribution of the present thesis in the big puzzle of alcohol addiction development. The present thesis focused on adolescents and young adults, demonstrating the *Vgluts/VGLUT2, Dnmt1* and *Mecp2* role in the potential transition from initial alcohol use to misuse and integrating the effect of early-life experiences as well as the nicotine co-exposure. Colored arrows within the brain show the direction of each effect on gene expression. Black arrows show the direction of methylation changes that accompanied these effects, which can vary depending on the targeted gene region. In carriers of the *rs2290045* minor allele (T), increasing quality of parenting was associated with higher and lower alcohol misuse in the absence and presence of previous maltreatment (early-life stress), respectively. ELS: early-life stress; *Dnmt1:* DNA-methyltransferase 1; *Mecp2:* Methyl-CpG-binding protein 2; *Vglut1-3*: Vesicular Glutamate Transporters 1-3;

Methodological considerations

Early life stress

Animal studies are useful for demonstrating causality between exposure to ELS and its long-lasting effects, furthering our understanding and translating findings to human research regarding the genetic, environmental, and gene-by-environment (GxE) impact on psychopathology in adulthood (Teicher et al., 2006). Nonetheless, caution needs to be taken when interpreting and comparing results of preclinical studies and even further when translating findings from rodents to humans. Conceptualization of ELS is important; that is, taking account of duration, number of times and severity of ELS, time-point when ELS is experienced, as well as ELS-mediated effect on maternal care along with the different maturation velocity of each brain region (Molet et al., 2014, Teicher et al., 2016).

Regarding the maternal separation paradigm employed in Papers I and II, a variety of MS models can be found in the literature. Differences concern the duration of prolonged separation (180 or 360 min), litter separation from the dam: i.e. whether it is individual or litter-wise, choice of control group (MS0/MS15 vs. AFR or non-handling), time of MS exposure, sex-differences, and housing conditions during adolescence (Nylander and Roman, 2013). The pups can be separated and kept in the separation cage individually or litter-wise. Contrary to the latter, individual separation deprives the pups from tactile contact with the littermates, which can be an additional stressor, confounding the MS effects (Nylander and Roman, 2013). Standard rearing (AFR) has been commonly used in the literature as control for prolonged MS (180 to 360 min) in alcohol-drinking paradigms. However, AFR animals are not exposed to the same daily handling as MS animals, therefore they cannot be considered as a reliable control. Further, different labs employ different conventional rearing protocols, thus making the comparison of results more difficult. More pronounced differences have been found in gene expression levels of MS360 rats when AFR were used as control group, compared to MS15 rats as control (Todkar et al., 2015). These differences could be a result of extra effects of different handling between the groups, rather than ELS.

The MS paradigm has high predictive and construct validity for ELS-mediated adult alcohol drinking (Moffett et al., 2007). One example of other ELS models in rodents, with potentially better face validity, is the chronic early stress (CES) model which refers to stress occurred due to limited bedding and

nesting material, a condition that stresses the dam and leads to fragmented and unpredictable maternal care towards the pups (Molet et al., 2014). This inconsistent pattern of maternal care is suggested to mimic in a more naturalistic way the ELS experienced by humans (Molet et al., 2014). That is, because ELS in humans is usually chronic and not intermittent (as in an MS model) and is usually caused by disrupted maternal care, for example in situations of drug-users and/or abusive mothers (Molet et al., 2014). Indeed, highly heterogeneous maternal care can further impact infant's brain programming, and consequently affect individual's mental health (Baram et al., 2012). Nonetheless, the MS procedure also leads to aberrant maternal care (i.e. licking/grooming) after the separations (Meaney, 2001), which is similar between prolonged-MS and control (short-MS) dams, but the distribution of maternal care within the day differs (Macri et al., 2004), implying fragmentation and unpredictability (in maternal care) of prolonged-MS dams. MS could be interpreted as physical and emotional neglect and its effects could be related to poor parental care in humans (Meaney, 2001). A supportive environment in rodents can be modeled by environmental enrichment and studies have demonstrated its impact on maternal care (Connors et al., 2015). However, the effect of a supportive environment on *Vgluts* expression or methylation was not assessed in the present thesis, therefore the applicability of the 'environmental sensitivity' framework remains to be explored in this animal model.

