Translational psychiatry; the twists and turns of early life stress and serotonin transporter gene variation

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Rick H.A. van der Doelen, 2015; Translational psychiatry; the twists and turns of early life stress and serotonin transporter gene variation.
PhD Thesis, Radboud university medical center

The research described in this thesis has received funding from the Netherlands Organisation for Scientific Research (NWO), ALW grant 819.02.022

The publication of this thesis was financially supported by the Department of Anatomy, Donders Institute for Brain, Cognition and Behaviour, Radboud university medical center

Cover design and lay-out
Promotie In Zicht, Arnhem

Printing
Ipskamp Drukkers BV, Enschede

ISBN
978-94-62840-15-7

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Chapter 1

General Introduction

Rick H.A. van der Doelen, Tamás Kozicz and Judith R. Homberg
1. Depression

Major depressive disorder, commonly referred to as (unipolar) depression, is a psychiatric disorder that inflicts a huge personal and societal burden (Schmidt et al., 2011; Morava and Kozicz, 2013; Belzung, 2014). The lifetime prevalence of depression has been reported to range between 15% and 20%, with women having a twofold higher risk than men (Kendler et al., 2002; Wittchen and Jacobi, 2005). Depression often becomes chronic, shows considerable comorbidity with anxiety and other psychiatric disorders, and also somatic diseases (Paile-Hyvärinen et al., 2007). Furthermore, according to the World Health Organization (WHO, 2008), depression is among the leading causes of disability worldwide, in third place in 2008 and predicted to be number one in 2030. In Europe, the financial costs associated with depression have been estimated to equal to about 1% of the total gross economic product of the European Union (Schmidt et al., 2011).

Depressed patients are typically treated with selective serotonin reuptake inhibitors (SSRIs) or selective noradrenalin reuptake inhibitors (SNRIs). These drugs block the transporter proteins that are located on axon terminals of the central nervous system (CNS), and are major regulators of serotonin and noradrenalin levels in the synaptic cleft. The SSRIs and SNRIs are the refined successors of the antidepressants that were discovered serendipitously in the 1950s. Whereas they have considerable less side effects, the SSRIs/SNRIs have provided little improvement regarding efficacy, remaining sub-optimal with regard to latency of onset of effect, remission and recurrence (Kirsch et al., 2008; Fournier et al., 2010); only about 50% of all patients demonstrate complete remission, with relapse rates of more than 40% (Huynh and McIntyre, 2008). The failure to improve the efficacy of pharmacological treatment is mirrored by the failure to provide molecular targets for the development of novel antidepressant therapies (Pryce and Seifritz, 2011; Hyman, 2014). At the same time, depressed patients can be successfully treated with psychotherapy and electroconvulsive shocks, or their combination with pharmacological treatment (UK ECT Review Group, 2003; Khan et al., 2012). Nevertheless, it has been urged to improve the current pharmacological treatment options, and for this it is necessary to elucidate novel molecular targets that are at the core of the biological causes of depression (Nestler et al., 2002; Pryce and Seifritz, 2011; Schmidt et al., 2011; Hyman, 2014).

As the knowledge of the pathophysiology of depression is limited, diagnosis is entirely based on the symptoms and course of the disease state. The most widely used manuals for the diagnosis of psychiatric disorders are the Diagnostic and Statistical Manual of Mental Disorders (DSM, 5th Ed 2013) of the American Psychiatric Association and the International Classification of Diseases (ICD, 10th Ed 2010) of the WHO. Based on the well-described and universally observable symptoms, the categorical approaches of DSM and ICD have enabled a standardized, unambiguous diagnostic system. However, patients can qualify for the diagnosis of depression by displaying variable sets of symptoms, and therefore, patients can vary considerably in clinical presentation (Box 1).
Consequently, depression is not a single disease, but a heterogeneous syndrome of likely distinct causes and pathophysiologicals (Nestler et al., 2002; Pryce and Seifritz, 2011). Furthermore, the all-or-none (qualitative) nature of the diagnosis of depression is incompatible with the psychological and neurobiological research (quantitative) that aims to elucidate the pathophysiology of depression. Indeed, during the development of the 5th edition of DSM (DSM-5, 2013) a conceptual change has been made by the adoption of a spectrum of phenotypes for diagnosis of certain disorders, also called the dimensional criteria for diagnosis. However, the categorical approach is still dominant over a spectrum of phenotypes for diagnosis of certain disorders, also called the dimensional criteria for diagnosis. Furthermore, the National Institute for Mental Health (USA) has launched a new approach to classification of depression and other psychiatric disorders, called the Research Domain Criteria (RDoC) project (Insel et al., 2010). Others have also supported the shift in psychiatric research from categorical to dimensional concepts of psychopathology, i.e. from the study of binary disease entities to that of endophenotypes (Hasler et al., 2004; Hyman, 2007; Miller and Rockstroh, 2013; Pryce and Klaus, 2013; Davis and Isles, 2014).

Endophenotypes and RDoCs can be regarded as equivalent, being biological or psychological characteristics that are quantifiable, and that are considered to occupy the terrain between etiological factors and disease symptoms. Important advantages of the endophenotype approach are the expected improvements in the obtainment of homogeneous study samples, and the amenability of animal models for depression. Therefore, studying endophenotypes is predicted to stimulate the identification of relevant pathophysiological changes, as well as genetic and environmental risk factors for depressive symptomatology (Homberg, 2013; Miller and Rockstroh, 2013; Pryce and Klaus, 2013).

The heritability of depression has been estimated to contribute to 30-50% of the risk to develop depression (Sullivan et al., 2000; Kendler et al., 2002, 2006), with the remaining liability accounted for by non-genetic factors as stress and traumatic events, but also viral infections and even stochastic processes during brain development (Sullivan et al., 2000; Nestler et al., 2002; Kendler et al., 2003). Despite the considerable levels of heritability for depression, the replicability of genetic association studies has been lacking (Bogdan et al., 2013; MDD Working Group of the Psychiatric GWAS Consortium, 2013). This ‘missing heritability’ could be explained by limitations of the current technology, stringent statistical approaches, the small effect sizes of genetic variants, and the lack of power to detect epistasis (gene x gene – GxG – interaction) (Davis and Isles, 2014). In addition, a likely explanation is that the effects of genetic variants are not independent of environmental factors, and therefore, epidemiological studies should incorporate the interactive nature of genome and environment (Uher, 2010; Bogdan et al., 2013). Indeed, it is becoming increasingly clear that vulnerability to depression consists of a complex interplay of genome and environment. Gene x environment (GxE) interactions are generally referred to as genetic variants that confer differential susceptibility to particular environments (Ellis et al., 2011; Pluess and Belsky, 2012). Furthermore, by affecting behaviour and personality characteristics, genetic variation is also thought to influence the exposure to particular environments (Jaffee and Price, 2007).

On the other hand, environmental events can affect gene expression by affecting epigenetic mechanisms as DNA methylation and hydroxymethylation, histone modifications (e.g. acetylation, methylation), and downstream of transcription, the actions of noncoding and microRNAs which act by modulating mRNA stability and translation (Mill and Petronis, 2007; O’Connor et al., 2012; Sun et al., 2013). Moreover, epigenetic programming of gene expression is considered to be a key interface for GxE interactions (Bale et al., 2010; Meaney, 2010; Van Ijzendoorn et al., 2010; Bogdan et al., 2013; Zannas and West, 2014). DNA methylation, catalyzed by DNA methyltransferases, generally occurs at cytosine-phosphate-guanine sites and is associated with repression of gene expression (Moore et al., 2013). DNA methylation is a relatively stable epigenetic mark, as exposure to ELS has
been shown to alter the DNA methylation and transcription of stress-related genes in the hippocampus of adult mice, rats and humans (Weaver et al., 2004; Mueller and Bale, 2008; McGowan et al., 2009; Chen et al., 2012).

2. Stress

Perhaps the best studied and validated environmental risk factor for depression is the experience of stress, particularly the exposure to chronic stress or traumatic events across development (Heim et al., 2008; Schmidt et al., 2011). Stress has been defined as any disturbance of the physiological as well as the psychological integrity (homeostasis) of an individual (Gally, 1936; De Kloet, 2013). Stress involves a stimulus – the stressor – that is recognized as a threat to homeostasis and that elicits specific response mechanisms (Levine, 2005; Scharf and Schmidt, 2012). A psychological stressor can generate a stress response that is clearly distinct from physical stressors, not in the last place because it can already occur in anticipation. The most severe psychological condition involves ambiguity and uncertainty as this renders the individual unable to predict or control the situation (De Kloet, 2013). Depressive patients frequently report hopelessness, helplessness and the inability to cope with environmental events (Beck et al., 1974). Although these feelings are not diagnostic symptoms of depression, stressors that are (perceived to be) unpredictable and uncontrollable appear to be major environmental etiological factors in depression (Koolhaas et al., 2011; Pryce and Seifritz, 2011).

The adverse consequences of chronic stress exposure have been explained by the concepts of allostatic load and allostatic load. Allostasis, stability through change, is an essential component of maintaining homeostasis as it encompasses the adaptations that are induced in response to a (stressful) challenge. The costs of the adaptations associated with allostatic load have been termed allostatic load. In circumstances where challenges are not adequately met (maladaptation), chronic stress exposure will increase this allostatic load, which ultimately can lead to exhaustion of adaptive mechanisms, and disease (Sterling and Eyer, 1988; McEwen, 1998). It has recently been proposed that, in certain cases, mitochondrial dysfunction could be a major predisposition to psychopathology that arises from maladaptation because of a lack of energy availability (Morava and Kozicz, 2013).

2.1 Early life stress

Early life stress (ELS) in the form of childhood maltreatment has been associated with increased risk to develop depression, but also bipolar disorder, anxiety disorders, post-traumatic stress disorder (PTSD), substance abuse, personality disorders and psychoses (Mullen et al., 1996; McCauley et al., 1997; MacMillan et al., 2001; Scott et al., 2010; Teicher and Samson, 2013). Childhood maltreatment is characterized by sustained or repeated exposure to events that usually involve a breach of trust (De Bellis, 2001). Active examples include childhood sexual, physical and various forms of emotional abuse, while passive examples include emotional and physical neglect. Teicher and Samson (2013), among others, have provided extensive and well-described assessment criteria and definitions for the subtypes of childhood maltreatment (Box 2).

**Box 2** Assessment criteria for childhood maltreatment (adapted from Teicher and Samson, 2013; before the age of 18 years: sustained or repeated exposure to events involving a betrayal of trust by caretakers or other significant individuals in the child’s life

**Active maltreatment**

- Emotional abuse
  - Verbal aggression (communications intended to inflict intense humiliation, denigration, or extreme fear)
  - Emotional manipulation (placing the child in a situation intended to elicit shame, guilt or fear in order to serve the emotional needs of the perpetrator or to persuade the child to perform actions against his or her will or denigrating or destroying things of value to the child)
  - Witnessing domestic violence (witnessing adults in the household intentionally humiliating, demeaning, or threatening to harm one another or other family members or actively engaged in physically harming family members)
  - Physical abuse (hitting with objects, intentionally inflicting harm that results in bruises, welts, or need for medical attention, forcing child to remove clothing or otherwise humiliate him- or herself in front of others)
  - Sexual abuse (adults or older children touching or fondling the child’s body in a sexual way or forcing the child to remove clothing or otherwise humiliate him- or herself in front of others)
- Sexual abuse (adults or older children touching or fondling the child’s body in a sexual way or forcing the child to touch or fondle the perpetrator’s body in a sexual way, or forcing the child to engage in other activities with a sexual content or attempted or actual sexual intercourse)

**Passive maltreatment**

- Emotional neglect (failure to provide for the child’s basic emotional needs, being emotionally unresponsive to the child’s distress, not attending to the child’s distress, not attending to the child’s social and emotional development or expecting the child to routinely manage situations that are beyond his or her maturity level or are not safe)
- Physical neglect (failure to provide for the child’s basic needs, such as for food, clothing, physical safety, adequate supervision, physical health)
It has been suggested that ELS confers increased vulnerability for later life stressors that trigger depressive episodes (Heim and Nemeroff, 2001). Furthermore, maltreated individuals that develop depression display an earlier age at onset, greater symptom severity, more comorbidity and poorer treatment response than non-maltreated depressed individuals (Bernet and Stein, 1999; Nemeroff et al, 2003; Nanni et al, 2012; Teicher and Samson, 2013). Therefore, the combination of ELS and depression may represent a depressive subtype with distinct pathophysiology. In addition to maltreatment, exposure to ELS can constitute the loss of a parent, or exposure to prenatal stress through adverse experiences of the mother (Heim and Nemeroff, 2001).

### 2.2 Resilience to stress

Thus far, it has only been discussed how stressors convey vulnerability for the development of psychiatric diseases. Yet, although the majority of the general population (50-60 %) experiences a severe trauma during their lifetime, the prevalence of PTSD has been estimated to be only 7.8% (Kessler et al., 1995). Also among children, despite a range of ELS events, resilience is the rule rather than the exception (Masten, 2001). Indeed, it has been suggested that overcoming stress-inducing situations can have a pro-resilience effect. Accordingly, low and high levels of stress exposure during development would impair coping, whereas moderate and temporally confined stress exposures would promote adaptive coping responses (Russo et al., 2012; Scharf and Schmidt, 2012). Notably, resilience is regarded as an active process, not just the absence of pathology (Bonanno and Mancini, 2008; Feder et al., 2009).

In squirrel monkeys, a potentially related phenomenon has been observed, which has been termed “stress inoculation” (SI). These socially housed monkeys were subjected to 10 weekly separations of 1 hour, or an unseparated (US) control treatment, starting at 17 weeks of age (juvenile stage). With 8 weeks after the final separation, the monkeys were placed in a moderately stressful, novel environment for 30 minutes, for 5 consecutive days. Observations showed that the SI monkeys displayed reduced anxiety, expressed by decreased maternal clinging, enhanced exploratory behaviour, and increased food consumption compared with the US offspring. Interestingly, the behaviour of SI and US monkeys was initially observed to be similar, but differences emerged over repeated test sessions. Moreover, another 15 weeks later, the SI monkeys were still observed to show reduced anxiety-like behaviour (Parker et al., 2004; Lyons and Parker, 2007).

In laboratory experiments, frequently only single exposures to a stressor are examined, but in real life this is the exception rather than the rule. Most individuals experience multiple significant stressful life events across different developmental time windows, as well as in adulthood (Scharf and Schmidt, 2012). A dominant view has been that the exposure to (early life) stress predisposes individuals to increased vulnerability to subsequent periods of stress (cumulative stress hypothesis), based on the earlier introduced notion of allostatic load. In this view, multiple stress exposures would increase the allostatic load over time and thereby increase the individual’s vulnerability to stress-related disorders (Scharf and Schmidt, 2012). Recently, an alternative model, the match/mismatch hypothesis, was introduced (Champagne et al., 2009; Schmidt, 2011; Nederhof and Schmidt, 2012; Daskalakis et al., 2013). This model proposes that individuals can use the experience of past stressors to adaptively respond to future challenges (predictive adaptive response, Gluckman et al., 2007). Specifically, individuals with high programming sensitivity are predicted to be capable of phenotypic adaptation that is beneficial if the environmental challenges remain the same or similar (match). Therefore, individuals exposed to ELS could benefit from a match of the environment between different stages of life, but would be maladapted in a mismatched environment (Homborg and Van den Hove, 2012; Scharf and Schmidt, 2012). It should be noted that there is considerable empirical evidence for both the cumulative stress as well as match/mismatch hypotheses, and it is therefore conceivable that these models may co-apply in varying degrees, dependent on the individual programming sensitivity and the severity of stressful life events (Nederhof and Schmidt, 2012).

Genetic variation inherently underlies individual differences in sensitivity to ELS exposure. Specifically, the depressogenic effects of ELS have been shown to be moderated by (single nucleotide) polymorphisms in several genes, of which a number of candidate genes have arguably received the most attention: BDNF (Gatt et al., 2009), CRFR (Bradley et al., 2008), FBRPS (Zimmermann et al., 2011) and SLCT6A4 (Caspi et al., 2003). Not surprisingly, these polymorphisms have also been shown to display epistatic (GxG) interactions in their moderation of the effects of ELS exposure (GxGxE) (Kaufman et al., 2006; Wichers et al., 2008; Reissler et al., 2009; Cicchetti et al., 2011). In this thesis, the focus is primarily on a polymorphic region of the serotonin transporter gene, SLC6A4.

### 3. 5-HTTLPR

Serotonin or 5-hydroxytryptamine (5-HT) is primarily present in blood platelets, the gastrointestinal tract and the CNS (Lucki, 1998). The neurotransmitter 5-HT is produced by neurons in the raphe nuclei of the brainstem, of which the dorsal and median raphe (DR, MR) are the main forebrain-projecting nuclei. The DR especially has been implicated in the behavioural and physiological stress response (Hale et al., 2012). When released from the nerve terminals of the presynaptic neuron into the extracellular space of the synaptic cleft, 5-HT can exert its actions via a wide variety of receptors (Hoyer et al., 2002), which modulate signal transduction in the post-synaptic neuron, affecting its electrophysiology and/or gene expression (Sibille and Lewis, 2006). The serotonin/5-HT transporter (5-HTT) plays a critical role in the termination of 5-HT neurotransmission by the re-uptake of 5-HT into the presynaptic neuron (Amara and Kuhar, 1993), and it is the target of the most widely prescribed class of antidepressants (SSRIs, Mandrioli et al., 2012).
The human 5-HTT gene (SLC6A4) was cloned in the early 1990s and was mapped to chromosome 17q11.2-q12 (Lesch et al., 1993; Ramamoorthy et al., 1993). The protein product of the human 5-HTT gene is 630 amino acids in length and is 92% homologous to the rat 5-HTT gene (Lesch et al., 1994). The 5-HTT-linked polymorphic region (5-HTTLPR) is a variable repeat sequence in the promoter region of the gene, which constitutes two genetic variants: a short (S) allele comprising 14 copies of a 20–23 bp repetitive sequence, and a long (L) allele comprising 16 copies. The deletion (S-allele) has an overall length of 44 bp and is located 1212-1255 bp upstream of the transcription start site (Figure 1) (Heils et al., 1995; 1996; Hariri and Holmes, 2006). The basal as well as stimulus-induced promoter activity of the 5-HTT gene was found to be significantly lower in vitro with the S-allele compared to the L-allele. In addition, in vitro 5-HT uptake and 5-HTT binding were found to be significantly lower in cell lines and platelets carrying the S-allele compared to the L-allele. In all of these studies, the S/S and S/L genotypes were found to produce similar phenotypic measurements (e.g., both approximately two-fold lower transcriptional efficiency compared to L/L homozygotes), suggesting that the polymorphism may have a dominant-recessive effect (Heils et al., 1996; Lesch et al., 1996; Greenberg et al., 1999). With regard to 5-HTT binding in the brain, results are not unambiguous, as some studies reported the S-allele to confer lower 5-HTT binding levels compared to the L-allele (Little et al., 1998; Heinz et al., 2000; Praschak-Rieder et al., 2007; Reimold et al., 2007), while others found no significant differences (Willeit et al., 2001; Shoe et al., 2003; Parsey et al., 2006a; Murthy et al., 2010). Interestingly, a recent study found that 5-HTTLPR interacted with the subjects being chronic smokers or not (Kobiella et al., 2011), indicating that beyond genetic impact, the expression of 5-HTT is influenced by various environmental factors (Cosgrove et al., 2009; Reimold et al., 2011), possibly explaining the discrepant studies.

