Genetic determinants of cortisol responses to daily life stressors

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Eindverhandeling voorgedragen tot het bekomen van de graad
Master in de biomedische wetenschappen klinische en
moleculaire wetenschappen

universiteit hasselt

Melancolia I

by

Albrecht Dürer

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List of abbreviations

5-HIAA	5-Hydroxyindolacetic
5-HT	5-Hydroxytryptamine (Serotonin)
5-HTP	5-Hydroxytryptophan
5-HTR	Serotonin receptor
5-HTT	Serotonin transporter
6-FAM	6-Carboxyfluorescein
AADC	Aromatic amino acid decarboxylase
ACTH	Adrenocorticotropin
APTD	Acute phenylalanine/tyrosine depletion
ATD	Acute tryptophan depletion
BDNF	Brain derived neurotrophic factor
BBB	Blood brain barrier
CNS	Central nervous system
COMT	Catechol-O-methyltransferase
CRF	Corticotrophin-releasing factor
DA	Dopamine
DEX	Dexamethasone
DSMIV	Diagnostic and Statistical Manual Fourth Edition
DST	Dexamethasone test
DZ	Dizygotic
E	Epinephrine
EFPTS	East Flanders Prospective Twin Survey
ESM	Experience Sampling Method
GPCR	G-protein coupled receptor
GR	Glucocorticoid receptor
HPA-axis	Hypothalamic-pituitary-adrenal axis
IRS	Inflammatory response system
MAO	Monoamine oxidase
MAO-A	Monoamine oxidase A
MAOI	Monoamine oxidase inhibitor

List of Abbreviations

MD	Major depression
MDD	Major depressive disorder
MR	Mineralcorticoid receptor
MZ	Monozygotic
NE	Norepinephrine
NSS	Neurotransmitter sodium symporter
PCR	Polymerase Chain Reaction
PVN	Paraventricular nucleus
RIA	Radio-immunoassay
RFLP	Restriction fragment length polymorphism
SCL-90	Symptom Checklist 90
SCN	Suprachiasmatic nucleus
SLE	Stressful life event
SNP	Single nucleotide polymorphism
SNRI	Serotonin and noradrenalin reuptake inhibitor
SSRI	Selective serotonin reuptake inhibitor
STR	Short tandem repeats
TCA	Tricyclic antidepressant
TPH	Tryptophan hydroxylase
VMAT2	Vesicular monoamine transporter 2
VNTR	Variable number of tandem repeats
VP	Vasopressin

Preface

This thesis recapitulates my 6-month internship at the department of 'Psychiatry and Neuropsychology' at Maastricht University. With this period coming to an end, I cannot stop from thinking that another period of my life will soon be ended. Four years combining both effort and the occasional leisure are ultimately culminating in a degree as master in 'Clinical Molecular Life sciences'.
Of course, I would not be writing this master thesis if it was not for the support and guidance of some special persons. Therefore, I would like to take this opportunity to thank all of them.

First, I would like to thank my promotor Prof. dr. Harry Steinbusch for giving me the opportunity to explore the world of Neuroscience and providing me with this fascinating project. I would also really much like to thank my co-promoter dr. Gunter Kenis. If it was not for your infinite knowledge and interest of the genetic world, I would not have been able to come this far. You skilled me in things I will remember and apply for the rest of my scientific career. I also would like to thank Marianne Markerink for her 'joyful' assistance in the lab.

Next, I would like to say thank you to all my close friends. Thanks Annelies, Kathleen and Eveline for making carpooling such a real pleasure. Listening to 'Q-music' in the future will always remember of those early mornings. Thanks also to my fellow students for the good laughs and interesting discussions. Thank you to all the guys of 't Stiemerke for that much needed short break at the end of each working day.
But especially thank you Liesbet. If it was not for you I don not think I would be writing my thesis at this moment. Thanks for always being there for me, thanks for listening, thanks for sharing some beautiful years, thanks for being you. You will always have a special place in my heart 'moppie'.

Finally, I would like to thank my parents. Mama and papa, thank you for giving me the opportunity to study and making my life during this period as easy as possible. I can believe that it was not always easy giving advice and guidance to such a stubborn son. But thanks for your patience and for always listening to me. Thank you for being my parents.

Thank you all for the support,

Olivier

Abstract

Introduction: Studies consistently link stress with an increased risk for major depression (MD). The underlying causality proposes a dysfunctional regulation of the neuroendocrine stress response, in particular the hypothalamic-pituitary-adrenal-axis (HPA-axis). However, the link between HPA-axis dysfunction and MD is not completely straightforward since not all individuals succumb to depressional onset after experiencing stressful events. Epidemiologic studies explain this variance by showing that MD is subject to specific gene-environment interactions, providing each individual a unique reactivity to experienced stressful life events (SLEs). This imposes the search for genetic variations at the basis of HPA-axis regulation, rendering some individuals more susceptible to MD onset after experiencing SLEs. To overcome methodological difficulties often encountered in genetic association studies, our study examined the influence of genetic variations on individual stress sensitivity, a previously described endophenotype of depression. Genetic mutations found in the serotonergic system (5-HT_{1A}C-1019-G, MAO-A uVNTR) were primarily studied, since this system is a principal regulator of the HPA-axis. It is hypothesized that genetic determinants, which have previously been associated with a higher risk for MD, will influence HPA-axis reactivity providing individual variability in stress sensitivity and MD risk.

Materials and Methods: Participants were recruited from the East Flanders Prospective Twin Survey (EFPTS), a longitudinal female twin population used to investigate gene-environment interactions in affective disorders. From this population, saliva samples for cortisol measures were collected in conjunction with registration of minor daily life stressors. To assess the effects of these stressors, the experience sampling method (ESM) was used. ESM measures fluctuations in three different daily life stressors: social, activity- and event-related stress. The influence of genetic variations on salivary cortisol levels in response stressors was examined by multilevel regression analysis.

Results: Social stress, but not activity- and event-related stress, significantly elevated salivary cortisol levels. Moreover, there was a significant association between the 5-HT_{1A} genotype and diurnal cortisol variations. Also, a significant elevation in social stress-induced cortisol levels was observed in function of 5-HT_{1A} genotype. In contrast to 5-HT_{1A} genotype, no significant association between the MAO-A genotype and HPA-axis reactivity after experiencing minor daily life stressors was found.

Conclusion: Social stressors appear to be essential predictors of cortisol response to minor daily life stressors in our female population. Additionally, our findings link a polymorphism in the 5-HT_{1A} gene with an increased sensitivity and responsiveness of the HPA-axis to daily life stressors. This association could not be found for variations in the MAOA gene, another important regulator of the serotonergic system. These data suggest that 5-HT_{1A} receptors play a primary role in serotonergic regulation of HPA-axis reactivity to minor daily life stressors. In addition, these data further support the use of reliable endophenotypes for analyzing the contribution of genetic variations to the development of psychiatric disorders like MD.

1 Introduction

1.1 Major Depression (MD)

Around 400 B.C. Hippocrates was the first to conceptually describe depression by introducing the term melancholia. He related this state of restlessness with the term 'black bile', representing a permanent fictional entanglement in the body. It was not until the 17th century that this ancient theory eclipsed by a scientific shift towards more chemical explanations (1). Nowadays, major *unipolar* depression (MD) or major depressive disorder (MDD) is defined as a complex psychiatric disorder with an ambiguous neuropathological basis marked by behavioural and motor abnormalities, feelings of hopelessness and suicidal thoughts (2).

MD represents one of the most prevalent forms of mental illness in the elderly but is also becoming increasingly familiar in adolescents and children (3). The disorder finds itself among the top five leading causes of disease burden throughout the world, having severe social and economical implications. Besides a worldwide incidence of approximately 2-5%, lifetime prevalence of MD is estimated between 10-15% (4). However, in these statistics discrimination has to be made when taking the role of gender into account because studies consistently provide strong support for both quantitative and qualitative sex differences in MD onset. More particularly, greater risk (2:1 ratio) has been attributed to women for developing MD when compared to men. The source for this female excess in depression is still unclear but can possibly be explained by i) genetic differences providing greater heritability of depression to women (5), ii) hormonal differences showing that oestrogen elevates gene expression in women rendering them more vulnerable to stress (6), iii) social differences showing a greater tendency of women to empathize providing them a greater preference for intense social relationships (6).

Table 1 The Diagnostic Criteria for Major Depression (Source: Nestler et al. Neurobiology of Depression. Neuron 2002)

Depressed mood
Irritability
Low self esteem
Feelings of hopelessness, worthlessness, and guilt
Decreased ability to concentrate and think
Decreased or increased appetite
Weight loss or weight gain
Insomnia or hypersomnia
Low energy, fatigue, or increased agitation
Decreased interest in pleasurable stimuli (e.g., sex, food, social interactions)
Recurrent thoughts of death and suicide
A diagnosis of major depression is made when a certain number of the above symptoms are reported for longer than a 2 week period of time, and when the symptoms disrupt normal social and occupational functioning

Specific brain regions (hippocampus, hypothalamus, amygdala) and neurotransmitter systems (serotonergic, noradrenergic, dopaminergic) (*see chapter 1.2.2*) have been proposed to play an essential role in the pathology of this emotional and behavioural disorder. This led to several promising hypotheses throughout the past decades, with the monoamine hypothesis being the most prominent in literature (*see chapter 1.2.1*) (7). However due to a heterogeneous and complex clinical picture, the monoamine hypothesis on its own cannot provide a consensus concerning the mechanisms of major depression making the understanding of MD neuropathology rudimentary.
To date, the diagnosis of depressive disorders is based on a diverse set of symptoms established by the "Diagnostic and Statistical Manual Fourth Edition (DSMIV)" (8) (see table 1). This manual distinguishes MD from other mood disorders like bipolar depression, dysthymia and cyclothymia.

Although the precise aetiology of depression remains elementary, recent years brought forth new scientific advantages with the opportunity of 'shedding light' on the neuropathology of MD (7). The advent of genetics and pharmacogenomics provided new means for revealing the (genetic) basis of MD, but until now genetic studies provided inconsistent results and limited success. To clarify, genes and the clinical outcome of MD are possibly not associated in a simplistic true relationship, contradicting the presence of a single or collection of gene(s) singularly coding for the clinical outcome MD (9). To overcome this methodological difficulty, Hasler et al. proposed the utilization of endophenotypes *(see chapter 1.4.6)* which represent internal phenotypes able to fill the gap between genes and the clinical outcome in depression (10).
In this study a possible endophenotype for MD, namely 'reactivity of the hypothalamic-pituitary-adrenal axis (HPA axis)', is studied **(figure 1)**. Recent studies show that, besides a monoaminergic dysfunction, stressful life events (SLE) *(see chapter 1.3*) increase risk of MD by overactivating the HPA-axis (11,12). However, because only a small fraction of the exposed individuals develop MD upon exposure to SLEs, it can be hypothesized that some persons may be more susceptible to depression-inducing effects of SLEs. These data support the search for genetic variations moderating HPA-axis 'reactivity', hereby having the potential to alter stress vulnerability and heighten the risk for MD onset (*see chapter 1.4*). This will ultimately open new opportunities for designing possible reliable tests for the early detection of individual MD risk and development of more efficient therapeutics.

1.2 Monoamine dysfunction in MD

Monoaminergic pathways are widely distributed throughout the central nervous system (CNS) showing high responsivity to aversive stimuli like stress. The fundamental monoaminergic subclasses which are implicated in receptive neurotransmission are the catecholamines dopamine (DA), epinephrine (E) and norepinephrine (NE) and the indoleamine serotonin (5-hydroxytryptamine or 5-HT) (13).

Upon neuronal stimulation these monoaminergic neurotransmitters play a crucial role in the regulation of behaviour, cognition, endocrine secretion, motor function and other factors which are

found to be profoundly disrupted in depressive states (2). Considering these disturbances it is not surprising that an association between monoaminergic (especially 5-HT-ergic) perturbations and neuropathology of MD has been enshrined in literature for decades.

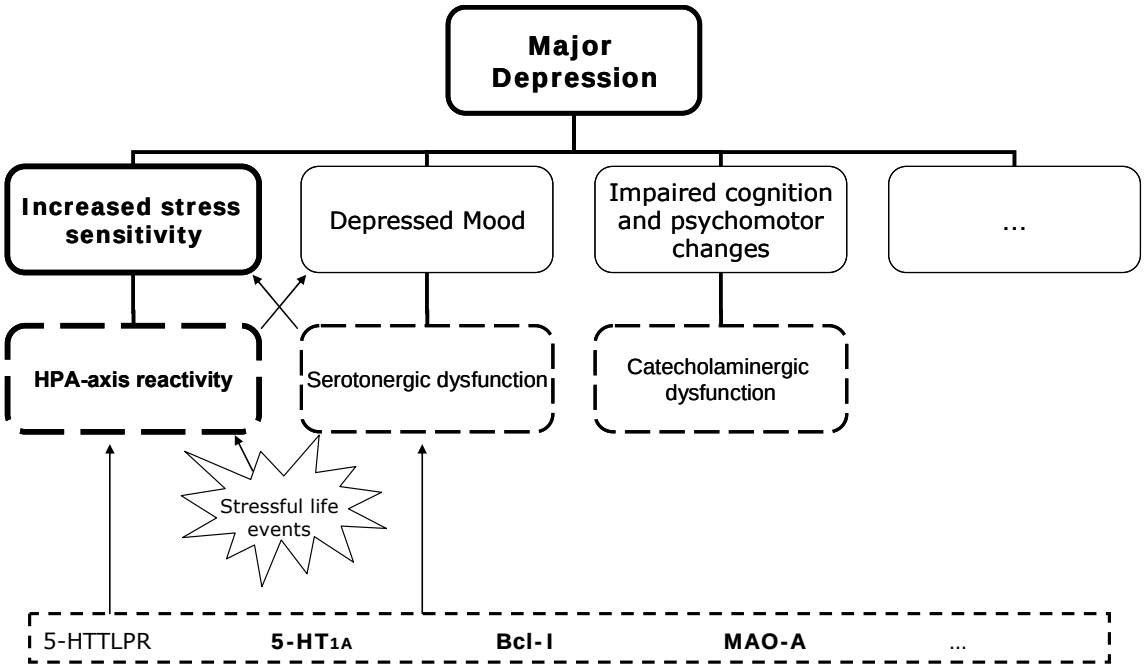

Figure 1: A possible endophenotypic model illustrating how genetic mutations may moderate key components of MD. Not all functional possibilities are indicated for maintaining clarity of the figure.

