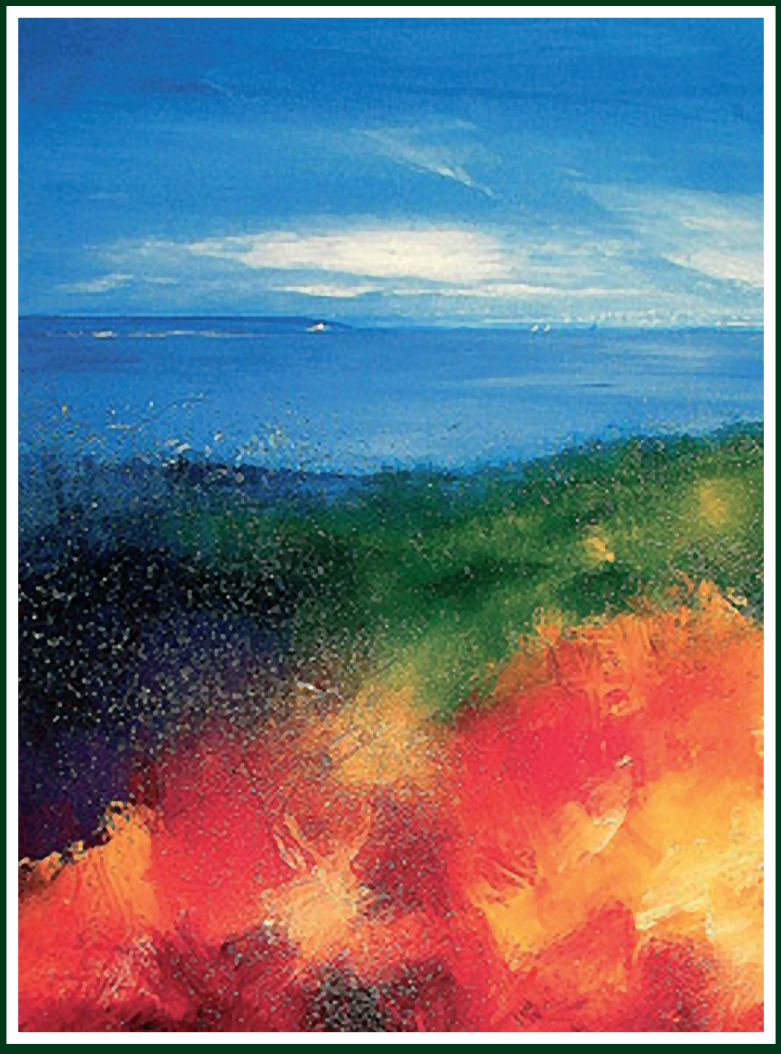
Cortisol exposure, cognition and clinical course of bipolar disorder



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Content

1. Introduction

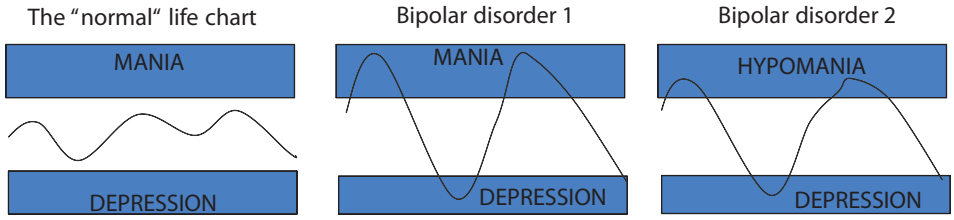
Bipolar disorder (BD) is a common mood disorder, with an estimated prevalence of 2.4 % in the Netherlands (1). According to the Trimbos institute and the most recent World Health Organization's report "The world health report 2001 - Mental Health: New Understanding, New Hope", in the Netherlands the burden of the disease is 30.000-40.000 Disability-Adjusted Life Years (DALY's), indicating a chronic disease disabling patients in normal functioning (2). Worldwide, BD is estimated to stay in the top ten causes of Years Lived with Disability (YLDs), accounting for 2.5% of total global YLDs (3). BD was once considered an episodic illness with a favorable outcome, however, nowadays seems to be a disease with a more chronic course with cognitive deficits in between episodes, residual mood symptoms and impaired functioning in daily life roles (4).

History

Bipolar disorder is characterized by mood episodes, with cycling patterns of both depression and mania or hypomania. Mania and hypomania are episodes of abnormal elevations of mood or irritability for at least 1 week (mania) or 4 days (hypomania), with raised activity level, inflated self-esteem, increased risk taking behavior, involvement in pleasant activities, and other features. Mania and hypomania differ with respect to severity of symptoms; when psychotic symptoms occur or admission is required, criteria for mania are met by definition. Furthermore, mania and hypomania can be distinguished in severity and duration of symptoms. Mania has a greater negative impact than hypomania in daily life with e.g. social and financial consequences. Depression is characterized by for at least 2 weeks of depressed mood or anhedonia for most of the time during the day. Several types of the disorder are distinguished according to the Diagnostic and Statistical Manual (DSM)-IV criteria: BD1 includes a history of at least one manic episode, though also depressive and hypo manic episodes are frequent, BD2 is marked by at least one depressive episode and one hypo manic episode. In figure 1, differences are shown. Furthermore, the DSM-IV includes cyclothymic disorder, consisting of a cycling pattern of hypomania and depressive symptoms, not fulfilling the criteria for full depressive episodes. Finally, BD not otherwise specified as rest category for patients with drastic mood changes regarded as mood disease, but not meeting enough criteria for hypomania and depressive episodes.

Manic and depressive episodes have already been described in ancient Greek Hippocrates. In his description of the Greek woman Thasos suffering from racing thoughts, sleeplessness, euphoria or irritable mood, and in severe cases hallucinations, he referred to this condition as a maniac state. The German doctor Emil Kraepelin (1856–1926), described manic depressive psychosis, as an illness with acute episodes, characterized

Figure 1: schematic example of "normal" mood chart and examples of mood charts in BD1 and BD2.



by depression or manic psychosis, with largely symptom-free intervals in between (5). Only until 1957 unipolar depressive and BD were distinguished into two different entities by K. Leonhard (6). In 1976, Goodwin and colleagues introduced hypomania and BD2 as a separate diagnosis. BD as such was officially introduced as diagnosis in the DSM-III in 1980. Goodwin and Redfield note that a complicating factor in research is the poor reliability of BD2 diagnosis resulting from the difficulties of assessing the history of hypo manic symptoms, especially by depressed patients (6). It is under current debate in what respect BD2 and recurrent unipolar depression differ with respect to cycling patterns and polarity. Long-term studies to differentiate between and validate diagnostic boundaries are highly needed.

Within this historical context, it is clear that the diagnosis and course of BD according to its modern criteria are only recently on their way to be further described and validated with respect to clinical course and treatment of the disease. Specifically validation of the "diagnostic" entity BD is subject to criticism (6), indicating the need for further research to the boundaries and criteria of this diagnosis.

In addition to the diagnostic difficulties, one must to realize the clinical course of the disease as highly variable and unpredictable. The DSM-IV criteria for BD predominantly focus on mood, with characteristics of mood episodes. Number of mood episodes, and recovery in between episodes, are thus key features in evaluating course of disease. However, in the past years evidence is increasing that impaired daily life functioning in patients with BD is caused by for example mood disturbance, especially residual depressive symptoms, as well as cognitive deficits.

Risk factors influencing clinical course

In identifying risk factors influencing clinical course, distal and proximal risk factors can be distinguished. Proximal factors are more immediately related to the actual episode of the disorder (preceding stress, interruptions daily rhythms, medication changes)

than distal factors which play a background role from conception and early childhood increasing imminent risk (maternal stress, childhood abuse). Several risk factors known to be associated with a worse long-term course of BD are already identified. In a landmark retrospective study of almost 1,000 BD patients in the Systematic Treatment Enhancement Program for Bipolar Disorder (STEP-BD) (7), the following was found: early onset was identified as risk factor, predicting higher rates of co morbid anxiety disorders, substance abuse, more recurrences, shorter periods of euthymia, greater likelihood of suicide attempts and violence. In longitudinal studies a relation was found between number of episodes in the index year and prior course of illness (history of rapid cycling), family with substance abuse, and poor occupational functioning (8, 9). In other studies, early childhood trauma was repeatedly associated with earlier disease onset and more severe course (10, 11). It has been thought that alcohol use can cause vicious circles leading to depression and vice versa, influencing the course of illness. Alcohol use as "self medication" strategy during (bipolar) depression occurs frequently (41% of bipolar patients) (12), increasing the risk on developing alcohol dependence. However, in a recent longitudinal study, no relation was found between alcohol use and longitudinal course of BD (13). Other environmental factors known to influence BD are life events and daily life stress. Life events are found to be of major impact in the year before the first depressive or manic episode (14). There are several, though somewhat contradictory, studies underlining the influence of life events on course of BD in longitudinal studies (15). Stressful life events may trigger depressive episodes in BD by inducing changes in social "Zeitgebers", circadian rhythms and the regulation of the stress response (15, 16). Thus, illness history, early childhood trauma, alcohol abuse and stressful life events influence course of the illness. In this study, we included social support, life events, medication use as proximal factors, and childhood trauma as distal factor. However, in this dissertation we will step back towards a preceding level, namely the underpinnings of the disease. In order to be able to further improve the understanding of the disease, we will first focus on two endophenotypes, namely stress hormone exposure and cognitive performance, in relation with clinical course of the disease.

Clinical course: endophenotypes as intermediate factors

Although clinical correlates associated with the course of the disease are known, the causes of the disease have still not been elucidated. Genetic risks seem to overlap with schizophrenia (17) in genome-wide association studies (GWAS), and currently, due to lack of convincing evidence, samples for a GWAS are now collected in 2,500 BD1 patients in the Dutch study named Bipolar Genetics. A major limitation of genetic association studies is that DNA nucleotide sequences themselves are not predictive for "in vivo"

gene expression, RNA transcription and proteome function. Research to protein function in relation with mental diseases is promising by detailing pathophysiological processes, which could lead to understanding of the biochemical overlap and differences between for example schizophrenia and BD (18). However, core pathophysiological underpinnings of BD have to be elucidated to be able to understand genetic, epigenetic and proteomic findings. One of the possible entrances in finding pathophysiological processes is the introduction of endophenotypes as simpler clues to find genetic underpinnings than complex phenotypic diseases. Endophenotypes are known as sub clinical quantitative traits that exist in affected and unaffected relatives independent of the disorder (19). The traits can differ in their presentation as they can be neurophysiological, biochemical, endocrine, neuroanatomical, or cognitive in nature (20).

Endophenotypes are generally less complex than their associated phenotype in a disorder as they have fewer genes, allowing easier linkage due to their higher signal-to-noise ratio. They are therefore useful indicators of processes that mediate between genotype and phenotype (21). The concept of endophenotypes was originally introduced by Gottesman and Shields in the early 1970s. A renewed interest has emerged due to the limited success of genetic linkage and association studies. For an endophenotype to be useful in genetically identifying a psychiatric disorder, it should meet a set of criteria as proposed by Gottesman and Gould (22): first, it must be associated with the disorder; second, it should be primarily independent of clinical state (i.e. it manifests whether or not illness is active); third, it should be heritable (i.e. more common in the non-affected relatives than in the general population) and fourth, it must be associated with a candidate gene or gene region. A valid endophenotype should hence have trait-like properties (21); in contrast to a state variable, an endophenotype it is not only affected by mood phase. Because having a stable and permanent presentation, endophenotypes may be more reliable than mood symptoms in predicting the long-term course of BD.

However, other concepts such as “biomarker” and “vulnerability trait” indicate the same need for better defined biological substrates for complex disease phenotypes. These terms refer to the search for biological underpinnings of diseases, while endophenotypes aim to serve as intermediate factors in finding genetic underpinnings of diseases. In BD, biological endophenotypes such as the status of the Hypothalamic- Pituitary- Adrenal (HPA-)-axis, could serve as endophenotype (23, 24). In addition, cognitive performance has been proposed to serve as a cognitive endophenotype (25-27) in BD, which also appears to be under influence of cortisol effects in the hippocampus (28, 29) and prefrontal cortex (30).

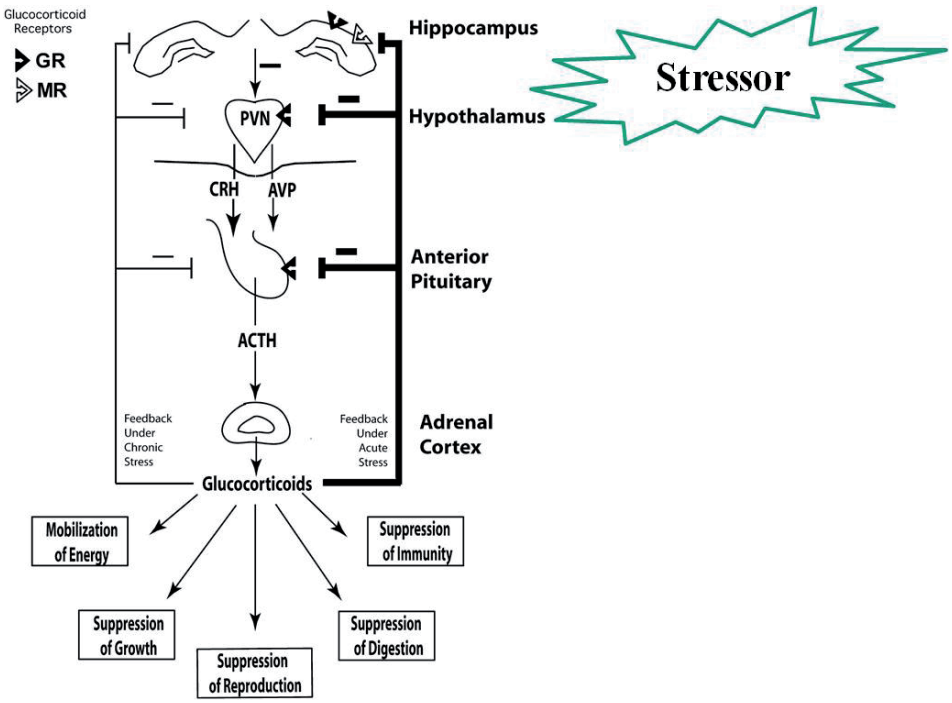
In the next paragraphs the HPA-axis as a biological endophenotype will be described in relation to BD. Furthermore, an overview will be provided with current assessments of

the HPA-axis, and new developments will be described. As a second endophenotype, cognition will be described in its relation both with the clinical course of BD and in its relation with the functioning of the HPA-axis. Although in this thesis the discussion of the role of endophenotypes will be limited, the introduction of this concept is needed to introduce the theoretical background and subsequently the choices made in our study design. The articles in this thesis focus mainly on the results of the cross sectional data.

Endophenotype 1: the HPA-axis

The biological stress response consists of a precisely regulated cascade of release of hormones and neurotransmitters. The direct route is stimulating the adrenergic pathways, inducing the sympathetic response; the slower hormonal route is regulating the HPA-axis. To maintain stability in a continuously changing environment, the human

Figure 2: Schematic overview of the HPA-axis, the major impacts on body processes, and the glucocorticoid (GC) feedback in the brain through Glucocorticoid Receptors (GRs) and Mineralocorticoid Receptors (MRs) (31) . GR and MR in the brain is included in this figure .Copied and adjusted with permission from Boonstra, 2004, in accordance with the Scientific Technical and Medical publishers permissions guidelines 2012.



body and mind are required to respond with flexibility, in which the HPA-axis plays an important role. The stress response is triggered by a stressor of any kind (psychological, physical). In figure 2 a schematic overview is provided.

Corticotropin Releasing Hormone (CRH) is a hormone that fulfils a central role in the stress response and is released by the hypothalamic paraventricular nucleus (PVN) under influence of a stressor. It is able to rapidly activate the sympathic response and plays an important role in orchestrating the so -called "fight, freeze or flight" reaction. In a second, slower way, CRH activates through ACTH- release in the pituitary, the release of cortisol in the adrenal glands. The combined system of CRH-ACTH-cortisol release is referred to as HPA-axis. Cortisol stimulates and regulates the mobilization of glucose in the liver, supporting the fight or flight reaction by stimulatory effects on the muscles, heart and blood pressure. Cortisol also affects cognitive processes such as memory of interpretation and decision-making in different situations. Furthermore, cortisol has suppressive effects on different organs and hormone systems not directly needed during

Table 1: Cortisol effects in and outside the brain

Cortisol	Central/ Brain effects	Systemic effects
Acute effects	Negative feedback of cortisol production by inhibition of CRH and ACTH production	Mobilization of glucose
		Increased blood pressure
	Suppression of growth hormone and sex hormone production	Suppression of bone re-calcification and immunity/inflammation.
	Inhibition of long-term potentiation neurons	
Chronic effects	Affective dysregulation, depression, euphoria	Hypertension, cardiovascular diseases
	Neurotoxic effects: cognitive deficits	Fatigue, myopathy
	Anxiety, eating problems, insomnia	Amenorrhea and impotency
		Impaired immune defenses, increased risk of regular infectious diseases
		Osteoporosis
		Diabetes

Based on PhD Thesis E. Van Rossum (2005) and "Stress, the brain and depression" – H.M. Van Praag, R. De Kloet and J.H. Van Os (2004).

the stress response, such as the gastrointestinal system, the immune system, the gonadal hormones, growth hormone and bone calcification (32). In sum, cortisol acts at multiple sites within the body to maintain homeostasis. Because of the damaging effects of chronic exposure to cortisol, the HPA axis is tightly regulated through negative feedback on glucocorticoid receptors: cortisol inhibits its own release and thereby finalizes the stress response. Chronic raised cortisol levels could be very damaging. Long-term effects of increased cortisol levels are: osteoporosis, myopathy, fatigue, abdominal obesity, diabetes and dyslipidemia leading to increased risk of cardiovascular disease, impaired immune defenses, affective symptoms and neurotoxicity, especially in the hippocampus area associated with cognitive symptoms such as memory deficits and problems with concentration and executive functioning. In table 1, cortisol effects are summarized.

Dysregulation of the HPA-axis in BD as endophenotype

Dysregulation of the HPA-axis is known to occur in several psychiatric disorders such as anxiety disorders like post traumatic stress disorder (33) and mood disorders including BD (34-40), in patients as well as in healthy offspring of bipolar parents (24, 37). Activation of the HPA-axis during depressive episodes exists in more than 80% of the patients (41), this association seems at least partly state independent in bipolar patients (39), indicating a trait phenomenon: remitted patients have higher overall cortisol levels, reduced cortisol reactivity to negative daily events, and flatter diurnal slopes associate all with more episodes (42), reflecting subtle but on the long-term clinically relevant influence. Daban (43) and Watson (39), reviewed the abnormalities in HPA-axis functioning in patients with BD. Both conclude that the Dex/CRH test, the most sensitive challenge test provoking a stress response and measure HPA axis abnormalities, is abnormal in remitted and non-remitted bipolar patients, indicating that this could serve as a trait marker in BD as well as in MDD (40). In chapter 1 and 2, this will be discussed in further detail. All mentioned arguments plead to the HPA-axis functioning as endophenotype in BD, following the aforementioned criteria according to Gottesman and Gould: it is associated with BD, it is at least partly independent of clinical state, it is found in healthy family members. In the next section we will focus on the final criteria, namely the genetic basis of the HPA axis regulation in relation with BD.

Dysregulation of the HPA-axis and the role of cortisol receptors: link to genetic underpinning.

There are two main cortisol receptors by which cortisol exerts its effect: the Mineralocorticoid Receptor (MR) and the Glucocorticoid Receptor (GR). In the brain, MR

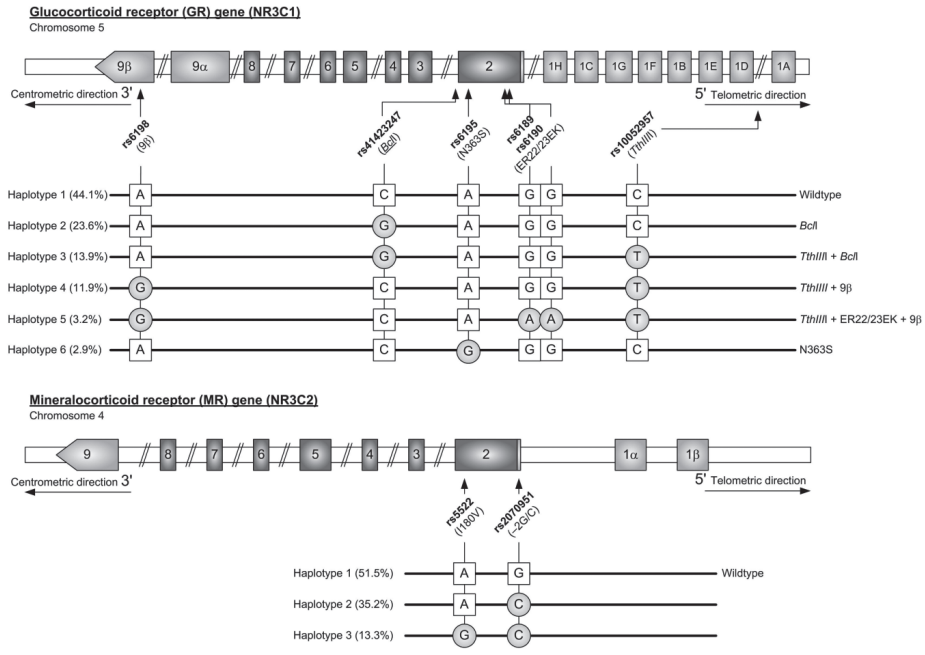
is predominantly localized in the hippocampus, the amygdala and the prefrontal cortex, and has a dominant function in controlling sensitivity of the stress response system, limiting disturbance of cellular homeostasis and for example acquisition and appraisal of information, and behavioral selection (prefrontal) (44).The GR is widespread throughout the brain, but has a high density in the hippocampus, the prefrontal cortex and the periventricular nucleus and is involved in facilitating recovery of cellular homeostasis, restraining stress induced responses and learning and memory processes promoting behavioral adaptation for future events (44). The affinity of the MR for cortisol is 10 times higher than the affinity of the GR for cortisol. In normal circumstances cortisol predominantly binds to the MR, during the stress response cortisol also binds to the GR, whereas the GR is important for the negative feedback thereby determining the regulation of the HPA-axis (45-47). During periods of stress and elevated plasma cortisol, there is increased occupation of GR.

The lipophilic cortisol is thought to enter the cell by passive diffusion through the cell membrane. In the cytoplasm it is binding with the GR or MR, after which the complex travels through the nuclear membrane into the nucleus. The MR and the GR function through several modes of action. An important route to affect gene transcription is the direct binding of GR or MR dimers to target genes. Metabolic effects and the feedback mechanism are exerted by stimulation of gene transcription, also transcription inhibition can occur through this mechanism. The second route is through to regulate gene expression by binding to gene regulating proteins like AP-1 (activating protein-1) and NF-κB (nuclear factor- κB) (48). These pathways influencing gene expression are time-consuming, as it is clear that changing protein production by altered gene expression is a slow process, applicable in regulatory but not acute processes. In animal studies, the MR is also thought to be active as membrane MR in regulating the HPA-axis in a fast way, by regulating the threshold for stress induced surge and neuronal excitability (49). The effects of GR and MR dimers are complex and divers, and can modulate the signal transduction of for example serotonin and adrenaline. However, these hypotheses are based on animal studies, in which the predominant glucocorticoid is corticosterone; in humans it is cortisol; it is unclear to what extend these findings remain the same in humans. The therapeutic applications of these receptors is currently under debate in trials with GR-antagonists, such as mifepristone in treatment of depression with psychotic features (50), which is continued in a placebo controlled trial in depressed patients with psychotic symptoms (ClinicalTrials.gov: NCT00637494), planned until the end of 2013. In bipolar depressed patients it has been found to improve mood and neurocognitive functioning (51). However, it was not effective for treatment of major depressive episodes in previous trials. In the current treatment of BD, lithium en valproate take a central place. These drugs seem to have an effect through GR-co-chaperone proteins,

that attenuates GR translocation in the nucleus, and indirectly reduce hypercortisolemic effects at tissue level (52).

Several different MR gene and GR gene variants (polymorphisms), appear to alter glucocorticoid sensitivity, which are described in figure 3. These polymorphisms are known to result in clinically relevant consequences (53), and discussed in detail in chapter 1.

Figure 3: Overview of the GR gene and MR gene, showing the single nucleotide polymorphisms (SNPs) and haplotypes



In sum, these haplotypes have been known to lead to altered HPA-axis regulation with clinical consequences:

GR gene haplotypes:

- Haplotype 2 (*Bcl*I) and haplotype 3 (*TthIII*I+ *Bcl*I): both involving a C to G single nucleotide polymorphism (rs6189/rs6190) at a polymorphic *Bcl*I restriction site, were associated with hypersensitivity to GCs (54). Several previous studies reported associations with unfavorable metabolic characteristics, such as increased body

mass index (BMI) (55), increased abdominal visceral fat(56, 57), hypertension(58), and lower amount of lean mass in the elderly (54).

- Haplotype 4 (*TthIII*I + 9β): The 9β polymorphism (rs6198) is a single nucleotide polymorphism (SNP) in exon 9β of the *GR* gene. An ATTTA sequence in the 3' UTR is changed into GTTTA. This polymorphism is associated with an increased stability of the mRNA of the inactive GR-β isoform and may lead to a relative glucocorticoid resistance by increased inhibition of the active GR-α isoform (59). This polymorphism results in reduced transrepressional effects (by which, for example, genes important in the immune system are regulated), whereas the transactivational effects appeared to be normal(60). The 9β polymorphism has also been reported to be associated with rheumatoid arthritis (59), and a 68% reduced risk of carriage of nasal Staphylococcus aureus in carriers of this polymorphism (61).

- Haplotype 5 (*TthIII*I + 9β + ER22/23EK): is also associated with a relative resistance to GCs, but in contrast to haplotype 4, this resistance is linked to the transactivational effects of the GR, by which most of the adverse effects of GCs are being mediated. The ER22/23EK polymorphism (rs6189/rs6190) is located in the transactivation domain of the GR gene, and involves two nucleotide changes in codons 22 and 23 (GAG AGG -> GAA AAG), which have been shown to be linked. This variant has been found to be associated with a healthy metabolic profile (lower C-reactive protein (CRP) levels, lower cholesterol levels and increased insulin sensitivity), longevity in the elderly and a beneficial body composition in young adults (48, 53).

- Haplotype 6 (N363S): this polymorphism (rs6195) is located in codon 363 of exon 2 of the *GR* gene. The nucleotide change (AAT -> AGT) results in an asparagine to serine amino acid. It is associated with lower cortisol levels and higher insulin levels after DST, suggesting increased GC sensitivity (62).

MR gene haplotypes:

- Haplotype 2 (-2G/C): the C allele of this polymorphism (rs2070951) is in vitro resulting in more MR protein. These carriers have a more sensitive suppression of cortisol after low dose (0.25 mg) Dexamethasone, with a highest decrease in women (63). The Cortisol Awakening Rise was not affected (63). During the Trier Social Stress Test, these persons showed highest saliva cortisol and heart rate, indicating that more MR protein leads to a higher acute stress response (64).

- Haplotype 3 (-2G/C and I180V): this haplotype is consisting of the C allele of the -2G/C polymorphism in combination with the A -> G allele of the I180V polymorphism

(rs5522). The G allele of the I180V polymorphism is associated with higher cortisol levels in men (63).

The genetic basis of HPA-axis functioning is fulfilling the final criteria to serve as endophenotype according to Gottesman and Gould. Recently, the GR polymorphisms *Bcl*I and ER22/23EK have been associated with unipolar depression in several studies (65, 66). In addition, the ER22/23EK polymorphism seems to be associated with a decreased risk of dementia in healthy individuals. MR haplotype 2 has been found to correlate with optimism and a lower risk of depression, both in women (66b).

In this thesis we investigated the association between GR and MR polymorphisms and clinical characteristics of BD. The future plan is to study the possible associations between these genetic variations and cognitive performance.

Analyzing the HPA-axis: how to assess a system in continuous change?

Until now, there are several methods to investigate the HPA-axis functioning in vivo. Multiple consecutive saliva and serum cortisol levels can provide insight in cortisol curves, total daily cortisol production and the cortisol awakening response (CAR). Circulatory cortisol levels are known to depend on chronobiological rhythms (ultradian, circadian as well as seasonal rhythms) and daily life stress.

Both the ultradian and circadian pacemakers reside in the hypothalamus (67), influencing hormonal release and hence cortisol level variability. First, ultradian rhythm is caused by oscillatory neurons in the hypothalamus, leading to CRH release and consequently, ACTH pulses and cortisol pulses, with a normal hourly rhythm (68). Contrary to GC binding to the MR (t1/2= 45 minutes), GR function is depending on this pulsatility, with a rapid dissociation of GCs after a rapid pulse (t1/2 = 5 min) (68). The timing of a stressor in a decreasing or increasing point of the pulse, has consequences for the magnitude of the stress response, which is absent in rats in the falling phase and vice versa in the increasing phase of GCs during the pulse. Second, circadian rhythms influence cortisol levels. Moreover, they are known to be disturbed during mood episodes, which in rats have been shown to be restored after fluoxetine (69). Third, seasonal variations influence HPA-axis activity, with higher cortisol levels in the morning and higher sensitivity of the stress response (increased feedback) during winter months, protecting against energy waste during winter sleeps in animals (70, 71). Therefore, it is crucial to be aware of the fact that the timing of an assessment during the day has limitations due to chronobiological rhythms in providing information about the HPA-axis functioning.

Table 2: Methods to evaluate HPA-axis functioning – pros and cons

Test	Method	Assessment	+	-
Cortisol day curve (73)	Saliva or serum sampling during the day	Assessment of circadian rhythm	Robust impression of day rhythm Mean cortisol level during the day	Low sensitivity for detecting psychopathology in psychiatric patients No evaluation of stress response Intensive for patients, serum sampling requires admission.
Urinary free cortisol (74)	Mean daily cortisol in 24h urine sampling	Reflection of total cortisol production as diagnostic tool for Cushings' syndrome	Comparison inter individual differences Information on total cortisol production	No information on stress response, no information on ultradian or circadian changes Increasingly unpopular due to low sensitivity as well as specificity in detecting Cushings' syndrome, however still broadly applied for diagnostic purposes
Scalp hair cortisol (73)	Cortisol levels in scalp hair strands	Long-term cortisol: mean cortisol level over months to years (depending on length of hair)	Easy, non invasive, information on long-term mean systemic cortisol levels. Not affected by daily fluctuations Possibility to assess cortisol in retrospect	New method: clinical relevance?
CAR (75)	First hour following awakening saliva or serum collection.	Assessing basal activity as well as response to awakening.	Associated with mood disorders Non invasive, outpatients, home and non-stress situations	Highly dependent on time of awakening Compliance
TSST (76)	Psychosocial stress test under laboratory conditions	Possibility to evaluate cortisol levels as well as other HPA-axis parameters pre, during and post acute stress	Induced "real" moderate to severe stress Responsiveness of endocrine and immunological parameters	Ethical considerations in patient populations Time consuming, expensive
ESM (77)	Saliva sampling after beeps (e.g. 10 beeps per day) in daily life, simultaneous report of daily life experiences	Insight in HPA-axis responsivity in daily life experiences	No recall bias Associated with reduced flexibility of stress response and higher overall cortisol levels in bipolar patients (42)	Depending on compliance Time consuming and demanding for participants

DST (41)	0.25 mg, 0.5 mg or 1.0 mg Dex in evening; morning awakening cortisol level	Testing negative feedback of the central GR	Easy to perform 1-mg DST is valuable in diagnosing Cushing's syndrome 0.25 mg DST is valuable in determining GR sensitivity in normal individuals.	Low sensitivity in psychiatric populations (<45%)
DEX-CRH test (34)	1.5 mg Dex at 11 pm followed by 100 µg CRH at 3 pm next day, blood sampling from 2 pm- 6 pm	Testing negative feedback of the central GR	High sensitivity in psychiatric inpatients (80-90%) Higher cortisol levels predictive for relapse depression (78)	Not suited for outpatients due to frequent sampling Expensive in large cohorts
Metapyrone test	Blocking cortisol production by inhibition of 11-deoxycortisol into cortisol	Diagnostic tool for testing the HPA-axis functioning	Widely used in endocrinology to diagnose secondary adrenal insufficiency Compensatory increasing ACTH levels indicate good functioning of HPA-axis on pituitary level	Not suited for subtle changes in HPA-axis regulation
Insulin tolerance test	By inducing hypoglycemia the HPA-axis is strongly activated	Diagnostic tool for testing the HPA-axis functioning	Diagnosing adrenal insufficiency	Risk for lethal hypoglycemia, should always be performed in clinical settings with closely monitoring of vital functions.
ACTH stimulation test, or, Synacthen test (79)	Administration of intravenous or intramuscular synthetic ACTH	Diagnostic tool for primary adrenal insufficiency	High sensitivity and specificity	Narrow indication, no information about feedback functioning.

Abbreviations: ESM: Experience Sampling Method; CAR: Cortisol Awakening Rise; DST: Dexamethasone Suppression Test; DEX/CRH test: Dexamethasone/ Cortisol Releasing Hormone test; TSST: Trier Social Stress Test

To get insight in set point, negative feedback sensitivity and functioning of cortisol receptors, challenge tests are needed to evaluate HPA-axis functioning during the acute stress response. Mean cortisol levels during the day, and as a new method, mean cortisol levels over months may provide information concerning the long-term cortisol levels as result of HPA axis sensitivity in relation to psychopathology. The most sensitive way to establish sensitivity of the GR is to perform the earlier mentioned Dex/CRH tests (72). Other methods include the Trier Social Stress Test and the Dexamethasone Suppression Test. Alternative ways to assess the functioning of the HPA-axis in basal and daily life conditions are by measuring cortisol levels in saliva, morning awakening responses, or by the Experience Sampling Method. In table 2 these different assessments are summarized.

Worldwide, we are the first to use scalp hairs to measure cortisol levels in patients with BD. This recently developed method is feasible to determine long-term cortisol levels and appears to yield a reliable estimate of long-term HPA-axis activity (80-82), with promising potential to inform about the consequences of increased long-term cortisol levels (83). A positive correlation has been found between cortisol levels in hair and waist circumference and Waist Hip Ratio (73), and higher cortisol levels have been found in patients with high endogenous cortisol levels (e.g. Cushings' syndrome). Since hair grows with an average rate of 1 cm per month, a hair segment of e.g. 3 cm would reflect mean cortisol levels over a period of approximately 3 months. This long-term cortisol measurement is therefore not influenced by the time of sample collection or acute stress due to daily circumstances or the research setting. Decreased cortisol levels are found in hair of patients with generalized anxiety disorder (GAD), but no differences in salivary cortisol levels between GAD patients and healthy controls (84). This suggests that hair cortisol levels may reflect the chronic cortisol secretion, whereas the results found with saliva or serum cortisol levels might also include acute responses to the measurement circumstances. Several other studies have shown that hair cortisol is indeed a marker of long-term cortisol exposure (80, 85-88). Previously, hair cortisol was used to provide a tool for doping control regarding chronic glucocorticoid use by top athletes (89). Van Uum et al used it to provide a technique to assess the cortisol levels due to chronic stress (88), which was confirmed one year later to be a reliable retrospective marker for cortisol levels for at least 6 months up to several years (90). This method proves promising in providing a retrospective calendar of cortisol production (73). Very recently, a relation has been found between higher cortisol levels over 6 months and depression (91), whereas lower hair cortisol levels relate to generalized anxiety disorder (92).

In order to explore the possibilities and relevance of the new available long-term cortisol assessment in hairs in patients with BD, we decided to use this technique to assess cortisol levels in scalp hair in our cohort. Previous studies did not report on hair cortisol levels in BD patients yet, the results of the patients in our cohort are described in chapter 5. Furthermore, we collected saliva samples at two consecutive evenings at 22.00h. Several studies have shown that saliva samples taken in the late evening on different days show the lowest variation in cortisol levels compared to diurnal cortisol measurements and the cortisol awakening response (93-95). This suggests that late evening cortisol levels are not as much influenced by acute stressors and daily influences than e.g. the cortisol awakening response or other daytime cortisol measurements. Therefore, it is most likely that *if* there is a correlation between hair and saliva cortisol measurements, it may have been with evening salivary cortisol measurements.

Endophenotype 2: Cognitive functioning

The word cognition refers to mental functions or processes including attention, memory, language skills, planning and supervising behavior. It is a widely used term with different meanings depending of the field it is used in. Here, we use cognition to refer to mental processes in the prefrontal cortex (executive functions), and hippocampus (memory). Additionally, attention is necessary to select information for further processing. This might be located in parietal lobes, where sensory information is selected to be transformed into "noticed" information. To avoid pollution of the meaning of these 3 terms, the content is described in Box 1. Cognitive functions like attention, memory and executive functions are not stringent different, but show overlap: without focused information selection there is nothing to remember. Without working memory it is not possible to plan and coordinate information into behavioral selection.

