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DEPRESSION:
GENETIC, EPIGENETIC AND DNA BIOBANK STUDIES

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To my beloved family

ABSTRACT

Depression is a disease that has an estimated lifetime prevalence of ~15% and a heritability of ~36%. There is support for a heterogeneous etiology of depression, which includes a) numerous genetic loci, b) various epigenetic contributors, and c) different environmental risk factors. The first five papers included in the present thesis investigate these three disease-contributing categories by studying a) the association of *P11*, *NPY*, *MAOA* and *NR3C1*, with depression, b) epigenetic marks like DNA methylation and histone modifications, and c) environmental influences, like childhood adversities, that may interact with certain genotypes and modulate the risk of depression. In two of these studies, there is also an attempt to pinpoint some targets and mechanisms of a current antidepressant drug and to examine the molecular effects of novel potential therapeutics. The thesis also includes a paper which investigates reasons behind public refusal to consent to participation in a human genetics repository; a so-called DNA biobank. Achieving high participation rates in DNA biobanks is a prerequisite for the identification of new genetic loci, already known to have small effect sizes, which are associated with complex disorders like depression. However, as addressed in this last paper, solidarity (i.e. the participation in research for the common good) seems to be at stake for DNA biobanks and is an issue that needs to be raised both by the scientific community and national policy-makers. Specifically, the data of this thesis 1) confirm a genetic association between *NPY* and depression, 2) show the existence of a *MAOA* x childhood-adversity interaction that increases the risk of depression, 3) demonstrate DNA methylation differences of *P11* in depression-like states and of *MAOA* in depression, 4) verify the effect of childhood trauma on *NR3C1* DNA methylation, 5) provide new insights into how *Npy* is transcriptionally regulated via an allele-specific epigenetic programming and describe an alternatively spliced *Npy* mRNA variant, 6) suggest that escitalopram (a selective serotonin reuptake inhibitor; SSRI) may exert part of its antidepressant function by affecting the expression of DNA methyltransferases (DNMTs) and DNA methylation levels, 7) support the antidepressant effect of running, and 8) provide awareness of the ethical problems posed by large-scale genomic studies that rely on DNA biobanking.

LIST OF PUBLICATIONS/MANUSCRIPTS

I. **Melas PA**, Rogdaki M, Lennartsson A, Björk K, Qi H, Witasp A, Werme M, Wegener G, Mathé AA, Svenningsson P, Lavebratt C.
Antidepressant treatment is associated with epigenetic alterations in the promoter of *P11* in a genetic model of depression.
Int J Neuropsychopharmacol. 2011 Jun 20:1-11.

II. Sjöholm LK, **Melas PA**, Forsell Y, Lavebratt C.
PreproNPY Pro7 protects against depression despite exposure to environmental risk factors.
J Affect Disord. 2009 Nov; 118(1-3):124-30.

III. **Melas PA**, Lennartsson A, Vakifahmetoglu-Norberg H, Åberg E, Werme M, Rogdaki M, Mannervik M, Brené S, Wegener G, Mathé AA, Lavebratt C.
Allele-specific epigenetic programming of neuropeptide Y (*Npy*) in depression-like states.
Manuscript.

IV. **Melas PA**, Mannervik M, Mathé AA, Lavebratt C.
Neuropeptide Y: Identification of a novel rat mRNA splice-variant that is downregulated in the hippocampus and the prefrontal cortex of a depression-like model.
Peptides. 2012 Mar 3.

V. **Melas PA**, Wei Y, Wong CCY, Sjöholm LK, Åberg E, Mill J, Schalling M, Forsell Y, Lavebratt1 C.
Monoamine oxidase A (*MAOA*) gene-environment and epigenetic associations with depression in females, and *MAOA*'s action as a mediator of the association between childhood adversities and hypermethylation of the glucocorticoid receptor.
Manuscript.

VI. **Melas PA**, Sjöholm LK, Forsner T, Edhborg M, Juth N, Forsell Y, Lavebratt C.
Examining the public refusal to consent to DNA biobanking: empirical data from a Swedish population-based study.
J Med Ethics. 2010 Feb; 36(2):93-8.

LIST OF ADDITIONAL PUBLICATIONS/MANUSCRIPTS

I. **Melas PA**, Rogdaki M, Ösby U, Schalling M, Lavebratt C, Ekström TJ.
Epigenetic aberrations in leukocytes of patients with schizophrenia: association of global DNA methylation with antipsychotic drug treatment and disease onset.
FASEB J. 2012 Mar 16.

II. **Melas PA**, Georgsson Öhman S, Juth N, Bui TH.
Information Related to Prenatal Genetic Counseling: Interpretation by Adolescents, Effects on Risk Perception and Ethical Implications.
J Genet Couns. 2011 Oct 25.

III. Amstadter AB, Balachandar V, Bergen SE, Ceulemans S, Christensen JH, Cole J, Dagdan E, De Luca V, Ducci F, Tee SF, Hartz S, Keers R, Medland S, **Melas PA**, Mühleisen TW, Ozomaro U, Pidsley R, Scott AP, Sha L, Talati A, Teltsh O, Videtic A, Wang K, Wong CC, Delisi LE.
Selected summaries from the XVII World Congress of Psychiatric Genetics, San Diego, California, USA, 4-8 November 2009.
Psychiatr Genet. 2010 Oct; 20(5):229-68.

IV. **Melas PA**, Tartani E, Edhborg M, Forsner T, Forsell Y.
Mental health literacy about depression and schizophrenia among Swedish adolescents
Manuscript.

CONTENTS

LIST OF ABBREVIATIONS

a.a.	amino acid
ASHG	American Society of Human Genetics
ATF	activating transcription factor
bp	base pairs
BDNF	brain-derived neurotrophic factor
ChIP	chromatin immunoprecipitation
CNS	central nervous system
CREB	cAMP responsive element binding protein
DNA	deoxyribonucleic acid
DNMT	DNA methyltransferase
DSM-IV	fourth edition of the Diagnostic and Statistical Manual of Mental Disorders
EMSA	electrophoretic mobility shift assay
Ep300	E1A binding protein p300
FC	frontal cortex
FRL	Flinders Resistant Line
FSL	Flinders Sensitive Line
FST	forced swimming test
G x E	gene-by-environment
GADD45b	growth arrest and DNA-damage-inducible, beta
GWAS	genome-wide association study
H3K18ac	histone 3 lysine 18 acetylation
HAT	histone acetyltransferase
HDAC	histone deacetylase
HDACi	histone deacetylase inhibitor
HIP	Hippocampus
HPA	hypothalamic-pituitary-adrenal
ICD-10	10^{th} revision of the International Statistical Classification of Diseases and Related Health Problems
ISH	*in situ* hybridization
LUMA	LUminometric Methylation Assay
MALDI-TOF	matrix-assisted laser desorption/ionization time-of-flight
MAOA	monoamine oxidase A
mRNA	messenger RNA
miRNA	microRNA
NaB	sodium butyrate
NGF	nerve growth factor
NGFI-A	nerve growth factor-induced protein A
NIH	National Institutes of Health
NPY	neuropeptide Y
NR3C1	nuclear receptor subfamily 3, group C, member 1
nt	nucleotides
NT-3	neurotrophin-3
S100a10	S100 calcium binding protein A10

PCAF	p300/CBP-associated factor
PCR	polymerase chain reaction
PFC	prefrontal cortex
qRT-PCR	quantitative real time-polymerase chain reaction
RACE	Rapid Amplification of cDNA Ends
RNA	ribonucleic acid
SNP	single nucleotide polymorphism
SSRI	selective serotonin reuptake inhibitor
TET	ten-eleven translocation
TF	transcription factor
u-VNTR	upstream variable-number tandem repeat
VEGF	vascular endothelial growth factor
WHO	World Health Organization
XCI	X-chromosome inactivation

1. INTRODUCTION

The concept of depression was coined by the ancient Greek physician Hippocrates (c. 460 BC – c. 370 BC) who referred to this state using the term *melancholy*, as it was thought to be caused by an excess of black bile [melas (μέλας) = black and choly (χολή) = bile; (1)]. Today, depression is defined as a mental disorder that is characterized by low mood and self-esteem, and loss of interest or pleasure in commonly enjoyable activities (2, 3). Up to 75% of those with depression also suffer from anxiety (4, 5), and other types of comorbidity include those of substance abuse (6), post-traumatic stress disorder (7), cardiovascular disease and diabetes (8). Depression occurs more often in females than in males [female-to-male ratio of about 2:1 (9)] and its lifetime prevalence is ~13% in Europe (10) and ~17% in North America (11). However, recent reports indicate that the latter estimates may actually be too low (12). The importance of studying depression is also indicated by the global projections of burden of disease provided by the World Health Organization [WHO; (13)]. More specifically, the three leading causes of burden of disease in 2030, ranked in descending order, are projected to include acquired immunodeficiency syndrome (HIV/AIDS), depression (unipolar depressive disorder), and ischemic heart disease (14).

