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GENE-ENVIRONMENT INTERACTIONS BETWEEN HPA AXIS REGULATORY GENES AND STRESSFUL LIFE EVENTS IN SUICIDE ATTEMPTS

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ABSTRACT

Suicide is a leading cause of death. In the future, treatment for suicidal behavior, as well as public health prevention and/or intervention efforts, may be guided by genetic epidemiology. This research is informed by biological alterations which have been previously observed in suicidal behavior. Such alterations include dysregulation of the cortisol response to psychosocial stress, which is in large part mediated by the hypothalamic-pituitary-adrenal (HPA) axis. HPA axis functioning is influenced e.g. by heritable variation in regulatory genes, by exposure to stressful life events (SLEs), and/or by gene-environment interactions (G x Es) of regulatory genetic variants with SLEs. Using a family-based design of proband offspring who have made a suicide attempt (SA) and their parents, we tested and characterized associations of transmitted variants in candidate genes that regulate HPA axis activation, as well as G x Es of these variants with SLEs, with SA and secondary outcomes.

In Study 1, we showed a G x E between a variant in corticotropin releasing hormone receptor type-1 (*CRHR1*), a gene which has a major role in mediating HPA axis activation, and physical assault in childhood/adolescence. We further observed a novel and independent G x E between another unlinked variant and physical assault in adulthood. These findings confirmed and extended previous findings on adulthood depressive symptoms, cortisol response, and alcohol misuse by other groups.

In Study 2, we investigated 98% of currently known common single nucleotide polymorphisms (SNPs) and certain low-frequency SNPs in serotonin receptor type-2a (*HTR2A*), a gene involved in serotonergic system modulation of HPA axis activation, and showed novel genetic linkage/association of a promoter SNP and a low frequency exon 2 SNP, as well as a novel G x E between a well-studied exon 1 SNP and exposure to cumulative lifetime SLEs. We further characterized this G x E, revealing a complex parent-of-origin effect in females, which may partly explain inconsistent findings in the literature.

In study 3, we investigated twenty-four genes in the glutamatergic, γ-aminobutyric acid (GABA)-ergic, and polyaminergic systems that are known to link several brain areas involved in emotional processing with HPA axis activation. We showed linkage/association of 3 SNPs in an *N*-methyl-D-aspartate (NMDA) receptor subunit type-2B gene (*GRIN2B*), and 2 SNPs and 1 haplotype in a gene which codes for a rate limiting enzyme in polyamine biosynthesis (*ODC1*). A G x E was also observed between another *ODC1* SNP and physical assault in childhood/adolescence.

In study 4, we investigated 100% of currently known common SNPs and a low frequency SNP in arginine vasopressin receptor type-1B (*AVPR1B*), a gene involved with *CRHR1* in partially overlapping roles in HPA axis activation. We showed linkage/association of two SNPs and a corresponding major allele haplotype across the gene predominantly on

current depressive symptoms in SA. Interestingly, we found no evidence of a G x E between *AVPR1B* variants and SLEs, or any gene-gene interaction effects with *CRHR1* variants.

In all studies, the findings were complemented with case-control re-analysis of SA offspring with healthy volunteers; characterized with SA-concomitant outcomes, as well as lifetime psychiatric diagnoses, and/or other descriptive variables; and discussed with regard to previous findings by our group or others, and to potential mechanistic roles. These findings support a stress-diathesis model of suicidal behavior, and a potential etiological role in the suicidal process for novel genetic variants and variants that have previously associated with stress-related HPA axis dysregulation, maladaptive behaviors and neuropsychiatric outcomes. The strength of these results is supported by the family-based design, which is robust to population substructure, and relatively comprehensive investigations of genetic variation, signaling pathway, and/or G x E. Further investigation and consistent replication across samples are warranted before utility in clinical diagnosis, treatment and/or public health efforts.

LIST OF PUBLICATIONS

I. Ben-Efraim YJ, Wasserman D, Wasserman J, Sokolowski M (2011) Gene–environment interactions between *CRHR1* variants and physical assault in suicide attempts. *Genes, Brain, and Behavior* **10**(6): 663-72.

II. Ben-Efraim YJ, Wasserman D, Wasserman J, Sokolowski M (2012) Family-based study of *HTR2A* in suicide attempts: observed gene, gene x environment and parent-of-origin associations. *Molecular Psychiatry* in press.

III. Sokolowski M, Ben-Efraim YJ, Wasserman J, Wasserman D (2012) Glutamatergic *GRIN2B* and polyaminergic *ODC1* genes in suicide attempts: associations and gene-environment interactions with childhood/adolescent physical assault. *Molecular Psychiatry* in press.

IV. Ben-Efraim YJ, Wasserman D, Wasserman J, Sokolowski M (2012) Family-based study of *AVPR1B* association and interaction with stressful life events and *CRHR1* on depression and anxiety in suicide attempts. *Submitted manuscript*.

CONTENTS

LIST OF ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
AP	Attributable proportion of risk
AVP	Arginine vasopressin
AVPR1B	AVP receptor type-1B gene
BDI	Beck depression inventory
CI	Confidence interval
CIDI	Composite international diagnostic interview
CLR	Conditional logistic regression
CRH	Corticotropin releasing hormone
CRHBP	CRH binding protein gene
CRHR1	CRH receptor type-1 gene
DSM	Diagnostic and statistical manual for mental disorders
FBAT	Family-based association test
FDR	False discovery rate
FKBP5	FK506 binding protein 5 gene
GABA	γ-aminobutyric acid
GISS	Genetic investigation of suicide and suicide attempt
GRIN2B	NMDA receptor subunit type-2B gene
GWAS	Genome wide association study
G x E	Gene x environment interaction
G x G	Gene x gene interaction
HBAT	Haplotype FBAT
HPA	Hypothamalmic-pituitary-adrenal
HTR2A	Serotonin receptor type-2a gene
HV	Healthy volunteer
ICD-10	International classification of diseases tenth revision
LD	Linkage disequilibrium
MAOA	Monoamine oxidase A
MC2R	Melanocortin 2 receptor gene
MDRS	Medical damage rating scale
NEO PI-R	Neuroticism, extraversion, and openness personality inventory-revised
NMDA	*N*-methyl-D-aspartate
NR3C1	Glucocorticoid receptor gene
ODC1	Ornithine decarboxylase gene
OR	Odds ratio
PKU	Phenylketonuria
POMC	Proopiomelanocortin gene
PTSD	Post-traumatic stress disorder
PVN	Paraventricular nucleus of the hypothalamus
RERI	Relative excess risk due to interaction
SA	Suicide attempt(er)
SLC6A4	Serotonin transporter gene
SLE	Stressful life event

SNP	Single nucleotide polymorphism
SSAT	Spermidine/spermine N(1)-acetyltransferase
TDT	Transmission disequilibrium test
WHO	World health organization

1 OUTCOME DEFINITIONS AND DESCRIPTIONS

1.1 SUICIDE ATTEMPT (SA)

While multiple definitions of suicide attempt (SA) exist, at least two standard definitions are in widespread usage. According to the World Health Organization (WHO) (Platt *et al*, 1992), the definition of a SA is "an act with a nonfatal outcome, in which an individual deliberately initiates a non-habitual behaviour that, without intervention from others, will cause self-harm, or deliberately ingests a substance in excess of the prescribed or generally realized therapeutic dosage, and which is aimed at realizing changes which the subject desired via the actual or expected physical consequences." The definition used by the Food and Drug Administration (FDA, 2012), which sets guidelines for outcome assessment in clinical trials, was taken from the Columbia Classification Algorithm of Suicide Assessment (Posner *et al*, 2007), which states a SA is "a potentially self-injurious behavior, associated with at least some intent to die as a result of the act. Evidence that the individual intended to kill him- or herself, at least to some degree, can be explicit or inferred from the behavior or circumstance. A *SA* may or may not result in actual injury." In research, this definition varies between studies, e.g. with regard to intent and physical consequences. For this thesis, medical professionals were trained by the study coordinator in the Ukraine to assess SA according to the WHO definition. As described in Chapter 6.2, the inclusion criteria used for proband collection included a further criterion that the SA should have serious physical consequences. During the analysis stage, the SAs were parsed into more homogenous subgroups according to measures of SA severity, various neuropsychiatric outcomes, as well as clinical and behavioral characteristics. We did not ascertain on intent according to the Columbia Classification Algorithm, however, a clinical intent scale was subsequently included among these subgroup assessment measures.

1.2 COMMON PSYCHIATRIC CO-MORBIDITY

Suicide is accompanied by co-morbid psychiatric disorder in at least 90% of cases (Lönnqvist, 2009). Conversely, by far the vast majority of persons with these disorders do not make a SA (Brent and Mann, 2005). Briefly, this can be explained by a complex, multifactorial process in psychiatric pathogenesis that involves probabilistic factors rather than deterministic causes. The factors underlying specific outcomes, e.g. unique and overlapping symptoms in distinct psychiatric disorders (Figure 1), may include unique and overlapping genes, environmental exposures, and gene-environment interactions (G x Es). Thus, while psychiatric disorders are commonly understood to be intricate pathways in the suicidal process and important factors that increase suicidal risk (Table 1), they clearly do not completely explain a large component of the pathogenesis of suicidal behavior, as will be explained further in Chapter 3: Genetic epidemiology of SA.

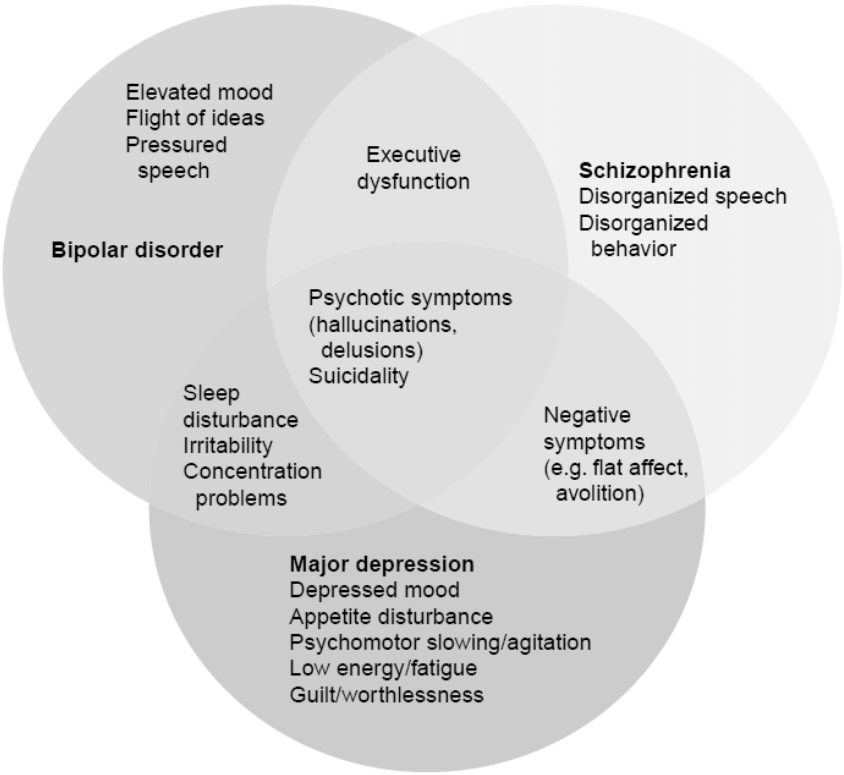


Figure 1. An illustration of symptom overlap between diagnoses of mood disorder (major depressive disorder and/or bipolar disorder) and schizophrenia. Distinct and overlapping phenotypic features of major psychiatric syndromes. Reprinted from *Curr Opin Genet Dev* **14**, Bearden *et al*, Why genetic investigation of psychiatric disorders is so difficult, Pages No. 280-286, (Copyright © 2004) with permission from Elsevier.

The following sections are devoted to describing the symptoms of a selection of some of the most prevalent psychiatric disorders in suicidal behavior (American Psychiatric Association, 2003), i.e. depressive and anxiety disorders; alcohol and/or drug use disorders; schizophrenia and other psychoses; and Cluster B personality disorders. The symptoms are described according to diagnostic guidelines from the WHO's International Classification of Diseases - 10th edition (ICD-10) section on Classification of Mental and Behavioral Disorders (World Health Organization, 1993). These are likely to change somewhat in 2013 with the expected release of the fifth edition of the American Psychiatric Association's Diagnostic and Statistical Manual of Mental Disorders (DSM) (http://www.dsm5.org), which will be closely related with the eleventh edition of the ICD (Kupfer *et al*, 2008), expected in 2015.

Table 1. Risk of suicide in persons with previous SAs and selected mental disorders based on a meta-analysis of 249 reports published between 1966-1993 (Harris and Barraclough, 1997). The standardized mortality ratio by suicide for the general population is 100. *Adapted with permission from Harris and Barraclough (1997) Br J Psych* ***170****:205-228.*

Mental disorder	Standardized mortality ratio	95% CI
Suicide attempt	3836	3403-4308
Major depression	2035	1827–2259
Bipolar disorder	1505	1225–1844
Schizophrenia	845	798–895
Personality disorders	708	477–1010
Anxiety disorders	629	533–738
Alcohol or substance use disorders	574	541–609
Organic disorders	332	293-375

Certain caveats are in order. Not all of the following symptoms are necessary for a diagnosis and therefore the following descriptions should not be interpreted as complete diagnostic guidelines. The diagnostic guidelines have additional instructions e.g. with regard to onset, duration, and severity of symptoms, and whether they should overlap with other diagnostic categories, e.g. drug-use induced psychoses. Furthermore, the following diagnostic categories may themselves be comorbid, and this mandates a new diagnosis, e.g. schizoaffective disorder, or multiple diagnoses. Even if multiple disorders are not diagnosed, the core symptoms of distinct diagnoses may be overlapping e.g. loss of pleasure (anhedonia) in depression and schizophrenia. Therefore, the following subsections should be read as a basic description of observable clinical and behavioral characteristics that may or may not be present in commonly comorbid psychiatric disorders in suicidal behavior. We intend to provide the uninitiated reader with a selection of the vast range of possible combinations of psychiatric outcomes underlying the diagnoses that associate with suicidal behavior, according to current diagnostic categories. It is noteworthy that a major criticism of the current diagnostic framework has been that diagnostic distinctions may not accurately represent underlying biology and genetics (Insel *et al*, 2010), and this criticism has influenced the proposal of a new framework for diagnostic classification based on coupling psychiatric domains and constructs to dysfunction in underlying neural circuits (Insel *et al*, 2010). That said, however, the present diagnostic framework also has certain advantages which will

continue to support its utility for the foreseeable future e.g. international inter-rater reliability (Regier *et al*, 2009).