Box 1: Definitions of cognitive functions used in this thesis

Attention:

Attention is the spotlight on non selective sensory information, enabling persons to select and subsequently process selected information efficiently. This function is useful in preventing sensory overload.

Working memory:

Short term memory is useful for "holding" information (visual, phonological) in the present with direct past and direct future, with limited capacity. The information held is coordinated by executive skills like retrieving memories, selection and initiation of behaviour, etc. "This interaction between flexible executive functions and passive processing routines is an essential characteristic of working memory" .

Executive functions:

These functions act as the brain's chairmen, coordinating information, silencing input, activating and synchronizing other brain regions. Prefrontal located; not one specific domain, but using for example attention, working memory and language. It is distinguished from "automatic" selection of behaviour; executive functions are needed when automatic behaviour appears to be insufficient. Five situations are identified (Ward, 2006, p.286): 1) planning, decision making; 2)

error correction and troubleshooting; 3) not well learned or novel sequences of actions; 4) dangerous or difficult actions; 5) need to overcome habitual actions or resisting temptation.

Based on:"The Student's Guide to cognitive neuroscience" – Jamie Ward; 2006; Psychology Press, Hove, East Sussex.

Until recently, due to Kraepelin's influence, BD was regarded as an episodic illness with the assumption that patients resumed complete recovery, cognitive function included, between episodes (96). Modern clinical neuropsychological batteries, have found that in BD there is a clear impact on cognitive function between episodes (21), with a clinically relevant relation between for example processing speed and a poorer outcome with respect to social and global functional (97), and verbal memory as a significant factor in determining functioning at work. This is in line with the finding that light depression and impairments in memory and executive functioning are predicting long-term (5 year) functional (negative) outcome of the disease (98). Poor outcome is highly associated with cognitive impairments, particularly executive dysfunction, persisting during euthymic phases, leading to impairment of psychosocial and occupational functioning (99). These studies underline that in addition to the clinical mood episodes (especially sub-threshold depression) also cognitive impairments should be taken into account to establish a functional prognosis of the clinical course of the disease.

Several domains of cognitive function are recently recommended to focus on attention, processing speed, executive functioning, working memory and visual and verbal learning memory (21, 100-102). These appear to influence daily life functioning significantly (98, 99). In 2010 the International Society of Bipolar Disorders (ISBD) reached consensus about the preference of assessment of these cognitive functions in BD (102). The ISBD Battery for Assessment of Neurocognition (ISBD-BANC) is proposed as still preliminary, still needing empirical validation towards a further delineation of a core set of cognitive tests. In 2005 however, we decided to use the Test for Attentional Performance(www.psytest.net). This instrument is developed to assess attention performance, working memory and executive functions in adults and children with brain damage, and later applied in psychiatric patient populations. The development of these tests was based primarily on the needs of low complex, easy understandable neuropsychological diagnostics, which could be applied easily to patient groups and investigators (103).

Until now, cognitive domains which are found to be disturbed in BD differ somehow in different mood phases of the disease. During euthymia, mainly deficits in sustained attention, divided attention, verbal and working memory, processing speed, and impairment of executive functioning appear to be most consistently observed according to a recent meta-analysis (100). However, these deficits are not limited to euthymic phases and seem to exacerbate by depressive and/or manic symptoms as well. Furthermore, depressive mood states are characterized by lack of concentration, deficits in working memory, and executive functioning (104). (Hypo)manic states are accompanied by lack of sustained and selective attention, and long-term working memory. However, with the aforementioned criteria of Gottesman and Gould (22) in mind, cognitive deficits are associated with BD, and seem at least partly state independent. Genetic factors are probably most important in explaining both cognitive impairments (105). The final criteria, namely the prevalence in unaffected family members, is met in some other studies (26), arguing that cognitive functioning could serve as an endophenotype in future research. However, this is still under debate, as cognitive functioning in unaffected family members is not convincingly found to be disturbed (106) and is still in need for future studies.

While cognitive abnormalities are recognized as an important feature of BD (107), the nature and extent of these are less clear in terms of their existence before onset of affective symptoms, their etiology, their relationship to underlying neuroanatomical abnormalities and how they are affected as illness progresses. Here we discuss the influence of medication on cognition, the hypothesis of cognitive damage due to HPA axis dysregulation and the scar hypothesis.

Cognition and medication

However, besides being a disease trait, cognitive performance is known to be influenced by other factors as well. Well known influences of better cognitive performance are younger age, higher level of education and euthymia. Furthermore, the influence of treatment and specifically medication on cognitive performance are other important factors. Effective medication became available in the early fifties of the twentieth century. Together with the development of more standardized diagnostic procedures, psychopharmacological treatment options were developed firstly to treat acute episodes and later to prevent new episodes. Lithium was first used in 1949 by John Cade for treatment of mania (108), and approved by the American Food and Drug Administration in 1970. Since then, drug treatment became more and more common in

the treatment of acute mood episodes as well as after recovery. Maintenance treatment preventing new episodes is a relative new phenomenon, influencing the long-term outcome of the disease. However, results have been contradictory; for example, in 2008 it was convincingly argued that (long-term) antidepressant use is increasing the risk on developing a rapid cycling pattern of BD on the long-term (109). In a naturalistic setting, patients with a history of psychotic episodes including 31 bipolar patients, were followed during 15 years and compared off and on medication use. On the long-term, it appeared that a subgroup of these patients did very well on symptom scores and global functioning (110). The influence of medication use on the long-term course of BD has to be further entangled, with respect to clinical observable mood episodes as well as cognitive functioning and physical health. It is already known that cognitive performance can be influenced by psychotropic medication. Although genetic factors are considered as most important influences on cognition in BD, the role of residual mood symptoms and of medication use is also topic of discussion (105). The impact of Lithium use on cognition concerns mainly a slight slowing of processing speed and subjective impairment of cognitive functioning; no other strong significant influences on cognitive performance have been found (111-113). Antipsychotic use seems associated with level of memory and executive functioning in a group of 40 BD patients compared with 40 healthy controls (112). Despite all the research, little attention has been given to the consequences for cognition of using polypharmacy, which is common practice in the treatment of BD.

To be able to investigate cognitive performance as endophenotype in relation with clinical course and with cortisol exposure, the role of medication cannot be neglected. Therefore, in chapter 6 the association between medication and cognition is investigated.

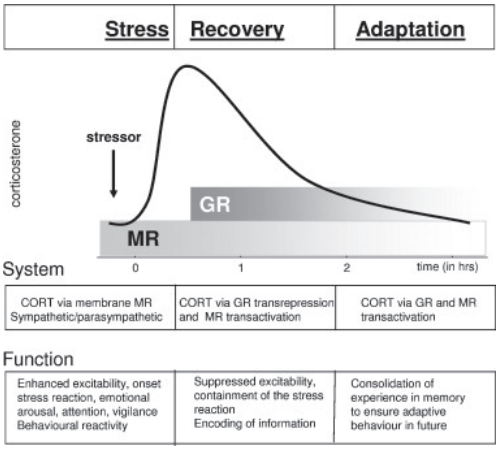
Cognition and impact of cortisol exposure: relation between two endophenotypes?

Recent studies suggest that abnormalities in the HPA-axis of BD patients may cause or exacerbate the cognitive impairments (114, 115). The negative influence of cortisol on neuroplasticity , which is disrupted in mood disorders, may well play a role in the development of cognitive deficits (116). In general, acute stress or the so called ‘fight or flight’ response improves cognitive functions such as attention and memory for the time the stressor is present. However chronic or excessive activation of the stress-system has disruptive effects on cognitive functioning (75). Patients with Cushings’ syndrome with excess of ACTH as well as cortisol production, are known to suffer from cognitive deficits. Furthermore, cortisol excess causes hippocampal atrophy in these patients (117, 118), which is reversible after successful treatment (119). The cognitive deficits found in BD

patients might therefore be related to the dysregulation of the HPA-axis. Recently, a relationship between higher cortisol levels and hippocampal related cognitive functions such as verbal memory, and executive functioning in unipolar depressed patients (120). The proposed relationship between cognition and HPA-axis functioning as described above, is giving ground to the choice of assessing both HPA-axis functioning as well as cognitive functioning in this study. However, in this thesis, the relationship will not be further described.

Having responsibility for the onset and termination of the stress response, the GR and MR also seem to be important in cognitive functioning. The MR as well as the GR are involved in learning and working memory (121-123). In figure 3, different functions of MR and GR are shown.

Figure 3: MR and GR- level of activity and cognitive function.



Based on (with permission): Brain development (in animals) under stress: Hypotheses of glucocorticoid actions revisited (30).

Van Rossum and colleagues reported an association between a GR polymorphism (ER22/23EK) and reduced risk for dementia in the general population (124) and less divided attention disturbances in depressed patients (65) indicating that this GR polymorphism may influence neuro-cognitive processes. It is hypothesized that one explanation could be a decreased direct effect of cortisol on brain tissue is mediated by a relatively insensitive GR (124). The MR is known to be involved in appraisal of critical situations and selection of behavioral adaptation (30). At a genetic level, MR haplotype 2 (the -2G/C SNP) is known to be associated with reduced depression, reduced hopelessness and more optimism (66b).

There are a number of indications that MR correlates to cognition; in patients with Addison's disease, a role of MR in encoding learning material has been found (125). The authors show the interaction between GR and MR, with optimal performance on working memory tests, when both activated. Further evidence for a role of the MR in cognitive performance has been found in blocking the MR with spironolactone in healthy individuals, which leads to impairment of attention, visuospatial memory and mental flexibility (126). Still, the connection of the MR with cognition has been mainly studied in depression: the MR polymorphism rs5522 (I180V) moderates reward processing and stress-induced reward learning deficits (127). However, the complex interaction of GR and MR in cognitive performance in health and disease needs to be further clarified. One hypothesis is, due to a shift in the MR:GR balance by chronic stress, MR-mediated behaviors are altered and consequently, GR mediates the consolidation of these altered behaviors (30).

The HPA-axis functioning and cognitive functioning: the scar hypothesis

A new approach to explaining brain damage in mood disorders is the scar theory (128), describing long lasting changes in the function of the brain (cognition, biological) following depression, increasing the risk for developing future depressive episodes. The pre-morbid regulated "set-point" of the HPA-axis is thought to be changed by epigenetic changes in DNA methylation of the GR caused by for example childhood abuse in suicide victims (129) or motherly depression during pregnancy (130). This can make an individual in advance more prone to be sensitized by stress. However, it is still questioned to what extend causes of depression lead to scarring, including sensitivity for stress (as measured by the ESM, TSST, cortisol assessments), and a changed set-point of the HPA-axis due to DNA methylation of the GR; on the other hand it is thought that depressive episodes themselves lead to further scarring of the functioning of the brain with respect to cognition and HPA-axis sensitivity. This scarring process is possibly a structural phenomenon developed during life, starting at early childhood and never ending, probably influenced by protective, as well as harmful circumstances.

The Bipolar Stress Study

In 2006 a study named The Bipolar Stress Study, was started at the outpatient department for Bipolar Disorders of PsyQ The Hague. The general topic of the Bipolar Stress Study is to identify risk factors, that have impact on the clinical course of BD and treatment of these patients. In the study approach three levels and their interactions with the environment (stressful life events and social support) were distinguished: 1.

clinical functioning (phenotype) , 2. genetic variations and vulnerability (genotype) and 3. cortisol exposure and cognitive functioning (endophenotypes).

In this dissertation, a part of the results is presented with the emphasis on the role of genotypes of the cortisol receptors and two endophenotypes, namely cortisol exposure and cognitive functioning. The results of the Bipolar Stress Study may help to develop clinically relevant interventions to improve the course of BD and subsequently the quality of life of patients with BD.

In general, there is a trend in psychiatric research towards early detection of disease and early identification of people at risk, also in research in the field of BD (131). It is expected that diagnostic processes will be more and more supported by genetic, biochemical, and neuropsychological assessments, to enable focused treatment strategies. As Hyman already pointed out that "defining a rational nosology for disorders of the brain, the body's most complex organ, is clearly one of the great challenges for modern medical science. Nonetheless, fundamental advances in our understanding of the genetic and environmental determinants of disease risk, and of the neural circuitry supporting normal and pathological mental processes promises to form the basis of improved classification in the coming decades" (132).

The Bipolar Stress Study is a project that consists of a cross sectional study followed by a 24 month longitudinal study, in part of the original patient group. The data analysis of the longitudinal study are ongoing.

For the cross sectional study the data of 366 patients were collected (genotype , phenotype and endophenotype). In the 24 month longitudinal study 189 patients participated.

The first patients were enrolled in 2006, data collection stopped at the end of 2011.

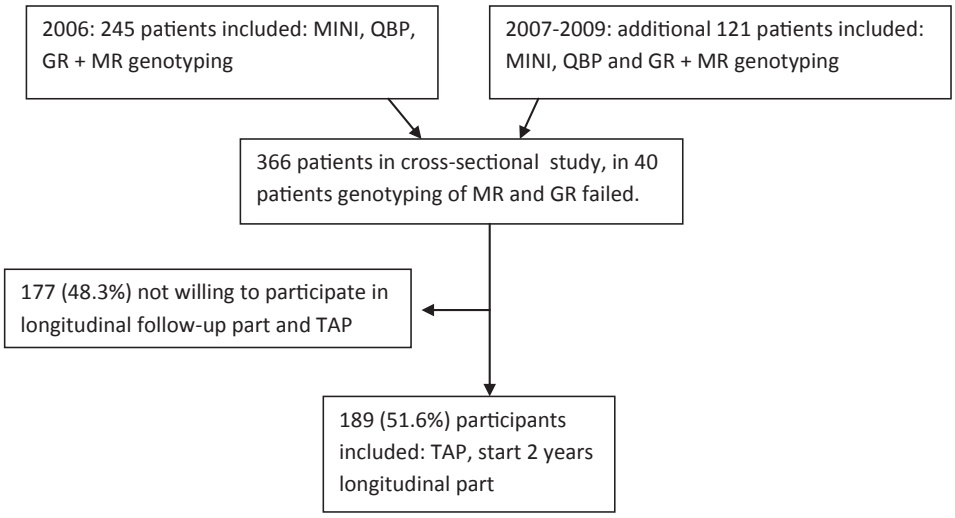
In figure 4, a flow chart is shown of our cohort.

Although in this thesis the cross sectional but not the longitudinal collected data are presented, a description of both the cross-sectional and longitudinal part is provided to clarify the study design and to be able to discuss the results within a consistent framework.

I. Cross-sectional part

First, in 2006, the first 245 patients were enrolled. They started with the assessment of the MINI, the QBP and GR and MR gene genotyping. In 2006 we extended the protocol, and recruited an additional 121 patients for baseline measurements. Furthermore

Figure 4: Flow chart of patient numbers and dropout rates in the Bipolar Stress Study.



we asked the first 245 patients to participate again in the extended study. In total we included 366 patients for the cross-sectional baseline measurements.

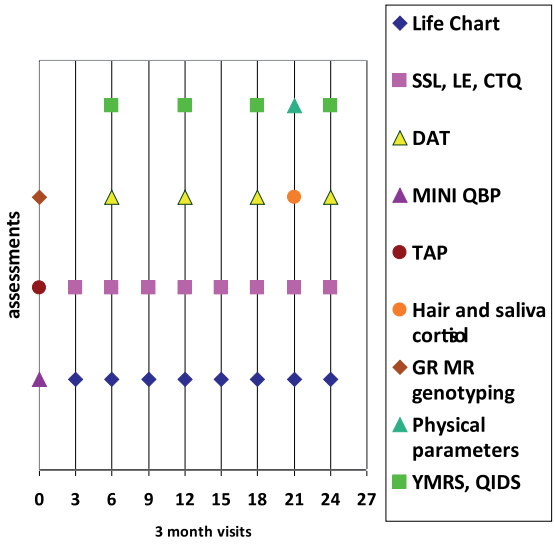
In 2007-2009, of these 366 patients, 189 participants were enrolled in the longitudinal study which also included the Test for Attentional Performance (TAP), which was not available for the complete 366 patients in the cross-sectional part. Please take note of the fact that the first visit includes the TAP and the MINI + QBP for most patients; however, the cross-sectional measurements with the patients of the cohort included in 2006 were separated in time with the MINI QBP and genotyping measured by entrance of the study, the TAP was assessed by continuing in the extended study the next year.

II. Longitudinal part

In the follow-up period of 24 months, all patients filled in a monthly Life Chart, and every three months the Social Support List and the Life Event List. Every 6 months they did a subtest of the TAP, namely the Divided Attention Test, together with measurement of current mood (the QIDS and the YMRS). Data on cortisol levels and physical health were collected in a subgroup of 100 patients in hair and saliva during the 7th visit at 21 months.

In figure 5, a timeline is provided with the various assessments during each visit in the longitudinal part of the study.

Figure 5: Timeline of longitudinal measurements



Abbreviations: MINI: Mini International Neuropsychiatric Interview; QBP-NL: Questionnaire for Bipolar Illnes- Dutch version; TAP: Test for Attentional Performance; YMRS: Young Mania Rating Scale; QIDS: Quick Inventory for Depressive Symptoms; LCM: Life Chart Method; SSL: Social Support List; LE: Life Events list; DAT: Divided Attention Test.

In this thesis, the following results will be described:

- The cross-sectional data of the first visit (t0) with emphasis on the genotype data and the role of GR and MR polymorphisms on the clinical course of BD;
- The cortisol levels in hair and saliva (21 month visit) in relation with clinical course of BD;
- The results of the divided attention test after 6, 12, 18 and 24 months, in relation to the QIDS and YMRS scores, as first analysis of the longitudinal data set.

In chapter 1 and 2 an overview of current studies is provided in two reviews, thereby giving the further theoretical underpinning of our research project. Chapter 1 focuses on the regulation of the HPA-axis in mood disorders, and especially on how to assess the HPA-axis. Furthermore, in this chapter findings of deregulation of the HPA-axis and mood will be discussed. Chapter 2 will discuss findings regarding genetic polymorphisms in the GR and MR gene in relation to mood and cognition.

In Chapter 3 and 4 we investigated the GR gene polymorphisms (chapter 3 and 4) and MR gene polymorphisms (chapter 4) in relation to cross-sectional retrospective collected data regarding previous illness course and clinical characteristics. These articles intend to identify the risk for the course of BD of these genetic variations influencing stress sensitivity.

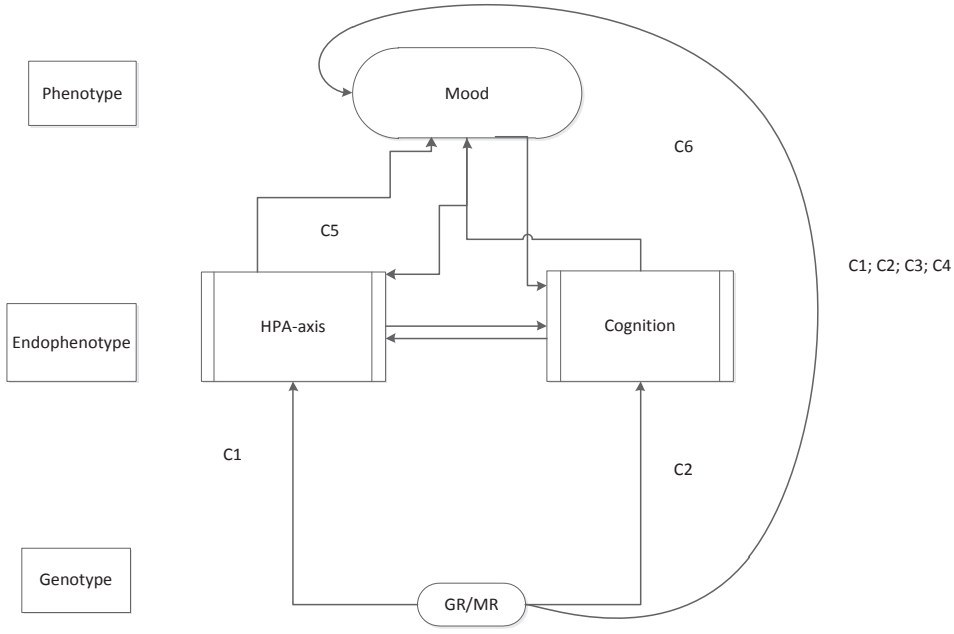
In Chapter 5 hair and saliva cortisol levels are described and related to clinical correlates of BD.

In Chapter 6 the cognitive functioning in relation to is described, in relation to clinical characteristics and medication use in BD patients is described.

Although the results presented in this dissertation are part of the larger ongoing Bipolar Stress Study, this thesis must be seen as a step forward in elucidating the role of cortisol exposure in relation to illness course and cognition. This may lead to new directions in future research and subsequent treatment. This will be discussed in more detail in the General Discussion.

In figure 6 an overview is provided from the content of this thesis.

Figure 6: Chapter contents (C1-C6) schematically shown



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2. Glucocorticoid sensitivity in Mood Disorders - Review

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Abstract:

In this review we provide an overview of recent literature on glucocorticoid (GC) sensitivity in mood disorders. Assessing GC sensitivity is often performed by measuring the Cortisol Awakening Rise (CAR), by challenging the Hypothalamic- Pituitary- Adrenal (HPA) -axis using a Dexamethasone Suppression Test (DST) or a Dexamethasone/ Cortisol Releasing Hormone test (DEX/CRH); more recently by measuring cortisol as a retrospective calendar in scalp hair. The main findings in mood disorders are higher mean cortisol levels in hair samples and in the CAR, showing a hyperactivity of the HPA-axis. This is in line with the mild resistance for GCs previously observed in challenge tests during mood episodes. GC sensitivity is partly determined by polymorphisms in the genes encoding receptors and other proteins involved in the regulation of the HPA-axis. We shortly discuss the Glucocorticoid Receptor, as well as the Mineralocorticoid Receptor, the CRH-Receptor 1, and the GR co-chaperone FKBP5. Data clearly indicate genetic changes, along with epigenetic changes which influence the set point and regulation of the HPA-axis. Early trauma, as well as influences in utero, appears to be important. Future research is necessary to further clarify the biological background and consequences of an individual's cortisol exposure in relation to mood.

Content

1. Introduction

During daily life, mood is known to be largely influenced by circadian rhythms and stress. An individual's risk to develop a mood disorder can depend on one or a combination of factors including vulnerability, defined by genetics, early life stress, and consequences of life events. The biological stress response has an important function in coping with life events, and differs between individuals with a genetically and epigenetically determined set-point during youth. The key systems in the stress response are regulated by fast adrenergic neurotransmitters (the sympathetic nervous system) and slower glucocorticoid hormones (the Hypothalamic-Pituitary-Adrenal-axis, the HPA-axis), of which cortisol is the main hormone in humans. It is complex to properly measure and assess the functioning HPA-axis in humans. However, it is important to evaluate the role of the stress response in the flexibility of the individual to cope with physical and mental changes in life in order to identify risk factors defining health and disease. In addition, an individual's level of chronic cortisol exposure in brain areas related to affection and cognition, may be very important in mood disorders. Aside from the stress system reactivity, it is also important to mention the consequences of absolute high and low cortisol levels. The consequences of absolute high cortisol levels is best observed in the clinically well known Cushing's syndrome, where cortisol levels are usually extremely high due to exogenous or endogenous causes. These symptoms can vary between physical symptoms, for example weight gain, increase abdominal fat, hyperhidrosis and hirsutism, and psychiatric symptoms, for example manic and depressive episodes, psychosis and anxiety. Also hypocortisolism as seen in Morbus Addison can have serious consequences on mental health, making patients vulnerable to mood disorders and anxiety.

Cortisol is known to exert its effect through two receptors: the Glucocorticoid Receptor (GR) and the Mineralocorticoid Receptor (MR). Both receptors are also present in the brain. The GR is found throughout the brain, with a very high density in the hippocampus, the PreFrontal Cortex (PFC) , the paraventricular nucleus of the hypothalamus, the amygdala and the dentate gyrus. The MR is predominantly found in the hippocampus, the PFC and the amygdala (1). The GR is known to be an important regulator during the acute stress response, while both GR and MR are active under basal conditions. Both corticosteroid receptors are co-expressed in the limbic system and it seems obvious that both receptor systems have a balanced function in regulating the stress response (2). FKBP5, a co-chaperone of the GR, also influences GR activity (3). Finally, the Cortisol Releasing Hormone Receptor type 1 (CRHR1) is important in initiating the stress response.

2. Assessment of Glucocorticoid Sensitivity in relation to mood

Functional evaluation of the HPA-axis is complex. The HPA-axis is characterized by daily rhythms, seasonal rhythms, and pulsation leading to varying cortisol levels in blood and saliva. The two most common approaches to evaluate HPA-axis functioning are the measurement of basal cortisol levels in response to awakening, as a model for an endogenous stress response; and the measurement of the HPA-axis functioning during challenging conditions, which gives an impression about the reactivity of the stress response itself. This measurement is characterized by negative feedback at the pituitary level on the production of Adrenocorticotropic hormone (ACTH), and hence by diminished stimulation of the cortisol production in the adrenal glands.

a. The Cortisol Awakening Rise

It is best to collect samples immediately upon waking to evaluate the Cortisol Awakening Rise (CAR), which reflects the natural response to awakening with an increase in cortisol levels of 50-75% within half an hour after awakening (4). Several influences on the CAR are defined by Vreeburg et al., including sleep patterns (duration, awakening time), season of sampling, activities (working day, physical activity) and health indicators (smoking, cardiovascular disease, physical activity) (5). Also psychosocial stressors and job stress have been found to result in higher CARs, whereas exhaustion and burnout resulted in lower CARs (6).

In patients with mood disorders, a higher CAR was observed in both remitted (n=579) and currently depressed (n=701) patients (7). In acutely depressed outpatients cortisol levels were 25% higher compared with healthy controls (8). In addition to this finding, a study among 230 late adolescents revealed that a higher CAR was predictive for developing a Major Depressive Disorder (MDD) within a year after sampling (9). In addition, subjects without a history of depression but with parents diagnosed with MDD had higher CARs, which were equal to the subjects with a current depression (10). It seems that these findings reflect higher basal cortisol levels as a trait phenomenon irrespective of current status.

b. Challenging the HPA-axis

The Dexamethasone Suppression Test (DST) is a neuroendocrine test measuring GR-mediated negative feedback. This test consists of a low dose administration (1 mg or 0.25 mg) of dexamethasone, a synthetical glucocorticoid hormone, at 11 pm and the measurement of cortisol levels the following morning. Due to the negative feedback

action at the pituitary and hypothalamic levels subsequent cortisol levels are suppressed the next day. Non-suppression of cortisol levels after dexamethasone indicates GR resistance. The strength of cortisol suppression reflects the negative feedback mechanism, which is largely variable between but rather stable within individuals (11). The test is very easy, however an important restriction to consider for use in psychiatry is the limited sensitivity in studying MDD (12). In particular in outpatients the sensitivity is low. It was reported that only 12 % of outpatients with non melancholic depression showed non suppression in the DST, while 64% patients with psychotic depression showed non suppression (13). Low percentages of non suppression after 1-2 mg dexamethasone in moderately depressed patients (44%) were found, in contrast with severely depressed psychotic patients and bipolar patients (67-78%) (14). Heuser et al developed the combined DEX/Cortisol Releasing Hormone- test (DEX/CRH- test) as a refinement of the DST (15). This challenge test consists of administration of 1.5 mg dexamethasone at 11:00 pm followed by administration of 100 microgram CRH at 3:00 pm on the next day. Cortisol and ACTH levels are sampled every 15 minutes from 2:00 pm until 6:00 pm. This test was found to be more sensitive for MDD (about 80%), and is even above 90% when the cohort was stratified for age.

However, the DEX/CRH-test is more intrusive for patients, which limits the use in research among large cohorts of outpatients. Zobel et al found that in a cohort of 74 remitted patients, the DEX/CRH test was able to predict relapse of depression within six months (16). In patients with a relapse within 6 months after discharge, there was a 4- to 6-fold increased cortisol response in the DEX/CRH-test just before discharge. In accordance to, Appelhof et al found in a sample of 45 outpatients with remitted major depression that higher cortisol levels in the DEX/CRH test were associated with relapse (17), which was confirmed by Ising et al (18). Rybakowski et al. also stressed that number of episodes was associated with more non-suppression. Bipolar patients in the same study were found to have most non-suppression when compared with healthy controls and unipolar depressed patients in remission. Watson *et al* confirmed that in bipolar patients, cortisol levels in response to the DEX/CRH test are increased, with no difference between a current depressive episode and remission (19).

The issue whether this is a state or trait phenomenon is not yet solved. The studies including healthy family members suggest that hyperactivity of the HPA-axis is not only a reflection of current mood state (20). A new approach to this problem is the scar theory (21), describing long lasting changes in the function of the brain (cognition, biological) following depression, increasing the risk for developing future depressive episodes. The pre-morbid regulated “set-point” of the HPA-axis is thought to be changed through depression by for example epigenetic changes in DNA methylation in depressed suicide victims (22) and children of mothers who were depressed during pregnancy (23). This

scarring process is possibly a structural phenomenon developed during life. In section 4 we will briefly discuss the influence of early life trauma on epigenetic phenomena.

c. Cortisol in scalp hairs

A novel and non-invasive parameter is measuring cortisol in scalp hair. Hair grows with an average of 1 cm per month, and it has been shown that cortisol can be reliably measured in hair (24-26). The use of hair provides the opportunity to measure long term cortisol levels (reflecting mean levels of the past months) in an easy way without limitations caused by the pulsatility and circadian rhythm of cortisol or acute circumstances. Strong correlations of hair cortisol have been observed with tissue effects of cortisol in healthy individuals (e.g. waist circumference), as well as with cortisol exposure in patients with hyper-or hypocortisolism (26).This method has only preliminary been applied in psychiatry. Steudte et al found a decreased cortisol level in patients with generalized anxiety disorder (27). Our group recently found that cortisol levels were increased in patients with BD when having a co-morbid psychiatric diagnosis (28). Interestingly, hair cortisol levels were decreased when patients with BD were also diagnosed with panic disorder. Moreover, we found an association of higher hair cortisol levels with adult onset (older than 30 years) of BD, and impaired executive functioning, compared to patients with puberty onset, normal executive functioning and normal cortisol levels (29).

3. Genetics of HPA-axis: consequences for physical and mental health

a. GR polymorphisms: physical health

There are several known genetic variations in the GR Gene *NR3C1* with consequences for cortisol sensitivity (30). Subtle changes in cortisol signaling leading to relative resistance or hypersensitivity for Glucocorticoids (GCs) can have long term consequences. It is known that these changes can affect metabolic and inflammatory status and body composition. Cognitive performance and mental health can also be influenced by altered HPA-axis regulation.

Haplotype 4 (*TthIII*I + 9β) and haplotype 5 (*TthIII*I + 9β + ER22/23EK) are both associated with a relative resistance for GCs (31-34). The ER22/23EK polymorphism is associated with a healthy metabolic and inflammatory profile, characterized by lower total cholesterol and low-density lipoprotein cholesterol levels as well as lower fasting insulin concentrations, a better insulin sensitivity and lower C-reactive protein levels (32, 35).

This GR variant is also associated with a beneficial body composition, shown by young male ER22/23EK carriers (taller, stronger and more muscle mass than non-carriers) and female ER22/23EK carriers (tendencies for smaller waist and hip circumferences, lower body weight (33) and protective effect on weight gain during pregnancy (36). These associations of the ER22/23EK polymorphism are in line with a mild GR resistance. The clinical data are supported by in vitro experiments showing reduction of transactivating capacity in transfection experiments and in peripheral blood mononuclear lymphocytes of carriers of this polymorphism (37). In addition the underlying molecular mechanism of the GR gene variant has been revealed (38).

The 9β-polymorphism seems to increase the stability of mRNA of GR-β, an alternative splice variant of the GR-gene (31). GR- β is thought to exert a dominant negative effect on the active GR-α. The association of the 9β polymorphism and the immune system has been shown by the higher risk of developing rheumatoid arthritis in carriers (31, 39). Patients with Multiple Sclerosis (MS) have been found to have a more aggressive course of disease when they carry at least one allele of haplotype 5 (*TthIII*I + 9β + ER22/23EK), which is possibly related with an altered inflammatory state due to GC resistance (40). Interestingly, in 2008 van den Akker et al reported that the 9β polymorphism is related to a more active pro-inflammatory system, and subsequently associated with the risk of cardiovascular disease (41). In line with these findings the Heart and Soul Study showed that the 9β SNP is associated with reduced heart function, partly mediated by low-grade inflammation (42).

Haplotype 2 (*Bcl*I), haplotype 3 (*TthIII*I + *Bcl*I) and haplotype 6 (N363S) have all been associated with a relative hypersensitivity to GCs and clinical signs of hypersensitivity to cortisol in various tissues (43). Carriers of N363S have in addition to increased cortisol suppression also an increased insulin response in the DST, a tendency towards lower bone mineral density, and increased body mass index (BMI) (33). Although other studies have reported associations with increased BMI, as expected as a result of glucocorticoid hypersensitivity, these findings have not been consistently confirmed (30, 44). The *Bcl*I polymorphism has been found to be associated with abdominal obesity (33), lower bone mineral density (45) and unhealthy body composition in young boys (46).

b. GR polymorphisms: mental health

Recently we reviewed the GR and MR SNPs in relation to mood disorders (47). The most important findings will be summarized and supplemented by recent progress in this area of research. The ER22/23EK polymorphism has repeatedly been associated with a higher risk on developing a depressive episode (48-50), and a faster response

after antidepressant treatment (48). In the study of Bet et al. an association to this polymorphism and clinically relevant depressive symptoms in an elderly population was only found in combination with childhood adversity, indicating a gene-environment interaction. Recently, attention has been directed to the 9β SNP in relation to mood. In a sample of 245 bipolar patients, we found an association between the 9β polymorphism and reduced risk on (hypo) mania (51). In the aforementioned study of Bet et al. a relationship between this SNP and clinically relevant depressive symptoms, in combination with childhood adversity was found (50). Recently, in a sample of 173 patients with bipolar I depressive episodes, the response to lamotrigine (anti-epileptic medication used in treatment of bipolar depression) in a subgroup of 88 patients was associated with the GR polymorphisms rs258747 and rs6198 (9β) (52). Finally, in a group of 526 outpatients with coronary heart disease the prevalence of depression was increased with an allele-dosage effect (from 24.4% of the non-carriers to 52.9% of the homozygous 9β carriers) (53). The *Bcl*I polymorphism is also associated with an increased risk on developing a depressive episode (48, 54-56), as well as with a reduced response after antidepressant treatment (54), which was not confirmed by Lee et al (56). Remarkably, in the latter study, it was found that in the Korean population there were no carriers of ER22/23EK and N363S. This is consistent with other reports in Asian populations (57).

c. MR polymorphisms

In figure 1, 2 SNPs in the MR Gene and 3 haplotypes are shown. The V allele in the MRI180V SNP is associated with higher cortisol levels in saliva and plasma in healthy subjects performing the Trier Social Stress Test (a validated psychological procedure inducing acute stress under laboratory circumstances, allowing evaluation of biological measurements of differences in stress levels between individuals). In vitro testing, using transactivational assays, this I180V variant was shown to have a slight loss of function using cortisol as a ligand (58). This SNP was associated with higher frequency of depressive symptoms in an elderly cohort (participants aged > 85 years) (59) and with neuroticism in depressed patients (60). Another MR SNP, the -2G/C variant also affects the transactivational capacity of the MR in vitro in response to cortisol. Both SNPs modified cortisol suppression in a DST (0.25 mg DEX) in a sex specific manner (61).

d. FKBP5 and CRH-R1 polymorphisms

An important co-chaperone protein functionally interacting with the GR is FK506 binding protein 5, better known as FKBP5, a member of the immunophilin protein family. Genetic

variations in the FKBP5 gene lead to increased intracellular FKBP5 protein expression, which in turn leads to adaptation of the GR function. Healthy subjects carrying these SNPs show GR resistance and diminished negative feedback of the HPA-axis. Carriers of these variations have been found to be overrepresented in patients with mood disorders (MDD and BD) and post-traumatic stress disorder (62), as well as respond faster to antidepressant treatment (63). Another gene, which is a key factor in the HPA-axis is the CRH1- receptor (CRH-R1). This receptor has been considered as a mediator in initiating the stress response. The CRH-R1 is located in the paraventricular nucleus (PVN) of the hypothalamus, the hippocampus as well as widely distributed beyond the hypothalamus. It interacts with a wide range of neurotransmitters, for example, influences the activity of the $5\text{-}HT_{2A/C}$ receptor (64, 65). Several SNPs in the CRH-R1 gene have been recently identified and explored in relation with mood disorders. Liu et al found an over-representation of rs242939 in patients with major depression compared to healthy controls (66). They also found that rs242941 carriers with major depression and high levels of anxiety responded faster after treatment with fluoxetine in 127 Han Chinese patients (67). This was not confirmed by Dong et al in a population of 536 unrelated Mexican Americans from Los Angeles (68). In male suicide attempters a relation between illness severity and a haplotype of the CRHR1 has been found (69), as well as in their offspring with CRH-R1 haplotypes, who were found to score higher on the Beck Depression Inventory, which was found in the same study.