The introductory section of this dissertation will provide some main definitions and background information on the terms and topics that are referred to and studied in the constituent papers. Hopefully, this will assist each reader in understanding the presented data and their putative clinical significance, in addition to the thesis' contribution to the field of psychiatric genetics and to the deciphering of the molecular etiology of depression.

"If you are depressed you are living in the past.
If you are anxious you are living in the future.
If you are at peace you are living in the present."
– Lao Tzu

1.1. DEPRESSION – IN GENERAL

This section will provide a general overview of how depression is diagnosed, what is known about the factors that contribute to its pathogenesis, the main brain structures that are affected, and the primary treatment options.

1.1.1. Definition and diagnosis

Depression is a disease that is classified under the mood (affective) disorders and is diagnosed by general practitioners, psychiatrists or psychologists based on the criteria of the American Psychiatric Association's revised fourth edition of the Diagnostic and Statistical Manual of Mental Disorders [DSM-IV; (2)] or the World Health Organization's 10th revision of the International Statistical Classification of Diseases and Related Health Problems [ICD-10; (3)]. Based on DSM-IV and ICD-10, a diagnosis of depression is given when main depressive symptoms (depressed mood, anhedonia, and reduced energy) persist for at least two weeks. While the use of these two instruments involves subjective self-reports and observations, there is also promising ongoing research focusing on the development of clinical tests that can detect biological markers (biomarkers) associated with this disease (15). Screening for biomarkers in e.g. blood will potentially provide an objective diagnosis method in the future. Finally, a distinction must be made between unipolar disorder (a.k.a. clinical or major or unipolar depression; which is the research topic of this thesis and will from now on only be referred to as *depression*) and bipolar disorder [a.k.a. manic-depressive disorder and usually defined by the presence of at least one manic or hypomanic episode, with or without depressive episodes; (2, 3)].

1.1.2. Etiology

There is an etiological relationship between mood disorders, like depression, and a number of behavioral, biological and psychosocial factors (16). With regard to *biology*, depression belongs to the so-called complex genetic disorders, meaning that multiple genes, in combination with chance, lifestyle, epigenetic and environmental factors, play a role in the disorder's pathogenesis (17-19). With regard to the genetic component, a Swedish national twin study estimated that the heritability of liability to depression is ~36%, which averages two highly different figures of 42% for women and 29% for men (20). With regard to the environmental influences, stressful life events (e.g. death, assault, marital problems, job loss, threat, humiliation and financial problems) are known to have depressogenic effects (21-23). The interplay between genetic and environmental factors has also been demonstrated using gene-by-environment interaction (G x E) studies, in which individuals with a high-risk genetic makeup exposed to stressful experiences are at an increased risk of developing depressive symptomatology (24, 25). Last but not least, epigenetics provides a mechanistic path through which the environment can affect the genome and has emerged as an additional disease-explanatory candidate of depression (18). The present thesis includes data from epigenetic, genetic and G x E studies of depression.

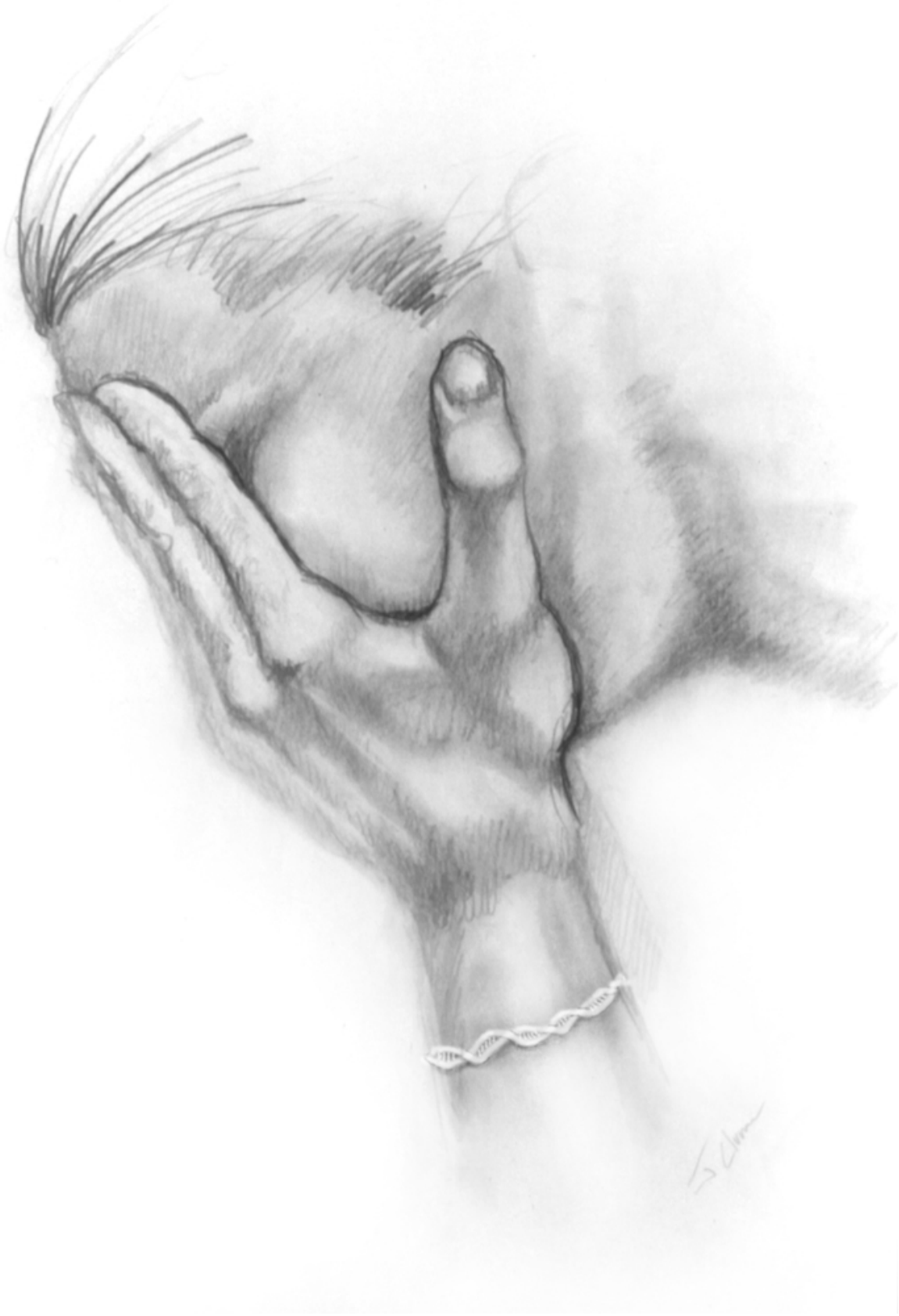

Image courtesy of John Clum. Grief. (Modified with a DNA helix)

1.1.3. Neuroanatomy

Neuroimaging studies have demonstrated a number of structural brain abnormalities in depressed patients compared to healthy controls. More specifically, patients have been found to have increased volumes of the lateral ventricles and decreased volumes of the basal ganglia, cingulate cortex, frontal lobe [including the (pre)frontal cortex, orbitofrontal cortex and gyrus rectus], hippocampus, striatum, and thalamus (26-28). This thesis contains studies investigating two of these regions: the *hippocampus* (HIP) and the *prefrontal cortex* (PFC). The hippocampus plays an important role in learning, memory function and spatial navigation (29 - 31), and its atrophy has been associated with depression (32). In particular, the subgranular zone of HIP's dentate gyrus is one of the two established brain regions where adult *neurogenesis* (i.e. the growth of new neurons from neural stem cells) occurs (33). [The second region where adult neurogenesis occurs is the subventricular zone which lines the lateral ventricles; (34)] Hippocampal adult neurogenesis has been suggested to be important for HIP's role in learning and memory (35), to be stimulated by antidepressant treatment (36) and to be required for the behavioral effects of antidepressants (37). The PFC, in its turn, plays an important role in higher cognitive functions and cognitive control, such as in orchestrating thoughts and actions to fulfill an individual's internal goals (38-40). Data from patients with brain lesions in this region have shown that the PFC is both critically and causally involved in the development of depression (41).