The 5-HTTLPR polymorphism is very common, as the allele frequency of the S-allele is around 40% and heterozygosity around 50% in Caucasian populations (Heils et al., 1996; Lesch et al., 1996). Notably, there are considerable ethnical differences (Gelernter et al., 1999), with S-allele frequencies being lower in African-Americans (20-25%, Lotrich et al., 2003; Odgerel et al., 2013) and higher in Asian populations (75-80%, Kunugi et al., 1997; Kim et al., 2000; Lee and Ham, 2008). Furthermore, within 5-HTTLPR two single nucleotide polymorphisms (SNPs), rs25531 and rs25532, have been identified. The rs25531 polymorphism has a minor-allele frequency of 9-15% in Caucasians and 24% in African Americans. Importantly, rs25531 (as well as rs25532) is present only in the L-allele, with the L0 variant and the S-allele showing nearly equivalent transcriptional efficiency, while the L1 variant is associated with higher levels of S-HTT gene expression (Hu et al., 2006; Wendland et al., 2006). Accordingly, subsequent studies have incorporated the rs25531 polymorphism in their analyses. In this thesis, the S- and L0 variant will be jointly referred to as the S-allele (low-expressing S-HTTLPR) and the L1 variant will be denoted as the L-allele (high-expressing S-HTTLPR). Additional variants within the S-HTT gene include a variable number of tandem repeats in intron 2 and several rare SNPs that modulate the structure and/or function of S-HTT (Lesch et al., 1994; Murphy and Lesch, 2008).

Figure 1 The human 5-HTT gene (SLC6A4) is located at chromosome 17q11.2-q12 (Lesch et al., 1993; Ramamoorthy et al., 1993) and its promoter region contains the 5-HTT-linked polymorphic region (5-HTTLPR). This polymorphism is generated by a deletion (lowercase letters, bottom-left of the figure) of a 44 bp sequence located 1212-1255 bp upstream of the transcription start site, and thus results in a short (S) allele versus a long (L) allele (insertion). The S-allele has been associated with reduced transcriptional efficiency of the gene and in vivo 5-HTT binding levels (although the latter is not consistently reported). Therefore, the S-allele is thought to result from compromised 5-HTT function across ontogeny, leading to prolonged 5-HT (5-hydroxytryptamine; serotonin) neurotransmission, and functional and structural alterations in the brain, because of the important developmental role of 5-HT (Heils et al., 1996; Gaspar et al., 2003; Hariri and Holmes, 2006). MAO-A: monoamine oxidase A, enzyme that degrades 5-HT. This figure has been adapted and reprinted with permission from Macmillan Publishers Ltd [Nature Neuroscience] (T. Canli and K-P Lesch), copyright (2007).

3.1 Moderation of stress-precipitated depression
In 2003, a longitudinal prospective study reported that 5-HTTLPR moderates the association of stressful life events (SLEs) with depression (Caspici et al., 2003). Compared to L/L homozygotes, S-allele carriers were found to have increased risk (2-3 times) to develop depression when they had endured 3 or more SLEs (life history calendar; events included employment, financial, housing, health and relationship stressors). Since then, numerous follow-up studies have been published, confirming the vulnerability of S-allele carriers or S/S homozygotes, or presenting null findings (e.g. Kaufman et al., 2004; Gillespie et al, 2006; de Geus et al., 2006; Wendland et al., 2006). In 2006, a follow-up study reported that 5-HTTLPR moderates the association of stressful life events (SLEs) with depression (Kaufman et al., 2006). Compared to L/L homozygotes, S-allele carriers were found to have increased risk (2-3 times) to develop depression when they had endured 3 or more SLEs (life history calendar; events included employment, financial, housing, health and relationship stressors). Since then, numerous follow-up studies have been published, confirming the vulnerability of S-allele carriers or S/S homozygotes, or presenting null findings (e.g. Kaufman et al., 2004; Gillespie et al, 2006; de Geus et al., 2006; Wendland et al., 2006). In 2006, a follow-up study reported that 5-HTTLPR moderates the association of stressful life events (SLEs) with depression (Kaufman et al., 2006). Compared to L/L homozygotes, S-allele carriers were found to have increased risk (2-3 times) to develop depression when they had endured 3 or more SLEs (life history calendar; events included employment, financial, housing, health and relationship stressors). Since then, numerous follow-up studies have been published, confirming the vulnerability of S-allele carriers or S/S homozygotes, or presenting null findings (e.g. Kaufman et al., 2004; Gillespie et al, 2006; de Geus et al., 2006; Wendland et al., 2006). In 2006, a follow-up study reported that 5-HTTLPR moderates the association of stressful life events (SLEs) with depression (Kaufman et al., 2006). Compared to L/L homozygotes, S-allele carriers were found to have increased risk (2-3 times) to develop depression when they had endured 3 or more SLEs (life history calendar; events included employment, financial, housing, health and relationship stressors). Since then, numerous follow-up studies have been published, confirming the vulnerability of S-allele carriers or S/S homozygotes, or presenting null findings (e.g. Kaufman et al., 2004; Gillespie et al, 2006; de Geus et al., 2006; Wendland et al., 2006). In 2006, a follow-up study reported that 5-HTTLPR moderates the association of stressful life events (SLEs) with depression (Kaufman et al., 2006). Compared to L/L homozygotes, S-allele carriers were found to have increased risk (2-3 times) to develop depression when they had endured 3 or more SLEs (life history calendar; events included employment, financial, housing, health and relationship stressors). Since then, numerous follow-up studies have been published, confirming the vulnerability of S-allele carriers or S/S homozygotes, or presenting null findings (e.g. Kaufman et al., 2004; Gillespie et al, 2006; de Geus et al., 2006; Wendland et al., 2006).
Some controversy arose in 2009 when two meta-analyses reported that the statistical significance of the 5-HTTLPR x SLE interaction regarding depression could not be supported (Munafo et al., 2009; Risch et al., 2009). In the discussion in the literature that followed these meta-analyses were criticized not to adhere to ‘best practice’ (Caspi et al., 2010). Specifically, the meta-analyses disregarded exposure to multiple SLEs, included only a subset of available studies, and these selections were found to be biased towards the inclusion of negative studies (Kaufman et al., 2010, Uher and McGuffin, 2010). Moreover, the 2009 meta-analyses focused on studies that explored an interaction of 5-HTTLPR with SLEs, excluding the studies that have assessed 5-HTTLPR x ELS interaction.

In 2011, a comprehensive meta-analysis was published that may have ended the debate on “arguably the most and least loved gene of psychiatric genetics” (Blakely and Veenstra-VanderWeele, 2011; Karg et al., 2011). In this study, Karg and colleagues reported a highly significant moderation by 5-HTTLPR of the relationship between stress and depression. When the studies were stratified by stressor, 5-HTTLPR was found to have a particularly strong association with stress sensitivity to childhood maltreatment, compared to its moderation of the relationship between SLEs and depression (Karg et al., 2011). Recently, a new meta-analysis was published comprising another 27 studies that were published until June 2013. This study has confirmed the significance of 5-HTTLPR in moderating the relation between stress and depression (Sharpley et al., 2014).

3.2 Differential susceptibility

Importantly, the increased environmental sensitivity of S-allele carriers is not restricted to adverse events, as S-allele carriers also show increased benefit from positive, supportive environments (Kaufman et al., 2004; Taylor et al., 2006; Pluess et al., 2010; Van IJzendoorn et al., 2012). For example, social support was shown to ameliorate the depressogenic effects of genetic predispositions, the differential susceptibility hypothesis provides a theoretical model. Whereas the diathesis-stress hypothesis focusses solely on the adverse consequences of developing depression has given rise to a conundrum that has been referred to as ‘the 5-HTT paradox’ (Sibille and Lewis, 2006). Because, how can this finding be reconciled with the clinical observation that SSRIs improve depressive symptoms by reducing 5-HTT function? The most probable answer is that the altered availability of 5-HTT from conception onwards profoundly affects ontogeny. The importance of the 5-HT system in neurodevelopment has long been recognized, affecting developmental events as neurotransgenesis, neurite outgrowth and apoptosis (Gaspar et al., 2003). For instance, the development of the rodent somatosensory cortex is affected by knockout of the 5-HTT gene (Persico et al., 2001; Esaki et al., 2005; Miceli et al., 2013). In rats, 5-HTT levels have been found to peak early in the postnatal period (postnatal day 0-21), followed by lower levels in the adult brain (Galnieau et al., 2004), and early life (postnatal day 4-21) blockade of 5-HTT has been shown to recapature the adult anxiety-like phenotype of knockout of the 5-HT gene in mice (Ansorge et al., 2004). Therefore, alterations in 5-HTT levels early in development likely affect the formation of neural networks into adulthood. The neural consequences of 5-HTTLPR indeed extend beyond alterations in the functioning of the 5-HT system (Hariri and Holmes, 2006). Furthermore, the discrepant 5-HTTLPR studies on in vivo 5-HTT binding, and the finding that the cognitive impact of 5-HTTLPR is associated with differential brain morphology rather than changes in the 5-HT system (Jedema et al., 2010), has led some researchers to suggest that the increased stress sensitivity of 5-HTTLPR S-allele carriers may be due to an altered development of stress-related neurocircuitry rather than an altered adult functioning of the 5-HT system (Hariri and Holmes, 2006).

Functional magnetic resonance imaging (fMRI) studies have provided some initial insight into the influence of 5-HTTLPR on stress-related neurocircuitry. Firstly, it was shown that S-allele carriers exhibit greater activity of the amygdala in response to fearful stimuli (Hariri et al., 2002; Munafo et al., 2008; Murphy et al., 2013), which could in part be mediated by alterations in amygdala volume (Kobriella et al., 2011). Furthermore, a model was proposed in which the stress x 5-HTTLPR interaction increases the resting state activity of the amygdala and hippocampus, leading to a heightened state of vigilance, and as such an increased sensitivity to the environment (Canli et al., 2005, 2006). Additional studies have indicated that the medial prefrontal cortex (mPFC) and the anterior cingulate cortex S-allele carriers exhibit increased anxiety-like traits (Lesch et al., 1996; Sen et al., 2004; Stein et al., 2008), increased acquisition of conditioned fear responses (Garpenstrad et al., 2001; Lonsdorf et al., 2009; Klumpers et al., 2012) and an elevated attentional bias towards negative stimuli (Williams et al., 2009; Fox et al., 2011; Peremalin-Hisht et al., 2012). But, S-allele carriers have also been shown to outperform L/L homozygotes on various cognitive tasks (Homberg and Lesch, 2011) and to exhibit increased attentional bias towards positive stimuli (Beeevers et al., 2009; Fox et al., 2011).

3.3 The 5-HTT paradox; altered neurodevelopment

The finding that genetically-conferred low availability of 5-HTT increases the probability of developing depression has given rise to a conundrum that has been referred to as ‘the 5-HTT paradox’ (Sibille and Lewis, 2006). Because, how can this finding be reconciled with the clinical observation that SSRIs improve depressive symptoms by reducing 5-HTT function? The most probable answer is that the altered availability of 5-HTT from conception onwards profoundly affects ontogeny. The importance of the 5-HT system in neurodevelopment has long been recognized, affecting developmental events as neurotransgenesis, neurite outgrowth and apoptosis (Gaspar et al., 2003). For instance, the development of the rodent somatosensory cortex is affected by knockout of the 5-HTT gene (Persico et al., 2001; Esaki et al., 2005; Miceli et al., 2013). In rats, 5-HTT levels have been found to peak early in the postnatal period (postnatal day 0-21), followed by lower levels in the adult brain (Galnieau et al., 2004), and early life (postnatal day 4-21) blockade of 5-HTT has been shown to recaputure the adult anxiety-like phenotype of knockout of the 5-HT gene in mice (Ansorge et al., 2004). Therefore, alterations in 5-HTT levels early in development likely affect the formation of neural networks into adulthood. The neural consequences of 5-HTTLPR indeed extend beyond alterations in the functioning of the 5-HT system (Hariri and Holmes, 2006). Furthermore, the discrepant 5-HTTLPR studies on in vivo 5-HTT binding, and the finding that the cognitive impact of 5-HTTLPR is associated with differential brain morphology rather than changes in the 5-HT system (Jedema et al., 2010), has led some researchers to suggest that the increased stress sensitivity of 5-HTTLPR S-allele carriers may be due to an altered development of stress-related neurocircuitry rather than an altered adult functioning of the 5-HT system (Hariri and Holmes, 2006).

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(ACC) regulate amygdala activity during the processing of fearful stimuli. Specifically, the amygdala of S-allele carriers was found to show increased and decreased functional connectivity with respectively the ventral mPFC and ACC (Heinz et al., 2005; Pezawas et al., 2005). It has been proposed that decreased amygdala-ACC coupling leads to amygdala hyperreactivity and increased anxiety-related traits, which would induce compensatory overactivity of the ventral mPFC (Pezawas et al., 2005). In addition, S-allele carriers have been found to display decreased structural integrity of the uncinate fasciculus, the white matter tract that connects the amygdala with the mPFC (Pacheco et al., 2009). Furthermore, dynamic causal modeling of fMRI data suggested that the increased amygdala activity displayed by S-allele carriers during the processing of fearful stimuli is the result of reduced prefrontal inhibitory regulation (Volman et al., 2013).

With regard to the hypothesis that altered neurocircuitry underlies ELS x 5-HTTLPR interaction, the epistasis with the BDNFVal66Met polymorphism could be highly relevant (Kauffman et al., 2008; Wichier et al., 2008). Brain-derived neurotrophic factor (BDNF) is the most abundant and widely distributed neurotrophin in the CNS. While it was initially recognized as an important mediator of cell survival and growth during ontogeny, the main function of BDNF in the adult brain is considered to be the regulation of synaptic plasticity. As such, BDNF has been implicated in behaviour, and cognitive functions as memory acquisition and consolidation (Martinowich and Lu, 2008). The Val66Met polymorphism (substitution of the valine for a methionine residue at amino acid position 66 of the predomain of BDNF) impairs the dendritic trafficking, synaptic localization and secretion of BDNF (Egan et al., 2003). The transcriptional regulation and cellular localization of BDNF is furthermore complicated by differential exon usage and polyadenylation of the 3’-untranslated region (3’-UTR) (Pattabiraman et al., 2005; An et al., 2008).

The epistatic interaction between S-HTTLPR and BDNFVal66Met is not surprising, given the interactions of BDNF and the S-HT system at the molecular and cellular level. For instance, BDNF is known to promote the survival and morphological differentiation of 5-HT neurons. Moreover, the therapeutic effect of SSRIs is thought to be mediated in part by the upregulation of BDNF expression, and the subsequent induction of neurogenesis and altered synaptic plasticity (Duman et al., 1997; Castren, 2004; Martinowich and Lu, 2008; Homberg et al., 2014). In this regard, BDNF is also thought to play a major role in the neural and behavioural alterations that underlie the increased sensitivity of S-HTTLPR S-allele carriers to stress (Homberg et al., 2014).

4. Translational psychiatry

Most studies with human subjects are limited because they can only look for correlations between behavioural phenotypes and genotypes (or GxE interactions), while meta-analyses are hampered by the heterogeneity of studies with human populations. Experiments are necessary to move towards causal relationships, and for such experiments, animal models are needed. In rhesus monkeys, an orthologue of S-HTTLPR has been identified (rh5-HTTLPR), of which the S-allele is also associated with decreased transcriptional efficiency (Lesch et al., 1997; Bennett et al., 2002). Similar to the human situation however, the rh5-HTTLPR is not unequivocally associated with altered in vivo 5-HT binding levels (Christian et al., 2009, 2013; Jedema et al., 2010; Embree et al., 2013). The influence of rh5-HTTLPR on the sensitivity to ELS exposure has been studied by separating infant rhesus monkeys from their mothers and rearing them with other infants (peer-rearing; PR). The interaction of ELS and rh5-HTTLPR has been shown to affect behavioural responses to social separation; of the monkeys that experienced PR, S-allele carriers were found to display increased agitation and stereotypic behaviour, while L/L homozygotes showed increased self-directed behaviour and withdrawal (Spinelli et al., 2007). Furthermore, S-allele carriers subjected to PR were found to exhibit increased ethanol preference (Barr et al., 2004a), increased neuroendocrine activity in response to social isolation (Barr et al., 2004b, 2004c), and lower 5-HT metabolite levels in the cerebrospinal fluid, indicative of increased 5-HT neurotransmission (Bennett et al., 2002).

Research using rodents allows the control over genetic background and the environment to a degree that is practically or ethically feasible in human or even nonhuman primate studies (Caspi et al., 2010). Furthermore, the use of rodents in biomedical research allows for invasive experiments and detailed molecular/physiological analyses, while additional advantages are the short life cycle and relatively low costs for housing and caretaking. In the case of complex psychiatric diseases as depression, an important question is however whether or not its etiology and pathophysiology can actually be reliably modeled in rats or mice. This is a critical issue as some of the symptoms of depression, e.g. low self-esteem, feelings of guilt and suicidality do not seem to have a rodent equivalent. Yet, metabolic and sleep disturbances, and alterations in hedonic, psychomotor and cognitive behaviour can be inferred from rodent models (Nestler and Hyman, 2010; Schmidt et al., 2011).