Interestingly, these SNPs in the CRHR1 gene are also studied in relation with the environment and with other genes. Experiencing childhood abuse leads to a higher risk for developing a lifetime depressive episode specifically in carriers of polymorphisms of the CRH-R1 *in combination with* the short serotonin transporter gene 5-HTTLPR (70). In parallel, Bradley et al found an interaction between SNPS in the CRHR1 gene and childhood abuse as predictor for depressive episodes (71). In one cohort of more than 1,000 female participants, Polanczyk et al found that carriers of a haplotype formed by rs7209436, rs110402, and rs242924 who were abused during childhood as measured by the Childhood Trauma Questionnaire (CTQ) are protected against depression in adulthood (72). This was not replicated in another cohort described in the same study, where childhood abuse was not measured by the CTQ.

A recent review by Binder and Nemeroff extensively summarized the relation between genetics of the CRH system in relation with psychopathology (64).

4. Epigenetics of the HPA-axis: adversity in childhood

The influence of genetic variations in the DNA sequences of HPA-axis related genes on mood disorders is clear, but may only be one brick in the building of our understanding of mood disorders. Of all other influences, it is important to mention epigenetic changes in regulating the "set-point" of the HPA-axis. Epigenetic changes comprise changes in gene expression which remain stable during cell divisions, but do not affect the DNA sequence itself. Epigenetic changes are heritable and could be caused by changes such as those found in DNA methylation, the modeling of chromatin and the de-acetylation of histones in the DNA.

Several circumstances can lead to epigenetic changes, for example intra-uterine influences and changes in early youth due to childhood adversity. A well-known example of intra-uterine effects on health in adulthood is the Dutch Famine Birth Cohort study. Children of the women who were pregnant during the famine in World-War II scored lower in mental health, and this effect was repeated in their children's children, suggesting an epigenetic effect (73). However, there was no relation found with changes in HPA-axis regulation (74). Other studies emphasize the importance of regulation of the HPA-axis in utero. Raised GC concentrations during pregnancy are associated with lower birth weight and later during childhood and adulthood with an increased cortisol response during HPA-axis activation (75). As a consequence these patients are at higher risk to develop obesity and/or diabetes. Yehuda et al report that women who were pregnant during the World Trade Centre Attack and developed Post Traumatic Stress Disorder had lower salivary cortisol levels, as did their 1-yr old offspring, suggesting they had already developed a risk factor for PTSD in later life (76). Animal studies show that prenatal stressed offspring developed hyper activation of the HPA-axis through epigenetic programming, and develop high anxiety levels and depression-like behavior (77, 78).

In early childhood the regulation of the HPA-axis is further developed. Early life trauma could have devastating consequences for the HPA-axis "set-point". In a recent review, the relation between early life trauma and the HPA-axis is characterized by hypocortisolism and an attenuated cortisol response during acute stress (79), which suggests a dysregulation of the negative feedback mechanism in the HPA-axis. This diminished stress response is continued throughout life and tends to worsen with ageing (80). Animal studies have shown that mouse pups that have been separated for several days from their mothers, have an increased GR expression in frontal cortical and hippocampal areas during the separation. After the separation they showed a diminished GR expression (81). In humans this was recently confirmed by McGowan et al, showing that suicide victims with a history of childhood abuse had decreased GR mRNA levels in the hippocampus, as well as increased cytosine methylation of the GR (82). Secure attachment is a central theme

in the work of Bowlby and Ainsworth. The "strange situation" is a classic test, comprising a short separation between mother and child. After reunion the response of the child towards the mother is categorized in attachment styles, reflecting the involvement or neglect/abuse of the mother for her child (83). Neglect by the mother is devastating for the development of a secure attachment style of the child, with definite changes (e.g. alterations of methylation pattern of the GR) for the rest of its life.

5. Conclusive remarks

In conclusion, while accumulating evidence indicates that alterations in the HPA-axis are important biological factors in mood disorders, the exact pathophysiological mechanisms are at present not completely understood. This is partly due to the difficulties in assessing the HPA-axis. One of the promising future techniques could be the assessment of the long term cortisol levels through analysis of scalp hairs. The "set-point" of the HPA-axis is influenced by genetic changes in the GR gene, MR gene, CRH-R1 gene and FKBP5 gene, as well as polymorphisms in other genes involved in cortisol signaling. These changes have been associated with mood disturbances. During life this set-point is further defined by epigenetic changes due to intrauterine influences and/or childhood adversity. Finally, during life this set-point could be influenced by mood episodes. As a result, hyperactivity of the HPA-axis may increase the vulnerability for future mood episodes.

Future research should focus on new tools in order to obtain a clear indication of an individual's cortisol status. This may provide better opportunities to understand possible causal relationships between cortisol exposure (in the brain) and mood disorders which may yield new treatment strategies.

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3. Glucocorticoid Receptor Polymorphisms in Major Depression: Focus on glucocorticoid sensitivity and neurocognitive functioning

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Abstract:

Previously, it has been suggested that hypothalamic pituitary adrenal (HPA) axis dysregulation and as a consequence increased cortisol levels, is not only a state phenomenon, but may also be a trait phenomenon in mood disorders. Cortisol exerts its effects mainly by binding to the glucocorticoid receptor (GR) and, in particular of interest in certain brain regions, the mineralocorticoid receptor (MR). Several GR polymorphisms have been shown to be associated with altered sensitivity of the HPA axis. Recently, the GR polymorphisms *Bcl*I and ER22/23EK have been associated with unipolar depression in several studies. In addition, the ER22/23EK polymorphism seems to be associated with a decreased risk of dementia in healthy individuals. Also during a depressive episode carriers of this ER22/23EK variant demonstrated a tendency towards better cognition, as measured by divided attention tests.

In this overview, currently known clinically relevant GR and MR polymorphisms are discussed in relation to mood disorders (both unipolar depression and bipolar disorder) and cognitive functioning.

1. Introduction: Glucocorticoids and Depression

Stressful life events often precede a major depressive episode. In the past decades a strong association between dysregulation of the hypothalamic-pituitary-adrenocortical (HPA) axis and depression has been found (1-6). This association is even stronger in patients with either severe unipolar depressive disorder with psychotic symptoms or with bipolar disorder (BD) (4, 5). In recent literature this association has been cautiously considered to be a trait phenomenon of depressive episodes, rather than simply a state phenomenon (2, 7, 8). This was supported by Holsboer et al. who demonstrated that first degree family members of patients with unipolar depression display a similar, although quantitatively more modest, pattern of dysregulation then that observed during a major depressive episode (9). Also Ellenbogen et al found higher cortisol levels in the morning and afternoon in offspring of patients with BD compared to offspring of patients with MDD and healthy controls (10). In the past years, data concerning the associations between mood disorders and HPA-axis have been reviewed extensively (11, 12). The most consistent finding is hyperactivity of the HPA-axis. The combined dexamethasone-suppression/ corticotropin-releasing hormone-stimulation (CRH) test (DEX/CRH test) has been reported to be the most sensitive test for detecting HPA-axis dysregulation, with approximately 80% of patients with a major depressive episode (uni- or bipolar) found to be non-suppressors (13). The test is not very specific for depression, as in all psychiatric patients cortisol levels were elevated in comparison with age-matched controls. Previous studies showed that the DEX/CRH test may also be useful as a predictor of relapse (14, 15).

In the brain, the dysregulation of the HPA-axis in major depressive disorder (MDD) and BD is associated with neuro-endocrine and anatomical changes. The neuro-endocrine changes mainly occur in catecholaminergic systems, like dopamine and adrenalin, influencing heart rate and blood pressure (16), and serotonergic systems influencing behavior, mood and cognitive functioning (17). Animal studies have shown that glucocorticoids (GCs) enhance the effects of dopamine in the reward system of the brain, the mesolimbic region, thus influencing goal directed behavior in stressful situations (18). Sustained overdrive of GCs is negatively associated with activity of serotonergic neurons, reduced expression of 5-HT-1A receptors and reduced 5-HT-1A receptor binding, thus believed to influence mood (7).

Another regulatory hormone in the brain which can be affected in depression through diminished negative feedback action of GCs, is CRH. Overproduction of CRH (1) leads to a series of stress-like physiological and behavioral phenomena (7). The behavioral features indicate increased anxiety, while some phenomena are also observed in animal models of depression (7, 19). In the review of Van Praag, a role is suggested for CRH in the

pathophysiology of states of anxiety and depression, conditions that in humans often occur simultaneously. However, this overdrive is not sufficiently understood yet. It could be the result of a primacy of CRH overdrive, or a consequence of a down regulation of centrally localized glucocorticoid receptors (GRs).

Anatomical changes in the brain in patients with depression are found mainly in the prefrontal cortex (PFC), the amygdala and the hippocampal area consisting of smaller volumes and reduced metabolic activity (20, 21). These brain areas regulate executive function and memory. Sassi et al (22) found a significantly smaller pituitary volume in patients with BD compared with patients with unipolar depressive disorder and healthy controls. No differences between the latter two groups were found. Recently, Aihara et al (23) studied 24 patients with MDD and found 75% non-suppressors on the DEX/CRH test. In positron emission tomography (PET) scans with a radiotracer, these patients also showed a hypo metabolism in various frontal regions and hyper metabolism in the right hippocampus and parahippocampal gyrus, both normalizing after successful antidepressant treatment.

In depressed patients almost all abnormalities returned to a normal pattern after response to medication, indicating the influence of altered GC regulation in these brain regions. These findings are in line with results from animal studies showing the negative effects of stress on neuroplasticity, mainly in the hippocampal area (where the effects are largely reversible after stress has ended), the PFC and the amygdale (24, 25). MacMaster et al found in a pediatric population that children with a family history of MDD had significantly smaller hippocampal volumes compared to healthy controls (26), indicating that this could be a risk factor for developing MDD. In addition, patients with Cushing's disease, characterized by hypercortisolism, can also develop psychiatric syndromes like MDD, anxiety disorders and cognitive dysfunction, as well as a reduction of hippocampal volume (27). It has been shown that the decrease of hippocampal volume in these patients can be reversed after successful treatment of the Cushing's disease and the co morbid psychiatric symptoms (28). It is still under discussion whether these structural changes in depression are reversible and what their exact relationship is with elevated cortisol levels (26, 29).

In summary, these findings are indicating that in the pathophysiology of MDD, GCs play an important role, affecting mood, executive function and memory through different neuronal networks in the brain as well as affecting several neuroendocrine systems.

2. Glucocorticoid sensitivity during a depressive episode

The functioning of the HPA axis is characterized by negative feedback at the pituitary level on the production of ACTH, and consequently on the production of cortisol in the adrenal glands. A strong indication of this feedback functioning is obtained through an HPA axis challenge test. The dexamethasone suppression test (DST) was for years the most frequently used challenge test. Due to its simple procedure, it is widely used in psychiatry by researchers and clinicians. Cortisol levels are measured after administration of 0.25 mg or 1-2 mg of the exogenous GC dexamethasone, providing an indication of adrenal negative feedback at the pituitary level. Sensitivity to exogenous GCs is highly variable between normal individuals (30), but within individuals it is rather stable, suggesting a set point of the HPA axis with respect to feedback action, which is possibly genetically determined. One of the main problems with the DST is its limited sensitivity in major depression (about 44%). In bipolar depression and psychotic depression the sensitivity is higher (67%-78%)(31). Also in manic states, non-suppression was found after a DST (32), as well as after a DEX/CRH test (4).

Heuser et al developed the combined DEX/CRH test as a refinement of the DST (13). This challenge test consists of administration of 1.5 mg dexamethasone at 11:00 pm followed by administration of 100 microgram CRH at 3:00 pm on the next day. Cortisol and ACTH levels are sampled every 15 minutes from 2:00 pm until 6:00 pm. This test is more sensitive for major depression (about 80%), and even more sensitive when clustering patients according to age groups. Zobel et al showed that the DEX/CRH test also may be used to predict relapse of depression within six months (15). Despite the observation that cortisol and ACTH responses to the DEX/CRH test did not differ between the patients with major depression on admission, the responses were found to be significantly different just before discharge. The relapse rate in this study was found to be 4 to 6-fold increased in patients with an elevated cortisol response in the DEX/CRH test just before discharge as compared to patients with a normal cortisol response. In accordance, Appelhof et al recently showed that in a sample of 45 outpatients with major depression in remission, higher cortisol levels in the DEX/CRH test were associated with relapse (14). In a group of patients with BD the cortisol response to a DEX/CRH test was significantly higher than in a group of unipolar MDD patients (5). This difference was not only found during a depressive episode, but persisted after remission. Watson et al confirmed that in BD patients cortisol levels in response to the DEX/CRH test are increased, both during a depressive episode and in remission (33).

3. The role of Glucocorticoid Receptors and Mineralocorticoid Receptors in Depression

GCs exert their effects by binding to the glucocorticoid receptor (GR) and mineralocorticoid receptor (MR). In the brain the MR is predominantly localized in the hippocampus, the amygdala and the prefrontal cortex. The GR is widespread throughout the brain, but is expressed in high density in the hippocampus, the prefrontal cortex and the paraventricular nucleus of the hypothalamus, the dentate gyrus and amygdale (34). The affinity of the MR for cortisol is ten times higher than that for GR (35). Under normal non-stress circumstances, cortisol binds to the MR in the mentioned brain areas, whereas in stress situations it also binds to the GR. In the limbic system both MR and GR are co-expressed, thereby fine tuning the central effects of GCs (36, 37). The MR seems to be involved in the onset of the stress response, while the GR terminates the stress reactions and promotes memory storage preparing for future events (38). In situations of chronic stress, this balancing system is disturbed, with damaging consequences for brain functions like mood and cognition. Matsubara et al found a reduced expression of GR mRNA in peripheral blood cells in a population of bipolar as well as unipolar depressed patients. This finding seems to be, at least partially, a trait phenomenon, as they found the same results in remitted patients (bipolar and unipolar) and in first degree family members of bipolar patients (39). Pariante stresses the importance of the dysfunction of the GR and the decreased expression of this receptor in the brain in patients with MDD [see chapter in this volume]. As a compensation for this GR dysfunction, cortisol levels increase to overcome the relative resistance (12, 40). Holsboer summarizes in his review the preclinical and clinical data supporting the hypothesis that GR signaling is impaired in MDD, yielding increased CRH levels in various brain regions, which in turn may lead to MDD (11) [see chapter in this volume].

In line with this model are the effects of GR antagonists and agonists on the one hand and the effects of (other) antidepressants on the GR on the other hand. The GR antagonist RU-486, the well known abortifacient mifepristone, has antidepressant effects in patients with BD (41) or MDD with psychotic features (42, 43). In a study with chronically stressed rats, it has been shown that a 4-day treatment with mifepristone normalized the stress-induced reductions in neurogenesis (44), possibly indicating the mechanism of action through blocking the GR. Interestingly, not only GR antagonists, but also GR agonists like hydrocortisone have been shown to exert antidepressant effects (45).

GR function is also positively stimulated by antidepressants. Pepin et al found *in vitro* that addition of tricyclic antidepressants (desipramine, amitriptyline, imipramine and maprotiline) or lithium to primary cultures of rat hypothalamic, hippocampal or amygdaloid neurons, GR mRNA was stimulated (46, 47). However, it is interesting that in

rats the selective serotonin reuptake inhibitors (SSRIs) such as citalopram and fluoxetine had no effect on GR mRNA (48, 49), whereas MR mRNA was stimulated by citalopram, desipramine and amitriptyline (48). It is unclear to what extent these animal data can be extrapolated to the human brain.

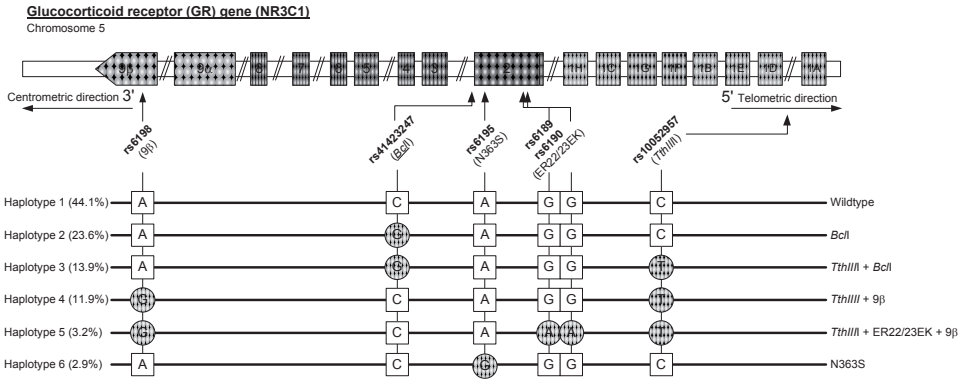
Thus, summarizing these data, although GR is more extensively studied than MR, it seems that both GR and MR are important in balancing GC levels in the brain. Imbalance in this system through dysfunction of GR and/or MR, could lead to a compensatory elevation of cortisol levels. Although still under discussion, the GR is thought to be an etiological factor in developing MDD. Studies with GR antagonists, GR agonists, and tricylic antidepressants seem to confirm this hypothesis (50). The clues suggestive of a genetic trait contributing to the development of MDD and the alterations in GR expression, as well as the dysregulation of the HPA axis during a depressive episode make the *GR* gene an important candidate gene for associations with susceptibility to MDD and response to antidepressant treatment. This article provides an overview of polymorphisms of corticosteroid receptors (both GR and MR), which in previous studies have been shown to be functionally relevant, and their associations with unipolar and bipolar depression, as well as with cognitive functioning during a depressive episode.

4. The relationship between GR polymorphisms and GC sensitivity

Previously, a few rare mutations of the *GR* gene in humans have been described, which lead to a generalized cortisol resistance. Patients present with hypertension and hypokalemic alkalosis (signs of adrenal overproduction of mineralocorticoids) and, in females, hirsutism, male pattern baldness and menstrual irregularities, as a consequence of overproduction of adrenal androgens. As a result of defects in the GR, the central negative feedback of GCs is reduced, GC production by the adrenal gland is increased, and cortisol binds with high affinity to the MR. The consequence is a hyperactivity of the HPA axis (51). These mutations, known to lead to the classical syndrome of GC resistance, are predominantly located in the ligand-binding and DNA-binding domains of the *GR* gene. Importantly, however, also in normal individuals, GC sensitivity, as measured with a low dose DST, varies considerably. Polymorphisms of the *GR* gene have shown to, at least partially, explain this variability in GC sensitivity (52).

Several important *GR* gene polymorphisms have been reported to yield altered GC sensitivity, and as a consequence, result in specific clinical features (see figure 1) (52):

Figure 1: Overview of the GR gene showing the single nucleotide polymorphisms (SNPs) and haplotypes



- Haplotype 2 (*TthIII*I + 9β). The 9β polymorphism is a single nucleotide polymorphism (SNP) in exon 9β of the *GR* gene. An ATTTA sequence in the 3' UTR is changed into GTTTA. This polymorphism is associated with an increased stability of the mRNA of the inactive GR-β isoform and may lead to a relative glucocorticoid resistance by increased inhibition of the active GR-α isoform (53). An *in vitro* study demonstrated that this polymorphism results in reduced transrepressional effects (by which, for example, genes important in the immune system are regulated), whereas the transactivational effects appeared to be normal (54). The 9β polymorphism has also been reported to be associated with rheumatoid arthritis (53), and a 68% reduced risk of carriage of nasal *Staphylococcus aureus* in carriers of this polymorphism (55), consistent with this variant's possible effect on activity of the immune system. It is known that IL-6 and IL-1β have activating effects on the HPA-axis. Interestingly, non-lithium treated bipolar patients have a low IL-1β and a high IL-6 production, which returns to normal after lithium treatment, suggesting an association of dysregulation in the immune system and the HPA-axis in these BD patients (56), *TthIII*I is a restriction fragment length polymorphism (RFLP) in the promoter region of the *GR* gene, which has not been found to be associated with changes in cortisol sensitivity. However, a previous report showed an association with elevated basal cortisol levels (57).

- Haplotype 3 (*TthIII*I + 9β + ER22/23EK) is also associated with a relative resistance to GCs, but in contrast to haplotype 2, this resistance is linked to the transactivational effects of the GR, by which most of the adverse effects of GCs are being mediated. The ER22/23EK polymorphism is located in the transactivation domain of the GR gene, and involves two nucleotide changes in codons 22 and 23 (GAG AGG -> GAA

AAG), which have been shown to be linked. This variant has been found to be associated with a healthy metabolic profile (lower C-reactive protein (CRP) levels, lower cholesterol levels and increased insulin sensitivity), longevity in the elderly and a beneficial body composition in young adults (52, 58). After a DST, carriers of this polymorphism showed a significantly smaller cortisol suppression, indicative of a relative GC resistance (58, 59). In addition, these ER22/23EK carriers displayed lower fasting insulin levels and lower total and LDL cholesterol levels than noncarriers (58, 59). In a recent study, this polymorphism has also been associated with an increased risk of recurrent depression, and a faster response to antidepressant medication, as we will discuss later in more detail (60,83). A relationship with cognitive performance has also been demonstrated. Carriers of the ER22/23EK polymorphism in a healthy elderly population were found to have a lower risk of developing dementia, and performed better on psychomotor speed tasks (61). No differences were observed in memory tasks. The ER22/23EK polymorphism was also studied in patients with multiple sclerosis and seemed to be associated with a more aggressive course of disease (as was observed clinically, as well as radiographically) (62). This might be due to an imbalance of the immune system and uncontrolled immune response against structural components of the central nervous system, since GCs are important factors in the pro-inflammatory signal transduction system.

- Haplotype 4 (*TthIII*I+ *Bcl*I) and haplotype 6 (*Bcl*I), both involving a C to G single nucleotide polymorphism at a polymorphic *Bcl*I restriction site, were associated with hypersensitivity to GCs (63). After both a 1 mg DST and a 0.25 mg DST both heterozygous (C/G) and homozygous (G/G) G-allele carriers had lower cortisol levels than CC-carriers, indicating increased sensitivity to the effects of GCs. This was confirmed in another study investigating GR haplotypes in relation to GC sensitivity (64). One GR haplotype, a G-A-T haplotype consisting of the G allele of the *Bcl*I polymorphism, the A allele of the intron B 33389, and the T allele of the intron B 33388, was found to be associated with increased sensitivity to GCs as well (64).

  Several previous studies reported associations with unfavorable metabolic characteristics, such as increased body mass index (BMI), (65) increased abdominal visceral fat (66, 67), hypertension (68), and lower amount of lean mass in the elderly (63). Recently, Syed et al reported another polymorphism in intron 2 (rs2918419), which was associated with hyperinsulinaemia and insulin resistance in males, but not in females. The authors suggest that previous articles reporting an association of *Bcl*I polymorphism with obesity-related characteristics may reflect linkage to rs2918419, since they found that carriage of *Bcl*I variant alleles without the rs2918419 variant appeared not to be related to insulin resistance (69).

- Haplotype 5 (N363S). This polymorphism is located in codon 363 of exon 2 of the *GR* gene. The nucleotide change (AAT -> AGT) results in an asparagine to serine amino acid. It is associated with lower cortisol levels and higher insulin levels after DST, suggesting increased GC sensitivity (70). Several reports demonstrated an association between the N363S polymorphism and obesity as could be expected if sensitivity to GCs is increased. (70) (71-74) However, in some other studies no associations were found with BMI (75, 76).

In conclusion, these GR genotypes seem to have consequences for cortisol sensitivity, both at the level of the feedback regulation at the pituitary gland and numerous target tissues. Therefore, they may be expected to induce subtle alterations in the set point of the HPA axis and may be involved in a genetic profile related to a hereditary susceptibility for depression. In the next sections the relationship between these GR polymorphisms, depression and cognition are discussed. Some relevant MR polymorphisms will also be briefly addressed.

5. GR polymorphisms and depression

A summary of data and an overview of studies concerning the associations between GR polymorphisms and depression are provided in Table 1. Research involving GR polymorphisms has only recently been focused on psychopathology and psychological stress (60, 77). Data are preliminary yet, but seem promising for future research. So far, several findings appear to be relevant in understanding the physiopathology of depression and will be discussed.

BclI polymorphism

In 2006 the *Bcl*I polymorphism was shown to be significantly associated with major depression (60). In this study in a large Caucasian study population the frequency of homozygous carriers of the minor *Bcl*I allele were significantly higher in the depressed group compared to the healthy control group (15.5% versus 9.9% homozygotes, respectively). Recently, this finding was confirmed in two other studies (78, 79). Zobel et al found similar frequencies: respectively 15.5% and 12.2% homozygotes *Bcl*I minor allele carriers in depressive patients and controls (79). No differences in hippocampal or amygdala volumes were observed for carriers of the *Bcl*I polymorphism in this study. In addition, in a recent study in premenopausal women a significantly higher number of homozygous carriers of the minor allele of the *Bcl*I polymorphism was observed in

Table 1: Overview of studies and summary of data concerning associations of GR polymorphisms and depression.

Author, year	Characterization participants	Associations	HPA axis parameters
Van Rossum *et al*, 2006(60)	490 inpatients with MDD (86% unipolar depression and 14% BD) 496 Healthy controls	Association between *Bcl*I polymorphism and depression Association between ER22/23EK and recurrent depression Association between ER22/23EK and faster response to antidepressants, as well as a slightly better cognition with respect to the divided attention test	No associations between SNPs and DEX/CRH test
Van West *et al*, 2006(84)	180 Belgian patients with MDD, uni- and bipolar 134 Swedish patients with MDD, unipolar 354 Healthy controls	Association between promoter region (NR3C1-1) SNP and MDD (Belgian sample) Association between ER22/23EK polymorphism (Swedish sample) and MDD	Not tested
Brouwer *et al*, 2006 (82)	98 outpatients with unipolar MDD	Trend of *Bcl*I SNP towards reduced response rate to antidepressants. In particular in a subpopulation of patients with the highest ACTH levels in the DEX/CRH test	*Bcl*I variant carriers have increased ACTH responses in the DEX/CRH test
Krishnamurthy *et al*, 2008(78)	53 premenopausal women with unipolar MDD, aged 21-45 29 healthy controls	Association between *Bcl*I SNP and depression Association between *Bcl*I SNP and higher WHR in depressive women	No differences in plasma and urinary cortisol.
Zobel *et al*, 2008(79)	322 patients with unipolar MDD 298 healthy controls	*TthIII*I and rs1866388 SNPs less frequent in patient group, and associated with larger hippocampal volumes.	Not tested
Spijker *et al*, 2008(85)	241 patients with BD 532 healthy controls	*TthIII*I SNP less frequent in patient group, and a trend for a lower frequency of the 9β polymorphism in BD patients. Association between the 9β polymorphism and less manic episodes.	Not tested
Moutsatsou *et al*, 2000(86)	15 patients with BD 12 healthy controls	No associations were found	Not tested
Feng *et al*, 2000(117)	100 patients with schizophrenia 40 patients with puerperal psychosis	No associations were found	Not tested
Bet *et al*, 2008(81)	221 elderly subjects (aged > 65 years) with depressive symptoms 685 healthy controls (aged >65 years)	ER22/23EK and 9β: in combination with childhood adversity associated with an increased risk of clinically relevant depressive symptoms.	*Bcl*I heterozygotes have lower serum cortisol binding globulin levels. ER22/23EK carriers with childhood adversity have a lower free cortisol index

Abbreviations; ACTH adrenocorticotrophic hormone; BD bipolar disorder; DEX/CRH test dexamethasone/ corticotrophin releasing hormone test; HRSD Hamilton Rating Scale for Depression; MDD major depressive disorder; SNP single nucleotide polymorphism; WHR waist hip ratio.

women with unipolar MDD (78). Furthermore, in the study of Krishnamurthy a positive association between this polymorphism and abdominal fat, as measured by a higher WHR, was reported. This is of particular interest, since it has been well recognized that a relationship exists between MDD and obesity (80). Bet et al recently observed lower levels of cortisol binding globulin in heterozygous *Bcl*I carriers (aged older than 65), but in this study no association between the *Bcl*I polymorphism and depressive symptoms was found (81).

Interestingly, Brouwer et al demonstrated a reduced response rate to antidepressants in depressive outpatients carrying the *Bcl*I variant (82). This finding applied in particular to a subpopulation of patients with the highest ACTH concentrations in the DEX/CRH test. It was suggested that the combination of both increased ACTH levels after administration of DEX/CRH, and carriage of the minor allele of the *Bcl*I polymorphism may be a more sensitive predictor of poor response to antidepressant treatment than these factors separately.

It should be noted that the single nucleotide change at the polymorphic *Bcl*I restriction site in intron 2 is referred to as a C (major allele) to G (minor allele) alteration in some articles (63, 78, 82), while in other reports a G (major allele) to C (minor allele) change is described (79, 83). This variability in the presentation can be explained by the direction in which the sequence of the *GR* gene is read (starting from the p-telomere to the q-telomere or vice-versa). To avoid confusion and to enable comparison of several studies we also used the term “minor allele”.

ER22/23EK polymorphism

The combined ER22/23EK variant was found to be associated with recurrent depression (60). The frequency of this polymorphism was 5.7% when all patients, unipolar and bipolar, were included, and did not differ statistically from the control group. However, comparison of the control group to patients with a recurrent unipolar MDD yielded a significant difference. In the healthy control group 4.2% was found to be carrier of the ER22/23EK polymorphism, whereas a carriage frequency of 7.7% in the patient group with a recurrent unipolar MDD was observed. In accordance, Van West et al showed a significant relationship between the ER22/23EK variant and recurrent MDD (84). In a Swedish population 11% of the unipolar MDD patients were found to be carriers of the ER22/23EK polymorphism, while in their control group this was only 4%. In a Belgian population, which was studied by the same authors, no association was found with the ER22/23EK variant. Recently, Bet et al found in presence of childhood adversity an association between the ER22/23EK and 9β polymorphisms and an increased risk of

clinically relevant depressive symptoms in a Dutch population of subjects aged 65 years and over (81). In absence of childhood adversity there was no association between these polymorphisms and depressive symptoms. This study demonstrates clearly the importance of gene-environment interactions.

Other GR polymorphisms

In several recent studies associations between other GR polymorphisms and unipolar or bipolar depression have been found. However, until now they have not been replicated. In this paragraph we will describe them briefly. In our recent study involving 241 patients with BD, we found a significantly lower frequency of the *TthIII*I polymorphism and a trend towards a lower frequency of a polymorphism in exon 9β (85). In addition, presence of the exon 9β polymorphism was significantly associated with less frequent manic episodes in this study, but no differences were found in frequencies of the other polymorphisms. Moutsatsou et al studied two *GR* gene isoforms (GR-a and GR-b) for the presence of mutations in 15 patients with BD and 12 normal subjects (86). They did not detect any mutations in the *GR* gene, which might have been due to the small number of patients in that study. Van West et al also found a negative association with another polymorphism (NR3C1-1) in the promoter region of the *GR* gene in a Belgian sample (84). This finding is not confirmed yet in other studies. In a Swedish sample, which was investigated in the same study, no association with this SNP was observed. Recent studies reported no associations between depression and the N363S polymorphism (60, 78, 85).

GR polymorphisms and response to antidepressant treatment

In several of the earlier mentioned studies there is some evidence of an association between GR polymorphisms and prediction of response. In the study of Brouwer et al, response rate was defined as more than 50% reduction of Hamilton Rating Scale for Depression (HRSD) scores after 8 weeks treatment with paroxetine (82). In this population, carriers of the minor allele of the *Bcl*I polymorphism had significantly higher ACTH levels measured in a DEX/CRH test, which was additionally correlated with a lower response rate. In a large German group of depressive patients, an association between the ER22/23EK polymorphism and a faster clinical response to antidepressant treatment was observed (60). Clinical response was defined as a reduction of the HRSD in 5 weeks of antidepressant treatment with tricyclic antidepressant or SSRIs, whereas remission was defined as a score below 10 on the HRSD. A significant effect on the HRSD score was found and carriers of the ER22/23EK polymorphism were found to be in remission on average 5 days earlier. At admission and at discharge, however, the Hamilton scores did

not differ significantly between carriers and non-carriers. Unexpectedly, DEX/CRH tests yielded no differences between carriers of the polymorphisms. This may be explained by an overriding effect of the state-dependent increased activity of CRH and arginine vasopressin and GR resistance, yielding increased HPA axis activity, which is presumed to be present in all genotypes (11).

To summarize, some evidence exists that unipolar MDD is associated with the *Bcl*I and the ER22/23EK polymorphisms. Additionally, the minor allele of the SNP NR3C1-1 in the promoter region of the *GR* gene was found to have a lower frequency in a mixed population of unipolar and bipolar depressed patients. Also, in a large group of patients with bipolar disorder a lower frequency of the *TthIII*I polymorphism and a trend towards lower frequency of the 9β polymorphism was found.

6. GR polymorphisms and cognition

Mood disorders

In both unipolar MDD and bipolar disorder, cognitive dysfunction can occur. In fact, cognitive symptoms such as reduced ability to think, lack of concentration and indecisiveness are, according to the Diagnostic Statistic Manual (DSM)-IV, some of the core symptoms in diagnosing a depressive episode. Also in the definition of a manic episode, cognitive symptoms are included. During a depressive episode, most well known cognitive deficits include diminished performance on shifting attention tasks, memory impairments, and problems with executive function (87-89).

While it is clear that cognitive impairment occurs during disturbed mood episodes, there is also growing evidence that these symptoms can persist in the euthymic phase of unipolar MDD and BD. In euthymic patients with unipolar recurrent MDD a persistent impairment of executive function was found (90). Persistent neuropsychological deficits present in BD also in the euthymic state, particularly impairment in sustained attention and working memory (91). This suggests that such deficits could be vulnerability trait markers of the illness (87). Additional evidence for this hypothesis is presented in studies of first degree family members of patients with BD, who show cognitive impairments in executive function, psychomotor speed and verbal ability, although milder than in the patient groups (90, 92). Some studies found that the deficits correlate with both the duration of illness and the number of episodes (93). However, Mur et al argue that these deficits, especially in executive function, are amongst the core symptoms of the disease, independent of course and severity of the disease (94). In BD, poor outcome is highly associated with cognitive impairment in remission, particularly executive dysfunction,

leading to impairment of psychosocial and occupational functioning (95). In a review on this topic, Glahn et al conclude that there are deficits in sustained attention, executive function and working memory in euthymic BD patients (96). These deficits are more severe in BD patients in comparison with unipolar patients, and could be trait markers of the illness. To enable interpretation of future studies on this topic, it is important to differentiate between patient groups (97), since patients with unipolar recurrent MDD seem to have different neurocognitive profiles compared to patients with BD and elderly patients with MDD (98).

Recently, some studies focused on the association between cognitive impairment, structural cerebral changes and GR polymorphisms. Zobel et al found in 64 patients with unipolar MDD, that during treatment with citalopram, the cortisol patterns in a DEX/CRH test changed and this finding was correlated with improvement of working memory. Other cognitive functions were not related to changes in HPA axis function in this study (99). In the earlier mentioned study of Zobel (see table), associations between the GR gene polymorphisms *TthIII*1 and rs1866388 and hippocampal volumes were found, possibly related to cognitive impairment, but this was not tested in this study (79).

In the German depressed patients a test for divided attention was performed (60). Impaired performance on this test in a group of depressed patients has been shown to be related to therapy resistance, as well as an elevated risk on to relapse within 6 months (100). During a depressive episode, ER22/23EK carriers showed a shorter reaction time with respect to the divided attention test than non-carriers. At discharge they also tended to perform better, although this effect was not statistically significant. This suggests that carriers of the ER22/23EK polymorphism may be relatively protected from potentially harmful consequences of increased HPA axis activity on cognitive functioning.

Dementia

In elderly patients with recurrent MDD, there is an overlap between depression and Alzheimer's disease. This is mainly because these patients exhibit a generalized pattern of cognitive deficits. One diagnostic feature is the reversibility of these symptoms of depression, which is clearly not the case in Alzheimer's disease (101). Over activity of the HPA axis has been associated with neurocognitive decline and dementia (102, 103). This decline in neurocognitive functioning could possibly be explained by elevated cortisol levels, which is also associated with increased risk of cardiovascular pathology. Cortisol levels tend to increase with aging, both in healthy and demented elderly (104, 105), but to a greater extent in demented patients. Magri et al conclude in their review that, besides the harmful effects of elevated cortisol levels on cognition, hippocampal

neuronal impairment may in turn be responsible for reactive elevation of cortisol levels (106).