1.1.4. Treatment

The most common ways of treating depression include pharmacotherapy [i.e. psychiatric medications like different antidepressant drugs; (42)], psychotherapy [i.e. treatment using psychological means like cognitive-behavioral therapy, a.k.a. CBT; (43)], electroconvulsive therapy [i.e. electrically induced seizures; (44)], or their combination (45). Experimental data also suggest an antidepressant efficacy of drugs that can influence certain epigenetic mechanisms (46-48). Additionally, physical exercise has been suggested to alleviate depressive symptoms (49). This thesis includes studies that examine the molecular and behavioral effects of a) a widely-prescribed drug (escitalopram) that belongs to the class of selective serotonin reuptake inhibitors (SSRIs), b) an experimental drug (sodium butyrate) that inhibits epigenetic processes, and c) physical exercise (running). One of the dissertation's studies also investigates a certain genetic variation that is present in the gene coding for monoamine oxidase A (*MAOA*). Monoamine oxidase inhibitors (MAOIs) belong to a class of antidepressants that act by inhibiting the activity of monoamine oxidases (like MAOA) and thus prevent the breakdown of monoamine neurotransmitters.

1.2. DEPRESSION – THE NEUROCHEMICAL PERSPECTIVE

Based on biochemical and psychopharmacological data derived from experimental and clinical studies of depression, a number of theories and models have been proposed to explain its neurochemical basis (50, 51). The present thesis includes studies that are related to the *monoamine*, the *stress-diathesis*, and the *neurogenesis* hypotheses of depression.

1.2.1. The monoamine neurotransmitter hypothesis

Monoamine neurotransmitters regulate the central nervous system and assist in the transmittance of signals –across a synapse– from a neuron to a target cell. They are termed monoamines as they contain one amino group, which is connected to an aromatic ring by a two-carbon chain. Based on the monoamine hypothesis, depression arises from a neurochemical imbalance that leads to a defective monoamine neurotransmission. This imbalance may occur as a result of a deficiency of major monoamine neurotransmitters [such as dopamine, serotonin, and norepinephrine (a.k.a. noradrenaline)] in the synaptic cleft or of their corresponding receptors and regulators. In line with this hypothesis, the majority of antidepressants act by increasing the brain levels of one or more of these neurotransmitters. For instance, SSRIs and MAOIs block the reuptake of serotonin from the synaptic cleft and hinder the breakdown of neurotransmitters, respectively. Two genes that are involved in the monoamine hypothesis and are studied in the constituent papers of this thesis are *P11* (a.k.a. *S100A10*) and *MAOA*. *P11* regulates the efficacy of serotonergic neurotransmission (52) by interacting with and enhancing the availability of serotonin receptors (especially 5-HT1B and 5-HT4) at the cell membrane (53, 54). *MAOA*, on the other hand, codes for a mitochondrial enzyme that catabolizes the major aforementioned neurotransmitters [dopamine, serotonin, and norepinephrine; (55)].

1.2.2. The HPA axis and the stress-diathesis hypothesis

The hypothalamic-pituitary-adrenal (HPA) axis is thought to mediate the psychological and/or physical experience of stress (56). In particular, the corticotropin-releasing hormone (CRH) can be released by the hypothalamus in response to stressful events. CRH stimulates the release of adrenocorticotropic hormone (ACTH) from the pituitary, which activates the HPA axis and ultimately leads to the release of glucocorticoids (e.g. cortisol). Glucocorticoids, in their turn, bind to their receptor [the nuclear receptor subfamily 3, group C, member 1; NR3C1 (a.k.a. glucocorticoid receptor; GR)] which, in healthy conditions, leads to a negative-feedback regulation to maintain homeostasis. According to the stress-diathesis model, depression is thought to develop as a result of a biological/genetic vulnerability of the HPA axis in combination with stress from life experiences. For instance, an HPA axis overdrive has been observed in depression (57, 58) and one of the underlying causes may be the reduced levels of NR3C1 (59, 60). The transcriptional activity of *NR3C1* is known to be regulated by epigenetic mechanisms that are studied in the present thesis. Agents acting as agonists for NR3C1 (e.g. dexamethasones) have shown better effectiveness than placebos in treating depression (61) and CRF receptor antagonists are being tested in clinical trials (62).

1.2.3. Neuropeptides, neurotrophins and the neurogenesis hypothesis

Neuropeptides are signaling molecules (e.g. the already mentioned CRH) that mediate or modulate neuronal communication and neuronal function, and neurotrophins are proteins that induce neuronal survival, growth and plasticity. With regard to neuropeptides, galanin, neuropeptide S (NPS), neuropeptide Y (NPY), substance P and vasopressin, belong to those that have been shown to affect stress responses (63-65), and some of them are currently serving as the base for the development of potential novel therapeutics for the treatment of depression and anxiety disorders (66). With regard to neurotrophins, brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin-3 (NT-3), and vascular endothelial growth factor (VEGF), belong to those that have been associated with mood disorders (67). A common function of certain neuropeptides and most neurotrophins, and which has been linked to depression, is the promotion of neurogenesis (68). The neuropeptide that is studied within the present thesis is NPY, which has been shown both to promote hippocampal neurogenesis (69-72) and to be associated with anxiety, depression and stress (73, 74). NPY is highly conserved among species, is widely expressed in the central nervous system (CNS) and is thought to confer mental resilience (75-78) through the interaction with its receptors [particularly NPY-Y1 receptors; (79)].

1.3. DEPRESSION – THE GENETIC PERSPECTIVE

The comparison of monozygotic and dizygotic twins, with regard to the concordance rates of psychiatric disorders, has shown that depression has a heritability of ~36% (19, 20). The latter represents a numerical estimate of the contribution of genetics to disease manifestation. Although not always consistent, genetic studies have been able to pinpoint some genetic loci that are associated with depression (80). The present thesis includes genetic association and G x E interaction studies of depression with respect to *NPY* and *MAOA*, respectively.

1.3.1. The neuropeptide Y gene

The *NPY* gene codes for a premature form of NPY (preproNPY) that consists of a signal peptide, followed by the mature NPY, a processing site, and a C-terminal peptide (81). A functional single nucleotide polymorphism (SNP) has been identified in the human *NPY* gene that leads to an amino acid (a.a.) substitution in NPY's signal peptide and affects preproNPY's processing into mature NPY (82). More specifically, the so-called Leu7/Pro7 genotype has been shown to lead to ~40% higher NPY levels in the plasma compared to the Leu7/Leu7 genotype (82). Genetic studies investigating this genetic polymorphism have found an association between this SNP and depression (83-85). The human *NPY* promoter has also been shown to harbor a functional SNP that controls *NPY*'s transcriptional activity (86) and is known to affect emotional processing and stress responses (86-88). The effects of *NPY*'s genetic variations have also been shown to be modulated by childhood adversities, in line with a G x E interaction model (89-92). In the present thesis, *Npy* was also studied with respect to *alternative splicing*; a pre-messenger RNA (pre-mRNA) regulatory mechanism which produces a number of different mRNAs using the same DNA template. Alternative splicing is important in enhancing an organism's transcriptome and proteome repertoires (93), and its dysregulation is associated with human disease (94).

1.3.2. The monoamine oxidase A gene

Whereas *P11*, *NR3C1* and *NPY* are all autosomal genes, the *MAOA* gene is X-linked. This means that the gene is located on the X chromosome, making females carriers of two *MAOA* copies while males have only one copy of the gene. In order for females not to have twice as many X-linked gene products as males, female organisms employ a process termed X-chromosome inactivation (XCI). During this procedure, one of the X chromosomes is silenced mainly through epigenetic procedures (95, 96). Even if some genes have been proposed to escape XCI (97), most reports on *MAOA* suggest that it is subjected to this event (98-101). *MAOA* is also known to contain a functional genetic length polymorphism in its promoter region (termed upstream variable-number tandem repeat; u-VNTR) that affects transcriptional activity *in vitro* (102-104). The *MAOA* u-VNTR has been associated with conduct (e.g. aggressive and antisocial) behavior in male individuals, particularly when exposed to childhood maltreatment, demonstrating a G x E interaction (105-110). The *MAOA* u-VNTR has also been associated with depressive symptomatology both alone (111-114) and in combination with childhood adversities (25).