1.2.1 Depressive and anxiety disorders

The following is a description of depressive and anxiety disorders, according to the ICD-10 (World Health Organization, 1993). Depressive symptoms include sustained depressed mood; loss of interest or pleasure (anhedonia); reduced energy leading to increased fatigability and diminished activity; reduced concentration and attention; reduced self-esteem and self-confidence; change in psychomotor activity, with agitation or retardation (either subjective or objective); disturbed sleep; change in appetite (decrease or increase) with corresponding weight change; and ideas or acts of self-harm or suicide. Anxiety symptoms include apprehension; motor tension; and autonomic hyperactivity. Anxiety disorders typically involve fearful reactions to environmental stressors including immediate reactions to specific phobias, but may also occur within 1 month of exposure to the stressor or more e.g. intrusive memories of traumatic events in post-traumatic stress disorder (PTSD). The stressor, however, may not be restricted to any particular situation or set of circumstances as in panic disorder, or may be the patient's own repetitive and irresistible thoughts or acts as in obsessive-compulsive disorder. Depression and anxiety are often co-morbid, and depression may also follow anxiety in quick succession. Anxiety disorders are present in about 11% of suicides, but true estimates may be distorted by co-morbidity with depression and/or other disorders, e.g. alcohol use disorders (American Psychiatric Association, 2003). Therefore, it's likely that the significance of suicide in anxiety disorders has, until now, been underestimated (Lönnqvist, 2009).

1.2.2 Alcohol and substance use disorders

Alcohol and/or substance use disorders are observed in about 25-50% of suicides (American Psychiatric Association, 2003; Lönnqvist, 2009). The following is a description of harmful use or dependence of alcohol and substances, according to the ICD-10 (World Health Organization, 1993). Harmful use suggests the alcohol or drug was responsible for (or substantially contributed to) physical or psychological harm that is clearly identifiable; and a persistent pattern of use. Dependence involves sustained strong desire or sense of compulsion to take the alcohol/substance; impaired capacity to control alcohol/substance-taking behavior; a physiological withdrawal state when alcohol/substance use is reduced or ceased; evidence of tolerance to the effects of the alcohol/substance; preoccupation with use; and persisting with use despite clear evidence of harmful consequences.

1.2.3 Schizophrenia and other psychoses

The following is a description of schizophrenia, according to the ICD-10 (World Health Organization, 1993). No uniquely characteristic symptoms can be identified, but symptoms are grouped according to special

importance for the diagnosis and because they often occur together, such as thought echo, thought insertion or withdrawal, and thought broadcasting; delusions of control, influence, or passivity, clearly referred to body or limb movements or specific thoughts, actions, or sensations, or delusional perception; hallucinatory voices giving a running commentary on the patient's behavior, or discussing the patient amongst themselves, or other types of hallucinatory voices coming from some part of the body; persistent delusions of other kinds that are culturally inappropriate and completely unrealistic; persistent hallucinations in any modality, when accompanied either by fleeting or half-formed delusions without clear affective content, or by persistent over-valued ideas, or when occurring every day for weeks or months on end; breaks or interpolations in the train of thought, resulting in incoherence or irrelevant speech, or neologisms; catatonic behavior, such as excitement, posturing, or waxy flexibility, negativism, mutism, and stupor; "negative" symptoms such as marked apathy, paucity of speech, and blunting or incongruity of emotional responses, usually resulting in social withdrawal and lowering of social performance; a significant and consistent change in the overall quality of some aspects of personal behavior, manifest as loss of interest, aimlessness, idleness, a self-absorbed attitude, and social withdrawal.

Mood symptoms may be present such as in depression, anxiety, or bipolar disorder, a mood disorder which is diagnosed in part by at least one episode of mania. Mania is an elevation in mood, and an increase in the quantity and speed of physical and mental activity, and may be accompanied by hallucinations and delusions (World Health Organization, 1993). As previously mentioned, in such cases where psychotic and mood symptoms are present to varying degrees, diagnoses may include e.g. schizoaffective disorder, depression or mania with psychotic symptoms, etc. The diagnostic differentiation of these disorders is a common problem (World Health Organization, 1993). Therefore, in research, individuals with these symptoms are often categorized as having psychoses, and schizophrenia and bipolar disorder categorized as major psychoses, as indicated by the presence of hallucinations, delusions, or a limited number of severe abnormalities of behavior, such as gross excitement and hyperactivity, marked psychomotor retardation, and catatonic behavior (World Health Organization, 1993).

1.2.4 Cluster B personality disorders

One-third to one-half of suicides have a personality disorder (American Psychiatric Association, 2003), while this is observed in 55-70% of SA (Stanley and Jones, 2009). Certain personality disorders belonging to Cluster B (dissocial, borderline, narcissistic, and histrionic personality disorders) are commonly observed in SA and completed suicide. Among these disorders, dissocial and borderline personality disorders are observed most frequently. The personality traits common in these disorders are dramatic, emotional, and erratic behavior (Stanley *et al*, 2009), including impulsivity, anger, aggression (including violent behavior).

The following is a description of dissocial and borderline personality disorders, according to the ICD-10 (World Health Organization, 1993). Specific traits that are observed in dissocial personality disorder include callous unconcern for the feelings of others; gross and persistent attitude of irresponsibility and disregard for social norms, rules, and obligations; incapacity to maintain enduring relationships, though having no difficulty to establish them; very low tolerance to frustration and a low threshold for discharge of aggression, including violence; incapacity to experience guilt, or to profit from adverse experience, particularly punishment; marked proneness to blame others, or to offer plausible rationalizations for the behavior bringing the subject into conflict with society; conduct disorder often present during childhood.

Borderline personality disorder involves a marked tendency to act unexpectedly and without consideration of the consequences, or to quarrelsome behavior and to conflicts with others, especially when impulsive acts are thwarted or criticized; liability to outbursts of anger or violence, with inability to control the resulting behavioral explosions; difficulty in maintaining any course of action that offers no immediate reward; unstable and capricious mood.

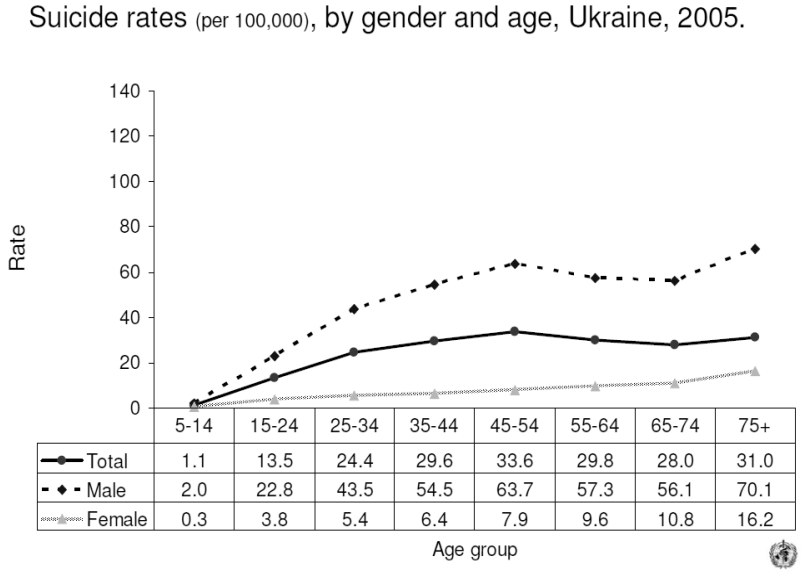
1.3 OTHER CORRELATED OUTCOMES

While the previously mentioned psychiatric disorders are common, additional psychiatric disorders and neuropsychiatric outcomes also have increased risk for suicidal behavior (American Psychiatric Association, 2003; Lönnqvist, 2009). The following outcomes are also selected because they are commonly observed in suicidal behavior, and a comprehensive description is provided elsewhere (Wasserman and Wasserman, 2009b). By far the greatest predictor of future suicide is a personal history of previous SA (Table 1). A family history of suicidal behavior also increases risk with patterns of aggregation suggesting genetic risk (Tidemalm *et al*, 2011). This correlation will be further explained in Chapter 3: Genetic epidemiology of SA. In addition to alcohol use disorders, alcohol intoxication has been shown in 20-50% of suicides, and estimated to occur in more than 50% of SAs (American Psychiatric Association, 2003). Additional SA-concomitant severity measures e.g. violent method (Runeson *et al*, 2010), are known to be strong predictors of future suicide. SA may thus also be viewed in a dimensional framework rather than as a categorical outcome, similarly to psychiatric disorders (Kraemer, 2007).

Suicidal behavior is observed to be correlated with many other outcomes that are suspected of playing a role in pathogenesis of suicidal acts (American Psychiatric Association, 2003; Lönnqvist, 2009). These may include psychological features, e.g. hopelessness, and neurocognitive deficits, especially those related to attention (American Psychiatric Association, 2003). Somatic diseases and other mental illness (neurological disorders, traumatic brain injuries, etc.) are also positively correlated with SA through e.g. coping responses that may lead to mood disorder or organic

changes that may affect emotional processing and decision making (Stenager and Stenager, 2009).

Approximately one million people commit suicide each year (World Health Organization, 2002), and it is estimated that at least ten times as many people per year make a SA (Bertolote *et al*, 2009). Suicide rates are highly variable according to geographic region, sociodemographic and cultural factors, etc. For example, suicide is more common in males (Figure 2), while SA is more common in females. Depressive and anxiety disorders are more common in females, while sex differences are observed for certain symptoms of all the previously mentioned disorders (World Health Organization, 1993). The first population level manifestation of suicidal behavior typically occurs in early adolescence (Figure 2). Age of onset of underlying psychiatric outcomes is highly variable with depressive disorders starting in adolescence while psychotic symptoms or first manic episode may occur in late 20s or up to 40s, respectively (World Health Organization, 1993). In general, rates of suicidal behavior are highly variable according to country of origin and fluctuate over time according to many factors e.g. geopolitical events, economic crises, etc. (World Health Organization, 2002). Since it is relevant for this thesis, which focuses on an the Ukrainian population (Chapter 6: Methods), it is notable that a J-curve from Finland in the northeast to Slovenia in the southwest that includes the Ukraine contains the highest suicide rates in Europe (Marusic, 2005; World Health Organization, 2002), possibly explained by geographic as well as shared sociocultural factors, though also potentially by genes and alcohol misuse (Marusic, 2005).



	5-14	15-24	25-34	35-44	45-54	55-64	65-74	75+
Total	1.1	13.5	24.4	29.6	33.6	29.8	28.0	31.0
Male	2.0	22.8	43.5	54.5	63.7	57.3	56.1	70.1
Female	0.3	3.8	5.4	6.4	7.9	9.6	10.8	16.2

Figure 2. Suicide rates vary according to age and sex: an example from the Ukraine. As is typical in most populations, peaks are present in adolescence and in old age, and males have a higher rate of completed suicide than females. Source: WHO suicide statistics, country reports (World Health Organization, 2002).

2 NEUROBIOLOGY AND GENETICS OF SUICIDAL BEHAVIOR

Explanations of suicidal behavior have been proposed in social, psychological, psychiatric and other disciplines (Mäkinen *et al*, 2009), and may further vary according to political and sociocultural determinants (Wasserman *et al*, 2009b). However, the integration of all proximal and distal factors into the decision to make a SA, or any decision or behavior for that matter, can be viewed as ultimately being mediated by neurobiological processes. While the neurobiological correlates and genetics of SAs may differ according to the definition of SA, suicidal behavior is generally part of the same clinical phenotype as completed suicide (Brent *et al*, 2005). Suicidal ideation of course also involves neurobiology, but it is not clear that all SAs involve prior ideation, which may be more closely related to depression (Brent *et al*, 2005). If SA and completed suicide are in the same clinical spectrum, the proximity of severe SAs to completed suicide at the extreme end of the spectrum may imply common underlying neurobiological correlates and genetics (Lander and Schork, 1994).

While gross anatomical abnormalities are not observed in suicidal behavior (with the exception of organic disorders), certain structural and neurochemical changes have been observed in SAs and in the postmortem brain tissue of suicide victims. Morphological changes observed in postmortem brain tissue and in structural brain imaging include volumetric changes in limbic areas e.g. reduction in frontal cortical gray matter density and increase in amygdala volume (van Heeringen *et al*, 2011). Neurochemical abnormalities have been shown e.g. in studies using ligand binding (an indicator of the membrane expression and density of receptors which bind the ligand) and immunoreactivity (a measure of biochemical changes within cells that can also be used as a marker for characterizing neuronal cell types) (Mann, 2003). These have been described most thoroughly for monoamine systems, lipid metabolism, neurotrophins and stress system hormones, though other systems that are involved include e.g. glutamate, γ-aminobutyric acid (GABA), polyamines, opioids, acetlycholine, neurotrophins, cannabinoids, intracellular signal transduction mechanisms, and several others (Mann, 2003; Wasserman *et al*, 2009a). Many changes in systemic neurochemistry mediate signaling in brain areas that are important for neurocognition, and these brain areas are further implicated in functional neuroimaging studies on neurocognitive outcomes in suicidal behavior (Jollant *et al*, 2011). Central systemic neurochemical abnormalities have also been observed outside the brain e.g. reduced levels of a serotonin metabolite in cerebrospinal fluid (Asberg *et al*, 1976) and increased cortisol levels in plasma in a dexamethasone suppression test (Coryell and Schlesser, 2001). Altogether, the evidence shows disruption in neural signaling and homeostasis across multiple systems and brain areas involved in clinical and behavioral

characteristics of suicidal behavior, e.g. dysregulation of stress response systems, emotional processing, decision making, aggression, etc.

Altered expression of specific genes may contribute to the neurochemical alterations observed. Gene expression studies in the postmortem brain tissue of suicide victims have implicated some of the same systems: glutamate and GABA (the primary excitatory and inhibitory neurotransmitters of the central nervous system, respectively), growth factors (involved in cell proliferation, differentiation, and survival), polyamines (involved in numerous essential cellular functions and stress responses at both the cellular and behavioral levels), etc. (Fiori and Turecki, 2012). However, other genes that have also been shown to have altered expression include those involved in presynaptic vesicle mediated neurotransmitter release, and those which are specific for astrocytes and oligodendrocytes (non-neuronal brain cells involved e.g. in development and homeostasis, and supporting and modulatory roles in synaptic neurotransmission) (Fiori *et al*, 2012). Epigenetics may mediate the effect of environmental exposures on gene expression, and epigenetic methylation changes have also been observed in the postmortem brain tissue of suicide victims for the glucocorticoid receptor gene (*NR3C1*) which is involved in stress hormone signaling (McGowan *et al*, 2009). Epigenetic changes have now been observed for several genes in postmortem brain tissue from suicide victims in association with changes in gene expression (Turecki *et al*, 2012). Most genes that were found to associate with suicidal behavior had already previously been shown in reverse genetic or association studies to play a role in mediating physiological and behavioral outcomes that may be important in the suicidal process (Wasserman *et al*, 2009a). However, a deterministic causal relationship of any gene with suicidal behavior has not been shown, as may perhaps be explained by a polygenic and multifactorial process of pathogenesis.