To our knowledge, only two studies have focused on the relationship between aging, neurocognitive decline and GR polymorphisms. First, in a Dutch population of more than 6000 elderly, carriers of the ER22/23EK polymorphism were found to have a lower risk of developing dementia (61). At baseline, dementia was less prevalent in this group (with an 86% risk reduction). In a second group of more than 1000 Dutch elderly in the same study, a cerebral MRI was also performed. ER22/23EK carriers showed significantly less cerebral white matter lesions, as well as a decreased risk of progression of these lesions. In this study, non-demented ER22/23EK carriers performed better on psychomotor speed tests. However, no differences in memory tasks between genotypes were found (61). Another study of Kuningas et al confirmed in a cohort of 563 participants of 85 years and over the association between cortisol levels and impaired global cognitive functioning, and in particular impairments in attention and psychomotor speed. No associations between these test performances and GR polymorphisms were observed (107).

7. MR polymorphisms

Theoretically, it seems plausible that MR polymorphisms could also affect vulnerability for mood disorders, but until now this hypothesis has not been extensively studied. Kuningas et al found an association in the afore mentioned study between the MR SNP I180V, consisting of a GTT to ATT change in exon 2 (codon 180) and the prevalence of depressive symptoms in the elderly (107). Interestingly, this SNP is also associated with increased saliva cortisol and plasma cortisol responses and higher heart rate in reaction to a psychosocial stressor (the Trier Social Stress Test) in normal subjects (108, 109).

8. Future perspectives

To summarize, evidence is accumulating showing that carriers of GR polymorphisms *Bcl*I and ER22/23EK may be more vulnerable to developing MDD. In addition, carriers of the ER22/23EK polymorphism appear to be protected against dementia, but these data on cognitive functioning have not yet been confirmed. Other studies focusing on GR and MR polymorphisms showed associations with uni- or bipolar depression, but these data also need replication, preferably in larger populations. Future research should therefore invest in replicating these association studies in additional and larger patient groups. Another important aspect in future studies should be the proper definition of patient groups. Bipolar depressed and unipolar depressed patients seem to differ with respect

to the severity in HPA axis dysregulation and the clinical effects of GR polymorphisms (5, 10, 22). Therefore, it could be argued that these two types of patients should be investigated separately. On the other hand, others have demonstrated that BD and MDD patients are endophenotypically rather similar and even may be considered as one disease entity with both showing increased cortisol and ACTH release after stimulation with CRH following DEX suppression (11).

Many discrepancies exist with respect to the relationship of GR polymorphisms with HPA dysregulation in various brain areas. It remains intriguing that the two GR polymorphisms (*Bcl*I and ER22/23EK) with opposite effects with respect to GC sensitivity to adrenal feedback and in peripheral tissues are both associated with depression. It is also known that increased as well as decreased levels of glucocorticoids in the brain can be associated with depression. Supporting this is the finding that GR antagonists as well as GR agonists have been shown to have antidepressant effects. These apparent contradictory findings could possibly be explained by the complex mechanism of action of the GR. GR acts like a transcription factor, and is able to both positively and negatively regulate target genes. The production of CRH in the brain is regulated by GCs through binding to GRs in different brain regions. While the GR stimulates the CRH expression in limbic regions like the amygdala, it inhibits CRH production through a negative feedback mechanism in the hypothalamus (110, 111). An important focus is the balancing system of GR as well as MR. Future research should elucidate the importance of the MR in the pathophysiology of MDD. Studies in rats have shown MR is up regulated in the brain after psychological stress, associated with inhibition of the HPA-axis (112). Blockade of the MR in the brain leads to activation of the HPA-axis, under basal and stressful conditions. This is reflected in the decrease in blood pressure, heart rate and corticosterone response in rats after administration of a MR antagonist (113, 114). Besides these autonomic responses, MR also enhances cognitive responses like spatial learning in rats (115) as well as behavioral responses, such as a dramatic decrease in aggressive behavior in rats towards an intruder after treatment with a MR antagonist (116). In humans, brain MR function has not yet been extensively studied, however, animal studies seem encouraging for future research in this field.

It is of paramount importance to obtain more insight in the pathophysiology of depression and its clinical consequences, such as cognitive impairment. Identification of genetic markers for prediction of response to treatment and relapse is important in order to develop new treatment strategies. It will also be important in future research to differentiate between patient groups, since different neurocognitive profiles have been identified in unipolar, bipolar and elderly patients with unipolar MDD. This may also have implications for treatment possibilities. Elucidating the connections between GR and MR polymorphisms and depression may provide new insights from a combined

endocrine-genetic perspective and may in the future offer possibilities to classify patients in more homogeneous groups for whom treatment and risk of relapse predictions can be made and in addition, may provide new therapeutic targets to influence this system in these illnesses.

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4. Functional Polymorphism of the Glucocorticoid Receptor Gene associates with mania and hypomania in Bipolar Disorder

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Abstract

Objectives

In affective disorders, dysregulation of the hypothalamus-pituitary-adrenal (HPA)-axis is a frequently observed phenomenon. Subtle changes in Glucocorticoid Receptor (GR) functioning caused by polymorphisms of the GR gene (*NR3C1*) may be at the base of the altered reaction of the HPA-axis to stress and subsequently related to the development and course of affective disorders. The aim of our study is to evaluate associations between GR gene polymorphisms and bipolar disorder (BD).

Methods

In this study 245 patients with BD were interviewed to confirm diagnosis and BD subtype. Data on medication use and sociodemographic details were also collected. The control group consisted of 532 healthy blood donors, of which data on sex and age were collected. To perform genotyping blood was collected from all patients and healthy controls.

Results

A trend was found for a protective effect of the exon 9β-polymorphism ($p=0.14$) and the *TthIII*I-polymorphism ($p<0.05$) on the manifestation of the disease. These effects were significantly influenced by male gender for both polymorphisms. Patients with BD and the A/G variant in exon 9β had significantly less manic and hypomanic episodes than noncarriers ($p<0.05$).

No further associations were found with the other investigated GR gene polymorphisms and BD. These findings were not corrected for multiple comparisons.

Conclusions

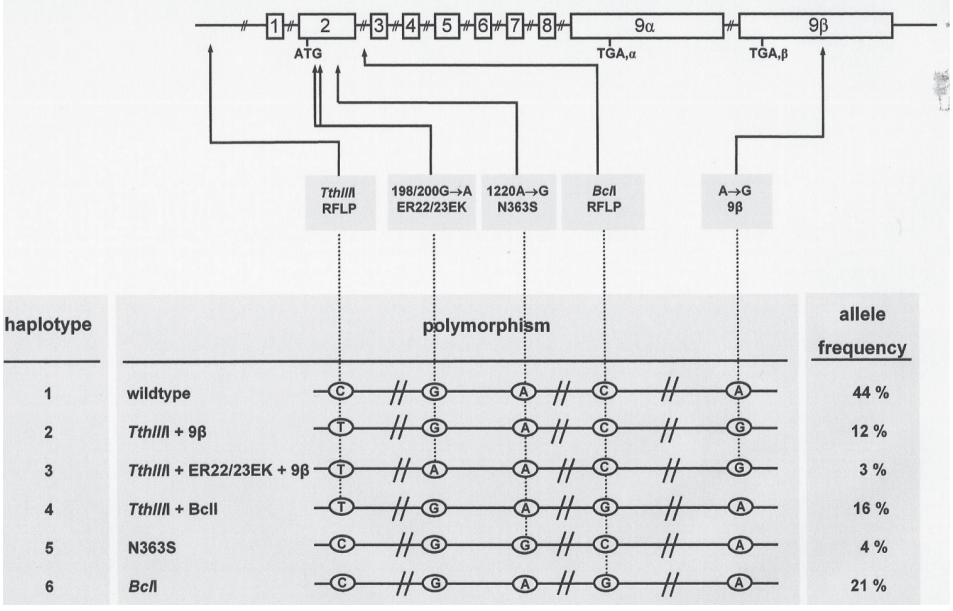
We conclude that the exon 9β-polymorphism and the *TthIII*I-polymorphism of the GR gene may be associated with a protective effect on the clinical manifestation and course in patients with BD. Furthermore no associations were found between the other studied GR gene polymorphisms and this disease.

Introduction

Bipolar Disorder (BD) is a common illness, with a lifetime prevalence of 1.9 % (1) to 2.4% (2). It is also associated with a high lifetime prevalence (82.8 %) of co morbid mental disorders, specifically anxiety disorders (31.9%) (3). The course of the disease is highly variable and unpredictable. As it is known that there is a dysregulation of the stress response in these patients, it is important to evaluate the influence of this dysregulation on the course of the disease.

Dysregulation of the stress response, or specifically dysregulation of the hypothalamus-pituitary-adrenal (HPA)-axis is known to occur in several psychiatric disorders, including BD (4-10). Cortisol exerts its effects through the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR). Recently, several polymorphisms of the GR and MR have been demonstrated to influence the response of the HPA-axis to stress (11-14). Figure 1 shows a schematic overview of the GR-gene *NR3C1* with the functional polymorphisms, as well as their haplotypes.

Figure 1 Schematically overview of the GR-gene, the different Single Nucleotide Polymorphisms and haplotypes.



Haplotypes 2-6 have been described as associated with a change in regulation of the HPA-axis. In two recent studies, haplotype 3 (*Tth*III*I* + 9β + ER22/23EK) was found to be associated with a higher risk of major depressive disorder (15, 16) and a faster response to antidepressant medication (15). In addition, the *Bcl*I-polymorphism (haplotypes 4 and 6) was more prevalent in depressed patients (15). In a recent study, carriers of the *Bcl*I-polymorphism with a major depressive episode had higher AdrenoCorticoTropic Hormone (ACTH) levels and a tendency to worse treatment outcome (17).

In this study, the prevalence of these polymorphisms in the GR-gene of patients with BD will be assessed, as well as their relationship with the course and severity of BD.

Patients and Methods

Subjects

This study is a cross-sectional, explorative case-control study of outpatients with BD. Patients with BD type 1, BD type 2 and BD Not Otherwise Specified (NOS), according to DSM-IV criteria were included. The design of the study was approved by the independent Dutch national medical ethics committee. All patients treated in the outpatient Clinic for Mood Disorders in The Hague were invited to participate. All 412 patients being treated for BD by the Outpatient Clinic for Mood Disorders in The Hague (Netherlands) were invited to participate in the study, either by letter or directly by their treating physician. After written informed consent was obtained, 245 patients were enrolled. To assess the diagnosis and co morbidity according to the DSM-IV criteria, patients were interviewed by trained research assistants using the Mini International Neuropsychiatric Interview Plus (18). Sociodemographic and ethnic data were collected, and the Clinical Questionnaire (CQBP-C) (Dutch translation Akkerhuis, Groenesteyn, Nolen 1997) was used to specify subtypes of BD and its course over time.

Blood samples from 532 healthy control subjects were randomly collected from the Rotterdam blood donation bank. Only the gender and age of these subjects are known.

Genotyping

From each patient, 40 ml of blood were collected in EDTA tubes and DNA was extracted from fresh blood using the Puregene whole blood DNA-extraction kit (Gentra Systems Inc; MN). Allelic discrimination was performed to genotype the subjects, using TaqMan Universal PCR master mix, and custom designed primers and probes (Applied Biosystems, Nieuwerkerk aan den IJssel, Netherlands, see also Table 1 and an Applied Biosystems

7900 HT Sequence Detection System as previously described (13). Reaction components and amplification parameters were based on the manufacturer's instructions. The genotypes were reanalyzed for all heterozygous and homozygous carriers of the single nucleotide polymorphisms (SNPs) and identical genotypes were identified. Both groups (controls and patients) were found to be in Hardy-Weinberg equilibrium with respect to all five polymorphisms. The accession number of the GR gene is NM_000176; the various polymorphisms have the following accession numbers: *TthIII*I - rs10052957; ER22/23EK - rs6189 and rs6190; N363S: rs6195; *Bcl*I - rs41423247; 9β - rs6198.

Table 1. *Sequences of primers and probes used for genotyping.*

Polymorphism	Primers	
Tth	Fw	5'-GGAGTGGGACATAAAGCTATGACAA-3'
	Rev	5'-GCAGAGGTGGAAATGAAGGTGAT-3'
N363S	Fw	5'-CAACAGCAGGATCAGAAGCCTAT- 3'
	Rev	5'-CCCAGAGAAGTCAAGTTGTCATCTC- 3'
ER22/23EK	Fw	5 '-TCCAAAGAATCATTAACTCCTGGTAGA- 3'
	Rev	5'-GCTCCTCCTCTTAGGGTTTTATAGAAG- 3'
*Bcl*I	Fw	5'-GCTCACAGGGTTCTTGCCATA- 3'
	Rev	5'-TTGCACCATGTTGACACCAAT- 3'
9-beta	Fw	5'-TCAGACTGTAAAACCTTGTGTGGAA-3'
	Rev	5'-CCAATTCGGTACAAATGTGTGGTT-3'

Polymorphism		Probes
Tth	Wt	5'-FAM-TTCAGACTCAATCAAGG-3'
	Mu	5'-VIC-TATTCAGACTCAGTCAAGG-3'
N363S	Wt	5'-FAM-CCTATTCCAAT<u></u>TTTCGGAACCAACGG- 3'
	Mu	5'-VIC- CCTATTCCAA<u>C</u>TTTCGGAACCAACGG - 3'
ER22/23EK	Wt	5'-FAM-ACATCTCCC<u>C</u>T<u>C</u>TCCTGAGCAAGC-3'
	Mu	5'-VIC-ACATCTCCC<u>TTT</u>TCCTGAGCAAGC-3 '
*Bcl*I	Wt	5'-FAM-TCTGCTGAT<u>C</u>AATCT -3'
	Mu	5'-VIC-TCTGCTGAT<u>G</u>AATCT - 3'
9-Beta	Wt	5'-FAM-TTTATTTTTTCGTTTAAATTT-3'
	Mu	5'-VIC-CTTTTATTTTTTCATTTAAATTT-3'

Fw=forward, Rev=reverse, Wt=wild type, Mu=mutant

Statistical analysis

Data were analyzed using SPSS (release 12.0.1 for Windows (SPSS, Chicago, IL)). Analyses for binary outcomes (case-control and dichotomous response variables) were performed with logistic regression analysis. The patient group and the control group significantly differed in age and gender. Therefore all analyses were corrected for age and gender.

First, frequencies of polymorphisms were analyzed in the patient and the control group. Because only age, gender and genotype were known from the control group, in the logistic regression analysis only these variables were added as independent explanatory variables.

Second, clinical data within the patient group were analyzed in relation to genotypes. Phenotyping was focused on current situation, and little was known about course and severity of the disease. Therefore, number of episodes was chosen as an indicator of severity of the disease. Groups concerning number of depressive or (hypo-) manic episodes were chosen based on the median number of episodes (see Table 2).

To reduce the problem of multiple testing, only the logistic regression analysis was performed for each genotype using frequency and number of episodes as dependent variables. As this is an explorative, hypothesis generating study, data mining is not completely avoidable. In this study the statistical power was not big enough to apply the Bonferroni's correction or the split sample method.

Haplotypes were not included in the analyses as the number of patients was not sufficient.

Results

Of the 245 patients enrolled in the study, genotyping failed in three patients and one patient did not have BD as defined by the MINI and the CQBP. The baseline characteristics of the remaining 241 patients and the control group are described in Table 2.

The *TthIII*I polymorphism was only analyzed in a subgroup of 320 controls. In 23 patients data on number of (hypo) manic episodes were missing.

Table 2 *Baseline Characteristics*

	Bipolar Patients	Controls
Number	241	532
Age (years)	47.8 ± 10.8	42.7 ± 12.2
Gender (% male)	102 (42%)	285 (54%)
Ethnicity	229 (95%) Caucasian	
Bipolar Disorder type 1	198 (82.2%)	
Bipolar Disorder type 2	41 (17%)	
Bipolar Disorder NOS	2 (0.8%)	
Age of onset (years)		
First depressive episode	25 ± 11,6	
First (hypo)manic episode	29 ± 11,7	
Depressive episodes		
Mean number	15 ± 21,2	
Median	6	
Manic episodes		
Mean number	12 ± 19,9	
Median	5	
Inter-episode functioning	142 (58,9%) well functioning	
	61 (25,4 %) some problems, most of the time well functioning	
	16 (6,6%) severely disturbed	
	22 (9,1%) no difference between episodes	
Use of medication	203 (84%) Lithium	
	31 (13%) Valproaat	
	8 (3%) Carbamazepine	
	11 (5%) Lamotrigine	
	71 (30%) Antidepressants	
Comorbidity	108 (47%) ≥1 Comorbid disorders	
	91 (39%) Anxiety disorder	
	10 (4%) Somatoform disorder	
	9 (4%) Pain disorder	
	6 (3%) Boulimia nervosa	
	5 (2%) Drug dependency	
	12 (5%) Alcohol dependency	
	6 (3%) ADHD	
	15 (11%) of all women have premenstrual dysphoria	

Comparison of frequencies of GR polymorphisms

In subjects of the patient group, a significantly lower frequency of heterozygous *TthIII*I carriers compared to the control group was found: 36.9% vs. 45.0% (p= 0.03, OR 0.51, 95% CI: 0.27-0.95). We found no differences in frequency of homozygous *TthIII*I variation, nor any other differences in genotype frequency between healthy controls and the group of bipolar patients (Table 3). Age had no significant effect: OR 1.0, 95%CI 0.98-1.01. Male gender appeared to have a significant effect in this analysis: OR 0.40, 95%CI 0.28-0.57, p<0.0001.

We found a non-significant trend for a lower frequency of the A/G or G/G variation in exon 9β in the patient group vs. the control group (respectively 24.5% vs. 31.0%, OR 0.76, 95%CI= 0.5-1.0; p=0.14, corrected for age and gender). Both age and gender had significant influences (for age: OR 1.04, 95%CI 1.02-1.05, p=0.000; for male gender: OR 0.52, 95%CI 0.37-0.72, p<0.0001).

Table 3 *Frequencies of five polymorphisms of the Glucocorticoid Receptor Gene in healthy control subjects and bipolar patients, and frequencies in patients with 0-5 hypomanic and manic episodes versus patients with >5 manic episodes; number (percentage)*

*Legend: *p= 0.03, OR 0.51 (95% CI: 0.27-0.95); ** p= 0.14, OR 0.76 (95% CI: 0.53-1.09)*

#p= 0.04, OR= 0.5 (95% CI: 0.27-0.98); ## p = 0.02, OR 0.46 (95% CI: 0.23-0.91)

	Healthy controls	**Bipolar patients**		
Polymorphism			with 0-5 manic and hypomanic episodes	>5 manic and hypomanic episodes
*TthIII*I				
CC	150 (46.9%)	126 (52.3%)	61 (51.3%)	53 (53.5%)
CT	144 (45.0%)	**89 (36.9%)***	46 (38.7%)	34 (34.3%)
TT	26 (8.1%)	26 (10.8%)	12 (10.1%)	12 (12.1%)
CT + TT	170 (53.1%)	115 (47.7%)	58 (48.7%)	46 (46.5%)
ER22/23EK				
GG	495 (93.0%)	227 (94.2%)	110 (92.4%)	94 (94.9%)
GA	36 (6.8%)	12 (5.0%)	8 (6.7%)	4 (4.1%)
AA	1 (0.2%)	2 (0.9%)	1 (0.9 %)	1 (1.0%)
GA + AA	37 (7.0%)	14 (5.8%)	9 (7.6%)	5 (5.1%)
N363S				
AA	493 (92.7%)	228 (94.6%)	111 (93.3%)	95 (96.0%)
AG	39 (7.3%)	13 (5.4%)	8 (6.7%)	4 (4.0%)
GG	0	0	0	0
AG + GG	39 (7.3%)	13 (5.4%)	8 (6.7%)	4 (4.0%)
*Bcl*I				
CC	199 (37.4%)	97 (40.2%)	53 (44.5%)	36 (36.4%)
CG	255 (47.9%)	110 (45.6%)	52 (43.7%)	48 (48.5%)
GG	78 (14.7%)	34 (14.1%)	14 (11.8%)	15 (15.2%)
CG + GG	333 (62.6%)	144 (59.8%)	66 (55.5%)	63 (63.6%)
9ß				
AA	367 (69.0%)	182 (75.5%)	82 (68.9%)	80 (80.8%)
AG	147 (27.6%)	51 (21.2%)	33 (27.7%)	**15 (15.2%)##**
GG	18 (3.4%)	8 (3.3%)	4 (3.4%)	4 (4.0%)
AG + GG	165 (31.0%)	**59 (24.5%)****	37 (31.1%)	**19 (19.2%)#**

Association of the GR polymorphisms with number of depressive and manic episodes

We further explored the association between number of mood episodes and the GR polymorphisms (Table 3). The number of episodes was dichotomously analyzed in four groups, based on the median number of episodes (see Table 2): the number of depressive episodes is 0 to 6 or more than 6, and the number of manic and hypomanic episodes is 0 to 5 or more than 5. Table 3 shows the frequencies of the GR polymorphisms in relation to number of manic and hypomanic episodes.

The most important finding is the difference in frequency of the exon 9β polymorphism between the group of patients with more and the group with less than 5 manic and hypo manic episodes (respectively 19.2% and 31.1%; p= 0.04, OR 0.5, 95%CI = 0.27-0.98). This difference is even more significant for heterozygous carriers (respectively15.2% and 27.7%; p=0.02, OR 0.46, 95%CI: 0.23-0.91). In both analyses age and gender had no significant effects. Additional analyses using nearby cut- off points yielded similar results. With respect to the number of depressive episodes, no significant differences between the group of patients with 0-6 episodes and the group of patients with >6 episodes, were found in the frequency of exon 9β-carriers.

None of the other comparisons (see Table 3) reached statistical significance.

Discussion

The major findings in this study are the lower prevalences of the *TthIII*I and exon 9β polymorphisms in the patient group compared to controls. These findings are consistent, since the 9β polymorphism is always present in combination with the *TthIII*I polymorphism, as is shown in Figure 1. Furthermore, those patients who carry the exon 9β polymorphism have significantly fewer manic episodes. This may indicate that the presence of the exon 9β-polymorphism has a (subtle) protective effect on both the prevalence and the clinical course of the disease. Clearly, these findings need replication to confirm the observations.

A possible limitation of the chosen cut-off points in number of episodes (mania more or less than 5, and depression more or less than 6) is that this is not based on clinical guidelines, but only on median number of episodes in our study population. However, other cut-off points in number of manic and hypo manic episodes yielded similar results. In future research, the course or severity of the disease will have to be carefully defined and prospectively followed to test these results.

We found no differences in the frequencies of the other GR gene polymorphisms between the controls and the patient group. In a recent study by Van Rossum (15) a higher prevalence of *Bcl*I and ER22/23EK polymorphisms was found in 495 hospitalized, severely depressed patients (13.2% with BD, 86.8% unipolar depressive disorder). In our study, patients differed considerably with respect to the severity of their illness, and none of the patients suffered from unipolar depressive disorder. Furthermore, the number of patients in our study was smaller, which potentially resulted in a lack of statistical power to detect minor differences.

This negative finding seems to indicate that these polymorphisms have no major effect on the etiology of the illness.

This study was the first to explore the theoretically relevant association between BD and genetically determined stress vulnerability as reflected by different GR polymorphisms. A limitation of this study is that the findings are not replicated yet in another population of patients with BD. It is clear that further research is needed to confirm these results.

Both findings in this study (the lower frequency of the *Tth*III*I* and exon 9β polymorphisms in the patient group, and the association of the G/A-polymorphism in exon 9β with less manic episodes) are in line with the modest conclusion that this haplotype may be associated with a protective effect on the illness. We found significant associations, but we cannot rule out that these associations are explained by a type I error.

The question remains, what could be the possible cause of the negative relationship between BD and the G/A-polymorphism in exon 9β of the GR gene. This polymorphism has a stabilizing effect on the mRNA levels of the GR-β isoform (19). This could result in a rise of GR-β levels, leading to a relative resistance to glucocorticoids since GR-b has been shown to exert a dominant negative inhibition of the active GR-α isoform. This polymorphism is also associated with the functioning of the immune system (19,20). This seems important, as several abnormalities in the functioning of the immune system are present in patients with BD. These include elevated IL-6 production, increased risk of autoimmune phenomena (such as autoimmune thyreoiditis, and raised thyroperoxidase auto-antibodies) and abnormalities in monocyte differentiation, indicating a dysregulation of the immune system (21, 22). However, the mechanisms for these associations among GR-β expression, changes in the functioning of the HPA-axis, and abnormalities in the immune system are largely unknown.

As mentioned in the introduction, there is ample evidence of dysregulation of the HPA-axis in patients with BD, possibly due to GR dysfunctioning. Our study found evidence that two commonly occurring GR gene polymorphisms are associated with the clinical manifestation and the course of bipolar disorder. Further research is needed to elucidate

the influence of stress vulnerability as revealed by GR and MR gene polymorphisms on the prognosis of BD. This might offer avenues for the development of new therapeutic interventions targeting the stress system.

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5. Glucocorticoid and mineralocorticoid receptor polymorphisms and clinical characteristics in bipolar disorder patients

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Abstract

Introduction

The Hypothalamus-Pituitary-Adrenal (HPA)-axis is often found to be dysregulated in Bipolar Disorder (BD) while stress and changes in day-night rhythms can trigger a new mood episode. Genetic variants of the Glucocorticoid Receptor (GR)- and Mineralocorticoid Receptor (MR)-gene influence both the reactivity of the stress-response and associate with changes in mood. In this study we tested the hypothesis that these polymorphisms associate with different clinical characteristics of BD.

Method. We studied 326 outpatients with BD and performed GR genotyping of the *TthIII*I, ER22/23EK, N363S, *Bcl*I, and 9β polymorphisms, as well as MR genotyping of the 2G/C and I180V variants. All patients were interviewed for clinical characteristics.

Results. Seasonal patterns of hypomania are related to the *Bcl*I haplotype and the TthIIII+9β haplotype of the GR gene (respectively crude p=.007 and crude p=.005). Carriers of the ER22/23EK polymorphism had an almost 8 years earlier onset of their first (hypo) manic episode than non-carriers (crude p=.004, after adjustment p=.016). No evidence for a role of the MR in modifying clinical manifestations was found.

Conclusion. Polymorphisms of the GR–gene are factors which influence some clinical manifestations of BD, with respect to seasonal pattern of (hypo) mania and age of onset.

1. Introduction

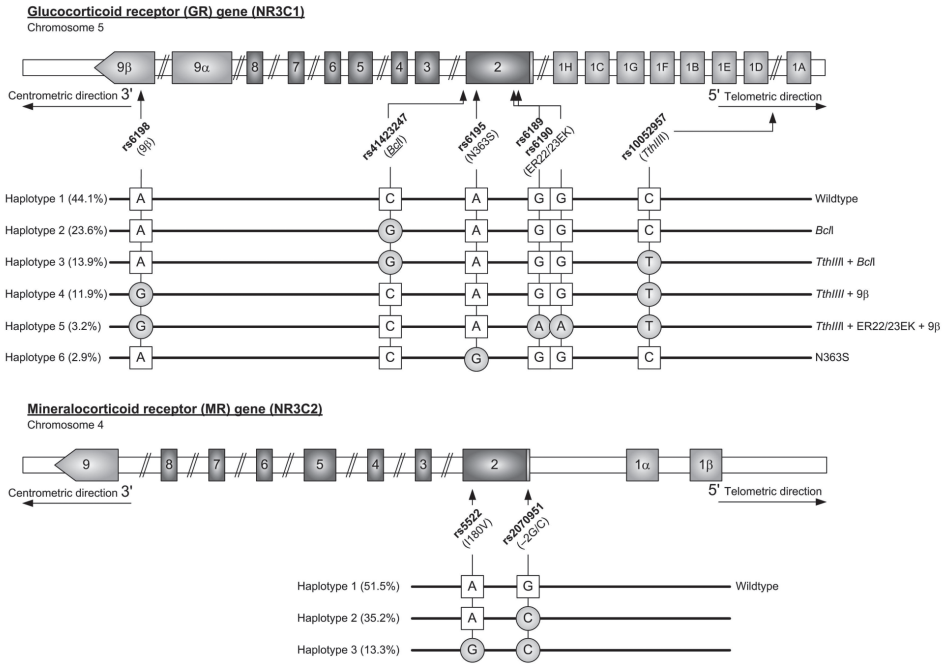
Bipolar disorder (BD) is a common mood disorder with a prevalence of around 2% in the general population. Several subtypes are distinguishable, of which Bipolar Disorder type 1 (BD1) is more prevalent than Bipolar Disorder type 2 (BD2*)* (1). BD1 is characterized by at least 1 manic episode and BD2 is characterized by at least one hypomanic episode and one depressive episode. The course and characteristics of the disease are highly variable and unpredictable. A clinically remarkable characteristic of BD are seasonal patterns of mood episodes in around 25% of all BD patients (2). Currently known predictors of an adverse course and higher severity include incomplete recovery between episodes, a history of rapid cycling and more than 10 episodes in the medical history, a history of childhood abuse, co-morbid panic disorder, substance abuse (3) and suicide attempts (4).

Stress and (abrupt) changes in day-night rhythms are common triggers of new mood episodes (5-7). The Hypothalamic Pituitary Adrenal (HPA)-axis is a central component of the stress-response and there is ample evidence that in BD the HPA-axis is dysregulated (8, 9). The most prominent finding is hypercortisolism in challenge tests like the Dexamethason Suppression Test (DST) and the Dexamethason/ Corticotropin Releasing Hormone (DEX/CRH)-Test. Moreover, hypercortisolism after the DEX/CRH-test is found during depressive episodes, manic episodes, as well as during remission, as compared to healthy controls (Schmider et al.,to healthy controls and family members (10), indicating that hypercortisolism is a feature predominantly observed during the stress responses and during active disease, but not in basal conditions.

The cortisol signal is mediated by two receptors, the low affinity Glucocorticoid Receptor (GR) and the high affinity Mineralocorticoid Receptor (MR). Under non-stress conditions, the low levels of cortisol already occupy a substantial number of the MR and GR, while only during stressful situations high levels of cortisol predominantly bind to the GR (11, 12). The activity of both these receptors regulates the stress-response, including the HPA axis and the autonomic output (13-15). Furthermore, the clinical relevance of these findings has become clear in the therapeutic effect of GR-antagonists, like mifepristone, improving mood and neurocognitive functioning in bipolar depression (16). Administration of the MR-agonist fludrocortisone as add-on to antidepressants greatly speeded up treatment response in depressed patients (17). The mood stabilizers lithium and valproate may mediate their therapeutic effects by up regulation of central GR (18) and by influencing GR-co-chaperone proteins (19).

Several polymorphisms of the GR- and MR-gene are involved in regulating the HPA-axis and may contribute to differences in the sensitivity of the stress-response (12, 20, 21), they are summarized in Figure 1.

Figure 1. *Schematic overview of the GR and MR gene structures with their respective most frequent haplotypes. The orientation of the gene is marked by arrows, with the genes transcribed from left to right. Some exons are not translated into protein (light gray). The gene encoding the GR consists of 17 exons, with the untranslated exons 1A-1H and 9a and 9b resulting in different mRNA splice variants. The five SNPs (rs 10052957 [Tth///I], rs6189/rs6190 [ER22/23EK], rs6195 [N363S], rs41423247 [BclI], and rs6198 [9β]) that were genotyped are indicated with arrows. Six main haplotypes are known (52). The gene encoding the MR consists of ten exons, exon 1α till exon 9. The two SNPs that were genotyped are indicated with arrows. The functional MR -2 G/C SNP (rs2070951) is located in exon 2, two nucleotides before the first translation start site. The functional MR I180V SNP (rs5522) is located in exon 2 and results in an Isoleucine to Valine amino-acid change. Both SNPs are located in a haplotype bin that extends into the promoter region. Three main haplotypes are known (53).*



GR polymorphisms have repeatedly been associated with mood disorders; the *Bcl*I and the ER22/23EK polymorphisms of the GR gene and, to a lesser extent the 9β polymorphism, may be most prominently associated with psychopathology (for review: (22)). In addition, two polymorphisms in the MR gene, the I180V SNP and the -2G/C SNP, have recently been found to be associated with the regulation of the stress-response in healthy subjects (13, 23). The I180V SNP was found to be associated with a higher frequency of depressive symptoms in an elderly cohort of participants aged 85 years and above (24) and with neuroticism in adult depressed patients (25).

Together these findings suggest a role of these GR- and MR-gene polymorphisms in the course and severity of bipolar mood disorders. To our knowledge no clinical study focused on the relationship between GR and MR polymorphisms and clinical characteristics of BD. We investigated whether different polymorphisms are associated with different clinical manifestations of BD.

2. Materials and Methods

2.1 Study design

This is a cross-sectional study in patients with BD. The study protocol was approved by the Medical Ethical Committee in Utrecht (METiGG) and has been carried out in accordance with the Declaration of Helsinki. All participants signed for informed consent.

2.2 Study population

All 702 patients, diagnosed and treated in the Department of Mood Disorders, PsyQ The Hague, with BD type 1, BD type 2, cyclothymia (a cyclic pattern of mood episodes which do not completely fulfill DSM-IV criteria), or bipolar disorder Not Otherwise Specified according DSM-IV-TR diagnosis, and older than 18 were selected to be asked to participate in this study. They were requested during a time frame of two years, by their psychiatrist and psychologist or by letter. Of these 702 patients 328 patients (46.7%) who responded and agreed to participate were enrolled in the study. The other 374 patients did not respond for largely unknown reasons, as they did not reply on repeated invitation by letter. Two more participants were excluded from the analysis, because only genotypic data and no clinical data were collected. Therefore, the present analyses were based on 326 (46.4%) subjects, yet some variables were missing in some participants due to logistical reasons. The included 326 patients did not differ from the 304 non-participating patients with respect to gender (39.9% male versus 41.4%

male respectively, p=.53). The included patients differed from the non-responders with respect to BD subtype (80% BD1, 19% BD2 and 1% BD NOS/cyclothymia versus 68% BD1, 26% BD2 and 6% BD NOS/cyclothymia, respectively, p<.001) and with respect to age (mean 48.0 ± 11.2 years versus 45.7 ± 12.5 respectively, p=.02) .

Data on seasonal patterns were complete in 301 (92.3%) subjects for depressive episodes, 298 (91.4%) subjects for manic episodes and 295 (90.5%) subjects for hypomanic episodes. Data on number of episodes and age of onset for mania were complete in 289 (88.7%) subjects and for depressive episodes in 292 (89.6%) subjects. Data on BD subtype were complete in 309 (94.8%) subjects. Age, gender and the presence of an anxiety disorder were assessed in all participants.

2.3 Assessments

All patients were diagnosed with bipolar disorder by a psychiatrist during their first visit on our Department. For the purpose of this study they were subsequently interviewed by one out of three trained psychologists who collected sociodemographic and disease characteristics, under supervision of a psychiatrist. Diagnostic status according to the DSM-IV-TR was assessed with a standardized diagnostic interview the Dutch version of the MINI International Neuropsychiatric Interview Plus (version 5.00-R; MINI-PLUS (26)). The age of onset of first symptoms of either (hypo)mania or depression, the number of episodes of (hypo)mania and depression, and the presence of co morbid anxiety disorders (according DSM-IV-TR) were assessed with the MINI. Psychiatric co-morbidity was assessed with the MINI and included lifetime and current anxiety disorders, somatoform disorders, eating disorders, and substance disorders. Current anxiety disorder was summarized into one dichotomous variable representing the presence or absence of any anxiety disorder. Suicide risk was classified according to the MINI in low, moderate and high risk.

Bipolar Disorder was further characterized by the Questionnaire for Bipolar Illness-Dutch Translation (QBP-NL) (27, 28). The QBP-NL BD was used to subtype BD into BD1, BD2, cyclothymia or BD Not Otherwise Specified (BD NOS). Due to small numbers the last two variables were aggregated in BD NOS. All patients were questioned in detail about seasonal patterns of depressive, hypomanic and manic episodes with the QBP-NL. Mood episodes were classified according to seasonal patterns, which was defined as whether the onset of the episode (i.e., depressive, hypomanic, or manic) was predominantly in the same season (winter, spring, summer or autumn). Age of onset and number of episodes were nominal variables, all other variables were categorical.

2.4 DNA analysis

From all 326 patients, blood samples of 40 ml were drawn with standard venapuncture techniques, collected in EDTA tubes, to analyze GR and MR gene polymorphisms (see Figure 1). Genomic DNA was isolated from fresh blood samples using the Puregene whole blood DNA-extraction kit (Gentra Systems Inc; MN). Genotyping was performed through PCR-based techniques. GR genotyping was performed in the Laboratory of Endocrinology of the Erasmus MC Rotterdam. MR genotyping was performed by Department of Pharmacology in Leiden in collaboration with the aforementioned laboratory in Rotterdam. Allelic discrimination was performed to genotype the subjects, using TaqMan Universal PCR master mix, primers and probes (Applied Biosystems, Nieuwerkerk aan den IJssel, Netherlands) and a Taqman ABI Prism 7900 HT Sequence Detection System as previously described (15, 29). Reaction components and amplification parameters were based on the manufacturer's instructions using an annealing temperature of 60°C and optimized concentrations for primers of 400 nmol/L for each polymorphism. The genotypes of all heterozygous and homozygous carriers of the polymorphisms were re-analyzed and yielded identical genotypes.