1.2.1 'The monoamine hypothesis': A potential paradigm for unraveling MD

Before discovery of the first psychotropics, the consideration that depression could be successfully managed by pharmacological treatment was unimaginable. The serendipitous discovery of the monoamine oxidase inhibitor (MAOI) *iproniazid* (14) and the tricyclic antidepressant (TCA) *imipramine* (15) in the late 1950s revolutionized psychiatry and was seen as an essential therapeutic breakthrough in depression research. These first generation antidepressants displayed potent effects on mood and behaviour mediated by a mutual potential to modify monoamine levels in the CNS, suggesting MD to be a pure biochemical phenomenon. These findings swiftly led to the establishment of a 'monoamine hypothesis of depression' in the early 1960s (16). The theory assumes that MD develops due to a multi-level deficiency of central monoaminergic activity and that this deficit can be efficiently corrected by antidepressants.

The foundation of the monoamine hypothesis launched a new paradigm, in which considerable efforts were made to unravel the underlying monoaminergic mechanisms causing MD. Predominantly for 5-HT, and to a lesser extent for NE and DA, considerable evidence was found supporting a monoamine role in the pathogenesis of depression.

The contribution of serotonergic pathways has been subject to intensive research for decades, providing substantial evidence for an altered brain serotonergic transmission. Firstly, when comparing depressed patients against healthy controls, a decrease in plasma thryptophan levels

(17), cerebrospinal fluid 5-hydroxyindolacetic (5-HIAA) levels (18) and platelet serotonin uptake (19) were found in depressed individuals. Second, cognitive deficits reported in patients with MD were found to be associated with serotonergic dysfunction (20). Lastly, acute tryptophan depletion (ATD) a method which acutely lowers serotonin in the brain by depleting its precursor tryptophan, provided additional evidence. The ATD studies showed an increased vulnerability to lowered mood in healthy subjects, especially in women with a familial history of MD (21). Furthermore, ATD did not exacerbate depression symptoms in unmedicated depressed subjects already suggesting the presence of a maximum dysfunction of the serotonergic system in depressed patients (22).

The involvement of NE, was primarily studied by applying acute phenylalanine/tyrosine depletion (APTD), a similar method to ATD, which acutely depletes the availability of catecholamine precursors phenylalanine and tyrosine lowering catecholamine neurotransmission (23). APTD studies showed that reduced NE neurotransmission increases vulnerability to lowered mood in healthy subjects (24) and lowers mood in healthy patients with a familial history of MD (25). As was the case for ATD, APTD also does not exacerbate depression symptoms in unmedicated depressed subjects (26).

All these observations etiologically link pertubations in 5-HT and NE neurotransmission with depression. However, due to the fact that to date research focused on the association of genetic variations in the serotonergic system and MD pathology, this study does not incorporate genetic analysis of the catecholaminergic system. Consequently, emphasis in this paper will lie on the central serotonergic system and its potential association with MD development.

1.2.2 The central serotonergic system

Serotonin (5-Hydroxytryptamine, 5-HT) was first detected in the CNS by Twarog and Page during the mid-fifties (27). Since 'the monoamine hypothesis', 5-HT is extensively studied and believed to be implicated in the pathophysiology of several affective mental disorders such as MD (28). This monoaminergic neurotransmitter is principally synthesized in two steps from L-tryptophan, an essential amino acid which is obtained via normal nutrition. Due to its hydrophilic structure 5-HT is not able to cross the blood-brain-barrier (BBB), so apart from the serotonin pool originally found in the gastrointestinal tract (29) there is a separate pool of 5-HT synthesized in the CNS (27). For this 5-HT brain synthesis to occur, L-tryptophan first has to cross the blood-brain barrier via specific transporters. This entry is directly dependent on the availability of L-tryptophan, which competes with other large neutral amino acids for BBB transportation (30). After entry into the brain, L-tryptophan is primarily converted by the enzyme tryptophan hydroxylase (TPH) to 5-hydroxytryptophan (5-HTP), which is the rate-limiting step of 5-HT synthesis. 5-HTP is subsequently converted by aromatic amino acid decarboxylase (AADC) to form serotonin (5-HT) **(figure 2)**.

5-HT brain synthesis occurs in distinct serotonin-containing neuronal cell bodies and their nerve terminals. These serotonergic soma are almost all restricted to discrete cell clusters along the midline of the brainstem, the raphe nuclei. Especially the dorsal and median raphe nuclei, located in the area of the caudal midbrain and rostral pons, give rise to the majority of serotonergic fibers in the human brain (31). Within these dorsal and median raphe nuclei, serotonergic neurons are organized in particular zones or groups that send axons to virtually all areas of the human brain,

including thalamus, hypothalamus, amygdala and hippocampus (32) **(figure 3 left)**. These anatomic regions, as mentioned earlier, play an important role in emotional and behavioural disorders like MD. After synthesis, serotonin is transported into synaptic vesicles at the nerve terminal by a specific vesicular monoamine transporter, VMAT2 **(figure 3 right)**. VMAT2 represents the predominant monoamine vesicular transporter in the central nervous system (CNS) transporting not only serotonin, but also NE and DA (33).

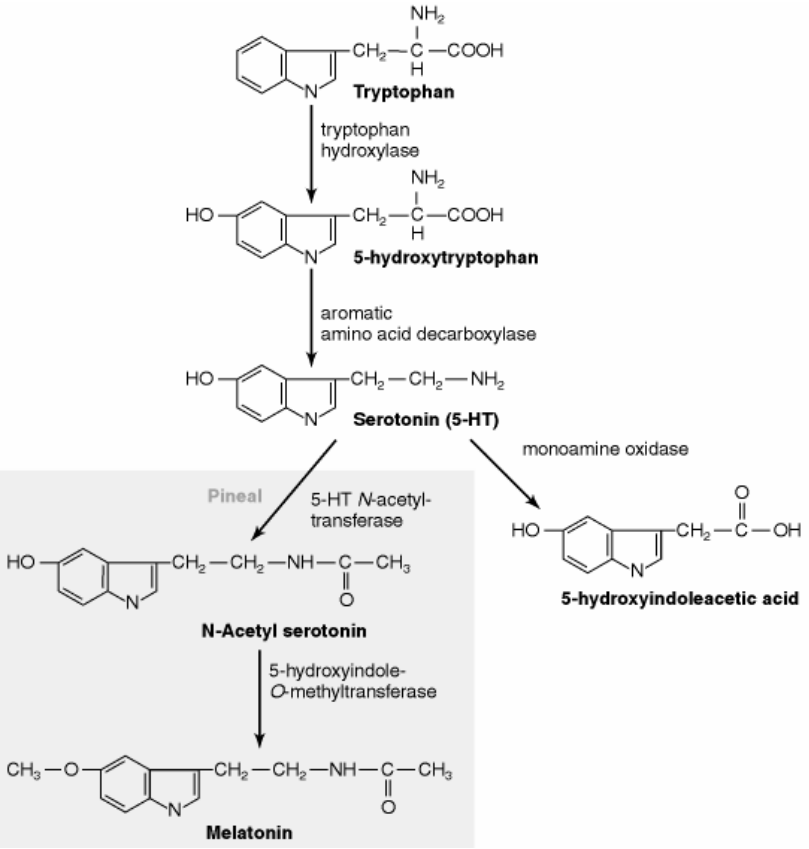

Figure 2: The biosynthesis and catabolism of serotonin. Note that besides enzymatic breakdown in the nerve terminal, serotonin is converted enzymatically to melatonin in the pineal gland (Adapted from: Siegel et al. 199, Basic Neurochemistry Sixth Edition)

5-HT exerts its diverse effects via a distinct set of 5-HT receptors (5-HTR). The existence of at least 14 receptor subtypes, divided into seven classes ($5HT_{1-7}$), has been shown (34). With the exception of the 5-HT_3 receptor, which is a ligand-gated ion channel, all 5-HT receptors belong to the G-protein-coupled receptor (GPCR) superfamily (35).

The 5-HT_1 receptors form the largest class comprising five receptor subtypes ($5HT_{1A, B, D, E, F}$). Upon 5-HT binding they preferentially couple to $G\alpha_{i/o}$, thereby inhibiting adenyl cyclase and reducing cAMP formation (36). The best known subtype, the 5-HT_{1A} receptor, is largely distributed throughout the CNS. In the raphe nuclei, 5-HT_{1A} receptors are mainly located somatodendritic acting as autoreceptors regulating 5-HT release through inhibition of neuronal firing. This inhibition is achieved by neuronal hyperpolarisation mediated by G-protein coupled K^+ channels (37). In contrast, postsynaptic 5-HT_{1A} receptors are present in a number of limbic structures particularly hippocampus, but also amygdala and hypothalamus, suggesting a role for 5-HT_{1A} in 5-HTergic

mood and behaviour mediation (38). This role is fortified by evidence implicating 5-HT_{1A} receptors in a number of behavioural and emotional actions and/or disorders. On the one hand, 5-HT_{1A} was found to assist in the regulation of the HPA-axis, principally via acting on adrenocorticotropin (ACTH) (39). On the other hand, 5-HT_{1A} receptors were revealed to be reduced in the hippocampus of patients suffering from major depression (40).

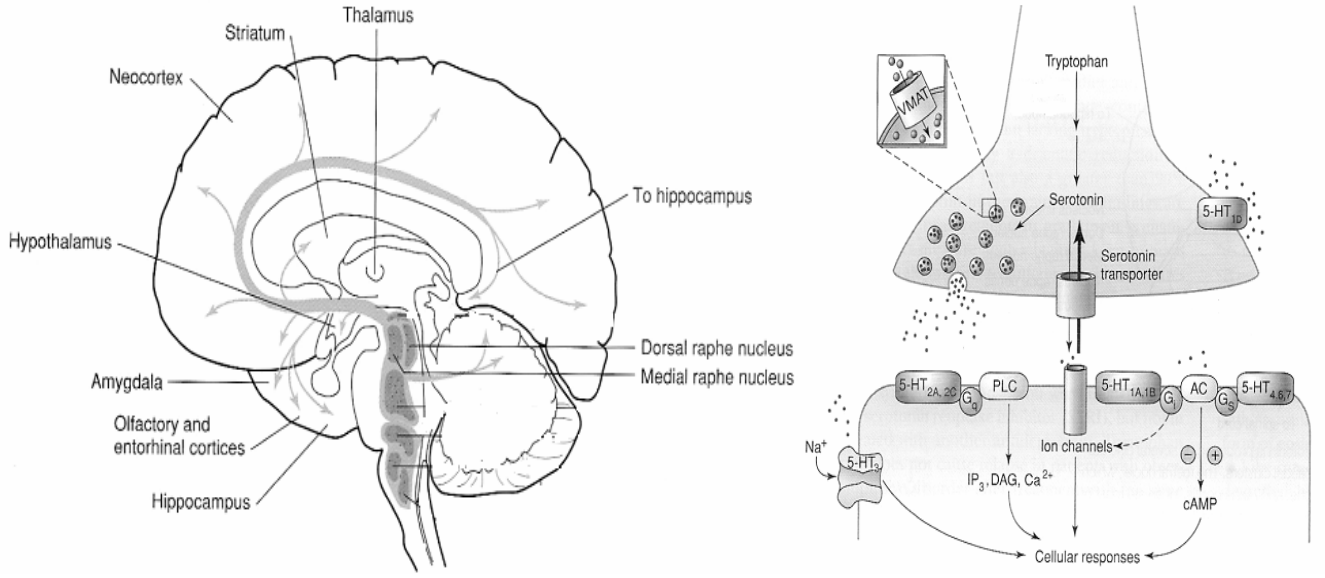

Figure 3: ***Left.*** Distribution of serotonergic nerve terminals to distinct brain regions. ***Right.*** Serotonin at the nerve terminal. (Source: Nestler et al. (2001), Molecular Neurophamacology: A Foundation for Clinical Neuroscience)

Besides the regulation of 5-HT release by presynaptic autoreceptors, the action of 5-HT is also controlled (even terminated) by active reuptake from the synaptic cleft into the presynaptic nerve terminal and/or glial cells. This reuptake mechanism utilizes a Na^+/Cl^- dependent serotonin transporter (5-HTT), a member of the neurotransmitter sodium symporter (NSS) family of sodium-coupled neurotransmitter transporters, which assist in controlling the concentration of serotonin (and catecholamine) release (41).

After reuptake, or when present in the presynaptic nerve terminal in an unsequestered state, 5-HT is metabolized to 5-HIAA (42). Two enzymes are important in the metabolic transformation of monoamines, being monoamine oxidase (MAO) and catechol-O-methyltransferase (COMT), both important for their respective monoamine subclass. Little or no COMT is found in serotonergic neurons (42), leaving MAO to be the principal enzyme acting on 5-HT. Two isoforms of MAO (MAO-A and MAO-B) exist, but of the two isoforms MAO-A preferentially metabolizes 5-HT.

The finding that first generation antidepressants demonstrated the potential to modify monoamine levels in the CNS through inhibition of 5-HT and NE reuptake (TCAs) and inhibition of MAO (MAOIs), provided a foundation for the development of more efficient second generation

antidepressant agents. Throughout the years, new insights into the composition of the central serotonergic system opened the way for, i) selective serotonin reuptake inhibitors (SSRIs) which selectively acted upon the 5-HTT, thereby inhibiting the neuronal reuptake of serotonin (43), and ii) "dual action" serotonin and noradrenalin reuptake inhibitors (SNRIs) inhibiting both 5-HT and NE reuptake from the synaptic clefts (44). The SSRIs were shown to have the same clinical efficacy as the first generation antidepressants, yet appeared to be better tolerated, explaining their enormous commercial success (45).

1.2.3 'The monoamine hypothesis': An insufficient model for MD

Although many studies link serotonergic dysfunction with major depression, 'the monoamine hypothesis' in itself could neither explain the whole mechanism of antidepressants actions, nor the pathogenesis of MD. From the start, the theory suffered from several shortcomings. For example, it was seen that after administration of antidepressants the expected elevation of monoamine levels in the CNS took place almost immediately. But strangely, the therapeutic response required chronic administration for weeks of these antidepressants (46). This suggests that secondary adaptive alterations, rather than a primary biochemical effect, may be the reason for the observed clinical improvement. Numerous neuropharmacological models in addition to 'the monoamine hypothesis' were developed, trying to explain this delayed effects of antidepressant. Dumas et al. pinpointed to intracellular mechanisms which decrease or increase the generation of neurotrophic factors necessary for neuronal survival (47). In this hypothesis, administration of antidepressants upregulates the intracellular second messenger cAMP leading to an increased expression of brain derived neurotrophic factor (BDNF) found to be decreased in depressed patients (47). Evidence was also found linking MD with an activation of the inflammatory response system (IRS), owing to an increased production of proinflammatroy cytokines. Research on this topic showed increased numbers of blood leukocytes including monocytes and T-lymphocytes (48). Antidepressive treatment were shown to normalize these signs of inflammation, suggesting antidepressants to have negative immunoregulatory effects contributing to a delayed treatment effect (49). To date however, none of these models is able to provide conclusive evidence concerning MD pathology and antidepressant action.