To assess whether an animal model appropriately models disease symptoms, three types of validators are classically used: construct, face and predictive validity (Willner, 1984). Construct validity refers to the relevance of the methods by which the model is set up, and is generally considered the most compelling and useful approach. Ideally, construct validity would be achieved by recreating the entire etiological process that causes depression in humans. Therefore, by incorporating known genetic and environmental risk factors of depression a rodent model would be regarded to possess construct validity (Nestler and Hyman, 2010; Schmidt et al., 2011; Homberg, 2013). Face validity indicates that a model recapitulates important anatomical, biochemical, physiological or behavioural features of a human disease. In the case of depression, face validity of animal models is largely limited to behavioural features because of the limited knowledge on the pathophysiology of depression. Unfortunately, at the level of behaviour,
the translational gap between rodent and human subjects is probably the largest (Nestler and Hyman, 2010). To improve the face validity of rodent behavioural readouts, an important improvement could therefore be the implementation of human task parameters in rodent tests (Homberg, 2013). Predictive validity is concerned with the extent to which an animal model displays responses to treatments that are predictive for therapeutic effects in humans. The lack of knowledge of the mechanisms by which psychiatric drugs exert their therapeutic effect was the reason for pharmacologists to develop rodent behavioural tests which are sensitive to drugs known to be effective in humans (Hyman, 2014), such as the forced swim test (FST) for SSRIs (Porso et al., 1977; Lucki et al., 2001) and the elevated plus maze for the anxiolytic benzodiazepines (Steru et al., 1985). A concern has been though that these assays only detect drugs with the same underlying mechanism and thus not detect potentially effective drugs that act by different mechanisms (Hyman, 2014). Moreover, whereas SSRIs acutely increase active coping behaviour in the FST, depressed patients only show clinical improvement after several weeks of treatment, suggestive of significantly different underlying mechanisms. Whereas the FST was initially used only as a screening test for novel antidepressants, it has later increasingly been used as a readout of depressive-like behaviour, as the immobility that develops across time in depressed patients only show clinical improvement after several weeks of treatment, suggestive of significantly different underlying mechanisms. Whereas the FST was initially used only as a screening test for novel antidepressants, it has later increasingly been used as a readout of depressive-like behaviour, as the immobility that develops across time in the FST has been interpreted as a measure of behavioural despair. The problem however is that immobility in the FST, but also other tests, may in fact be an adaptive coping strategy (to save energy) under inescapable stress conditions (Krishnan and Nestler, 2010; Homberg, 2013; Pryce and Klaus, 2013).

In the translation of rodent behavioural studies to human clinical phenotypes there is the inherent risk for anthropomorphic interpretation, and the difficulty in judging what is adaptive or maladaptive behaviour in a given context. In the learned helplessness (LH) paradigm however, the testing phase actually confers an unambiguous advantage towards active, goal-directed compared to passive, enduring behaviour. In this paradigm, rodents are exposed to a series of unpredictable and inescapable electrical (usually foot) shocks, after which the animals are tested for their coping behaviour in a shuttle-box where the shocks are escapable. After the exposure to inescapable shocks, a subset of the animals will however display maladaptive behaviour as they fail to escape from the shocks in a majority of the trials, despite the presence of a clear escape route (Vollmayr and Henn, 2001). It has been shown that the lack of (perceived) control - not the exposure to shocks by itself - is central to the development of this passive coping behaviour, which has been termed “learned helplessness” (Maier and Seligman, 1976; Amat et al., 2005). For this thesis, we have used the LH paradigm as a behavioural readout for stress coping behaviour and adult stress vulnerability. We have applied it to experiments in which we combined a model of ELS exposure with a model of low vs high availability of S-HTT in order to produce a rat model of ELS x S-HTT genotype interaction. In addition to stress coping behaviour, we have addressed the physiological changes that underlie ELS x S-HTT interaction. We have focused a large part of our studies on the hypothala-

mo-pituitary-adrenal (HPA) axis, the main stress-responsive neuroendocrine system of the body, which is considered to mediate the influence of ELS on the risk for later life depression (Lupien et al., 2009). Therefore, we first introduce the HPA-axis, before going into the selected rodent models of ELS exposure and 5-HTTLPR genotype.

### 5. Hypothalamo-pituitary-adrenal (HPA) axis

The HPA-axis ultimately functions to regulate the cortex of the adrenal glands with regard to the synthesis and release of glucocorticoids in the bloodstream. In response to stress, glucocorticoids, mainly cortisol in primates and corticosterone in rodents (collectively referred to as CORT), mobilize energy resources together with catecholamines (released by the adrenal medulla and sympathetic nerve terminals) by elevating circulating glucose levels, increasing heart rate and blood pressure, and decreasing the activity of the immune and digestive systems. Furthermore, in addition to peripheral tissues, CORT acts on the brain to affect neural plasticity, cognition and behaviour (Roozendaal, 2000; de Kloet et al., 2005, Joëls et al., 2011, 2012; Hermans et al., 2014).

A stress response of the HPA-axis is initiated when, from other parts of the CNS, signals encoding physical or psychological stressors stimulate the activity of paravascular neurons in the paraventricular nucleus (PVN) of the hypothalamus. These neurons secrete corticotropin-releasing factor/hormone (CRF/CRH) and arginine vasopressin in the external zone of the median eminence, at the base of the third ventricle. From here, these peptides enter the hypophysial portal system and stimulate the anterior pituitary to synthesize and release adrenocorticotropic hormone (ACTH), which itself stimulates the synthesis and release of CORT from the adrenal cortex (Figure 2) (De Kloet et al., 1998; Ladd et al., 2000; Joëls et al., 2012). In response to a stressor, circulating CORT levels usually reach peak levels after 15-30 minutes and return to baseline 60-90 min later. Under basal conditions, CORT levels furthermore show an ultradian rhythm by hourly secretory pulses from the adrenal cortex. The amplitude of these ultradian pulses actually determine the circadian peak and trough of CORT levels, with the largest pulses underlying the awakening response and onset of circadian activity (night-time in nocturnal animals such as rodents) (Young et al., 2004; Lightman and Conway-Campbell, 2010). Interestingly, it was recently shown that pulsatility of CORT levels is needed for adequate HPA responses to stress, and that stressors applied during the falling phase of a CORT pulse result in lower ACTH responses than those applied during the rising phase (Sarabdajitsingh et al., 2010a, 2010b).

The activity of the HPA-axis is regulated through direct feedback action of CORT at the level of the pituitary and the PVN, but also via extra-hypothalamic brain areas such as the medial prefrontal cortex, hippocampus and extended amygdala (Ulrich-Lai and Herman, 2009). This feedback action is mediated via the mineralocorticoid and
glucocorticoid receptors (MR, GR), with MR being mainly involved in the maintenance of basal HPA activity and GR with the recovery from stress-induced activity (De Kloet et al., 1998, 2005). The balance between GR and MR functioning has been proposed to be central to stress-related psychopathology, as GR and MR serve such complementary CORT functions during the stress response (De Kloet et al., 1998; De Kloet, 2014). Of note, GR and MR are classically known to be nuclear receptors and therefore to exert their effects via the modulation of gene expression. However, whereas the genomic effects of CORT are slow in onset, many rapid – putatively non-genomic – effects of CORT have been reported in the literature (Hermans et al., 2014). Indeed, a number of studies have recently indicated the existence of membrane-associated forms of GR and MR (Karst et al., 2005, 2010; Groeneweg et al., 2011, 2012).

Besides the PVN, major sources of CRF include the amygdala and the bed nucleus of the stria terminalis (BNST), which are thought to contribute to the regulation of HPA-axis activity (Korosi and Baram, 2008). The central amygdaloid nucleus (CeA) and the oval subdivision of the BNST (BNStov) contain the major populations of CRF neurons in the rat amygdala and BNST, respectively (Merchenthaler et al., 1982; Morin et al., 1999; Sterrenburg et al., 2012). These CRF populations are responsive to acute and chronic exposure to stress (Rouwette et al., 2011; Sterrenburg et al., 2011), and their modulation has been shown to affect the HPA-axis as well as stress-related behaviour (Keen-Rhinehart et al., 2009; Regev et al., 2011; Flandreau et al., 2012; Callahan et al., 2013; Sink et al., 2013).

In addition, evidence has accumulated suggesting that other members of the CRF family of neuropeptides, certainly urocortin 1 (Ucn1), but also urocortin 2 and urocortin 3, complement the actions of CRF in the regulation of the stress response (Kuperman and Chen, 2008; Kozicz et al., 2011a; Ryabinin et al., 2012). The actions of CRF and the urocortins are mediated by two G-protein coupled receptors, corticotropin-releasing factor receptor 1 and 2 (CRF-R1, CRF-R2). Ucn1 binds both receptors with high affinity, while CRF has low affinity for CRF-R1, and Ucn2 and Ucn3 selectively bind to CRF-R2 (Vaughan et al., 1995; Hsu and Hsueh, 2001; Lewis et al., 2001; Reyes et al., 2001). In the mammalian brain, Ucn1 is most abundantly expressed by neurons of the centrally projecting Edinger-Westphal nucleus (EWcp) in the midbrain (Vaughan et al., 1995; Kozicz et al., 1998, 2011a; Bittencourt et al., 1999; Lino et al., 1999). In contrast to the HPA-axis, EWcp-Ucn1 neurons do not show habituation upon chronic stress exposure (Viau and Sawchenko, 2002; Korosi et al., 2005; Xu et al., 2010). It has therefore been proposed that Ucn1 is involved in the termination of the central stress response (Kozicz et al., 2011a, Ryabinin et al., 2012).

5.1 The HPA-axis; depression and 5-HTTLPR

In depression, about 50% of patients display basal hypercortisolemia and resistance to GR-mediated negative feedback (Checkley, 1996; Pariante and Miller, 2001), possibly representing the melancholic clinical subtype of depression (Gold and Chrousos, 2002; Lamers et al., 2013). In contrast, HPA hypoactivity has been observed for atypical depression...
and PTSD (Gold and Chrousos, 2002; Yehuda, 2009). The observation that remission of depression coincides with normalization of the HPA-axis underscores its pathophysiological role (Heuser et al., 1996; Binder et al., 2009). In addition, postmortem studies have found an increased number of CRF neurons and CRF mRNA content in the PVN of depressed subjects (Raadsheer et al., 1994; Raadsheer et al., 1998; Wang et al., 2008), and these patients display increased levels of CRF in their cerebrospinal fluid, which can be reduced by treatment with antidepressants or electroconvulsive shocks (Nemeroff et al., 1984; Nemeroff et al., 1991; Heuser et al., 1998). Furthermore, single nucleotide polymorphisms and altered expression levels of the genes encoding GR (NR3C1) and MR (NR3C2) have been associated with depression (Webster et al., 2002; Van Rossum et al., 2006; Klok et al., 2011a, 2011b; Medina et al., 2013; Qi et al., 2013) which might be caused by impaired glucocorticoid signaling. Glucocorticoids act through the glucocorticoid receptor (GR). In addition, FK506-binding protein 51 (FKBP5) has recently warranted attention, as genetic variation of FKBP5 was found to interact with ELS to modulate the risk of depression and PTSD, mediated by epigenetic regulation of GR expression (Binder, 2009; Zimmermann et al., 2011; Klengel et al., 2013).

The 5-HTTLPR S-allele has been associated with increased basal CORT levels (O’Hara et al., 2007; Chen et al., 2009; Goodyer et al., 2009; Wust et al., 2009; Wankerl et al., 2010), and S/S homozygotes show increased CORT stress responses to social separation (Barr et al., 2004a, 2004b). There have been reports of functional variation in the mouse S-HTT gene (Carneiro et al., 2009), but there is no rodent orthologue of (Rh)5-HTTLPR. Therefore, several mouse 5-HTT knockout (5-HTT−/−) lines (C57BL/6J, 129S6 and CD-1 backgrounds) have been generated by the genetic engineering of embryonic stem cells (Bengel et al., 1998; Holmes et al., 2003a; Lira et al., 2003). The 5-HTTLPR S-allele has been associated with increased basal CORT levels (O’Hara et al., 2007; Chen et al., 2009; Goodyer et al., 2009; Wust et al., 2009; Wankerl et al., 2010), and S/S homozygotes show increased CORT stress reactivity compared with individuals carrying a long (L) allele of the 5-HTTLPR (Gotlib et al., 2008; Way and Taylor, 2010; Miller et al., 2013). In the case of ELS × S-HTTLPR genotype interaction, only a history of severe stress has been shown to trigger increased CORT responses in human S-allele carriers (Alexander et al., 2009; Mueller et al., 2011). In contrast, in macaques the combination of the S-allele with adverse rearing conditions results in increased ACTH but unaffected CORT responses to social separation (Barr et al., 2004a, 2004b). There have been reports of functional variation in the mouse S-HTT gene (Carneiro et al., 2009), but there is no rodent orthologue of (Rh)5-HTTLPR. Therefore, several mouse 5-HTT knockout (5-HTT−/−) lines (C57BL/6J, 129S6 and CD-1 backgrounds) have been generated by the genetic engineering of embryonic stem cells (Bengel et al., 1998; Holmes et al., 2003a; Lira et al., 2003; Zhao et al., 2006). The 5-HTT−/− mice show increased adrenomedullary but not CORT responses to stress, and basal plasma CORT levels have been reported to be unaltered or lower in 5-HTT−/− mice (Li et al., 1999; Lanfumey et al., 2000; Tjurmina et al., 2002, 2004; Bartolomucci et al., 2010; Jansen et al., 2010; Van den Hove et al., 2011; Hohoff et al., 2013; Spinelli et al., 2013). Furthermore, 5-HTT−/− mice have been reported to exhibit decreased CRF and GR mRNA levels in the PVN, and decreased GR mRNA in the pituitary gland (Jiang et al., 2009). Extrahypothalamic CRF, GR, and FKBP5 mRNA have not been assessed in 5-HTT−/− mice, but they have been found to display decreased expression of Ucn1 in the EWcp (Fabre et al., 2011).

6. 5-HTT knockout

In addition to the mouse, a 5-HTT−/− rat line has been obtained by ENU (N-ethyl-N-nitrosourea)-driven, target-selected mutagenesis. In short, high-throughput screening of genomic targets in offspring of mutagenized Wistar rats revealed an ENU-induced mutation that resulted in a premature stop codon in exon 3 of the 5-HTT gene (Slcdav) (Smits et al., 2006; Homberg et al., 2007). Both 5-HTT−/− mice and 5-HTT−/− rats have been shown to display reduced 5-HT uptake (synaptosomes, primary neurons), increased extracellular and decreased tissue 5-HT levels across the brain, decreased spontaneous firing of raphe neurons, and decreased expression and sensitivity of 5-HT1A inhibitory autoreceptors (Bengel et al., 1998; Li et al., 1999; Fabre et al., 2000; Gobbi et al., 2001; Pan et al., 2001; Mathews et al., 2004; Homberg et al., 2007, 2008a). No gross health abnormalities are observed for 5-HTT−/− rodents, as they are similar to their wild-type (5-HTT+/+) counterparts on measures as locomotor activity, coat and whisker condition, neurological reflexes and piloerection (Holmes et al., 2002; Carroll et al., 2007; Homberg et al., 2007).

With regard to stress coping behaviour, both 5-HTT−/− mice and rats show increased immobility in the FST (Holmes et al., 2002; Lira et al., 2003; Carroll et al., 2007, Olivier et al., 2008a; Popa et al., 2008; Schipper et al., 2011a, 2011b), increased anxiety-like behaviour in the elevated plus maze (EPM), light-dark exploration (LDE), open field (OF), marble burying and novelty-suppressed feeding (NSF) tests (Holmes et al., 2003a, 2003b, Anzorge et al., 2004; Zhao et al., 2006; Carroll et al., 2007; Olivier et al., 2008a; Popa et al., 2008; Jansen et al., 2010; Schipper et al., 2011a, 2011b), and impaired extinction recall of fear memory (Wellman et al., 2007; Narayanan et al., 2011; Schipper et al., 2011a; Nonkes et al., 2012a). There is no difference in sensitivity to electric shocks (measured by startle amplitude) or mechanical allodynia between 5-HTT−/− and 5-HTT+/− mice (Lira et al., 2003; Vogel et al., 2009), but it has been noted that 5-HTT−/− mice display diminished thermal hyperalgesia upon nerve injury or hind paw inflammation (Vogel et al., 2003; Palm et al., 2008).