GR genotyping was completed for *TthIII*I in 307 (94.2%) subjects, for the ER22/23EK in 310 (95.1%) subjects, for N363S in 313 (96.0%) subjects, for *Bcl*1 in 310 (95.1%) subjects and for 9**β** in 314 (96.3%) subjects. MR genotyping was completed for -2G/C in 302 (92.6%) subjects and for I180V in 305 (93.6%) subjects. Due to small numbers, frequencies of the ER22/23EK (GA=17, AA=2), N363S (AG=20, GG=0) and I180V (AG=67, GG=10) Single Nucleotide Polymorphisms (SNPs) were combined into one group for the hetero- and homozygote carriers of the minor allele.

2.5 Statistical analysis

Haplotypes were created with SNPHAP (version 1.3, www-gene.cimr.cam.ac.uk/clayton/software/snphap.txt). All SNPs were tested for Hardy-Weinberg equilibrium. Thesias (Tregouet & Garelle, 2007) was used to assess inter-marker linkage disequilibrium scores (LD scores), expressed as D'. All further analyses were performed with Statistical Packages for Social Sciences (SPSS) version 17.

For analyses of baseline data t-tests and chi-square tests were used, when appropriate. All variables were normally distributed, except for "number of manic episodes" and "number of depressive episodes". These two positively skewed variables were therefore log transformed in all analyses and back-transformed geometric means values were presented in our results. Analyses of disease characteristics in relation to SNPs and haplotypes (see Figure 1) were performed with t-tests, Analysis of Variance (ANOVA)

and Chi Square tests. Adjustment for age, sex and subtype of BD was performed with Analysis of Covariance (ANCOVA) and multivariable logistic regression analysis. Results were considered statistical significant with p-value < 0.01 for GR SNPs and haplotypes and with a p-value <.025 for MR SNPs and haplotypes, to take multiple testing into account for SNP analyses. Post hoc Dunnett's method was used in post hoc tests to correct for multiple testing in haplotype analyses.

3. Results

3.1 Patient characteristics

Sociodemographic and disease characteristics are summarized in Table 1. In total 326 patients were enrolled in this study, of whom 80% were diagnosed with BD1, 19% with BD2, and 1% with BD NOS. Age ranged from 18-80 years, with a mean of 48 years (SD 11.2). In a total of 94 patients (31.2%) a seasonal pattern was present. Of all patients with seasonal depressive episodes, 87% had autumn and/or winter episodes. Eighty per cent of all seasonal manic episodes and 93% of all seasonal hypomanic episodes were spring/summer episodes. In further analyses seasonal patterns were dichotomized for hypomanic, manic and depressive episodes.

3.2 Genotypes and haplotypes

All SNPs were in Hardy-Weinberg equilibrium, except for ER22/23EK (χ^2 = 8.15, p<.01). The frequencies of the GR and MR Single Nucleotide Polymorphisms (SNPs) are presented in Tables 2 and 3. Comparisons between the GR SNPs resulted in different LD scores, D' ranged from 0.2-1.0 with an average of 0.9. Linkage of the two MR SNPs was high, with D'=1.0. Haplotypes created with SNPHAP was successful in 99.9% for the GR gene and 100% for the MR gene. Haplotypes are described in Figure 1, and numbering as used in Table 4 is defined there. Both GR and MR haplotypes 1 were the most prevalent wild-type haplotypes, and were therefore used as the reference category in further analyses. Age and sex distributions did not differ among the GR and MR haplotypes.

***Table 1**. Demographic and clinical characteristics of 326 BD patients*

Variable	Total group
No. (n)	326
Male n(%)	130 (39.9)
Age (yr); mean (SD)	48.0 (11.2)
Bipolar disorder subtype; n (%)	
Type 1	246 (80)
Type 2	60 (19)
NOS	3 (1)
Rapid cycling; n(%)	84 (27.2)
Seasonal pattern; n(%)	94 (31.2)
Depressive episodes	83 (27.6)
Manic episodes	41 (13.9)
Hypomanic episodes	28 (9.6)
Current drug use; n(%)	28 (9)
Current alcohol use ≥3/day; n(%)	32 (16)
Psychiatric co morbidity; n(%)	
Current co morbid disorder	118 (36)
Current anxiety disorder	80 (25)
Suicide risk moderate/ high	43 (13)
Physical morbidity; n(%)	
Cardiovascular disease	26 (10)
Endocrine disease	58 (22)
Medication use; n(%)	
Lithium	259 (80%)
Valproate	36 (11%)
Antidepressant	91 (28%)
Number of depressive episodes; median (IQR)	5.5 (3.0-15.0)
Number of manic episodes; median (IQR)	5.0 (2.0-12.0)
Age of onset of depression; mean (SD)	25.6 (11.7)
Age of onset of mania; mean (SD)	29.3 (11.8)

IQR denotes interquartile range.

Table 2. Association of GR and MR gene polymorphisms with co-morbid anxiety disorder and seasonal pattern in 326 BD patients

	Geno-type	N(%)	Current anxiety disorder		Seasonal pattern:					
				p	Depres-sion	p	Mania	p	Hypoma-nia	p
GR SNP										
rs10052957 (Tth///I)	CC	155 (50.5)	36 (23.2)	.12	39 (26.7)	.64	18 (12.7)	.67	10 (7.1)	.25
	CT	118 (38.4)	25 (21.2)		34 (31.5)		15 (13.9)		14 (13.2)	
	TT	34 (11.1)	13 (38.2)		8 (25.0)		6 (18.8)		4 (12.5)	
rs6189 (ER22/23EK)	GG	291 (93.9)	73 (25.1)	.05	78 (28.7)	.32	36 (13.5)	.70	28 (10.6)	.15
	GA+AA	19(6.1)	1 (5.3)		3 (17.6)		3 (16.7)		0	
rs6195 (N363S)	AA	293 (93.6)	70 (23.9)	.91	80 (29.5)	.06	38 (14.3)	.59	27 (10.2)	.48
	AG	20(6.4)	5 (25.0)		2 (10.0)		2 (10.0)		1 (5.3)	
rs41423247 (Bc/1)	CC	125 (40.3)	27 (21.6)	.74	28 (24.1)	.29	17 (15.0)	.70	7 (6.2)	.05
	CG	138 (44.5)	35 (25.4)		38 (29.5)		16 (12.3)		13 (10.2)	
	GG	47 (15.2)	12 (25.5)		16 (36.4)		7 (17.1)		8 (19.0)	
rs6198 (9β)	AA	227 (72.3)	53 (23.3)	.25	58 (27.4)	.49	26 (12.4)	.45	16 (7.7)	.02
	AG	75 (23.9)	22 (29.3)		22 (32.4)		12 (18.2)		12(18.2)	
	GG	12 (3.8)	1 (8.3)		2 (16.7)		2 (18.2)		0	
MR SNP										
rs2070951 (–2G/C)	GG	86 (28.5)	21 (24.4)	.85	22 (27.5)	.98	9 (11.3)	.79	10 (12.3)	.70
	GC	140 (46.4)	34 (24.3)		35 (26.7)		18 (14.1)		11 (8.7)	
	CC	76 (25.1)	21 (27.6)		20 (28.2)		10 (14.7)		7 (10.3)	
rs5522 (I180V)	AA	229 (75.1)	56 (24.5)	.75	62 (29.0)	.29	29 (13.8)	.63	22 (10.6)	.63
	AG+GG	76 (24.9)	20 (26.3)		16 (22.5)		8 (11.6)		6 (8.6)	

Associations are tested with Chi square tests and logistic regression analyses with age, sex and subtype of BD as covariates. P values of Chi square analyses are mentioned. All results are written as n(%).

Table 3. *Association of GR and MR gene polymorphisms and clinical characteristics in 326 BD patients*

	Geno-type	N (%)	Age of onset of depression		Age of onset of (hypo)mania		Number of (hypo) manic episodes		Number of depressive episodes	
			mean (SD)	p	mean (SD)	p	mean (95% CI)	p	mean (95% CI)	p
GR SNP										
rs10052957 (Tth*III*)	CC	155 (50.5)	24.5 (11.8)	.53	28.6 (11.0)	.74	6.4 (5.2-7.8)	.84	7.7 (6.4-9.3)	.76
	CT	118 (38.4)	28.8 (11.9)		29.5 (12.8)		6.2 (4.9-8.0)		7.8 (6.1-9.8)	
	TT	34 (11.1)	26.5 (11.8)		30.4 (12.3)		7.3 (4.1-12.5)		9.2 (5.7-14.4)	
rs6189 (ER22/23EK)	GG	291 (93.9)	26.1 (12.0)	.26	29.7 (12.1)	.004	6.4 (5.4-7.6)	.73	8.2 (7.0-9.6)	.05
	GA+AA	19(6.1)	22.8 (10.9)		21.9 (9.3)		5.8 (2.5-12.5)		4.3 (2.0-8.4)	
rs6195 (N363S)	AA	293 (93.6)	26.0 (11.9)	.71	29.4 (12.0)	.15	6.3 (5.4-7.5)	.76	8.0 (6.9-9.4)	.65
	AG	20(6.4)	24.9 (11.5)		25.6 (10.6)		6.9 (3.2-14.0)		6.4 (3.1-12.2)	
rs41423247 (B*c*l1)	CC	125 (40.3)	25.0 (11.9)	.24	27.4 (11.5)	.05	5.8 (4.7-7.2)	.62	7.8 (6.1-9.9)	.30
	CG	138 (44.5)	27.1 (11.9)		31.2 (11.7)		6.5 (5.2-8.1)		7.3 (6.0-8.9)	
	GG	47 (15.2)	23.0 (11.2)		27.7 (12.7)		7.2 (4.4-11.3)		10.1 (7.0-14.5)	
rs6198 (9β)	AA	227 (72.3)	25.0 (11.7)	.59	29.0 (11.7)	.96	6.5 (5.5-7.8)	.87	8.0 (6.8-9.4)	.71
	AG	75 (23.9)	27.0 (12.0)		29.3 (12.1)		6.1 (4.3-8.4)		7.2 (5.2-9.9)	
	GG	12 (3.8)	28.2 (13.3)		30.5 (15.0)		5.7 (2.2-13.0)		9.4 (3.9-21.2)	
MR SNP										
rs2070951 (–2G/C)	GG	86 (28.5)	26.4 (12.3)	.52	29.4 (12.5)	.54	5.4 (4.0-7.3)	.36	7.4 (5.6-9.8)	.75
	GC	140 (46.4)	24.7 (11.3)		28.3 (11.4)		6.5 (5.3-8.1)		8.5 (6.8-10.5)	
	CC	76 (25.1)	26.3 (12.2)		30.6 (11.9)		7.4 (5.3-10.2)		7.7 (5.7-10.1)	
rs5522 (I180V)	AA	229 (75.1)	25.5 (12.2)	.45	28.9 (12.0)	.52	6.1 (5.0-7.3)	.44	7.9 (7.7-9.5)	.71
	AG+GG	76 (24.9)	26.8 (11.5)		30.0 (11.9)		7.4 (5.3-10.3)		7.8 (5.6-10.7)	

Associations are tested with ANOVA and t-test analyses, and ANCOVA to adjust for age (number of episodes), sex and BD subtype (age of onset and number of episodes). P-values of t-tests and ANOVA's are mentioned. Number of episodes variables were both log transformed during analyses, and back transformed in this table.

Table 4. *Association of GR and MR gene haplotypes with clinical characteristics in BD 326 patients*

Variables	Haplotype 1	Haplotype 2	Haplotype 3	Haplotype 4	Haplotype 5	Haplotype 6	P value
No. of haplotypes; n (%)	289 (44.3)	155 (23.7)	91 (13.9)	78 (11.9)	21 (3.2)	19 (2.9)	
Age (yr); mean (SD)	47.3 (11.2)	47.9 (10.7)	50.0 (11.4)	49.1 (11.9)	46.2 (10.3)	46.7 (11.1)	.34
Male sex; n (%)	111 (38.7)	64 (41.6)	39 (42.9)	25 (32.5)	12 (57.1)	8 (42.1)	.40
Age of onset of depression; mean (SD)	25.6 (11.8)	25.8 (12.2)	26.0 (11.7)	28.2 (12.0)	22.7 (10.8)	25.5 (11.5)	.53
Age of onset of (hypo)mania; mean (SD)	28.8 (11.5)	30.1 (12.4)	30.1 (12.4)	31.1 (12.4)	21.7 (9.1)	26.0 (10.8)	.03
Number of (hypo) manic episodes; mean (95%CI)	6.1 (5.3-7.0)	6.4 (5.1-8.1)	7.1 (5.2-9.5)	5.6 (4.0-7.7)	7.7 (4.1-14.0)	6.8 (3.2-13.6)	.85
Number of depressive episodes; mean (95% CI)	7.5 (6.4-8.0)	7.6 (6.3-9.2)	9.0 (6.9-11.8)	8.8 (6.3-12.0)	4.7 (2.7-7.9)	7.0 (3.4-13.3)	.35
Current anxiety disorder; n (%)	67 (23.3)	37 (24.0)	27 (29.7)	23 (29.9)	1 (4.8)	5 (26.3)	.21
Seasonal pattern depression; n (%)	67 (25.5)	46 (32.2)	25 (30.1)	21 (29.2)	5 (26.3)	2 (10.5)	.39
Seasonal pattern mania; n (%)	33 (12.6)	19 (14.0)	12 (14.1)	11 (16.4)	5 (25.0)	2 (10.5)	.71
Seasonal pattern hypomania; n (%)	14 (5.4)	19 (14.0)**	10 (12.0)*	12 (17.1)**	0	1 (5.6)	.008

Associations of haplotypes are always in comparison with haplotype 1 (wildtype). Associations of haplotypes and dichotomous variables are tested with chi square tests and adjusted in logistic regression analyses for age, sex and subtype of BD. P-values are considered significant when <.01 to correct for multiple testing. Associations of haplotypes and continuous variables are tested with ANOVA. ANCOVA is applied to adjust for age, sex and subtype of BD. Post Hoc Dunnett's methods were used in post-hoc tests, correcting for multiple testing. Overall P values by ANOVA of chi-squared tests are presented.

** p<.01 **p<.001*

3.3 Glucocorticoid receptor (GR) genotype and bipolar phenotypic characteristics

Table 3 shows that the ER22/23EK carriers had slightly different characteristics with respect to age of onset. These carriers were significantly younger during the first (hypo) manic episode (crude 29.7 vs. 21.9, p<.004; however, after adjustment this was only a statistical trend with ages of respectively 29.5 vs. 23.1, p=.016). The onset of the first depressive episode also showed a tendency to a lower mean age in ER22/23EK carriers vs. noncarriers, but did not reach statistical significance.

Analyses on haplotype level (table 4) revealed that there was a tendency for a difference in age of onset of (hypo) mania among GR haplotypes (p=.03). Further analysis revealed a trend for an earlier age of onset of mania in subjects with GR haplotype 5 (crude 28.8 in GR haplotype 1 vs. 21.7 in GR haplotype 5, p=.06; after adjustment 29.0 vs. 22.2, p=.02). Number of episodes and age of onset of depressive episodes did not differ significantly among haplotypes.

Analyses at haplotype level showed that a seasonal pattern of hypomania was more frequent in carriers of the GR haplotypes 2 and 4 (Table 4). In GR haplotype 1 the frequency of seasonal hypomania was 5.4%. In GR haplotype 2 this was 14.0% (crude p=.004; after adjustment p=.007, OR= 2.83, 95%Confidence Interval [CI]:1.3-6.0), in GR haplotype 4 this was 17.1% (crude p=.001; after adjustment p=.005, OR=1.50, 95% CI: 1.13-1.98). GR haplotype 3 revealed a tendency for an association with seasonal pattern of hypomania with a frequency of 12.0% (crude p=.04; after adjustment p=.06, OR= 1.52, 95% CI: 0.98 - 2.34).

3.4 Mineralocorticoid receptor (MR) genotype and bipolar phenotypic characteristics

Table 2 and 3 present the associations between disease characteristics in relation to MR SNP analyses. No statistically significant associations between clinical disease characteristics of BD and any of the MR SNPs or haplotypes were found.

4. Discussion

In this study we investigated whether GR- en MR-gene polymorphisms were correlated with clinical manifestations of BD. We found evidence for a minor role of GR polymorphisms in influencing clinical aspects of BD. First, we found an association between seasonal patterns of hypomania and the *Bcl*I haplotype 2 and the T*thIII*I+9β

haplotype 4. This result was also found as a statistical trend association at the SNP level for the *Bcl*I SNP and the 9β SNP. Second, carriers of the ER22/23EK SNPs (located in haplotype 5) had an almost 8 years earlier onset of their first (hypo) manic episode than non carriers. Similar results were obtained in the haplotype analysis, showing that the T*thIII*I+9β+ER22/23EK haplotype 5 was associated with 7 years earlier age at the onset of the first manic episode. No evidence for a role of the MR in modifying clinical characteristics was found.

To our knowledge this is the first study showing a relationship between seasonal patterns of mood episodes in BD and genetic variation in the GR gene. Several studies have investigated the relation between seasonal changes of the HPA-axis responsivity in animals, healthy humans and depressed inpatients. Some studies found elevated morning cortisol levels in healthy humans during winter (30, 31). However, in a study with depressed patients a decreased sensitivity to Dexamethasone feedback of the HPA-axis was measured in autumn and spring, and an increased sensitivity in summer and winter (32). In mice the highest GR expression was found in January (33), also illustrating an increased GC sensitivity during winter. In parallel, mice studies showed which higher GR expression in the hippocampus of mice during short days and a faster return to basal cortisol levels after restraint (34). One of the mechanisms leading to seasonal changes in cortisol sensitivity is thought to be the direct effect of change in day lengths on the activity of the suprachiasmatic nucleus (SCN) in the hypothalamus (35, 36). This is thought to be mediated by reduced expression of clock genes in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus and the hippocampus, which was found in European hamsters (37). In order to understand the relation between seasonal changes of the HPA-axis and mood episodes in BD patients with GR SNPs, it is important to emphasize that these SNPs alter HPA-axis sensitivity. The *Bcl*I and the N363S SNP are known to increase Glucocorticoid sensitivity (38-40). The 9β and the ER22/23EK SNP are both associated with a mild resistance for GCs (41, 42). We hypothesized that these genetic variants in corticosteroid receptor genes confer susceptibility for the precipitation of psychiatric disorders under less favorable conditions (43). In the context of seasonal changes these SNPs could bring the precisely regulated HPA-axis off balance when adjusting for seasonal changes. This gene-variant – environment interaction might well play a role in patients with BD where seasonal changes of daylight are known to influence mood (44), which could be worsened by the described SNPs. We found associations between haplotypes 2 (*Bcl*I) and 4 (T*thIII*I+9β) only with seasonal patterns of hypomanic episodes, and not with manic episodes. This is in line with the finding of a 10-year prospective study among 302 bipolar patients, revealing more seasonal episodes in patients with BD2 (2). Besides, when looking at seasonal patterns in mood episodes, irrespective of subtype of bipolar

disorder, a clear association with depression as well as with hypomania was found, but not with mania.

The finding that carriers of the ER22/23EK SNPs had an earlier onset of the first (hypo) manic episode has not been reported earlier. Earlier onset may be related to a worse outcome (27, 45). The finding that carriers of the ER22/23EK SNP were younger when the first symptoms of BD appeared and may consequently have had a higher risk on a poorer outcome with more mood episodes, is in accordance with previous studies demonstrating that the ER22/23EK variant correlated to an increased susceptibility for dysregulation of mood (17, 46-49). On the other hand these studies indicate a more beneficial outcome for carriers of these SNPs (or this haplotype). In line with the hypothesis of a beneficial outcome, we found a statistical trend towards less prevalent anxiety disorders in patients with the ER22/23EK variant (5.3% vs. 25.1% in non carriers). Previously, Russcher et al showed that the transcriptional activity of the GR in ER22/23EK carriers is decreased because more of the less transcriptionally active GR-A isoform is formed, which seems to be caused by altered secondary mRNA structure (50). The subtle resistance for GCs in ER22/23EK carriers may result in compensatory higher cortisol levels, which may have tissue-specific increased effects in the limbic system yielding mood disturbances. Another explanation we could speculate on is that the insensitive GR signaling due to the ER22/23EK variant leads to intracellular deficit of cortisol in the limbic system yielding an increased vulnerability for mood disturbance, and at the same time a protective effect on the long-term with respect to other brain areas related to anxiety. However, further research is needed to confirm these speculations and elucidate the neurobiological pathways.

The MR is known to be involved in the regulation of both low levels of cortisol and the reactivity of the stress-response following a challenge (13, 15). There is some evidence for an association between MR gene polymorphisms and social stress situations in adults. In one study (24) an association between depressive symptoms and the V-allele has been found, and another study provides evidence for an association between MR-SNPs and neuroticism (25). No such associations were found in adolescents (51). Since we found no evidence for a role of the MR in modifying number of episodes, age of onset and co morbidity in BD this might indicate that environmental influences predominantly interact with gene-variants of the GR but less so with the MR.

There are several limitations in this study. First, the ER22/23EK SNPs were not in Hardy Weinberg equilibrium, which may be explained as a chance finding in combination with a low allele frequency. Yet, it is unlikely that this deviation influenced the outcomes of the analyses. A second limitation of this study is the absence of measurements of serum cortisol levels and other endocrinological clinical parameters. Serum cortisol could

provide information about the potential mediating effects of cortisol on the relationship between GR and MR SNPs and clinical characteristics in BD patients. However, serum cortisol measurements may fail to adequately reflect cortisol's true impact because of the circadian rhythm of cortisol levels and the pulsatile way cortisol is secreted. Also, large daily variations due to e.g. acute stress or infection are also an important limitation of measuring cortisol levels. Using information from genetic variations known to be associated with altered cortisol sensitivity may better contribute to understanding the relationship between glucocorticoids and BD. Third, we used a relatively small sample size for a genetic association study, although we used functionally characterized genetic variants. Moreover, we were not able to perform a replication study in a different sample. Fourth, in this study no data were obtained with regard to premorbid functioning, known to be an important predictor for outcome of first episode psychosis. However, information on premorbid functioning is often based on patients recalling and difficult to assess in an unbiased way. Fifth, our study design was cross-sectional with clinical data gathered retrospectively, leading to potential recall bias. This could have affected the reliability of the retrospectively collected data on age of onset. Patients were questioned about their first illness symptoms, which hamper a sharp differentiation between hypomania and mania symptoms. Finally, BD is genetically a very complex disease. Besides HPA-axis related factors, numerous other environmental and genetic factors also influence clinical BD characteristics. Therefore, findings on number of episodes and seasonality need to be further explored and replicated in larger and prospective studies. Future prospective studies should include clinical characteristics like cognition and physical health. The ultimate goal is to obtain more insight in risk factors for poor outcome and subsequently develop individualized therapeutic interventions.

We conclude that GR SNPs affect several clinical manifestations of BD. Seasonal mood episodes are likely to develop in BD patients with altered HPA-axis regulation, at least partly caused by genetic variation in the GR. Together with natural changes of GC sensitivity throughout the year this could increase vulnerability for mood episodes.

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6. Long-term cortisol in Bipolar Disorder: Associations with age of onset and psychiatric co-morbidity

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Abstract

Introduction:

Dysregulation of the hypothalamic-pituitary-adrenal (HPA-)axis is hypothesized to play a role in the pathogenesis of bipolar disorder (BD). Conflicting results have been reported when saliva or serum was used to measure cortisol levels. A recently developed method is to measure cortisol in scalp hair, with one cm of scalp hair representing one month. We studied whether there are differences in long-term hair cortisol levels between BD patients and healthy individuals and whether there are associations between hair cortisol and disease characteristics.

Methods

Hair samples were collected in 100 BD patients and 195 healthy controls. Long-term cortisol levels were determined in 3 cm hair segments. Saliva samples were collected on two consecutive evenings. Documented disease characteristics were disease state, age of onset and psychiatric co-morbidity.

Results

Hair cortisol levels were not statistically different in BD patients compared to healthy controls ($p=0.233$) and were not associated with the disease state at the moment of sample collection ($p=0.978$). In the subgroup of patients with age of onset ≥30 years, hair cortisol levels were significantly elevated compared to the subgroup with age of onset <30 years and to healthy controls ($p=0.004$). Psychiatric co-morbidity was associated with elevated cortisol levels (44.87 versus 31.41 pg/mg hair; $p=0.021$), with the exclusion of panic disorder, which was associated with decreased cortisol levels (22.13 versus 34.67 pg/mg hair; $p=0.019$).

Conclusions

Elevated long-term cortisol levels might play a role in a subgroup of patients with BD. There may be differences in pathogenesis of younger and older onset BD suggesting two different disease entities.

Introduction

Cortisol, the main glucocorticoid in humans, is released in response to stress as part of the hypothalamic-pituitary-adrenal (HPA) axis that affects mineralocorticoid and glucocorticoid receptors throughout the brain. Dysregulation of the HPA-axis is known to occur in several psychiatric disorders, such as anxiety disorder (e.g. post traumatic stress disorder) (1) and mood disorders including bipolar disorder (BD) (2-7). In addition, depression and mania are common psychiatric symptoms seen in patients treated with corticosteroids or suffering from Cushing's Syndrome, a disorder caused by cortisol excess (8, 9). Dysregulation of the HPA-axis in BD is also supported by several studies that have found more non-suppression after dexamethasone (dex) administration and increased cortisol secretion after corticotrophin releasing hormone (CRH) in patients with BD (10, 11). However, conflicting data have been published concerning HPA-axis dysfunction during episodes and during euthymic phases of the disease. Furthermore, there are only a few studies reporting dexamethasone suppression tests (DSTs) or combined dex/CRH tests in patients with bipolar disorder (10, 11).

In the majority of previous studies salivary or serum cortisol levels have been used to evaluate HPA-axis functioning. However, these studies yielded conflicting findings. Some studies reported no differences in basal salivary or serum cortisol levels in patients with BD (12-15), whereas others described elevated cortisol levels (16, 17). These conflicting findings might be the result of methodological differences between the studies. Some studies reported cortisol levels in patients in remission, while others measured cortisol levels during active depression or mania. Furthermore, there is a large discrepancy in the methods used to evaluate cortisol levels in BD patients. Some studies evaluated the cortisol awakening response, whereas others investigated diurnal rhythms of cortisol secretion or cortisol levels at single time points. Cortisol is secreted in a circadian rhythm and with a pulsatile pattern, which complicates the interpretation of serum and salivary cortisol levels. Furthermore, in blood, only 10% of the circulating cortisol is free, 75% is bound to cortisol binding globulin (CBG) and 10% to albumin (18). In most studies describing serum cortisol levels, the concentration of CBG is not taken into account. Therefore the concentrations of unbound, biologically active, cortisol are not known. In addition, since cortisol is a stress hormone, acute psychological or physical stress will also influence serum and saliva cortisol levels. A recently developed method to measure cortisol levels in scalp hair is a feasible method to determine long-term cortisol levels and appears to yield a reliable estimate of long-term HPA-axis activity (19, 20). Since hair grows with an average rate of 1 cm per month, a hair segment of e.g. 3 cm would reflect mean cortisol levels over a period of approximately 3 months. This long-term cortisol measurement is therefore not influenced by the time of sample collection or acute

stress due to daily circumstances or the research setting. Steudte et al. (21) used hair cortisol levels to measure HPA-axis activity in generalized anxiety disorder (GAD) and found decreased cortisol levels in hair of patients with GAD, but no differences in salivary cortisol levels between GAD patients and healthy controls (22). This suggests that hair cortisol levels may reflect the chronic cortisol exposure, whereas the results found with saliva or serum cortisol levels might also include acute responses to the measurement circumstances. Several other studies have shown that hair cortisol is indeed a marker of long-term cortisol exposure (19, 23-26).

The aim of this study was to explore the role of long-term endogenous cortisol exposure by comparing long-term cortisol levels in BD patients with healthy controls using cortisol measurements in scalp hair. We aim to obtain insight in the long-term assessment of cortisol in patients with bipolar disorder, since chronic subtle changes in HPA-axis functioning appears to be involved in the pathophysiological processes leading to mood episodes. We hypothesized that hair cortisol levels are higher in BD patients compared to healthy individuals. In addition, we explored the relation between cortisol levels in hair and clinical course of the disease, with characteristics like disease state, age of onset, and psychiatric co-morbidity. Furthermore, we measured saliva cortisol levels in order to compare the observed findings of hair cortisol.

Materials and methods

Study design

This is a cross-sectional study involving outpatients with BD. The study was approved by the local medical ethics committee and is carried out in accordance with the declaration of Helsinki. After complete description of the study to the participants, all participants gave informed consent.

Participants

Bipolar Disorder patients

Patients with BD (type I, type II and Not Otherwise Specified) included in our previous study (27) concerning the role of glucocorticoid and mineralocorticoid receptor polymorphisms were asked to participate and the first 100 eligible patients were included in this study. Patients were eligible for inclusion if they had not been using glucocorticoids

in the six months prior to hair sample collection and if they had sufficient hair growth at the posterior vertex. Detailed description of the assessment methods of the patients has been described elsewhere (27, 28). In brief, participants were interviewed by trained psychologist to collect socio-demographic data and disease characteristics. Diagnoses of BD and psychiatric co-morbidities were based on DSM-IV criteria and were assessed with a standardized diagnostic interview developed by Sheehan et al. (29) using the Dutch version of the MINI International Neuropsychiatric Interview Plus (version 5.00-R; MINI-PLUS) (30). The Questionnaire for Bipolar Illness, Dutch translation (31, 32) was used to specify subtypes of BD, its course over time and detailed information about age of onset of first symptoms regarding hypomanic, manic and depressive episodes. The Questionnaire for Bipolar Illness, Dutch translation is a widely used questionnaire, developed by the National Institute of Mental Health. In addition, detailed information was gathered to define whether patients suffered from depression, mania or a combined episode during the three months prior to hair sample collection (this timeframe corresponds with cortisol measurements in 3 cm hair segments). Based on this information**, disease** state was categorized as stable disease, depressive episode, manic episode or mixed episode. **Data** on age of onset were present in 84 patients and data concerning psychiatric co-morbidities were present in 99 patients.

Healthy controls

A group of 195 healthy controls were used as control group. Detailed information of this study group has been reported previously (19). Both patients and healthy controls filled out a questionnaire concerning hair treatment (dyeing/bleaching/permanent waving/straightening), the use of hair products (gel, wax, hair spray) and frequency of hair wash.

Sample collection

In all patients and healthy controls, a lock of hair of approximately 100-150 hairs was cut of from the posterior vertex as close to the scalp as possible. Hair was taped to a paper and stored until preparation. In addition, in 90 patients with BD also saliva samples were collected on two consecutive evenings at 2200h. Collection at 2200h was chosen since several studies showed that saliva samples collected in the late evening on separate days show the lowest variation in cortisol levels compared to diurnal cortisol measurements and the cortisol awakening response (33-35). This suggests that late evening cortisol levels are slightly less influenced by acute stressors and daily influences than e.g. the cortisol awakening response, and may thereby be a better reflection of basal cortisol status. Participants were instructed not to eat, drink or brush their teeth 30 minutes prior

to the saliva collection. Saliva was collected by spitting into a Salicap plastic tube. Saliva samples were stored at -20°C until analysis. Cortisol levels were measured separately in both saliva samples and we calculated the mean salivary cortisol levels afterwards. We used the mean salivary cortisol levels in the statistical analyses.

Hair sample preparation

Hair samples were measured, weighted and put into separate glass vials. The 3 cm segment most proximal to the scalp was used in the analysis, which corresponds roughly to a period of three months. In the glass vial, hair segments were cut into small pieces and 1 mL of methanol was added to extract cortisol from the hair samples. After overnight incubation for 16 hours at 52°C while gently shaking, the methanol was transferred to another glass vial and was evaporated under a nitrogen stream and the samples were dissolved in 250 µL phosphate buffered saline (pH 8.0).

Cortisol measurement

Saliva samples were thawed and vortexed. After this, saliva samples were centrifuged for 5 minutes to separate the mucins. Cortisol in saliva as well as in the hair extracts was measured using a commercially available salivary ELISA cortisol kit (DRG Instruments GmbH, Marburg, Germany). Cross reactivity of the kit's antibodies with other steroids was reported as follows: Corticosterone (29.00%), Cortisone (3.00%), 11-Deoxycortisol (<1.00%), 17-OH Progesterone (<0.50%), other hormones (<0.10%). Intra-assay variation was below 5% and the inter-assay variation below 8% as stated by the manufacturer. The low end detection limit for this assay is 1.5 nmol/L. To test the recovery of the assay, we created cortisol standards in PBS with concentrations of 5, 10, 20, 40, 80 and 160 nmol/L and measured the recovery in duplicate. We also spiked two hair samples with 20 nmol/L hydrocortisone, to measure recovery when hydrocortisone was dissolved in hair extract. The recovery of 5, 10, 20, 40, 80 and 160 nmol/L cortisol standards from PBS was 96.0%, 94.0%, 94.0%, 95.0%, 96.8% and 89.9% respectively. When hydrocortisone was added to hair extracts, the mean recovery was 101.3%.

Statistical analysis

SPSS 17.0 for Windows and GraphPad 5.0 were used for statistical analysis. Differences between group characteristics were tested with Chi-square and Kruskal Wallis tests. After log transformation hair and saliva cortisol levels were normally distributed. Differences in cortisol levels between healthy controls and BD patients and between

disease characteristics of BD patients were tested with ANCOVA and linear regression. The association between saliva cortisol and hair cortisol levels was tested using linear regression analysis. All hair cortisol analyses were adjusted for gender, age, frequency of hair wash, hair treatment (dyeing, bleaching, permanent straightening or waving) and the use of hair products. Saliva cortisol analyses were adjusted for gender and age.

Results

Hair cortisol measurements were completed in 100 BD patients and 195 healthy controls. Saliva cortisol levels were available in 90 BD patients. Group characteristics are shown in Table 1. All BD patients were treated in the outpatient Department of Mood Disorders in The Hague, the Netherlands. BD patients were significantly older than healthy controls and had significantly higher BMI. Furthermore, BD patients washed their hairs less frequently and used less hair products, but dyed/bleached their hairs more often compared to the healthy control group (Table 1).

The number of patients on lithium, antidepressants, antipsychotics and other mood stabilizers are shown in Table 1. There were only 2 (2.0%) BD patients that did not use anti-depressants, antipsychotics, lithium or other mood stabilizers, 30 patients (30.0%) used only 1 type of medication, 31 patients (31.0%) used 2 types of medication and 19 patients (19.0%) used 3 types of medication. There was no correlation between log-transformed saliva cortisol and hair cortisol measurements (b=0.157, p=0.140) (Figure 1).