1.4. DEPRESSION – THE EPIGENETIC PERSPECTIVE

Epigenetics is considered as one of the most rapidly expanding biological fields and its definition is constantly evolving as new epigenetic marks are being discovered and their function is becoming clear. A recent scientific meeting proposed a definition of "an epigenetic trait (being) a stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence" [p. 781 of (115)]. Epigenetic marks include DNA methylation, histone modifications, microRNAs (miRNAs) and nucleosome positioning, all of which are critical for the regulation of cellular processes such as differentiation, DNA-protein interactions, embryogenesis, gene expression, genomic stability, imprinting, and X-chromosome inactivation (116). Two types of epigenetic modifications have been studied with respect to depression in the present thesis: a) *DNA methylation*, and b) *histone modifications*.

1.4.1. DNA methylation

DNA methylation involves the covalent binding of a methyl group to the 5-carbon position of the cytosine residues within CpG dinucleotides of the mammalian genome and, to date, a number of human diseases have been associated with aberrations in this type of epigenetic mark (117). In general, genome-wide DNA methylation is associated with genomic stability (118) and DNA hypermethylation within a gene's promoter region and first exon is linked to gene expression silencing (119, 120). With regard to genomic stability, DNA methylation is known to silence transposable elements, i.e. DNA sequences that have the ability to "jump around" the genome (121). The ways by which DNA methylation can affect gene expression have been summarized by two main models; the *critical site* and the *methylation density* models (122). The first model refers to distinct DNA elements –usually located in a gene's promoter region– whose methylation leads to gene silencing through the binding inhibition of a transcription activator [e.g. a transcription factor (TF)]. The second model collectively investigates numerous CpG sites [e.g. at CpG-rich regions, usually referred to as CpG islands; (123)] and regards high methylation levels as an inactivating chromatin structure determinant. DNA methylation is catalyzed by three main enzymes [DNMT1, DNMT3a and DNMT3b; (124, 125)] and can be inhibited by certain pharmacological agents, such as 5-aza-2'deoxycytidine (126). DNA demethylation, on the other hand, is thought to occur through the action of proteins of the ten-eleven translocation (TET) family (127, 128) and to be promoted by proteins like the growth arrest and DNA-damage-inducible, beta [GADD45b; (129)]. With regard to psychopathology, perhaps the most prominent findings to date originate from DNA methylation studies of *NR3C1*. More specifically, childhood adversities in the form of maternal separation in rodents (130, 131) and abuse in humans (132) have been associated with DNA hypermethylation of the promoter region of *NR3C1*. The primary methylation model examined in these studies has been that of the critical site model, with CpG methylation in the *NR3C1* region interfering with the binding of the nerve growth factor-induced protein A (NGFI-A) TF that leads to decreased expression levels of this HPA-axis-related component (131, 132).

1.4.2. Histone modifications

Nucleosomes serve as the basic DNA packaging units in the cell nuclei of eukaryotes, and each nucleosome consists of ~147 base pairs (bp) of DNA wrapped around an octamer of two copies of the core histone proteins: H2A, H2B, H3, and H4 (133). A second widely studied epigenetic mark is that of the post-translational modifications of the histone proteins' N-terminal tails. These modifications include acetylation, methylation, phosphorylation and ubiquitination, and they can all alter the chromatin architecture in a way that either increases or decreases the permissiveness of the transcriptional machinery that controls gene-expression activity (134-136). In the present thesis, the modification that has primarily been studied is histone acetylation; a histone modification that neutralizes the positive charge of histones' lysine residues. The latter decreases the histone interactions with the negatively charged DNA, and is associated with decondensed chromatin (so-called euchromatin) and increased gene activity (134-136). Deacetylation, on the other hand, leads to condensed chromatin (so-called heterochromatin) that is associated with transcriptional repression (134-136). Acetylation and deacetylation reactions are catalyzed by histone acetyltransferases (HATs) and histone deacetylases (HDACs), respectively, and can be pharmacologically manipulated with the use of appropriate agents (e.g. trichostatin A and sodium butyrate which act as HDAC inhibitors; HDACi). With regard to depression, it has been shown that electroconvulsive therapy and other antidepressant agents (e.g. imipramine) lead to activating histone modifications, decreased expression of certain HDACs, and increased mRNA levels of neurotrophins like *Bdnf* (48, 137). It has also been shown that HDACi, like sodium butyrate, have antidepressant-like effects (47) and physical exercise (running) increases histone acetylation (138).

1.5. BIOBANKS

The Swedish Act on Biobanks (SF 2002:297) defines the concept *biobank* as "biological material from one or several human beings collected and stored indefinitely or for a specified time and whose origin can be traced to the human or humans from whom it originates" [(139); translation from (140)] and The Swedish National Biobank Program states that the usage of a biobank is primarily to: 1) enhance patient disease diagnostics, 2) control epidemic infectious diseases, 3) educate medical personnel, 4) document clinical analyses and allow for re-analyses, and 5) enhance medical research and quality follow-up of medical care.

1.5.1. DNA biobanks and complex disorders

With the human genome available (141, 142), biobank repositories of DNA samples – preferably combined with health history data– have acquired a crucial role in the identification of genetic elements associated with complex disorders like depression (143). Indeed, the human studies included in the present thesis were performed with genetic material from *DNA biobanks*. The latter collect, store and handle DNA from human subjects, with the primary purpose in this dissertation's studies being the enhancement of medical research particularly in the field of psychiatric genetics and epigenetics. However, the success of these genetic databases in promoting research depends on public participation and acquiescence, as thousands of individuals are needed for detecting depression-associated genetic loci that are known to have low effect-sizes (144). As only few DNA biobanks can achieve this number of samples on their own, access to genetic data across different studies has been recognized as an important factor for identifying new associations, via so-called genome-wide association studies [GWAS; (145)].

1.5.2. Legal and ethical issues

The legal and ethical requirements when setting up a biobank for medical research usually entail the acquisition of an *informed consent* through which the individual, whose biological samples will be gathered and used, is informed about the purpose of the research and how the provided material will be used, and gives his/her approval (146). To seek an informed consent is viewed as an act of respect for the subject's autonomy, whereas signing the consent is an indication of public trust in biobank research (147). However, according to WHO "no regulatory framework for genetic databases has been developed to date that is global in scope yet developed with regional input, while being specific enough to provide practical guidance" (148). There is thus a risk of failing to attain public acceptance due to insufficient forms of confidentiality, anti-discriminatory and privacy measures, which brings the principle of *solidarity*, i.e. research participation for the common good, at stake for DNA biobanks (149, 150). A means of building trust and reciprocity with potential participants is *benefit sharing*; broadly defined as the equitable distribution of benefits deriving from the utilization of genetic resources (151). The last paper in the present thesis, examines the reasons for why certain individuals bypass solidarity and refuse to consent to DNA biobanking.

2. AIMS

The general aim of this thesis was to increase the understanding of depression's pathophysiology and to raise some ethical issues associated with human genetic biobanking. Several specific aims were also defined *a priori*, or arose during the course of the experimental procedures.

These specific aims were to:

- Study the association of *P11* with depression using a genetic animal model, and explore the epigenetic mechanisms that govern *P11* gene expression both in naïve (untreated) states and also in response to antidepressant treatment with SSRIs.
- Try to replicate the association of a functional SNP, in the human *NPY* gene, with depression.
- Investigate the genetic and/or epigenetic components responsible for the decreased levels of *Npy* in an animal model of depression-like states, and study the effects of different treatment interventions on *Npy* expression.
- Characterize novel mRNA splice-variants of *Npy* that are present in the rat brain.
- Test whether the human *MAOA* u-VNTR interacts with childhood adversities to increase the risk of depression, and whether the u-VNTR may mediate the association between childhood adversities and DNA methylation of *NR3C1*.
- Quantify the DNA methylation of a *MAOA* region and examine the putative association with depression in humans.
- Examine the concordance of DNA methylation levels, of *MAOA* and *NR3C1*, between DNA extracted from human blood and saliva.
- Identify the reasons behind the public's decision to refuse to provide DNA when requested to do so for research purposes.

3. MATERIAL AND METHODS

The following section provides a brief overview of the material and methods that were used in the thesis' constituent papers. If not otherwise stated, all generated data were analyzed using appropriate statistical methods. All experiments and methodologies were reviewed and approved by appropriate ethical committees.

3.1. HUMAN STUDIES

Human investigations presented in this thesis included genetic and epigenetic analyses, as well as the study of the motivations for not consenting to DNA biobanking. All human studies were performed using participants from the PART study.