With regard to cognitive functioning, both 5-HTT−/− mice and rats show improved reversal learning (Brigman et al., 2010; Schipper et al., 2011b; Nonkes et al., 2013). In addition, 5-HTT−/− rats have been shown to perform better than their 5-HTT+/+ counterparts on strategy set-shifting (Nonkes et al., 2012b) and a rodent version of the Iowa Gambling task (Homberg et al., 2008a), but perform worse on the object recognition task (Olivier et al., 2009). No sex differences have been noted with regard to the behavioural phenotype of 5-HTT−/− rodents (Holmes et al., 2003b; Alexandre et al., 2006), although admittedly, many of the above studies did not directly compare male to female subjects. It does seem that the genetic background has a strong influence on the behavioural consequences of 5-HTT knockout (Homberg et al., 2010a), as also evidenced by direct comparison of anxiety-like behaviour displayed by inbred mouse 5-HTT−/− strains (C57BL/6J, 129S6) (Holmes et al., 2003a).
The 5-HTT-/- mice and rats have a complete absence of 5-HTT expression, although in rats some residual synaptosomal 5-HT uptake has been noted, probably by the noradrenaline transporter (Bengel et al., 1998; Homberg et al., 2007). A complete knockout of 5-HTT does not exist in humans, and the reduction in 5-HTT expression conferred by the 5-HTTLPR S-allele appears to be approximately 50% (Lesch et al., 1996; Heinz et al., 2000). Yet, because of the behavioural similarities between 5-HTT-/- rodents and 5-allele carriers in terms of negative emotionality and cognitive functioning, it is argued that 5-HTT-/- rodents are a robust phenotypic model for the 5-HTTLPR (Kalueff et al., 2010). From the perspective of rodents, when exposure to ELS and 5-HTT deficiency come together. For this purpose, however, knowledge has still been very limited, and therefore, the goal of this thesis was to explore the observation of amygdala-prefrontal connectivity that underlies ELS x 5-HTTLPR interaction. All by all, 5-HTT+/- mice have been reported to show intermediate (significantly different from 5-HTT+/- mice) anxiety-like behaviour in the LDE (Holmes et al., 2003a), but not in the EPM or NSF tests (Holmes et al., 2003b; Ansorge et al., 2004). Considering the increased stress sensitivity of 5-HTTLPR S-allele carriers, behavioural abnormalities (as observed in 5-HTT-/- rodents) may only become apparent in 5-HTT+/- rodents after exposure to stress. A number of studies have conducted such experiments in which the consequences of stressor exposure were compared between 5-HTT+/- and 5-HTT+/- mice (Carola et al., 2008; Heiming et al., 2009; Bartolomucci et al., 2010; Narayanan et al., 2011; Van den Hove et al., 2011; Kloke et al., 2013; Spinelli et al., 2013). These studies showed that 5-HTT+/- mice are indeed more sensitive to stressors than 5-HTT+/- mice across different phases of life. Furthermore, they have provided some starting points for the elucidation of the neurobiology (BDNF, amygdala-prefrontal connectivity) that underlies ELS x 5-HTTLPR interaction. All by all, however, knowledge has still been very limited, and therefore, the goal of this thesis was to further explore how (neuro)physiology and stress coping behaviour are affected in rodents, when exposure to ELS and 5-HTT deficiency come together. For this purpose, we have exposed 5-HTT+/-, 5-HTT+/- and 5-HTT+/- rats to a frequently used model of ELS exposure, repeated maternal separation.

7. Maternal separation

The maternal separation (MS) paradigm entails the repeated separation of the pups from their mother, during the early postnatal period. As in this period the developing brain is undergoing many changes and shows a high degree of plasticity, it is considered as one of the ‘vulnerable’ developmental time windows (Levine, 2005). The timing and sequence of early events in brain development have been found to be remarkably conserved across mammals (Clancy et al., 2007). As such, it has been estimated that the maturity of the cerebral cortex of humans at birth corresponds approximately with PND12-13 in rodents (Romijn et al., 1991; Homberg et al., 2010b). In addition, compared to primates and other mammals, the HPA-axis of rodents is relatively immature at birth (Owen et al., 2005). With regard to developmental timing, the MS model may therefore be more suitable for prenatal compared to postnatal/childhood ELS experiences.

The first demonstration that early postnatal manipulation of rodents can have significant influence on adult stress reactivity came from studies by the late Seymour Levine and others, who demonstrated that daily handling of the pups attenuated the effects of adulthood stressors, compared to rats in undisturbed laboratory conditions, i.e., non-handled (NH) rats (Levine et al., 1956; Levine, 1957; Denenberg, 1964). This treatment is often called early handling (EH), but it actually also comprises the isolation in a novel environment and therefore separation from the dam for up to 15 minutes (MS15). Adult rats subjected to MS15, compared to NH, were shown to exhibit decreased anxiety-like behaviour and HPA responses to stress (Levine and Lewis, 1959; Vau et al., 1993; Meerlo et al., 1999; Caldji et al., 2000). Although the MS15 procedure was considered as a stressful experience, the associated phenotype could plausibly be interpreted as beneficial. Therefore, the findings with the MS15 experiments indicated that exposure to moderate stress could provide resilience to later life stressors, just as was later suggested by the ‘stress inoculation’ procedure of squirrel monkeys (Glaessens et al., 2011).

In the laboratory setting, maternal care is observed to occur in bouts of retrieving, nursing and L/G of the pups, and otherwise the dam is usually off the nest for periods of typically 20-25 min (Jans and Woodside, 1990). Therefore, the MS15 procedure does not result in an abnormal period of separation. In seminaturalistic conditions, subordinate dams are often forced to build their nests far from nutritional sources, and this environmental challenge results in periods of separation for 2-3 h (Calhoun, 1962; Meaney, 2001). Therefore, to obtain an ethologically relevant model of ELS exposure, the MS15 procedure has been adjusted by prolonging the repeated MS to typically 180 min (MS180), although in the literature repeated separations of up to 8 h, or a single separation of 24 h (maternal deprivation) are used as well. Further, several studies on the effect of MS180 and MS15 procedure have been adjusted by prolonging the repeated MS to typically 180 min (MS180), although in the literature repeated separations of up to 8 h, or a single separation of 24 h (maternal deprivation) are used as well. Further, several studies on the effect of MS180 and MS15 have also incorporated an additional reference group (next to NH), that of ‘standard’ animal facility rearing (AFR), which constitutes handling once or twice a week due to the cleaning of cages and changing of bedding material.

Overall, rats that underwent MS180 show a phenotype reminiscent of increased stress sensitivity (HPA responses, anxiety-like behaviour), while NH rats show an intermediate phenotype, in comparison to rats with an early life history of MS15 or AFR (Plotzsky and Meaney, 1993; Caldji et al., 2000; Liu et al., 2000; Huot et al., 2001, 2004; Ladd et al., 2000, 2004; Plotzsky et al., 2005; Lee et al., 2007; Lippmann et al., 2007; Toda et al., 2014). Therefore, although it should be noted that findings have not always been consistent, the MS180 treatment is generally regarded as a robust model for ELS exposure (Ladd et al., 2000,
Levine, Pryce et al., Schmidt, 2010), while it has been proposed that the intermediate NH phenotype is related to understimulation of the pups (for an extended discussion see Pryce et al., 2005).

The differential findings in the literature with regard to MS are likely related to different experimental procedures across different laboratories; duration, frequency, and age of onset of the separation. Furthermore, the genetic background of the rodent is highly relevant as well, given the differential effects of MS in different rat and mouse strains (Anisman et al., 1998; Ellenbroek and Cools, 2000; Kember et al., 2012). For this thesis, we have adopted the protocol of Plotsky, Meaney and co-workers who have contributed many key studies in the field of prolonged-MS experimentation. This procedure is performed for PND2-14 and consists of daily 3 h separations of the pups from the dam, during which the complete litter is transferred to an adjacent room and placed in a novel cage with clean bedding, that is heated to maintain the body temperature of the pups (Plotsky and Meaney, 1993; Francis et al., 2002; Huot et al., 2004; Ladd et al., 2004; Plotsky et al., 2005). The exposure to novelty presumably enhances the long-term impact of prolonged-MS (Tang, 2001; Tang et al., 2006; Enthoven et al., 2008). The comparison treatments that are typically used are AFR, MS15 or a treatment which consists of the exact same handling as MS15/MS180 but in which the pups and dam are immediately reunited (MS0). Although the MS0 treatment has been used less frequently, the advantage over MS15 is that it closely resembles a naïve comparison group, as the pups do not experience separation nor exposure to novelty. We have furthermore chosen to use the MS0 treatment over the use of an AFR group. The rationale for this was two-fold: 1) in comparison to MS15, the MS0 represents a ‘cleaner’ comparison group as the AFR pups would not receive the same degree of handling, and 2) although the term would imply otherwise, AFR procedures (cage cleaning, bedding changes, way of handling the animals, number of care takers, frequency of entering the room for daily controls) are far from consistent across laboratories (Crabbe et al., 1999), and hence the choice of the MS0 treatment (with no involvement of animal care takers other than the experimenter) actually offers increased standardization and chances of replication by other laboratories.

8. Aims and outline of the thesis

The aim of this thesis has been to come to a further understanding of the interaction between ELS and 5-HTTLPR, and its relation with adult stress-related behaviour and the pathophysiology of depression. Alterations in the regulation of HPA-axis activity and of hippocampal BDNF expression have been related to the increased stress sensitivity displayed by 5-HTTLPR 5-allele carriers (e.g. Kaufman et al., 2006; Carola et al., 2008; Mueller et al., 2011; Alexander et al., 2012). It has been unknown however which functional transcripts (3’-UTR, differential exon usage) of BDNF are affected by ELS x 5-HTT genotype interaction. Furthermore, it has thus far been unclear at what level of the HPA-axis and through what mechanisms the activity of the HPA-axis is programmed by ELS x 5-HTTLPR interactions. This programming could be mainly present within the HPA-axis itself, but also likely involves altered gene expression of CRF, Ucn1, GR, MR and FKBP5 in extra-hypothalamic brain areas. Therefore, we have subjected 5-HTT+/-, 5-HTT+ and 5-HTT- rats to daily 3 h maternal separations (MS180) or a comparison/control treatment (MS0) during PND2-14 and examined their adult levels of stress coping behaviour, HPA-axis activity, and gene expression in the mPFC, hippocampus, central amygdala, BNSTov, and the HPA-axis itself. In chapter 2, we have assessed adult stress coping behaviour in the LH paradigm. Specifically, escape latencies and escape failures were recorded in a shuttle-box escape test after exposure to inescapable, unpredictable foot shocks. In chapter 3, we examined if, and at what level, the HPA-axis is affected in our animal model of ELS x 5-HTTLPR interaction, by measuring plasma levels of ACTH, CORT and adrenaline and assessing gene expression of main mediators at the level of the hypothalamus, pituitary and adrenal glands. In chapter 4, we examined if the alterations of the HPA-axis that are paralleled by adaptations of the mRNA levels of GR, MR and FKBP5 in the mPFC, hippocampus, central amygdala and BNSTov. In chapter 5, we have examined the expression of CRF at the mRNA and protein level in the PVN, central amygdala and BNSTov of the rats that underwent the LH paradigm and rats that were naïve in terms of adult stress exposure. Furthermore, we assessed the role of DNA methylation of the promoter region of the CRF gene. In chapter 6, we have examined the immunocytochemical expression of S-HT in the DR, together with the measurement of mRNA levels of 5-HT1A, GR, CRF-R and CRF-R in the DR and EWcp. Furthermore, DNA methylation of the promoter region of the Ucn1 gene and its expression at the mRNA and protein level in the EWcp was included. In chapter 7 different functional transcripts (short/long 3’-UTR, exon IV, V) of the BDNF gene were measured in the mPFC and hippocampus of our rat model of ELS x 5-HTTLPR interaction. Finally, chapters 8 and 9 are dedicated to a summary and a general discussion of the findings that are presented in chapter 2-7.
Adaptive fitness; early life adversity improves adult stress coping in heterozygous serotonin transporter knockout rats

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Molecular Psychiatry 18: 1244-1245 (2013)
Early life adversity in the form of childhood abuse has been associated with increased risk for the development of psychiatric disorders such as major depression (Mullen et al., 1996; McCauley et al., 1997). Recent reviews, however, have postulated the match/mismatch hypothesis of disease, which states that early life stress (ELS) is not necessarily pathological but can be of adaptive value (Schmidt, 2011; Homberg, 2012). Central to this hypothesis is the concept of the predictive adaptive response (PAR) (Gluckman et al., 2007), which entails that an individual uses the experience of past stressors to adaptively respond to future stressors. PAR is expected to be most pronounced in stress-sensitive individuals, similar to humans carrying the low activity short (S) allelic variant of the serotonin transporter (S-HTT) promoter polymorphism (5-HTTLPR) and S-HTT knockout rodents modeling this polymorphism (Homberg and Van den Hove, 2012). In this study, we used the maternal separation paradigm to test the hypothesis that ELS can improve adult stress coping behaviour as a function of serotonin transporter gene variation.

The methods are fully available in the Supplementary Materials. Briefly, from postnatal day 2 to 14 litters were either exposed to a daily 180 min maternal separation (MS180) or a control treatment, which involved the exact same handling, but immediate reunion of dam and pups (MS0). Adult male offspring was subjected to inescapable (IS) shock stress in a shuttle-box (model ENV-010MD, Med Associates, St Albans, VT, USA). For 2 consecutive days, rats received 50 IS, unpredictable 0.6 mA foot shocks. Exactly 24 h after the start of the second exposure to IS, the rats were tested for 30 consecutive trials in which they could escape shocks by moving to the opposite end of the shuttle-box. The latency to escape the shocks was automatically recorded and the maximum of 15 s was registered in case of escape failure.

The escape latencies were found to be affected by both genotype ($F_{2,105} = 4.013, p < 0.05$, Figure 1) and early life treatment (ELT; $F_{1,105} = 5.160, p < 0.05$, Figure 1). The genotype effect was attributed to the S-HTT homozygous knockout (S-HTT $-/-$) rats, which displayed lower escape latencies irrespective of their ELT ($p < 0.05$ for pairwise comparison with the other two genotypes). On the other hand, the effect of ELT was only significant for the S-HTT heterozygous knockout (S-HTT $+/-$) rats ($p < 0.05$, Figure 1).

This study supports the PAR concept by showing that ELS can lead to improved stress-coping behaviour in later life. Interestingly, this adaptive effect of ELS was only significant for S-HTT $+/-$ rats, which fits our previous prediction that behaviour of S-HTT $+/-$ rats is strongly modified by environmental conditions (Kalueff et al., 2010; Homberg and Van den Hove, 2012). Furthermore, S-HTT $+/-$ rats showed lower escape latencies regardless of ELT, supporting the notion that S-HTT $+/-$ rats display increased attentional vigilance under stressful conditions (Pergamin-Hight et al., 2012). Notably, earlier studies revealed increased escape latencies in S-HTT $+/-$ mice compared with wild-type controls (Muller et al., 2010). However, these mice underwent several behavioural tests before the final escape test, which may have influenced the behavioural response.
In conclusion, our results nuance the prevailing theory that 5-HTTLPR s-allele carriers have an increased risk to develop depression when exposed to ELS (Caspi et al., 2003), and suggest that carrying the S-allele does not inevitably have negative consequences. Rather, the increased sensitivity of S-allele carriers to a respective match or mismatch between the early and adult life environment may govern their adaptive or maladaptive responses to stress.

Figure 1 The average escape latencies of the shock escape test. Serotonin transporter homozygous (S-HTT\(^+\)) and heterozygous (S-HTT\(^+\)) knockout rats and their wild-type littermates (S-HTT\(^+\)) (n = 14-23) with an early life history of maternal separation (MS180) or control treatment (MS0) were exposed to two sessions of inescapable (IS) foot shocks. This was followed by a third session in which the foot shocks were escapable. Data show mean ± SEM escape latencies (s). G, genotype effect, p < 0.05; E, early life treatment effect, p < 0.05; *MS180 S-HTT\(^+\) rats different from MS0 S-HTT\(^+\) rats, p < 0.05.

Supplementary material: methods

Animals
Serotonin transporter knockout rats (Slc6a4\(^{\text{null}}\)) were generated by ENU-induced mutagenesis (Smits et al., 2006). Experimental animals were derived from crossing 3 month old heterozygous 5-HTT knockout (5-HTT +/-) rats that were outcrossed for at least eight generations with commercial (Harlan, Ter Horst, The Netherlands) wild-type Wistar rats. The pregnant dams were housed in standard polypolypropylene cages (40 x 20 x 18 cm) with sawdust bedding and ad libitum access to water and rodent chow (Ssniff Spezialdiäten, Soest, Germany) in a temperature (21 ± 1 °C) and humidity-controlled room (45-60% relative humidity), with a 12:12 h light:dark cycle (lights on at 07:00 a.m.). The dams were inspected daily for delivery at 5:00 p.m. and day of birth was designated as postnatal day (PND) 0. At PND1, two paper towels (22.5 x 24.5 cm) were supplied to the dam for nest construction. Further, the litters were culled to a maximum of 10 pups, with gender ratios in favor of a male majority to maximally 7:3, and litters were randomly allocated to one of the following rearing conditions (from PND 2 to 14): maternal separation for 180 min (MS180) or a control treatment with immediate reunion of dam and pups (MS0). MS180 was started daily between 08.30 and 09.00 a.m., and consisted of the following procedure: The dam was removed from the home cage and placed into an identical cage until the end of the separation period. Pups were then removed from the nest as complete litters and placed into a cage (24 x 15 x 14 cm) with only sawdust bedding, and then transferred to an adjacent room. There, the cages were placed on heat pads, which were set to maintain a bedding temperature of 31-33 °C for PND 2-7 and at 29-31 °C at PND8-14. At the end of the separation period, litters were returned to their home cage by first rolling them in the home cage bedding material and then placing them in the nests. This was followed by reunion with the dams. During PND 0-22, half of the bedding material of the home cages was refreshed every week. At PND 22, the pups were weaned and housed in groups of 2-3 littermates of the same sex, under the same conditions as mentioned above. Only the male offspring were used for subsequent experimental testing. All experiments were approved by the Committee for Animal Experiments of the Radboud University Nijmegen, The Netherlands, and all efforts were made to minimize animal suffering and to reduce the number of animals used.

Inescapable shock stress
When the male rats reached adulthood, they were subject to inescapable shock stress in a shuttle box (model ENV-010MD, Med Associates, St. Albans, VT, USA) located within a sound-attenuating cubicule. The box was equipped with eight infrared beams to detect the position of the animal, and separated into two identical chambers by an automated door that opened vertically. The grid floor of the apparatus was connected to a scrambled shock generator (model ENV-412, Med Associates), which enabled the variation of potential
differences between the bars of the grid floor in order to prevent animals from avoiding the foot shocks. For two consecutive days, between 08.30 and 12.00 a.m., rats received 50 inescapable, unpredictable 0.6 mA foot shocks. After a 5 min (day 1) or 2.5 min (day 2) habituation period, the IS session started with varying interval durations (10-18 s) and shock durations (6-14 s), amounting to a total session duration of 25 or 22.5 min, respectively. During the intervals, the door separating the chambers was raised, allowing the rats to move freely across the shuttle box.

**Escape testing**
Exactly 24 h after the start of the second exposure to IS, the rats were assessed for their ability to escape shocks in the same apparatus. The difference with the IS sessions was that the door was now raised 1 s after the onset of the shock exposure and that rats could escape the shock by moving to the opposite end of the shuttle box. The rats were tested for 30 consecutive trials with a fixed interval duration (25 s) and a maximum shock duration of 15 s. The latency of the rats to escape the shocks was automatically determined by use of the infrared beams. If the rat failed to escape, the maximum 15 s was registered as the escape latency score.