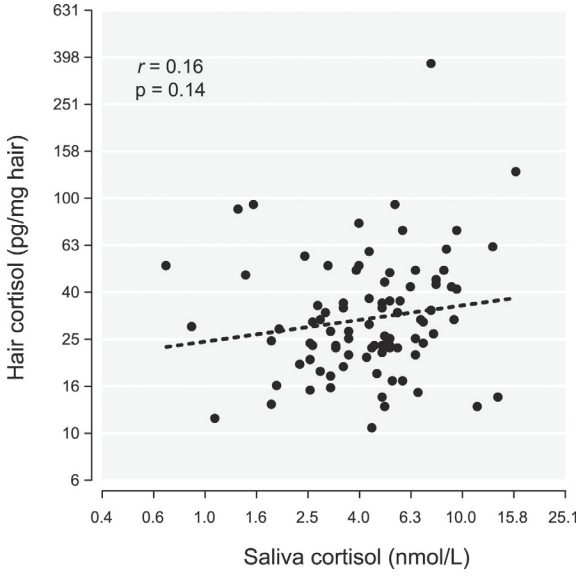
Table 1. Group characteristics

	Bipolar Disorder (n=100)	Healthy Controls (n=195)	p-value
Age (years) – median (range)	52.0 (20-82)	32.0 (18-63)	<0.001
Number of women – n (%)	62 (62.0%)	103 (52.8%)	0.18
BMI (kg/^{2}) – median (IQR)	25.3 (23.5-28.0)	23.7 (21.7-26.5)	<0.001
Frequency of hair wash – n (%)			
≤ 2 times/week	53 (53.0%)	50 (25.6%)	
≥ 3 times/week	47 (47.0%)	143 (73.3%)	<0.001
Hair treatment*	40 (40.0%)	43 (22.1%)	0.001
Use of hair products**	32 (32.0%)	90 (46.2%)	0.018
Age of onset Bipolar Disorder			
median (IQR)	21.0 (15.5-35.0)		
< 30 years of age – n (%)	55 (55%)		
> 30 years of age – n (%)	29 (29%)		
One or more psychiatric co morbidities – n (%)	42 (42.0%)		
Co morbid panic disorder	14 (14.0%)		
Co morbid anxiety disorder	16 (16.0%)		
Co morbid agoraphobia	21 (21.0%)		
Co morbid pain disorder	1 (1.0%)		
Co morbid somatoform disorder	2 (2.0%)		
Medication			
Lithium – n (%)	68 (68.0%)		
Other mood stabilizers – n (%)	17 (17.0%)		
Antidepressants – n (%)	31 (31.0%)		
Antipsychotics – n (%)	34 (34.0%)		
Disease state in the period covered by the hair sample			
Stable disease– n (%)	43 (43.0%)		
Depressive episode – n (%)	29 (29.0%)		
Manic episode – n (%)	3 (3.0%)		
Mixed episode – n (%)	8 (8.0%)		

* *Hair treatment: dyeing, bleaching, permanent waving, permanent straightening of hairs*

*** Hair products: includes hair spray, wax, mouse and gel and other not wash-related hair products.*

Figure 1. *Relationship between the mean cortisol level in two evening saliva samples (collected at 22.00h) and the hair cortisol levels on logarithmic scales. The Pearson's correlation coefficient and its accompanying p-value are given with a regression line.*

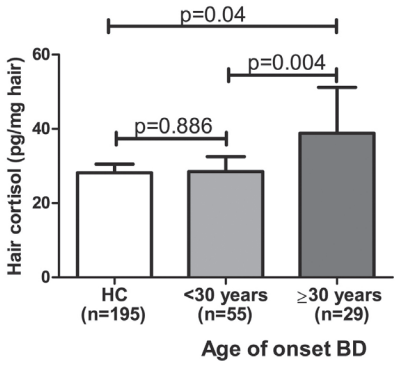


Hair cortisol

There were no correlations between hair cortisol levels and age in healthy controls (r=0.062, p=0.39) or BD patients (r=-0.047, p=0.64). There were also no differences in hair cortisol levels between men and women in healthy controls (p=0.87) or BD patients (p=0.12) and hair cortisol levels did not correlate with BMI in BD patients (r=0.163, p=0.11) or in healthy controls (r=0.042, p=0.59). Hair cortisol levels in the total group of BD patients were not different from hair cortisol levels in healthy controls (31.84 pg/mg hair (95% Confidence interval (CI): 28.38 – 35.81) versus 28.18 pg/mg hair (95% CI: 25.94 – 30.62); p=0.233, adjusted for gender, age, hair treatment, hair products and frequency of hair wash). Since there was variability in the state of disease at the moment of sample collection, the group of BD patients was split up into groups with a stable period (n=43), depressive episode (n=29), manic episode (n=3) or combined episode (n=8) in the 3 months prior to sample collection. There were no significant differences between hair cortisol levels in the groups with various disease states in the three months prior to hair sample collection (unadjusted p=0.868; adjusted for gender, age, hair treatment, hair products and frequency of hair wash p=0.978). In addition, there was no effect of lithium (p=0.357), other mood stabilizers (p=0.388), antidepressants (p=0.816) or antipsychotics (p=0.278) on cortisol levels.

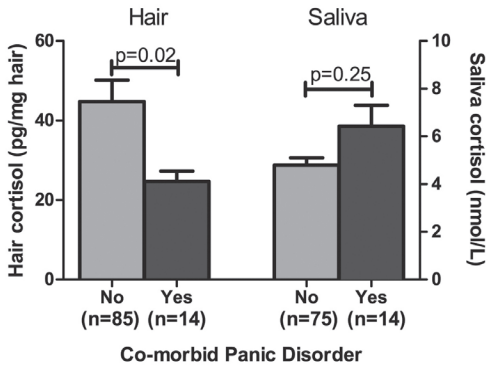
We found no significant correlation between age of onset of BD (age at which the first episode of (hypo)mania or depression presented) and hair cortisol levels (b=0.156, p=0.179). . However, when assessing the distribution of hair cortisol throughout the different age of onset groups divided in decades, we found significantly elevated hair cortisol levels in the group of patients with their first depression or mania ≥ 30 years of age compared to the group of patients with their first depression or mania before 30 years of age (p=0.004) (Figure 2). Hair cortisol levels in the group with onset of BD < 30 years of age were similar to those in the healthy controls (p=0.886, adjusted for gender, age, hair treatment, hair products and frequency of hair wash) (Figure 2). There were no differences in use of lithium (p=0.536), other mood stabilizers (p=0.384), antidepressants (p=0.397) and antipsychotics (p=0.278) between the group of patients with an older age of onset of BD compared to the group of patients with an age of onset <30 years. Furthermore, there was no correlation between duration of the disease and hair cortisol levels (r=-0.105, p=0.34).

Figure 2. *Hair cortisol levels in healthy controls, bipolar disorder patients with younger and older age of onset. HC, healthy controls; p-values are adjusted for age, gender, use of hair products, hair treatment and frequency of hair wash. Error bars represent SEM.*



Interestingly, in patients with co-morbid panic disorder (n=14) hair cortisol levels were significantly lower than in BD patients without panic disorder (22.13 versus 34.67 pg/mg hair, p=0.019, adjusted for gender, age, hair treatment, hair products and frequency of hair wash) (Figure 3). These hair cortisol levels in patients with co-morbid panic disorder were even lower than hair cortisol levels in healthy individuals (p=0.05, adjusted for gender, age, hair treatment, hair products and frequency of hair wash). Furthermore, we found elevated hair cortisol levels in the group of BD patients with other psychiatric co-morbidities (e.g. anxiety disorders, agoraphobia, pain disorder, somatoform disorder) compared to the BD patients without psychiatric co-morbidities (44.87 pg/mg hair (95% CI: 34.36-58.61) versus 31.41 pg/mg hair (95% CI: 26.06-37.76) in patients without co-morbidities, p=0.021). When patients with co-morbid panic disorder were included in the group of total co-morbidities, there was no significant difference in hair cortisol levels between BD patients with and without psychiatric co-morbidities anymore (34.67 versus 31.33 pg/mg, p=0.448). Additional adjustment for BMI did not change any of the results.

Figure 3. *Hair cortisol and salivary cortisol levels in bipolar disorder patients with and without co-morbid panic disorder. Hair cortisol analyses are adjusted for age, gender, use of hair products, hair treatment and frequency of hair wash. Saliva cortisol analyses are adjusted for gender and age. Error bars represent SEM.*



Saliva cortisol

There were no significant differences in salivary cortisol levels between BD patients with a depressive episode, manic episode or stable disease at the moment of saliva collection (p=0.304). In addition, we found no differences in salivary cortisol levels between age of onset of disease before the age of 30 years and after the age of 30 years (p=0.155). In contrast to the results with hair cortisol levels, co-morbid panic disorder was associated with a trend towards increased salivary cortisol levels (5.5 (95% CI: 4.0-7.5) nmol/L versus 4.1 (95% CI: 3.6-4.7) nmol/L, unadjusted p=0.094). However, this was not statistically significant after adjustment for gender and age (p=0.245) (Figure 3). Furthermore, we found no differences between the group of BD patients with psychiatric co-morbidities (panic disorder excluded) and without psychiatric co-morbidity (p=0.562). Also, when panic disorder was included in the total group of psychiatric co-morbidities, there were no significant differences in salivary cortisol levels between the BD patients with and without co-morbidities (p=0.306). An overview of all results for hair and salivary cortisol is given in Table 2.

Table 2. Overview of the hair cortisol and saliva cortisol results of disease characteristics in patients with Bipolar Disorder

	Hair cortisol				Salivary cortisol			
	Crude b	p	Adjusted b	Adjusted p	Crude b	p	Adjusted b	Adjusted p
Age of onset Bipolar Disorder (<30 versus ≥ 30 years)	0.249	*0.022*	0.335	*0.004*	0.166	0.146	0.163	0.155
Co morbid panic disorder	-0.211	*0.036*	-0.243	*0.019*	0.187	0.094	0.122	0.245
Psychiatric co morbidity (excl. panic disorder)	0.227	*0.036*	0.253	*0.021*	0.020	0.865	0.068	0.562
Psychiatric co morbidity	0.087	0.389	0.078	0.448	0.153	0.329	0.142	0.306

The Beta was calculated with linear regression. Hair cortisol analyses were adjusted for age, gender, use of hair products, hair treatment and frequency of hair wash. Saliva cortisol analyses were adjusted for gender and age

Discussion

In this study, we measured long-term hair cortisol levels of patients with bipolar disorder and healthy individuals. In addition, we measured evening salivary cortisol levels in bipolar disorder patients. The main finding of this study is the elevated hair cortisol level in patients with older age of onset BD compared to the group with a younger age of onset and healthy controls. We also found that BD patients with co-morbid psychiatric disorders (excluding panic disorder) had higher hair cortisol levels than patients with only BD. Interestingly, in patients with co-morbid panic disorder, hair cortisol levels were significantly decreased, whereas saliva cortisol levels were slightly elevated. No further correlations between hair and salivary cortisol measurements were found.

This is the first study that measured long-term hair cortisol levels of patients with BD. Several studies have measured cortisol levels in serum and saliva and results have been inconclusive with reports of increased (16, 17) and normal cortisol levels (12-15) compared to healthy individuals. Serum and saliva cortisol measurements reflect cortisol levels at one time point and are influenced by the pulsatile pattern and circadian rhythm of cortisol secretion, as well as by acute stress due to daily circumstances. These limitations do not apply to hair cortisol measurements which makes hair cortisol a better marker of HPA-axis functioning on the long-term. Recently, two studies measured hair cortisol levels and related them to mood disorders (36, 37). Dettenborn et al. showed that hair cortisol levels were elevated in medicated patients with major depression (37) and Dowlati et al. showed that there was no difference in hair cortisol levels between depressed and non-depressed patients with coronary artery disease (36). However, hair cortisol levels of both depressed and non-depressed patients with coronary artery disease were higher than those in healthy controls. The possible psychological stress associated with suffering from coronary artery disease and the presence of coronary artery disease itself could have abolished the differences in hair cortisol between depressed and non-depressed patients, resulting in different results than the observations of Dettenborn et al. Since both studies only included patients with major depression and not BD, it is difficult to compare their results to our findings.

In our study, we found that patients with younger (<30 years of age) and older (≥30 years of age) onset BD clearly differed in their mean hair cortisol levels. Our finding of elevated hair cortisol levels in BD patients with older age of onset supports the hypothesis of dysfunction and hyperactivity of the HPA-axis which has been described in BD patients and their offspring (15, 38-43). However, cortisol levels in patients with earlier age of onset BD were comparable to hair cortisol levels in healthy individuals. In the past decade, several studies have found that there might be differences in disease characteristics and pathogenesis of early and late onset BD. Early onset of BD seems to be associated with

greater severity and a poorer long term outcome (44, 45). In addition, several genetic differences have been described between early and late onset BD (46, 47). Differences in cortisol levels and HPA-axis functioning between subtypes of BD based on age of onset have not been studied previously. Our hair cortisol data suggest that elevated cortisol levels may play a role in late onset BD, but not in early onset BD. This suggests that BD onset at younger age may be less influenced by dysregulation of the HPA-axis, but more by other mechanisms e.g. changing regulation of sex hormones or differences in genetics of the disease. Later onset of the disease might be influenced by dysregulation of the HPA-axis through e.g. stressful life events, which are thought to be a trigger for manifestation of the disease (48). Furthermore, it can also be hypothesized, that long-term elevations in cortisol affect proneness for mood disorders by neuronal damage in the brain (49), which may lead to a higher risk of developing mood disorders. As seen in patients with endogenous hypercortisolism, psychopathology continues even after cure (8, 9), suggesting that long-term elevations in cortisol can have irreversible effects on the brain, resulting in mood disorders.

We did not observe these differences in age of onset with saliva cortisol, suggesting that saliva cortisol may be an acute marker of cortisol rather than an estimate of the HPA-axis activity on the long-term. It supports the additional value of measuring hair cortisol levels. Furthermore, we found no differences in hair cortisol levels between patients in different disease states. The same applied to the observed findings of salivary cortisol in relation to BD, which is consistent with several other studies (15, 16). This suggests that the increased HPA-axis activity may be a trait phenomenon rather than a state phenomenon in the subgroup of patients with older age of onset.

Moreover, we found that psychiatric co-morbidity was associated with elevated hair cortisol levels within the group of BD patients. With respect to the different types of co-morbidities, we found significantly lower cortisol levels in BD patients with co-morbid panic disorder. In contrast to this, evening saliva cortisol levels tended to be higher in this group. Our study is the first study that describes both hair and saliva cortisol levels in patients with panic disorder. These data again suggest that hair cortisol levels may reflect cortisol levels over a larger time frame (about three months is this study, since we used three cm length of hair), whereas saliva cortisol may provide an estimate of one time point including potential acute effects of the circumstances. Steudte et al. (22) previously demonstrated similar results in a group of patients with generalized anxiety disorder. In this group they found that hair cortisol levels were decreased compared to healthy individuals, whereas there was no difference between saliva samples of

GAD patients and healthy individuals (22). The increased saliva cortisol levels in our BD patients with panic disorder compared to those without co morbid panic disorder, might be explained by more perceived stress in the participants with panic disorder prior to a visit to the research center than BD patients without panic disorder. Reports of saliva, serum and urine cortisol levels in patients with panic disorder have shown conflicting results (50-55), which emphasizes the need for a new reliable measurement of long-term cortisol exposure.

There are several limitations of this study. First, the healthy individuals were significantly younger than the BD patients, which may have affected our findings. However, there was no effect of age on cortisol levels in the control group (19) or in the bipolar disorder group. In addition, other studies reported no effect of age on hair cortisol levels (56, 57), and all analyses were adjusted for age. Second, several studies have shown that other factors, such as suicide attempts and childhood trauma can have a significant effect on the HPA-axis (58, 59). In our study population, there was no significant effect of childhood abuse (sexual, physical and verbal) on hair cortisol levels (data not shown), but we did not collect data concerning suicide attempts. In future studies, other factors that might influence the HPA-axis such as childhood trauma and suicide attempts should be taken into account as well.

Third, our study lacked data concerning smoking status and somatic health. However, we found no differences in hair cortisol levels between smokers and non-smokers in our group of healthy individuals (data not shown), and a recent study of Dettenborn et al (37) did not find differences in hair cortisol levels between smokers and non-smokers as well. Furthermore, our results of possible increased saliva cortisol levels with decreased hair cortisol levels in patients with panic disorder were found in a group of only 14 patients with co-morbid panic disorder in addition to bipolar disorder. The pathogenesis of panic disorder in BD patients might be different from the pathogenesis of panic disorder in patients without BD or another psychiatric disorder. Therefore, these results have to be interpreted carefully and it is not certain whether the results can be extrapolated to patients with panic disorder without other psychiatric disorders. Finally, measurement of cortisol in scalp hair is a relatively new and promising method but many details concerning hair growth rate and incorporation of cortisol in hair are still unknown.

Despite these limitations, our results support the hypothesis that elevated long-term cortisol levels play a role in a subgroup of patients with bipolar disorder. Since we found that cortisol levels were only elevated in the group of patients with a relatively late age of onset of BD, we hypothesize that there may be differences in pathogenesis among patients with early and late onset BD yielding two different disease entities. Future

research should focus on exploring these subtypes of BD based on age of onset with potentially a differential neuro-endocrine background.

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7. The impact of medication use and clinical characteristics on cognitive functioning in bipolar disorder

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submitted

Abstract

Objective

Attention, memory and executive cognitive functions are frequently impaired in patients with Bipolar Disorder (BD). These impairments seem to be a trait phenomenon, but may also be influenced by medication and alcohol use. Approximately one-third of BD patients use more than one drug simultaneously, known as polypharmacy. In this study, the associations between medication use as well as clinical characteristics were investigated in relation to cognitive performance in 187 euthymic BD patients.

Methods

Sociodemographic and illness characteristics, as well as medication use were related to 3 cognitive domains (i.e., attention, memory and executive function) of the Test for Attentional Performance (TAP) using multivariable regression analyses.

Results

The main finding was that the use of multiple types of medication was associated with poorer cognitive performance in all three cognitive domains, but only statistically significant for executive functioning (β= -0.19, *P*=0.004). Particularly the use of antipsychotics was associated with impaired executive functioning, whereas Lithium use was not associated with cognitive performance. As we included only euthymic patients, our findings cannot be extrapolated to chronically unstable patients.

Conclusions

Our findings suggest that the use of multiple types of medication adversely affects diverse cognitive domains in BD patients, but specifically executive functions. Clinicians should be aware of these potential side effects when prescribing medication to BD patients.

Introduction

Bipolar Disorder (BD) is a common mood disorder, characterized by mood swings like depression and (hypo) mania. In addition to mood episodes, recent evidence shows that cognitive deficits are important clinical features of BD (1) related with poorer social and global functioning. In BD, attention problems, slowing of processing speed, and deficits in working memory and executive functioning appear to be most consistently observed (2), and have negative influence on treatment adherence and outcome of the disease(3). The disturbances are partly state-dependent and partly determined by trait factors of BD that may have a genetic origin, however, cognitive performance seems mostly linked to specific sociodemographic and clinical factors like age, level of education, medication use, residual mood symptoms and early adversity (4). The impact of Lithium use on cognition appears to be minimal (5, 6), but antipsychotic use seems associated with deficits in memory and executive functioning in BD patients (5). Despite available research, little attention has been given to the cognitive side effects of the number of different types of medication, known as polypharmacy, which is common practice in the treatment of BD (7). Other clinical characteristics of BD related to cognitive performance include BD subtype (8), and with some controversy, substance abuse (9).

However, studies are small, with a number of included patients between 10 -76 (10). In this study we aimed to further disentangle the associations between cognitive performance in a large cohort of 187 BD patients, medication use and a number of sociodemographic and clinical characteristics. We hypothesize that medication use and specifically use of multiple types of medication would adversely associate with specific domains of cognitive functioning.

Patients and Methods

Study design

This is a cross-sectional study involving outpatients with BD. This study was approved by the Medical Ethical Committee (METTIG) in Utrecht, The Netherlands, and was performed in accordance with the declaration of Helsinki.

Out of a cohort existing of 364 patients with BD, a subgroup of 187 (51.4%) agreed to perform cognitive tasks. The cohort of 326 patients has been described in a previous publication (11), currently extended to 364 patients. These 187 patients fulfilled the inclusion criteria of being diagnosed with BD1, BD2, cyclothymia or BD not otherwise specified, all according DSM-IV-TR diagnosis and being older than 18 years. Due to small numbers, cyclothymia and BD no otherwise specified were included in the BD2 group in all analyses. They were all euthymic at the time of the investigation. All patients are treated in the Program for Mood Disorders at PsyQ in The Hague, The Netherlands. An exclusion criterion in this study were schizo-affective disorder and age below 18 years old. Participants were comparable with the non participants with regard to age and gender and clinical characteristics, but differed with respect to subtype of BD (respectively 66.8% and 80.4% BD1; p=.05), number of depressive episodes (median respectively 6.5 and 5; p=.02), Lithium use (respectively 27.3% and 15.7% used no Lithium; p=.006) and alcohol use (respectively 35.1% and 46.7% used no alcohol; p=.03).

Assessment of clinical characteristics and medication use

Detailed information about the protocol regarding data collection of diagnostic status and sociodemographic information, is described in our previous publication (11). In addition, to assess euthymia, all patients were questioned about current mood with the Quick Inventory of Depressive Symptomatology- Self Report (QIDS-SR) (http://www.ids-qids.org) and the Young Mania Rating Scale (YMRS) (12). Euthymia was prospectively defined as score ≤7 for the YMRS and a score of ≤12 for the QIDS-SR. Early adversity was assessed with questions of the Childhood Trauma Questionnaire (CTQ) (13).

The psychotropic medication types that were used most frequently, were coded according to the Anatomical Therapeutic Chemical Classification System (http://www.whocc.no/) and included Lithium (ATC code: N05AN01), anti-epileptics (ATC code: N03AF01, N03AG01, N03AX09) antipsychotic medication (ATC code: N05Ax with exclusion of N05AN01), benzodiazepines (ATC codes: N05BA, N05CD, N03AE01, N05CF), and antidepressants (N06A). Lithium is regarded as drug of first choice in the treatment

of Bipolar Disorder, therefore it was not included in antipsychotic medication but coded separately. In case of more than 1 used medications of the same type (for example the use of 2 antidepressants), this was counted as 1 type.

Test for Attentional Performance (TAP)

Cognitive performance was assessed by means of the Test for Attentional Performance ('Testbatterie für Aufmerksamkeitsprüfung' (TAP), version 2.1, http://www.psytest.net/ ; Zimmermann & Fimm, 2002). The TAP is a widely used computer based standardized test battery and is easy to use in clinical practice (5, 14, 15). In this study, 8 out of 12 subtests were used to evaluate cognitive performance in the patient group, including attention (phasic and tonic), sustained attention, divided attention, working memory, cross-modal integration, flexibility, go/no-go and incompatibility. The other four subtests included eye movement, visual neglect, visual field scanning and vigilance. These subtests were initially developed for patients with brain damage, but are of less interest for our research question and were therefore excluded. In previous studies raw scores as well as compound scores were used in further analyses (for references see http://www.psytest.net/). All skewed variables were log transformed to fulfill normal distribution criteria. To summarize the results in further analyses and avoid multiple testing problems, compound scores and z-scores were created and averaged for 3 domains of closely related cognitive component item test results, namely attention (11 component items, including alertness median reaction time in two series, alertness errors and total performance index; sustained attention errors in four series, sustained attention omissions in four series), working memory (2 component items: errors and omissions) and executive functioning (13 component items, including flexibility, total performance scores of 2 series, divided attention errors, omissions and correct scores in two series, go/ no go errors and median, incompatibility median and errors in two series, cross modal integration errors and omissions). Intercorrelations between the component items of each compound z-score were highly significant, with the exception of two component items in the z-score for executive functioning. However, when re-analyzing the data excluding these 2 component items, results remained largely similar. Lower z-scores reflect more mistakes, longer reaction times and more omissions. Attention includes phasic and tonic attention, as well as a separate subtest for sustained attention. Executive functioning comprises divided attention, flexibility, Go/No go, incompatibility and cross modal integration.

Statistical analysis

All of the analyses were conducted using SPSS version 17.0 statistical software (SPSS Inc, Chicago, Illinois). First, analyses were performed to describe the clinical characteristics, with use of t-tests and Chi-square tests when appropriate to compare participants with non-participants. For TAP results z-scores were created, tested for normality, and used to analyze the possible relationships between domains of cognition and baseline characteristics. For assessing correlations between z-scores, Pearson's correlation scores were used. Based on linear regression analysis with z–scores as dependent variables and baseline characteristics as independent variables, we decided to use age, gender and level of education as covariates in multivariate regression analysis. Variables with positively skewed distributions (e.g., number of depressive episodes and number of (hypo) manic episodes) were logarithmic transformed for all analyses. Back-transformed geometric medians with Inter Quartile Rates are presented in the tables, when appropriate. To correct for multiple testing with 10 analyzed variables per cognitive domain (medication use; BD subtype; childhood adversity; current anxiety disorder; psychiatric co-morbidity; alcohol use; drug use; smoking; age of onset; current mood), we used the compound scores in our analyses. Results are considered significant with a p-value < .05.

Results

Clinical characteristics

In table 1 all population characteristics are summarized.

Anxiety disorder was mentioned separately, due to the high prevalence. All participants were euthymic with a mean QIDS score of 7.7 (SD=5.0), reflecting no or mild symptoms (http://www.ids-qids.org) and a mean YMRS score of 1.6 (SD=2.9), meaning no manic or hypomanic symptoms. The Pearson's correlation scores between the three cognitive domains were respectively r=.45, p<.001 (for the association between working memory and attention); r=.40, p<.001 (for attention- executive functioning); and r=.35, p<.001 (for working memory and executive functioning). Out of 187 participants, 184 (98%) completed the attention tests, 147 (79%) completed the working memory subtest and 166 (89%) completed all executive function subtests. All these patients were included, however for the in domain attention 42 patients and for the domain executive functioning 26 patients had 3 or more missing component tap items. When re-analyzing the data with exclusion of patients with >3 missing variables, effects reflected by β- coefficients were largely similar, though of slightly less strength.

On all component items of the TAP, the patient group showed longer mean reaction times with larger standard deviations, more omissions and more mistakes in comparison with persons from the general population described in the TAP manual as reference populations. They showed longer mean reaction times with larger standard deviations, more omissions and more mistakes in comparison with persons from the general population. As norm scores for sustained attention are unknown, no comparison was possible.

Table 1: *Characteristics in 187 participants with BD*

	Participants
Total N	187
Male sex; n(%)	75 (40.1)
Mean age; (SD)	48.6 (11.1)
Level of education;	
N (%):	
- primary	41 (21.9)
- secondary	55 (29.4)
- higher	91 (48.7)
Clinical characteristics:	
Diagnostic information; N(%)	
BD1	125 (66.8)
Childhood adversity	56 (30.4)
Current anxiety disorder	54 (28.9)
Psychiatric co-morbidity	75 (40.1)
Age of onset; mean (SD)	
Age of onset first (hypo-) mania	29.0 (11.9)
Age of onset first depression	24.9 (12.2)
Age of onset disease	26.7 (9.5)
Number of episodes; median (IQR)	
No. of manic episodes	5 (2-10)
No. of depressive episodes	6.5 (4-20)
QIDS; mean (SD)	7.7 (5.0)
YMRS; mean (SD)	1.6 (2.9)
Medication use; N(%):	
Lithium	
No lithium (ref)*	51 (27.3)
Only lithium	49 (26.2)
Lithium + other medication	87 (46.5)
Anti-epileptics	40 (21.4)
Anti-psychotics	43 (23.2)
Benzodiazepines	54 (29.2)
Antidepressants	64 (34.4)
No. of psychotropic med.ty pes; mean (SD)	2.1 (1.2)
Substance use; N(%):	
Alcohol use	
None	65 (35.1)
1-2 units/day	100 (54.1)
≥3 units/day	20 (10.8)
Drug use	14 (7.7)
Smoking	78 (42.2)

Abbreviations: BD indicates Bipolar Disorder; IQR indicates Inter Quartile Range; YMRS indicates Young Mania Rating Scale; QIDS indicates Quick Inventory of Depressive Symptomatology.

Influence of sociodemographic and clinical characteristics, medication use and substance use on cognition

In table 2, the sociodemographic and clinical characteristics are presented in relation to the three described cognitive domains.

Older age was significantly associated with all three cognitive domains: poorer attention (unadjusted β=-.35; p<.001), working memory (unadjusted β=-.19; p=.02), and executive functioning (unadjusted β=-.38; p<.001). Higher level of education was associated with better working memory (unadjusted β=.35; p< .001) and executive functioning (unadjusted β=.45; p<.001), but not with better attention. Mood scores on QIDS and YMRS were not associated with TAP scores. In table 3, β-coefficients with the accompanying p-values are presented, adjusted for gender, age and level of education.

With respect to clinical characteristics, we found that BD 2 was related to better attention (unadjusted β=.19; p=.009; adjusted β=.16; p=.03) and we found a trend towards better executive functioning in BD2 patients (unadjusted β=.15; p=.05; adjusted β=.11; p=.10). The number of manic episodes was also positively related to executive functioning (unadjusted β=.12; p=.13; adjusted β=.14; p=.04).

With respect to medication use, we found that the number of different types of psychotropic medication was associated with poorer performance on executive functioning (unadjusted β=-.19; p=.01; adjusted β=-.19; p=.004). When analyzed per medication type, Lithium use versus no Lithium use was not associated with cognitive function on any domain. However, when Lithium use was compared with Lithium plus other medication types, a statistical trend was observed that showed lower performance on executive functioning in the group using Lithium and other medication (unadjusted β=-.13; p=.13; adjusted β=-.19; p=.01). The use of anti-psychotics was related with lower scores on executive functioning (unadjusted β=-.22; p=002; adjusted β=-.20; p =.002). A statistically significant association was found for the use of antidepressants and lower attention scores (unadjusted β=-.16; p=.03; adjusted β=-.16; p=.02). Figure 1 shows the associations between the number of different types of psychotropic medication and cognitive function on the three domains.

When trying to gain insight in whether medication worsens cognitive function or whether the severity of illness confounded the association, all analyses with the number

of medication were additionally adjusted for severity of illness course variables age of first episode, number of depressive and number of manic episodes, QIDS score and YMRS score. Most associations persisted with beta-coefficients that did not decrease in strength.

Finally, with respect to substance use, we found an association between moderate alcohol use and attention, that remained significant after adjustment for gender, age, level of education, indicating that moderate alcohol use (of 1-2 units per day) was associated with slightly better attention versus no alcohol use. Smoking was also associated with better attention (unadjusted β=.15; p=.05; adjusted β=.16; p=.03).

Table 2: *Clinical characteristics in relation with cognitive performance in 3 domains*

	Attention (n=184)			**Working memory** (n=147)			**Executive functioning** (n=185)		
	N	b	**P**	**N**	b	**P**	**N**	b	**P**
Female (vs male gender)	111	.02	.84	90	.04	.67	111	-.07	.37
Age	184	**-.35**	<.**001**	147	**-.19**	.**02**	185	**-.38**	<.**001**
Level of education::									
- primary (ref)*	40	Ref.		25	Ref.		40	Ref.	
- secondary	55	.08	.44	44	.21	.08	55	.29	.**01**
- higher	89	.13	.15	78	**.35**	<.**001**	90	**.45**	<.**001**
Clinical characteristics									
Diagnostic information:									
BD 2 (BD 1 ref.)	122	**.19**	**.01**	101	.16	.06	123	.15	.05
Childhood adversity	54	-.08	.31	39	.01	.91	55	-.02	.82
Current anxiety disorder	55	-.10	.18	42	-.04	.65	55	.05	.55
Psychiatric co-morbidity	75	-.03	.67	61	-.04	.68	75	.07	.34
Age of onset:									
Age of onset first mania	163	-.15	.05	130	-.05	.58	164	**-.21**	**.01**
Age of onset first depression	167	**-.24**	.002	131	-.01	.94	168	-.14	.07
Age of onset disease	183	**-.21**	**.004**	146	-.05	.52	184	-.12	.10
Number of episodes:									
No. of manic episodes	163	.02	.76	130	-.01	.95	164	.12	.13
No. of depressive episodes	167	-.00	.96	131	.00	.97	168	-.01	.85
QIDS	180	-.02	.80	143	-.06	.51	181	-.04	.62
YMRS	181	-.01	.85	144	-.03	.76	182	.08	.23
Medication use:									
Lithium									
- no lithium (ref)*	50	Ref.		38	Ref.		50	Ref.	
- only lithium	49	-.02	.88	40	-.11	.36	48	-.00	.97
- lithium + other medication	85	-.06	.50	69	-.06	.57	87	-.13	.13
Anti-epileptics	38	.04	.58	30	.03	.73	40	.02	.83
Anti-psychotics	42	-.06	.44	35	-.12	.16	43	**-.22**	.**002**
Benzodiazepines	53	-.09	.25	40	-.06	.45	54	-.18	.**01**
Antidepressant	64	**-.16**	**.03**	48	-.06	.48	64	-.08	.29
No. of psychotropic med. types	184	-.10	.18	147	-.09	.30	185	**-.19**	**.01**
Substance use:									
Alcohol:									
- none (ref.)*	63	Ref.		43	Ref.		63	Ref.	
- 1-2 units/day	99	**.20**	**.01**	84	**.18**	.**04**	100	.16	.05
- ≥3 units/day	20	.09	.44	18	-.11	.40	20	.01	.96
Drug use (no drug use ref.)	14	-.08	.31	12	.08	.37	14	.12	.10
Smoking (non-smoking ref.)	77	.15	.05	61	.06	.48	77	-.02	.78

* For reference category no β can be estimated

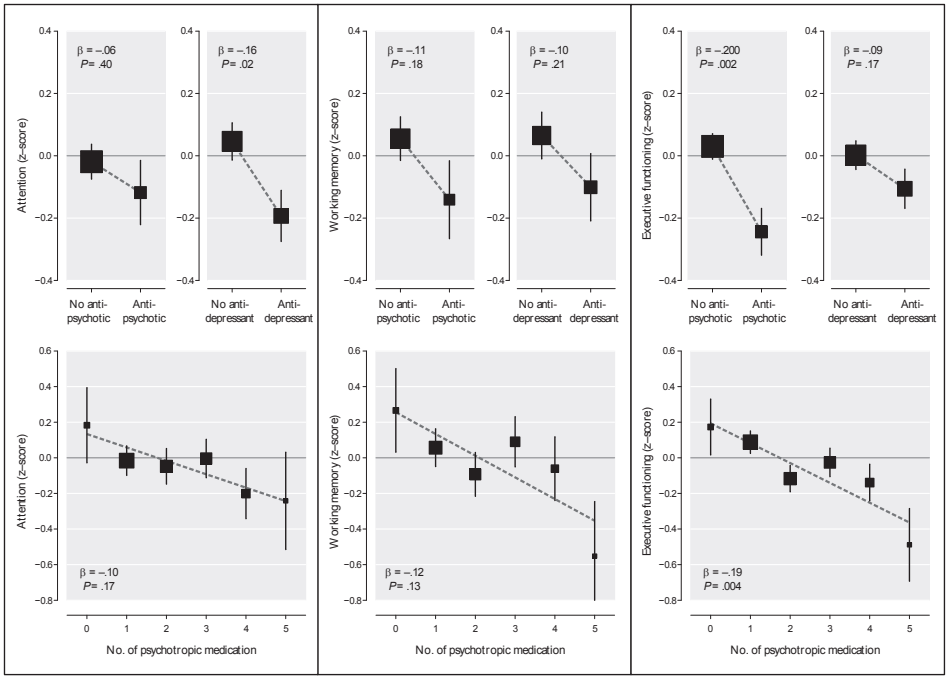
Table 3: *Clinical characteristics in relation with cognitive performance in 3 domains, adjusted for covariates age, gender and level of education*

	Attention (n=184)			**Working memory** (n=147)			**Executive functioning** (n=185)		
	N	b	**P**	**N**	b	**P**	**N**	b	**P**
Clinical characteristics									
Diagnostic information:									
BD 2 (BD 1 ref.)	59	**.16**	**.03**	45	.12	.13	59	.11	.10
Childhood adversity	54	-.10	.16	39	.03	.76	55	.01	.90
Current anxiety disorder	54	-.13	.06	42	.01	.94	55	.09	.18
Psychiatric co-morbidity	75	-.09	.24	61	.01	.95	75	.08	.24
Age of onset:									
Age of onset first mania	163	-.01	.87	130	.01	.97	164	-.03	.74
Age of onset first depression	167	-.15	.06	131	.03	.74	168	.01	.92
Age of onset disease	183	-.11	.12	146	-.03	.72	184	.02	.80
Number of episodes:									
No. of depressive episodes	167	.02	.80	131	.05	.53	168	.06	.42
No. of manic episodes	163	.02	.76	130	.04	.62	164	.14	**.04**
QIDS	180	-.02	.79	143	-.03	.72	181	.00	.99
YMRS	181	-.04	.54	144	-.04	.59	182	.06	.36
Medication use:									
Lithium use:									
- no lithium (ref)*	50	Ref.		38	Ref.		50	Ref.	
- only lithium	49	-.01	.95	40	-.07	.56	48	-.05	.58
- lithium + other medication	85	-.07	.38	69	-.07	.44	87	**-.19**	**.01**
Anti-epileptics	38	.03	.39	30	-.02	.83	40	.00	.95
Anti-psychotics	42	-.06	.40	35	-.11	.18	43	-.**20**	.**002**
Benzodiazepines	53	-.02	.75	40	-.02	.80	54	-.08	.26
Antidepressant	64	**-.16**	**.02**	48	-.10	.21	64	-.09	.17
No. of psychotropic med types	184	-.10	.17	147	-.12	.13	185	**-.19**	**.004**
Substance use:									
Alcohol use:									
- none (ref.) *	63	Ref		43	Ref		63	Ref	.40
- 1-2 units/day	99	**.16**	**.04**	84	.14	.10	100	.06	.79
- ≥3 units/day	20	.10	.35	18	-.08	.56	20	-.03	.07
Drug use (no drug use ref.)	14	.06	.43	12	.09	.25	14	.12	.85
Smoking (non-smoking ref.)	77	**.16**	**.03**	61	.08	.31	77	-.01	.74

* For reference category no β can be estimated

Figure 1.

Mean standard scores (with error bars representing standard errors) for attention (n=184), working memory (n=147), and executive functioning (n=185) in BD patients according to the use of antipsychotic medication, the use of antidepressant medication, and the total number of psychotropic medication used. The grey reference line shows the mean cognitive score. The size of each square is proportional to the number of patients. Scores are adjusted for sex, age, and education. Beta-coefficients and P values are calculated by multivariate linear regression analysis.



Discussion

We studied the relationship between cognitive performance and sociodemographic and clinical characteristics of BD, medication use and substance use. In this study we found that the non-illness related variables age and level of education were strongly associated with cognitive performance assessed with the TAP. With respect to clinical characteristics we found that BD2 is associated with better attention and more manic episodes are associated with better executive functioning. Second, the number of different types of medication was inversely associated with executive functioning, and non-significantly with poorer levels of attention and working memory. When focusing on the individual types of medication, antipsychotics were in particular associated with poorer executive functioning, while antidepressant use was associated with poorer attentional performance. Remarkably, Lithium had no influence on cognitive performance. Smoking and moderate alcohol use (1-2 units/day) was related to better attention, but was not associated with working memory or executive functioning.