3.1.1. The PART study

PART (Psykisk hälsa, Arbete, RelaTioner) is a longitudinal (three waves) population-based study of mental health, work and relation conditions, performed in Stockholm County, Sweden. It started in 1998 and has been based on questionnaires and subgroup psychiatric interviews (152). The questionnaires covered demographic, socioeconomic and somatic-health data, and screening instruments for psychological wellbeing, psychiatric symptoms, social disability and alcohol/drug abuse. The interviews evaluated psychiatric symptoms, life events and working conditions. The PART database has also been enriched with complementary data deriving from diverse national registries. PART's first wave (PART I) was conducted during the period 1998-2000 and responses were obtained from 10441 participants leading to a participation rate of 53%. When non-participants were contacted by telephone, the main reason given for not responding was that the questionnaire concerned sensitive issues (153). PART's second wave (PART II) was conducted during the period 2001-2003 and used a supplemented version of PART I's questionnaire. Out of 10303 eligible PART I participants, 8613 responded, leading to a participation rate of 84%. An extensive non-participation analysis was performed using available official registries. Participation was associated with female gender, higher age, income and education, being born in the Nordic countries, and having no psychiatric diagnosis in the hospital discharge and the early retirement registries. The associations between age, gender, income, country of origin, and in-patient hospital care due to psychiatric diagnosis were calculated for participants and non-participants, separately, and the OR's for these associations were similar among the two groups (153, 154). PART's third wave (PART III) was conducted during 2011. No kind of incentive has been offered throughout the PART study, making participation rely on a pure voluntary act.

3.1.2. DNA biobanking of PART

During the period 2006-2007 a total of 5527 PART II subjects were requested to contribute genetic material to PART's DNA biobank that aimed at investigating genetic elements responsible for stress and wellbeing. All individuals received a mail package containing a self-administered saliva kit and a prepaid mail packet for specimen return. They also received instructions for using the saliva collection kit, a specified informed consent form and an information letter. The latter stated the confidentiality rules, the coding procedures and the DNA storage within the Karolinska Institutet, and according to the biobank law that protects the individual's integrity. The participation rate of the DNA biobanking wave was 54.6% (n = 3018 participants). A subset of individuals was

also requested to contribute blood samples. Thus, DNA extracted from saliva and blood was used for the genetic and epigenetic analyses described in the following sections.

3.1.3. Reasons behind non-participation in DNA biobanking

In order to reveal the public's reasons behind non-participation in DNA biobanking, PART's DNA biobanking wave was used. In particular, a self-administered structured questionnaire was first mailed to non-respondents asking them to state the reasons for non-participation (155). Subsequently, a subgroup of individuals who answered the questionnaire was interviewed, and responses were analyzed using qualitative content analyses methods (156, 157).

3.1.4. Genetic analyses

Genetic analyses were performed for *NPY* and *MAOA*. The genetic analysis of *NPY* investigated the association of a functional *NPY* SNP (rs16139) with depression and SNP genotyping was performed using the Pyrosequencing methodology (85). Pyrosequencing is a DNA sequencing method that relies on the detection of pyrophosphate release after nucleotide incorporation (158). The genetic analyses of *MAOA* included both DNA sequencing and fragment analyses. DNA sequencing was used to determine the exact number of *MAOA* u-VNTR repeats and was performed using dye-terminator sequencing. The latter utilizes four dideoxynucleotide chain terminators, each labeled with different fluorescent dyes, which assist in the generation of a DNA sequence trace chromatogram after capillary electrophoresis (159). Fragment analysis was used to study the *MAOA* u-VNTR (a minisatellite) and associate it with depression. The latter method is based on the amplified fragment length polymorphism technique (160). Following capillary electrophoresis, fragment analysis generates a size estimate of the length polymorphism under investigation by comparing it to a size standard of nucleotide fragments with known lengths. All genetic analyses, as well as the epigenetic analyses presented in the next section, incorporate the polymerase chain reaction (PCR) as an essential step in the experimental protocols. PCR is probably the most widely used technique in molecular biology and entails the amplification of a piece of DNA across several orders of magnitude (161).

3.1.5. Epigenetic analyses

The human epigenetic analyses investigated the DNA methylation levels of gene-specific regions of *MAOA* and *NR3C1*, and tested for association with depression and childhood adversities, respectively. These gene-specific DNA methylation studies were performed using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry of base-specifically cleaved amplification products of bisulphite-treated DNA. This technology allows a quantitative assessment of DNA methylation levels under high-throughput conditions (162) and incorporates the treatment of DNA with bisulfite as an essential preceding step. Bisulfite treatment of DNA converts cytosine residues to uracil and leaves methylated cytosine residues unaffected, thus allowing the distinction between unmethylated and methylated CpG sites of the genome (163).

3.2. ANIMAL STUDIES

Animal studies included genetic, epigenetic, gene-expression, alternative splicing, protein, DNA/protein-interaction and behavioral experiments. All animal studies used the Flinders Sensitive Line (FSL) as a model of depression-like states and the Flinders Resistant Line (FRL) as its controls.

3.2.1. The FSL model of depression-like states

The FSL/FRL model is a genetic rat model, meaning that selective breeding of animals was used to generate these lines. When compared to FRL, the FSL line resembles human depression and is thus referred to as a depression-like model (164). More specifically, the FSL line exhibits both face validity for depression (e.g. psychomotor retardation, appetite reduction, sleep disturbances, impaired emotional memory and anxiety) and also antidepressant predictive validity [e.g. is successfully used for behavioral pharmacological screen of antidepressant drugs; (164, 165)]. In addition, neurochemical and molecular data suggest that the FSL rat exhibits changes consistent with the monoamine (serotonergic and dopaminergic) and neuropeptide models of depression (164). However it should be noted that the FSL does not resemble human depression in all aspects. For instance, it shows only stress-induced anhedonic effects, and neurochemical data argue against an involvement of the noradrenergic and the HPA-axis models of depression (164, 166). The FSL/FRL tissue samples used for the experimental procedures included PFC and HIP regions.

3.2.2. Genetic analyses

The genetic analyses included DNA sequencing of *P11* and *Npy*, and SNP genotyping of *Npy*. As described previously for humans, DNA sequencing was performed using dye-terminator sequencing and SNP genotyping was performed through Pyrosequencing.

3.2.3. RNA and protein analyses

Analyses of RNA molecules included gene-expression (mRNA) analyses, of *P11* and *Npy*, and examination of alternative splicing, of *Npy* pre-mRNA. The gene-expression analyses included *in situ* hybridization (ISH) and quantitative Real-Time PCR (qRT-PCR) experiments. ISH is a hybridization technique that utilizes labeled probes which bind to complementary DNA or RNA and allows both for localization and quantification of their corresponding levels in a tissue section (167-169). The second gene-expression technique, i.e. qRT-PCR, is a PCR-based method that amplifies and simultaneously quantifies a targeted DNA or RNA molecule (the RNA exists in the form of complementary DNA; cDNA) with the help of fluorescent dyes or reporters (170). Alternative splicing of pre-mRNA was examined using Rapid Amplification of cDNA Ends (RACE); a method used to obtain the full length sequences of mRNA transcripts and allows for the identification of putative splice variants (171). The protein analyses were performed by immunoblotting [a.k.a. Western blotting; (172)] techniques to detect and quantify protein levels of P11 and to detect putative protein isoforms corresponding to the *Npy* mRNAs splice variants.

3.2.4. Transcriptional activity and DNA/protein interaction analyses

To assess transcriptional activity of the different genetic variations present in the *Npy* promoter region, a luciferase reporter assay was utilized. In these bioluminescent experiments, cells are transfected with a genetic construct that contains the promoter region under investigation, placed upstream of the luciferase gene, which leads to the emission of light whose level is positively correlated with the gene-activity (173). To investigate putative DNA/protein interactions related to *P11* and *Npy*, three types of analyses were performed: *in silico*, *in vitro* and *in vivo* analyses. The *in silico* analyses were performed with the help of web-based tools that assist in the prediction of cis-regulatory elements and may provide clues about the proteins binding at a specific nucleotide sequence (174). The *in vitro* analyses included electrophoretic mobility shift assays (EMSA). An EMSA will reveal a DNA/protein interaction in the form of a gel shift following an electrophoretic separation of a mixture containing the probe/sequence of interest and a protein extract (175). The *in vivo* interactions analyses were performed using chromatin immunoprecipitation (ChIP). ChIP involves the cross-linking between chromatin and an associated protein, which is followed by an immunoprecipitation stage, a reversal of the cross-linking and a DNA quantification stage that yields data corresponding to the presumed interaction (176).