3 GENETIC EPIDEMIOLOGY OF SA

In comparison with completed suicide, studying the role of genetics in brains of SA individuals is limited by ethical and practical reasons. This may be overcome to a certain extent by e.g. using peripheral tissues such as platelets (Khait *et al*, 2005), and imaging genetics studies that aim to characterize the role of candidate genes in neural circuit dysfunction in SA (Desmyter *et al*, 2011). However, genetic epidemiology may use additional approaches to discover and characterize genes involved with suicidal behavior, such as their variation across the population, and how they interact with other factors, e.g. environment (Thomas, 2004a). Since genes may be mediators of SA and/or moderators of the effect of other genes or stressful life events (SLEs) on SA (Kraemer *et al*, 2001), characterizing interaction effects is particularly important. This approach aims to describe the association of genetic variants with SA using associative measures of occurrence. Based on consistency and magnitude of risk estimates across studies, it may be possible to infer causal mechanisms that can later be confirmed with reverse genetic and mechanistic research. Conversely, associations with SA may suggest a role for functional genetic variants that were previously identified in reverse genetic and mechanistic research as further being important in the suicidal process.

The heritability of suicidal behavior has been established by twin, adoption, and family studies (Brent and Melhem, 2008; Voracek and Loibl, 2007). Between 38-55% of population variance for suicide and SA is attributable to genetic factors (Fu *et al*, 2002; Glowinski *et al*, 2001; Statham *et al*, 1998), an effect that is persistent even after adjusting for co-morbid psychiatric disorder and other risk factors (Brent *et al*, 2005; Fu *et al*, 2002). Familial loading is stronger in families with an earlier onset of suicidal behavior, suggesting a stronger genetic component within these families (Brent *et al*, 2005). Environment and personality traits may also be heritable and increase the risk for suicidal behavior (Brodsky and Stanley, 2008). A unique heritable component of suicidal behavior has been shown (Brent *et al*, 2005) and may be the attempt, itself, or rather "the tendency to act upon suicidal thoughts" (Mann *et al*, 1999).

Despite the unique genetic component, it is difficult to separate the heritability of SAs from comorbid psychopathology. Because of the polygenic nature of psychiatric genetics and pleiotropic gene function, it is also possible that "a gene for suicidal behavior" does not exist (Kendler, 2005). As for other neuropsychiatric outcomes, the genes that contribute specifically to suicidal behavior are currently unknown. Neuropsychiatric outcomes themselves have highly complex genetic components that may lead to SA via unique pathogenic pathways (Wasserman *et al*, 2010). Thus, the genetics of SAs can be partially explained by the genetics of distinct or comorbid psychiatric pathologies, as well as certain personalities, which precede all SAs to a certain degree with few exceptions.

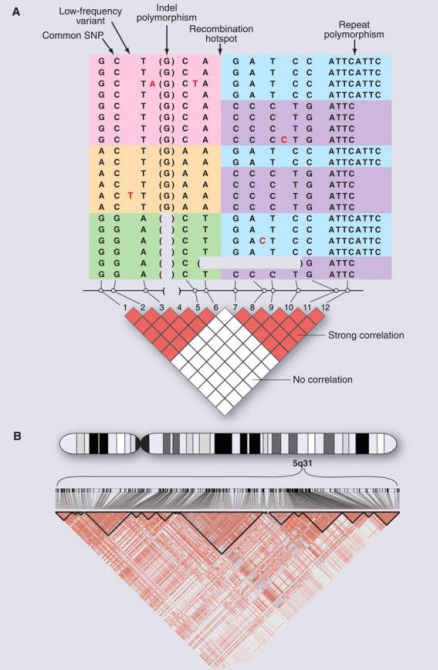


Figure 3. Genetic variants and linkage disequilibrium (LD). A) Different types of genetic variants, including single nucleotide polymorphisms (SNPs) and haplotypes, as studied here. Common haplotypes are identified by colors B) LD map showing regions of strong correlation (red), i.e. high LD, and no correlation (white) between SNPs in a closeup of chromosomal region 5q31. Reprinted from Altshuler et al. (2008) Genetic Mapping in Human Disease, *Science* **322**: 881-888. Reprinted with permission from AAAS.

One of the principal aims of genetic epidemiology is to discover genetic variants that predispose one to disease. This is accomplished by linkage studies, positional cloning, etc. that attempt to narrow the position of a causal variants by co-transmission ("linkage disquilibrium"; LD) with known markers in the genome since high LD indicates markers are close (Figure 3) (Thomas, 2004b). In comparison with Mendelian disorders, which show strong linkage peaks at one or few positions, psychiatric disorders are observed to have multiple peaks of smaller magnitude spread out throughout the genome. This indicates that they are polygenic, and that heritability cannot be explained by Mendelian genetics, i.e. they are complex disorders. Further factors that may explain complexity include interactions with environment and other genes, as well as by the heterogeneity of the psychiatric disorders *per se*.

To counteract the problems of psychiatric heterogeneity, identifying genetic variants that underlie heritability of complex diseases and behaviors such as SAs may be aided by studying heritable, intermediate phenotypes, i.e. "endophenotypes" (Gottesman and Gould, 2003) and severity measures (Lander *et al*, 1994). Endophenotypes such as cortisol response and aggression may involve genes that are involved in certain biological systems, i.e. the hypothalamic-pituitary-adrenal (HPA) axis and serotonin (Asberg *et al*, 1976; Coryell *et al*, 2001; Merali *et al*, 2006; Nemeroff *et al*, 1988; Stanley *et al*, 1982). Certain severity measures may predict completed suicide among SA, e.g. a history of previous SA (Table 1), a SA with high lethality (Suokas and Lonnqvist, 1991), the method used (especially violent method) (Runeson *et al*, 2010), and precautions against discovery (Beck and Steer, 1989). Distinguishing

subtypes based on comorbidity and correlated outcomes (Chapter 1) may further help aggregate any overlapping genetic susceptibility.

Among endophenotypes of suicidal behavior (Table 2), the dysregulated cortisol response to psychosocial stress can be viewed as directly supporting a stress diathesis model of suicidal behavior (Mann *et al*, 1999). Cortisol response to psychosocial stress is mediated by genes of the HPA axis (Derijk, 2009), and is differentiated by sex (Uhart *et al*, 2006) as well as age (Lupien *et al*, 2009). G x Es of HPA axis genes and SLEs may explain variability in dysregulation of cortisol reactivity (Heim *et al*, 2009; Tyrka *et al*, 2009). Thus, genetic epidemiology research that includes such G x Es is a potentially valuable research tool to explain the role of specific genes involved in stress reactivity in SAs.

Table 2. Endophenotypes of suicidal behavior.
Reprinted from *Biological Psychiatry* **65**(7), Mann et al., Candidate endophenotypes for genetic studies of suicidal behavior, Pages No. 556-563, © 2009 with permission from Elsevier.

	Endophenotype Criteria				
	Association with Suicidal Behavior	Heritability (>20%)	State-Independent	Cosegregate in Families (Gene Association)	More Frequent in Nonaffected Relatives
Endophenotypes					
Aggression/Impulsivity	YES	YES	YES	YES	YES
Early-onset major depression	YES	YES	YES	YES	No data
Neurocognitive function	YES	YES	YES	YES	YES
Cortisol stress response	YES	YES	YES	YES	No data
Candidate Endophenotypes					
5-HT in postmortem brain	YES	No data	No data	Insufficient data	No data
CSF 5-HIAA	YES	YES	YES[a]	Insufficient data	No data
5-HT in vivo (imaging)	YES	No data	No data	No data	No data
Brain imaging (rCMRGlu)	YES	No data	No data	No data	No data
Second messengers	YES	No data	No data	No data	No data
BPD					
Interpersonal reactivity	No data	YES	YES	No data	No data
Affect dysregulation	YES	YES	YES	No data	No data

BPD, borderline personality disorder; CSF, cerebrospinal fluid; 5-HIAA, 5-hydroxyindoleacetic acid; 5-HT, serotonin; rCMRGlu, regional cerebral glucose metabolic rates.
[a]Animal studies.

3.1 ENVIRONMENTAL FACTORS

Certain SLEs are commonly associated with suicidal behavior. Exposure to traumatic SLEs during childhood/adolescence, in particular childhood sexual and physical abuse, are frequently observed in suicidality (Brodsky *et al*, 2008; Santa Mina and Gallop, 1998). Childhood sexual abuse, which is associated with feelings of shame or internal attributions of blame, may explain up to 20% of the population attributable risk for suicidal behavior in young people, along with intermediate outcomes of early-onset major depression, PTSD, and Cluster B personality disorders (Brodsky *et al*, 2008).

Many environmental risk factors for SA do not depend on the family environment (Brent *et al*, 2008). Although abuse related SLEs certainly occur within families, these types of SLEs may not necessarily be shared among offspring (if at all) as closely as more distal factors such as socioeconomic status, urban vs. rural residence, etc. Exposure to various SLEs throughout life, however, may act on a background of heightened environmental susceptibility. This susceptibility can be caused by repeated

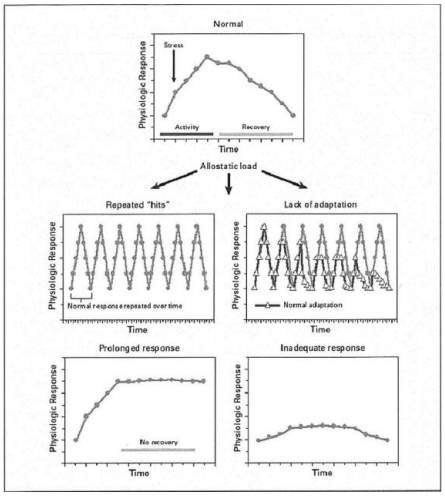


Figure 4. Three types of allostatic load. The top panel illustrates the normal allostatic response, in which a response is initiated by a stressor, sustained for an appropriate interval, and then turned off. The remaining panels illustrate four conditions that lead to allostatic load: repeated "hits" from multiple stressors; lack of adaptation; prolonged response due to delayed shutdown; and inadequate response that leads to compensatory hyperactivity of other mediators. Reproduced with permission from McEwen (1998) Protective and Damaging Effects of Stress Mediators *N Eng J Med* **338**:171-179, Copyright Massachusetts Medical Society.

exposure to SLEs that are less severe than childhood abuse, but that nevertheless may wear down the body's homeostatic mechanism of responding to stress (McEwen, 2007). This breakdown in homeostasis is called allostasis, and can be caused by genetic factors in addition to SLEs of varying dimensions (Figure 4).

Environmental risk factors may be offset by protective factors. These may include proximal factors such as social support within one's personal network, or distal factors such as public health policy that limits access to alcohol or suicidal means, e.g. pesticides. Additionally, exposures which were previously explained as risk factors, e.g. SLEs and alcohol, may be protective in other contexts. For example, exposure to levels of mild stress which do not create an allostatic overload may prime the body's ability to deal with larger threats, while alcohol may moderate the effects of stress on suicidal behavior differently in Mediterranean vs. Easter European countries.. On a genetic level, differential responses to risk and protective environmental factors may be supported by alleles that confer susceptibility to the overall environmental allostatic load, rather than vulnerability to certain risk factors *per se* (Belsky *et al*, 2009).

The context of environmental exposure may be measured with several dimensions. These may include severity, frequency, duration, and relationships for interpersonal environmental factors (Brodsky *et al*, 2008). Ultimately, it is how helpful vs. stressful an environmental factor is perceived which moderates its effect on risk for SA. This perception differs for most individuals, and thus environmental factors are notoriously difficult to standardize for research, though may be more reliable across studies for acute, severe SLEs (Caspi *et al*, 2010).

3.2 GENETIC FACTORS

Candidate genetic variant and G x E associations on suicidal behavior were previously summarized (Wasserman *et al*, 2009a). More recently three

genome wide association studies (GWAS) on SA (as well as ideation) have been published in mood disorder subjects (bipolar disorder or depression), though no variant associations passed significance after correction for multiple comparisons or an attempt to replicate in a second cohort (Perlis *et al*, 2010; Schosser *et al*, 2011; Willour *et al*, 2012). All studies concluded that larger samples are necessary, while none of the studies used a heterogeneous sample of SA as in this thesis (Chapter 6: Methods).

3.2.1 Hypothalamic-pituitary-adrenal (HPA) axis

Many HPA axis genes have been discovered by expression level changes in tissue samples from completed suicides. These reflect genes that mediate HPA axis signaling at the level of the paraventricular nucleus of the hypothalamus (PVN), anterior pituitary corticotropes, adrenal medulla, and limbic feedback circuits which regulate PVN excitability e.g. the hippocampus, or cortisol-mediated feedback mechanisms that directly attenuate PVN secretion of corticotropin releasing hormone (CRH) (Table 3). For parsimony, I shall designate these genes to be those involved in mediating secretion and reception of adrenocorticotripic hormone (ACTH) and cortisol. CRH and arginine vasopressin (AVP) in the PVN parvocellular neurons, genes that mediate the CRH receptor type-1 (CRHR1) and AVP receptor type-1B (AVPR1B) driven ACTH secretory mechanism via corticotrope innervation of anterior pituitary via the median eminence. CRHR1 and AVPR1B signal transduction mechanisms are not completely clear, but include downstream transcriptional effects, e.g. pro-opriomelanocortin, i.e. POMC, which encodes the precursor polypeptide in ACTH synthesis. ACTH, in turn, mediates the release of cortisol from the adrenal medulla via the melanocortin receptor type-2, MC2R (Figure 5). Negative feedback modulation of HPA axis signaling is then mediated directly at the level of the PVN or via inputs from many brain areas, e.g. limbic system. This is mediated by cortisol receptors, i.e. the highly sensitive mineralocorcticoid receptor and NR3C1, which is a transcriptionally active nuclear hormone receptor.

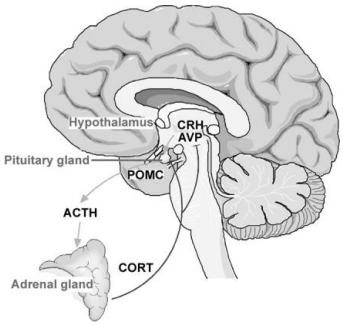


Figure 5. Signaling in the hypothalamic-pituitary-adrenal (HPA) axis. Source: Murgatroyd and Spengler (2011), Epigenetics of early child development, *Front Psychiatry* **2**:16. Copyright: © 2011 Murgatroyd and Spengler.