Besides a delayed treatment effect of antidepressants, another observation shows that not all depressed patients respond equally to the same antidepressant (50). This led to a general consensus as to the existence of individual vulnerability for MD risk. Yet again, numerous models were established to explain this individual variation, probably originating from environmental and/or genetic factors. In 1978, Brown and Harris demonstrated that MD was likely to be antedated by SLEs. From that moment the challenge initiated to reveal depression as a stress-related disorder, with SLEs playing a essential causative or contributing factor of MD risk in addition to the presence of a serotonergic dysfunction (51).

1.3 Stressful life events and Major Depression

1.3.1 The 'stress' concept

Hans Selye was the first to imply that so called 'stressors' play an inevitable part in our daily life (52). All existing organisms attempt to continuously uphold their bodily functions in a dynamic equilibrium called homeostasis. In Selye's 'biological stress concept' this equilibrium is challenged by stressful physical and/or psychological events. This results in a human 'stress response', which is found to be mediated by several neuropeptide-secreting systems. On the one hand, activation of the sympathetic part of the autonomic nervous system takes place. This causes a release of NE from widely distributed synapses and secretion of adrenaline from the adrenal medulla, instantly activating the behavioural 'fight-or flight' response (53) . On the other hand, the HPA-axis is activated, culminating in the secretion of glucocorticoids from the adrenal cortex (54).

1.3.2 HPA-axis in mediating the stress response

The HPA-axis operates throughout two essential settings of everyday life. The level of HPA-axis reactivity is genetically set at birth and its sensitivity will be modulated by environmental experiences throughout life. Under relatively unstressed conditions, HPA-axis activity follows a circadian rhythm controlled by the suprachiasmatic nuclei (SCN) of the hypothalamus, with peak glucocorticoid secretion at initiation of the waking cycle and then dropping throughout the day. This permits circulating cortisol to optimize the functional tone of several homeostatic systems (55).

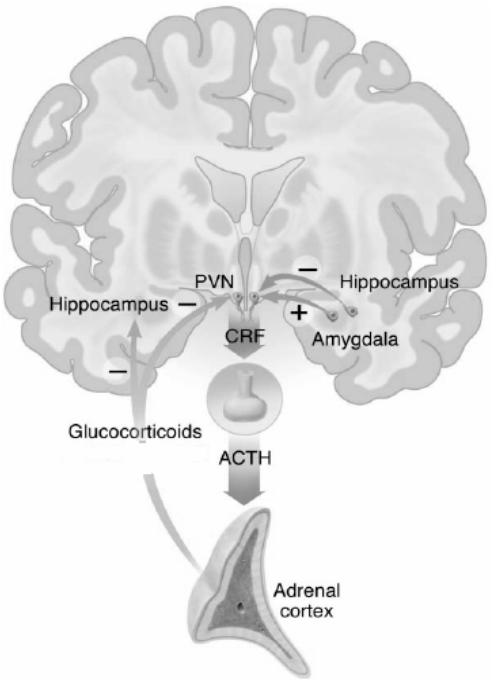

Figure 4: Active pathways and 'negative feedback' regulation of the HPA-axis (Source: Nestler et al. (2002). Neurobiology of depression, Neuron)

But as already mentioned, the brain also responds to episodes of acute and chronic stress by activation of the HPA-axis **(figure 4)**. In both conditions, the HPA-axis acts via a set of hypophysiotrophic neurons in the medial parvocellular division of the hypothalamic paraventricular nucleus (PVN). These neurons synthesize and secrete corticotrophin-releasing factor (CRF) in combination with vasopressin (VP) into the hypophysial portal veins. Via this portal tractus, the secretagogues stimulate the anterior pituitary corticotrophs, which release of ACTH into the systemic circulation. Upon binding to ACTH receptors of the adrenal gland, this hormone stimulates the synthesis and release of glucocorticoids (principally cortisol) from the adrenal cortex (56). These steroid hormones alter transcription of key regulatory proteins, which will ultimately result in the activation of homeostatic processes including glycogenolysis, suppression of the innate immune system, inhibition of bone and muscle growth, proteolysis and lipolysis (57), preparing the body for the changing environment. However, these processes are generally catabolic, pointing out that cortisol hypersecretion is deleterious and thereby not surprisingly, implicated in several metabolic and affective disorders, including MD (58). For this reason, activity of the HPA-axis is tightly controlled by several 'negative feedback' mechanisms **(figure 4)**. Firstly, a direct glucocorticoid 'negative feedback' mechanism exists, whereby cortisol primarily acts on the hippocampus and prefrontal cortex, which subsequently exerts an inhibitory influence on hypothalamic CRF-containing neurons (59). Secondly, an until present unknown glucorticoid-independent inhibition from neuronal sources is present. This was revealed by Jacobson et al. who showed that andrenalectomized animals can still inhibit HPA-axis activity, thus lacking glucocorticoid feedback (60). Contradictory to these inhibitory mechanism, another limbic region, the amygdala, is implicated in the activation of glucocorticoid secretion (59). It is important to note that the limbic regions implicated in HPA-axis regulation (hippocampus, amygdala) are also implicated in MD.

1.3.3 Cortisol: A special neuroendocrine hormone in stress mediation

Hormones play a critical role in the development and expression of a wide range of behaviours. The glucocorticoid cortisol, constituting the hormonal end product of the HPA-axis, is able to reach every organ via the circulation. Under relatively unstressed conditions, well-defined pulses of cortisol are secreted during a 24h period, representing the well-known circadian rhythm. This diurnal tone is characterized by peak levels of cortisol secretion in the early morning and a nadir around midnight (55). Cortisol is especially important in that it exerts the final effects of the human stress response on distinct organs, whereby an excess of cortisol is secreted from the adrenal cortex in addition to the basal levels secreted during the circadian cycle (56). It was found that large individual differences exist in the diurnal levels of cortisol, with a possible cause being an individual's genetic makeup. By genetic model fitting, a substantial heritability for cortisol levels during the awakening period was revealed. However, cortisol levels during the day were shown to be primarily influenced by environmental factors (61). Steroid hormones like cortisol are small, lipid-soluble ligands that diffuse across the cell membrane to bind on its respective receptors, localized in the cytoplasm. Cortisol specifically, mediates its action through two distinct intracellular corticosteroid receptor subtypes, referred to as mineralcorticoid (MR) and glucocorticoid receptors (GR) (9). Attributable to an approximately 5-10 fold greater affinity of the MR for cortisol, this receptor subtype has been postulated in the regulation of basal HPA tone, whereas the GR is seen

to be important in i) the mediation of cortisol through negative feedback following stress and ii) the modulation of target organs following acute stress (58). GR density is highest in neurons of ascending serotonergic HPA-axis pathways and in limbic neurons that modulate HPA-axis activity such as the hippocampus, where both GR and MR are colocalized (62). The underlying mechanism of cortisol action involves an, upon binding induced change in receptor conformation, which ultimately leads to modulations of gene transcription inhibiting the ongoing stress reaction (58).

1.3.4 "HPA-axis reactivity": An important hallmark of depression

In 1978, Brown and Harris showed that SLEs are associated with MD (51). As a result, research trying to elucidate the relation between stress and depression intensified. Consequently, a large body of evidence emerged relating the occurrence of SLEs with an increased vulnerability to MD. Whilst trying to explain this association, data almost consistently pointed into the direction of a dysfunctional HPA-axis in depressed patients. Depressed patients showed elevations of CRF (63), ACTH (64), increased urinary excretion of glucocorticoid metabolites (65) and elevations in peripheral cortisol secretion (57). When looking into the history of MD research, high circulating levels of cortisol were documented even before an association between stress and depression was suggested. However, this was initially seen as an epiphenomenon, reflecting the stressful experience of depression itself. To further assess a possible dysfunction of the HPA-axis in depressed patients, Carroll et al. utilized the dexamethasone-suppression test (DST), an established procedure which makes it possible to diagnose hypercortisolemia in Cushing's syndrome (66). In this test, a synthetic glucocorticoid, dexamethasone (DEX), is orally administered. This synthetic hormone exerts its effects on the HPA-axis, more specifically at the level of the anterior pituitary, where it reduces the secretion of ACTH (67). In normal subjects, this results in a decrease of cortisol release from the adrenal cortex. However, failure of DEX to suppress plasma cortisol suggests impaired feedback resulting in hyperactivity of the HPA-axis. Carroll et al. demonstrated that a large percentage of drug-free depressed patients indeed exhibited failure to suppress secretion of cortisol following DEX administration, suggesting a role for HPA-axis dysfunction and subsequent hypercortisolemia in MD (66).

These data ultimately led to the establishment of the 'glucocorticoid cascade hypothesis', stating that prolonged overproduction of glucocorticoids, as a result of HPA-axis hyperactivity, damages brain structures (especially the hippocampus) essential in HPA-axis 'negative feedback' inhibition. Such damage will subsequently result in indefinite glucocorticoid overproduction, ultimately leading to the development MD (9). However, as was the case for 'the monoamine hypothesis', this hypothesis could not fully explain the heterogeneous clinical picture of MD, as it was shown that SLEs per se are not sufficient to cause MD (4). But as mentioned earlier, HPA-axis reactivity and subsequent cortisol secretion are not solely regulated by environmental factors like stress. They also comprise an important genetic basis, which suggests the existence of gene-environment interactions causing individual variability to stress sensitivity (5). This individual discrepancy may render some subjects more vulnerable to developing MD after experiencing SLEs.

1.4 Genetics of depression

1.4.1 Psychiatric genetics: Epidemiologic, linkage and association studies

Genes have the incomparable potential to manage all levels of human biology. The discovery that stress sensitivity is mediated by genes generating an unique HPA-axis reactivity for each individual, underlined the importance of genetic analysis in psychiatric research. Genetic analysis has the potential to identify individuals at risk by looking for specific gene-environment interactions with the potential to mediate biological systems implicated in psychiatric disorders (68).

When looking in the context of MD, this specifically opens the possibility to initiate the search for genetic variants that make an individual more susceptible to SLEs by mediating HPA-axis reactivity, thereby heightening the risk for MD onset. Early epidemiologic studies on the contribution of genetics to depression concluded that genetic factors account for approximately 40% of the variance observed in depressive patients, with most of the remainder being attributed to an individual's unique environment (69). Kendler et al. showed that this heritability of depression is even greater in women (42%) compared to men (29%) with some genetic factors being sex-specific in their effect (5).

The presence of this moderate genetic involvement initiated the search for specific genetic markers by whole-genome linkage analysis. However, to date genetic linkage studies in MD provided little success, possibly attributed to the fact that i) MD is only moderate genetically influenced and ii) genetic liability to MD is contributed by multiple genes, each having a too small individual effect to be detected by linkage analysis. Nonetheless, with completion of the reference human genome sequence the opportunity to look for specific genetic markers by association analysis arose, providing more power than previously used linkage studies (70).

To date, most known genetic association studies in MD have focused on genetic variations in loci encoding for components of the serotonergic system (71). This is a logic starting point, because this system is strongly linked with MD pathology and target of several antidepressants. As already pointed out, serotonergic function in the CNS is determined by complex interaction among proteins which regulate reuptake after release, enzymatically mediated breakdown, and effects on 5-HT receptors. Many of the genes encoding these proteins were found to incorporate functional polymorphisms which influence their function, thereby altering 5-HT functioning.

In human genetics, essentially four types of polymorphisms exist:

i) Insertions or deletions ('indels'), which are extra base pairs that may be added (insertion) or removed (deletion) from a gene. These 'indels' can have devastating consequences for the gene by causing translation of the gene to be 'frameshifted'.

ii) Single Nucleotide Polymorphisms (SNPs), characterized by a base point mutation causing a restriction site. SNPs are known to be widely distributed across the human genome, and are generally examined by using Restriction Fragment Length Polymorphism (RFLP).

iii) Variable Number of Tandem Repeats (VNTRs), which represent a short sequence of DNA (so called mini-satellites of 20-50 nucleotides) repeated a variable number of times in the DNA sequence. VNTRs primarily arise from unequal crossing-over during meiosis.
iv) Short Tandem Repeats (STRs), also representing a short sequence of DNA (so called micro satellites of 2-6 nucleotides) typically arising from strand mispairing.
In our study, focus will primarily lie on genetic mutations found in the serotonergic system (5-HT_{1A}C-1019-G, MAO-A VNTR), important in the regulation of HPA-axis stress mediation (38-40), and in the glucorticoid receptor (Bcl-I), which plays an important role in 'negative feedback' regulation of HPA-axis activity (9, 79).

1.4.2 5-HTTLPR

The most intensively studied polymorphism in the pathogenesis of MD is a 44-base pair insertion/deletion (5-HTTLPR) in the flanking regulatory region of the serotonin transporter gene (SCL6A4) located on chromosome 17 (q11.1-q12). The serotonin transporter, as already mentioned, is involved in reuptake of serotonin at brain synapses. Lesch et al. showed this polymorphism to cause differential transcriptional efficiency, with the long 'L' variant (528 bp) having twice the transcriptional activity as the short 'S' variant (484 bp) (72). Caspi et al. examined whether this polymorphism buffers or exacerbates the effects of life stress on depression. This study showed that individuals with the short 'S' variant were more likely to develop depression after exposure to SLEs than individuals with the long 'L' variant. However, no direct relationship between SLEs and depression was found, stating that the 5-HTTLPR genotype only moderated the association between SLEs and depression (73). A possible explanation for this finding is that this 5-HTT polymorphism is a factor that moderates the psychological impact of SLEs rather then just influencing the relationship between SLEs and MD (74).

1.4.3 5-HT1A

Inhibition of serotonergic neurons is mediated by the somatodendritic 5-HT_{1A} autoreceptors. This receptor is encoded by an intronless gene (HTR1A) located on chromosome 5 (q12.3). A functional C-1019-G single nucleotide polymorphism (SNP) in the transcriptional control region of the gene (HTR1A-1019) is found to be associated with depression. This due to the fact that the G-variant displays differential binding efficiency of transcriptional regulators consequently elevating transcriptional activity. In this manner, the G-variant increases the presence of somatodendritic autoreceptors, which inhibits 5-HT-ergic neurotransmission and thus heightens the risk for MD by serotonergic neurotransmission dysfunction (75).