3.2.5. Epigenetic analyses

The epigenetic analyses included both DNA methylation and histone modification experiments. DNA methylation was performed both for gene-specific regions and globally for the entire genome. The gene-specific DNA methylation analyses were conducted for a *P11* promoter region with the help of Pyrosequencing technology after bisulfite treatment of the DNA (177). Global DNA methylation was assessed at CCGG sites throughout the genome using the LUminometric Methylation Assay (LUMA), which involves the DNA cleavage with methylation-sensitive and methylation-insensitive restriction enzymes followed by a quantification using Pyrosequencing (178). ChIP, described in the previous section, can also be modified for the study of histone modifications using appropriate antibodies (179) and was utilized for the investigation of certain histone marks at the *Npy* locus.

3.2.6. Treatments and behavioral models

Three types of treatment interventions and one type of behavioral experiment were utilized. Specifically, animals were subjected to SSRI (escitalopram), HDACi (sodium butyrate), and physical exercise (running) interventions. The behavioral model of depression used was the Porsolt test (a.k.a. behavioral despair test or forced swimming test; FST) that is considered to have an excellent predictive validity for antidepressants (180) and to be analogous to behavioral despair that is observed in depression (181). During this experiment, the rat is placed in a cylindrical glass that is filled with water and the swimming, climbing, and floating (immobility) times are scored. The expected outcome of an effective antidepressant is to decrease the floating time and/or increase the swimming or climbing time.

4. SUMMARY OF PAPERS AND RESULTS

This section provides a summary of the thesis' constituent papers by presenting their titles, background, purpose, main findings and conclusions.

4.1. PAPER I

4.1.1. Title

Antidepressant treatment is associated with epigenetic alterations in the promoter of P11 in a genetic model of depression.

4.1.2. Background

P11 levels have been found to be decreased in postmortem brain tissues of depressed subjects and in an animal model of depression (53, 182). In addition, *p11* knockout mice display a depression-like phenotype and *p11* gene therapy reverses depressed behaviors in animals (53, 183). Different antidepressant therapies have been associated with increased P11 levels (53, 184, 185) and DNA methylation has been shown to plays a role in *p11*'s transcriptional regulation (186, 187).

4.1.3. Purpose

To investigate potential P11 mRNA and protein differences in the PFC between the FSL and the FRL lines and explore possible DNA methylation contributors, as well as, effects of SSRI treatment.

4.1.4. Main findings

FSL had decreased levels of P11 in the PFC which were associated with higher DNA methylation levels in *P11*'s promoter region. SSRI treatment in FSL was associated with an increase of *p11* mRNA levels, a restoration of the DNA methylation pattern, and a reduction of *Dnmt1* and *Dnmt3a* mRNA levels.

4.1.5. Conclusion

P11 is associated with depression-like states and appears to be controlled by DNA methylation mechanisms that can be affected by antidepressant treatment with SSRIs.

4.2. PAPER II

4.2.1. Title

PreproNPY Pro7 protects against depression despite exposure to environmental risk factors.

4.2.2. Background

The functional SNP rs16139 (Leu7Pro, T1128C) has been associated with depression in humans (83, 84). This a.a. substitution lies within NPY's signal peptide and affects the processing of NPY's premature form (preproNPY) into mature NPY, with the C allele (Pro) leading to higher plasma NPY levels (82).

4.2.3. Purpose

To investigate the putative association of this SNP with depression and/or anxiety using the PART cohort.

4.2.4. Main findings

The Leu7Pro SNP was associated with depression and there was a tendency for association with anxiety. Specifically, Pro7 was rarer in subjects with depression suggesting a protective role of NPY that was also evident by studying healthy individuals exposed to environmental risk factors of depression.

4.2.5. Conclusion

Given that PreproNPY Pro7 leads to increased levels of NPY, this investigation is in line with the literature supporting a role for this neuropeptide in promoting mental resiliency.

4.3. PAPER III

4.3.1. Title

Allele-specific epigenetic programming of neuropeptide Y (*Npy*) in depression-like states.

4.3.2. Background

The human *NPY* promoter contains a functional SNP (C/T; rs16147) that controls *NPY*'s transcriptional activity (86) and affects emotional processing and stress response (86-88). The molecular mechanisms that underlie this transcriptional regulation remain however unknown. The FSL rat model is known to have decreased *Npy* levels compared to FRL (188-192).

4.3.3. Purpose

To investigate the putative genetic and/or epigenetic regulatory mechanisms leading to the aberrant *Npy* expression in FSL, in addition to testing the effects of HDACi administration and physical exercise (running) on *Npy* expression.

4.3.4. Main findings

The FSL/FRL model harbors a functional rat SNP in the *Npy* promoter region, similar to the one present in humans, which appears to control gene expression in an allele-specific manner. This allele-specific regulation was mediated by the binding of the CREB2 transcription factor and the Ep300 co-activator. Accordingly, Ep300's histone acetyltransferase activity was associated with specific histone modifications. Running, but not HDACi administration, increased *Npy* gene expression.

4.3.5. Conclusion

Npy gene expression is associated with epigenetic modifications that are influenced by SNPs in the promoter region and may be affected by physical exercise.

4.4. PAPER IV

4.4.1. Title

Neuropeptide Y: Identification of a novel rat mRNA splice-variant that is downregulated in the hippocampus and the prefrontal cortex of a depression-like model.

4.4.2. Background

Most genes are considered to undergo alternative splicing and dysregulation of this post-translation process can cause disease (94). However, there is no knowledge about the existence of *Npy* splice variants.

4.4.3. Purpose

To investigate the presence of novel *Npy* splice variants in different rat brain regions and examine their association with depression-like states.

4.4.4. Main findings

A novel "short" mRNA splice variant of *Npy* was found in five examined rat brain regions. This "short" *Npy* mRNA appeared to be non-coding, to have the same transcription start site (TSS), and to be expressed in lower levels, compared the longer mRNA counterpart. The FSL model had reduced mRNA levels of both *Npy* mRNA variants.

4.4.5. Conclusion

Npy undergoes alternative splicing, but with an unknown function of the newly identified non-coding "short" mRNA.

4.5. PAPER V

4.5.1. Title

Monoamine oxidase A (*MAOA*) gene-environment and epigenetic associations with depression in females, and *MAOA*'s action as a mediator of the association between childhood adversities and hypermethylation of the glucocorticoid receptor.

4.5.2. Background

MAOA's promoter contains a functional length polymorphism (u-VNTR) that, given the presence of childhood maltreatment, has been associated with conduct behavior in males (105-110) and with depression in youth (25). In addition, *MAOA* has been found to be regulated by DNA methylation (193). Childhood adversities have been associated with DNA hypermethylation and decreased expression levels of *NR3C1* (130-132, 194). There is little knowledge about how the DNA methylation statuses of genes correlate between different tissues.

4.5.3. Purpose

To investigate a putative G x E interaction between the *MAOA* u-VNTR and childhood adversities that potentially increases the risk of depression. To examine the DNA methylation status of *MAOA* and test for an association with depression. To test whether the *MAOA* u-VNTR may mediate the effect of early-life adversities on the DNA methylation status of *NR3C1*. To examine the concordance of *MAOA* and *NR3C1* DNA methylation between saliva and blood samples.

4.5.4. Main findings

Female individuals with childhood adversities that carried the *MAOA* u-VNTR leading to low *in vitro* gene expression, had an increased risk of developing depression. In addition, *MAOA* was hypomethylated in depressed female individuals compared to controls, and the u-VNTR appeared to mediate the association of parental death with hypermethylation of *NR3C1*. When comparing the mean DNA methylation levels of *MAOA* and *NR3C1* between saliva and blood, some gene- and gender-specific differences were found.

4.5.5. Conclusion

Both a G x E interaction and an epigenetic dysregulation of *MAOA* are associated with depression. In addition, an *NR3C1* hypermethylation resulting from early-life trauma may be mediated by variations of the *MAOA* u-VNTR, suggesting a common link between these two loci. Finally, when conducting gene-specific epigenetic analyses, it is important to take into consideration factors like gender and tissue type.

4.6. PAPER VI

4.6.1. Title

Examining the public refusal to consent to DNA biobanking: empirical data from a Swedish population-based study.

4.6.2. Background

Extensive biomedical datasets and DNA biobanks are essential in order to identify genes, gene-gene and gene-environment interactions contributing to complex human disorders like depression. Public acceptance and participation is thus a mandatory component, but most studies have only explored the *in theory* public opinion towards tissue donation, DNA information usage and genetic discrimination (195-199).

4.6.3. Purpose

To explore, in a pragmatic and empirical way, non-participation in DNA biobanking.