These and other genes that regulate HPA axis signaling have been reviewed for mood and anxiety disorders (Bao and Swaab, 2010; Binder and Nemeroff, 2010a) and alcohol use (Clarke *et al*, 2008), and are prime candidates to investigate for association with SA. *CRHR1* variants interacted with childhood maltreatment in association with non-suppression in a dexamethasone suppression /CRH combined test (Heim *et al*, 2009; Tyrka *et al*, 2009) in these disorders or in healthy volunteers

Table 3. Substances currently known to directly influence PVN parvocellular neuron excitability and synaptic function. Reprinted with permission from Wamsteeker and Bains (2010), A synaptocentric view of the neuroendocrine response to stress, Eur J Neurosci **32**(12): 2011-2021. Publisher: John Wiley and Sons. © (2010) The Authors. European Journal of Neuroscience © (2010) Federation of European Neuroscience Societies and Blackwell Publishing Ltd

Increase excitability	Decrease excitability
2-B40 (Oomura *et al.*, 2003) 5-HT (5HT2CR) (Heisler *et al.*, 2007) Acidic-FGF (Sasaki *et al.*, 1995) Adiponectin (Hoyda & Ferguson, 2010) Adrenomedullin (Follwell & Ferguson, 2002a) Angiotensin II (Latchford & Ferguson, 2005) CORT (Kasai *et al.*, 1988; Saphier & Feldman, 1988; Zaki & Barrett-Jolley, 2002) CRF (Qiu *et al.*, 2005) Ghrelin (Cowley *et al.*, 2003) Interleukin1B/PGE2 (Ferri & Ferguson, 2005) Leptin (Powis *et al.*, 1998) Noradrenaline (α1) (Kasai & Yamashita, 1988a; Yang *et al.*, 2007) Nesfatin (Price *et al.*, 2008) Neuromedin U (Qiu *et al.*, 2003) Neuropeptide W (Taylor *et al.*, 2005) Nitric oxide (Bains & Ferguson, 1997) Orexin A (Follwell & Ferguson, 2002b) Orexin B (Shirasaka *et al.*, 2001a) Oxytocin/vasopressin (Inenaga & Yamashita, 1986) Prokineticin (Yuill *et al.*, 2007) StresscopinRP (Davidowa & Plagemann, 2004)	CORT (Kasai & Yamashita, 1988b; Saphier & Feldman, 1988) GABA (GABA-AR) unstressed (Hewitt *et al.*, 2009) GABA (GABA-BR) (Wang *et al.*, 2003) Noradrenaline (α2) (Shirasaka *et al.*, 2007) Opioids (Pittman *et al.*, 1980; Shirasaka *et al.*, 2001b)
Alter glutamate	**Alter GABA**
CORT (Di *et al.*, 2003) CRH (NMDAR) (Kuzmiski *et al.*, 2010) Endocannabinoids (Di *et al.*, 2003; Wamsteeker *et al.*, 2010) GABA-BR (Wang *et al.*, 2003) Ghrelin (Kola *et al.*, 2008) Hypertonic saline (Chu *et al.*, 2010) Noradrenaline (Daftary *et al.*, 2000)	BDNF (Hewitt & Bains, 2006) CORT (Verkuyl *et al.*, 2005) Endocannabinoids (Wamsteeker *et al.*, 2010) GABA-BR (Wang *et al.*, 2003) MCR (Cowley *et al.*, 1999) Noradrenaline (Han *et al.*, 2002) NPFF VF (Jhamandas *et al.*, 2007) NPY (Cowley *et al.*, 1999) PGE2 (Ferri & Ferguson, 2005)

(HVs). *NR3C1* is furthermore a target of epigenetic modification, and was observed with methylation changes in the post-mortem hippocampus of suicide victims with a history childhood exposure to severe SLEs (McGowan *et al*, 2009).

Certain HPA axis genetic variants have associated directly with suicidal behavior, as previously described (Ben-Efraim *et al*, 2011). Genetic polymorphisms in *CRHR1*, a gene which has major roles in HPA-activation and which is expressed in pituitary, as well as several brain nuclei coupled to HPA axis functioning, are linked and associated with SA in depressed males (Wasserman *et al*, 2008). G x Es for CRHR1 have been shown for SA in depressed males exposed to low lifetime SLEs (Wasserman *et al*, 2009c), and gene-gene interaction (G x G) with the functionally coupled CRH binding protein gene, *CRHBP*, has been shown for suicidal behavior in schizophrenia (De Luca *et al*, 2010). A G x E of FK506 binding protein 5 gene (*FKBP5*), another HPA axis regulatory gene involved in moderating NR3C1 signaling, and childhood trauma has been shown to associate with SA (Roy *et al*, 2010).

CRH and AVP have synergistic roles in HPA axis activation (Scott and Dinan, 1998) and are implicated in certain intermediate outcomes of the suicidal process e.g. depressive and anxiety disorders (Bao *et al*, 2010; Binder *et al*, 2010a), alcohol or substance abuse (Clarke *et al*, 2008), and aggression (Veenema, 2009). In suicide post-mortem brain tissue, levels of AVP-immunoreactivity were increased in several brain regions including PVN (Merali *et al*, 2006). AVP secreted from PVN

potentiates stress-induced CRH stimulation of anterior pituitary ACTH secretion via AVPR1B (Antoni *et al*, 1984; Gillies *et al*, 1982; Sugimoto *et al*, 1994), and AVPR1B signaling is the main ACTH secretagog in animals models of certain stress paradigms (Roper *et al*, 2011). CRHR1 and AVPR1B are the only known mediators of ACTH secretion. AVPR1B antagonists are a target of drug development for stress-related disorders (Griebel *et al*, 2005; Roper *et al*, 2011). While *AVPR1B* antagonists are not yet available for *in vivo* human studies (Roper *et al*, 2011), intranasal AVP administration has shown a role for AVP in physiological responses to social stressors or perception of angry/threatening faces (Ebstein *et al*, 2009; Shalev *et al*, 2011; Thompson *et al*, 2004; Thompson *et al*, 2006), and is partially supported by *AVPR1B* antagonists or gene knockout in animal models linking stress and social behavior, e.g. aggression (Stevenson and Caldwell, 2012). *AVPR1B* variants were previously observed to alter risk for having stress-related disorders (Dempster *et al*, 2007; Leszczynska-Rodziewicz *et al*, 2012; van West *et al*, 2004; van West *et al*, 2009), but evidence in the context of pharmacogenetics (Binder *et al*, 2010b) or psychosocial stressors (van West *et al*, 2010) is currently lacking.

The role of the CRH and AVP systems in mediating HPA axis activation at the level of the hypothalamus and pituitary makes them primary targets for candidate genes. Additionally, G x Gs have previously been studied between variants of *AVPR1B* and *CRHR1*, with inconclusive results (Keck *et al*, 2008). AVPR1B and CRHR1 are also capable of physically interacting (Young *et al*, 2007), and heterodimerization affects their pharmacological properties *in vitro* (Murat *et al*, 2012), perhaps in part explaining synergism of AVP and CRH e.g. in pituitary signaling and HPA axis activation (Scott *et al*, 1998). Thus, the role of *AVPR1B* and *CRHR1* genetic variation in SA with regard to different types SLEs, sex, and age is potentially very interesting.

3.2.2 HPA axis modulation

HPA axis signaling is additionally modulated by innervating neurotransmitters, neuromodulators, neurotrophic factors, etc.. At the level of the PVN, the final site of integration for HPA axis activation, several such systems provide input from many brain areas to modulate excitability (Table 3) (Wamsteeker and Bains, 2010) and affect transcription of *CRH* and *AVP* (Kageyama and Suda, 2009). The serotonergic system has major inputs to the HPA axis at all levels (Lowry, 2002), influencing the secretion of CRH and AVP, ACTH, and cortisol, as well as limbic feedback circuits. Serotonergic system dysregulation has been consistently associated with suicidal behavior severity measures (Asberg *et al*, 1976; Pare *et al*, 1969) and the above-mentioned endophenotypes (Mann *et al*, 2009), and alteration in the level of a cerebrospinal fluid serotonin metabolite is one of the best known biological predictors of future suicide (Asberg *et al*, 1976; Mann and Currier, 2007).

Changes in brain expression of serotonin levels (Bourne *et al*, 1968; Pare *et al*, 1969; Shaw *et al*, 1967) and receptors (Stanley *et al*, 1982)

have long been observed in suicidality, including binding potential alterations *in vivo* (Audenaert *et al*, 2001) and in suicide post-mortem tissue (Stanley and Mann, 1983) of HTR2A, a serotonin receptor. The serotonin receptor type-2a (HTR2A) has long been associated with suicidal behavior (Stanley *et al*, 1982), but with inconsistent findings (Norton and Owen, 2005; Serretti *et al*, 2007). Positive associations with three common SNPs were found for HPA axis stress reactivity (Falkenberg and Rajeevan, 2010; Fiocco *et al*, 2007; Rosmond *et al*, 2002), brain expression of HTR2A, suicidality (Du *et al*, 2000) and outcomes related to the above-mentioned endophenotypes and severity measures: early-onset mood disorder (Burt and Mikolajewski, 2008; Dickel *et al*, 2007; Manchia *et al*, 2010; Zalsman *et al*, 2006), attention deficits (Fiocco *et al*, 2007; Quist *et al*, 2000; Ucok *et al*, 2007), alcohol dependence (Nakamura *et al*, 1999), impulsivity (Bjork *et al*, 2002; Preuss *et al*, 2001), aggression (Assal *et al*, 2004; Lam *et al*, 2004), and related personality traits in suicidality (Geijer *et al*, 2000; Giegling *et al*, 2006). Since these initial discoveries, subsequent studies have shown inconsistent associations (Norton *et al*, 2005; Serretti *et al*, 2007), though one meta-analysis found an association of rs6311 with suicidal behavior (Li *et al*, 2006). Given there are currently 170 verified SNPs in the *HTR2A* coding region (HapMap database, release 28) (The International HapMap Consortium, 2003), further exploration of linkage disequilibrium (LD) in this region is warranted in samples that are sufficiently powered.

Table 4. Some major sources of glutamatergic (Glu) and GABAergic innervation of medial parvocellular PVN.

Reprinted with permission from Herman et al. (2004) Role of GABA and glutamate circuitry in hypothalamo-pituitary-adrenocortical stress integration, *Ann. N.Y. Acad. Sci.* **1018:** 35–45. Publisher: John Wiley and Sons © 2004 New York Academy of Sciences

Region	Transmitters	Major Limbic Afferents[a]
SPZ	GABA	vSUB, il/plPFC, MeA, LS
PeriPVN	GABA	vSUB, il/plPFC, MeA, LS
BSTif	GABA	MeA, vSUB, il/plPFC
BSTtr	GABA	MeA, vSUB, il/plPFC
BSTam	GABA	MeA, vSUB, il/plPFC
BSTfu	GABA, CRH	CeA
POAm	GABA, some Glu	MeA, vSUB
LHA	GABA, Glu	il/plPFC, vSUB, MeA, LS
DMHvl	GABA, some Glu	CeA, vSUB
NTS	Glu	il/plPFC, CeA

[a] Limbic structures are listed in approximate order of the density of innervation to the respective PVN-projecting regions. See Refs. 15, 20, 32–34, 43, 44, 47, 51, 54, 57–62.

ABBREVIATIONS: SPZ, subparaventricular zone; vSUB, ventral subiculum; ilPFC, infralimbic region of the medial prefrontal cortex; plPFC, prelimbic region of the medial prefrontal cortex; MeA, medial amygdaloid nucleus; LS, lateral septum; BSTif, bed nucleus of the stria terminalis, intrafascicular subnucleus; BSTtr, bed nucleus of the stria terminalis, transverse subnucleus; BSTam, bed nucleus of the stria terminalis, anteromedial subnucleus; BSTfu, bed nucleus of the stria terminalis, fusiform subnucleus; CeA, central amygdaloid nucleus; POAm, medial preoptic area; LHA, lateral hypothalamic area; DMHvl, dorsomedial nucleus, ventrolateral region; NTS, nucleus of the solitary tract.

Alterations in other types of HPA axis modulating brain synaptic processes are indicated, for example, excitatory glutamate and inhibitory GABA signaling modulate HPA axis activation via a complicated network of inputs which further partially mediate cortisol-mediated negative feedback control (Table 4). Glutamate receptors are divided into into *N*-methyl-D-aspartate (NMDA) and non-NMDA receptors (NMDARs and non-NMDARs), and can be ionotropic and metabortopic. The glutamatergic system is furthermore modulated by polyamines, which may exert direct modulatory functions on ionotropic glutamate receptors (Figure 6) (Mony *et al*, 2009; Stromgaard and Mellor, 2004). Polyamines are important modulators of ion channels, cell-cell interactions, the cytoskeleton, signaling via phosphorylation, transcription, etc. (Pegg, 2009). Dysregulation of the glutamatergic system may occur in depressive and anxiety disorders (Gao and Bao, 2011; Hashimoto, 2009; Riaza Bermudo-Soriano *et al*, 2012), alcohol use disorders (McCool *et al*, 2010), and major psychoses (Cherlyn *et al*, 2010). Postmortem brain tissue alterations of glutamate and GABA receptors, and more recently polyamines, have been observed in suicide. Many of these genes are furthermore involved in polyamine biosynthesis (Figure 7) (Fiori *et al*, 2011).

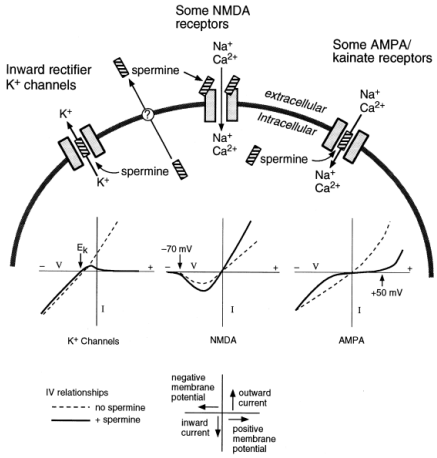


Figure 6. Polyamine effects on glutamate receptor signaling.
Reprinted from *Cell Signal* **9**(1), Williams, Modulation and block of ion channels: a new biology of polyamines, Pages No. 1-13, © 1997 with permission from Elsevier.

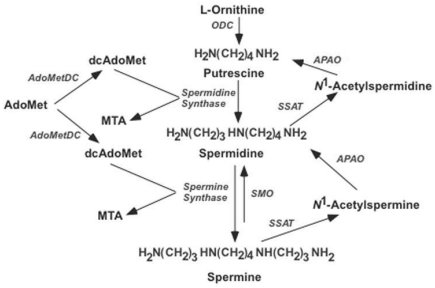


Figure 7. Polyamine biosynthesis. Ornithine decarboxylase (ODC) and S-adenosylmethionine decarboxylase (AdoMetDC) are the rate-limiting enzymes in polyamine biosynthesis while spermidine/spermine N(1)-acetyltransferase (SSAT) is the rate-limiting enzyme in catabolism.
Reprinted with permission from Pegg (2009) Mammalian polyamine metabolism and function, *IUBMB Life* **61**(9)**:** 880-894. Publisher: John Wiley and Sons. Copyright © 2009 International Union of Biochemistry and Molecular Biology, Inc.