**Statistical analysis**
The results are presented as the mean and the standard error of the mean (SEM) for all experimental groups. Mean escape latencies were analyzed with factorial analysis of variance (ANOVA), and if a significant main effect (“genotype”, “treatment”) or interaction (“genotype x treatment”) was found, appropriate a posteriori tests were performed (one-way ANOVA and independent samples t-test). If doubt about the normality of the sample distributions existed, bootstrapping was applied to test the robustness of the parametric tests. Statistical significance was set at p < 0.05. All statistical tests were carried out using SPSS (version 20, IBM corporation, Armonk, NY, USA).
Chapter 3

Early life adversity and serotonin transporter gene variation interact at the level of the adrenal gland to affect the adult hypothalamo-pituitary-adrenal axis

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Translational Psychiatry 4: e409 (2014)
Abstract

The short allelic variant of the serotonin transporter (5-HTT) promoter-linked polymorphic region (5-HTTLPR) has been associated with the etiology of major depression by interaction with early life stress (ELS). Furthermore, 5-HTTLPR has been associated with abnormal functioning of the stress-responsive hypothalamo-pituitary-adrenal (HPA) axis. Here, we examined if, and at what level, the HPA-axis is affected in an animal model for ELS x 5-HTTLPR interactions. Heterozygous and homozygous 5-HTT knockout rats and their wild-type littermates were exposed daily at postnatal days 2–14 to 3 h of maternal separation. When grown to adulthood, plasma levels of adrenocorticotropic hormone (ACTH), and the major rat glucocorticoid, corticosterone (CORT), were measured. Furthermore, the gene expression of key HPA-axis players at the level of the hypothalamus, pituitary and adrenal glands was assessed. No 5-HTT genotype x ELS interaction effects on gene expression were observed at the level of the hypothalamus or pituitary. However, we found significant 5-HTT genotype x ELS interaction effects for plasma CORT levels and adrenal mRNA levels of the ACTH receptor, such that 5-HTT deficiency was associated under control conditions with increased, but after ELS with decreased basal HPA-axis activity. With the use of an in vitro adrenal assay, naïve 5-HTT knockout rats were furthermore shown to display increased adrenal ACTH sensitivity. Therefore, we conclude that basal HPA-axis activity is affected by the interaction of 5-HTT genotype and ELS, and is programmed, within the axis itself, predominantly at the level of the adrenal gland. This study therefore emphasizes the importance of the adrenal gland for HPA-related psychiatric disorders.

Introduction

The risk to develop depression is largely determined by both genetic and environmental factors, and understanding the precise mechanisms is essential to design personalized treatments. Although severe adverse events such as childhood abuse and neglect have been convincingly associated with depression (Heim and Nemeroff, 2001), a discrepancy exists between the high heritability estimates of depression and the replicability of genetic association studies (Nestler et al., 2002; Bogdan et al., 2013). It has become apparent that the effects of genetic and environmental factors should not merely be regarded as independent, but should be considered to have an interactive nature. For instance, the effects of stressful life events on the individual risk to develop depression have been shown to be dependent on serotonin transporter (5-HTT) promoter-linked polymorphic region (5-HTTLPR) genotype (Caspi et al., 2003). Although some meta-analyses could not confirm this gene x environment (GxE) interaction (Munafò et al., 2009; Risch et al., 2009), others have shown that it is especially significant after a history of early life stress (ELS) (Karg et al., 2011). Specifically, individuals with the short (S) allele of the 5-HTTLPR polymorphism were found to be more sensitive to the depressogenic effects of stress (Clarke et al., 2010; Kyoshara and Yoshimasu, 2010; Karg et al., 2011).

One biological system through which the 5-HTTLPR may interact with stress is the stressor-responsive hypothalamo-pituitary-adrenal (HPA) axis (Caspi et al., 2010). A stress response of the HPA-axis is initiated by parvocellular neurons in the paraventricular nucleus (PVN) of the hypothalamus, by secreting corticotropin-releasing factor (CRF) at the median eminence to stimulate the synthesis and release of adrenocorticotropic hormone (ACTH), which itself stimulates the synthesis and release of glucocorticoids from the adrenal cortex (De Kloet et al., 2005). The major glucocorticoid in humans is cortisol, whereas in rodents it is corticosterone (both referred to as CORT).

For 5-HTTLPR as an independent factor, it has been reported that S-allele carriers display increased basal activity of the HPA-axis (O’Hara et al., 2007; Chen et al., 2009; Gooddy et al., 2009; Wüst et al., 2009, Wankel et al., 2010), and that S/S homozygotes show increased CORT stress reactivity compared with individuals carrying a long (L) allele of the 5-HTTLPR (Gotlib et al., 2008; Way and Taylor, 2010; Miller et al., 2013). In macaques, however, 5-HTTLPR genotype has not been shown to affect basal and stress-induced CORT levels (Barr et al., 2004b, 2004c). In mice, 5-HTT knockout (5-HTT−/−) leads to increased adrenomedullary but not CORT responses to stress, and basal plasma CORT levels have been reported to be unaltered or lower in 5-HTT−/− mice (Li et al., 1999; Lanfumey et al., 2000; Tjurmina et al., 2002, 2004; Bartolomucci et al., 2010, Jansen et al., 2010, Van den Hove et al., 2011; Hohoff et al., 2013; Spinelli et al., 2013). In the case of ELS x 5-HTTLPR genotype interaction, only a history of severe stress has been shown to trigger increased CORT responses in human S-allele carriers (Alexander et al., 2009; Mueller et al., 2011). In contrast, in macaques the combination of the S-allele with adverse rearing conditions results in
increased ACTH but unaffected CORT responses to social separation (Barr et al., 2004b, 2004c).

Despite the relatively large body of literature it is yet unclear at what level of the HPA-axis and through what mechanisms the activity of the HPA-axis is programmed by 5-HTTLPR x ELS interactions. Therefore, we assessed the HPA-axis at both central and peripheral levels in 5-HTT knockout rats, which model the 5-HTTLPR S-allele and display depression-related behaviour (Olivier et al., 2008a). Specifically, we tested the effect of ELS — that is, maternal separation – on plasma stress hormone, PVN, pituitary and adrenal gene expression levels, and we measured ACTH sensitivity of the adrenal gland as a function of 5-HTT genotype. The outcome of this study is potentially important, because whether to target central or peripheral components of the HPA-axis is essential for future drug design, due to the constraints of the blood–brain barrier.

Materials and Methods

Animals

All experiments were approved by the Committee for Experimental Animals of the Radboud University Nijmegen, The Netherlands, and all efforts were made to minimize animal suffering and to reduce the number of animals used. Serotonin transporter knockout rats (Slc6a41Hubr) were generated by N-ethyl-N-nitrosourea-induced mutagenesis (Smits et al., 2006). Experimental animals (5-HTT homozygous knockout (5-HTT −/−), 5-HTT heterozygous knockout (5-HTT +/−), 5-HTT heterozygous knockout (5-HTT +/−) and wild-type (5-HTT +/+) were derived from crossing 3-month–old 5-HTT +/− rats that were outcrossed for at least 12 generations with commercial (Harlan, Ter Horst, The Netherlands) wild-type Wistar rats. The pregnant dams were housed in standard polypropylene cages (40 × 20 × 18 cm) with sawdust bedding and ad libitum access to water and rodent chow (Sniff Spezialdiäten, Soest, Germany) in a temperature (21 ± 1 °C) and humidity-controlled room (45–60% relative humidity), with a 12:12 h light:dark cycle (lights on at 0700 hours). The dams were inspected daily for delivery at 1700 hours and day of birth was designated as postnatal day (PND) 0. At PND 1, two paper towels were put on ice and the brain and pituitary were dissected. The blood samples were put on ice and subsequently centrifuged (3400 r.p.m., 15 min) to obtain plasma samples, which were then stored at −80 °C until measurements. The brains and pituitaries were frozen in aluminum foil on dry ice and also stored at −80 °C.

Early life stress

We used repeated and prolonged maternal separation as a model for ELS, as this paradigm has previously been shown to affect adult HPA-axis functioning (Smotherman et al., 1977; Plotsky and Meaney, 1993). Litters were randomly allocated to one of two rearing conditions (from PND 2 to 14): maternal separation for 180 min (MS180) or a control treatment with immediate reunion of mother and pups (MS0). A detailed description of the procedure can be found in the Supplementary Material. From PND 2 to 8, the mothers were observed to score their maternal care behaviour outside the maternal separation period. The scoring of maternal care was performed daily at 0700, 1300, 1700 and 2000 hours. The distribution of the observation periods was based on the finding that nursing in rats occurs more frequently during the light period (Champagne et al., 2003). The observation periods lasted 1 h, with 20 single, focal observations spaced by 3 min. The observations were scored within 5–10 s by a trained observer in front of the cages. The following behaviours were scored (not mutually exclusive): (1) mother away from the pups, (2) mother in any type of contact with the pups, (3) mother licking/grooming any pup, (4) arched-back nursing (ABN), (5) blanket-posture nursing and (6) passive-nursing posture. The scoring of the nursing postures was based on the descriptions by Myers et al (Myers et al., 1989). The frequency of the (combinations of) behaviours across each observation period was calculated by dividing the number of times the specific behaviour was observed (0–20) by the total number of observations in that period (20). At PND 14, ear punches were taken of the pups for identification and genotyping, which was performed by Kbiosciences (Hoddesdon, UK). The procedure of genotyping has been described previously (Homberg et al., 2007). At PND 22, the pups were weaned, weighed and housed in groups of 2–3 littermates of the same sex, under the same conditions as mentioned above. From weaning until adulthood, the rats were regularly weighed (PND 30, 38, 46, 58, 65, 72, 79).

Tissue collection

For the collection of tissues only adult (PND85–95) male rats were used. Of every litter, where possible, a single rat was selected of all three genotypes. The rats were sacrificed between 0900 and 1400 hours by either acute decapitation or by transcardial perfusion. Across this time period, the rats were randomized for their genotype and early life treatment.

For decapitations, the rats were taken from their home cage into a separate room and decapitated within 10 s. Immediately, the trunk blood was collected in EDTA-coated vials and the brain and pituitary were dissected. The blood samples were put on ice and subsequently centrifuged (3400 r.p.m., 15 min) to obtain plasma samples, which were then stored at −80 °C until measurements. The brains and pituitaries were frozen in aluminum foil on dry ice and also stored at −80 °C.

Before transcardial perfusion, rats received an intraperitoneal injection of sodium pentobarbital (50 mg kg−1 body weight). With anesthetization commencing within 3–5 min, the transcardial perfusion was performed with a clamp on the abdominal aorta to limit the perfusion to the upper body parts. The perfusion was performed with phosphate-buffered saline and followed by fixation with 4% paraformaldehyde in phosphate-buffered saline. Directly after the start of the perfusion (5 min), the adrenal glands were dissected, weighed and stored at −80 °C.
Plasma measurements

All plasma measurements were performed on samples derived from acutely decapitated rats. Plasma CORT was measured in duplicates using a colorimetric enzyme-linked immunosorbent assay kit (Demeditec Diagnostics GmbH, Kiel, Germany), ACTH with a luminescent enzyme-linked immunosorbent assay kit (Calbiotech, Spring Valley, CA, USA) and plasma adrenalin by analyzing 2,3-diphenyl quinoxalin derivatives using isocratic high-pressure liquid chromatography with fluorimetric detection after extraction from the plasma as described elsewhere (Willemsen et al., 1995).

RNA isolation & cDNA synthesis

Frozen brains were cut in 420 μm-thick coronal slices in a cryostat (−15 °C). From two of these slices (cut at Bregma −1.30 and −1.72 mm) the PVN was bilaterally punched out with a Miltex 1.0 mm biopsy puncher (Integra Miltex, York, PA, USA). The punched samples were collected in sterile vials, immediately placed on dry ice and stored at −80 °C. After punching was completed for all samples, PVN RNA was isolated with the NucleoSpin RNA II kit (Macherey-Nagel GmbH, Düren, Germany). For RNA isolation from the pituitary and adrenal glands, 800 μl of ice-cold TRIzol (Life Technologies, Carlsbad, CA, USA) was added to the samples, which were thereafter homogenized by sonication. After chloroform extraction and isopropyl alcohol precipitation, RNA was dissolved in 30 μl of DEPC-treated, RNase-free water. All RNA samples were stored at −80 °C. RNA concentrations were measured and RNA purity checked (A260/280 ratio between 1.8 and 2.0) with a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). First strand cDNA synthesis was performed using 40 ng of PVN RNA and 100 ng of pituitary and adrenal gland RNA. The RNA was dissolved in 12 μl of RNase-free DEPC containing 0.25 mM random hexamer primers (Roche Applied Science, Penzberg, Germany) and then incubated at 70 °C for 10 min, followed by double-strand synthesis in first strand buffer with 10 mM DTT, 100 U Superscript II (Life Technologies), 0.5 mM dNTPs (Roche Applied Science) and 20 U of RNasin (Promega, Fitchburg, WI, USA) at 37 °C for 75 min. The cDNA samples were stored at −20 °C.

Quantitative real-time PCR

Quantitative real-time PCR (qRT–PCR) was performed with the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). For the reactions a total volume of 25 μl buffer solution was used containing 5 μl template cDNA, 12.5 μl Power SYBR Green Master mix (Applied Biosystems, Foster City, CA, USA), 1.5 μl RNase-free DEPC and 0.6 μM of each primer. The sequences of the primers are available in Supplementary Table 1. Before analysis of the relative expression of the genes of interest, for each tissue it was evaluated whether Rn18S, Gapdh or Hprt1 would be the best internal control gene. The cycling protocol started with 10 min at 95 °C, followed by 39 reaction cycles with 15 s at 95 °C and 1 min at 60 °C. For each reaction, the Ct (cycle threshold) was determined, that is, the number of cycles needed to detect fluorescence above the arbitrary threshold. Relative expression of the genes of interest was calculated by the 2−ΔΔCt method (Schmittgen and Livak, 2008). The procedure was concluded with a melting curve protocol, from 65 °C to 95 °C, measuring fluorescence every 0.5 °C, to control for product specificity. All qRT–PCR analyses were carried out in triplicate, with newly synthesized cDNA.

Adrenal in vitro assay

Adult male 5-HTT+/+, 5-HTT+/− and 5-HTT−/− rats without any ELS were acutely decapitated, and trunk blood and adrenal glands were collected. After dissection, the adrenals were immediately placed in 1 ml of chilled (4 °C) Dulbecco’s modified Eagle’s medium (DMEM, Sigma-Aldrich, St. Louis, MO, USA) containing 3.7 g l−1 NaHCO3 and 2.5% BSA (one gland per tube). The adrenals were then processed into four pieces of equivalent size by two perpendicular cuts right through the medial axes of the adrenals, with the use of forceps, a sterile razor blade and a cutting mat. Then, the adrenal pieces were incubated at 37 °C in a 95% O2-5% CO2 atmosphere in 1 ml DMEM. After 2 × 60 min pre-incubation and refreshment of medium, 15-min samples were collected six times with ACTH-containing medium in the second fraction. The selected concentration of ACTH (10−12 M) was based upon experience with previous experiments (Zelena et al., 2008, 2011). After every 15-min incubation, medium was aspirated, centrifuged and the supernatant was used for measurement of CORT concentrations by the use of a radioimmunoassay, as described elsewhere (Zelena et al., 2011).

Statistical analysis

All statistical tests have been carried out using SPSS (version 20, IBM corporation, Armonk, NY, USA). The results are presented as the mean with the standard error of the mean (SEM). For the qRT–PCR results, the 2−ΔΔCt data have been expressed as a ratio compared with the average of the M0 wild-type group. For the adrenal assay, CORT levels were expressed as percentage of the basal secretion measured in the first two 15-min samples. For the maternal care scores, body weight data and the adrenal assay, analysis of variance (ANOVA) with repeated measures was performed. In case of violation of the assumption of sphericity, Greenhouse–Geisser correction was applied to determine the F-ratio. Factorial ANOVA was applied for data from the plasma hormone, adrenal weight and qRT–PCR measurements, and if a significant main effect (‘genotype’, ‘early life stress’) or interaction (‘genotype x early life stress’) was found, appropriate a posteriori tests were performed (one-way ANOVA and independent samples t-test). For the adrenal assay, we examined the a priori hypothesis of greater CORT response in 5-HTT+− versus 5-HTT++ rats with one-sided t-tests. If doubt about the normality of the sample distributions existed, logarithmic transformation and bootstrapping were applied to test the robustness of the parametric tests (see also Supplementary Material). Log-transformation was applied in the statistical analysis of plasma adrenalin, pituitary CRF, PVN MR and adrenal ACTH-R, CYP11B1/3 and StAR mRNA levels. Statistical significance was set at p < 0.05.
CHAPTER 3 THE HPA-AXIS AND ADRENAL PROGRAMMING

Plasma measurements

Plasma ACTH levels of the adult offspring (PND85-95) were not significantly affected in our experimental design, which was also the case for plasma adrenalin (Figures 2A and C), the major output of sympathetic-adrenal medulla activation. It should be noted that it is difficult to reliably obtain basal plasma adrenalin levels from non-catheterized animals, as adrenalin levels rise within seconds when handling animals.

In contrast, plasma CORT levels were significantly affected by an interaction of ELS with genotype ($F_{2,34} = 3.51, p < 0.05$), and not by either factor independently. Specifically, the GxE interaction comprised an opposite effect of 5-HTT genotype depending on ELS; 5-HTT−/− rats displayed the highest plasma CORT levels in the control group (MS0), which was absent after MS180 exposure. In contrast, the 5-HTT+/+ rats showed an up-regulation

Results

Maternal Care and Body Weight

Significant effects for both ELS ($F_{1,20} = 23.49, p < 0.001$) and time ($F_{11.14,222.85} = 8.51, p < 0.001$) on the percentage of time that the pups received ABN were found (MS0: 31.3 ± 2.0% versus MS180: 41.5 ± 0.9%). The main effect of ELS on ABN was found to be significant from PND 3–7 ($p < 0.05$, Figure 1). No interaction effects were found, nor main effects of ELS, on the other measures of maternal care including the licking/grooming of pups and its combination with ABN.