4.6.4. Main findings

Refusing to participate in DNA biobanking was motivated by two main reasons: lack of personal relevance and discomfort related to the DNA being used for alternative purposes. Interviews, with subjects feeling discomfort, revealed an underlying public mistrust of DNA biobanks that was associated with concerns about integrity, insecurity, privacy and suspiciousness.

4.6.5. Conclusion

Solidarity seems to be at stake for DNA biobanks and there is a need for stricter societal measures that include a solid and reinforced legislation to protect privacy, and clear guidelines on benefit sharing.

5. DISCUSSION

This section will provide a brief discussion that is primarily based on the papers' findings, their potential clinical relevance, and it will also present some future experimental perspectives.

5.1. IN GENERAL

Numerous studies have tried to unravel the pathophysiology of depression but have been hindered by the disease's heterogeneous nature and the need for thousands of depressed individuals to identify novel susceptibility genetic loci (50, 51). The first five constituent papers of this thesis have combined genetic, epigenetic, molecular and behavioral techniques to study depression and depression-like states in humans and animal models, respectively. Overall, these data support the complex etiological nature of this psychiatric disorder; with a number of genes being associated with depressive symptomatology. Interestingly, these associations do not only lie on the genetic level but also incorporate epigenetic modifications, like DNA methylation and histone modifications, which are in line with an epigenetic hypothesis of neuropsychopathology (200). The administration of agents with potential antidepressant effects, in combination with the study of their molecular outcomes, has also provided some additional insights into the epigenotypic architecture of depression. Finally, the last (sixth) constituent paper of the thesis, by studying why the public may refuse to donate DNA samples, may assist in the development of more comprehensive DNA biobanks that take into account, and do not diminish, core bioethical principles. Last but not least, as in almost all conducted research, it should be acknowledged that each paper and its corresponding study design comes with a number of technical and biological limitations that must be taken into consideration when interpreting the results. An effort to summarize these limitations has been made and they are presented in the discussion section of each paper.

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5.2. GENETIC FINDINGS

Even if achieving reproducibility in associations between candidate genes and depression has been a major scientific problem (83), the data generated by separate genetic studies have been able to increase the knowledge about the disorder's genetic component (80). For example, linkage has been proposed between different chromosomal locations and depression [e.g. 3p25-26 and 15q25-q26; (201, 202)]. Specific genes [e.g. *NPY*, *TNF*, and genes coding for neurotransmitter transporters; (83, 203, 204)] and G x E interactions [e.g. *5-HTT*LPR and/or *MAOA* u-VNTR interacting with childhood maltreatment; (24, 25)] have also been associated with depression. The genetic association data from this dissertation's papers replicated the involvement of *NPY* in depression (85) and provided some new evidence for a G x E interaction of *MAOA* that increased the risk of depression, particularly in females. With regard to *NPY*, the genetic data are in agreement with the neuropeptide's role in conferring mental resilience (75-78). In addition, a novel non-coding *Npy* mRNA splice variant was found in the rat brain (205) which, due to the high homology of *Npy* between rat and human (206), could indicate this variant's presence in humans, but with a currently unknown function. Finally, with regard to *MAOA*, the G x E data suggest that carrying a certain u-VNTR allele, with a low *in vitro* transcriptional activity, and having been exposed to childhood adversities will increase the risk of depression in adulthood, especially in females. This is also in agreement with previous *MAOA* studies suggesting that genetic factors predispose to psychopathology by affecting the sensitivity towards adverse and stressful environmental influences (25, 105-110).

5.3. EPIGENETIC FINDINGS

A general consensus in complex disorders, like depression, is that disease development usually requires the presence of environmental risk factors. In addition, data supporting an epigenetic dysregulation in different psychiatric disorders have been accumulating during the past decade (18, 200, 207). Thus, epigenetics has served as an attractive candidate in this mediatory interplay between environment and genome, as epigenetic modifications can be influenced by environmental and stochastic events, and can be transmitted both mitotically [i.e. from one somatic cell to another; (208)] and also transgenerationally [i.e. through the germline; (209)]. In line with an epigenetic dysregulation in depression, we found both DNA methylation and histone modification aberrations associated with depression or depression-like states. For instance, depressed females presented with a hypomethylated pattern of *MAOA*. This epigenetic modification theoretically leads to increased levels of MAOA, which metabolize their target neurotransmitters at a higher rate, and can lead to a neurochemical imbalance that is in line with the monoamine hypothesis of depression. Accordingly, a positron-emission tomographic study revealed elevated MAOA levels in the brain of depressed individuals (210). DNA methylation changes were also found with respect to *NR3C1* and the presence of certain childhood adversities. In particular, parental loss during early life, but not other types of adversities (e.g. parental divorce), was found to affect the DNA methylation status of the *NR3C1* region under investigation. Interestingly, this epigenetic outcome also appeared to be mediated by the *MAOA* locus. The *NR3C1* methylation data are in agreement with the psychobiological notion of "how adversity gets under the skin" (211) and support recent recommendations of trying to distinguish and study different types of adversities independently and not as a unitary construct

(212). The animal studies, in their turn, showed that DNA methylation changes of *P11* in the PFC are associated with depression-like states (213), and histone modifications of *Npy* in the HIP are dictated by genetic variations and are associated with *Npy* gene-activity aberrations. These epigenetic marks were present in the promoter region of the genes, and it is noteworthy that a weak human genetic association has been found between depression and a *P11* SNP located in the promoter region [close to a putative transcription factor binding site; (214)], and the same localization feature applies for a functional *NPY* SNP that has been associated with depressive psychopathology and anxiety (86-88). It is also of note that both of these epigenetic modifications were affected by antidepressant interventions, demonstrating the dynamic nature of epigenetic marks compared to the more static state of genetic variations. Even if DNA methylation and histone modifications were studied separately in this thesis, it should be mentioned that these two modifications are also known to be dependent on each other and their crosstalk is thought to be mediated by interactions between histone and DNA methyltransferases (215). The animal data also indicated that *Npy* is regulated by a transcription factor that is member of the CREB family, which has the ability of recruiting co-activators with histone modifying properties. The latter finding is in line with previous human genetic data which have linked CREB1 to depression in females (216, 217). CREB is also known to be upregulated by antidepressant treatments and an increase in its levels has an antidepressant-like effect in animal models (218). Finally, the comparison of the DNA methylation signature of specific genes between different peripheral tissues revealed some differences, and these tissue-type differences are important to consider especially when conducting epigenetic studies of traits primarily related to the brain. In psychiatric disorders, for instance, not only is it highly unknown how well epigenetic marks of peripheral tissues correlate with those of the brain, but the brain itself is known to acquire region-specific DNA methylation signatures (219).

5.4. NON-PARTICIPATION IN DNA BIOBANKS

While the contribution of genetic research to medical progress is widely acknowledged, the development of the scientific field itself has been controversial and overwhelming during the past decade. Bioethical and legislative measures have required revision but the difficulty in keeping pace with this constant development has been substantial. According to a former legal adviser to the US Senate, "we faced a paradox. Law is by definition local, but science is by definition global, and so are the legal problems that new technologies inspire" (220). In parallel, the ongoing public debate has made people realize the potential misuse of genomic data. Characteristically, in 2006 the National Institutes of Health (NIH) was described as an "ethical Potemkin village where a hollow system appears to provide the illusion of integrity" (221). Previous studies have mostly investigated the in theory public opinion towards tissue donation, DNA information usage and genetic discrimination, both in the Western (195-197, 199) and Eastern world (198). However, the paper presented in this thesis explored such attitudes in an empirical way by using the DNA biobanking wave of PART. The two main reasons among the public for not consenting to DNA biobanking, as shown by the questionnaire completion, were lack of personal relevance of DNA contribution and discomfort related to the DNA usage. Further examination of the latter reason, with the help of interviews, revealed a public mistrust of DNA biobanks. So even if all individuals who denied consenting to DNA biobanking had participated twice before in