1.4.4 MAO-A

Another functional polymorphism was found in the upstream regulatory region of a gene, mapped to the short arm of the X chromosome, encoding the mitochondrial outer membrane enzyme MAO-A which degrades 5-HT. In this MAO-A gene a variable-number-tandem-repeat (uVNTR) polymorphism in the promoter region of the gene has been identified by Sabol et al. (76). The

polymorphism, located 1.2 Kb upstream of the MAOA coding sequence consists of 30bp repeated sequences present in 2, 3, 3.5, 4 or 5 repeats. The 3.5 and 4 repeats were found to be functional by making the process of transcription 2 to 10 times more efficient than transcription with 2, 3 or 5 repeats. This additional transcription efficiency elevates concentrations of the enzyme MAO-A in the presynaptic neuron, leading to more degradation of 5-HT. This is hypothesized to result in 5-HT neurotransmission dysfunction, heightening risk for MD (76). In this perspective, the 3.5 and 4 repeats have already been associated with higher CSF levels of the serotonergic metabolite 5-HIAA in women, giving evidence for higher 5-HT turnover (77).

1.4.5 Bcl-I

The glucocorticoid receptor (GR) is a member of the steroid receptor superfamily. It mediates the effects of cortisol on target tissues by binding to responsive elements in the DNA, as well as negative feedback terminating HPA-axis activity. A common Bcl-I restrictrion fragment length polymorphism (RFLP) in the GR gene located 646bp from the exon 2/ intron 2 junction was found (78). An association between this C-to-G substitution and poor feedback regulation of the HPA-axis was reported, probably causing a dysfunctional HPA-axis, a hallmark of depression (79). The polymorphism has already been found to be associated with insulin resistance (80), obesitas (79) and altered adrenocortical responses to stress (81).

1.4.6 Endophenotypes in MD: A new methodological approach

While association studies in psychiatric genetics generated possible genetic markers for MD, these studies could not provide an understanding of the processes by which genetic variants bias clinical outcome of MD. This limited success of genetic studies in MD has resulted in considerable debate regarding current methodological approaches, specifically concerning the view of genetically relevant phenotypes. To clarify, genes and clinical outcome in MD are probably not associated in a simplistic true manner. It is more likely that the clinical outcome of MD is caused by susceptibility genes interacting with both environmental and developmental factors. In addition, gene-gene interactions and epigenetic alterations also contribute the susceptibility to develop MD throughout life. To overcome limited success in elucidating the genetic basis of MD, Hasler et al. proposed the use of putative endophenotypes. These are internal phenotypes that are not visible when observing the clinical symptoms of depression, but which are more proximal to the effect of genetic variations in the underlying genes (82). The evaluation of an endophenotype is based on the following criteria:

i) *Specificity*: The endophenotype is more strongly linked with MD than with other psychiatric disorders.
ii) *Heritability*: Variance in the endophenotype is associated with genetic variance
iii) *Familial association*: The endophenotype is more prevalent among subjects with a familial history of MD compared with an appropriate control group
iv) *Cosegregation*: The endophenotype is more prevalent among depressed subjects with a familial history of MD compared with the non-depressed subjects with a familial history
v) *Plausibility*: The endophenotype bears a conceptual relationship to MD.

When applying these criteria in the pathology of MD, possible neurochemical endophenotypes are recognized to be serotonergic and noradrenergic dysfunction, as well as HPA-axis hyperactivity (82). By assuming that the number of genes involved in each separate endophenotype is less than those involved in the clinical outcome of MD, these endophenotypes provide a means for efficiently researching pathways involved in MD. An interesting approach to apply this concept of endophenotypes is by looking at the association between SLEs and MD susceptibility. The presence of an association between SLEs and depression is widely accepted in the scientific community, with SLEs elevating MD risk. But not every stressed subject develops depressive symptoms, caused by individual variability in stress sensitivity (69). This opens scientific possibilities to look for precarious genetic mutations that could alter an individual's sensitivity to SLEs, characterized by altered HPA-axis reactivity.

2 Study hypothesis

Major depression (MD) is defined as a complex psychiatric disorder with an ambiguous neuropathological basis (2). Recent studies show that stressful life events (SLEs) are strongly associated with MD risk. In search of underlying mechanisms, data almost consistently propose an dysfunction in HPA-axis activity with the most important hallmark being hypercortisolemia (11,12). However, the relationship between SLEs and MD is not straightforward since only a small fraction of the exposed individuals actually experience depressive episodes. This implies that due to genetic variation, some individuals may be more susceptible to the depression-inducing effects of SLEs (68). To date, genetic linkage and association studies provide limited success in deciphering the genetic basis of MD, probably because genes and clinical outcome in MD are not associated in a simplistic manner. Moreover, epidemiologic studies show that MD is subject to specific gene-environment interactions, providing each individual a unique reactivity to experienced SLEs (69). In this study it is hypothesised that individual stress reactivity is mainly mediated by HPA-axis dysfunction. This imposes the search for polymorphisms moderating HPA-axis reactivity, thereby rendering an individual more sensitivity to MD onset when experiencing stressful events. To overcome methodological difficulties often encountered in genetic association studies, our study examined the influence of genetic variations on individual stress sensitivity, a previously described endophenotype of depression.

The focus will primarily lie on genetic mutations found in the serotonergic system (5-HT_{1A}C-1019-G, MAO-A uVNTR), important in the regulation of HPA-axis stress mediation (38-40), and in the glucorticoid receptor (Bcl-I), which plays an important role in 'negative feedback' regulation of HPA-axis activity (9, 79). The influence of these genetic variations on 'HPA-axis reactivity' are studied by looking at variations in salivary cortisol levels of individuals experiencing typical minor daily life stressors. These minor daily life stressors were recently shown to represent a true endophenotype for studying genetic liability in depression (83).

The following questions are important when studying this gene-environment interaction:

- Is there an effect of these polymorphisms on HPA-axis reactivity in non-depressed individuals upon exposure to minor daily life stressors?
- Are these polymorphisms associated with the occurrence of negative emotions in non-depressed individuals?

Participants were recruited from the East Flanders Prospective Twin Survey (EFPTS), a longitudinal female twin population. To assess the effects of daily life stressors the experience sampling method (ESM) was used. ESM measures fluctuations in three different daily life stressors: social, activity- and event-related stress. The influence of genetic variations on salivary cortisol levels in response to these stressors was examined by multilevel regression analysis.

It is hypothesized that these genetic determinants, which have previously been associated with a higher risk for MD, will influence HPA-axis reactivity rendering individuals more sensitive for MD onset when experiencing stressful episodes.

3 Materials and Methods

3.1 Samples

Participants were recruited from the East Flanders Prospective Twin Survey (EFPTS), a longitudinal female twin population used to investigate gene-environment interactions in affective disorders. This population-based survey has prospectively recorded all multiple births in the province of East-Flanders (Belgium) since 1964 (84). Zygosity was determined through sequential analysis based on fetal membranes, blood groups, and DNA fingerprints.

The study sample initially included 621 participants aged 18-46 years. The project was approved by the Local Ethics Committee, and all participants gave written informed consent. Participants were all white and of Belgian origin. The sample composition was strictly female given evidence of sex-specific genetic effects on neuroticism and depression (85).

Mean age of twins was 27 years (SD: 7.4 years). 63% had a college or university degree, 35% completed secondary education and 2 % had only a primary education. The majority was currently employed (63% employed, 32% student, 2% unemployed, 2% homemaker and 0.4% sick leave).

3.2 Stress Measure: Experience Sampling Method (ESM)

To assess the effects of SLEs in daily life, the experience sampling method (ESM) was used. ESM is a structured diary technique to assess subjects in the context of their daily living environment. It is a valid and reliable way to study immediate effects of stressors on mood, reducing biases in recall (86). Trained research assistants explained the ESM procedure to the participants during an initial briefing session and a practice form was completed to confirm that subjects understood the rating scales. Subjects could reach a member of the research team by phone if they had questions or problems during the ESM sampling period. Participants received a digital wristwatch together with ESM self-assessment forms collated in a booklet for each day. The wristwatch was programmed to emit a 'beep' at an unpredictable moment in each of ten 90-min time blocks between 7:30 and 22:30, on 5 consecutive days. After every signal, subjects were asked to fill out the ESM-self assessment forms concerning thoughts, current context (activity, persons present, location), appraisals of the current situation, and mood. All self-assessments were rated on 7-point Likert scales. Subjects were instructed to complete their reports immediately after the beep, thus minimizing memory distortions, and to record when they completed the from.

At the same time, participants collected a saliva sample with a cotton swab (Omniswab; Whatman, 'S-Hertogenbosch, Netherlands). After saliva collection, they were instructed to store the swab in the salivette tube and to record the exact time of collection on the label, on which subject code and date had been pre-printed. Samples were stored in subjects' home freezers until transport to the lab, were centrifuged samples were kept at -20° C until analysis. In the lab researcher transferred the swabs into a separate 100 cc bottle with electronic monitoring cap (eDEMk, Aardex Ltd., Switzerland) for each day.

To determine whether the subjects had completed the procedure within 15 minutes of the beep, the time at which subjects indicated they completed the report was compared to the actual time of the beep. All reports that were not completed within 15 minutes were excluded from the analysis. Previous work has shown that reports completed after this interval are less reliable and less valid (98).

3.3 SLEs in daily life

Appraisals of minor daily events, situational contexts and mood were collected at each beep within the Experience Sampling Method (ESM) framework. To assess the impact of SLEs both social stress, event-related stress, activity-related stress and negative affect were measured with the ESM.

For the measurement of *event-related stress*, subjects were asked to report the most important event that happened between the current and the previous beep. This event was subsequently rated on a 7-point bipolar scale (from -3 = very unpleasant, 0 = neutral, to 3 = very pleasant). *Social stress* was measured in the following way: first, subjects were asked whether or not they were alone at the time of the beep. Second, if not alone, they were asked whether they liked the company they were in at that moment. This was rated on a 7- point Likert scale (from 'not at all' (1) to 'very much' (7)). The scale was reversed so that higher scores represent higher disliking of being in that company.

Activity-related stress was assessed at each beep by the items 'I would rather be doing something else' and 'this activity requires effort', both rated on a 7-point Likert scale.

Negative affect (NA) was assessed at each beep with 6 mood adjectives (I feel 'insecure', 'lonely', 'anxious', 'low', 'guilty' and 'suspicious') rated on a 7-point Likert scale.

3.4 Depression Measure: Symptom Check List-90 (SCL-90)

Together with stress measurements also depression symptoms were scored. Because it was previously shown that results for 5-HTTLPR moderation were similar for self-reported or interview-based measurements of depression, a validated self-report measure was used (87). Self-report questionnaires have unique properties, as they rely on the judgment of the respondent. Trained research assistants explained the SCL-90 procedure to the participants during an initial briefing session. At baseline and at each of the 4 follow-up measurements, participants completed the Symptom Checklist 90 (SCL-90). The SCL-90 has been used widely as an outcome measure, a measure of mental status, and as a screening instrument. It has been shown to have a good reliability as its internal consistency is high. It is a psychiatric self-report inventory developed to measure 90 symptomatic items. Each item of the questionnaire is rated on a five-point Likert scale, from 0 (none) to 4 (extreme), indicating the point-in-time psychological symptom status. This is intended to measure symptom intensity in nine different dimensions. The dimension of depressive symptoms consists of 16 items, such as "feeling low on energy or slowed down", "feeling no interest in things", and "experiencing feelings of worthlessness". A continuous weighted depression

score was calculated at each measurement occasion. This measure was ultimately used in the statistical analysis.

3.5 DNA isolation and Cortisol measurement

For DNA isolation and subsequent analysis placental tissue for 156 participants, blood samples for 14 participants, and buccal swab samples for 208 participants were available. Genomic DNA of these subjects was extracted using QIAamp DNA Mini Kits (Qiagen, Venlo, The Netherlands) according to the appropriate protocol for each sample type, either Qiagen tissue protocol, Qiagen blood spin protocol, or Qiagen buccal swab spin protocol. For every monozygotic (MZ) twin in the sample with genotypic data, the same genotypic data were included for the co-twin, assuming that both twins had identical genotypes.

Salivary free-cortisol levels were determined in duplicate by direct radio-immunoassay (RIA). The lower detection limit of the assay was 0.2 nmol/L; interassay and intraassay coefficients of variation were less than 10%. Samples with cortisol lower then 44 nmol/L were excluded from statistical analysis.

3.6 Genotyping

3.6.1 Optimizing MAO-A genotyping

Prior to determination of the participants' MAO-A genotype, Polymerase Chain Reaction (PCR) conditions for amplifying this VNTR had to be optimized. Primarily, a starting selection of different PCR kits and machinery were chosen to compare PCR efficiency and develop optimal PCR conditions.

PCR kits tested were, *i)* the Taq DNA polymerase kit (Qiagen, Venlo, The Netherlands) including 250 units of Taq DNA Polymerase, 10x PCR Buffer (containing Tris-HCl, KCl, (NH4)2SO4 and 15mM MgCl2), 10x CoralLoad PCR Buffer, 5x Q-Solution and 25mM MgCl2. *ii)* the Super Taq kit (Sphaero-Q/HT-Biotechnology, Cambridge, England), which included 500 units of Super Taq and a 10x PCR buffer containing 100mM Tris-HCl, 15mM $MgCl_2$, 500mM KCl, 1% Triton X-100 and a 0.1% (w/v) stabilizer.

PCR run efficiency was tested on either the Geneamp PCR system 2700 (Applied Biosystems, Ijsel, Netherlands) or on the Biometra T1 thermocycler/Tgradient (Westburg, Leusden, Netherlands). Optimization was initially based on a previously described protocol from Deckert et al. (88) using primers MAOAFor2 (5'-CCCAGGCTGCTCCAGAAAC-3') and MAOARev2 (5'-GGACCTGGGCAGTTGTGC-3'), flanking the polymorphic region located approximately 1.1kb upstream of the initiation codon. The PCR was always performed in a final volume of 25 µl containing variable concentrations of genomic DNA, primers, MgCl2, Taq polymerase, Q-solution, DMSO and/or Tween-20. Standard PCR concentrations of the dNTP mix (200 µM) and the PCR buffer (1x) were kept invariable during the optimization period.

After optimization, amplification of genomic DNA was performed on 96-well plates and afterwards visualized by Genescan Analysis. For Genescan Analysis, a separate 15 µl mixture was prepared for

each individual PCR product, containing a 0.4 µl Genescan-500 Rox size standard (Applied Biosystems, Ijsel, Netherlands), 13.6 µl HiDi formamide (Applied Biosystems, Ijsel, Netherlands) and 1µl of the corresponding genomic PCR product. For Genescan analysis a MAOAfor2 primer labeled with 6-carboxyfluorescein (6-FAM) (Applied Biosystems, Ijsel, Netherlands) was used. The mixtures were prepared on 96-well-plates, denatured for 3 min. at 95° C, and finally size-resolved by the ABI3100 Genetic Analzyer (Applied Biosystems, Ijsel, Netherlands). Further analyses were done and pictures were taken with Genetic Analysis 2.0 software (Applied Biosystems).