Of great relevance to the translational effects of MS models is the diverse maturation trajectories of different brain regions and circuitries within and among species (Semple et al., 2013). In terms of brain development, the first three postnatal weeks in rodents correspond to a prenatal developmental time window in humans of the third gestational trimester (Clancy et al., 2007, Workman et al., 2013). Thus, it is important to bear in mind that MS in rodents during the first postnatal weeks may therefore reflect alterations of stress exposure not only during early childhood, but also perinatally in humans. Notwithstanding, the MS paradigm has been proven to show high construct and predictive validity for ELS-mediated adult alcohol drinking and has been extensively used in the addiction field (Moffett et al., 2007, Nylander and Roman, 2013).

In Paper IV, regarding humans, different types of maltreatment were grouped into one combined maltreatment variable, as it has been suggested that the effects of maltreatment on brain are not depending on type but rather to perception of the experience as stressful (Teicher et al., 2006). Indeed, it has been demonstrated that different types of child maltreatment usually co-occur and they have equivalent and universal, rather type-specific, effects (Vachon et al., 2015). More important than the type of maltreatment is the number of exposures to adverse experiences suggesting that the impact of juvenile maltreatment on later psychopathology is "dose-dependent" (Merrick et al., 2017). In the present study, this issue was attempted to be explored in GP-Adults and CS individuals, where multiple maltreatment types were as-

sessed, by creating a combined maltreatment variable with different levels depending on the types of maltreatment the individual was exposed to. By employing this multi-level variable in the model we saw that the explained variance was higher than when a dichotomous maltreatment variable was used. Furthermore, maltreatment was assessed separately at each time-point, as it has been suggested that using lifetime measures hinders the possibility of establishing temporal order between a hypothesized cause and an effect (Moffitt and Caspi, 2014), although in the present study temporal order could be assessed only to a limited extend.

The interpretation of the findings of Paper IV needs to be done with caution relative to the assessment of parenting. In GP-Adolescents, parenting style was assessed using the well-validated PASCQ instrument (Skinner et al., 2005). In the CS, a factor analysis was performed on a total of 58 questions regarding parent-child relationship. The dimensions comprised "child-parent openness", "parent-child affect" and "parent-child support", from which only the former was found to interact with maltreatment and genotype in the present study. This finding implies that how the adolescents perceive their relationship with their parents (i.e. to be open and trustworthy, to be able to share feelings and thoughts) is more important than the affect or support that is received. Indeed, parental communication, involvement and monitoring have been emerged as important aspects of parenting in regards to reduced adolescent alcohol drinking (Carver et al., 2017, Yap et al., 2017). Finally, in GP-Adults, parent-child relationship was assessed only by two questions relative to the participant's perceived relationship with the mother and father. Nonetheless, parenting is complex, multi-factorial and two questions may not be sufficient to describe it.

Alcohol and nicotine consumption

With regard to Papers I and II, models assessing alcohol consumption can differ significantly and thus findings from studies using distinct models cannot be directly comparable. For instance, in Paper I and II, the effect of alcohol alone or in interaction with ELS was not seen on *Vglut2* or *Vglut1* expression in the Acb, contrary to previous findings (Zhou et al., 2006, Truitt et al., 2014, McBride et al., 2013). Nevertheless, the animals used in the present studies had no genetic preference for alcohol as in the abovementioned ones, while the focus was on the initial alcohol consumption stage, during the transition from voluntary to more habitual drinking patterns and not on withdrawal phase as in the study of *Zhou et al*. (Zhou et al., 2006) or upon acute administration as in *Truitt et al.* (Truitt et al., 2014). These divergent results are likely to be driven by the diversity of the study design. Apart from the use of animals with genetic preference to alcohol or different stages of alcohol consumption, variations, among others, can regard also the period when alcohol

exposure begins (adolescence or adulthood), how much percentage of alcohol is given to the rats (5, 10 or 20%), continuous or intermittent access to alcohol, and 24-hours or limited access to alcohol (Sommer, 2013).