the PART study (by answering extensive questionnaires), people seem to reconsider and reevaluate when it comes to sharing their genetic makeup. In particular, a number of interviewees stated that questionnaires (which they had agreed on completing before) could not act as an identifiable mean in the same way as the DNA can, making questionnaire participation more acceptable. This reference is in accordance with the American Society of Human Genetics' (ASHG) response to the NIH regarding genome-wide association studies: "The ASHG is acutely aware that the most accurate individual identifier is the DNA sequence itself…It is clear that these available genotypes alone…are more accurate identifiers than demographic variables alone..." (222). *Identifiability* in genomic research has been acknowledged as a pivotal concern and a number of de-identifying tactics are currently utilized (223). Equally interesting was the citation of DNA's *nature* as a contributing factor to the distressing view of biobank studies. Indeed, compared to clinical data that entail individual phenotypic information, genetic material has some unique characteristics: it can predict future health risks for both a proband and its blood-related family members, it can be derived from minute physical traces and it is an immortal material that can be effectively replicated and stored, making its utilization open for endless purposes in the future if not exploited in a regulated manner (224). Interestingly, the interview topic aiming at identifying prerequisites for future DNA participation conveyed the importance of keeping an individual informed and updated. Whereas it is expected that such a process would increase the feeling of individual control and probably even the personal relevance, the potential problems with e.g. revealing scientific results sometimes outweigh this possibility (225). In some instances, when this latter issue was brought during the interviews, individuals automatically recognized the potential problems and suggested by themselves that being offered the opportunity of choosing whether to get to know the results or not would be the best option. In addition, even if interviewees mentioned the possible DNA utilization by governmental agencies (e.g. police), there was no respondent who referred to the interest of private sectors (e.g. insurance companies and employers) in acquiring genetic information. The implications of such a potential unawareness are obvious when genetic testing, for instance, can nowadays be ordered in an unrestricted way (226). Conclusively, *mistrust* was shown to be a determinant factor in DNA biobank participation, which is in accordance with previous studies showing that trust plays a major role in biobank participation (227). This mistrust may not only interfere with the acquisition of large cohorts in biological studies but, in the case of these feelings being stronger in certain groups, it may also introduce a bias in the selection of participants. As a solution, it has been suggested to "build greater trust and reciprocity with participants through the equitable approach of benefit-sharing" (151), but there is currently no legally binding framework regulating benefit sharing for human genetic resources (228) and this needs to be addressed in the near future.

5.5. CLINICAL SIGNIFICANCE

The four main genes under investigation (*P11*, *NPY*, *MAOA* and *NR3C1*) and the data generated by studying them, in relation to depression and depression-like states, can also be discussed with regard to their putative clinical significance.

- **P11:** Although an increase in serotonin levels occurs soon after SSRI administration (229), clinical studies have consistently shown an unexplained ~4-6 week therapeutic delay (230). The data from this thesis suggest a downstream action of escitalopram, which involves the epigenetic upregulation of *P11*. In combination with previous data that demonstrate the behavioral antidepressant effects of SSRIs in the FSL model (231-233), these results are both in line with the clinical efficacy of SSRIs and also suggest a putative epigenetic mode of action. This epigenetic process may involve multiple up/down-stream biochemical steps that collectively require more time than the period needed to increase the availability of serotonin in the synaptic cleft, and could thus explain the observed therapeutic delays. Interestingly, and in accord with the previous assumption, selective agonists of the serotonin 5-HT4 receptor (the increase of which, on the cell surface, is a downstream effect of P11's action) have been shown to produce rapid (3-4 days) antidepressant effects in animals (234). It is worth mentioning that antidepressants have also been shown to increase the levels of S100β (235-237). S100β has been associated with depression and belongs to the same protein family as P11 (238, 239). Therefore it has been proposed that the upregulation of different S100 proteins might be a common characteristic of antidepressant treatments (240). It can thus be concluded that a transcriptional deregulation of *P11*, e.g. via DNA hypermethylation, can lead to depression but this state appears to be reversible and to be affected by SSRI administration.
- **NPY:** The low remission rates of SSRIs [~30% for citalopram; (241)] have encouraged the search for more effective antidepressants. Investigating the factors that support psychological resilience may thus serve as an alternative approach in order to advance in the psychopharmacological field (51). NPY, for instance, belongs to one of the best candidates considered to confer mental resilience (75-78) and its intranasal administration as a putative antidepressant is currently under investigation (74). The data on NPY presented in this thesis both support its function in promoting mental resilience and also show that –at least in rats– *Npy* is controlled by histone modifications, highlighting the therapeutic potential of agents acting as histone remodelers. Importantly, the preliminary data on physical exercise (in the form of rat wheel-running) suggest an increase in *Npy* mRNA expression that is associated with a "rescued" behavioral phenotype according to the FST model. This is in line with previous studies showing that physical exercise alleviates depressive symptoms and increases both hippocampal neurogenesis and *Npy* expression (49, 242-247). Conclusively, these data indicate that running has the potential of serving as a non-pharmacological antidepressant substitute that putatively acts through the upregulation of NPY and hippocampal neurogenesis. However, further studies and trials are needed to determine the exact type and period of exercise needed, but also to obtain accurate estimates of effect sizes (248).
- **MAOA and NR3C1:** In contrast to the epigenetic analyses of *P11* and *Npy* that were conducted using brain regions of the FSL model, the DNA methylation analyses of *MAOA* and *NR3C1* were performed using DNA from human peripheral tissues. Even if epigenetic aberrations in a peripheral tissue may not

account for the actual pathogenesis of a mental illness, they may serve as suitable disease or pharmaco-epigenetic biomarkers. *MAOA*, for example, was found to be hypomethylated in depressed individuals, which putatively leads to increased MAOA levels which is in line with previous studies showing a higher-than-normal abundance of MAOA in the brain of individuals with depression (210). However, as MAOIs are not the first line of choice for the treatment of depression due to their increased side-effects compared to e.g. SSRIs (249), the DNA methylation data suggest a way to identify the individuals that would benefit the most from this type of antidepressant medication. With regard to *NR3C1*, the results showed that adult female individuals with depression and with a certain *MAOA* genotype, who had experienced parental loss during childhood, were hypermethylated in the *NR3C1* promoter region. These data indicate the possibility of using the *NR3C1* methylation status as a biomarker, as individuals with depression and a history of childhood adversities have been suggested to benefit more from a combinatorial treatment of psychotherapy and pharmacotherapy (250), which requires further investigation using randomized control trials.

"Healthy mind in a healthy body"
-Thales

5.6. FUTURE PERSPECTIVES

Some of the future planned experimental procedures and studies will be mentioned before ending this discussion.

- Recent evidence suggests that *P11* is regulated by BDNF, thereby proposing an additional role of the P11 protein through which neurotrophins may exert their antidepressant action (185). The *BDNF* mRNA exists in nine splice variants in the rat (251), which are planned to be examined using the FSL model of depression-like states.
- It was recently shown that methylated cytosine (5-methylcytosine) can be converted to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC); constituting a pathway for active DNA demethylation (127, 128). The enzymes responsible for these conversions are called Tet1, Tet2 and Tet3 (252-254). In particular, 5hmC has been found to be abundant in the brain (255) which could suggest a functionality that is related to neuronal processes. As the DNA methylation techniques employed in this thesis' papers did not have the capacity to distinguish between the different forms of methylated CpG residues (256), it is of importance to investigate the contribution of 5hmC in genes whose methylome was associated with depression (e.g. *P11*, *MAOA* and *NR3C1*).
- Besides DNA methylation and histone modifications, a third major epigenetic component is a class of RNAs called miRNAs. These are short non-coding gene-regulatory RNA sequences that have been implicated in psychiatric disorders including schizophrenia, bipolar disorder, and autism (257) and that have distinct expression patterns in brain regions critical in depression [e.g. FC and HIP; (258)]. For example, miR-22 has been shown to regulate some of the genes mentioned earlier in this thesis [e.g. BDNF and MAOA; (259)]. However, little is known about their association with depression which requires further investigation and is currently ongoing using PFC regions from the FSL model.
- Several of the papers included in this thesis were based on a rodent model of depression-like states. Even if depression models can show high face and antidepressant predictive validities, and are essential in translational neuropsychiatric research, they will never be able to completely model human depression as some symptoms (e.g. guilt and suicidality) are impossible to reproduce using such systems. This emphasizes the need for reproducing the animal findings and testing their relevance using appropriate human material (e.g. RNA samples for testing gene expression levels), which is also an ongoing project.
- Finally, benefit sharing was proposed as a solution to address the issues raised by non-participation in DNA biobanks. While this concept may be more straightforward in cases involving the private sector (e.g. sharing monetary amounts deriving from pharmaceutical sales), it is less evident how to deal with it in academic research where the direct benefit is nothing more than knowledge. The fourth-ranking alternative in the questionnaire of paper VI (Alternative D: "I would have wanted to get informed about the results from my specimen, but as that is not possible I don't want to participate") provides some answers to this issue. However, the ethical problems with revealing scientific results/knowledge of unknown clinical relevance will most often outweigh this possibility. Thus, finding the best ways to bring benefit sharing into practice in large-scale genetic research is probably the next area of debate and future study.

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