3.6.2 Optimizing Bcl-I genotyoping

Prior to determination of the Bcl-I genotype of the participants, PCR conditions for amplifying this RFLP had to be optimized. A starting selection of different PCR kits and machinery were chosen to compare PCR efficiency and develop optimal PCR conditions.

PCR kits tested were the i) Taq DNA polymerase kit (Qiagen, Venlo, The Netherlands) and ii) Super Taq kit (Sphaero-Q/HT-Biotechnology, Cambridge, England) similar to those for MAO-A. PCR run efficiency was tested on either the Geneamp PCR system 2700 (Applied Biosystems, Ijsel, Netherlands) or on the T1 thermocycler/Tgradient (Biometra (Westburg), Leusden, Netherlands). Additionally, a Faststart Taq DNA polymerase kit (Roche Applied Sciences, Almere, Netherlands) was tested, containing 250 units Faststart Taq DNA polymerase, 10x PCR buffer (500mM Tris-HCl, 100mM KCl, 50 mM $(NH_4)_2SO_4$, 20mM $MgCl_2$), 10x PCR buffer without $MgCl_2$, 25mM $MgCl_2$ and a 5x GC-rich solution. Optimization was initially based on a previously described protocol form Fleury et al. (78) using primers Bcl-I forward (5'AAATTGAAGCTTAACAATTTTGGC-3') and Bcl-I reverse (5' GCAGTGAACAGTGTACCAGACC-3'). The PCR was always performed in a final volume of 25 µl containing variable concentrations of genomic DNA, primers, MgCl2, Taq polymerase, Q-solution and DMSO. Standard PCR concentrations of the dNTP mix (200 µM) and the PCR buffer (1x) were kept invariable during the optimalisation period.

After optimization, amplification of genomic DNA was performed on 96-well plates. After PCR, the amplificated DNA was restricted with a Bcl-I restriction kit (New England Biolabs, Leusden, Netherlands) containing Bcl-I restriction enzyme and 10X NEBuffer 3. Prior to digestion, a separate 10 µl mixture was prepared for each individual PCR product, containing a 5.4 µl genomic DNA, 0.6 µl 10X NEBuffer 3 and 4µl of a Bcl-I digestion mixture. Digestion was also performed on 96-well-plates, overnight at 50° C. After digestion the resulting digested fragments were separated on an 2% agarose gel, and visualized by Gelstar Nucleic Acid gel stain (Cambrex, New Jersey, USA). Pictures were taken with Gel Doc 2000 (Biorad, California, USA) and Quantity 4.0.1 software (Biorad, California, USA).

3.6.3 5-HT_{1A} genotyoping

The C(-1019)G polymorphism in the promotor region of the 5-HT_{1A} receptor gene (rs6295) was determined with a pre-designed Taqman® SNP Genotyping Assay (Applied Biosystems, Nieuwerkerk a/d Ijsel, The Netherlands). The assay number is designated as: C__11904666_10. The principle of Taqman® assays is based on the 5' nuclease activity of the *Taq* polymerase, used to amplify the target region containing the SNP by PCR. Two unique probes are used: one being a

perfect match of the wild-type allele, the other of the mutant allele. Both probes are labelled with different fluorophores and hybridize between the primers. When the Taq polymerase reaches the probe during the extension phase of the PCR, the 5' nuclease activity cleaves the labelled 5' end of the probe, thereby separating the fluorophore and his quencher (attached to the 3' end). The resulting fluorescent signal is detected and recorded. In case of a single base mismatch, the probe-template hybridization is unstable and prevents nuclease activity, yielding lower fluorescent signals when compared to perfect matched probes. The use of two differently labelled, unique probes, allows to discriminate between homozygous samples of either allele and heterozygous samples.
The assay was performed on a 7900HT Fast Real-Time PCR system (Applied Biosystems, Nieuwerkerk a/d Ijsel, The Netherlands). Since this is a pre-designed assay, all necessary software and analysis tools are delivered with the assay and result analysis is done virtually automatic.

3.8 Statistical analysis

ESM data have a hierarchical structure. This implies that multiple observations (level 1) are clustered within subjects (level 2), who are part of twin pairs (level 3). To take this 3-level grouping structure into account, multilevel random regression analysis is necessary. Consequently, the association between genetic variations and cortisol/mood responses in the flow of daily life (i.e. stress sensitivity), was studied using the XTMIXED command in STATA 9.0 (StataCorp, 2005) to fit linear mixed models. Mixed models are characterized as containing fixed and random effects. The fixed effects are analogous to standard regression coefficients and can be estimated directly.
In the first basic model, main effects of minor daily life stressors and genotype on cortisol and mood were studied. Important confounders that had to be implicated into these models include 'koffie', 'age', 'tabak', 'voedsel', 'alcohol', 'posture' and 'anticonceptie' These variables possibly influence basal cortisol levels and were previously identified by factor analysis (Jacobs et al.). Second, to examine possible gene-environment interactions, the polymorphism-stress interaction term was regressed on either cortisol or mood. To obtain effect sizes of the different genotypes, basic regression models were stratified for the specific genotypes studied.
*Note: In the remainder of this manuscript, possible associations are examined by looking at regression coefficents. These 'effect sizes' **only** show the presence of a possible association without giving information about real quantitative differences in cortisol response or mood changes.*

4 Results

4.1 Optimization MAO-A genotyping conditions

Prior to genotyping, PCR conditions for the MAO-A VNTR had to be optimized. The Taq DNA polymerase kit (Qiagen) and the Super Taq kit (Sphaero-Q/HT-Biotechnology) were tested and compared. PCR run efficiency was examined primarily on the Biometra T1 thermocycler. The initial protocol was obtained from previously described conditions (88).

NOTE: Due to infinite optimalisation results, only a selection of results are included in this manuscript. The specific results were chosen to keep everything as coherent and explicable as possible. For specifics concerning the specific PCR kits: see Materials and Methods

The PCR technique is a method for selective amplification of DNA segments. It depends on the annealing of oligonucleotides to homologous DNA sequences by enzymatic synthesis. In MAO-A genotyping, the use of PCR suggest the specific amplification of the VNTRs present in our population.

Pilot testing of the Taq DNA polymerase kit, when executing the unaltered protocol derived from Deckert et al. (88), primarily generated inefficient amplification. PCR products were visualized through separation on a 3% agarose gel containing Nucleic Acid Gel Stain **(figure 5)** as suggested by Deckert et al. (88). The anticipated MAO-A VNTR was observed in every lane, except for lane 4 which contained a negative control (sterile Milli-Q) **(figure 5 lanes 1, 2 and 3)**. However, due to the presence of non-specific products in the form of primer dimerization **(figure 5 lanes 1-4)**, and unspecific bands **(figure 5 lanes 1-3)**, further optimization was required.

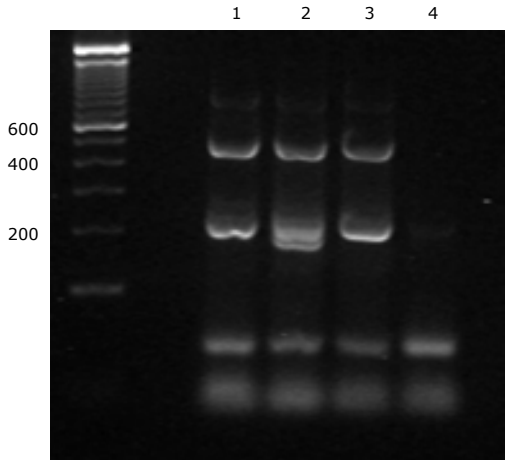

Figure 5: PCR-VNTR optimization of the MAO-A polymorphism using the Taq DNA polymerase kit (Qiagen), visualized on 3% agarose gel containing Nucleic Acid Gel Stain. Lane 4 represents a negative control (sterile Milli-Q). The MAO-A VNTR (in the range of 200bp) was observed in lane 1, 2 and 3. As can be observed, distinguishing between VNTRs can not be performed on a 3% agarose gel due to a lack of accuracy. In addition, non-specific products in the form of primer dimerization (bands below 100bp) and unspecific bands in lanes 1, 2 and 3 were observed (bands of approximately 500 bp).

To overcome the problem of non-specific amplification, a standard selection of conditions was carried out in subsequent testing: i) lowering the concentrations of forward and reverse primers in the master mix going from 0.4µM to 0.1µM, ii) elevating the annealing temperature from 58° C to 64° C (in steps of 2° C), iii) lowering the number of cycles of PCR conditions from 35 to 25 cycles, iv) using varying concentrations (1 to 10 %) of the cosolvent DMSO instead of Solution-Q.

Varying PCR conditions in further pilot testing of the Taq DNA polymerase kit could not provide optimal conditions due to permanent occurrence of non-specific products. Consequently, a different kit was tested for its potential to generate more efficient amplification. The Super Taq kit was chosen due to the fact that the 10x PCR buffer of this kit contained Triton X-100, a detergent suggested to assist in diminishing the amplification of non-specific products.
As was expected, pilot testing of the Super Taq kit provided optimal conditions with amplification of non-specific products being heavily reduced. Due to a lack of accuracy, 3% agarose gel could not distinguish between possible repeats of the MAO-A VNTR. Therefore, analyses of the PCR products were performed with Genescan Analysis, yielding more detailed separation.

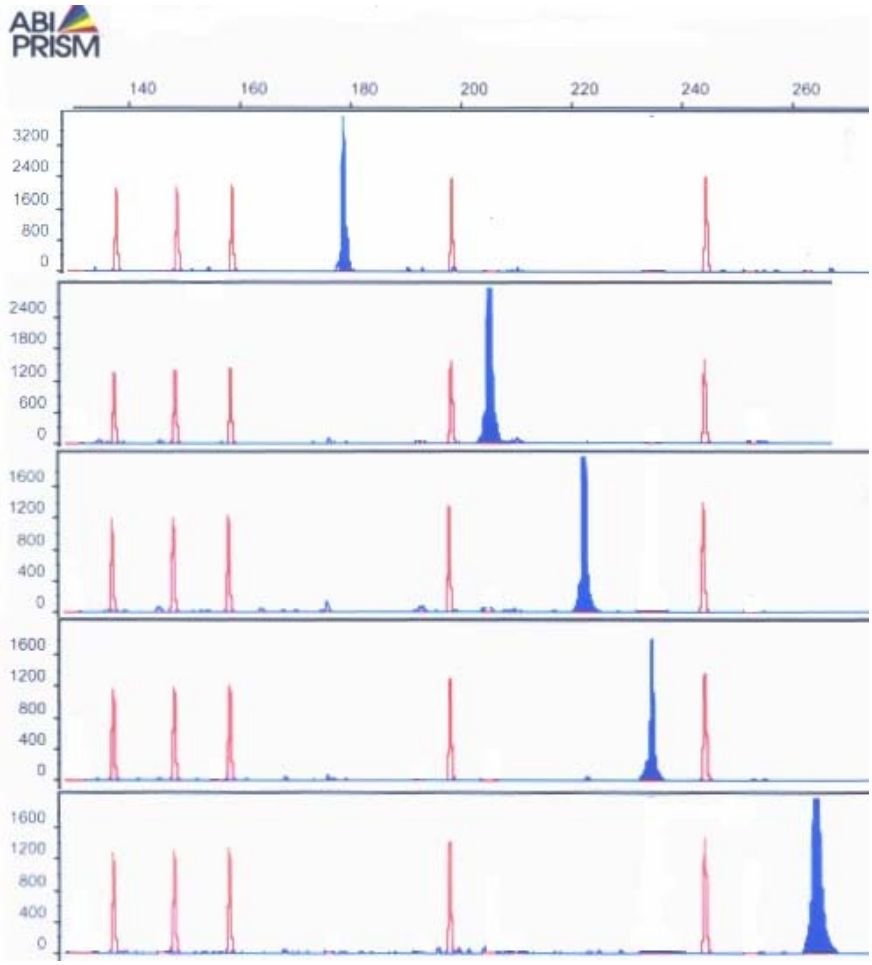

Figure 6: Allelic MAO-A repeats present in our female twin population, visualized by size-resolved Genescan Analysis. All repeats described in previous studies were found in our population (blue peaks). Both 2 repeats (179bp), 3 repeats (209bp), 3,5 repeats (227bp), 4 repeats (239bp) and 5 repeats (269bp) are visualized by the ABI3100 Genetic Analyzer using 6-carboxyfluorescein-labeled forward primers. Each peak is identified relative to an internal Genescan-Rox size standard (red peaks).

Optimal PCR amplification of the MAO-A in our female twin sample was carried out in a reaction mixture of 25µl containing a 0.2mM dNTP mix, 1x PCR buffer, 0.4µM forward primer, 0.4µM reverse primer, 1.5mM $MgCl_2$ and 1U of Super Taq polymerase. Final PCR conditions were: initial denaturation at 95° C for 5 min, 35 cycles of denaturation at 94° C for 40s, annealing at 59° C for 40s, and extension at 72° C for 60s; followed by a final extension at 72° C for 60s; followed by a final extension at 72° C for 10 min.

4.2 Optimization Bcl-I genotyping conditions

Furthermore, PCR conditions for the Bcl-I RFLP also had to be optimized prior to genotyping.
During PCR-RFLP run optimization, the Taq DNA polymerase kit (Qiagen), the Super Taq kit (Sphaero-Q/HT-Biotechnology), and the Faststart Taq DNA polymerase kit (Roche AS) were tested and compared. PCR run efficiency was examined mainly on the Biometra T1 thermocycler. The initial protocol was obtained from previously described conditions (78).
NOTE: Due to infinite optimalisation results, only a selection of results are included in this manuscript. The specific results were chosen to keep everything as coherent and explicable as possible. For specifics concerning the specific PCR kits: see Materials and Methods

Pilot testing of the Taq DNA polymerase kit, when employing the unaltered protocol derived from Fleury et al. (78), generated inefficient and non-specific amplification. Undigested PCR products of the pilot population were visualized through separation on a 2% agarose gel containing Nucleic Acid Gel Stain **(figure 7)**. The undigested Bcl-I SNP (335bp) was observed in every lane, except for lane 10 and 18 which contained a negative control (sterile Milli-Q) **(figure 7 lanes 2-9 and 11-17)**. However, a vast amount of primer dimerization **(figure 7 lanes 1-18)**, varying quantification of Bcl-I PCR products, and non-specific bands were observed **(figure 7 lane 3, 9 and 14)**, emphasizing the need for further optimization.