4 RATIONALE

To complement neuroendocrinological evidence of HPA axis dysregulation in SA, as well as of altered neurochemical levels and expression of genes related to HPA axis related stress hormone signaling in suicidal brains, the intention here was to study the HPA axis with a focus on the heritability of variants in genes that may regulate its functional activation. The genes, and in some studies the specific variants, were selected based on evidence that they associate with altered HPA axis stress reactivity, or based on previous association with diagnoses e.g. depression, alcohol use disorders, etc. that are commonly associated with HPA axis stress reactivity and suicidal behavior. Based on accumulated GWAS evidence since the completion of the human genome project, complex diseases (including neuropsychiatric outcomes) are now known to be associated with few common genetic variants of large effect (Figure 8) (Manolio *et al*, 2009). The missing heritability may be contributed in part by common variant G x Es, G x Gs (Uher, 2009), and/or by low-frequency variants (McCarthy *et al*, 2008). Studies that are adequately powered to detect these effects require larger samples and must statistically control for additional comparisons. These requirements may also be applied to candidate gene association studies that aim to fine map genes with dense sets of genetic variant markers, i.e. larger samples are necessary to

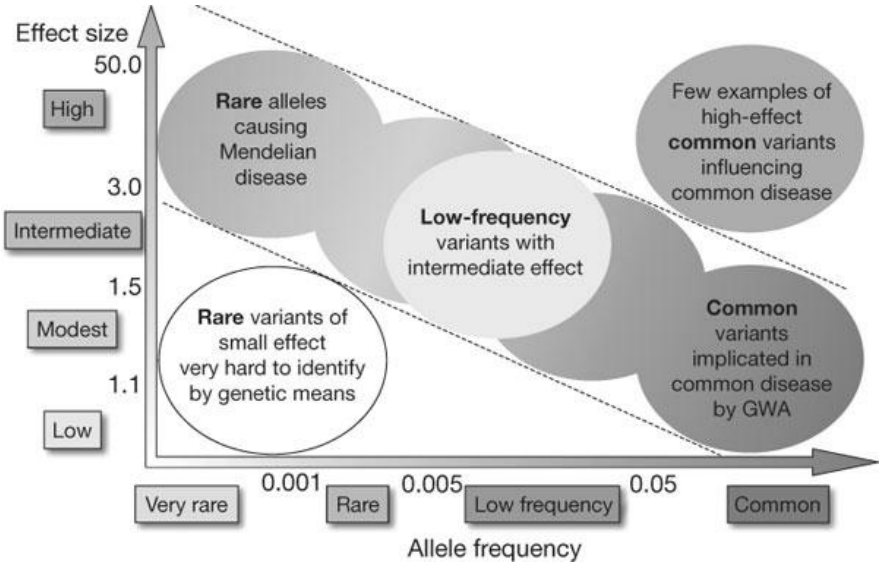


Figure 8. Feasibility of identifying genetic variants by risk allele frequency and strength of genetic effect (odds ratio).
Reprinted with permission from Macmillan Publishers Ltd: Nature and Nature Reviews Genetics, Manolio et al. (2009), Finding the missing heritability of complex diseases, *Nature* **461**(7265): 747-753 (Copyright © 2009) as adapted from McCarthy et al., Genome-wide association studies for complex traits: consensus, uncertainty and challenges, *Nat Rev Genet* **9**(5): 356-369 (Copyright © 2008).

accommodate more markers, low-frequency variants, and stratified analyses.

Family-based genetic association studies are robust to population substructure (due to e.g. inbreeding, stratification, admixture, etc.) a major source of potential confounding and spurious findings in population-based studies (Balding, 2006; Laird and Lange, 2006). In addition to genetic effects, they are also suitable for investigating G x Es, maternal genetic effects, and parent-of-origin effects. Maternal genetic effects may imply an *in utero* pathogenic mechanism, while parent-of-origin effects imply genomic imprinting, both potential sources of confounding in genetic association studies. Imprinting is increasingly understood to be important in the development and maturation of the brain (Wilkinson *et al*, 2007) and the pathogenesis of neuropsychiatric disorders (Kopsida *et al*, 2011), though such effects have not been investigated with regard to suicidal behavior. Few family-based candidate gene association studies with adequate power to detect the effect of low-frequency variants, G x Es, or G x Gs on neuropsychiatric outcomes have investigated HPA axis regulatory genes, and none have investigated SA as the primary outcome. Furthermore, since common variants are expected to have modest effects (Figure 1), it is possible that previous family-based studies on suicidal behavior did not have power to detect such effects, either.

Because of a hypothesized etiological role of a dysregulated stress response in the pathogenesis of suicidal behavior (stress-diathesis model), we are interested in studying the linkage/association of specific variants in genes that operate in these systems. Discovering that transmission of such variants occurs in SA would support a heritable genetic role in etiology, as well. While the HPA axis is the primary mediator of the neuroendocrinological response to psychosocial stressors, there are many additional systems e.g. neurotransmitters and neuromodulators, that may regulate the HPA axis by directly innervating the PVN. It is hoped that the penetrance of genetic variant effects or GxEs on SA may reveal the importance of specific genes in the suicidal process, thereby possibly revealing the involvement of specific genetic variants in specific systems. In addition to discovery, we are interested in characterizing the penetrance of specific genetic variants on occurrence of SA under different contexts and in different subgroups. The long-term goal of this line of research is to reveal the genetic variants in stress response systems mediating susceptibility to SA, as well as the variants that moderate differential effects of SLEs in heterogeneous SAs, at the population level. In follow-up to the initial goal, a secondary goal is to then explore any findings on subgroups of SA, to elucidate a role for genetic susceptibility in particular behaviors and clinical outcomes in SA pathogenesis. If consistent findings with large effects and/or with functional variants are observed in future studies using different samples, the cumulative evidence may one day be useful in guiding the development and usage of treatments, as well as in tailoring effective public health

prevention and intervention efforts to vulnerable subgroups of the population.

5 AIM AND OBJECTIVES

The aim of the dissertation was to characterize G x E effects between SLEs and genes modulating HPA axis stress reactivity in association with SAs. The ultimate goals of this line of research are to improve suicide prevention through delineating high risk groups on the basis of genetic vulnerability to stress, to characterize the genetic susceptibility of biological systems that are known to respond to stress, and to dissect the heterogeneity of suicide attempts by characterizing enrichment of association signals with secondary outcomes.

The dissertation focused on ***four specific study objectives***:

1. To investigate G x E between variants in the HPA axis regulatory gene *CRHR1* and SLEs on SAs.

2. To investigate G x E between variants in the HPA axis modulating gene *HTR2A* and SLEs on SAs.

3. To investigate genetic variants in the HPA axis modulating glutamatergic, GABA-ergic, and polyaminergic systems, and G x Es between these variants and SLEs on SAs.

4. To investigate G x Es between variants in the HPA axis regulatory gene *AVPR1B* and SLEs on SAs, and G x G between variants in *AVPR1B* and *CRHR1* on SAs.

6 METHODS

6.1 OVERALL STUDY DESIGN

The Genetic Investigation of Suicide and Suicide Attempt (GISS) project collected and investigates two interrelated samples with regard to genetic and psychological material using. The primary sample is family-based with SA probands, while an unrelated control sample is used for confirmatory case-control association analysis in comparison with SA probands. An index SA (the event by which the proband subject was ascertained) was assessed in emergency rooms where probands were treated. Non-suicidal controls and proband parents were also assessed for a personal history of SA during an interview. The family-based design allowed tests of variant linkage and association with SA and secondary outcomes, and confirmation of association with SA was provided by SA vs. HV comparison. The tests rely on LD, which may be caused by different time points of mutation occurring in the history of diverse population, or selective pressure leading to combination of disease and marker allele to persist (Thomas, 2004b). Control of the False Discovery Rate (FDR) (Benjamini and Hochberg, 1995) was used to minimize Type I that may occur with multiple comparisons as well as Type II error that may occur if one controls for Type I error too conservatively.

6.1.1 Family-based association tests (FBATs)

6.1.1.1 Genetic effects

FBATs are unbiased with regard to population substructure or model mis-specification because they condition on the observation that an allele is transmitted from a heterozygous parent and do not rely on using a real-life control sample. The null hypothesis is no association and linkage of polymorphisms (SNPs and haplotypes here; Figure 3) with outcomes of interest, e.g. SA, and they test for association in the presence of linkage (in the design used here, i.e. 1 offspring per complete trio). Assuming we know the genotypes of the parents and offspring, there is a dichotomous outcome e.g. SA, and all offspring are affected, it is possible to compare expected vs. observed transmissions from heterozygote parents to offspring using Mendel's laws of segregation. According to Mendel's laws, a difference should not be observed if an allele is not associated with the disease outcome. If there is association among parents, but no linkage of a causal allele with a non-causal marker allele in high LD, the causal allele is not transmitted with the marker so there will be no association in offspring. If there is linkage, but no association, different alleles will be transmitted with disease alleles in different families. Thus, the transmission disequilibrium test (Equation 1; "TDT") is a test of linkage and association (Spielman *et al*, 1993). Because it is a test that is conditional on parental genotypes, it is robust to confounding by population stratification. Because

it does not use controls and only affected offspring, it is robust to confounding by mis-specified models, e.g. regression models.

The FBAT software uses an approach which can accommodate families with missing genotype data, and analyses, e.g. different genetic models, as well as other circumstances (Laird *et al*, 2000). Since the family-based sample here used complete nuclear family trios, the main advantage of using it here for single SNP effects was to compare results with the TDT. The TDT is a special case of FBAT that uses complete nuclear families with only affected offspring with at least one heterozygous parent. The result is a Z score, which can be squared and tested for significance on a χ^2 distribution. FBAT algorithms are additionally adapted for other samples, e.g. They are calculated slightly differently than the TDT (Equation 2), but similarly use a score test, which is equivalent to the TDT when using complete nuclear family trios (as here). Extensions of the FBAT e.g. for haplotype analysis using unphased genotypes (HBAT), have been described (Horvath *et al*, 2004) and were also used here. HBAT accounts for unknown haplotype phase, however, this was inferred by other methods prior to using HBAT.

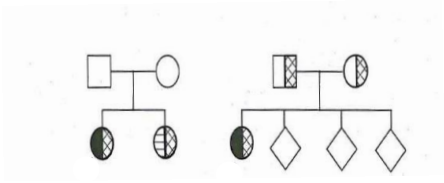
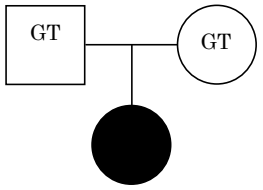


Figure 9. Left solid symbols indicate the case and left-hatched symbols indicate controls; right-hatched symbols indicate individuals to be genotyped. Circles indicate real individuals, diamonds indicate pseudosibs. Adapted from Thomas (2004), Testing candidate gene associations in *Statistical methods in genetic epidemiology*. Oxford University Press: New York, pp 253-282, with permission from Oxford University Press.

A transformation of the TDT into an asymptotic model provides a framework to calculate effect size and controlling for confounding variables. This is accomplished by matching the affected offspring with a pseudosibs created from the untransmitted allele (Self *et al*, 1991). This can also be used as a genotypic test if the pseudosibs are created from the three untransmitted genotypes (Figure 9). Conditional logistic regression (CLR) can then be used to compare the SA offspring with matched pseudosibs. CLR, which uses likelihood methods, is additionally more efficient for estimating genotypic effects than the TDT (Schaid, 1999). The TDT and CLR can be adjusted for 1 df genotypic tests of association, while CLR can also be used for 2 df full genotypic test of association .

The following is a brief summary of the statistical frameworks used herein to calculate genetic effects, as described in the previously cited articles. Their extensions, e.g. HBAT, are described in subsequent articles.

TDT



Transmitted allele	Non-transmitted allele	
	G	T
G	a	b
T	c	d

Figure 10. Allele transmission. Left: a pedigree chart for a complete nuclear family trio. Shading represents case. Square represents male, circle represents female. Right: a table showing the contribution of each heterozygous parent to the TDT statistic.

Using trios such as in Figure 10, each trio will contribute to the test statistic, which is a special case of the Mantel-Haenszel test (a cross-product calculation of relative risk using a 2 x 2 table), as follows:

$$U = b - \frac{b+c}{2} = \frac{b-c}{2} \qquad (1)$$

with variance (var) = (b + c)/4

The test statistic U follows a χ^2 distribution on 1 df, so that:

$$\chi^2 = U^2/\text{var} = (b-c)^2/(b+c) \qquad (2)$$

Families with one transmission contribute -1 or 1, and those with two transmissions contribute -2, 0, or 2, to the test statistic.

FBAT

Let X be genotype with coding of a minor allele G and major allele T according to a pre-specified genetic model (which is unknown). For dichotomous outcomes Y (all outcomes used here were dichotomous), the FBAT test statistic is defined, as follows:

$$U = \sum Y\left(X - E\left(X|P\right)\right) \qquad (3)$$

This is a covariance of offspring genotype and affection status that centers the genotype, X, with $E(X/P)$ to provide robustness to population substructure.

The test statistic distribution is defined, as follows:

$$Z = U/\sqrt{\text{var}\,U} \qquad (4)$$

where var(U) is computed from X/P under H_0, i.e. var$(U) = \sum T^2$var(X/P), *and* $Z \approx N(0,1)$, $Z^2 \approx \chi^2$ on 1 df under H_0. If Y is always equal to 1, i.e. all affected offspring, and if using an additive model for X, $Z^2_{FBAT} = \chi^2_{TDT}$.

Families with one transmission contribute +1/2 or -1/2 to U and variance of 1/4, and those with two transmissions contribute -1,0,1 to U and variance of 1/2.

CLR

The following is the likelihood corresponding to the allelic risk estimate using CLR, as previously described (Cordell and Clayton, 2002; Maestri *et al*, 1997), written in the notation of a generalized linear model as follows:

$$Y = \ln\left[\frac{P(transmission)}{1-P(transmission)}\right] = \alpha + \beta_1 allele \qquad (5)$$

This assumes equal transmission from parents, and is conditional on parental mating type like the TDT. When this comparison uses an additive model, $\chi^2_{CLR} = \chi^2_{TDT}$.

CLR models of genotypic relative risk follow1 df or 2 df tests according to coding of indicator variables for the model to be tested (Table 4), as follows:

$$\ln[P(case)/\{1-P(case)\}] = \beta_0 + \beta_1 X_1 + \beta_2 X_2 \qquad (6)$$

The likelihood ratio test can be used to test for genotypic effects on the log-odds of being the case by testing the H_0:$\beta_1 = \beta_2 = 0$ in a 2 df test. This approach provides convenient estimates of the OR of being a case as OR(*case*) = e^{β_1} for heterozygotes and OR(*case*) = e^{β_2} for homozygotes, identifying which genotypes are at increased risk.

Table 5. Coding of genotypic indicator variables according to minor allele effects

Genotype	Genetic model				df
	Additive	Dominant	Recessive	Overdominant	1
			Homozygote	Heterozygote	2
2 minor alleles	X = 2	X = 1	X = 1	X = 0	
1 minor allele	X = 1	X = 1	X = 0	X = 1	
0 minor alleles	X = 0	X = 0	X = 0	X = 0	

6.1.1.2 Interaction and joint tests

The primary family-based tests of G x E and G x G were designed for dichotomous exposures and outcomes, and use a permutation-based covariate correlation (Hoffmann *et al*, 2009) or CLR with a likelihood ratio test (Cordell *et al*, 2002; Schwender *et al*, 2011).