Depending on the alcohol-access models, MS-induced effects on alcohol intake can also differ (Nylander and Roman, 2013). Studies to date show consistent results relevant to MS-mediated propensity to higher alcohol consumption, regardless of continuous or intermittent alcohol exposure, when adult male rats that have been group-housed (during adolescence), and exposed to litter-wise prolonged MS, using as control the short MS are assessed (Nylander and Roman, 2013).

Voluntary alcohol-drinking paradigms show good face and construct validity as models of human alcohol consumption (Sanchis-Segura and Spanagel, 2006), and they are useful in the identification of pharmacological treatments to prevent excessive alcohol drinking, thus demonstrating also predictive validity. In these paradigms, single-housing is common in order to measure individual alcohol-intake. Nonetheless, single-housing could be an extra stressor confounding the MS and/or alcohol effects on gene expression (Palm and Nylander, 2014). Social interactions are important during the sensitive period of adolescence. Single-housing during this period leads to multiple neurobiological alterations due to social play deprivation (Fone and Porkess, 2008). This can further lead to vulnerability towards loss of control over alcohol seeking (Lesscher et al., 2017), a correlate of craving in humans. However, single-housing during adulthood does not have that detrimental effects (Schenk et al., 1990), and in the present study showed to not have an impact on gene expression in AFR animals. Yet, single-housing effects were not investigated in MS rats, and it would be interesting to explore them in future studies, considering that animals exposed to ELS could be more sensitive to stressors later in life (Murthy and Gould, 2018).

ELS has been linked to elevated alcohol consumption later in life in both rodents (Becker et al., 2011) and humans (Enoch, 2006). In the present studies of rodents, animals exposed to ELS (MS360) did not consume higher alcohol as compared to MS15 or AFR rats, as the animals were sacrificed before different patterns would be expected to emerge. However, consistent with previous findings (Nylander and Roman, 2013), distinct alcohol intake patterns were observed in MS360A group with ‘responders’ that elevated their alcohol consumption over time versus ‘non-responders’ that displayed stable or even decreased alcohol intake. This finding underlies that only a subgroup of rats was more sensitive to ELS relative to alcohol consumption reflecting the phenotypic heterogeneity seen in AUD patients and confirming the notion that not all individuals exposed to ELS will develop hazardous drinking patterns (Baram et al., 2012). Nonetheless, no differences in gene expression were noted between these subgroups and thus in the present study, the MS360A group was analyzed as a whole.

Yet, the ‘environmental sensitivity’ notion that susceptibility to rearing conditions varies among individuals depending on inherent characteristics

(i.e. *Vgluts* expression pattern) could also apply here. Although in these initial stages of alcohol-drinking significant alterations on the studied genes were not present between the MS360A subgroups, this does not exclude the possibility that other set of genes could be affected or that in later stages different gene expression patterns would emerge among the subgroups. In fact, in Paper II differences in *Vgluts* DNA methylation were seen between these subgroups hinting towards DNA methylation markers of different alcohol doses; yet whether these signatures precede or follow alcohol consumption remains to be clarified. Future exploration of behavioral and/or neurochemical correlates of these distinct subgroups would be of great interest, and it could contribute to further disentangle mechanisms behind vulnerability and resilience to ELS.

In Paper III, rats were exposed to subcutaneous nicotine injections and alcohol administration via gavage. Although forced drug administration cannot be translated into a human way of smoking or drinking, it is advantageous for studying drug effects compared to voluntary models as it minimizes inter-individual variations in drug intake. Moreover, the drug administration regimen during PNW3-9 was modelled to mimic episodic drinking patterns seen in adolescents and young adults. Blood alcohol concentration levels were not measured, but previous studies support achievement of binge-like alcohol levels (Lundberg, 2020). Last but not least, the animals were sacrificed two hours after the drug administration, a time point when the animals were still under the acute intoxicating effects of the drugs. Thus the present findings cannot exclude a possible confounding acute effect of the drugs, and therefore, the results should be interpreted under this prism. Nonetheless, it is still within the interest of the present study to examine the acute ethanol- and/or nicotine-induced effects in a brain that has been protractedly exposed during adolescence, focusing on the differences between the two drugs alone and the combination.