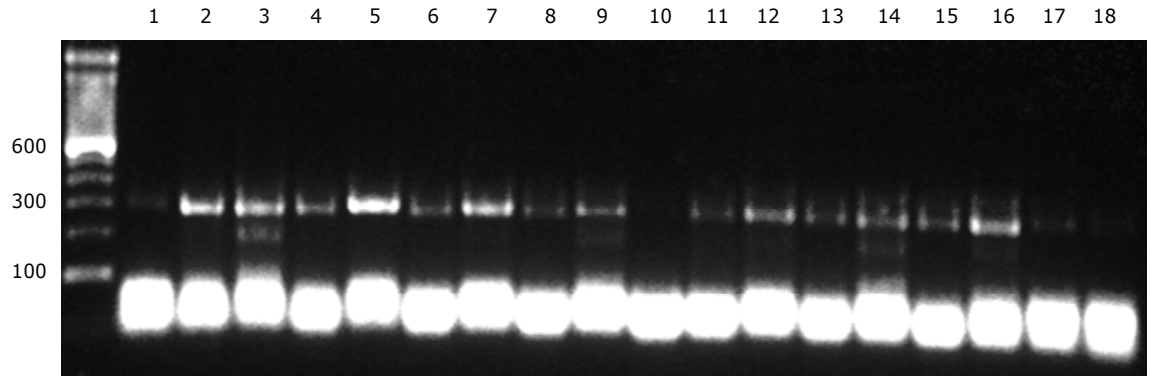

Figure 7: PCR-RFLP optimization of the Bcl-I polymorphism using the Taq DNA polymerase kit (Qiagen), visualized by separation on 2% agarose gel containing Nucleic Acid Gel Stain. Lanes 10 and 18 represent negative controls. The undigested PCR product (335bp) is visible in lanes 1-9 and 11-17, but varying in quantity. Vast amounts of primer dimerization are observed (bright white bands below 100bp).

To overcome the problem of primer dimerization a standard selection of conditions were carried out in subsequent testing: i) lowering the concentrations of forward and reverse primers in the master mix going from 0.4µM to 0.1µM, ii) elevating the annealing temperature from 59° C to 64° C (in steps of 2° C), iii) lowering the number of cycles of the PCR run from 40 to 30 cycles (in steps of 5 cycles), iv) using varying concentrations of the cosolvents DMSO (1-5%) instead of Solution-Q.

These varying PCR conditions for the Taq DNA polymerase kit could not provide optimal conditions during pilot testing, especially due to permanent primer dimerization. Consequently, the Super Taq kit was tested for its potential to generate more efficient amplification. This novel kit generated considerably improved results, however did not provide optimal settings. Undigested as well as digested PCR products of the pilot population were visualized on a 2% agarose gel containing Nucleic Acid Gel Stain **(figure 8)**. Undigested PCR product (335bp) was observed in lanes 1-4 **(figure 8 left)** with more consistent amplification when compared to the Qiagen kit **(figure 7)**. Also, previously observed unspecific bands were eliminated. Even though eradication of most unwanted effects was observed, primer dimerization was still present **(figure 8 left)** although not as extensive. Restriction of PCR products from the pilot sample was tested with a specific Bcl-I restriction enzyme. This was primarily done to examine if the presence of primer dimerization would obstruct genotyping. Digested PCR products were visualized on a 2% agarose gel. As anticipated, in lanes 1 and 3 digested bands were visible with a size of 335bp, 221bp and 117bp **(figure 8 right)**. However, in lanes 2 and 4 digested bands were not sufficiently apparent to make conclusions concerning a specific genotype. To clarify, bands with a size of 221bp in lanes 2 and 4 are hardly visible and their presence could even well be caused by carry-over contamination. Together with this carry-over effect, primer dimerization was still present.

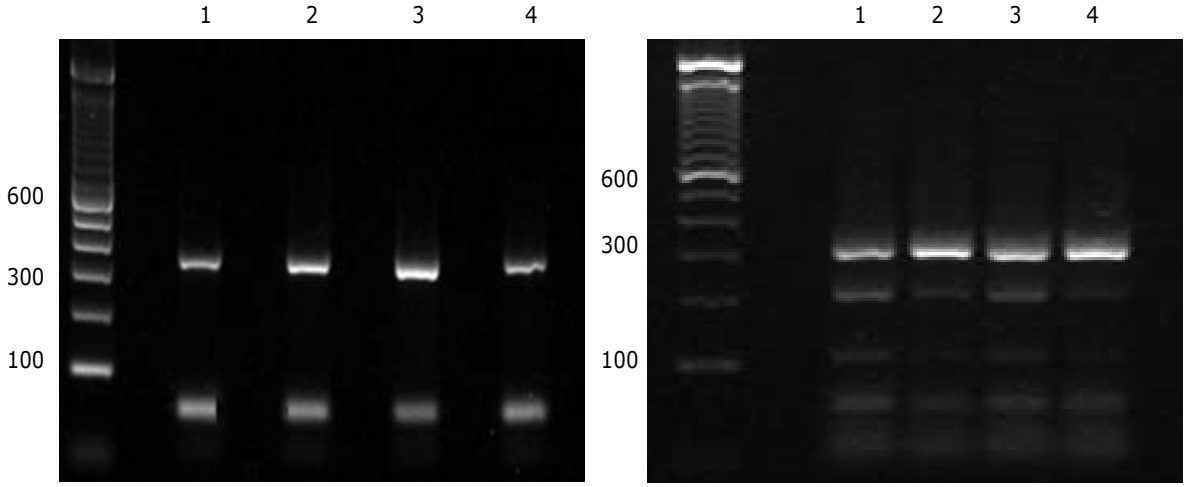

Figure 8: PCR-RFLP optimization of the Bcl-I polymorphism using the Super Taq kit (Sphaero-Q), visualized by separation on 2% agarose gel containing Nucleic Acid Gel Stain. ***Left.*** In lanes 1-4, undigested PCR product (335bp) is visible and steady in brightness. Primer dimerization is observed (bright white bands below 100bp). ***Right.*** In lanes 1-4, digested PCR products are visualized. Lanes 1 and 3 display digested bands with a size of 335bp, 221bp and 117bp. In lanes 2 and 4 no conclusions concerning a specific genotype are possible. Digested bands with a size of 221bp in these lanes are hardly visible, and due to this low specificity their presence could even well be caused by carry-over from other lanes.

Subsequent tests were performed, similar as those mentioned prior for the Taq DNA polymerase kit. However, the Super Taq kit could not provide optimal settings. The principal problem again appeared to be the permanent appearance of non-specific products in the form of primer dimerization.

In a final attempt to overcome these difficulties, the use of a Faststart Taq DNA polymerase (Roche) was suggested. This kit has several benefits when compared to standard PCR kits since it minimizes amplification of non-specific products owing to 'Hot Start' specificity, and its activity is not downregulated by cosolvents and detergents like DMSO. Pilot testing of the Faststart polymerase kit provided excellent results. Both complete elimination of primer dimerization and possible carry-over effects were obtained, finally allowing efficient genotyping **(figure 9 lanes 1-2)**. The pattern of digestion lanes 1 and 2 indicates a homozygous individual for the mutant allele constituting a GG genotype **(figure 9 lanes 3-4)**. On the other hand, lanes 3 and 4 contain heterozygous individuals containing both the mutant and wild-type allele constituting a CG genotype. Finally, lanes 5 an 6 represent individuals homozygous for the wild-type allele constituting a CC genotype **(figure 9 lanes 5-6)**.

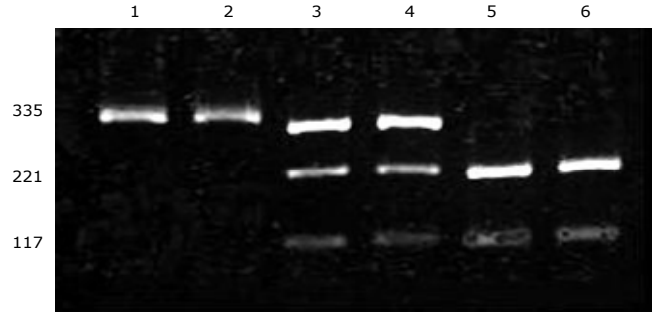

Figure 9: PCR-RFLP optimization of the Bcl-I polymorphism using the Faststart Taq DNA polymerase kit (Roche), visualized by size-resolved separation on 2% agarose gel containing Nucleic Acid Gel Stain. Lanes 1 and 2 indicate a homozygous individual for the mutant allele constituting a GG genotype. Lanes 3 and 4 represent heterozygous individuals containing both the small and large allele constituting a CG genotype. Finally, lanes 5 an 6 represent individuals homozygous for the wild-type allele constituting a CC genotype.

Optimal PCR amplification of the Bcl-I polymorphism in our twin sample was carried out in a reaction mixture of 25µl containing a 0.1mM dNTP mix, 1x PCR buffer, 0.4µM forward primer, 0.4µM reverse primer, 1.5mM $MgCl_2$ and 1U of Faststart Taq polymerase. Final PCR conditions were: initial denaturation at 95° C for 5 min, 40 cycles of denaturation at 94° C for 30s, annealing at 59° C for 30s, and extension at 72° C for 45s; followed by a final extension at 72° C for 7 min. Digestion of the amplificated PCR product was carried out in a final mixture of 10µl containing 5,4µl PCR product with 4U of Bcl-I enzyme for 16h at 50° C.

4.3 Sample characteristics

The study population initially included 621 participants. Out of these 621 participants, 129 refused genotyping leaving a sample of 492 subjects who gave informed consent for further genetic analysis. Out of these 492 consented participants, 412 provided samples suitable for DNA analysis. Additionally, 34 subjects were discarded either due to unreliable ESM measurements or due to a lack of cortisol measurements, leaving a final sample of 370 participants for genotyping (8 internal controls were not included in statistical analysis).
In this final population of 370 participants, the selected polymorphisms of the serotonergic system (5-HT_{1A}C-1019-G, MAO-A uVNTR) and the glucorticoid receptor (Bcl-I) were genotyped.

4.3.1 $5HT_{1A}$ measurements

354 participants yielded valid 5-HT_{1A}(C-1019-G) genotype measurements following Taqman assay. Allelic frequencies of the polymorphism the population are 50.7% (C allele) and 49.3% (G allele). In addition, **table 2** shows genotypic frequencies of the 5-HT_{1A} SNP in our female twin population. The homozygous wild-type CC genotype is presented in approximately 27% of the participants. The heterozygous mutant CG genotype constitutes the major part of the study population (46.6%), and the homozygous mutant GG genotype is represented by 26% of the participants. Reported genotypic frequencies are comparable to those previously reported by similar populations (89) and in Hardy-Weinberg equilibrium (x^2= 1.6; P= 0.21).

Table 2: Genotypic frequencies of the $5HT_{1A}$(C-1019-G) in our EFPTS population (n=354). Both the homozygous wild-type CC genotype, the heterozygous mutant CG genotype and the homozygous mutant GG genotype are represented in our population. The reported frequencies are comparable to those reported by Lemonde et al. (n=123 female subjects) (89)

5HT1A (C-G)	CC	CG	GG
EFPTS (%)	27.4	46.6	26.0
Lemonde et al. (%)	37.3	50.7	11.9

4.3.2 MAO-A VNTR measurements

327 participants yielded valid MAO-A genotype measurements. **Table 3** shows allelic frequencies of the MAO-A VNTR in our twin population. These frequencies are analogous to previously reported frequencies in similar samples (90).
The genotypic frequencies of the MAO-A VNTR in our population were as follows: 16.2%, *3-3*; 40.4 %, *3-4*; 40.4%, *4-4*; 0.6%, *3-5*; 1,2%, 3.5-4; 0.3%, *3-3.5*; 0.6%, *4-5*; 0.3%, *3.5-3.5*; 0.3% *2-4*. These frequencies could not be compared to previously reported samples due to the fact no other study found or described such a broad selection of MAO-A genotypes in their sample. Also Hardy-

Weinberg equilibrium could not be calculated since no STATA command was available for calculating a Hardy-Weinberg equilibrium for multiallelic polymorphisms.

Table 3: Allelic frequencies of the MAOA VNTR repeats in our EFPTS population (n=327). All These frequencies are comparable to allelic frequencies reported by Jacob et al. (n=418 female subjects) (90)

MAO-A VNTR	2	3	3.5	4	5
EFPTS (%)	0.15	36.63	1.80	61.55	0.61
Jacob et al. (%)	/	35.9	0.7	62.2	1.2

4.3.3 Bcl-I measurements

In contrast to the 370 participants providing DNA samples for genotyping polymorphisms of the serotonergic system, only a selection of 283 samples were available for genotyping the Bcl-I polymorphism. Out of these 283 participants, 236 generated valid Bcl-I genotyping measurements. Allelic frequencies comprise 64% for the wild-type C allele and 36% for the mutant G allele. In addition, **table 4** shows genotypic frequencies of the Bcl-I polymorphism. The homozygous wild-type CC genotype is presented in approximately 40% of the participants. The heterozygous mutant CG genotype constitutes the major part of our population (49.2%), and the homozygous mutant GG genotype is present in 11% of the participants. These frequencies are similar to genotype frequencies reported in analogous samples (91) and in Hardy-Weinberg equilibrium (x^2=1.2; P= 0.2683).

Note: Due to ongoing statistical analyses, no results concerning the Bcl-I genotype will be incorporated in this manuscript

Table 4: Genotypic frequencies of the Bcl-I (C-G) SNP in our EFPTS population (n=236). Both the homozygous wild-type CC genotype, the heterozygous mutant CG genotype, and the homozygous mutant GG genotype is represented in our population. These reported frequencies are similar to those found in a previous study from Bachmann et al. (n=42) (91)

Bcl-I (C-G)	CC	CG	GG
EFPTS (%)	39.8	49.2	11.0
Bachmann et al. (%)	36	48	16

4.3.4 ESM desciptives

Stress was conceptualized as subjective appraisals of different minor stressors that continually occur in the flow of daily life. Appropriate responsiveness to these minor daily life stressors is

crucial for maintaining homeostasis, as they were shown to represent a possible endophenotype of MD (83). After collection of ESM data, mean scores of minor stressors were calculated.

Ongoing activity-related stress was defined as the mean score (M) of 'I would rather be doing something else' and 'This activity requires effort' both rated on a 7-point Likert scale (Cronbach's alpha= 0.57). Data from activity-related stress generated a mean score of 2.239 (SE=0.010). Ongoing social stress was defined as the mean score (M) of the two items 'I don't like the present company) and 'I would rather be alone' both rated on a 7-point Likert scale (Cronbach's alpha= 0.45). Data for this stress parameter generated a mean score of 2.400 (SE=0.010).

Finally, for event-related stress subjects reported the most important event that happened between beeps rated on a bipolar 7-point Likert scale generating a mean score for this minor stressor of -1.104 (SE= 0.012). When recoding data from event-related stress to a 7-point Likert scale similar to other stress parameters, it is shown that occurrence of social stressors generate the highest stress scores in our female twin population **(figure 10)**. This might suggest that this minor stressor has the highest impact in our population.