Sociodemographic characteristics: Age and level of education

Age is a well known determinant of cognition, with mainly processing speed, reasoning, memory and executive functions declining with advancing age (16). This is referred to as cognitive ageing starting in early adulthood, which is in line with our results. The influence of the level of education on cognition has also been studied thoroughly in the elderly, whose educational level positive associated with late life cognition (17). This is in line with our findings that educational level was strongly associated with better cognitive performance.

Clinical characteristics

BD2 was found to be associated with a slightly better cognitive functioning on all three domains (though only significantly for attention) compared to patients with BD1. This finding was partly consistent with the findings from a meta-analysis (8), showing that BD2 patients performed slightly better only on memory tasks but furthermore the patients in this meta-analysis were equally severely impaired on other cognitive domains. Criteria to differentiate BD1 and BD2 are soft and hypomania is difficult to diagnose, leading to a risk of misclassification. For all these reasons, differences between BD1 and BD2 need to be more thoroughly studied. Regarding the influence of the number of mood episodes, it was recently found that a history with more manic and depressive episodes was associated with poorer working memory (18). However, no longitudinal studies

are available to further specify the influence of number of episodes. Our finding that number of manic episodes was positively associated with executive functioning should therefore be interpreted with caution. Longitudinal studies are needed to investigate the influence of the number of episodes.

Use of multiple types of medication

Many BD patients, even one third after one year medication use, use more than one drug simultaneously (7). In our patient group, an impressive percentage of 59.9% used 2 or more types of psychotropic medication. Kupfer et al. found that more than one third of bipolar patients even uses 3 or more different types of psychotropic medications (19). This could be explained by selection bias (see Strengths and weaknesses). Without taking into account the problematic side effects, interaction risks and health problems, use of multiple types of medication itself has previously been found to be negatively associated with cognition in 76 patients with bipolar (spectrum) disorder (18). Elie et al (20) also found negative effects on cognition in 56 patients who suffered from schizophrenia or schizoaffective disorder, possibly (partly) explained by high doses of antipsychotics. Although one explanation for the link may be that use of multiple types of medication adversely affects cognition in BD patients, the direction of causality may be more complex. It could indicate a higher degree of disease severity explaining both use of multiple types of medication and cognitive dysfunction. Another possibility is that patients with more cognitive deficits and subsequent worsening clinical outcomes, end up using more medication which may lead to use of multiple types of medication and as such to cognitive impairment. However, by adjusting our analyses for clinical severity parameters, all findings remained, thereby strengthening the hypothesis that medication use influences cognitive deficits.

Antipsychotic use and cognition

In observational research, it is not possible to completely disentangle the direction of this association. Besides a direct adverse effect of antipsychotics on cognition, it is again possible that third factors, like more severe disease activity or history of psychosis (21) could explain both the prescription of antipsychotics and poorer cognition. One argument supporting the hypothesis that cognitive deficits are adverse effects of antipsychotics is given by a recent study of Arts et al. (18), who followed cognitive performance in 76 bipolar patients over a period of 2 years. Use of second generation antipsychotics was associated with negative effects on cognition, mainly affecting motor speed and information processing. In particular antipsychotics were repeatedly correlated with worsening of cognitive performance and IQ in bipolar patients (22). Moreover, these findings seem to be rather specific for bipolar patients (5). Effects of antipsychotics in patients with schizophrenia were conflicting. However, Elie et al. (20) found that increasing antipsychotic daily doses as well as use of multiple types of medication were associated with poorer cognitive functioning.

Antidepressants and attention

Antidepressants may also be responsible for the occurrence of some of the side effects on cognitive dysfunction. Anticholinergic effects (for example resulting from the usage of paroxetine) are known to influence cognition, especially memory retrieval (23). The authors state that these effects might be even more pronounced in elderly patients. However, studies in patients with BD are scarce. In the study of Arts et al. no influence of antidepressants on cognition was found in BD patients (18). However, as antidepressants differ in pharmacological profiles it is difficult to assess this medication type as a group.

Lithium use and cognition

In our study, lithium use was not related to cognitive decline. In literature, short term and long-term use of lithium have been recognized to exert differential effects on cognition. Short term use could negatively affect processing speed (18). Subjective complaints of Lithium users clearly include mental slowness as well (24). Chronic lithium use was associated with minor (6) or no (25) cognitive problems. Recently, it has been shown that long-term lithium exposure in brain injured mice is leading to attenuation of neuronal degeneration in areas of the hippocampus (26), accompanied by improved performance in spatial learning and memory tasks during a 14 day follow-up. In human

patients with Bipolar Disorder, Arts et al. (18) also found that long term use of Lithium positively predicted longitudinal improvement in verbal learning.

Substance use

We found that smoking as well as daily alcohol use of 1-2 glasses is associated with improved attention. It is known that smoking is directly influencing the nicotinergic receptors, involved in for example attention, learning and working memory (27). Therefore, direct smoking effects cannot be ruled out. Light to moderate daily alcohol use is known to have preventive effects on (vascular) dementia (28). Our study included middle aged patients (mean age=48.6; SD=11.1). We hypothesize that light to moderate alcohol use could protect also against mild cognitive decline in these middle aged patients.

Strengths and weaknesses of this study

A strength of this study is the high number of patients compared to previous studies in BD that included a total of 10 to 76 subjects (10). Moreover, all patients were in a euthymic phase of the illness, thus ruling out the effects of current manic or depressed mood state and the median number of medication was high, suggesting a high disease severity on average. This limits the external validity of our findings and the possibility to extrapolate the conclusions to chronically unstable patients and patients with milder symptoms.

This study focuses on clinical characteristics and medication use. We are aware of the fact that other factors, for example, physical risk factors summarized in metabolic syndrome, are known to be related with worse performance on cognitive tasks (executive function, working memory) as well (29). This needs attention in future studies.

Two weaknesses should be noted. Firstly, because of the cross-sectional design, it was not possible to disentangle the direction of causality. More severe illness is likely associated to more extensive use of medication, and therefore it cannot be ruled out that disease severity rather than medication adversely affected cognition. However, antipsychotics and use of multiple types of medication are known to negatively influence cognition in other studies. In our study the findings remained alike after adjustment for disease severity parameters, suggesting a separate role for medication in influencing cognition. Secondly, selection bias is not ruled out, showed by the high percentage of patients using 2 or more different types of medications. This could have led to a more severe patient population included. Therefore, conclusions are only justified for a subgroup

of severely ill patients. Thirdly, information about the history of psychotic symptoms for example during manic episodes, was unavailable. Finally, it needs to be remarked that cognitive deficits is not automatically related with subjective cognitive complaints (30). Therefore, clinical relevance has to be further assessed in future research. It will also improve insight in clinical relevance, when it is possible to compare the cognitive performance of our group with a matched healthy control group.

Conclusion

We conclude that use of multiple types of medication, specifically with antipsychotic medication, is associated with poorer performance in three important cognitive domains in patients with BD. In the light of the aforementioned, we carefully recommend clinicians to take into account the long term side effects on cognition of antipsychotics and use of multiple types of medication when deciding about pharmacological treatment options in patients with BD.

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8. Discussion

In 2005, we started a study named The Bipolar Stress Study, at the outpatient department for Bipolar Disorders of PsyQ The Hague. The general topic of the Bipolar Stress Study was and is to identify risk factors that have impact on the clinical course and treatment of BD. In the study three levels and their interactions with the environment (stressful life events and social support) were distinguished: clinical functioning (phenotype), genetic variations and vulnerability (genotype) and two endophenotypes, namely cortisol exposure and cognitive functioning. For the cross-sectional study the data of 366 patients were collected (genotype and phenotype); 189 patients participated in the extended part of the study including the cognitive test at first visit and the 24 month longitudinal study.

In this dissertation, a part of the results is presented with the emphasis on the role of two endophenotypes, namely cortisol exposure and cognitive functioning. Here, the main findings will be critically presented, including the strengths and limitations.

Subsequently will be discussed:

1 GR /MR polymorphisms and clinical course of BD;

2 Cortisol levels in saliva and hair, the relation with clinical course of BD;

3 Associations of cognitive functioning with medication use in BD.

1. GR /MR polymorphisms and clinical course

Of the 366 patients enrolled in the cross-sectional part of the study, 326 patients were included in the genotypic assessments. Due to organizational reasons, the last 40 patients could not be genotyped. We found that several GR gene polymorphisms altering cortisol sensitivity associate with seasonal patterns of mood episodes, especially hypomania. In particular the 9β (rs6198), and, to a lesser extent, both the ER22/23EK (rs6189/rs6190) and the *Bcl*I (rs41423247) polymorphisms seem to associate with clinical characteristics of BD.

Even though this cohort is a large group with bipolar patients, for genetic association studies it is rather small. For example, only 8% of a healthy population (n=350; age is between 13-36 years old; not screened for psychiatric diagnoses), is carrier of the ER22/23EK on at least one allele. More frequent polymorphisms in healthy populations are the *Bcl*I (63% is carrier in the healthy population) and the 9β (31%) (1). However, contrary to Genome Wide Association Studies needing thousands of patients to find genetic associations with disease, this study is not designed to detect causes of BD. It is theory driven, expecting to unravel associations between several clinically relevant

genetic polymorphisms involved in regulation of cortisol sensitivity at the cellular level. The associations we found with clinical course of BD shed some light on the influence of changes in HPA-axis regulation on the disease. For example, the activity of the HPA-axis is known to be regulated by seasonal changes, with mild hypersensitivity for glucocorticoids during the winter. BD patients who also have a genetic vulnerability for dysregulation in the HPA-axis by GR polymorphisms may be more likely to develop seasonal mood episodes. However, several limitations have to be noticed. First, in this part of the study, the approach was cross-sectional with clinical data gathered retrospectively, leading to potential recall bias. This might have affected the reliability of the retrospectively collected data such as the reported age of onset of symptoms. On the other hand, we expect that due to the impact of first hypomania and mania these might be remembered quite well. Second, patients were questioned about their first illness symptoms, which hamper a sharp differentiation between symptoms and episodes. Third, results should be taken with caution, since we cannot rule out false positive genetic findings. Future studies are needed to replicate our findings.

Our findings of a relationship between GR polymorphisms and the course and characteristics of BD and the effect of treatment, can be added to a growing number of studies, in which the regulation and functioning of the GR has been shown to be related to clinically relevant aspects of mood disorders. One study showed a lower frequency of the G –allele of the *Bcl*I polymorphism in patients with excellent Lithium response (2). Other haplotypes are also more frequent in patients with partial or no Lithium response, further underlining the influence of differences in stress hormone (cortisol) sensitivity on medication response defining course of the disease(2). In another study, three GR gene polymorphisms in exon 9 (among which the 9β variant) were found to be associated with response to Lamotrigine but not to Olanzapine/Fluoxetine treatment (3). This is evidence in the body of literature supporting the relevance of GR polymorphisms in effect of antidepressant and mood stabilizing treatment. In addition to genetic variations in the GR, also changes of the GR function through gene activity and expression appears to be important (4-7). However, the exact mechanism is not elucidated yet. Direct evidence of involvement of the GR in BD has been shown by effects of GR antagonist mifepristone in bipolar depression, improving cognitive function and mood after one week treatment compared with placebo (8, 9, 9b).

In more detail it is interesting to notice the differences in direction of influence between polymorphisms. Specifically the 9β and the ER22/23EK polymorphisms are known to lead to mild glucocorticoid resistance, whereas other GR gene polymorphisms in this study (most important is the *Bcl*I polymorphism) are leading to mild glucocorticoid hypersensitivity (10-12). In trying to grasp this paradox, some issues have to be noted.

First, higher susceptibility to develop depressive episodes is found for both mild glucocorticoid hypersensitivity due to the *Bcl*I polymorphism (13), as well as mild glucocorticoid resistance due to the ER22/23EK polymorphism (13). This might indicate that the HPA-axis functioning knows an optimal set point with a U-shaped curve. Polymorphisms leading to relative resistance as well as SNPs leading to relative hypersensitivity in response to GCs, can both be involved in development of mood disorders. In this thesis we found that the ER22/23EK polymorphism is associated with an almost 8 years younger age of onset of BD (14). With the influence of GR polymorphisms, specifically the 9β or *Bcl*I polymorphism, on HPA-axis regulation, other influences, like seasonal induced changes in cortisol sensitivity, can further deregulate the system, leading to proneness for seasonal episodes as shown in chapter 4 (14).

Second, polymorphisms differentially affect intracellular signaling pathways of the GR, which might explain why for example both the ER22/23EK and 9β polymorphisms lead to resistance for GCs, but seem to differ in susceptibility for mood episodes. The 9β polymorphism has been shown to increase mRNA stability of the GR-β splice variant. This may result in more of the GR-β at the protein level, which dominantly inhibits the active GR-α isoform. Carriers of the 9β polymorphism have a higher susceptibility to develop rheumatoid arthritis (15, 16). Additionally, homozygous carriers of this polymorphism have been shown to be 70% less likely to be carrier of nasal S. Aureus (17). This indicates that the increased level of the GR-β splice variant leads to activation of inflammatory activity and resistance for GCs. The ER22/23EK polymorphism on the other hand is inducing decreased transcriptional activity because of an increase in a less transcriptional active translation isoform of the GR (the GR-A) (18), leading to diminished effects of GCs. This might be one of mechanisms involved in differences for susceptibility for developing mood episodes. However, further research is needed to shed light on exact consequences of GR signaling pathways in relation to mood.

Third, the already mentioned effects on the immune system should be taken into account to understand the effects of the polymorphism in relation to mood. In the afore mentioned study of Van Oosten et al. (15), in addition to the 9β polymorphism carriers, also the ER22/23EK carriers show a higher risk on developing rheumatoid arthritis, indicating a possible indirect effect on inflammation and the immune system. This is in line with the effect of the other polymorphisms, the *Bcl*I and N363S (rs6195), known to induce hypersensitivity for glucocorticoids, which lead to a lower risk on developing rheumatoid arthritis. Both may reflect a reduced (ER22/23EK and 9β) or increased (N363S and *Bcl*I) GC induced immunosuppression. Hypothetically, this may result in a more pro-inflammatory state of the immune system, which is also known to be the case during mood episodes and in BD in general (19, 20). The intertwining of the immune system and the HPA-axis is a complex and not fully understood field. However, it is important for

future research to study both systems in relation to each other. This may help to identify new pathways for treatment.

To conclude, further understanding is needed about the relation between functional polymorphisms of the GR gene, like *Bcl*I, 9β and the less frequent but influential ER22/23EK and clinical phenotypes in BD (and other mood disorders) like seasonal patterns, age of onset of disease and clinical course as well as therapy response. The role of MR gene polymorphisms in BD is not related to these factors in BD. However, future analyses will focus on elucidating the role of GR and MR polymorphisms in cognitive impairments in BD.

Furthermore, a complicating factor in understanding the role of GR and MR polymorphisms is that serum cortisol seems to fail in providing information about the potential mediating effects of cortisol on the relationship between GR and MR polymorphisms and clinical characteristics in bipolar patients. This might be due to the circadian rhythm of cortisol levels and the pulsatile way cortisol is secreted. Also, large daily variations due to e.g. acute stress or infection are also an important limitation of measuring serum cortisol levels. Using information about genetic variations known to be associated with altered cortisol sensitivity, results in a more adequately assessment of the lifelong true impact of cortisol at the tissue level. Taking GR genotypes into account may contribute to better understanding the relationship between glucocorticoids and BD. In the next paragraph this relation will be further discussed.

2. Cortisol levels in saliva and hair, the relation with clinical course of BD

In the introduction, an overview with different methods to evaluate HPA-axis functioning is provided. Previous studies used salivary or serum cortisol levels to evaluate HPA-axis functioning. With these assessment methods, insight in total cortisol levels, the Cortisol Awakening Response and changes in cortisol levels during the day can be obtained. However, these assessments do not provide insight in long-term functioning of the HPA-axis, due to the frequent fluctuations and influenceable cortisol levels. In BD studies, as well as in other studies with patients categorized according DSM-IV diagnoses, there were conflicting results. In addition to the changing levels of cortisol during the day, these results could reflect the limitations of the DSM-IV categories. Recently, clusters of symptoms in patients with depression and/or anxiety have been shown to correlate nonlinearly with the cortisol awakening rise (21), indicating the added value of analyzing symptom clusters above DSM-IV categories in depression and anxiety. In studies including bipolar patients, results vary from no differences in basal salivary or serum cortisol levels

in patients with BD versus healthy controls (22-24) to elevated cortisol levels in other studies(25, 26). Vreeburg et al, found that smoking has been found to associate with resistance for GCs, other factors influencing different HPA-axis measurements included sampling factors, health factors, sex and age. In order to minimize the influence of all factors involved in this variability, it is necessary to adjust analyses for confounders (27).

In order to obtain insight in long-term functioning of the HPA-axis, we used a newly developed assay with which cortisol can be measured in scalp hair. This method offers the opportunity to determine mean long-term cortisol levels and appears to yield a reliable estimate of long-term HPA-axis activity (28, 29). Furthermore, it is unique in its ability to retrospectively assess cortisol levels, an approach which can help evaluating the consequences of life events, or therapy interventions (28). Recent evidence shows that in veterans higher hair cortisol relates with PTSD and number of traumatic events (30). Additionally, long-term cortisol was higher in patients admitted for acute myocardial infarction compared to patients with other indications for admission on internal wards (31): cortisol levels were raised 3 months prior to the event. Shift work is also associated with higher long-term cortisol levels in hair, as well as with higher BMI in shift workers and healthy persons (32). This method seems promising for the future in following HPA-axis functioning over prolonged periods of time.

However, several issues regarding cortisol assessment in scalp hair have to be addressed:

First, it is not clear what is measured in hair: free or total cortisol. In the circulation approximately 75% of cortisol is bound to cortisol binding globulin (CBG), which is thought to be the biologically inactive state, circa 20% is bound to serum albumin, and around 4% is free cortisol. It is already convincingly found that total cortisol levels, vary significantly within and between individuals, thereby affecting the interpretation of HPA-axis test results (33). Still under debate but with support of recent evidence is the hypothesis that hair cortisol is reflecting free circulating cortisol levels. Evidence to support this is found in estrogen users. Oral contraceptives (OAC) stimulate CBG levels, but in OAC users, cortisol levels in scalp hair are similar to non users. This suggests that hair cortisol reflects free (biologically active) cortisol levels, and not total cortisol levels (28).

Second, long-term hair cortisol and short term saliva cortisol do not relate (34), which is also found in our study (chapter 5). The retrospective calendar provided by long-term hair cortisol could provide information about mean cortisol exposure and reflect long-term (weeks to months) gradual changes (28, 35-38), in contrast to saliva samples reflecting levels of acute stress response (minutes to hours). This indicates that these two methods could be nicely applied together, serving different purposes in evaluating the HPA-axis. The hair cortisol levels provide insight in long-term consequences for cortisol levels due

to life events, chronic stress, cardiovascular diseases, and treatments, e.g. treatment of Cushing's Syndrome (28). Saliva cortisol provides information of daily cortisol changes, with the CAR as awakening stress response, responsivity during the day by Experience Sampling Method and the cortisol day curve as information about the daily changes and diurnal rhythm. Challenge tests on the other hand, such as the Dexamethasone Suppression Test (DST) or the more sensitive Dex/CRH-Test, provide information about the magnitude and variability of the sensitivity of the negative feedback at pituitary level on cortisol production.

Third, the clinical relevance of this novel method of hair cortisol measurement has to be further investigated. Currently, evidence is swiftly mounting in a growing number of patient populations. For example, in generalized anxiety disorder (GAD), decreased cortisol levels in hairs have been found, but no differences in salivary cortisol levels between GAD patients and healthy controls (39). This is in line with our findings of lower hair cortisol levels in BD patients with co-morbid panic disorder and suggests also that hair cortisol levels may reflect the long-term cortisol secretion, whereas the results found with saliva or serum cortisol levels might include acute responses to the time and circumstances of assessment. Depressed medicated patients were found to have higher cortisol levels during 6 months before cutting hair strands, compared with healthy controls (40). In the future it would be very interesting to analyze hair cortisol levels in relation to clinical parameters such as medication effects, life events, remission and recurrence of mood episodes.

Fourth, it is not yet clear to what hair length retrospective cortisol levels can be measured reliably. The maximum length of hair strands differs between laboratories, differing between 6 cm (=6 months) (41) to several years depending on the length of the hair (28). This is probably due to differences in processing of the hair samples which slightly differs between laboratories.

Fifth, it seems that over time cortisol levels in scalp hairs remain stable. In over 2000 years old mummies changes in hair cortisol levels were assessed, with possibly one case of pathological cortisol raise (42), indicating the reliability over time of found cortisol levels. However, this needs further replication. Currently, a growing number of studies is validating this method and define the clinical relevance.

As already stated in the introduction of this thesis, in addition to medication use, the chronic course of the disease is also defined by another endophenotype, namely cognition. This endophenotype will be discussed below, but in this paragraph we will discuss the relation between cognition and long-term hair cortisol. In our study cognitive performance was measured at baseline, while the hair strands were in some cases cut almost 2 years later, at the end of the study period, which is leading to difficulties in

interpreting the results. Hair cortisol reflects long-term cortisol levels, and cognitive deficits are thought to be related with high cortisol levels on the long-term as well (43). It is known that patients with Cushing's Syndrome defined by chronic excessive cortisol levels suffer from early aging in cognitive performance and general cerebral loss of volume (44-46), specifically hippocampal atrophy.

In our preliminary analyses a negative relationship was found between executive functioning and hair cortisol levels. Patients with an above average score showed lower cortisol levels (p=.02; after adjustment for age, gender, and hair treatment, the use of hair products and frequency of hair wash, p=0.015). It has to be noted that these analyses need further replication and comparison with healthy controls. Although in healthy controls hair cortisol levels are stable over time, the fact that the cognitive tests in BD patients have been performed at baseline of the study and the hair analyses includes the period between the 18th and 21th month of the study complicates the interpretation of the results. Taken together, these findings underline the preliminary and explorative character of this new technique, which is in need for further validation and interpretation in a clinical relevant way.

To conclude, we and others found associations of hair cortisol with psychopathology (mood and anxiety), which are promising for future research. Hair cortisol measurements have a promise to serve as an endophenotype in identifying the role of regulation of the HPA-axis in the long-term course of BD. As we did not find any difference between healthy controls and BD as disease entity, we would recommend to study long-term hair cortisol levels in relation to cognitive performance over time, and also focus on symptom level like for example mood and anxiety over time. This is in line with recent studies focusing on symptoms clusters or dimensions in relation with for example HPA-axis functioning and metabolic syndrome (21, 47).

3. Associations of cognitive functioning with medication use in BD

In our study, cognitive performance was assessed by means of the Test for Attentional Performance ('Testbatterie zur Aufmerksamkeitsprüfung' (TAP), version 2.1, http://www.psytest.net/ ; Zimmermann & Fimm, 2002). The TAP is a widely used computer based standardized test battery and easy to use in clinical practice (48-50). However, when we started our study, no international consensus was reached about the use of instruments. Recently, the International Society for Bipolar Disorders (ISBD) proposed a more complete neurocognitive battery to asses global cognitive impairment and improvement, the ISBD Battery for Assessment of Neurocognition (ISBD-BANC)(51). This ISBD battery is composed of the neuropsychological tests that show the largest

patient-control effect size differences across the literature. Cognitive domains tested with this battery, include attention/ vigilance, processing speed, verbal learning and memory, executive functioning and working memory, visual learning and memory. Of these domains, the TAP considered attention, executive functioning and working memory. However, using a common set of standardized procedures will probably lead to more comparable results across different research groups spanning the international community.

As stated in the introduction, cognitive deficits are known to have “trait” as well as “state” features.

Cognitive deficits, as “trait” feature, are thought to be mainly caused by genetic factors and thus are an endophenotype of BD (52). This is in line with findings in unaffected siblings of BD patients performing worse on memory and executive functioning tasks compared with healthy controls (53), arguing that cognitive functioning could serve as an endophenotype in future research. This is also concluded in an earlier meta-analysis of Bora et al., finding that impairments of executive functioning, sustained attention and verbal memory were common both for patients as well as for relatives with larger effect sizes for the patient group (54).

However, in this meta-analysis deficits in processing speed and visual memory were only observed in patient groups, not in relative groups. Possibly, these functions reflect the influence of patient specific factors such as medication use (associated with psychomotor slowing) and earlier age of onset (associated with psychomotor slowing and verbal memory impairment). Thus, other, more “state-like” factors influencing cognitive abilities, seem important as well. This means that, in addition to the “trait” part of cognitive impairments in BD, it is clear that also “state” dependency like current mood, as well as other factors like medication use, are worsening cognitive performance (52). In this dissertation, before using cognition as endophenotype, we decided to first investigate the influences on the “state” of cognitive performance.

In chapter 6, we showed an association between use of number of medication types and worsening of executive functioning; antipsychotics and antidepressants associate respectively with poorer executive functioning and attention. Lithium showed no association with cognitive functioning. Regarding the literature about the relation between medication and cognition in BD, our results seem in line with previous findings: 1) Lithium induces mainly a slight slowing of processing speed and subjective impairment of cognitive functioning; no strong other significant influences on cognitive performance have been found (48, 55, 56). 2) Antipsychotic use was associated with level of memory and executive functioning in a group of 40 BD patients compared with 40 healthy controls (48). 3) Despite the large body of research, little attention has been

given to the long-term consequences for cognition of polypharmacy, a common practice in the treatment of BD. Moreover, long-term studies are scarce. One 15 year longitudinal study showed in patients with non-schizophrenic psychosis a more than twice better functioning and social adjustment in non medicated patients compared with patients on any psychiatric medication (46).

To conclude, the results in this thesis underline the importance of thoughtful prescription of medication, and especially caution in prescribing antipsychotics as well as in the use of 2 or more different types of medications. However, an important limitation of our study was the cross-sectional study design, which made it impossible to make statements about causality. For example, it could well be that patients with a more severe course of illness use more types of medication as well as suffer from more severe cognitive deficits. To be able to elucidate the direction of causality, we adjusted our analyses for all variables regarding severity of the disease, which did not change any finding. This supports our view that polypharmacy and especially antipsychotics, seem to negatively influence executive functioning.

In thinking about causes of cognitive impairments in BD, the “trait” as well as the “state” need attention. Moreover, as the scar hypothesis (57) suggests, cognitive performance can evolve during life under influence of for example number of mood episodes. This, together with medication use, current mood, and the genetically vulnerable profile (possibly caused by genetic vulnerability for BD) of cognitive performance could result in a detrimental evolution of cognition during the disease course. However, this topic should be considered more in detail, with attention for different domains of cognitive performance.

Strengths and limitations

In general, this thesis presents the first results of a cross-sectional and longitudinal study, based on a relatively large and well phenotyped cohort. Proper phenotyping is known to be crucial for this type of epidemiological genetic association studies (58). In addition to clinical information, data were also thoroughly collected with respect to endophenotypic (biological data, cognitive performance) and genotypic information, to explore the influence of cortisol exposure on BD. Selection of genetic polymorphisms was based on functionality of polymorphisms and was driven by the hypothesis that HPA-regulation (mediated by GR and MR) is influencing the parameters chosen on endophenotypic as well as phenotypic level. The functionality of the GR and MR polymorphisms has been convincingly shown, which is quite unique, for all polymorphisms in in vivo studies (with the DST, and somatic consequences of increased or decreased cortisol levels), and also

in in vitro bio-assays (GR: ER22/23EK, 9β, N363S; MR: I180V and -2G/C (59)) and even on molecular level (GR: ER22/23EK (18) and 9β(16)).

In the analyses of the cross-sectional data, several limitations have to be noticed:

First, data regarding illness characteristics were gathered by interviewing the patients with questionnaires, with a risk of recall bias.

Second, it is not possible yet at this point of the study to already claim clear causative conclusions, but only associative findings. However, our findings give direction to our future analyses of the longitudinal data. The longitudinal data hopefully will provide insight in the long-term influence of medication on cognition; furthermore, the influence of cortisol exposure on the longitudinal outcome will be analyzed.

Third, the clinical relevance of our genetic analyses of the MR and GR is yet limited. For a genetic association study, our cohort is relatively small. This is foremost of importance for less frequent polymorphisms like the ER22/23EK and the N363S SNP. Statistical power was appropriate for the more frequent polymorphisms (GR-9β, *Bcl*I). Our genetic association study suffers, like all such studies, several other possible fallacies. One of them is the risk on finding false positive or false negative results. In genetic studies, depending on SNP frequency in healthy populations, high numbers of participants are needed (58). To decrease the risk of having such false positive findings, we corrected all analyses for multiple testing. Nevertheless, it remains uncertain whether the finding in chapter 3, regarding lower number of hypomanic and manic episodes in carriers of the 9β polymorphism in patients with BD, is a false positive finding. After including more patients in the cohort, the strength of the association diminished to sub threshold significance level. Replication is needed in another independent cohort. Furthermore, besides false positive findings, and the importance of reliable phenotyping; other fallacies in genetic associations include influence of racial heterogeneity, and differences in gender and age (58). In our study, the cohort consisted of 95 % Caucasian people from Dutch ascend which could be regarded an asset of this study. Furthermore, patients were matched on age and gender with blood bank donors in this study (1). Notwithstanding these limitations these genetic studies merit continuation, as studies focusing on medication targeting the HPA-axis are promising and underline the relevance of the GR and MR function in mood disorders and cognition.

Fourth, the assay for cortisol assessment in scalp hair has only recently been developed. Although in the past years clinical data are rapidly accumulating that this is a reliable marker of long-term mean systemic cortisol levels, in this stage application in psychiatry as well as in other specialisms, is new. There are several issues that need to be addressed regarding this technique (60) : 1) the exact mechanism of how (through blood, sweat,

or sebum; probably most important through blood) and where (hair cortex or medulla) cortisol is incorporated in hair shafts, need to be further clarified; however, for our findings this is of no direct relevance, but in interpreting cortisol levels in relation with other HPA-axis assessments, this would be of relevance; 2) the grow speed of hairs is important in creating timelines of cortisol levels and therefore more research on possible interracial differences is important.

Fifth, hair cortisol analysis needs further embedding in clinical practice of mood disorders. Cortisol in hair can be regarded as a trait feature; however, severe stress, caused for instance by mood disorders, may lead to increased mean cortisol levels. Thus, the relation between life events and mood, but also between therapeutic success and mood, can be focus of research in the future. In our sample, numbers of patients with severe mood episodes were too low to be useful for analysis in this study. Our findings warrant further study of the use of hair cortisol in relation to BD.

Conclusive remarks

In this thesis several potential candidates are identified as risk factors influencing clinical course of BD. At the genotypic level, several GR gene polymorphisms changing cortisol exposure by sensitivity, associate with seasonal patterns of mood episodes, especially hypomania. In particular, the 9β polymorphism seems to associate with clinical characteristics of BD. At the endophenotypic level, higher cortisol exposure assessed by hair analysis is associated with more psychiatric co-morbidity and an older age of onset of the disease. However, due to our finding that no difference is found between the total group of patients with the broad phenotype BD and healthy controls, we would recommend to focus in future analyses with long-term cortisol on more defined and unambiguous long-term parameters like mood episodes in preceding months, anxiety symptoms, life events, and cognition, all in preceding months.

Cognitive function, as second endophenotype, is related to the number and types of medication used. It should be noted that while lithium had no effect on cognition, antipsychotics significantly did. In the analyses performed in this thesis regarding cognition, the main conclusion is that the rate impact of medication use on cognitive performance is still under debate. With these results in mind, the next phase in the analysis of this study is to investigate cognition as an endophenotype, in relation to e.g. GR and MR polymorphisms, cortisol in hair, and clinical course of BD.

Summarizing, cortisol exposure as determined by cortisol sensitivity at the genetic level and by measuring long-term cortisol levels using hair extracts, is associated with several clinically relevant phenomena defining the course of bipolar disorders. Furthermore,

cognitive functioning as second endophenotype, appears to relate to (number of different types of) medication.

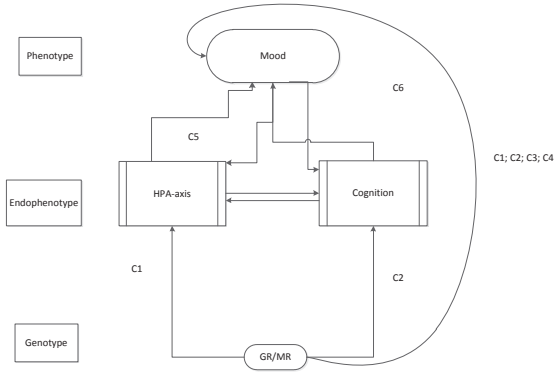
Future perspectives

First, as already noted, it would be very interesting to relate our data to the longitudinal course of BD. In box 1, the current findings are summarized in the light of the Bipolar Stress Study.

Box 1: findings and future directions with this thesis as part of the Bipolar Stress Study;

C1-C6 = Chapter 1 – Chapter 6.

Aims Bipolar Stress Study: *Identifying risk factors influencing clinical course of BD following figure 6 in the introduction:*



Current status:

In this figure knowledge is added on

1) *the relation of GR and MR polymorphisms to clinical characteristics of BD;*
2) *on the relation of endophenotype of the HPA axis to clinical characteristics of BD;*
3) *on influences like medication use on cognition to establish confounders in using this as endophenotype.*

Future directions: *The longitudinal part of the study will be investigated (life chart data):*

a. *What is the influence of genetic variations of the cortisol receptor on mood as measured by life chart? Is indeed the GR 9β and MRI180V protective? Can we confirm the relation to vulnerability for seasonal patterns?*
b. *How is the relationship between cortisol in hairs, environmental factors and course of mood, for example with sub analyses to patients with and without co-morbid panic disorder?*
c. *What is the influence of current mood as "state-factor" on cognitive performance?*
d. *What is the relation of cognitive performance to prospective course of the disease?*
e. *What is the influence of childhood trauma on endophenotypic as well as phenotypic level?*

Second, it would be very interesting to study immunological and inflammatory parameters in relation to the HPA-axis in BD and subsequent phenotypic data. The intertwining nature of both systems requires an integral vision to interpret the data in a meaningful way. Chronic stress is usually accompanied with raised pro-inflammatory cytokines (61), leading to higher risk of daily life infections and prolonged wound healing (62). In BD inflammatory changes are shown by a higher prevalence of organ auto-immunity (20) and pro-inflammatory activity in monocytes and anti-inflammatory activity in T cells (63), showing an imbalance in interleukin levels, which is normalized with Lithium treatment (19). An altered gene expression of inflammatory genes, found as a mRNA gene signature, was found in patients with BD (64), with a clear pro-inflammatory state of genes in monocytes (65). However, replication and meaning of those findings need further research. Possibly, these genes are also influenced by factors like altered GR sensitivity or medication use.

Third, a range of medical problems has been associated with BD (66). The most frequently described conditions are cardiovascular diseases, diabetes, and obesity (66). These are all related to what is referred to as the Metabolic Syndrome (MetS) (67), defined by the criteria from the National Cholesterol Education Program (NCEP) Adult Treatment Program III (ATP III)(68) these risk factors comprise three or more of the following five criteria: 1) an increased abdominal circumference (> 102 cm for men and > 88 cm for women), 2) hypertriglyceridemia (> 150 mg/dl), 3) a low level of high-density lipoprotein cholesterol (< 40 mg/dl for men and < 50 mg/dl for women), 4) hypertension (> 130/80 mmHg), and 5) a high fasting glucose level (> 100 mg/dl). Although several definitions of the MetS have been developed (69-71) the NCEP/ATPIII criteria are the most widely used in studies investigating the MetS. Taken into account the recent insights in metabolic changes due to for example atypical antipsychotics (commonly used in treatment of BD), it is urgently needed to understand shared mechanisms in psychopathology and hence be able to assess individual risks**.** The metabolic syndrome is highly prevalent in the general population (around 25% in the US (72) and around 15% in a Dutch population aged 28-59(73) to almost 20% in the healthy control cohort of the Netherlands Study to Anxiety and Depression (NESDA) (74). A relation with severity of depressive symptoms was predominantly found with abdominal obesity and dyslipidemia. MetS seems even more prevalent in bipolar patients (30% in the US study of Fagiolini (72) and 25.3% in an Italian sample (75)). In our sample almost 33% fulfilled the criteria of metabolic syndrome (unpublished analyses). The metabolic syndrome is repeatedly associated with increased sensitivity for glucocorticoids and with a pro-inflammatory state (76). Thus, physical health is important to evaluate in studies with attention for possible shared biological pathways.

And fourth, as a consequence of the above, the influence of medication has to be studied separately in longitudinal studies with respect to biological changes. It would be very relevant to study the consequences of different types of medication use on endophenotypes such as cortisol sensitivity and cognitive performance, but also on clinical phenotype with respect to number and severity of mood episodes. Iatrogenic damage due to antipsychotics or anti-epileptics in high risk patients should be avoided or minimalized.