In Paper IV, the AUDIT-C and AUDIT were used to measure alcohol consumption and alcohol-related problems, respectively. AUDIT has been proved the best screening tool for the whole range of alcohol-related problems as compared to other questionnaires such as CAGE (an acronym deriving from its four questions) and MAST (Michigan Alcohol Screening Test) (Babor, 2001). AUDIT-C has also been demonstrated to effectively screen for young adults (18-20 years old) with AUD in emergency departments (Kelly et al., 2009). Here, a modified version of AUDIT-C, tailored to the young age of adolescents was used to capture even slight differences in their alcohol consumption. In the present study, literature-recommended cut-offs (such as "8" for AUDIT (Conigrave et al., 1995, Babor, 2001) or "4" for AUDIT-C (Bush et al., 1998)) were not used, as the focus was on the change (increase or decrease) of the alcohol-consumption/alcohol-related problems continuum as a function of gene-by-environment interaction and not on screening for specific endpoints.

Genetic and epigenetic analyses

There are multiple methods for assessing gene expression (i.e. mRNA detection) such as RNA protection assays, *in situ* hybridization (ISH), microarrays, Northern blot, and quantitative PCR. The latter has been proven advantageous in comparison with the rest because of its high sensitivity, reproducibility and quantitative nature (Wong and Medrano, 2005); hence, it was chosen for assessing gene expression in Papers I and II. Nevertheless, it lacks anatomical information and impedes investigation of neuronal co-phenotype. ISH instead, although semi-quantitative, provides spatial information about localization of mRNA sequences *in situ* (Jensen, 2014), and it was thus chosen in Paper III. Notably, results from colorimetric and fluorescent ISH (CISH and FISH, respectively) were not directly comparable in this study. FISH seemed to allow higher contrast in detection of the targeted mRNA, which was also reflected in the consistently lower values for the area occupied by *Vglut2* expression compared to CISH. Double FISH, using *Th* expression as a marker for the VTA, allowed a more precise localization and selection of the bregma compared to CISH, as *Th* expression differed distinctly along the rostro-caudal axis. This observation led us to finer selection but also exclusion of a number of animals analyzed by CISH due to prior mis-estimation of bregma co-ordinates. Animals were excluded mainly from the groups exposed to either drug alone (alcohol or nicotine), thus impeding us from assessing (by CISH) whether there was an additive or synergistic effect of the drugs. Furthermore, only one slice per animal (at bregma -4.92mm) was analyzed in FISH images, while duplicates or triplicates of brain slices, possibly covering a slightly greater range around bregma -4.92mm, were used for CISH. Despite these differences, FISH analysis also resulted in similar (to CISH) elevation of *Vglut2* in the VTA; however, it did not reach statistical significance.

Finally, automated cell counting was applied using a standardized pipeline developed in CellProfiler for unbiased, automated image analysis (Carpenter et al., 2006), overcoming a potential error due to manual counting. The pipeline included computation of areal ratio occupied by the cells, controlled for potential bregma levels-selection bias, as well as over- or under-identification of cell number due potential over-declumping or over-merging of cells, respectively. However, the definition of a cell as stained is still within the judgement of the researcher and the criteria for unbiased and correct assignment of a cell as stained should be carefully applied. Considering this point, FISH images were advantageous in identification of stained cells, allowing for estimation of number of cells against DAPI-stained nuclei (Figure 17C).

Epigenetic markers (i.e. histone methylation, acetylation, microRNAs) other than DNA methylation have been demonstrated in ELS (Turner et al., 2015) and/or alcohol-related phenotypes (Berkel and Pandey, 2017, Ponomarev, 2013, Pucci et al., 2019). Nonetheless, DNA methylation is amongst the more intensely studied epigenetic mechanisms and one of the