For G x E using a multiplicative, non-parametric method, the FBAT-I test stratifies the sufficient statistic for parental mating type, as previously described (Hoffmann *et al*, 2009). Let s index the strata of sufficient statistics S, and let f index the offspring within the strata. Let $X_{sf} = X(g_{sf})$ be a univariate coding of the genotype (e.g. additive) for the f^{th} offspring in the s^{th} strata, and let Z_{sf} be the corresponding environmental exposure. The following test statistic using only the affected probands is proposed

$$Y = \sum_{S,f} \{X_{Sf} - \bar{X}_S\}\{Z_{Sf} - \bar{Z}_S\} \qquad (7)$$

where $\bar{X}_S$ and $\bar{Z}_S$ are sample means of X and Z of the affected offspring in strata s. The test statistic can be viewed as a stratified sample covariance. FBAT-I then uses a Monte-Carlo permutation test to break the correlation by permuting $\{x_{sf} - \bar{x}_s\}$ within each strata of sufficient statistics, or equivalently permuting $\{z_{sf} - \bar{z}_s\}$ as discussed previously (Lake and Laird, 2004).

For the parametric versions, the TDT was transformed to CLR using matched pseudo-sibs. Comparing this extended model to Eq. 6 provides a likelihood ratio test (with 2 df) to test for either G x E or for heterogeneity in the effect of genotype between two groups (e.g. between SA classified positive or negative for secondary outcomes). This method was also extended to detect an additive model of G x G at 2 unlinked loci for using 15 pseudo-sibs (Cordell *et al*, 2004). This approach can be extended to test for interaction with an observable covariate (Z) by including an additional term for each genotype. If Z = 1 for "exposed" trios and = 0 otherwise, then the model becomes:

$$\ln[P(case)/\{1-P(case)\}] = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 (X_1 * Z) + \beta_4 (X_2 * Z) \qquad (8)$$

We also used a joint test to screen for a joint effect e.g. of X_1 and X_1*Z using either an allelic (2 df) or genotypic (4 df) coding. The joint test was used primarily as a screening method for potential G x E by comparison of significance with the main genetic effect, as previously described (Hoffmann *et al*, 2009; Vansteelandt *et al*, 2008).

6.1.2 Population-based association tests

Case-control re-analysis using logistic regression was used to confirm associations originally found in FBATs. Logistic regression is a method to correlate an independent variable (genotype, environment, G x E) with a categorical outcome (affection status, i.e. SA). The attributable proportion of risk (AP) due to G x E was estimated with a 4 x 2 table (Table 6) (Zou, 2008).

If one assumes that like suicide, severe SAs are rare (population prevalence < 0.05%), several additional indicators of additive interaction may be calculated from case-control data since OR ≈ relative risk in rare outcomes (Rothman *et al*, 2008b). These include the relative excess risk due to interaction (RERI), as follows:

$$RERI = RR_{ge} - RR_g - RR_e + 1 \quad (9)$$

while the AP is annotated by

$$AP = RERI / RR_{ge} = 1 / RR_{ge} - RR_g / RR_{ge} - RR_e / RR_{ge} + 1 \quad (10)$$

Both of which may be parameterized for calculation of CI and adjustment for confounders using readily available regression software. The AP was used here to estimate the proportion of risk in the population due to G x Es observed with large effect (OR > 2) in trios.

Table 6. Calculation of crude odds ratio for G x E in a case-control design using a 4 x 2 table.

Reprinted from Khoury and Flanders, Nontraditional epidemiologic approaches in the analysis of gene-environment interaction: case-control studies with no controls!, *Am J Epidemiol* (1996) **144**(3): 207-213 with permission from Oxford University Press.

Exposure*	Susceptibility genotype	Cases	Controls	Odds ratio†
–	–	*a*	*b*	1.0
–	+	*c*	*d*	$OR_g = bc/ad$
+	–	*e*	*f*	$OR_e = be/af$
+	+	*g*	*h*	$OR_{ge} = bg/ah$

* –, absent; +, present.

† Under an additive model: $OR_{ge} = OR_g + OR_e - 1$.
Under a multiplicative model: $OR_{ge} = OR_g \times OR_e$.

6.2 STUDY PARTICIPANTS

Complete nuclear family trios (SA offspring proband and both parents) were collected by the GISS project, as previously described (Wasserman et al. 2005). SA subjects entering treatment through emergency wards at major hospitals were consecutively evaluated for the clinical severity of their self-inflicted injuries. All evaluators confirmed the occurrence of the index SA according to the WHO definition (Chapter 1: Outcome definitions and descriptions). In case the self-inflicted injuries required a certain level of medical treatment, i.e. a Medical Damage Rating Scale (MDRS) score (Beck *et al*, 1975) of ≥ 2, an attempt was subsequently made to contact both living parents for consent and approval concerning participation in the study. The MDRS is rated 0 (no damage) to 8 (death) and a score of 2 indicates medical treatment was necessary. It was estimated that approximately 20% of the SA offspring consecutively entering the emergency wards fulfilled all ascertainment criteria, i.e. an

offspring SA with MDRS ≥ 2 and access to all three subjects per trio (thus representing an intact nuclear family structure). In addition, the majority of SA were characterized by further occurrence of risk factors for completed suicide (Chapter 4). The collection period was between 2001-2006 in catchment areas spread out across 15 cities in the Ukraine except the ethnically diverse city of Lvov. HVs were community controls without history of SA or psychiatric diagnosis, and were collected in Odessa in 2003 and 2005.

After applying extensive quality-control procedures using both demographic (cross check verifications of data entries and various reasonability checks of entries), psychometric (for nay-saying, random responding, entry missingness or response-duplicates) and genetic (complete DNA of good quality for all of the three subjects [per trio], misclassified parenthood or sex, and any DNA duplicates) data, 85% complete trios remained (n = 796). Here, n = 660 (equal to 69% of the initial collection) of these complete trios where used for analysis, as they also fulfilled the additional demand on minimum DNA concentration. Although 672 trios were used previously (Wasserman *et al*, 2009c), 12 trios with incomplete data required for this study were subsequently removed, such that 660 complete and quality controlled trios were used for the first study of the this thesis (Ben-Efraim *et al*, 2011). A reduced set of HVs were subsequently age-matched by group and sex to SA offspring, such that n = 300 HVs (age 18-33 yrs) were matched to 660 SA offspring overall; n = 141 (age 18-34 yrs) males matched n = 337 SA male offspring; n = 143 (age 18-30 yrs) female HVs matched n = 323 SA female offspring. Regarding adult SA offspring (n = 561), n = 357 HVs (age range 18 - 38 yrs) were age-matched overall, male adult SA offspring (n = 299) were age-matched to n = 161 HVs (age range 18-38 yrs); female adult SA offspring (n = 262) were age-matched to n = 193 HV females (age range 18-37 yrs).

The ethnodemographic criteria were described previously in trios (Ben-Efraim *et al*, 2011, 2012), as well as in comparison with HVs (Ben-Efraim *et al*, 2011) The lifetime rate of SA in the Ukrainian population has been approximated to 1.8% (Bromet *et al*, 2007). In our sample, 22% of SA offspring reported about occurrences of suicidal behavior in the family (parent, sib or grandparent). The age of onset for SA in the Ukrainian population was approximately 25 and >30 years of age (Bromet *et al*, 2007), compared to the mean (S.D.) age of 23.9 (7.1) and 24.6 (7.3) among SA offspring recruited here, for females and males respectively, demonstrating that our sample had a comparatively higher representation of young onset SA. In the Ukrainian population, lifetime SA rates were equally common among males and females (Bromet *et al*, 2007), comparable to the female/male ratio of 48.9%/51.1% among SA offspring in the sample used here. In addition, 22% (n=142) of SA offspring had suicidal behavior in the family (attempt in parent/sib and/or completed suicide among grandparents).

6.3 ETHICAL CONSIDERATIONS

The collection of research subjects followed the code of ethics of the World Medical Association (Declaration of Helsinki), and written informed consent was obtained from all participants. The study was approved by the institutional review board at the Karolinska Institute at Huddinge Hospital in 1997 (document no. 97-188), and revised and re-approved between 1998-2001. The review board was later replaced by the Regional Ethic Review Board in Stockholm, and the ethical approval was confirmed in 2010. All necessary permissions were obtained in the Ukraine (Ministry of Health document no. 5.12-210/D/210, and document no. 5.12-3835).

6.4 INSTRUMENTS

6.4.1 Data management

Data was stored using Filemaker Pro v5.5 (Claris Corp., Santa Clara, CA, USA).

6.4.2 DNA

Blood was drawn by venipuncture into 10 ml vials containing ethylenediaminetetraacetic acid from each subject, and stored. DNA was extracted using standard protocol, as described previously (Geijer 1994). Genotyping was performed as described previously (Wasserman *et al*, 2005) using Illumina GoldenGate DNA genotyping platform (Illumina Inc., San Diego, CA, USA) at the SNP&SEQ facility in Uppsala, Sweden. Minimum DNA concentration was set to > 100 ng/µl in all of three subjects (per trio), as per Illumina protocol. The call rate was > 90% (median 99.9%, lower quartile 99.8%), and genotypes were subsequently checked for absence of Mendelian inheritance errors, 100% intra-assay reproducibility and an estimated 99.4% 1-year inter-assay reproducibility.

6.4.3 Interview

A structured psychiatric diagnostic interview, semi-structured GISS interview, and the Neuroticism Extraversion, and Openness Personality Inventory-Revised (NEO PI-R) interview (Costa and McCrae, 1992) were conducted days after the index SA by trained interviewers, under the guidance of Prof. Vsevolod Rozanov of Odessa National Mechnikov University, Odessa, Ukraine. The diagnostic interview was performed with WHO tools, i.e. the Composite International Diagnostic Interview (CIDI) v2.1 (World Health Organization, 1997), and mental and behavioral diagnostic codes were given according to the ICD-10. The content of the GISS interview were identification, demography, description of the index and any previous SA, family history of suicidality, physical health, SLEs, and special instruments. Exposure to SLEs were assessed according to the Life Events section of the European Parasuicide Study Interview Schedule v5.1 (Kerkhof *et al*, 1989), using 60 questions between

2001-2003 which were subsequently reduced to 21 questions in 2003. Exposure to 9 SLE types that may be relevant for PTSD were assessed according to CIDI v2.1 section K (PTSD section). All outcomes were assessed or reduced to dichotomous variables to reduce distribution effects on the data, and since certain tests of association (described below) were developed specifically for dichotomous variables.

6.4.3.1 Stressful life events (SLEs)

The reliability of specific questions was assessed in HVs by sex with Cohen's kappa measuring test-retest agreement over 2 weeks. Certain questions that were determined to represent overlapping SLE types according to those used in previously published G x E studies (Kendler *et al*, 2005) were combined with "or" combinations into single SLE types. An SLE checklist was constructed by aggregating exposure to cumulative SLE types with a minimum kappa of 0.40, indicating moderate-or-better agreement (Fleiss *et al*, 1969; Landis and Koch, 1977), totaling 19 possible SLEs in either sex (Ben-Efraim *et al*, 2011). In either sex, the median cut-off of the SLE checklist was 5 SLEs. In Study IV, 5 SLE types representing network-related, interpersonal SLEs were selected from the larger checklist and aggregated into a subscale with 3 or 2 median number of SLE exposures in males or females, respectively.

Certain SLEs were assessed using cutoffs for age of exposure, and were selected as per similarity with SLE types in the Childhood Trauma Questionnaire (Bernstein *et al*, 1994), which assesses several types of abuse and neglect, and which has been shown to be correlated with suicidal behavior (Brodsky *et al*, 2008). Three questions from CIDI PTSD section that matched these criteria were selected, i.e. "Were you ever seriously physically attacked or assaulted?", "Were you ever raped, that is someone had sexual intercourse with you when you did not want to, by threatening you, or using some degree of force?", and "Were you ever sexually molested, that is someone touched or felt your genitals when you did not want them to?". These questions were assessed with regard to SLE exposure occurring at age 18 or younger (childhood/adolescence), or age 18 or over (adulthood). Physical assault was used in Studies I-IV. Rape was used in Study I, however, after observing it was underpowered to study G x E with rape due to few exposed, a more prevalent sexual assault (rape or molestation) exposure was used in Studies II-IV for increased power.

6.4.3.2 Outcomes

The prevalence of lifetime psychiatric diagnoses was assessed by the diagnostic interview. Several special instruments were used to assess additional secondary outcomes in SA offspring using previously recommended dichotomization points. One question from the Beck Hopelessness Scale (Beck *et al*, 1974), "My future seems dark to me", was shown to represent a single component model of the scale (Aish *et al*, 2001) and was assessed by yes vs. no answer. The Global Assessment of

Functioning Scale, adapted from Axis V of the DSM, Fourth Edition-*Text Revised* (American Psychiatric Association, 2000), was used with a score ≤ 60 representing moderate-to-severe impairment of social functioning. The Beck Depression Inventory (BDI) assesses depressive symptoms from the past 2 weeks (Beck *et al*, 1961), and a score ≥ 17 indicated clinical depressive symptoms. Two scales were used with a median dichotomization, as recommended: the Past Feelings and Acts of Violence Scale was designed to assess violent behavior among psychiatric patients (Plutchik and van Praag, 1990), and was used here to assess aggression; the Trait Anger Scale assesses trait and state anger (Spielberger, 1988). A subscale of the Suicide Intent Scale (Beck *et al*, 1975) that was shown to predict future suicide in longitudinal follow-up was used, i.e. the Precautions against discovery subscale (Beck *et al*, 1989; Beck *et al*, 1976). The precautions subscale used here involved answering yes to three questions from the Suicide Intent Scale. Several variables were used to further describe the index SA, and were assessed at interview: whether the index attempt was a repeat attempt, any intoxication by alcohol and/or drugs immediately prior to SA, a Suicide Intent Scale question on prior planning used to assess impulsive vs. non-impulsive SA (Saiz *et al*, 2008).