As a final conclusive remark, the influence of the HPA-axis in relation with other biological systems, should be noticed to understand the clinical course of BD. Mood disorders are not limited to the brain, but involves wide bodily processes through hormones, cytokines and neural regulation.

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Summary

Introduction

Bipolar disorder (BD) is a common mood disorder, with an estimated prevalence of 2.4 % in the Netherlands. Worldwide, BD is estimated to stay in the top ten causes of Years Lived with Disability (YLDs), accounting for 2.5% of total global YLDs. BD was once considered as an episodic illness with a relatively favorable outcome, but nowadays more attention is given to the variant with a more chronic course of the disease with cognitive deficits in between episodes, residual mood symptoms and impaired functioning in daily life roles.

The core pathophysiological underpinnings of BD have to be elucidated to be able to understand the underlying processes leading to the clinical manifestations of the disease. One of the possible strategies in identifying biological underpinnings is the introduction of endophenotypes as alternative leads to find genetic vulnerability markers instead of the complex, phenotypical, disease symptoms and syndromes. Endophenotypes are known as subclinical quantifiable traits that exist in affected and unaffected relatives independent of the clinical manifestation of the disorder. The traits can be for example, neurophysiological, biochemical, endocrine, neuro-anatomical, or cognitive in nature. In this dissertation the focus will be on the role of cortisol exposure and cognitive performance as two possible endophenotypes influencing clinical course of BD.

Study aims

In order to identify risk factors, that have impact on the clinical course of BD and treatment of patients suffering from BD, we started the Bipolar Stress Study (BiSS). In this study three levels and their interactions with the environment (stressful life events and social support) were distinguished: clinical functioning (phenotype), genetic variations and vulnerability (genotype) and endophenotypes, specifically cortisol exposure and cognitive functioning.

In this dissertation, part of the results of this study is presented. The emphasis was on the role of genotypes of the cortisol receptors, and the endophenotypes, cortisol exposure and cognitive functioning. The results of the BiSS may help to develop clinically relevant diagnostic and treatment interventions to improve the course of BD and subsequently the quality of life of patients with BD.

Patients and Methods

The BiSS consisted of a cross sectional, and a prospective, longitudinal part. For the cross sectional study, data of 366 patients were collected with respect to genotype, phenotype

and endophenotype. In the 24 month longitudinal study, of these 366 patients, 189 patients participated. In this thesis, the cross-sectional data at baseline are analyzed. Genetic data were associated with clinical characteristics and retrospectively collected data on clinical course of BD. In a subpopulation of the 189 patients hair cortisol was analyzed in relation to clinical characteristics of BD at baseline.

In the longitudinal study, we first assessed cognitive performance of the 189 patients at the first visit of the longitudinal part. Cognitive functioning is regarded as endophenotype with trait-like characteristics. Additionally, it is known to be influenced by other, more state-like factors as well. In order to be able to properly investigate the role of cognitive performance as an endophenotype, it was necessary to first elucidate the influence of "state-factors", like medication use on the results of the cognitive tests. In this thesis, the influence of medication use on cognitive performance was analyzed with data of the first visit of the longitudinal part.

Results

1. Literature review (Chapter 1 and 2)

As a first step in approaching the relation between cortisol exposure and mood, the literature has been reviewed, regarding 1) glucocorticoid sensitivity and mood (chapter 1), and, 2) genetic variations, or polymorphisms, in the Glucocorticoid Receptor (GR) and Mineralocorticoid Receptor (MR) genes in relation to glucocorticoid sensitivity, mood and cognition (chapter 2).

Our main findings are:

Chapter 1:

- Hyperactivity of the HPA-axis occurs in 80% of the acutely depressed patients (either bipolar or unipolar) as measured by the Dexamethasone/ Corticotropin Releasing Hormone (DEX/CRH)-test, with higher cortisol levels after DEX/CRH administration.
- A long-term pattern of mild resistance for negative feedback signaling of glucocorticoids has been found, worsening during depressive episodes (not exclusively bipolar depression).
- Single nucleotide polymorphisms in the genes encoding the GR as well as the MR, influence the set-point and regulation of the HPA axis, both measured through the Dexamethasone Suppression Test (DST) with the use of a very low dose

Dexamethasone of 0.25 mg. This dosage is known to detect subtle changes in feedback response to cortisol elevation.

- The GR polymorphisms ER22/23EK (rs6189/rs6190) and 9β (rs6198) and the MR polymorphism MRI180V (rs5522) show a relative resistance for cortisol suppression after the DST. The GR polymorphisms *Bcl*I (rs41423247), N363S (rs6195) and the MR polymorphism -2G/C (rs2070951) show a relative hypersensitivity for cortisol suppression after the DST.
- In utero stress and early trauma are important (dys)regulators of this set-point, probably through epigenetic changes leading to different functional gene expression and subsequent protein synthesis.

Chapter 2:

- By looking at different polymorphisms in the GR gene and the relation with cognitive endophenotypic and phenotypic characteristics, it has been shown that the *Bcl*I (rs41423247) and ER22/23EK (rs6189/rs6190) polymorphisms are associated with risk on developing depression in several studies.
- The polymorphism ER22/23EK (rs6189/rs6190) associates with a decreased risk of dementia in healthy individuals.
- During a depressive episode, carriers of this ER22/23EK (rs6189/rs6190) variant demonstrated a tendency towards better cognition compared with non-carriers, as measured by divided attention tests.

Concluding, the above described polymorphisms in the GR and MR gene, are associated with phenotypic characteristics in mood and endophenotypic features in cognition, revealing a possible mediating mechanism through changing the HPA-axis set point. Mainly the *Bcl*I (rs41423247) and the ER22/23EK (rs6189/rs6190) are associated with higher risk for depressive episodes.

2. GR /MR polymorphisms and clinical course (chapter 3 and 4)

Due to findings in BD research that the HPA-axis is dysregulated, we investigated the GR and MR as important receptors in the regulation of the feedback loop in the HPA-axis on pituitary level. Hereby, we focused on genetic variations of the GR and MR gene in relation to clinical characteristics of BD. The results of the cross sectional study show that the GR and MR gene polymorphisms associate with clinical characteristics and retrospective course of BD, the exact findings are as follows:

Chapter 3

- In the first analysis (n= 245) no differences in frequencies of polymorphisms were found in bipolar patients compared to healthy controls.
- The 9β (rs6198) polymorphism in the GR gene is less frequent in bipolar patients with a history of more than a median number of 5 (hypo)manic episodes.

Chapter 4

- In the extended cohort (n=326, in 40 patients genotype failed), an association is found between the haplotype consisting of the 9β (rs6198) polymorphism in combination with the TthIIII (rs10052957) polymorphism in the GR gene and a higher risk on seasonal patterns of hypomania.
- For the BclI (rs41423247) haplotype, we observed a similar trend for higher risk of seasonal hypomania.
- Carriers of the ER22/23EK (rs6189/rs6190) polymorphism have an almost 8 years earlier onset of their first (hypo)manic episode than non carriers.
- No relation between MR gene polymorphisms and characteristics of BD was found.

Our findings indicate that 9β (rs6198), BclI (rs41423247), and ER22/23EK (rs6189/rs6190) influence clinical features of BD. This relationship of GR-polymorphisms with the course and characteristics of BD can be added to a growing number of studies, in which the regulation and functioning of the GR has been shown to be related to clinically relevant aspects of mood disorders. However, we found a non-linear relationship between GR polymorphisms and sensitivity of the central feedback on cortisol production. The idea is that the HPA-axis has an optimum set point in a U-shaped curve with regard to cortisol exposure at pituitary level. This is illustrated by the fact that some SNPs lead to relative resistance while others are involved in relative hypersensitivity in response to GCs: both can be involved in dysregulation of mood. Moreover, with the influence of GR polymorphisms, specifically 9β(rs6198) or *Bcl*I (rs41423247), on HPA-axis regulation, other factors, like seasonal induced changes in cortisol sensitivity, can further deregulate the system, leading to proneness for seasonal episodes as shown in chapter 4.

3. Cortisol levels in saliva and hair, and the relationship with clinical course of BD (chapter 5)

As first in the world, we applied a new technique for assessing cortisol levels in scalp hair in patients with BD. This could give us the opportunity to get insight in the influence of e.g. life events, mood episodes and effects of treatment on the long-term functioning of the HPA-axis. In chapter 5 we showed the following results:

- Compared to healthy controls, bipolar patients have similar cortisol levels in scalp hair.
- There is no association between hair and saliva cortisol levels assessed in the BD group.
- Within the BD patients with higher cortisol levels as measured in hair, we found an association with an older age at onset (>30 years).
- In addition, a higher rate of psychiatric co-morbidity with the exception of panic disorder was found in patients with higher cortisol levels.
- Remarkably, patients with co-morbid panic disorder reveal lower cortisol levels in hair, even when compared to healthy controls, whereas saliva cortisol was non-significantly increased.

Our findings raise the question whether changes in HPA-axis functioning might be limited to subgroups of patients or whether changes in HPA-axis functioning might be limited to the stress response itself and not to long-term mean cortisol levels. Additionally, the findings that saliva and hair cortisol did not correlate supports the concept that both analyses for cortisol indeed differ in what aspect of the HPA-axis functioning is assessed. Cortisol in hair reflects long-term free cortisol levels, whereas saliva reflects short-term free cortisol levels which are influenced by acute stress responses, diurnal variation, as well as pulsatility of cortisol secretion. Finally, several clinical characteristics correlate with hair cortisol levels; although results might be complex to interpret properly, our findings gives impetus to further research to the relationship of hair cortisol levels and retrospective clinical aspects of BD.

4. Associations of cognitive functioning with medication use in BD (chapter 6)

Currently, it has been convincingly found that cognitive deficits regarding attention, working memory and executive functioning, can have a negative impact on social

functioning and the clinical course of BD. Biologically seen, HPA-axis dysregulation can cause impairment of cognitive functioning. Before using cognitive functioning as endophenotype in relation to cortisol exposure and to clinical characteristics, we decided to first investigate the "state-like" effects on cognition. We started with analyzing the influence of medication use on cognitive performance, and continued with analyzing the influence of current mood on cognitive performance. Therefore, the Test for Attentional Performance (TAP) was explored in relation to medication use and clinical parameters. Our main findings in chapter 6 comprise:

- A significant association between use of multiple types of medication and poor cognitive performance regarding executive functioning.
- Attention and working memory showed a similar, however non-significant trend.
- Particularly the use of antipsychotics is associated with impaired executive functioning.
- Lithium use is *not* associated with diminished cognitive performance.

The question remains whether medication use, specifically use of multiple types of medication (polypharmacy), is causing cognitive impairments or is an expression of the severity of the disease. In other words, it might be possible that patients with a more severe form of BD need more medication. However, we adjusted the analyses with inclusion of disease severity, which did not alter the results, indicating that the medication use might be an independent factor. However, other study designs, like Randomized Controlled Trials, could investigate this important topic in more depth.

Conclusion

In this dissertation, several factors are identified as risk factors influencing clinical course of BD. At the genetic level, several GR gene polymorphisms altering cortisol sensitivity, associate with seasonal patterns of mood episodes, especially hypomania. In particular the 9β polymorphism (rs6198) seems to associate with clinical characteristics of BD. One hypothetical explanation could be the link with increased anti-inflammatory activity. These findings give further biological background to the importance of sensitivity of cortisol feedback response in relation to mood disorders.

At the endophenotypic level, higher cortisol exposure assessed by hair analysis is associated with more psychiatric co morbidity in BD patients, and an older age at onset of the disease. However, no difference is found between the total group of BD patients and healthy controls, indicating that long-term cortisol in scalp hairs is not changed

when comparing with a broad phenotypic entity like BD. This is underlining the need of precise defined and unambiguous long-term phenotypic parameters like mood episodes, anxiety symptoms, life events, and cognition, all in preceding months. This thesis gives impetus to further research with this technique, in order to identify consequences of chronic stress and chronic stress hormone elevations.

Cognitive function is another endophenotypic marker. Before using cognitive performance as endophenotype, we first investigated the influences of medication as potential confounder. We found that cognitive performance is related to the number and types of medication used. It should be noted that while lithium had no effect on cognition, the use of antipsychotics significantly did. With this result in mind, the next phase in the analysis of the Bipolar Stress Study will be to first investigate the influence of current mood on cognitive functioning as second potential confounder. Thereafter, we will study the relationship between cognition and GR and MR polymorphisms, as well as cognition as potential endophenotypic risk factor influencing course of BD.

Summarizing, cortisol exposure and cognition are both associated with several clinically relevant phenomena defining course of BD, thereby giving ground to further investigation of the consequences on the long-term effects of differences in cortisol exposure in BD patients in the Bipolar Stress-study.

Nederlandstalige samenvatting

Inleiding

Bipolaire stoornis,ook bekend als manisch depressieve stoornis, komt bij ongeveer 2% van de bevolking voor. De ziekte begint bij de meeste patiënten tussen het 20ste en 30ste levensjaar, en kan veel lijdensdruk geven en grote gevolgen hebben voor het functioneren op allerlei gebied. Onderzoek naar oorzaken, beloop, behandeling en gevolgen van de ziekte, zijn daarom van groot belang.

Bipolaire stoornis wordt gekenmerkt wordt door periodes van depressie afgewisseld met manie of hypomanie. Het ziektebeloop kan sterk verschillen tussen patiënten. Tussen de episodes door kunnen mensen volledig herstellen, en vaak met medicatiegebruik stabiel blijven. Ongeveer de helft van de patiënten is niet volledig klachtenvrij tussen episoden. Dit kan bijvoorbeeld betekenen dat ze lichte depressieve klachten houden, of veel stemmingswisselingen kennen. Sommige patiënten hebben last van cognitieve beperkingen ten gevolge van doorgemaakte episodes. Dit kan zich uiten in concentratieproblemen, vergeetachtigheid en moeite met complexere cognitieve taken, zoals plannen en overzicht houden. Dit kan in wisselende mate invloed hebben op het functioneren in de maatschappij en in de thuissituatie.

Tot nu toe is het niet mogelijk om in een vroeg stadium een voorspelling te doen over het verdere klinische beloop van de bipolaire stoornis. De biologische achtergrond en de genetische oorzaken zijn vooralsnog onvoldoende duidelijk om goed te kunnen begrijpen wat er gebeurt als een patiënt manisch of depressief wordt. Met andere woorden, de vraag is wat er eigenlijk gebeurt in het lichaam, inclusief de hersenen, als de stemming ontregelt.

Naast het kijken naar klinische verschijnselen en beloop (fenotype) kan gekeken worden naar endofenotypes. Endofenotypes kunnen een eenduidiger link geven naar genetische oorzaken, en bewegen zich als scharnier tussen genetica enerzijds en het heterogene klinische beloop anderzijds. Eén van de kenmerken van een endofenotype is dat het meetbaar is, en dat het relatief onafhankelijk van de fase van de ziekte is. In dit onderzoek zijn twee endofenotypes centraal gesteld, waarvan bekend is dat ze bij de bipolaire stoornis een rol spelen. Ten eerste is dat de gevoeligheid van de Hypothalamus-Hypofyse- Bijnier as (HPA-as) voor stresshormoon cortisol. Ten tweede is dat het cognitief functioneren van de patiënt dat mede bepaald wordt door de cortisol niveaus in de hersenen.

Doel en opzet studie

In dit proefschrift wordt een deel van het onderzoeksproject “The Bipolar Stress Study” gepresenteerd. Dat onderzoek kent een cross-sectioneel gedeelte en een prospectief longitudinale deel, waarbij de relatie tussen bovengenoemde endofenotypes en het ziektebeloop nader onderzocht wordt. In dit proefschrift ligt de nadruk op het eerste cross-sectionele gedeelte.

In dit gedeelte hebben we gekeken naar de mogelijke invloed van de verschillen in gevoeligheid van de HPA-as en van cognitief functioneren, op het beloop van de bipolaire stoornis. We zijn daartoe in 2005 begonnen met de “Bipolar Stress Study”, waarbij we gegevens verzameld hebben van in totaal 366 patiënten met een bipolaire stoornis die behandeld worden binnen het team Bipolaire Stoornissen van PsyQ Den Haag. De gevoeligheid van de HPA-as wordt bepaald door onder andere de genetische codering voor receptoren die zorgen voor de effecten van het stresshormoon cortisol, de cortisol receptoren. Verschillen in codering kunnen leiden tot een verhoogde gevoeligheid of juist resistentie (verminderde gevoeligheid) voor het cortisol signaal. We hebben de twee cortisol receptoren onderzocht, namelijk de Glucocorticoid Receptor (GR) en de Mineralocorticoid Receptor (MR). De GR is vooral actief als het cortisol niveau hoog is, dus tijdens de stress reactie. Het reguleert deze reactie door de aanmaak van cortisol af te remmen. In het brein is het betrokken bij de vorming van herinnering rond de gebeurtenis. De MR is actief als er lage cortisol niveaus zijn, dus in rust. In het brein heeft de MR een rol bij het behouden van stabiliteit, en het beïnvloeden van gedrag.

Daarnaast hebben we de hoeveelheid cortisol in hoofdhaar per tijdseenheid gemeten; dit als reflectie voor het lange termijn functioneren van de HPA-as. Tot slot hebben we gekeken naar het cognitief functioneren en de invloed van type en hoeveelheid medicatie gebruik door de patiënt.

Het doel van het onderzoek is om te kijken of verschillen tussen patiënten op het gebied van de stressrespons en van de cognitieve functie, van invloed zijn op het klinische beloop van de bipolaire stoornis. In dit proefschrift wordt een deel van deze vraagstelling beantwoord, namelijk:

a. Wat is de associatie tussen de genetisch variaties in de genen die coderen voor de MR en de GR, en de klinische kenmerken van bipolaire stoornis?

b. Wat is het verband tussen de lange-termijn cortisol niveaus en enkele belangrijke klinische kenmerken van bipolaire stoornis?

c. Wat is het verband tussen cognitieve functie en klinische kenmerken van bipolaire stoornis?

Resultaten

In **hoofdstuk 1** en **hoofdstuk 2** is de literatuur samengevat op het gebied van de biologische stressrespons. De gevoeligheid van de stressrespons wordt vooral bepaald door de gevoeligheid voor het stresshormoon cortisol. Het hormoon cortisol wordt in de bijnier geproduceerd en remt de eigen aanmaak, doordat het de productie van cortisol-stimulerende hormonen door de hersenen en de hypofyse remt. Dit wordt negatieve feedback genoemd. De acute stressrespons wordt dus ook weer actief beëindigd, wat een belangrijke, functionele actie is. Vermindering van de negatieve feedback leidt tot een chronische activatie van de stressrespons met als mogelijke negatieve gevolgen stemmingsklachten en cognitieve klachten, maar ook bijvoorbeeld vermoeidheid, suikerziekte en hoge bloeddruk. Om te begrijpen hoe het kan dat dit feedback mechanisme verminderd functioneert, is het van belang om te weten dat cortisol alleen werkt na binding met de cortisol receptor. Na binding met de cortisol receptor kan het cortisol door de celwand heen naar de celkern toe, en heeft dan direct effect op de activiteit van verschillende genen. Er zijn 2 verschillende cortisol receptoren, de mineralocorticoid receptor (MR) en de glucocorticoid receptor (GR). De MR bindt het makkelijkst met cortisol, met als gevolg dat bij lage cortisolconcentraties vooral de MR actief is. De MR in het brein is vooral gelokaliseerd in de hippocampus (belangrijke rol bij geheugenfuncties), de amygdala (centraal in de regulering van angst en emoties) en de prefrontale cortex (PFC; van belang bij hogere cognitieve functies). De GR is wijdverbreid in het brein, maar heeft eveneens een grotere aanwezigheid in onder meer de hippocampus en de PFC. De MR speelt een grote rol in het behouden van stabiliteit, met onder meer ook beoordelen van informatie, en selectie van gedrag. De GR is actief tijdens de stressrespons, als de cortisol niveaus fors stijgen, en heeft naast de negatieve feedback, een belangrijke functie bij het vormen van herinnering aan de stressor en de bijbehorende gedragsaanpassing in die situatie.

Er zijn als gevolg van een aantal bekende variaties in de genen die coderen voor de cortisolreceptor, kleine veranderingen in de receptor die kunnen leiden tot subtiele verschillen in de sterkte van de negatieve feedback. Deze subtiele verschillen kunnen echter op de lange termijn gevolgen hebben voor de lichamelijke en geestelijke gezondheid. Sommige genetische variaties, ofwel polymorfismen, in het gen dat codeert voor de GR kunnen leiden tot milde hypersensitiviteit (*Bcl*I), andere juist tot resistentie voor cortisol (9β, ER22/23EK). Ook de MR kent zowel een variatie die tot hypersensitiviteit leidt (-2G/C) als een die tot resistentie leidt (I180V). Zowel resistentie als hypersensitiviteit kan verband houden met depressieve episodes. De genoemde polymorfismen zijn allen in verband gebracht met verhoogd risico op depressie, met

uitzondering van MR -2G/C, die bij vrouwen juist associeert met optimisme en minder hopeloosheid.

In de **hoofdstukken 3 en 4** beschrijven we dat een aantal GR polymorfismen (*Bcl*I en 9β) associëren met een hoger risico op seizoensgebonden hypomanie. Daarnaast vonden we dat ER22/23EK dragers gemiddeld 8 jaar jonger waren bij eerste symptomen van de bipolaire stoornis.

Deze bevindingen laten zien dat kleine veranderingen in de genetische codering van de GR klinische invloed hebben op het verloop van de bipolaire stoornis. We vonden dit verband voor de meest voorkomende polymorfismen, en dan met name bij hypomanieën in de lente en zomer. De stress gevoeligheid is verschillend per seizoen; in de winter is er sprake van een milde resistentie voor het stresshormoon, en in de zomer juist een actievere respons bij stijging van het stresshormoon. Het kan zijn dat deze wisselingen van cortisol gevoeligheid in seizoenen versterkt wordt door klinische relevante GR polymorfismen. Het feit dat zowel grotere gevoeligheid als resistentie (respectievelijk *Bcl*I en 9β) voor cortisol leidt tot kwetsbaarheid voor het seizoensgebonden patroon van hypomanie, wijst erop dat de stressrespons een optimale+ balans kent.

De ER22/23EK is een apart polymorfisme, dat minder vaak voorkomt in de Westerse bevolking. Dit polymorfisme is zowel geassocieerd met een betere metabole status (dus smallere tailleomtrek, hogere botdichtheid, minder risico op diabetes) alswel met een verhoogd risico op depressie. De fors jongere leeftijd waarop dragers van ER22/23EK eerste symptomen kregen, zou overeenkomen met het idee dat de stemming kwetsbaarder is voor ontregeling bij deze mensen.

In **hoofdstuk 5** hebben we als eerste in de wereld een nieuwe methode toegepast voor bepaling van lange termijn cortisol niveaus bij patiënten met een bipolaire stoornis. In het hoofdhaar van patiënten is de hoeveelheid cortisol bepaald per 3 cm. Het haar groeit gemiddeld 1 cm per maand,wat betekent dat 3 cm een retrospectieve kalender verschaft van de gemiddelde cortisolwaarden van de afgelopen 3 maanden. In dit hoofdstuk laten we zien dat de hoeveelheid cortisol in haar bij patiënten met een bipolaire stoornis vergelijkbaar is met gezonde controles. Dit doet de vraag rijzen, of de in de literatuur gevonden ontregeling van de HPA-as wellicht beperkt is tot een subgroep van patiënten. We vonden inderdaad wel verschillen tussen bijvoorbeeld mensen met een bipolaire stoornis die wel of niet een paniekstoornis hadden. De groep patiënten met een paniekstoornis had een beduidend lagere hoeveelheid cortisol in het haar dan de patiënten zonder paniekstoornis. Patiënten echter met een andere co-morbide stoornis (dus niet paniekstoornis) naast de bipolaire stoornis, hadden juist een verhoogd cortisol vergeleken met de patiënten zonder co-morbide psychiatrische diagnose. Dit duidt erop dat de ziekere mensen een verhoogd cortisol hadden. Tot slot vonden we een verband

met de leeftijd waarop eerste symptomen van de bipolaire stoornis zich manifesteerden, en de hoogte van cortisol. Patiënten die ouder waren dan 30 jaar tijdens de eerste manifestaties van de ziekte, hadden een beduidend hoger cortisol, terwijl mensen die jonger waren dan 30 jaar bij eerste symptomen een met gezonde controles vergelijkbaar cortisol niveau hadden. Ons onderzoek geeft aanleiding om deze nieuwe methode van haarcortisol metingen verder te onderzoeken op betekenis en bruikbaarheid in dit veld.

Tot slot hebben we in **hoofdstuk 6** gekeken naar de resultaten van een test om cognitieve functies te meten: de Test for Attentional Performance. In het vervolg op dit promotie traject gaan we de relatie tussen de testresultaten en het klinische beloop van de bipolaire stoornis onderzoeken. Ten behoeve daarvan hebben we alvast gekeken naar de mogelijke invloeden van klinische factoren en medicatie op de testresultaten. Met deze test worden cognitieve functies gemeten, zoals concentratievermogen, geheugen en executieve functies (hogere complexe functies als bijvoorbeeld het vermogen te plannen, prikkels te verwerken en informatie te integreren; van groot belang bij allerlei dagelijkse processen). In dit hoofdstuk laten we zien dat vooral medicatiegebruik van invloed lijkt te zijn op executieve functies. Gebruik van antipsychotica blijkt vooral van invloed, en daarnaast ook het aantal verschillende soorten medicijnen die mensen gebruiken lijkt de executieve functies negatief te beïnvloeden. Hoewel we zo aanwijzingen hebben dat medicatie van invloed is op cognitieve functies, ook na correctie voor ernst van de ziekte, kan het verband ook andersom liggen: de ziekste mensen gebruiken gemiddeld meer verschillende soorten medicijnen en hebben meer cognitieve beperkingen. Om precies zicht te krijgen op de gevolgen van gebruik van verschillende soorten medicatie op cognitief functioneren, zou een andere onderzoeksopzet nodig zijn.

Conclusies uit dit proefschrift

De bevindingen in dit proefschrift wijzen op de betrokkenheid van de HPA-as bij de regulatie van de stemming van patiënten met een bipolaire stoornis. De volgende conclusies kunnen op basis van dit proefschrift getrokken worden:

1. Genetische variaties in het gen dat codeert voor de GR houden verband met klinische kenmerken van de bipolaire stoornis. Dragers van de *Bcl*I of de 9β variant hebben vaker last van seizoensgebonden patronen van hypomanie. De ER22/23EK dragers hebben een 8 jaar eerder ontstaan van symptomen. Deze bevindingen ondersteunen eerder onderzoek, en maken duidelijk dat genetische variaties op lange termijn, weliswaar subtiele, gevolgen kunnen hebben voor het beloop van de bipolaire stoornis.

2. Op endofenotypisch niveau is een voorzichtige conclusie te trekken dat een aantal subgroepen van patiënten met een bipolaire stoornis een verhoogd lange termijn cortisol (gemeten in hoofdhaar) hebben. Dit betreft patiënten die ouder dan 30 jaar waren bij de aanvang van stemmingsklachten en patiënten bij wie meerdere psychiatrische diagnoses gesteld zijn. Patiënten met een paniekstoornis naast de bipolaire stoornis hadden juist een verlaagd cortisol. Dit zijn wereldwijd de eerste bevindingen met deze techniek bij patiënten met een bipolaire stoornis, ze moeten daarom wel bevestigd worden in ander, onafhankelijk onderzoek. Wel maken deze resultaten duidelijk dat cortisol niveaus niet bij alle patiënten verhoogd zijn, maar een indicatie kunnen geven van een subgroep die mogelijk een ander beloop kent van de ziekte. Dit zou gevolgen kunnen hebben op de keuze van behandeling.
3. Cognitief functioneren is een belangrijk domein bij bipolaire stoornissen. Medicatie gebruik, waaronder ook de hoeveelheid verschillende soorten medicijnen, moet in toekomstig onderzoek zeker als co-variaat meegenomen worden. Lithium bleek geen verband te houden met cognitieve dysfunctie.

De twee endofenotypes, HPA-as regulering en cognitief functioneren, gaan we in de toekomst verder onderzoeken in relatie tot beloop en ernst van de bipolaire stoornis. De lange termijn gegevens die we verzameld hebben, zullen we hierbij gebruiken om te zien of de voorgaande conclusies stand houden. In dit kader willen we bijvoorbeeld kijken of er subpopulaties van patiënten zijn bij wie de stemming ontregelt in samenhang met een ontregeling van de HPA-as. Kortom, het beter kunnen voorspellen van het ziektebeloop van deze ziekte, die zich meestal zo vroeg in het volwassen leven openbaart en waarbij een groot risico bestaat op blijvende klachten, is van groot belang. Dit zou op termijn mogelijkheden kunnen geven om de behandeling hierop aan te passen en te verbeteren. Hiermee zou hopelijk de schade beperkt kunnen worden die depressies en manieën kunnen aanrichten.

Curriculum Vitae

Annet Spijker was born on July 31, 1975, in Rotterdam. She graduated from high school in 1993, at the Gereformeerde Scholengemeenschap Rotterdam. In september 1993, she started studying medicine at the Erasmus University Rotterdam, and finished in 1997. Subsequently, she graduated cum laude for her propaedeuse Philosophy at the Erasmus Universiteit Rotterdam. From 1998 until 2000, she followed medical internships and graduated for her MD exam. After working as MD in the Vlietland Hospital in Vlaardingen on the neurology, internal diseases and emergency wards, from 2001-2002, she worked in Delft, GGZ Delfland on a general psychiatric inpatient ward.

From 2002-2007, she completed her psychiatry residency in the Parnassia Group, The Hague. She graduated in 2007.

Since 2007, she worked on the outpatient department for Mood disorders, in PsyQ The Hague, with special focus on patients suffering from bipolar disorder. Besides patient care, and development of the Program for Bipolar Disorders, she started with the research project “Bipolar Stress Study”, of which the results partially are presented in this PhD thesis.

Dankwoord

In mijn eentje was dit proefschrift zeker nooit tot stand gekomen, en ik wil op deze plaats dan ook graag een heel aantal mensen bedanken.

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Margreet, Marieke en heel veel anderen: dank je wel voor alle gezelligheid, support en gesprekken door de jaren heen.

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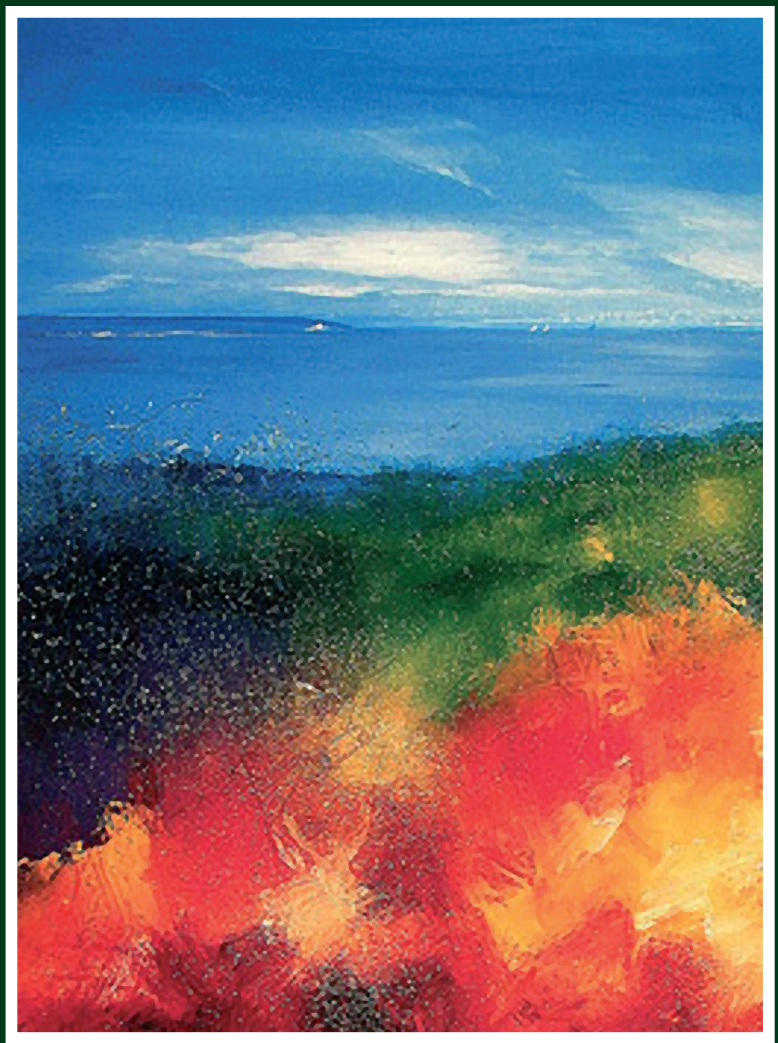

Stellingen behorende bij het proefschrift

Cortisol exposure, cognition and clinical course of bipolar disorder

1) De polymorfismen 9β en *Bcl*I van het gen coderend voor de glucocorticoid receptor (GR) kunnen leiden tot een verhoogde kans op hypomanie in het voorjaar (*dit proefschrift).*

2) Hoewel het polymorfisme ER22/23EK van het GR gen associeert met gezondere metabole status (*Van Rossum et al; JCEM,2004, 89(8): 4004-9)*, hebben dragers van deze variant wel een groter risico op depressie (*Van Rossum et al; 2006, Biol Psych, 59(8): 681-8)*, en een duidelijk eerdere aanvang van klachten van een bipolaire stoornis (*dit proefschrift*).

3) Cortisol bepaling in hoofdhaar geeft wezenlijk andere informatie over het functioneren van de Hypothalamus Hypofyse Bijnier- as dan alle eerder gebruikte cortisol testen (*Manenschijn et al, Steroids. 2011,76(10-11):1032-6).* Haar cortisol waarden reflecteren de systemische cortisol waarden van de maanden voorafgaande aan de haarafname en is daarmee een veelbelovende maat voor toekomstig onderzoek (*dit proefschrift*).

4) Het cortisol in hoofdhaar is hoger bij mensen die ouder dan 30 jaar waren bij eerste klachten van bipolaire stoornis; mensen die jonger waren dan 30 jaar hadden een cortisol wat vergelijkbaar is met gezonde controles. Dit wijst op het bestaan van een subgroep patiënten bij wie de Hypothalamus- Hypofyse- Bijnier- as functie een rol speelt (*dit proefschrift*).

5) Executieve functies, van belang bij planning en ordening van taken, zijn verminderd wanneer patiënten met een bipolaire stoornis meerdere psychofarmaca tegelijkertijd gebruiken. Vooral bij gebruik van antipsychotica speelt dit een rol (*dit proefschrift).*

6) Genetische variaties in het GR gen kunnen leiden tot hypersensitiviteit dan wel resistentie voor cortisol effecten (*Van Rossum et al,Recent Prog Horm Res. 2004, 59: 333-57*). Van beide soorten polymorfismen is een relatie met stemmingsstoornissen aangetoond. Dit is suggestief voor het belang van een optimale balans voor cortisol gevoeligheid met betrekking tot het functioneren van de stress respons (*Van Rossum et al; 2006, Biol Psych, 59(8): 681-8).*

7) De nieuwe huisartsenrichtlijn voor depressie en depressieve klachten waarin initieel leefstijlinterventies en stressreductie centraal staan (*NHG standaard Depressie*, *2012*), zou breder getrokken kunnen worden door het ontwikkelen van een algemene medische richtlijn “Gezond leven voor lichaam en geest”, ofwel “Mens sana in corpore sano” (*Juvenalis- Satire X*).

8) De vraag of psychofarmaca ook een rol zouden kunnen spelen bij het induceren van een chronisch verloop van psychiatrische ziekten moet serieus genomen moeten worden (*R. Withaker - The anatomy of an epidemic)*. Zie het toegenomen risico op een chronisch beloop van de bipolaire depressie bij gebruik van antidepressiva (*S. Nassir Ghaemi; Am J Psychiatry, 2008; 165:300-302)*.

9) Door de onvermijdelijke toename van online communicatie met patiënten, verandert de communicatie qua vorm (zien, bellen, mailen, chatfora, etc) maar vereist ook andere vaardigheden van de behandelaar. Dit verdient een kritische verkenning van grenzen, mogelijkheden maar ook aanpassing in het opleiding curriculum.

10) Het is stellig gemakkelijker zichzelf te veranderen dan anderen om te vormen *(Uitspraak van Zeno; Italo Svevo- Bekentenissen van Zeno)*.

11) Hoewel het als onbeleefd ervaren kan worden door medeweggebruikers, is het wel rationeel om op de weg volledig gebruik te maken van de invoegstrook; zo wordt de weg optimaal benut (*Tom Vanderbilt - Traffic*).

12) Verwende kinderen kunnen in het volwassen leven te maken krijgen met Vrouw Holle, die egocentrisme beloont met pek en veren (*sprookje Vrouw Holle*).