principal interfaces between the genome and the environment (Leenen et al., 2016), and it was thus the focus of Paper II. There are various techniques to assess DNA methylation, among those is mass spectrometry (the gold standard for array-based genome-wide methylation analyses), digestion-based assays which make use of selective digestion of DNA by restriction enzymes depending on DNA methylation status, pyrosequencing or methylated DNA immunoprecipitation. Bisulfite-treated DNA is widely used and bisulfite-sequencing is the gold standard in gene-specific DNA methylation studies (Kurdyukov and Bullock, 2016). Indeed, sequence-based techniques are preferential to array-based ones, as they allow access to the underlying genomic sequence (Leenen et al., 2016). Especially next generation sequencing, which was implemented in Paper II, allows investigation of subtle methylation changes in greater detail genome-wide (Leenen et al., 2016). However, although widely used, bisulfite-treated DNA cannot distinguish between 5-methyl- and 5-hydroxymethylcytosine (5mC and 5hmC, respectively) (Kurdyukov and Bullock, 2016). This limitation, has not been considered in studies on psychiatric disorders and needs to be addressed for a better disentangling of each marker's contribution and regulatory effect on the phenotype of complex diseases (Nestler et al., 2016). Furthermore, careful interpretation of the DNA methylation results should take into consideration the homogeneity of the analyzed cell population, as the resulting ratio is a snapshot of all DNA isolated from the sample, opposite to single cell analysis. Thus, cells with varying methylation status could have a dilution effect on methylation levels (Kurdyukov and Bullock, 2016).

Study design

There is a vast amount of studies repeatedly shown that there are sex differences in addiction (Becker et al., 2012, Becker et al., 2017), including AUD as recently reviewed in (Flores-Bonilla and Richardson, 2020). For several decades, preclinical studies were predominantly performed on males, with the argument of the increased amount of animals needed for a study on females, considering their hormonal state during difference phases of the estrous cycle (ter Horst et al., 2012). Nevertheless, the similarity of rodent hormonal patterns to humans, although estrous cycle in rodents is shorter (4-5 days), as is their lifespan, renders animal models valuable in comparing sex and hormonal influences from adolescent to adult ages, providing essential information about genetic and neurobiological factors in addictive behavior (Carroll and Lynch, 2016). Indeed, lately there has been an upsurge in clinical and preclinical studies including both sexes in the AUD field (Flores-Bonilla and Richardson, 2020). In the present animal studies, only male rats were used, impeding us from explore the *Vgluts*-correlates of sex differences relative to ELS and alcohol. Nevertheless, sex was considered in the human study and

findings were similar in both males and females, suggesting that sex does not influence the *VGLUT2* genotype-implication in ELS-mediated alcohol-related outcomes. Future human study designs however, should take into account not only sex, but also gender, considering that these two terms are not synonymous (Becker et al., 2017). Sex, referring to biological and morphological characteristics, and gender to a social construct of expected behaviors relative to biological sex, have both shown to influence addiction-related phenotypes (Becker et al., 2017). Neuroimaging studies taking into account both sex and gender have been conducted during the last decade, although research on AUD is still limited (Verplaetse et al., 2021). Sex influences also the stress response system (HPA-axis), while sex differences have been observed in ELS-mediated outcomes on brain neurocircuitries, including the glutamatergic system (Perry et al., 2020). Taken together, sex impacts both ELS and alcohol-related phenotypes and considering their interactive nature, it calls for a more in-depth exploration of sex-specific differences as well as an examination of the effects of age of onset and chronicity of stressors on multiple levels, including neurobiology, neurochemistry, brain function, structure and connectivity (Zik and Berkowitz, 2019, Teicher et al., 2016, Verplaetse et al., 2021, Ciccocioppo, 2017).

Longitudinal studies with repeated measures are useful for the evaluation of the relationship between risk factors and the development of a disease over different periods (Caruana et al., 2015). Cross-sectional studies, on the contrary, are considered less valid for examining causal relationships, as the collection of the data is performed at a specific time-point (Caruana et al., 2015). In paper I, gene expression levels were assessed at only one time-point, as the tissue of interest was the brain and the animals had to be sacrificed. The follow-up investigation in Paper II, could not assess causality between changes in CpG methylation and gene expression, thus the findings were primarily correlational in nature. Much evidence shows that DNA methylation ensures gene silencing (Jones 2012). Accordingly, DNA methylation could potentially follow transcriptional changes and function as a signature "lock" to these changes rather than a molecular mechanism that regulate gene expression. Future studies would be needed to further explore this hypothesis.