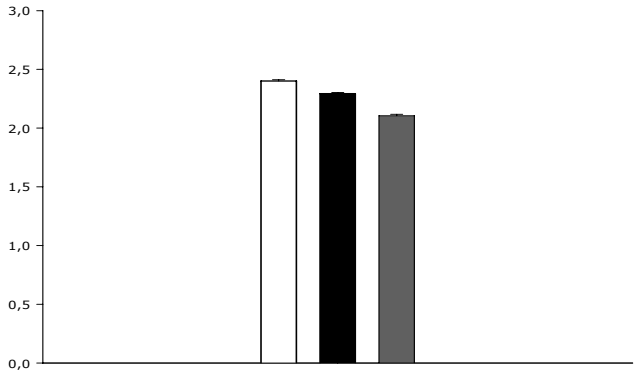

Figure 10: Mean ESM scores for minor daily life stressor in our EFPTS population. All ESM scores were rated on a 7-point Likert scale, generating the highest mean score for social stressors (white) when compared to activity-related stress (black) and event-related stressors (grey).

4.3.5 Effects of daily stressors on cortisol and mood

Figure 11 summarizes the effects of the previously described minor daily stressors on cortisol and mood. When incorporating the different minor stressors and basal cortisol levels in a regression model, it is clear that solely social stress is significantly associated with basal cortisol levels. Moreover, a considerably higher size effect of social stress in association with basal cortisol was found (p=0.017) **(figure 11 left)**. Both activity-related and event-related stress are not significantly associated with basal cortisol levels.

This indicates that, at least in our female twin population, social stress is the essential predictor of cortisol variations in response to minor daily life stressors. Besides the effects of minor stressors on cortisol, their impact on participants' mood was examined, by scoring the mood parameter 'negative affectivity' (NA). When looking at the association between minor stressors and NA, not

only social stress but all examined stressors are significantly associated with NA. This shows that minor daily life stressors have the potential to lower mood **(figure 11 right).**

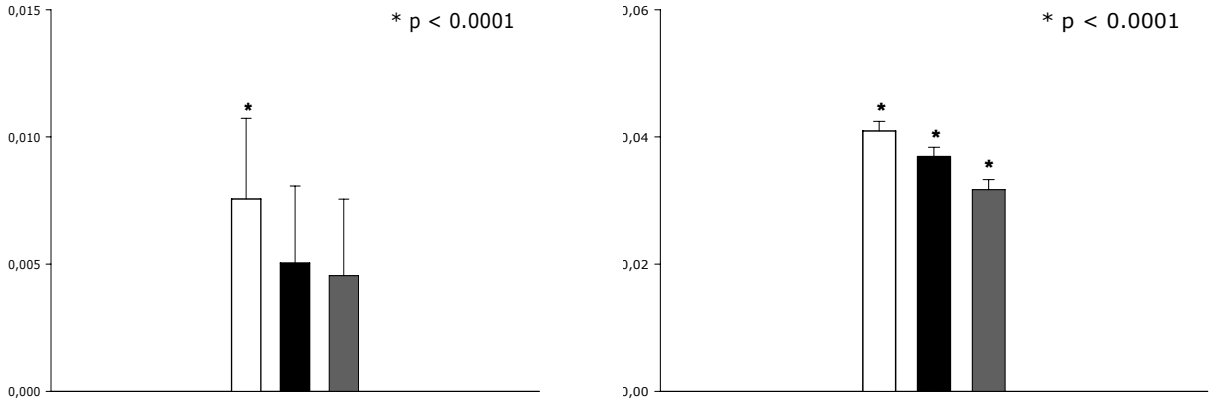

Figure 11: Left. The effects of minor daily life stressors on basal cortisol response in our EFPTS population. All minor stressors show a positive regression coefficient. However, only social stress (white) shows a significant elevation of effect size ($p<0.0001$) in comparison to activity-related (black) and event-related stress (grey). This shows that social stress can significantly elevate basal cortisol levels. **Right.** The effects of minor daily life stressors on negative affectivity (NA) in our EFPTS population. All minor daily stressor show significantly elevated size effects when studying their association with NA. (* $p < 0.001$)

4.3.6 Effects of genotype on basal cortisol levels

Before studying if the association between minor daily life stressors and cortisol and/or mood is mediated by specific genotypes, the direct impact of a participants' genotype on cortisol and mood was examined. Main effects of the MAO-A and 5-HT_{1A} on cortisol and mood were studied. Neither for MAO-A nor for 5-HT_{1A}, a significant association with basal cortisol levels was observed **(table 5).**

Table 5: Main effects of 5-HT1A and MAO-A genotype on basal cortisol in our EFPTS populations. Neither 5-HT_{1A} nor MAO-A significantly influenced cortisol.

Cortisol response	β (SE)	p-value
5-HT_{1A}	-0.0056 (0.0050)	0.269
MAO-A	0.0050 (0.0227)	0.827

Subsequently, the effect size of each individual genotype on basal cortisol was calculated. To achieve regression analysis of individual 5-HT_{1A} genotypes in our xtmixed model, heterozygous mutant CG and homozygous mutant GG genotypes were incorporated as variables and referenced against the homozygous wild-type genotype CC, integrated into the model as a dummy variable .

No significant difference in size effect was found between CC and CG (β=0.0371, p-value=0.497), or between CC and GG (β=-0.0366, p-value=0.567). Similar analysis of individual MAO-A genotypes occurred by a method previously created by Sabol et al (76). In this manner, efficient analysis is possible by discriminating between genotypes with a high, high-low or low transcriptional efficiency. This makes analysis of the MAO-A VNTR repeats showing high transcriptional efficacy much more efficient, since otherwise it was not possible to incorporate the heterozygous genotypes ('now high-low') into our model. To attain this discrimination in our regression model, three new variables were generated: i) MAO-A genotypes with high transcriptional efficiency (including MAO-A genotypes 4-4; 3,5-4; and 3,5-3,5), ii) MAO-A genotypes with high-low transcriptional efficiency (including MAO-A genotypes 3-4; 2-4; 4-5; 3-3,5), iii) MAO-A genotypes with low transcriptional efficiency (including MAO-A genotypes 3-3; 2-3; 3-5). Subsequently, 'MAO-A high' and 'MAO-A high-low' were incorporated as variables and referenced against 'MAO-A low', used as reference dummy variable. However, as was the case for 5-HT_{1A}, neither 'MAO-A high' (β=0.0041, p-value=0.945) nor 'MAO-A high-low' (β=-0.0716, p-value=0.301) showed significant size effect divergence compared to dummy variable 'MAO-A low'.
In addition to the association between genotype and basal cortisol, a regression model was constructed which made it possible to examine if genotypes had the potential to cause fluctuations in cortisol secretion throughout daytime. For analysis of the individual 5-HT_{1A} genotypes, the variables 'GG' and 'GC' were again referenced against the homozygous wild-type CC dummy variable. This yielded a significant effect size divergence between both CC and GG genotype (β=-0.0109, p-value=0.001), respectively CG and GG genotype (β=0.01, p-value=0.001), with the homozygous mutant GG genotype generating a significantly lower regression coefficient **(figure 12)**. There was no significant difference in effect size between the CC and the CG genotype (β=-0.0019, p-value=0.741). These statistics suggest that the 5-HT_{1A} the homozygous mutant GG genotype can mediate cortisol fluctuations throughout the day by causing lower cortisol values somewhere throughout the day.

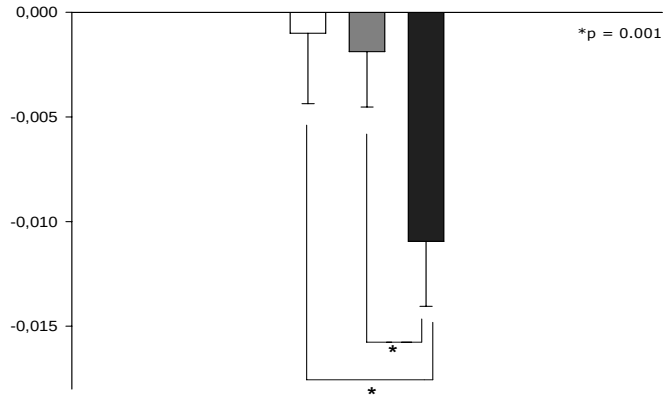

Figure 12: Effect of individual 5-HT1A genotypes on cortisol fluctuations throughout the day. Both CG (grey) and homozygous mutant GG genotypes (black) were referenced against the homozygous wild-type CC genotype (white). A significant lowering in size effect between both CC and GG genotype, respectively CG and GG genotype was observed. In contrast, between the CC and the CG genotype, no significant size effect difference exists. (* p=0.001).

Next, for individual MAO-A genotype analysis, MAO-A 'high' and 'high-low' genotypes were referenced against dummy variable MAO-A 'low'. In contrast to 5-HT_{1A} genotype, none of these MAO-A genotypes showed a significant difference in cortisol fluctuation throughout daytime **(table 6)**.

Table 6: Effect of individual MAO-A genotypes on cortisol fluctuations throughout the day. Both MAO-A 'high' and 'high-low' genotypes were referenced against dummy variable MAO-A 'low'. No significant size effects differences were observed, which suggests that MAO-A is not associated with subtle cortisol fluctuations throughout daytime.

	β (SE)	p-value
MAO-A 'high' vs. MAO-A 'low	0.0011495 (0.0033)	0.733
MAO-A 'high-low' vs. MAO-A 'low'	-0.003183 (0.0033)	0.925

4.3.7 Effects of genotype on mood

The possible association between MAO-A or 5-HT_{1A} and NA was also studied. Neither for MAO-A nor for 5-HT_{1A}, a significant association with NA was observed **(Table 7)**. However, 5-HT_{1A} showed higher size effect in the association with NA, indicating that when stratifying for this serotonergic structure, participants generated higher scores for NA. This proposes that 5-HT_{1A} shows a greater potential to influence negative emotions.

Table 7: Main effects of 5-HT_{1A} and MAO-A genotype on NA in our EFPTS populations. Neither 5-HT_{1A} nor MAO-A significantly directly influenced negative emotions. However, 5-HT_{1A} generated a higher regression coefficient in the association with NA, indicating that participants with this genotype generated higher scores for NA.

NA	β (SE)	p-value
5-HT_{1A}	0.00286 (0.0023)	0.213
MAO-A	-0.0116 (0.0092)	0.207

4.3.8 Does a specific genotype mediate cortisol responses after experiencing minor daily life stressors?

It is hypothesized that individual stress sensitivity is mainly mediated by HPA-axis dysfunction. In this paradigm, polymorphisms may exist that moderate HPA-axis reactivity, thereby having the potential to alter susceptibility to minor daily life stressors. To test this hypothesis, 5-HT_{1A} and MAO-A were stratified against the effects of minor daily life stressor on cortisol and mood. Because

only a significant association between social stress and cortisol was previously found **(figure 11)**, analyses were limited to the effects on cortisol and mood after experiencing this minor daily life stressor. Primarily, main effects of the MAO-A and 5-HT_{1A} genotype on HPA-axis reactivity were studied. This was done by regressing the polymorphism - social stress interaction term on cortisol. Both genotypes were incorporated into the previously described regression model, analyzing the effects of social stressors on cortisol. Analysis showed a significant interaction between 5-HT_{1A} genotype and social stress on cortisol levels (β= 0.0019347, p=0.021) **(figure 13 left)**. This suggests that 5-HT_{1A} can significantly mediate cortisol secretion after experiencing social stress. To observe if this main effect of 5-HT_{1A} genotype was arbitrated by the presence or absence of a certain risk allele, the regression model previously used was stratified for each individual 5-HT_{1A} genotype (CC, CG and GG). The presence of the mutant G allele in a participant's 5-HT_{1A} genotype seemed to be the crucial factor. When stratifying for the wild-type CC genotype no significant association with cortisol levels (β=0.0033263, p-value=0.646) after social stress were found. A dose-response in cortisol secretion was observed between the wild-type CC genotype, and CG and GG genotypes containing the mutant allele, with a significant difference between the homozygous wild-type CC and homozygous mutant GG genotype (β=0.02613, p-value = 0.013) **(figure 13 right).** Moreover, in both the CG (β=0.01549, p-value=0.004) and GG group (β=0.02833, p-value<0.001) there was a significant and positive association between social stress and cortisol. This indicates that the G-allele carriers have higher cortisol levels to social stress in daily life as compared to non-G carriers **(figure 13 right).** In contrast to 5-HT_{1A}, there was no significant interaction between MAO-A and social stress on cortisol (β= 0.0002575, p-value=0.712). This implies that MAO-A genotype is not associated with cortisol response after experiencing minor stressors.

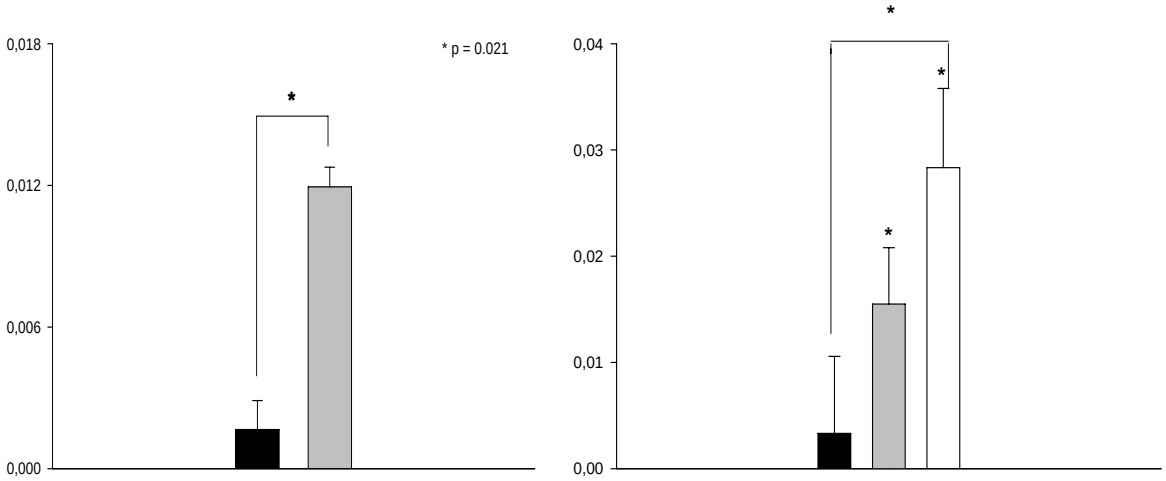

Figure 13: Left. The potential 5-HT_{1A} to mediate HPA-axis 'reactivity'. A significant elevation in size effect was observed when taking the main 5-HT_{1A} genotype into account (grey) (β= 0.0019347, p=0.021). **Right.** The effect of 5-HT_{1A} genotype is arbitrated by the presence or absence of the mutant G allele. Both the CG (grey) (β=0.01549, p-value=0.004) and GG genotype (white) (β=0.02833, p-value<0.001) displayed a significant elevation of the regression coefficient**.** A dose-response of cortisol secretion was observed between the CC, CG and GG genotype, with a significant difference between the homozygous wild-type CC and homozygous mutant GG genotype (p-value = 0.013). (* p < 0.05)

4.3.9 Does a specific genotype mediate mood after experiencing minor daily life stressors?

Finally, the presence of an association of the 5-HT1A genotype with negative emotions, when experiencing social stress, was examined. Also here a crucial role for the mutant G allele was observed. In the xtmixed regression model, the CG and GG genotype were referenced against the wild-type CC genotype. This generated a significant size effect elevation between the CC and GG genotype **(table 8).** However, no significant difference between the CC and CG genotypes were observed.