For the post-weaning body weight development, significant main effects of time ($F_{17,353.65} = 14.380.56, p < 0.001$), ELS ($F_{1,189} = 14.76, p < 0.001$) and genotype ($F_{2,189} = 17.57, p < 0.001$) were obtained. Further, significant interactions were present for time x ELS ($F_{17,353.65} = 9.56, p < 0.001$) and time x genotype ($F_{3,431,313.63} = 12.07, p < 0.001$), but not for ELS x genotype or time x ELS x genotype. The MS180 male offspring developed a significantly lower body weight than MS0 animals from PND30 onwards, whereas 5-HTT−/− rats had a significantly lower body weight compared with 5-HTT+/− and 5-HTT+/+ rats across all measurements ($p < 0.05$, Supplementary Figure 1).

Figure 1 The frequency of arched-back nursing (ABN) of the pups as displayed by the mother rats across postnatal days (PND) 2–8. The frequency of ABN by the mother was observed daily during four observation periods (7.00 a.m. and 1.00, 5.00 and 8.00 p.m.) in litters subjected daily to 3 h maternal separation (MS180, n = 13) or a control treatment (MS0, n = 12). Data show mean ± SEM percentage of time. *: p < 0.05 for MS180 vs MS0.

Figure 2 Plasma levels (± SEM) of adrenocorticotropic hormone (A, ACTH), corticosterone (B, CORT) and adrenalin (C) of serotonin transporter (5-HTT) homozygous knockout (5-HTT−/−), heterozygous knockout (5-HTT+/-) and wild-type (5-HTT+/+) rats (n = 5-8) exposed to daily 3 h separations (MS180) or a control treatment (MS0). The plasma CORT concentrations were found to be significantly affected by the interaction of 5-HTT genotype and early life treatment (G x E, p < 0.05).
of plasma CORT levels due to MS180, such that the 5-HTT<sup>+</sup> rats showed the highest plasma CORT levels after MS180 treatment (Figure 2B).

**PVN mRNA levels**

In the PVN we measured the mRNA levels of CRF, glucocorticoid receptor (GR), mineralocorticoid receptor and the GR chaperone FK506-binding protein 51 (FKBP5). Factorial ANOVA revealed a significant effect of 5-HTT genotype on GR mRNA levels (F<sub>2,36</sub> = 3.51, p < 0.05). It followed that 5-HTT<sup>-/-</sup> rats exhibited a significantly lower GR mRNA expression than 5-HTT<sup>+</sup> rats, independent of ELS (p < 0.05, Supplementary Figure 2). The PVN CRF, MR and FKBP5 mRNA levels were not affected (Supplementary Figure 3).

**Adrenal mRNA levels**

Interestingly, the qRT–PCR analysis of adrenal mRNA levels revealed gene expression patterns that resemble the plasma CORT levels as a function of ELS x 5-HTT interaction. Although we did not find independent effects of either factor on adrenal mRNA levels, the GxE interaction significantly affected the expression of the ACTH-receptor (F<sub>2,36</sub> = 7.91, p < 0.01) and the mitochondrial enzyme 11β-hydroxylase (F<sub>2,36</sub> = 15.38, p < 0.001) (Figures 3A and 3B), which is responsible for the last step in glucocorticoid biosynthesis (Zhou et al., 1995). Furthermore, ELS x 5-HTT genotype interaction significantly affected the mRNA levels of steroidogenic acute regulatory protein (StAR, F<sub>2,36</sub> = 3.61, p < 0.05) and 3β-hydroxysteroid dehydrogenase (3βHSD1, F<sub>2,36</sub> = 12.17, p < 0.001) (Supplementary Figure 6).

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**Figure 3** Adrenocorticotropic hormone (A, ACTH-R), 11β-hydroxylase (B, cytochrome P450 11B1/3, CYP11B1/3) and tyrosine hydroxylase (C) mRNA levels in the adrenal glands of serotonin transporter (5-HTT) homozygous knockout (5-HTT<sup>-/-</sup>), heterozygous knockout (5-HTT<sup>+</sup>−/−) and wild-type (5-HTT<sup>+</sup>+) rats (n = 7) exposed to daily 3 h separations (MS180) or a control treatment (MS0). The mRNA levels of both ACTH-R and CYP11B1/3 were found to be significantly affected by the interaction of 5-HTT genotype and early life treatment (p < 0.01, p < 0.001 respectively). Data were normalized to the average of the MS0-S-HTT<sup>+</sup> group. * p < 0.05, ** p < 0.01, *** p < 0.001.

**Figure 4** Corticosterone (CORT) response to 10<sup>-12</sup> M adrenocorticotropic hormone (ACTH), of adrenal tissue derived from serotonin transporter (5-HTT) homozygous knockout (5-HTT<sup>-/-</sup>), heterozygous knockout (5-HTT<sup>+</sup>−/−) and wild-type (5-HTT<sup>+</sup>+) rats (n = 6) which were reared in standard animal facility conditions. The CORT response was measured in vitro, in a static incubation system from which samples were collected every 15 min. * 5-HTT<sup>-/-</sup> significantly different from 5-HTT<sup>+</sup>+ (p < 0.05, one-sided t-test).

**Pituitary mRNA levels**

In the pituitary gland no significant effects on the expression of pro-opiomelanocortin (precursor protein of ACTH), GR and MR mRNA were found (Supplementary Figure 4). For the mRNA levels of CRF receptor 1 (CRF 1R) there was a trend towards a 5-HTT genotype effect (Supplementary Figure 5A) (F<sub>2,34</sub> = 2.63, p = 0.087), while pituitary FKBP5 mRNA levels were affected by a main effect of ELS (F<sub>1,34</sub> = 6.42, p < 0.05). The exposure of ELS led to a decrease of FKPB5 expression in the pituitary of both 5-HTT<sup>+</sup>+/+ and 5-HTT<sup>-/-</sup> rats (Supplementary Figure 5B).

**Adrenal mRNA levels**

Interestingly, the qRT–PCR analysis of adrenal mRNA levels revealed gene expression patterns that resemble the plasma CORT levels as a function of ELS x 5-HTT interaction. Although we did not find independent effects of either factor on adrenal mRNA levels, the GxE interaction significantly affected the expression of the ACTH-receptor (F<sub>2,36</sub> = 7.91, p < 0.01) and the mitochondrial enzyme 11β-hydroxylase (F<sub>2,36</sub> = 15.38, p < 0.001) (Figures 3A and 3B), which is responsible for the last step in glucocorticoid biosynthesis (Zhou et al., 1995). Furthermore, ELS x 5-HTT genotype interaction significantly affected the mRNA levels of steroidogenic acute regulatory protein (StAR, F<sub>2,36</sub> = 3.61, p < 0.05) and 3β-hydroxysteroid dehydrogenase (3βHSD1, F<sub>2,36</sub> = 12.17, p < 0.001) (Supplementary Figure 6). Steroidogenic acute regulatory protein transports the steroid precursor cholesterol into the mitochondria, a process regarded as the rate-limiting step in corticosterone synthesis.
Adrenal in vitro assay

As we found that the interaction of ELS and 5-HTT gene variation affected plasma CORT but not ACTH levels, as well as adrenal gene expression of the ACTH receptor, we hypothesized that the basal CORT levels were effected by programming of adrenal ACTH sensitivity. To provide a proof of concept, we performed an in vitro experiment with adrenal glands derived from adult male 5-HTT+/+, 5-HTT+/- and 5-HTT−/− rats without any early life environmental manipulation (standard animal facility rearing, AFR). As the AFR and MS0 groups are both considered to be control groups, we expected that, upon administration of ACTH, adrenal tissue derived from AFR 5-HTT−/− rats would show a greater in vitro release of CORT than adrenal tissue of AFR 5-HTT+/+ rats. The basal plasma CORT levels of AFR 5-HTT−/− rats were indeed, just as for the MSO group, found to be higher than that of 5-HTT+/+ rats (p < 0.05, Supplementary Figure 8).

For the in vitro assay, ANOVA-RM revealed that the adrenal explants showed a significant CORT response after the application of 10−12 M ACTH to the medium (time, F1,25 = 11.92, p < 0.001), with furthermore no main effect of 5-HTT genotype, but a significant interaction of time x 5-HTT genotype (F1,25,2,75 = 1.96, p < 0.05). Unfortunately, Mauchly’s test indicated a violation of sphericity and we therefore had to apply Greenhouse-Geisser correction, after which the effect of time remained significant (F1,18.45 = 11.92, p < 0.001) but the interaction of time x 5-HTT genotype did not (F1,18.31,2,65 = 1.96, p < 0.05). However, as we had an a priori hypothesis, we used one-sided t-tests to confirm that immediately after application of ACTH (fractions 3 and 4) 5-HTT−/− adrenal tissue shows a significantly higher CORT response than 5-HTT+/+ adrenal tissue (p < 0.05) (Figure 4). An area under the curve analysis was used to quantify the total CORT release upon ACTH stimulation, which also showed a higher CORT response of adrenal tissue derived from 5-HTT+/+ compared with 5-HTT−/− rats (Supplementary Figure 9).

Discussion

In this study, we show for the first time that ELS and 5-HTT genotype interact to program basal CORT levels and that this is paralleled by an equivalent G×E programming of adrenal mRNA levels of the ACTH-receptor, steroidogenic acute regulatory protein, 3ß-HSD1 and 11ß-Hydroxylase, which regulate the sensitivity of the adrenal glands to synthesize and release CORT upon stimulation by ACTH. In contrast to the adrenal gland, gene expression in the PVN and pituitary gland were not affected by ELS x 5-HTT genotype interaction. It therefore seems that the ELS x 5-HTT genotype programming of basal HPA-axis activity is, within the axis itself, predominantly effectuated at the level of the adrenal gland. It remains, however, to be investigated how the interaction of ELS and 5-HTT genotype can actually program adrenal ACTH sensitivity. Interestingly, there are numerous intra-adrenal, paracrine pathways that are involved in the regulation of adenocortical steroidogenesis, including the intra-adrenal CRF–ACTH and renin–angiotensin systems (Nussdorfer, 1996, Ehnhart-Borstein et al., 1998). Moreover, the chromaffin cells of the rat adrenal medulla are known to contain 5-HT, which potently stimulates CORT release by the adrenal cortex (Verhofstand and Jonsson, 1983; Lefebvre et al., 1992, Contesse et al., 1998). In humans and frogs, this stimulation is mediated by activation of 5-HT3 receptors, but for the rat the responsible 5-HT receptor subtype remains elusive (Contesse et al., 1998). In this study, we found no effect of ELS x 5-HTT genotype on the expression of the 5-HT3 receptor in the adrenals (data not shown), but so far we have not further explored the possibility of ELS x 5-HTT genotype programming of the intra-adrenal 5-HT-S system.

In human 5-HTT/LP 5-allele carriers basal CORT levels are increased (OHTara et al., 2007, Chen et al., 2009; Goodyer et al., 2009; Wüst et al., 2009, Wankerl et al., 2010), just as we found for 5-HTT−/− rats in control conditions. We expand this finding by showing that after exposure to ELS, the effect of 5-HTT deficiency is abolished, whereas 5-HTT+/+ rats show an up-regulation of their basal HPA-axis activity. Therefore, without a history of ELS, 5-HTT−/− rats show the highest CORT levels, while after ELS 5-HTT−/− rats display decreased and 5-HTT+/+ rats show increased levels of basal CORT. These results resonate with the finding that 5-HTT/LP S/S individuals displayed the highest basal CORT levels within a low-risk for depression group, whereas in the high-risk group the S/S subjects showed the lowest and the L/L subjects the highest baseline CORT levels (Jabbie et al., 2007). Accordingly, CORT levels could mediate the combined effects of (early life) stress and 5-HTTLPR on later life risk for psychopathology (Vinberg et al., 2014). However, although CORT is expected to have a significant role in the onset and course of depression, it is not exactly clear what this role is (Herbert, 2013). For instance, some studies have, whereas others have not, found a relation between basal CORT levels and the recurrence of depression in remitted patients (Goodyer et al., 2009, Bockting et al., 2012; Lok et al., 2012). Nevertheless, altered basal HPA activity seems to be an endophenotype that cuts across disorders, with lower CORT levels consistently observed for post-traumatic stress disorder (Yehuda, 2009), whereas elevated plasma CORT levels are found in a subset of depressive patients (Checkley, 1996), which possibly reflect the melancholic clinical subtype of depression (Gold and Chrousos, 2002). From animal studies, the perspective arises that the adaptive- or maladaptiveness of the early life programming of HPA activity is highly dependent upon the match or mismatch with the later life environment (Heiming and Sachser, 2010; Nederhof and Schmidt, 2012; Van der Doelen et al., 2013) likely due to the specific demands of a given stressful context (Myers et al., 2014). Indeed, ELS has been
predicted to depend on 5-HTT genotype (Heiming et al., 2011; Kloke et al., 2013). Moreover, the consequences of maternal perinatal stress might be an important mediator of the effects on the offspring, which are also linked to later life variables, such as obesity and psychiatric variables. Furthermore, in the case of perinatal stress, the maternal HPA-axis may be affected by including their possible interaction with later life adversity (Essex et al., 2011; Kloe et al., 2013).

Interestingly, in our study the interaction between ELS and 5-HTT gene variation determines basal HPA-axis output and matches an identical GxE programming of gene expression in the adrenal glands. Given that these genes include the ACTH receptor as well as several key enzymes involved in the biosynthesis of Cort and that plasma ACTH levels are simultaneously measured, we propose that the interaction between ELS and 5-HTT genotype programs the sensitivity of the adrenals to translate a stimulation of ACTH into the synthesis and release of Cort. In support, as 5-HTT+/− rats without a history of ELS (MSO/AFR) show increased basal Cort and adrenal ACTH-R mRNA compared with 5-HTT−/− rats, their adrenals also show an increased Cort release upon in vitro stimulation with ACTH. These findings strongly suggest that the ELS x 5-HTT genotype effect on basal Cort levels would influence stress-induced HPA-axis output activity as well. In addition, the limited adaptations within the HPA-axis (pituitary, PVN) to the programming of the adrenal glands found in this study predict that Cort would influence extra-hypothalamic sites (for example, hippocampus, amygdala and prefrontal cortex) involved in HPA regulation/programming.

As no previous studies have assessed the rodent HPA-axis after combining ELS exposure and 5-HTT knockout, our results have to be considered independently too for these factors to compare them to the literature. In our 5-HTT+/− rats, we confirm previous findings in 5-HTT−/− mice showing decreased GR mRNA levels in the PVN and unaltered pituitary CRF mRNA, adrenal tyrosine hydroxylase mRNA, plasma ACTH and adrenal levels. We however could not replicate the finding that 5-HTT−/− mice show decreased CRF mRNA in the PVN and GR mRNA in the pituitary gland (Li et al., 1999; Tjernim et al., 2002; Jiang et al., 2009). Regarding basal plasma Cort, both lower and unaltered levels have been reported in 5-HTT−/− mice (Li et al., 1999; Lanfumey et al., 2000, Tjernim et al., 2002, 2004; Bartolomucci et al., 2010; Jansen et al., 2010; Van den Hove et al., 2011; Hofhoff et al., 2013; Spinelli et al., 2013), complicating a comparison with the present data. For ELS exposure, we replicate here previous studies that showed that maternal separation leads to higher baseline plasma Cort levels in Wistar and Sprague–Dawley rats (Biagini et al., 1998; Lajud et al., 2004; Cotella et al., 2013), with unaltered CRF mRNA levels in the PVN of Sprague–Dawley rats (Bravo et al., 2011). In Long–Evans rats, however, maternal separation leads to an increase in PVN CRF gene expression with unaltered basal Cort levels (Plotsky and Meaney, 1993; Huot et al., 2004; Plotsky et al., 2005). These strain differences, in addition to 5-HTT gene variation, show that the effects of ELS are highly dependent on genetic variation.

Our GxE interaction findings on plasma Cort and adrenal mRNA levels consist of a strong and opposite regulation of 5-HTT+/− and 5-HTT−/− rats by the exposure to ELS. In contrast, 5-HTT−/− rats seem to be unaffected. Yet, it should be noted that 5-HTT−/− rats do consistently display an intermediate phenotype on these measures consistent with a gene dosage effect. Although 5-HTT−/− rodents have been proposed as the foremost model for human 5-HTTLPR S-allele carriers, 5-HTT−/− rodents are regarded as a robust model for the S-allele plus a history of stress exposure (Kaluff et al., 2010). Indeed, we have shown previously that when 5-HTT−/− rats are exposed in adulthood to an additional stressor the experience of early life adversity directs the stress coping behaviour of 5-HTT+−/− rats towards that as displayed by 5-HTT−/− rats (Van der Doelen et al., 2013). Therefore, the effects of ELS on the HPA-axis of 5-HTT−/− rats might only become apparent with exposure to additional stressors in later life.

For the interpretation of the effects of ELS on HPA-axis programming, we have considered the role of alterations in the care that the mother rats provide to their pups. The group of Michael Meaney and others have namely shown that a very specific part of maternal care, the licking and grooming of pups, can influence the programming of the HPA-axis into adulthood (Li et al., 1997; Weaver et al., 2004; Macrì and Würbel, 2006). However, the exposure of ELS was not found to affect the frequency of licking and grooming displayed by the mothers. In contrast, we found that ELS increased the frequency of ABN, but this maternal behaviour is not known to affect HPA-axis programming (Li et al., 1997; Weaver et al., 2004; Macrì and Würbel, 2006). Therefore, we conclude that the ELS-induced programming of the HPA-axis is not mediated by alterations in maternal care. The increased ABN due to maternal separation could be considered as an expression of nutritional compensation, although it did not prevent a decreased body weight from PND 30 onwards. The isolated, negative effects of ELS and 5-HTT deficiency on body weight development have both been documented before (Biagini et al., 1998; Homberg et al., 2010c).