In Paper IV, the longitudinal design of the studies of the GP-Adolescents and CS allowed us to monitor developmental trajectories of differential susceptibility. This was not possible in the GP-Adults due to its cross-sectional design; however, the current findings were similar in all three samples confirming their robustness. Attrition, a potential confounding factor in longitudinal and large studies (Gustavson et al., 2012), was considered in each sample; though, attrition related to follow-up variables has been shown to create bias mainly on mean estimates rather than on the association between variables (regression estimates) (Gustavson et al., 2012). The use of retrospective questionnaires to measure the environmental factors in paper IV could entail a possible memory or response bias (Newbury et al., 2018). Research has shown

that although false positive reports are probably rare, false negative reports could be substantial (Hardt and Rutter, 2004, Newbury et al., 2018). However, when possible, face-to-face interviews were also employed and answers of both adolescents and parents were considered (Cater et al., 2014). It has been in fact suggested that the combined use of self-reports and interviews is indeed optimal (Hopwood et al., 2008). Furthermore, interviews or filling of questionnaires that is done face-to-face are considered more reliable than interviews conducted by phone or questionnaires sent by mail (Moffitt and Caspi, 2014).

Ethical considerations

Animal studies

The animal studies followed the Swedish Animal Welfare Act guidelines, considering the 3Rs-principle, i.e. replacement, reduction, refinement, and complied with the EU directive regarding the performance of the animal experiment by a trained person, certified with the "Federation of European Laboratory Animal Science Associations" (FELASA). According to the 3Rs-principle, animals should be *replaced* by non-animal alternatives (i.e. computational models, cell lines, tissues) when possible. In the present study, the neurobiological correlates to ELS-mediated mechanisms of voluntary alcohol consumption were not possible to be explored in non-animal experiments. Moreover, the number of animals should be *reduced* to the minimum, sufficient for the research questions to be addressed. Accordingly, 10 animals per group were considered sufficient, except for the MS360A group, where 20 animals were included, based on previous studies of MS 'responders' and 'non-responders' relative to alcohol consumption (Nylander and Roman, 2013). Finally, the experimental procedures should be *refined* regarding pain and/or suffering, which should be minimized unless absolutely necessary. In the present study, procedures that would potentially cause pain or suffering of the animals were not involved; water and food were available *ad libitum,* whereas quick decapitation of the animals was performed in the end of the experiment considering the "humane endpoint". The study was approved by the Uppsala Animal Ethical Committee (C32/11) and followed the guidelines of the Swedish Legislation on Animal Experimentation (Animal Welfare Act SFS1998:56) and the European Communities Council Directive (86/609/EEC).

Human Study

In Paper IV, questions were asked regarding maltreatment during childhood and adolescence, including sexual and physical abuse, thus the participants were asked to answer very sensitive questions. Confidentiality was reassured, and participants were informed that they could leave the study at any time. In the case of CS, cases of abuse were reported to the authorities. Furthermore, the studies collected saliva samples to isolate DNA; data deriving from genetic

analyses have raised ethical considerations in regard to exchange of any genetic information with the participant or access to it via public databases, as well as the use of such information by third parties (Foster and Sharp, 2006). Due to the sensitive nature of the data, anonymity was ensured, and an informed consent to participate in the study was collected from all participants of all three samples. For GP-Adolescents, consent was obtained from their parents as well. The studies on GP samples were approved by the Regional Ethics in Uppsala, Sweden (GP-Adolescents: Dnr2012/187); (GP-Adults: Dnr 2010/463). The study on "high-risk" CS individuals was approved by the Karolinska Institute Research Ethics Committee Nord (Dnr 03-543), and the Regional Board for Research Ethics in Stockholm, Sweden (Dnr 2008/1934-31/3).

Conclusions

The studies of the present thesis investigated the relationship between the *Vglut/VGLUT* genes and early environmental experiences of negative, and positive, nature as well as alcohol consumption and nicotine co-exposure in an integrative translational approach including young adult rats and humans. The findings specifically highlight a potential *Vglut2/VGLUT2*-mediated molecular signature behind these associations.