In addition, the potential of MAO-A to mediate negative emotions after experiencing social stress were studied. But as was the case for cortisol responses, no interaction between MAO-A and social stress on the experience of negative emotions could be confirmed. These data reject a role for MAO-A in mediating cortisol and mood responses after experiencing minor daily life stressors.

Table 8: Association between 5-HT1A genotype and NA scores after experiencing social stress in daily life. Analysis showed a significant size effect elevation in participants with the GG genotype, suggesting that the mutant G allele can heighten negative emotions after experiencing minor stressors.

	β (SE)	p-value
CC vs. CG	-0.005167 (0.00436)	0.236
CC vs. GG	0.0108348 (0.0051)	***0.034****

4 Discussion

In every-day life, responsiveness to stressful experiences is efficiently managed by regulation of HPA-axis activity (54). However, evidence consistently link stressful experiences with an increased risk for MD onset. The underlying causality proposes a dysfunctional regulation of the neuroendocrine stress response, in particular the HPA-axis (11,12).
MD neuropathology is not only dominated by dysfunction of HPA-axis reactivity, but also by neurotransmitter pertubations of the 5-HTergic system (16). Nonetheless, neither system by itself can account for the clinical outcome of MD. To illustrate, the link between HPA-axis dysfunction and MD is not completely straightforward since not all individuals succumb to depressional onset after experiencing stressful events.
Epidemiologic studies explained this variance by showing that MD is subject to specific gene-environment interactions, providing each individual a unique reactivity to experienced SLEs (68). This imposes the search for genetic variations at the basis of HPA-axis regulation, making some individuals more susceptible to MD onset after experiencing stressful episodes. The present study primarily focused on genetic polymorphisms found in the serotonergic system (5-HT_{1A}C-1019-G, MAO-A uVNTR). We hypothesize that genetic variations in the serotonergic system have the ability to modify HPA-axis reactivity, rendering individuals more susceptible to MD-inducing effects of SLEs.

HPA-axis reactivity in our population was studied by looking at the effects of minor daily life stressors. Wichers et al. showed that genetic liability to MD is manifested as the tendency to display negative emotions in response to small stressors in the flow of daily life. This establishes the emotional sensitivity to minor daily life stressors as a true endophenotype of MD (83). To validate the model used in this study, the impact of minor daily life stressors on negative affectivity (NA) was studied in a healthy female twin population. All minor daily stressors were found to significantly elevate NA scores in our population. This supports the hypothesis of Wichers et al. and states that the NA response to stress sensitivity is a good mechanism to study a possible association with genetic liability to MD.

Basal and stress-induced cortisol secretion have been shown to depend on the specific nature of the experienced stressor (e.g. interoceptive or exteroceptive, minor or severe,…) (92). For this reason, the impact of the different minor daily life stressors on basal cortisol levels was examined. Cortisol was measured in saliva since it is comparable to plasma and CSF cortisol and easily obtainable in contrast to plasma cortisol (93). Both social, activity-related and event-related stress elevated cortisol levels, representing the human stress response. In contrast, discrepancy was found since only episodes of social stress significantly elevated saliva cortisol levels. Also, social stressors generated the highest mean stress score obtained from our ESM data. An explanation for this significant elevation of cortisol levels when experiencing social stressors can possibly be found in human personality and neuroendocrine studies. Kirschbaum et al. studied the effects of gender differences in the regulation of HPA-axis reactivity. They showed that for both male and female

subjects, HPA-axis reactivity was dependent on the type of stressor experienced (94). Moreover, activity and event-related situations induced more tension and stress in the male subjects, while problems in interpersonal and social situations evoked more stress in female subjects (94). This can be explained by female personality traits, showing a greater preference to empathize and embark in intense social relationships (6). These studies support our data since our population is strictly female. As a consequence, it can be concluded that at least in our female population social stressors exemplify the essential predictors of cortisol response when studying the effects of minor daily life stressors.

Subsequently, the direct effects of polymorphisms of the serotonergic system on cortisol levels and mood were examined. There are several reasons why we focussed primarily on genetic variations within the serotonergic system. First of all, pertubations of the serotonergic system are strongly associated with MD pathology (16). Secondly, currently used antidepressants act via specific components of the serotonergic system (14,15,43, 44). Third, genetic linkage and association studies have primarily focused on polymorphisms of the serotonergic system, generating numerous polymorphisms associated with MD onset (71).
Finally, but most importantly, evidence from animal and human studies shows a crucial role for serotonergic innervation in the regulation of HPA-axis reactivity, suggesting that correct serotonergic neurotransmission represents the basis for accurate HPA-axis responsiveness (95). Moreover, functional studies of serotonergic projections arising from the raphe nuclei have led to the hypothesis that the serotonergic system regulates resistance, adaptation and adequate responsiveness to acute or chronic stress (96). More specifically, the amygdala has been found to excitate the HPA-axis stimulation via serotonergic neurons from the raphe nuclei projecting to subdivisions of bed nucleus of stria terminalis (BNST), thereby enhancing HPA axis activity (97).
In contrast, the hippocampus exerts an important inhibitory role. Critical within this hippocampal inhibition pathway is thought to be the ventral subiculum, one of the primary projections from the septohippocampal system. This neural system is extensively innervated by serotonergic neurons arising from the raphe nuclei (98).
When considering these data, both the functional C-1019-G SNP located on the 5-HT_{1A} gene as well as the functional VNTR found the gene encoding the enzyme MAO-A represent interesting targets for studying their effect on HPA-axis reactivity, since these polymorphisms are revealed to cause serotonergic dysfunction (75,76).

When studying the effects of both genotypes on HPA-axis reactivity without accounting for minor stressors, no significant main effect of MAO-A or 5-HT_{1A} genotype on basal cortisol levels was found. This possibly reflects the indirect pathway by which both genotypes regulate HPA-axis activity and cortisol secretion, or might suggest that without accounting for the impact of minor stressor, no association with basal cortisol levels will be found. This nicely illustrates the shortcomings of previous genetic association studies in MD by showing that allelic variants in serotonergic genes exert such small and indirect effects that they even can't be observed by looking at molecular changes.

Subsequently, we examined if the MAO-A and 5-HT_{1A} genotypes had the potential to cause subtle fluctuations in cortisol secretion throughout daytime. This yielded a significant association between the homozygous mutant GG genotype and subtle reductions in cortisol throughout the day. This suggest that the 5-HT_{1A} mutant G allele, by efficiently inhibiting serotonergic neurotransmission can directly cause subtle reductions in cortisol secretion throughout the day. This association illustrates the pathway by which serotonin directly acts upon HPA-axis activity, giving a neurochemical explanation for the observation of stress-induced hormonal changes.

Could polymorphisms of the serotonergic system indeed mediate HPA-axis reactivity after experiencing minor daily life stressors? The interaction between 5-HT_{1A} or MAO-A and social stress on cortisol levels was examined. Analyses were limited to the effects of social stress, since this stressor was previously proposed as the essential predictor of cortisol response in our population.
A significant elevation in social stress-induced cortisol levels was observed in function of 5-HT_{1A} genotype. This partially supports our hypothesis, by showing that, in our population, at least a gene-environment interaction exists which can significantly mediate cortisol secretion. In addition however, the presence of the mutant G allele in a participant's 5-HT_{1A} genotype seemed to be a crucial factor. To clarify, in both the heterozygous CG and homozygous mutant GG genotype, a significant elevation of cortisol secretion was observed when experiencing episodes of social stress. Moreover, a dose-response in cortisol secretion was present between the wild-type CC, mutant CG and mutant GG genotypes, with a significant elevation between the homozygous wild-type CC and homozygous mutant GG genotype. This indicates that the G-allele carriers have higher cortisol levels to social stress in daily life as compared to non-G carriers, and that this association is 'dose'-dependent.
A possible mechanism by which the mutant 5-HT_{1A} genotype leads to higher HPA-axis sensitivity when experiencing stress could be disruption of serotonergic neurotransmission projecting to the inhibition pathway of the hippocampus, thereby diminishing efficient 'negative feedback' regulation of the HPA-axis. However, a direct relationship between serotonin neurotransmission and cortisol may be more plausible. In particular, it has already been shown that hypercortisolemia can efficiently reduce 5-HT1A receptor function (99). When taking this into account a possible interplay may exist in which both the 5-HT_{1A} mutant G allele and subsequent hypercortisolemia act upon each other causing a deleterious cascade culminating in dysregulation of HPA-axis function. However, this consideration remains as yet speculative. Nonetheless, these data entirely support our hypothesis by showing that genetic variations associated with serotonergic dysfunction in MD, can mediate stress-induced HPA-axis reactivity.

In contrast to 5-HT_{1A}, no significant association between the MAO-A genotype and HPA-axis reactivity after experiencing minor daily life stressors was found. This does not subside our hypothesis, but implies that 5-HT_{1A}, and not MAO-A, is a crucial component in the serotonergic regulation of HPA-axis reactivity when experiencing stressful episodes. Supporting evidence for this hypothesis can be found in animal studies. More specifically, 5-HT_{1A} receptors play an important role in the regulation of the HPA-axis (100). Numbers of 5-HT_{1A} receptors were also found to be reduced in the hypothalamus of animals subjected to experimental depression models, where they were shown to be directly involved in the regulation of stress hormone secretion (101). In addition,

both amygdala and dorsal raphe nuclei showed reduced numbers of 5-HT_{1A} receptors in these animals (102). Data from Li et al. further supports a principal role for 5-HT_{1A} in stress mediation, by suggesting that hypothalamic 5-HT_{1A} receptors are involved in the regulation of stress-induced ACTH secretion via acting on CRF neurons (103). This effect can be mediated by direct acting on CRF neurons or by indirect acting via interneurons which innervate CRF neurons and inhibit their activity. All these data further support our findings proposing a principal role for 5-HT_{1A} in stress mediation.

However, a hypothesis is never made alone. During the course of our study, Jabbi et al. published a paper linking the endocrine stress response in human subjects with polymorphic variations in the 5-HTT, COMT and MAO-A gene (104). They tested the role of these genes by looking at effects of polymorphisms on peripheral cortisol and ACTH response to experimental psychological stressors. A significant main effect of MAO-A on cortisol response in controls was found when experiencing a psychological stressors. In addition, a significant elevation of the cortisol response was observed when comparing MAO-A genotypes with high expression to the MAO-A genotypes with low expression.
This is very interesting since these findings are in total contrast with our MAO-A data. A number of explanations can be given to explain this divergence. Firstly, the sample used in the study by Jabbi et al. is rather small (n=70), suggesting that the sample size of our sample (n=327) is more fit for a reliable assessment of genetic contribution without compromising statistical power. Secondly, the paradigm in which they studied the genetic contribution to HPA-axis reactivity was quite different from our paradigm. More specifically, in contrast to looking at the effects of minor daily life stressors they induced a experimental stress challenge in the form of a speech test. In comparison to minor daily life stressors, this type of induced stress is very intensive and can therefore not be compared with the effects and impact of minor stressors in the course of daily life.
However, this could give an explanation for the inconsistent role of the MAO-A genotype in stress mediation, since it has been shown that different types of stressors clearly utilise different neural systems in HPA-axis activity modulation (105). Based on electrophysiological studies in animals, it became apparent that subpopulations of serotonergic neurones with unique functional properties exist (106). This might suggest that subpopulations of serotonergic neurons display specific stress-type related properties. Moreover, it has been shown that intense stressors lead to enhanced serotonin release and metabolism when compared less intense stressors (107). This has an important implication when considering the genetic contribution of the MAO-A gene in HPA-axis reactivity. More specifically, it could be that the genetic contribution of the MAO-A gene only becomes visible when an individual is challenged with severe stressors, consequently leading to an elevation in serotonin metabolism. This might explain the fact the in our study no significant association between MAO-A genotype and HPA-axis reactivity was found.
Consequently, the effects of minor daily life stressors might therefore only be observed in serotonergic components that are intensely associated with HPA-axis regulation, like the 5-HT_{1A} receptor.

Before drawing any final conclusions is has to be noted that our study has some limitations. Our sample included strictly female individuals. Previous studies showed that women report a greater

distress in relation to daily life stressors (108). In addition, studies have reported significant gender differences in cortisol responses to stress, implying that our findings might not apply for male individuals (109).

The study sample was recruited from the general population of female twins living in Belgium. In theory, this could limit the generalizability of the results. Moreover, twins are more likely to have lower birthweigths when compared to singletons which could have an effect on future HPA-axis reactivity (110). However, preliminary analyses indicated twins in the current sample did not differ from their singleton siblings in either mood or cortisol reactivity, suggesting that similar results can be expected in the general population. Finally, as shown by previous studies, missing data in naturalistic studies are inevitable. Problems may arise in the ESM procedure as it depends on the compliance of subjects. In particular, fixed time sampling protocols may be problematic and can bias results. However, missing reports and saliva samples appeared to be randomly distributed over the day so that data used in the analysis can be expected to represent a relatively unbiased sample of daily life activities and contexts (111).

Analyses were performed on the effects of serotonergic genes on HPA-axis reactivity when experiencing minor daily life stressors. Social stressors appear to be essential predictors of cortisol response to minor daily life stressors in our female population. Additionally, our findings link a polymorphism in the 5-HT_{1A} gene with an increased sensitivity and responsiveness of the HPA-axis to daily life stressors. This association could not be found for variations in the MAOA gene, another important regulator of the serotonergic system. These data suggest that 5-HT_{1A} receptors play a primary role in serotonergic regulation of HPA-axis reactivity to minor daily life stressors. In addition, these data further support the use of reliable endophenotypes for analyzing the contribution of genetic variations to the development of psychiatric disorders like MD.

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