In conclusion, we report here that early life programming of basal HPA-axis activity is moderated by 5-HTT genotype and that this interaction seems to be effectuated predominantly by the regulation of adrenocortical gene expression. Altered HPA activity is an endophenotype that is widely relevant across the spectrum of psychiatric disorders, therefore, this study emphasizes the importance of the adrenal gland in stress-related psychopathology.

Acknowledgements
We thank Anthonieke Middelman, Debbie van Tilburg-Ouwens, Peter Cruijzen, Ron Engels and Zsuzsa Merli for technical assistance.
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Supplementary methods

Maternal separation

Litters were randomly allocated to one of two rearing conditions (from PND 2 to 14): maternal separation for 180 min (MS180) or a control treatment with immediate reunion of mother and pups (MS0). MS180 was started daily between 08:30 and 09:00 a.m., and consisted of the following procedure: The mother was removed from the home cage and placed into an identical cage until the end of the separation period. Pups were then removed from the nest as complete litters and placed into a cage (24 x 15 x 14 cm) with only sawdust bedding, and then transferred to an adjacent room. There, the cages were placed on heat pads, which were set to maintain a bedding temperature of 31-33°C for PND 2-7 and 29-31°C for PND 8-14. At the end of the separation period, litters were returned to their home cage by first rolling them in the home cage bedding material and then placing them in the nests. This was followed by reunion with the mothers. During PND 0-22, half of the bedding material of the home cages was refreshed every week.

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Table S1 Primer pairs used for quantitative real-time PCR. Primers were designed using NCBI Primer-Blast (www.ncbi.nlm.nih.gov/tools/primer-blast/) and synthesized by Biolegio BV (Nijmegen, The Netherlands). The primer pair for 11β-hydroxylase does not discern between the isoforms CYP11B1 and CYP11B3 and will thus be referred to as CYP11B1/3. With standard curve analysis all primer pairs were confirmed to have reaction efficiencies of > 1.8. The efficiency of the primer pairs was determined separately for cDNA samples of the paraventricular nucleus of the hypothalamus (PVN), the pituitary (PIT) and adrenal (ADR) glands.
Figure S1 Body weight (± SEM) development from weaning, postnatal day (PND) 22, until adulthood (PND79). Independently, early life stress (ELS) and serotonin transporter (5-HTT) genotype affected body weight. The maternally separated (MS180, n = 50) animals developed a significantly lower body weight compared to control animals (MS0, n = 45) from PND30 onwards (A). Furthermore, while serotonin transporter (5-HTT) homozygous knockout (5-HTT−/−, n = 24) rats had significantly lower body weight than 5-HTT heterozygous knockout (5-HTT+/−, n = 42) and wild-type (5-HTT+/+, n = 29) rats at every time point (B) *p < 0.05.

Figure S2 Glucocorticoid receptor (GR) mRNA levels in the paraventricular nucleus of the hypothalamus of serotonin transporter (5-HTT) homozygous knockout (5-HTT−/−), heterozygous knockout (5-HTT+/−) and wild-type (5-HTT+/+) rats (n = 5-9) exposed to daily 3 h separations (MS180) or a control treatment (MS0). Factorial ANOVA revealed a main effect of 5-HTT genotype (G: p < 0.05) and post-hoc testing showed that 5-HTT−/− rats have significantly lower GR mRNA levels than 5-HTT+/+ rats (p < 0.05). Data were normalized to the average of the MS0-5-HTT+/+ group.

Figure S3 Corticotropin-releasing factor (CRF), mineralocorticoid receptor (MR), and FK506-binding protein 51 (FKBP5) mRNA levels in the paraventricular nucleus of the hypothalamus of serotonin transporter (5-HTT) homozygous knockout (5-HTT−/−), heterozygous knockout (5-HTT+/−) and wild-type (5-HTT+/+) rats (n = 5-9) exposed to 3 h daily separations (MS180) or a control treatment (MS0). Data were normalized to the average of the MS0-5-HTT+/+ group.
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Figure S4 Pro-opiomelanocortin (POMC), glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) mRNA levels in the pituitary glands of serotonin transporter (5-HTT) homozygous knockout (5-HTT−/−), heterozygous knockout (5-HTT+/−) and wild-type (5-HTT+/+) rats (n = 5-9) exposed to 3 h daily separations (MS180) or a control treatment (MS0). Data were normalized to the average of the MS0-5-HTT+/+ group.

Figure S5 Corticotropin-releasing factor receptor 1 and FK506-binding protein S1 (FKBPS) mRNA levels in the pituitary glands of serotonin transporter (5-HTT) homozygous knockout (5-HTT−/−), heterozygous knockout (5-HTT+/−) and wild-type (5-HTT+/+) rats (n = 5-9) exposed to daily 3 h separations (MS180) or a control treatment (MS0). Factorial ANOVA revealed a main effect of early life treatment (E: p < 0.05), with 5-HTT+/+ and 5-HTT−/− rats showing decreased FKBPS mRNA levels after exposure to early life stress. Data were normalized to the average of the MS0-5-HTT+/+ group.

Figure S6 Steroidogenic acute regulatory protein (StAR) and 3β-hydroxysteroid dehydrogenase 1 (3β-HSD1) mRNA levels in the adrenal glands of serotonin transporter (5-HTT) homozygous knockout (5-HTT−/−), heterozygous knockout (5-HTT+/−) and wild-type (5-HTT+/+) rats (n = 7) exposed to 3 h daily separations (MS180) or a control treatment (MS0). The mRNA levels of both StAR and 3β-HSD1 were found to be significantly affected by the interaction of 5-HTT genotype and ELS (p < 0.05, p < 0.001 respectively). Data were normalized to the average of the MS0-5-HTT+/+ group. *: p < 0.05, **: p < 0.01.
**Figure S7** Adrenal weight expressed as percentage of body weight of serotonin transporter (5-HTT) homozygous knockout (5-HTT−/−), heterozygous knockout (5-HTT+/−) and wild-type (5-HTT+/+) rats (n = 5–9) exposed to 3 h daily separations (MS180) or a control treatment (MS0).

**Figure S8** Plasma corticosterone (CORT) levels of serotonin transporter (5-HTT) homozygous knockout (5-HTT−/−), heterozygous knockout (5-HTT+/−) and wild-type (5-HTT+/+) rats (n = 7) which were reared in standard animal facility conditions. The plasma CORT levels were significantly affected by 5-HTT genotype (F2,20 = 5.029, p < 0.05), with 5-HTT−/− rats showing higher levels than 5-HTT+/+ (p = 0.053) and 5-HTT+/+ rats (p < 0.05).

**Figure S9** Plasma corticosterone (CORT) response to 10^-12 M adrenocorticotropic hormone, of adrenal tissue derived from serotonin transporter (5-HTT) homozygous knockout (5-HTT−/−), heterozygous knockout (5-HTT+/−) and wild-type (5-HTT+/+) rats (n = 6) which were reared in standard animal facility conditions. The total amount of secreted CORT, i.e. the area under the curve is shown, which was not found to be significantly altered by 5-HTT genotype (F2,17 = 2.368, p > 0.05). However, as it was hypothesized that adrenal tissue derived from AFR 5-HTT−/− rats would show a significantly higher CORT response than adrenal tissue derived from AFR 5-HTT+/+ rats we also performed a one-sided t-test, confirming the a priori hypothesis (p < 0.05).
Early life stress and serotonin transporter gene variation interact to affect the transcription of the glucocorticoid and mineralocorticoid receptors, and the co-chaperone FKBP5, in the adult rat brain

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Frontiers in Behavioral Neuroscience & Article 355 (2014)
Abstract

The short allelic variant of the serotonin transporter (5-HTT) promoter-linked polymorphic region (5-HTTLPR) has been associated with the etiology of major depression by interaction with early life stress (ELS). A frequently observed endophenotype in depression is the abnormal regulation of levels of stress hormones such as glucocorticoids. It is hypothesized that altered central glucocorticoid influence on stress-related behaviour and memory processes could underlie the depressogenic interaction of 5-HTTLPR and ELS. One possible mechanism could be the altered expression of the genes encoding the glucocorticoid and mineralocorticoid receptors (GR, MR) and their inhibitory regulator FK506-binding protein 51 (FKBPS) in stress-related forebrain areas. To test this notion, we exposed heterozygous (5-HTT+/-) and homozygous (5-HTT-/-) serotonin transporter knockout rats and their wildtype littermates (5-HTT +/-) to daily 3 h maternal separations from postnatal day 2 to 14. In the medial prefrontal cortex (mPFC) and hippocampus of the adult male offspring, we found that GR, MR, and FKBPS mRNA levels were affected by ELS x 5-HTT genotype interaction. Specifically, 5-HTT-/- rats exposed to ELS showed decreased GR and FKBPS mRNA in the dorsal and ventral mPFC, respectively. In contrast, 5-HTT+/- rats showed increased MR mRNA levels in the hippocampus and 5-HTT-/- rats showed increased FKBPS mRNA in the ventral mPFC after ELS exposure. These findings indicate that 5-HTT genotype determines the specific adaptation of GR, MR, and FKBPS expression in response to early life adversity. Therefore, altered extra-hypothalamic glucocorticoid signaling should be considered to play a role in the depressogenic interaction of ELS and 5-HTTLPR.

Introduction

Vulnerability to stress-related psychiatric disease is determined by a complex interplay of genome and environment. The moderation of the effects of stressful life events by the serotonin transporter (5-HTT) gene-linked polymorphic region (5-HTTLPR) is a well-known example of such a gene x environment (GxE) interaction (Caspi et al., 2003). Specifically, the short (S) allele of 5-HTTLPR has been associated with a significantly increased risk to develop depression in interaction with adverse events such as childhood abuse (Karg et al., 2011, but also see Risch et al., 2009). Compared to L/L homozygotes, individuals with the S-allele have an approximate two-fold reduction in the promoter activity of the 5-HTT gene (Heils et al., 1996; Lesch et al., 1996; Greenberg et al., 1999). The consequences of lower 5-HTT availability can be studied by use of homozygous and heterozygous 5-HTT knockout (5-HTT-/-, 5-HTT+/-) rodents. The 5-HTT+/- rodents are considered to be the superior genotype model of the S-allele, as they display a two-fold reduction in 5-HTT availability (Bengel et al., 1998; Homberg et al., 2007), and increased sensitivity to stressful events (Carola et al., 2008; Narayanan et al., 2011; Van der Doelen et al., 2013), just as 5-HTTLPR S-allele carriers. Yet, at baseline, 5-HTT-/- rodents show superior behavioural similarity with S-allele healthy controls, as expressed by anxiety- and depressive-like behaviour (Holmes et al., 2003a; Lira et al., 2003a; Olivier et al., 2008a; Schipper et al., 2011b). Therefore, it has been argued that both 5-HTT+/- and 5-HTT-/- rodents are useful to study the underlying biology of ELS x 5-HTTLPR interaction (Caspi et al., 2010; Kaluëff et al., 2010; Homberg and Van den Hove, 2012).

One of the candidate biological systems that could underlie this depressogenic GxE interaction is the stress-responsive hypothalamo-pituitary-adrenal (HPA) axis. A stress response of the HPA-axis is initiated by parvocellular neurons in the paraventricular nucleus (PVN) of the hypothalamus, by secreting corticotropin-releasing factor (CRF) at the median eminence to stimulate the anterior pituitary to synthesize and release adreno-corticotropic hormone (ACTH), which itself stimulates the synthesis and release of glucocorticoids (cortisol in humans, corticosterone in rodents) from the adrenal cortex. The HPA-axis is regulated through direct feedback action of glucocorticoids at the level of the pituitary and the PVN, but importantly also by extra-hypothalamic brain areas such as the medial prefrontal cortex, hippocampus and extended amygdala (Ulrich-Lai and Herman, 2009). This feedback action is mediated via the mineralocorticoid and glucocorticoid receptors (MR, GR), with MR mainly involved in maintaining basal HPA activity and GR with recovery from stress-induced activity. Glucocorticoids furthermore act via these receptors on peripheral tissues as well as the brain to affect physiology and behaviour, and to facilitate an integrative stress response (De Kloet et al., 1998, 2005; Champagne et al., 2009).

In depression, about 50% of patients display hyperactivity of the HPA-axis as represented by basal hypercortisolemia and resistance to GR-mediated suppression of glucocorticoid levels (Checkley, 1996; Pariante and Miller, 2001). In contrast, HPA hypoactivity has been
observed for atypical depression and post-traumatic stress disorder, the latter being associated with increased GR sensitivity (Gold and Chrousos, 2002; Yehuda, 2009). Further, single nucleotide polymorphisms and expression levels of the genes encoding GR (NR3C1) and MR (NR3C2) have been associated with depression (Webster et al., 2002; Van Rossum et al., 2006; Klok et al., 2011a, 2011b; Medina et al., 2013; Qi et al., 2013) which might be caused by impaired glucocorticoid signaling. Glucocorticoids act through the glucocorticoid receptor (GR). Specifically, in major depression postmortem studies have documented decreased hippocampal GR and MR mRNA levels (Webster et al., 2002; Klok et al., 2011a). The decrease in hippocampal MR expression in major depression could be restricted to the anterior hippocampus (Medina et al., 2012). Furthermore, lower MR mRNA levels in different areas of the prefrontal cortex have been reported for depressed compared to non-depressed subjects (Klok et al., 2011a; Qi et al., 2013).

Therefore, there is convincing evidence that altered glucocorticoid signaling through altered expression of GR and MR in forebrain areas is highly relevant in the pathophysiology of depression (Holboer, 2000). Recently, FK506-binding protein 51 (FKBP5), a co-chaperone of steroid hormone receptors, has emerged as an important regulator of stress-induced GR-mediated effects (Binder, 2009). Genetic variation of FKBP5 has additionally been shown to interact with early life stress (ELS) to epigenetically program GR-induced transcription of FKBP5, leading to increased risk for the development of stress-related psychiatric disorders (Klengel et al., 2013). Furthermore, the expression levels of Fkbp5 and Nr3c1 in the adult rat brain have recently been reported to be sensitive to chronic stress (CS) exposure and antidepressant treatment (Guidotti et al., 2013), while Fkbp5 knockout mice seem to be less vulnerable to CS exposure (Hartmann et al., 2012). In addition, CS has been shown to lead to a disruption of the extra-hypothalamic control of HPA function (Radley et al., 2013).

Altogether, altered central glucocorticoid signaling is a plausible contributing factor to the increased vulnerability of childhood maltreatment-exposed 5-HTTLPR S-allele carriers to psychopathology. Previously, we have shown that our animal model of ELS x 5-HTT genotype interaction displays differential susceptibility to inescapable stress and altered activity of the HPA-axis (Van der Doelen et al., 2013, 2014a). In the HPA-axis, we predominantly found GxE programming of the adrenal gland, while gene expression in the pituitary and PVN was largely unaffected (Van der Doelen et al., 2014a). Therefore, we hypothesized that if there are adaptations in the expression of GR, MR, and/or FKBP5 in our animal model of ELS x 5-HTT genotype interaction, these adaptations would take place in extra-hypothalamic brain regions. To test this hypothesis we exposed 5-HTT heterozygous (S-HTT+) and homozygous (S-HTT−) knockout rats to ELS, i.e. maternal separation, and examined the expression of GR, MR, and FKBP5 in the medial prefrontal cortex, hippocampus, amygdala, and bed nucleus of the stria terminals. In addition to their regulatory function of the HPA-axis (Ulrich-Lai and Herman, 2009), these brain areas are known for their involvement in stress-related behavioural processes such as cognitive control, learning and memory, and fear and anxiety output (De Kloet et al., 1998, 2005; LeDoux, 2000; Amat et al., 2005; Kim et al., 2013).

Materials and Methods

Animals

The experiments were approved by the Committee for Animal Experiments of the Radboud University Nijmegen, The Netherlands, and all efforts were made to minimize animal suffering, to reduce the number of animals and to utilize alternatives to in vivo techniques. Serotonin transporter knockout rats (Slc6a4−/−) were generated by N-ethyl-N-nitrosurea (ENU)-induced mutagenesis (Smits et al., 2006). Experimental animals (S-HTT+, S-HTT− and S-HTT−/− rats) were derived from crossing 3 month old S-HTT− rats that were outcrossed for at least twelve generations with commercial (Harlan, Ter Horst, The Netherlands) wild-type Wistar rats. The pregnant dams were housed in standard polypropylene cages (40 x 20 x 18 cm) with sawdust bedding and ad libitum access to water and rodent chow (Sniff Spezialdiäten, Soest, Germany) in a temperature (21 ± 1 °C) and humidity-controlled room (45-60% relative humidity), with a 12:12 h light:dark cycle (lights on at 07.00 a.m.). The dams were inspected daily for delivery at 5.00 p.m. and day of birth was designated as postnatal day (PND) 0. At PND1, two paper towels (22.5 x 24.5 cm) were supplied to the mother for nest construction. Further, the litters were culled to a maximum of 10 pups (one litter had only 9 pups, another only 8 pups), with gender ratios in favor of a male majority (5:5 to maximally 7:3).

Early life stress

We used repeated and prolonged maternal separation as a model for ELS, as this paradigm has previously been shown to affect the HPA-axis functioning and stress coping behaviour of the offspring (Plotsky and Meaney, 1993; Francis et al., 2002, Ladd et al., 2004; Levine, 2005; Plotsky et al., 2005; Macrì and Würbel, 2006; Van der Doelen et al., 2014a). Litters were randomly allocated to one of two rearing conditions (from PND 2 to 14): maternal separation for 180 min (MS180) or a control treatment with immediate reunion of mother and pups (MS0). MS180 was started daily between 08.30 and 09.00 a.m., and consisted of the following procedure: The mother was removed from the home cage and placed into an identical cage until the end of the separation period. Pups were then removed from the nest as complete litters and placed into a cage (24 x 15 x 14 cm) with clean sawdust bedding, and then transferred to an adjacent room. There, the cages were placed on heat pads, which were set to maintain a bedding temperature of 31-33 °C for PND 2-7 and 29-31 °C for PND 8-14. At the end of the separation period, litters were returned to their home cage by placing them in the nests and sprinkling soiled home cage bedding over them. This was followed by reunion with the mothers. We have previously reported that this
procedure affects maternal care behaviour across PND2-8 (Van der Doelen et al., 2014a). During PND 0-22, half of the bedding material of the home cages was refreshed every week. At PND 14, ear punches were taken of the pups for identification and genotyping, which was performed by kbisciences (Hoddesdon, United Kingdom). The procedure of genotyping has been described previously (Homberg et al., 2007). At PND 22, the pups were weaned and housed in groups of 2-3 littermates of the same sex and rearing, under the same conditions as mentioned above.