The results presented in Papers I, II and III showed the following in non-preferring, non-dependent outbred young adult male rats:

- Voluntary alcohol consumption affects *Vglut1-3* expression and methylation, as well as *Dnmt1* and *Mecp2* expression, in the VTA and the striatal parts of the brain, depending on ELS; ELS rats were more sensitive to alcohol-induced effects
- ELS and alcohol interactive effects partly influenced the correlation patterns of these genes among themselves and with their methylation levels
- CpG-specific *Vglut1-2* methylation did not moderate the effect of ELS and alcohol on *Vglut1-2* expression in the VTA
- Different alcohol intake doses affected *Vglut1-2* DNA methylation and its correlation with gene expression, but did not affect gene expression *per se*
- Single-housing in adulthood, which is common in voluntary alcohol-drinking paradigms, did not the confound alcohol-drinking effects on the investigated genes
- Adolescent repeated co-administration of alcohol and nicotine was associated with higher *Vglut2* expression in the lateral aVTA*;* this difference was driven by *Vglut2*-only neurons, and not the neurons co-expressing *Vglut2* and *Th*
- *Vglut2* expression in the MeA, VMH and PVT was insensitive to adolescent episodic alcohol and nicotine consumption

The findings reported in Paper IV have demonstrated the following:

- *VGLUT2* rs2290045 genotype moderated environmental sensitivity to alcohol-related problems among adolescents and young adults
- Carriers of the minor allele (T) displayed differential susceptibility to the environment; neither sex nor nicotine confounded this finding
- The interactive effect between *rs2290045,* maltreatment and parenting, was present in all three samples, despite differences in age, alcohol-drinking patterns (i.e., initial, habitual and risky) or assessment of the environmental factors

Future perspectives

ELS is highly correlated with adverse health outcomes (Kirsch and Lippard, 2020), though a high percentage of those exposed to ELS does not develop any psychopathology (Rehan et al., 2017), but may develop sub-symptoms of psychopathology (Teicher et al., 2016). In fact, maltreated individuals with or without any psychopathology show a similar *ecophenotype,* sharing neurobiological, developmental and structural correlates in the brain (Teicher et al., 2016). It is of considerable interest to study this "resilient" population (maltreated individuals without psychopathology) in greater depth to understand the protective factors behind the compensatory mechanisms and how they could be nurtured for the benefit of the general population (Teicher et al., 2016, Murthy and Gould, 2018). By contrast, maltreated individuals differ in their brain structure, function and connectivity from non-maltreated individuals with the same psychopathology, and it is important that these differences are defined, from a molecular perspective, for a better understanding on how ELS shapes the brain (Teicher et al., 2016). For instance, following-up the human cohorts included in Paper IV, would shed light on the trajectories of alcohol consumption and problems, confirming or rejecting our hypothesis about experimentation vs. "drink to cope" alcohol use and subsequent development or not of AUD, further determining the role of different *ecophenotypes*. Using the "environmental sensitivity" approach in animal studies as well, will allow a better conceptualization of human environmental experiences in preclinical research, ultimately leading to an improved understanding of the complex interactive mechanisms between environment and genotype, and potentially causality.

A deterministic functional role in gene activity relevant to single epigenetic modifications has yet to be found (Leenen et al., 2016, Hamilton and Nestler, 2019). It appears that it is rather the combination of multiple modifications that contributes to any observed phenotype (Hamilton and Nestler, 2019), suggesting a clear need for determining the epigenetic profile that underlies complex phenotypes. Prospective human studies and translational animal studies are greatly needed to unravel the epigenetic profiles of alcohol-related phenotypes and to determine whether these profiles are the cause or the consequence of alcohol misuse. Functional studies on *Vgluts* are needed to demonstrate the role of epigenetic modifications as well as the potential biological significance of even subtle changes (Leenen et al., 2016), using sequence-specific tran-

scription molecules or the CRISPR/Cas9 technology to target particular modifications in a specific gene and brain region (Hamilton and Nestler, 2019). Epigenetic signatures have the potential to be utilized within a clinical set-up to assess AUD status and treatment response, while their presumably reversible nature renders them encouraging targets for treatment (Zhang and Gelernter, 2017).