6.4.4 Statistical and software tools for association analysis

CLR with matched pseudosibs (Beaty *et al*, 2002; Cordell *et al*, 2002; Maestri *et al*, 1997; Schaid, 1999; Self *et al*, 1991)
FBAT-I in PBAT-R (Hoffmann *et al*, 2009)
GENASSOC (Cordell *et al*, 2002)
Haploview v4.1-4.2 (Barrett *et al*, 2005)
FBAT v2.0-2.0.4 (Laird *et al*, 2000)
fdrtool (Strimmer, 2008)
PBAT v3.6 (Lange *et al*, 2004)
QVALUE (Storey and Tibshirani, 2003)
R software environment for statistical computing v2.12-2.15 (http://www.r-project.org/
SNP and matrix spectral decomposition (Nyholt, 2004)
STATA v9-11 (StataCorp, College Station, TX, USA)
TRIO (Schwender *et al*, 2011)

6.4.5 Other statistical and bioinformatics tools

1000 Genomes Project genomic database (The 1000 Genomes Project Consortium, 2010)
BEAGLE v3.3 (Browning and Browning, 2009)
Genecards
Ensembl
HapMap genomic database (The International HapMap Consortium, 2003)
LEM (Vermunt, 1997)
National Center for Biotechnology Information (NCBI) PubMed
NCBI SNP database (Sherry *et al*, 2001)

PHASE (Stephens *et al*, 2001)
QUANTO v1.2.4 (Gauderman and Morrison, 2006)
UNPHASED (Dudbridge, 2008)

7 RESULTS

7.1 STUDY I: THE CORTICOTROPIN-RELEASING HORMONE RECEPTOR TYPE-1 GENE (*CRHR1*) (BEN-EFRAIM *ET AL*, 2011)

Study 1 expanded on previous studies that showed linkage/association of *CRHR1* SNPs on SA using the same trios described here (Wasserman *et al*, 2008; Wasserman *et al*, 2009c). The SNPs to be studied in G x Es were selected according to previous association with depression and alcohol use outcomes (Bradley *et al*, 2008; Licinio *et al*, 2004; Liu *et al*, 2006; Papiol *et al*, 2007; Treutlein *et al*, 2006), or on SA (Wasserman *et al*, 2008). In these previous studies, a main effect was observed for one low frequency exonic SNP and one common intron 1 SNP that was overtransmitted in SA males with elevated BDI scores, and this transmission distortion was observed with greatest effect among those exposed to 1-3 lifetime SLEs (Wasserman *et al*, 2008; Wasserman *et al*, 2009c), with diminishing effects observed for lower and higher exposures. The present study used a new, median-dichotomized SLE checklist (median 5 SLEs) and a G x E was observed for the same common SNP (rs4792887) and same outcome, with transmission distortion observed for low (fewer than 5) SLEs (Ben-Efraim *et al*, 2011), in agreement with the previous observation (Wasserman *et al*, 2009c). We further identified G x Es between two independent common SNPs (by low LD), 5' SNP rs7209436 and 3' SNP rs16940665, and physical assault in childhood/adolescence vs. adulthood, respectively. Secondary outcome subgroup analyses were performed using either univariate combinations or 2 multivariate clusters of 8 selected secondary outcomes (one cluster primarily defined by high anger/aggression and another primarily defined by low anger/aggression and high BDI). The findings were similar in either case: both the G x E of the 5' SNP and childhood/adolescent physical assault, and the G x E of the 3' SNP and adulthood physical assault were best described by association with high anger and aggression as well as several predictors of future suicide. On the other hand, the G x E previously identified (Wasserman *et al*, 2009c) was now further described by association with low anger/aggression. Evidence for sex-specificity was not supported for rs7209436 but the G x E involving rs16940665 was only observed in males. All findings were confirmed by using an SA vs. HV case-control design, and passed the FDR control threshold.

7.2 STUDY II: THE SEROTONIN RECEPTOR TYPE-2A GENE (*HTR2A*) (BEN-EFRAIM *ET AL*, 2012)

Despite the long history of association studies of *HTR2A* variation on SA, only one previous study has investigated all common SNPs in the gene (Brezo *et al*, 2010) and few family-based studies investigated candidate SNPs (De Luca *et al*, 2007; De Luca *et al*, 2009; Zalsman *et al*, 2005). In Study 2, we investigated 98% of currently known common SNPs

and certain low-frequency SNPs in *HTR2A*, and showed novel genetic linkage/association of a promoter SNP, rs6310, and a low-frequency synonymous exon 2 SNP, rs6305 (Ben-Efraim *et al*, 2012). In G x E testing, we also showed a novel G x E between an exon 1 SNP, rs6313, with exposure to cumulative lifetime SLEs on SA and this was the main finding of the study. Upon further characterization of this G x E we revealed a complex parent-of-origin effect in females, which may imply paternal imprinting of the gene (i.e. expression from the maternally-inherited chromosome). In additional results that were not previously published, the allelic direction of 4 of 7 SNPs were confirmed in case-control SA vs. HV re-analysis. These include the main finding (rs6313 x lifetime SLEs), both findings in low-frequency SNPs (rs6305 and rs2070036) and a G x E of rs1928042 x lifetime SLEs in males. With regard to rs7322347, there were fewer than 5 minor allele homozygotes in both cases and controls, thus re-analysis of the recessive model observed in trios was inconclusive. The findings were characterized by 19 secondary outcome covariates, however, robust covariation with these outcomes was not observed.

7.3 STUDY III: GLUTAMATE, Γ-AMINOBUTYRIC ACID (GABA), AND POLYAMINE CANDIDATE GENE PATHWAYS (SOKOLOWSKI *ET AL*, 2012)

In study 3, we investigated twenty four genes in the glutamatergic, GABAergic, and polyaminergic systems. These genes were selected according to evidence that they innervate PVN and therefore potententially modulate HPA axis activation. We showed linkage and association of two SNPs (rs2268115 and rs220557) in an N-methyl D-aspartate (NMDA) receptor 2B-subunit (*GRIN2B*), and two SNPs (rs1049500 and rs2302614) and a haplotype (rs1805247-rs1806201-rs1805482-rs2268115 AGGC) in a rate-limiting enzyme in polyamine biosynthesis (*ODC1*) (Figure 7) (Sokolowski *et al*, 2012). We also observed a G x E between another *ODC1* SNP (rs7559979) and physical assault in childhood/adolescence. All findings were characterized by past year alcohol and/or drug use disorders, violent method of index SA, and/or transdiagnostic trait anger.

7.4 STUDY IV: THE ARGININE VASOPRESSIN RECEPTOR TYPE-1B GENE (*AVPR1B*)

In study 4, we investigated 100% of currently known common SNPs and a low-frequency SNP in *AVPR1B*. We showed association, for the first time in suicidal behavior, of two SNPs (rs33911258 and rs33990840) and a corresponding major-allele haplotype across the gene, predominantly on current clinical depressive symptoms in SA. Interestingly, we found neither evidence of G x E between *AVPR1B* variants and SLEs, nor observed any G x E with *CRHR1* variants. We discussed the possibility that rs33990840, a non-synonymous exon 1 SNP, may affect AVPR1B

pharmacological properties, and we also speculated upon a potentially important role for intra-European ethnic in *AVPR1B* genetics of mood disorders. The association of the putative functional SNP was further observed predominantly in SA using a non-violent method.

7.5 SUMMARY

Genetic effects on SA were observed in Studies 1-4. G x Es on SA were observed in Studies 1-3. Significant haplotype effects on SA were observed in Studies 1, 3, and 4, while haplotype tagging SNPs were observed to associate in Studies 2 and 4. A parent-of-origin effect on SA was observed in Study 2. G x G was investigated in Study 4. Case-control confirmation of family-based findings was observed for all findings in Studies 1 and 3, and some but not all findings in Studies 2 and 4.
A ranking of findings from all four studies (Table 7) shows large effect sizes (OR $\geq$ 2) were observed in association with SA for G x Es of common SNPs in *CRHR1*, *ODC1*, and *HTR2A*, as well as for genetic effects of common SNPs in *ODC1* and a low-frequency variant in *HTR2A*.

Table 7. Summary of all significant findings on SA in the thesis which passed correction for multiple comparisons, ranked according to effect size.

Gene	SNP (x SLE)	Sex	MAF	Best-fit model		P-value
				model	effect size	
CRHR1	rs16940665 (x PA in adulthood)	m	0.13	additive	2.73 (1.28, 5.81)	0.0095
CRHR1	rs4792887 (x lifetime SLEs)	m	0.10	additive	2.67 (1.24,5.74)	0.0203
HTR2A	rs6313 (x lifetime SLEs)	f	0.38	over-dominant	2.38 (1.40,4.03)	0.0011
ODC1	rs7559979 (x PA in childhood/adolescence)	tot	0.36	additive	2.32 (1.51,3.56)	9.6×10^{-5}
ODC1	rs1049500; rs2302614	f	0.07	additive	2.03 (1.30,3.17)	0.0014
CRHR1	rs7209436 (x PA in childhood/adolescence)	f	0.49	additive	2.08 (1.14,3.81)	0.0159
HTR2A	rs6305	tot	0.03	additive	2.03 (1.22,3.38)	0.0047
HTR2A	rs6313 (x lifetime SLEs)	tot	0.38	over-dominant	2.00 (1.39,2.90)	0.0002
GRIN2B	rs1805247-rs1806201-rs1805482-rs2268115 (AGGC-haplotype)	tot	0.16	additive	1.63 (1.31,2.02)	8.4×10^{-6}
HTR2A	rs6310	tot	0.07	additive	1.61 (1.18,2.20)	0.0025
AVPR1B	rs33990840	tot	0.07	additive	1.39 (1.03,1.85)	0.0311
GRIN2B	rs2268115	f	0.47	additive	1.36 (1.09,1.69)	0.0058
AVPR1B	rs33911258	tot	0.18	additive	1.34 (1.08,1.65)	0.0083
GRIN2B	rs2268115	tot	0.46	additive	1.25 (1.07,1.46)	0.0043
GRIN2B	rs220557	tot	0.35	additive	1.25 (1.06,1.47)	0.0076

Abbreviations: f, female; m, male; MAF, minor allele frequency; PA, physical assault; tot, total sample

8 DISCUSSION

8.1 CONTEXT OF FINDINGS

8.1.1 HPA axis genes

CRHR1: The G x E of rs7209436 x physical assault in childhood/adolescence was congruent with previous results on adulthood depressive symptoms (Bradley *et al*, 2008; Polanczyk *et al*, 2009). This SNP was in LD with rs110402, with which we observed a non-significant tendency, and which was observed to associate in a G x E with childhood maltreatment on altered cortisol stress reactivity (Heim *et al*, 2009; Tyrka *et al*, 2009). However, we observed reduced evidence for sex-specificity in comparison to previous results in our follow-up subgroup analysis. The G x E of rs16940665 x physical assault in adulthood was novel with regard to exposure. Interestingly, similar G x Es with more recent exposures were observed in alcohol misuse in adolescents (Blomeyer *et al*, 2008). The male sex-specificity we observed as well as the allelic direction of association was not congruent with the previous studies or another on alcohol misuse (Nelson *et al*, 2010). While we also observed secondary outcome association with depressive symptoms (rs7209436) and past year alcohol and/or drug use disorders (rs16940665), both G x Es were associated most prominently with anger/aggression as well as several SA-concomitant clinical predictors of future suicide, e.g. a personal history of previous SA, an index SA with increased medical lethality as well as precautions taken against discovery. The G x E of rs4792887 and lifetime SLEs, on the other hand, was driven robustly by high depressive symptoms and male sex-specificity and low anger/aggression. These results may partially explain SA heterogeneity as well as susceptibility of the HPA axis in vulnerable individuals to SLEs with different life timing.

AVPR1B: The association observed with the 12-SNP major allele haplotype on depressive symptoms in SA was congruent with certain studies but not congruent with others. The opposing effects were discussed with regard to potential population effects, i.e. study design (case-control vs. families), and ethnicity (Southcentral/eastern vs. Northcentral/western and Northern Europeans). The association of rs33911258 was observed to stem from the same signal as the 12-SNP major haplotype. The association of rs33990840 was novel as our study was the first to investigate it. Interestingly, this SNP may affect pharmacological properties of the receptor and therefore it would be useful to study it further in molecular pharmacology studies using site-specific mutagenesis and/or pharmacogenetic studies of AVPR1B antagonists (should these drugs ever come to market). Furthermore, the association of rs33990840 in SA using a non-violent method supports a role for this SNP in depression though not necessarily completed suicide (violent method is a strong predictor of future suicide). Despite adequate power to detect many G x Es and G x G with *CRHR1* variants, none were observed. Although the role of AVPR1B in stress reactivity is not

questioned by these results, the role of *AVPR1B genetic variation* in stress reactivity was not supported in our material. Nevertheless, we did not have 80% power to observe sex-specific effects, as well as interaction effects involving low-frequency variants and/or exposures that were less prevalent in our sample, e.g. sexual assault, repeat exposures in childhood and adulthood, etc. such that G x Es and G x Gs may be observed in larger samples.

8.1.2 HPA axis modulatory genes

An important caveat in labeling the following genes according to their role in potentially modulating excitability of PVN (and thus HPA axis activation) is that these genes are not only expressed in PVN or innervating neurons. They have important function in many aspects of the suicidal process, e.g. impulsivity, aggression, neurocognitive aspects, etc. The following genes are furthermore, according to present knowledge, not the core genes mediating HPA axis functioning, which is mediated by certain neuropeptides and their receptors. Thus, while our results suggested that specific variants in the serotonergic, glutamatergic and polyaminergic systems were associated with SA, a role in HPA axis stress reactivity would be important to support with functional studies. G x Es with SLEs, in particular, imply a relationship with stress reactivity indicating the most interesting findings to follow up in functional analysis, e.g. candidate polymorphism association studies with cortisol levels in stress reactivity.

HTR2A: This was an initial report on *HTR2A* with regard to: 1) G x E with adulthood or cumulative lifetime SLE exposures, 2) low-frequency variants, and 3) the largest family-based sample of SA, to our knowledge. Our findings extended previous knowledge of the inheritance of *HTR2A* variants by presenting transmission distortion associations observed with 6 SNPs, and at least 7 true associations. Since none of the SNPs studied were in high LD with each other ($r^2 < 0.80$), they were possibly independent barring mutual high LD with unstudied variants. With regard to the primary finding, this study was first to show a G x E of rs6313 and lifetime SLEs on any neuropsychiatric outcome. The parent-of-origin effect observed in females exposed to low lifetime SLEs was the first evidence of the complex "polar overdominant" transmission pattern on any neuropsychiatric outcome, and implies that *HTR2A* may be imprinted in certain individuals. If true, this would be an important confounder in genetic association studies, and may explain inconsistency in the literature.

The main finding (the G x E of rs6313 x lifetime SLEs in the total sample best fit by an overdominant genetic model) and two secondary finding were furthermore confirmed in case control reanalysis. Furthermore, at least 7 of 10 findings were true positive associations following FDR control. Among the findings, some SNPs had never previously associated with any neuropsychiatric outcome, i.e. rs6310, rs17289304, and low-frequency rs2070036. Importantly, the HapMap reference panel and previous studies show that SNPs tagged by rs17289304

and rs6310 were functional. Moreover, while rs6313 is a well-studied SNP, the other findings were not previously associated in a family-based design.