There is no successful pharmacotherapy to date for AUD (Heilig et al., 2016, Cannella et al., 2019). Acamprosate, one of the four approved medications for AUD, targets the glutamatergic system, although its exact mechanism of action (Spanagel et al., 2014), and its efficiency (Franck and Jayaram-Lindström, 2013, Holmes et al., 2013) remain elusive. Two of the other clinically approved medications (nalmefene and naltrexone) target the opioid system, and the third (disulfiram) targets the activity of acetaldehyde dehydrogenase; all have shown moderate levels of efficacy as well (Franck and Jayaram-Lindström, 2013). This inadequacy of treatment for AUD is due to the polygenic nature of the disorder and the complex interplay between genetic and environmental factors in its development (Spanagel, 2009), subsequently resulting in high heterogeneity among patient populations (Cannella et al., 2019). Indeed, in humans, exposure to a single factor is not the norm (Nilsson et al., 2018); for instance, most of the maltreated individuals have been exposed to more than one type of maltreatment (Vachon et al., 2015). Furthermore, other life stressors, social injustice and even the increased distress, e.g., due to COVID19 pandemic may affect individuals' behavior towards misuse of alcohol and other substances (Kalin, 2020). Likewise, alcohol misuse or AUD usually co-occurs with the use of other substances, and also with other psychiatric disorders (Kalin, 2020). Not only is AUD highly comorbid with mood and anxiety disorders, but it has also been suggested that the latter mediate the risk for AUD (Kirsch and Lippard, 2020).

Adding to all this variability, there are also large sex differences in many psychiatric disorders, including AUD, and despite the recent upsurge in clinical and preclinical studies including both males and females, sex differences in AUD remain largely understudied (Verplaetse et al., 2021). Taking into account that sex influences behavior, response to stress/ELS and/or drugs, there is a clear need for studying both sexes in this field (Ciccocioppo, 2017). Preclinical studies with animal models, which serve as an excellent tool in psychiatric research, should include females despite the increased sample size that would be required for each experiment, to enable the delineation of sex-attributable genetic and epigenetic signatures (Nestler et al., 2016, Ciccocioppo, 2017). Taken together, it is evident that the field of alcohol research should explore new directions to identify novel therapeutics, taking into account sex, maltreatment history, polysubstance use and comorbidity with other psychiatric illnesses, tailored to specific patient subgroups that may share *eco-* or *endophenotypes* reflecting differential responses to pharmacological treatments.

Pharmacological studies on animal models that replicate specific alcohol-related phenotypes are important, but caution should be taken in regards to their predictive validity for drug efficiency in humans (Cannella et al., 2019). To that end, neuroimaging studies have started to shed light on the effect of ELS on the human brain (Teicher et al., 2016) and addiction-related phenotypes (Koob and Volkow, 2016), including AUD (Verplaetse et al., 2021). For example, brain imaging techniques can be used during human laboratory experiments of alcohol consumption to clarify the neurobiological correlates of different alcohol-related phenotypes (e.g., craving or relapse) and to determine subgroups of patients that share *endophenotypes* reflecting differential responses to pharmacological treatments (Cannella et al., 2019). Such techniques can be non-invasive, like magnetic resonance imaging (MRI) or functional MRI, or make use of positron emission tomography (PET) markers, which are radioactively labelled molecules (i.e., radiotracers) that measure receptors and other chemicals, as well as fluctuations in neurotransmitter levels, in the living brain. To date, dopaminergic and opioid PET markers have been widely used in AUD research (Koob and Volkow, 2016), but glutamatergic PET markers are still limited, and alcohol-related effects are understudied (Moeller et al., 2016, Fu et al., 2019). The development of PET tracers targeting the glutamatergic system, including the VGLUTs, applied to both animals and humans, would provide valuable information on disease development, diagnosis and therapeutic intervention. In conclusion, there are many potential research pathways within the AUD field. A multimodal approach considering all the above-mentioned variability will help to delineate the complex nature of AUD and, it is hoped, will take us a step forward in improving the lives of all those individuals who suffer from this deleterious disorder.

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