With regard to suicidality, *per se*, our study is additionally the first to show inheritance of SNPs rs6310 and low-frequency rs6305 in association with SA, and the first to show inheritance of secondary finding SNPs rs17289304, rs2070036, and rs1928042. The large sample and a more comprehensive SNP coverage were important advantages that provided an opportunity to find these associations, many of which had moderate effect sizes. This was also the first confirmation of an association previously observed of recessive inheritance of a 3' SNP in G x E with severe childhood/adolescence SLE exposure on SA in females (Brezo *et al*, 2010). Many interesting trends on secondary outcome associations were observed.

GRIN2B and *ODC1*: Neither of these genes were previously associated with SA, though a wealth of molecular evidence is available regarding glutamatergic and polyaminergic gene expression changes in postmortem brain tissue of suicide victims and candidate polymorphism association studies with SA and SA-related neuropsychiatric outcomes (Sokolowski *et al*, 2012). Thus, this study provided the first evidence of genetic variation in these two genes, and a G x E of *ODC1* with physical assault in childhood/adolescence, in association with SA. Furthermore, a tendency for association with SA of a SNP in *SAT1*, the gene coding for SSAT (Figure 7), confirmed the allelic direction of an association previously only observed in completed suicide (Sokolowski *et al*, 2012).

8.1.3 Classic studies

Three classic examples of genetic susceptibility to the environment in mental illness are worthy of discussion in the context of prevention (Figure 11). Figure 11(a) shows the discovery of variants in a gene which codes for a hepatic enzyme that converts the amino acid phenylalanine to tyrosine in a trio with proband offspring affected with phenylketonuria (PKU) (Woo *et al*, 1983). PKU is an autosomal, recessive disease in which elevated plasma levels of phenylalanine (hyperphenylalaninemia) lead to brain toxicity and dose dependent cognitive deficits. Few genes are known to be involved in this pathway, and deleterious mutations in both copies of core genes lead to PKU (Scriver, 2007). However, newborn testing may prevent PKU onset by preventive intervention of phenylalanine ingestion. While it is informative as a classic example of Mendelian susceptibility to environment (diet), PKU is not a paragon of G x E in epidemiological terms (Ottman, 1996) since it is possible to achieve the metabolic syndrome via several mechanisms unrelated to the genotype of the affected individual e.g. maternal genotype and diet in pregnant mothers can also lead to hyperphenylalaninemia *in utero* (Scriver, 2007).

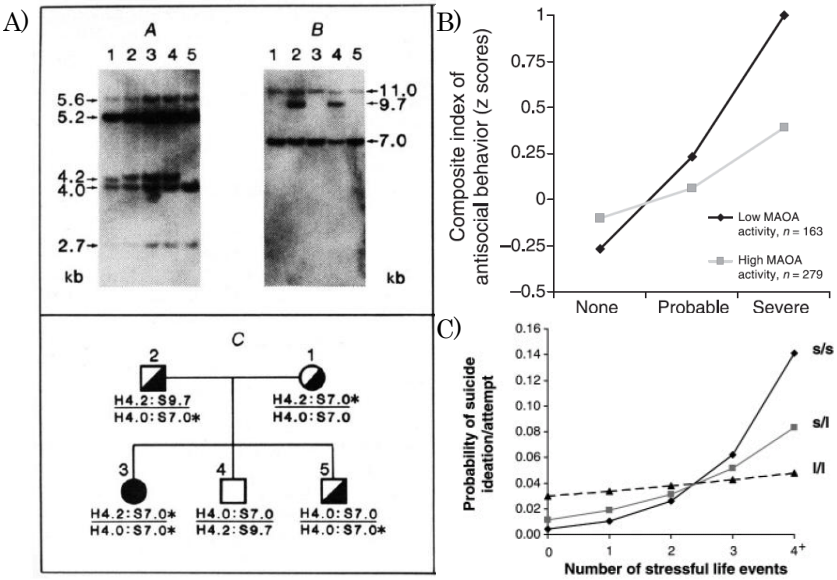


Figure 11. Genetic susceptibility to the environment in mental illness. A) Haplotype analysis of the phenylalanine hydroxylase gene in a Danish PKU family with restriction fragment length polymorphisms. B) G x E of monoamine oxidase type-A gene and childhood maltreatment on antisocial behavior. C) G x E of the serotonin transporter gene and number of recent exposures to SLEs on risk of SA/suicide ideation. Figure A is reprinted with permission from Macmillan Publishers Ltd: Nature, Woo et al. (1983), Cloned human phenylalanine hydroxylase gene allows prenatal diagnosis and carrier detection of classical phenylketonuria, *Nature* **306**(5939): 151-155, (Copyright © 1983). Figure B is reprinted from Caspi et al. (2002) Role of genotype in the cycle of violence in maltreated children, *Science* **297**(5582): 851-854; and Figure C is reprinted from Caspi et al. (2003) Influence of Life Stress on Depression: Moderation by a Polymorphism in the 5-HTT Gene, *Science* **301**(5631): 386-389. Reprinted with permission from AAAS.

Figure 11(b) shows a G x E of childhood maltreatment and an insertion-deletion polymorphism leading to amino acid substitution and reduced function of the Monoamine oxidase type-A gene (*MAOA*), which is important in monoamine catabolism (Caspi *et al*, 2002). Figure 11(c) shows a G x E of recent exposure to SLEs and a promoter repeat polymorphism which leads to changes in expression of the serotonin transporter gene (*SLC6A4*) (Caspi *et al*, 2003). Another finding from the same article of a G x E between SLC6A4 and SLEs on depressive symptoms is the most often studied G x E in psychiatric research, and the center of a recent debate in the field as to significance (Karg *et al*, 2011) or non-significance (Risch *et al*, 2009) of the accumulated findings across studies and the utility of the candidate gene G x E approach in psychiatric genetic epidemiological research (Caspi *et al*, 2010; Duncan and Keller, 2011).

8.1.4 Genetic mapping vs. clinical diagnosis

Can the previous findings on PKU be informative with regard to the findings here on SAs? The simplicity of a single gene and exposure causing a single outcome may serve as an interesting reference point for G x E studies of environmental susceptibility genes in complex, multifactorial behaviors such as SAs. Over 50 mutations with deleterious effects in 2 genes have been mapped, and there are several known modifiers (Scriver, 2007). Yet, the prevailing diagnostic measure of PKU remains testing amino acid levels via the Guthrie test (plasma phenylalanine) or tandem mass spectrometry. While genetic variants in the gene for the primary enzyme has high specificity, variants in another gene (an enzyme co-factor) are not sensitive enough to predict important deleterious effects of protein folding (Scriver, 2007). Furthermore, there appears to be a dosage effect of both the gene and exposure on phenotype, which varies according to variant penetrance and strictness of dietary restriction (Scriver, 2007). Thus, genetic mapping may be more important in confirming evidence of heritability and involvement of specific genetic components than in actually diagnosing or treating the disorder. However, one can envision that gene therapy may one day overtake dietary restriction in treating the disorder.

Certain candidate genes investigated here, e.g. *HTR2A*, were also observed to have multiple mutations with various effect size. Multiple effects in a single gene may imply a node in the pathway to disease onset, and at least can support the involvement of the serotonergic system in the pathogenesis of suicidal behavior. In contrast to PKU, Mendelian or G x E effects involving a single gene may be insufficient to explain SAs (Kendler, 2005). Even for a disease like PKU, genetic effects may be susceptible to myriad unobserved downstream effects that can compensate for deleterious mutations or amplify mutations with modest effect. Therefore, it is recommended to "treat the actual phenotype not the one predicted from genotype at the major locus" (Scriver, 2007). It is possible, however, that over time evidence of penetrance across genes, G x Es and G x Gs will be accumulated and may aid in clinical assessment, treatment, and public health intervention. When combined with other markers e.g. the Guthrie or dexamethasone suppression test, a goal of using genetic and G x E biomarkers could thus be to enhance sensitivity and specificity of measuring and treating disease phenotypes.

The results on SA here may be understood even more clearly in the context of the two articles by Caspi and colleagues. Our approach was slightly different in that not all variants that were tested for association were shown to have functional outcomes, e.g. association with cortisol response, altered gene expression, etc. For many associations observed here, a mechanistic relationship of the gene to the observed outcome remains to be clarified by reverse genetic experimental work, e.g. the deleterious effects of the *MAOA* polymorphism on enzyme activity, or stronger hypotheses of biological interaction, e.g. SLC6A4 is the putative biological target of serotonin selective reuptake inhibitor antidepressants. However, a direct relationship of biological to statistical interaction is not

necessary for statistical interaction to be relevant. For example, downregulation of HTR2A has been proposed to moderate the long term effect of pharmacologically distinct antidepressants that do not target the receptor *per se* (Eison and Mullins, 1996). Furthermore, in studies 2-4 a discovery oriented fine mapping approach was deemed more appropriate than selecting candidate functional polymorphisms, which were not so well established. Altogether, however, our research supports the study design used by Caspi and colleagues to study common genetic variants using G x E methods on psychiatric outcomes, which may reveal more penetrant associations than main effects of common genetic variants (Uher, 2009).

8.2 METHODOLOGICAL CONSIDERATIONS

8.2.1 Strengths

The family-based design is robust to population stratification for Mendelian genetic effects but is susceptible to spurious findings due to genotyping errors. However, we were able to reduce potential effects of genotyping errors by confirmation of trio results in case-control analysis and by genotyping testing for association signals in high LD SNPs, which were also genotyped. In HV recruitment for case control re-analyses, the exclusion of psychiatric diagnoses may have actually reduced bias possibly present when recruiting HVs who were dependent on the exposures under study to a certain extent, since many psychiatric diagnoses are dependent on increased SLE exposures (Rothman, 2002). Regarding the SLEs, specific severe stressors e.g. physical assault in childhood/adolescence, have been replicable across G x E studies (Caspi *et al*, 2010), and our lifetime exposure checklist consisted of widely studied SLE types (Kendler *et al*, 2005; World Health Organization, 1997).

As discussed below, gene-environment correlation may be problematic for our G x E tests. The assumption that gene-environment correlation does not occur within strata of parental genotypes is weaker than the assumption that it does not occur in the source population (Thomas, 2010).

8.2.2 Limitations

Retrospective designs are susceptible to recall bias, as well as reverse causation between certain SLE exposures and secondary outcomes (Rothman *et al*, 2008c). This differential misclassification due to recall bias may introduce distortions of G x E effect size (Greenland, 1980) or reduce power to detect a G x E (Garcia-Closas *et al*, 1998). Furthermore, the environmental exposures may be correlated with other commonly measured stressors, e.g. psychological abuse, emotional and physical neglect, which may also be important in suicidal behavior (Brodsky *et al*, 2008). However, due to the low frequency of SA in the study population, a prospective study design would be very costly.

The family-based G x E methods used here assume independence of the genetic variant and SLEs, i.e. that the genetic variant

does not regulate exposure to the SLE, conditional on parental genotypes (Umbach and Weinberg, 2000). Family-based G x E methods are also susceptible to population stratification (Shi *et al*, 2011), however, error is only predicted when gene-environment correlation is unusually strong (Kraft, 2011; Lindström *et al*, 2009).

HVs were recruited from specific workplaces, and there were also fewer HVs collected than cases. Control for population stratification, e.g. by using genomic control markers, was not performed. The predicted effect on measures of association are reduced precision of effect size estimation, and possible distortions (Rothman *et al*, 2008a). For this reason, case-control re-analysis was primarily used to confirm the allelic direction of association, genetic models, and sex-specificity, which are likely less susceptible to bias than the magnitude of effects, *per se*.

8.2.3 Reliability and validity

All diagnostic and psychometric instruments used here have high internal reliability, as discussed in the referenced articles. The ICD-10 is the international diagnostic classification standard (http://www.who.int/classifications/icd/factsheet/en/index.html). All interviewers were trained by principal investigators from the Karolinska Institute. The two principal interviewers from the Ukraine who assessed CIDI diagnoses participated in a CIDI training course in the UK and then went on to train the local interviewers in Ukraine. All interviewers were trained by the study coordinators to conduct the interviews in Russian. The high validity of the NEO PI-R in the Russian language has been previously described (Martin *et al*, 2002). Other self-report scales were translated into Russian by the study coordinators. SA-concomitant variables and psychometric scales followed commonly used clinical score cut-offs or subscales were extracted according to cited references.

During sample collection, SLE type categories were aggregated into a shorter version of the interview. One third of the sample was interviewed with the longer version and two thirds was interviewed with the shorter version. The two week test-retest reliability of the long vs. short versions of the interview was assessed in HVs. The questions from the CIDI PTSD section, including questions about exposure to sexual and physical assault, were exactly the same in both versions of the interview. All SLEs from the CIDI PTSD section had kappa ≥ 0.80, indicating good-to-excellent agreement (Fleiss *et al*, 1969; Landis *et al*, 1977). SLEs with low reliability (kappa < 0.40) were removed from the SLE checklist. Nevertheless, some misclassification is possible since reliability of the SLE checklist may differ in SA offspring vs. HVs due to disease related memory bias, age, etc (Rothman *et al*, 2008c). Therefore, the SLE checklist was also median dichotomized to further protect it from exposure misclassification (Fardo *et al*, 2007).

8.2.4 Type I and Type II error

We controlled for study-wide Type I error by correcting the significance threshold for multiple comparisons by mainly using the FDR method. We used FDR to also control for Type II error. In contrast to Bonferroni-type methods that only aim to avoid reporting any false positive, FDR is less conservative and permits a rate of false positive findings in order to avoid false negative findings. We additionally attempted to control for sampling bias. For example, results in Studies 2 and 3 confirmed previous associations by others in a highly detailed manner, e.g. sex-specificity, allelic direction, G x E, genetic model, etc. Some of these findings did not pass our FDR threshold. We chose to report these results in the interest of avoiding false negatives due to potential sampling bias. Since the gold standard is not a statistical threshold but rather consistent findings across studies, all reported findings should also be replicated in other samples, if possible.

8.3 CONCLUSION

In four articles, genetic and/or G x E associations were reported for genes that mediate HPA axis funcitoning as well as for genes in the serotonergic system, the glutamatergic system, and the polyaminergic system. The findings support a role for variants in HPA axis regulatory genes as moderators of SLE effects on SA and a stress-diathesis model of suicidal behavior (Mann *et al*, 1999). These findings reveal the importance of the underlying genetic variation of the HPA axis and innervating systems in diagnoses and behaviors which may be intermediate phenotypes in the suicidal process (Mann *et al*, 2009). This research further supports G x E analysis as a useful method to study genetic heritability of stress-related disorders. G x E characterizations may ultimately aid public health prevention efforts by e.g. defining vulnerable individuals or subgroups of the population, and guiding intervention on reducing the effect of certain SLEs in the appropriate time windows of increased pathogenicity, e.g. in childhood/adolescence (Brodsky *et al*, 2008). Since many of the genes studied here are targets of drug development for stress-related disorders, it may be possible to pharmacologically treat intermediate phenotypes that share genetic etiology with suicidal behavior as a means to help prevent suicide. The results also support using G x E analysis for discovery and characterization of associated risk and protective variants.

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