**Tissue collection**

For the collection of tissues only adult (PND85-95) male rats were used. These rats were derived of 13 litters that were subjected to MS180 and 12 litters that received the control treatment (MS0). Of every litter, where possible, a single rat was selected of all three genotypes. The rats were sacrificed between 9:00 a.m. and 2:00 p.m. by acute decapitation. Across this time period, the rats were randomized for their genotype and early life treatment. Immediately after decapitation, the brains were isolated, frozen in aluminum foil on dry ice and stored at -80 °C. In a cryostat (-15 °C), the brains were prepared in 420 µm-thick coronal slices in order to obtain punches from dorsal and ventral parts (prelimbic, infralimbic respectively) of the medial prefrontal cortex (mPFC, Bregma +3.72 and +3.30 mm), the anterodorsal part of the bed nucleus of the stria terminalis (BNST, Bregma +0.24 and -0.18 mm), central amygdala (Bregma -1.72 and -2.14 mm) and dorsal (Bregma -2.14 and -2.56 mm) and ventral hippocampus (Bregma -4.80 and -5.22 mm); the brain areas were bilaterally punched out with a Mitexx 1.5 (hippocampal samples) or 1.0 mm (other areas) biopsy puncher (Integra Miltex, York, PA, USA), collected in sterile vials, immediately placed on dry ice and stored at -80 °C.

**RNA isolation and gene expression analysis by quantitative real-time PCR**

Total RNA was isolated by a single step of guanidinium isothiocyanate/phenol extraction using PureZol RNA isolation reagent (Fisher Scientific, Waltham, MA, USA). Samples were treated with DNase to avoid DNA contamination. As the study was a collaborative effort, real-time PCR (RT-PCR) was performed both in Milan (mPFC and hippocampal samples) and Nijmegen (anterodorsal BNST and central amygdala). To exclude possible differential results depending on the methods, adrenal gene expression was assessed in both labs and yielded identical statistical conclusions as previously published (Van der Doelen et al., 2014a). In Nijmegen first strand cDNA synthesis was performed by incubating 40 ng of RNA dissolved in 12 µl of RNase-free water containing 0.25 mM random hexamer primers (Roche Applied Science, Penzberg, Germany) at 70 °C for 10 min, followed by double-strand synthesis in 1st strand buffer with 10 mM DTT, 100 U Superscript II (Life Technologies), 0.5 mM dNTPs (Roche Applied Science) and 20 U of rRNasin (Promega Corp., Fitchburg, WI, USA) at 37 °C for 75 min. In Milan, RNA was analyzed using the iScript™ one-step RT-PCR kit for probes (Bio-Rad), with RT-PCR performed in multiplexed reactions with a normalizing internal control (36B4) by use of the CFX384 real time system (Bio-Rad). Thermal cycling was initiated with an incubation at 50 °C for 10 min (RNA retrotranscription), followed by 5 min at 95 °C (TaqMan polymerase activation) and 39 reaction cycles with 10 s at 95 °C and 30 s at 60°C. In Nijmegen, RT-PCR was performed with the CFX96 real time system (Bio-Rad). Prior to analysis of the relative expression of Nr3c1, Nr3c2 and Fkbp5, it was evaluated whether Rn18S, Gapdh or Hprt1 would be the best internal control gene (also see Derks et al., 2008). Gapdh expression was found to be unaffected by our experimental design in all brain areas, and was therefore used as the internal control for this study. Furthermore, all primer pairs were tested for reaction efficiency. For the reactions a total volume of 2.5 µl of buffer solution was used containing 5 µl of template cDNA, 12.5 µl Power SYBR Green Master mix (Applied Biosystems, Foster City, CA, USA), 1.5 µl RNase-free water and 0.6 µM of each primer. The cycling protocol started with 10 min at 95 °C, followed by 39 reaction cycles with 15 s at 95 °C and 1 min at 60°C. The results of the experiment were already noted before the statistical analysis. The statistical analysis of the remaining data was performed (one-way ANOVA and independent samples t-test). Statistical analysis

All statistical tests have been carried out using SPSS (version 20, IBM corporation, Armonk, NY, USA). The results are presented as the mean with the standard error of the mean (SEM). The RT-PCR 2-ΔCt data have been normalized to the average of the MS0-wild-type group and have been examined with factorial ANOVA. If a significant main effect ("genotype", "early life stress") or interaction ("genotype x early life stress") was found, appropriate *posterior* tests were performed (one-way ANOVA and independent samples t-test). Statistical significance was set at *p* < 0.05. Group sizes are available in the supplementary material.
Results

GR mRNA levels
GR mRNA levels were found to be significantly affected by the interaction of ELS and 5-HTT genotype in the dorsal mPFC ($F_{2,31} = 7.08, p < 0.01$) and dorsal hippocampus ($F_{2,33} = 4.57, p < 0.05$). In the dorsal hippocampus, a significant main effect of 5-HTT genotype ($F_{2,33} = 4.57, p < 0.05$) was found in addition.

The exposure to ELS selectively decreased GR expression in the dorsal mPFC of 5-HTT++ rats ($p < 0.01$), leading to significantly lower GR mRNA levels for 5-HTT++ rats in comparison to 5-HTT+/- ($p < 0.01$) and 5-HTT-/- rats within the MS180 group ($p < 0.05$) (Figure 1A). In the dorsal hippocampus, 5-HTT-/- rats displayed higher GR mRNA levels compared to 5-HTT+/- and 5-HTT++ rats within the MS0 group ($p < 0.01$), but this effect of 5-HTT deficiency was not present in the case of ELS exposure (MS180 group) (Figure 1B).

MR mRNA levels
MR mRNA levels in the dorsal hippocampus ($F_{2,33} = 14.09, p < 0.001$) and ventral hippocampus ($F_{2,33} = 7.10, p < 0.01$) were found to be significantly affected by the interaction of ELS and 5-HTT genotype. These interaction effects were found together with main effects of ELS ($F_{1,29} = 11.59, p < 0.01$) and 5-HTT genotype ($F_{2,29} = 26.92, p < 0.001$) in the ventral hippocampus and a main effect of 5-HTT genotype ($F_{2,26} = 9.87, p < 0.01$) in the dorsal hippocampus.
For the dorsal hippocampus, MR mRNA levels were selectively increased for 5-HTT<sup>+</sup>+ rats due to exposure to ELS (p < 0.01), leading to significantly higher MR expression for 5-HTT<sup>+</sup> rats compared to 5-HTT<sup>-/-</sup> (p < 0.01) and 5-HTT<sup>-/-</sup> rats (p < 0.001) (Figure 2B). In the ventral hippocampus, MR mRNA levels were significantly lower for 5-HTT<sup>-/-</sup> rats in comparison to 5-HTT<sup>+</sup> (p < 0.05) and 5-HTT<sup>-/-</sup> rats (p < 0.05). The exposure to ELS led to a selective increase of MR expression for 5-HTT<sup>-/-</sup> rats (p < 0.01), leading to significantly higher MR mRNA levels for 5-HTT<sup>-/-</sup> rats compared to 5-HTT<sup>-/-</sup> rats within the MS180 group (p < 0.001) and further increasing the significantly lower MR expression in the ventral hippocampus of 5-HTT<sup>-/-</sup> rats compared to 5-HTT<sup>-/-</sup> rats (p < 0.001) (Figure 2E).

In the dorsal MPFC, MR mRNA levels were found to be affected by main effects of both ELS (F<sub>1,32</sub> = 4.26, p < 0.05) and 5-HTT genotype (F<sub>2,32</sub> = 13.01, p < 0.001). The exposure to ELS led to a decrease in MR expression (MS0 > MS180), while 5-HTT<sup>-/-</sup> and 5-HTT<sup>-/-</sup> rats were both found to display higher MR mRNA levels than 5-HTT<sup>-/-</sup> rats (p < 0.01 and p < 0.001, respectively) (Figure 2A).

Figure 2. Mineralocorticoid (MR) mRNA levels in the dorsal medial prefrontal cortex (mPFC) (A), dorsal hippocampus (B), central amygdala (C), ventral mPFC (D), ventral hippocampus (E) and the anterodorsal bed nucleus of the stria terminalis (BNST) (F) of serotonin transporter (5-HTT) homozygous knockout (5-HTT<sup>-/-</sup>), heterozygous knockout (5-HTT<sup>+</sup>-/-) and wild-type (5-HTT<sup>++</sup>) rats exposed to daily 3 h separations (MS180) or a control treatment (MS0). MR mRNA levels in the dorsal and ventral hippocampus were found to be affected by a significant interaction of early life stress (ELS) and 5-HTT genotype (p < 0.001, p < 0.01, respectively), with asterisks indicating significant pairwise comparisons (*: p < 0.05, **: p < 0.01 and ***: p < 0.001). Furthermore, MR mRNA levels were affected by independent effects of 5-HTT genotype (G, p < 0.001) and ELS (E, p < 0.05) in the dorsal mPFC. Post-hoc analysis revealed MR mRNA levels in the dorsal mPFC to be higher for both 5-HTT<sup>-/-</sup> and 5-HTT<sup>-/-</sup> rats compared to 5-HTT<sup>-/-</sup> rats (p < 0.01 and p < 0.001, respectively). Data were normalized to the average of the MS0-5-HTT<sup>++</sup> group.

In the central amygdala, ventral mPFC, and anterodorsal BNST MR mRNA levels were not found to be affected by ELS, 5-HTT genotype or their interaction (Figure 2C, D, F).

FKBP5 mRNA levels
FKBP5 mRNA levels were found to be significantly affected by the interaction of ELS and 5-HTT genotype in the dorsal mPFC (F<sub>1,28</sub> = 7.08, p < 0.01) and ventral mPFC (F<sub>2,28</sub> = 4.57, p < 0.05). In addition, main effects of ELS in the dorsal mPFC (F<sub>1,29</sub> = 8.87, p < 0.01) and 5-HTT genotype in the ventral mPFC (F<sub>2,29</sub> = 7.89, p < 0.01) were identified.

For the dorsal mPFC, within the MS0 group 5-HTT<sup>-/-</sup> rats displayed increased FKBP5 mRNA levels compared to 5-HTT<sup>-/-</sup> rats (p < 0.05). After exposure to ELS however, FKBP5 expression decreased only in 5-HTT<sup>-/-</sup> rats (p < 0.01), and no difference was found between 5-HTT<sup>-/-</sup> and 5-HTT<sup>-/-</sup> rats (Figure 3A). In contrast, FKBP5 mRNA levels in the ventral mPFC were found to be increased for 5-HTT<sup>-/-</sup> rats (p < 0.01), but decreased for 5-HTT<sup>-/-</sup> rats (p < 0.05), after exposure to ELS. Therefore, within the MS180 group 5-HTT<sup>-/-</sup> and 5-HTT<sup>-/-</sup> rats displayed higher FKBP5 mRNA levels compared to 5-HTT<sup>-/-</sup> rats (p < 0.01 and p < 0.001, respectively). Data were normalized to the average of the MS0-5-HTT<sup>++</sup> group.
Discussion

In this study, we found that ELS exposure differentially induced adaptations of GR, MR, and FKBP5 mRNA levels at extra-hypothalamic sites in adult 5-HTT+/+, 5-HTT+/- and 5-HTT-/- rats. It remains to be experimentally determined how these findings could be related to the altered activity of the HPA-axis (Van der Doelen et al., 2014) and stress coping behaviour (Van der Doelen et al., 2013) that we previously reported of our animal model of ELS x 5-HTTLPR interaction. Moreover, as we and others have recently shown (Daskalakis et al., 2012; Van der Doelen et al., 2013; Santarelli et al., 2014), the exposure to stress does not necessarily need to have negative consequences, but can rather induce phenotypic changes that have adaptive value in coping with later life stressors. As such, these findings support the recently postulated match/mismatch hypothesis (Champagne et al., 2009; Nederhof and Schmidt, 2012), which states that individuals can use the experience of past stressors to adaptively respond to future challenges (predictive adaptive response).
The match/mismatch hypothesis predicts that such phenotypic changes are beneficial when the individuals face similar environments later in life (match), but could be maladaptive if the environment changes significantly (mismatch). The match/mismatch hypothesis is not compatible with a deterministic view as it implies that the prior life history and the specific demands of current/future stressors (Myers et al., 2014) will dictate whether a given phenotype is adaptive or maladaptive. Therefore, it remains to be elucidated under which specific conditions the differential expression of GR, MR, and FKBP5 mRNA levels in our animal model of ELS x 5-HTTLPR interaction will turn out to be adaptive or maladaptive. For now, we will discuss the current findings from different perspectives in the following sections.

**ELS x 5-HTT genotype: anatomical specificities**

Interestingly, the effects of ELS x 5-HTT genotype interaction showed a gene-dependent anatomical distinction, with MR mRNA levels affected only in the dorsal regions of the mPFC and hippocampus. In contrast to these higher order/processing areas of the limbic system, for the extended amygdala areas (central amygdala and anterodorsal BNST) we found that ELS exposure, irrespective of genotype, affected only GR mRNA levels in the anterodorsal BNST. The influence of ELS x 5-HTT genotype interaction was therefore clearly stronger in the mPFC and hippocampus compared to the central amygdala and anterodorsal BNST.

Another interesting observation is that the effects of ELS and 5-HTT genotype on GR, MR, and FKBP5 expression were markedly different for dorsal vs. ventral subregions of both mPFC and hippocampus. The dorsal and ventral mPFC punches in this study correspond to the functionally distinct prelimbic and infralimbic cortices, which have been linked to opposite roles in the control of fear responses (Sotres-Bayon and Quirk, 2010). Here, we found that the dorsal and ventral mPFC of 5-HTT-/- rats also showed opposite regulation of FKBP5 mRNA levels after ELS exposure. Regarding the hippocampus, it has been argued that the dorsal regions perform primarily cognitive functions, while ventral subregions are related to stress, emotion and affect (Fanselow and Dong, 2010). In our model, we found that the ventral hippocampus showed more alterations in GR, MR, and FKBP5 mRNA levels by the interaction of ELS and 5-HTT genotype than the dorsal hippocampus, although the latter was certainly not unaffected. The physiological and behavioral consequences of distinct programming of dorsal vs. ventral mPFC and hippocampus subregions should be investigated further, both in our and other animal models of stress-related diseases.

**ELS x 5-HTT genotype: GR/MR balance**

The balance between GR and MR functioning has been proposed to be central to stress-related psychopathology, as GR and MR serve such complementary glucocorticoid functions during the stress response (De Kloet et al., 2005). For instance, hippocampal GR is involved in the consolidation of emotional memory and disinhibition of the HPA-axis (Roozendaal and McGaugh, 1997, De Kloet et al., 1998), while MR is important for maintaining basal HPA activity and memory retrieval (De Kloet et al., 1998, Dorey et al., 2011). Essential to this functional segregation is the 10-fold higher affinity of glucocorticoid binding by MRs compared to GRs. Low basal corticosterone levels therefore predominantly occupy MR, while GR is activated by stress-induced as well as ultradian peaks of glucocorticoid levels (De Kloet et al., 1998; Lightman et al., 2006). To explore the relevance of a functional GR/MR disbalance in ELS x 5-HTTLPR interactions, we will discuss our findings here in terms of the balance between programmed expression levels of GR and MR.

In addition to the selective effects of ELS on different 5-HTT genotypes, the exposure to ELS was found to lead in all genotypes to decreased MR mRNA levels in the dorsal mPFC, decreased GR mRNA levels in the anterodorsal BNST and decreased GR and FKBP5 mRNA levels in the ventral hippocampus. As FKBP5 has mainly been characterized as an inhibitory co-chaperone for GR and not MR (Binder, 2009), it is assumed at present that FKBP5 chiefly regulates GR function. In the ventral hippocampus, ELS exposure decreased both GR and FKBP5 mRNA levels, and it can therefore be assumed that GR/MR balance in the ventral hippocampus is not affected by a general effect of ELS.

For the anterodorsal BNST, it seems that ELS exposure only leads to a decrease in GR mRNA in 5-HTT+/- rats, although the ELS x 5-HTT genotype interaction was not found to significantly affect anterodorsal BNST GR mRNA levels. In the 5-HTT-/- rats, ELS exposure furthermore selectively decreased ventral mPFC FKBP5 and dorsal mPFC GR mRNA levels. The former could affect local GR/MR balance, but the latter would be expected to counterbalance the general ELS effect of decreased dorsal mPFC MR mRNA levels. Therefore, after ELS exposure, transcriptional GR/MR balance in 5-HTT+/- rats only seems to be affected in the anterodorsal BNST and the ventral mPFC, with a relative increase in MR over GR transcription in the anterodorsal BNST and the opposite in the ventral mPFC.

For 5-HTT-/- rats, ELS exposure led to increased MR mRNA levels in both the dorsal and ventral hippocampus, while increased dorsal mPFC MR expression is present both under control conditions and after ELS exposure in 5-HTT-/- rats. Therefore, MR levels in the anterodorsal BNST and the ventral mPFC in 5-HTT-/- rats may not lead to an increase in GR mRNA levels. Interestingly, the effects of ELS on different 5-HTT genotypes, the exposure to ELS was found to lead in all genotypes to decreased MR mRNA levels in the dorsal mPFC, decreased GR mRNA levels in the anterodorsal BNST and decreased GR and FKBP5 mRNA levels in the ventral hippocampus. As FKBP5 has mainly been characterized as an inhibitory co-chaperone for GR and not MR (Binder, 2009), it is assumed at present that FKBP5 chiefly regulates GR function. In the ventral hippocampus, ELS exposure decreased both GR and FKBP5 mRNA levels, and it can therefore be assumed that GR/MR balance in the ventral hippocampus is not affected by a general effect of ELS.

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tional GR/MR balance in 5-HTT-/- rats after ELS exposure seems to be only affected in the ventral hippocampus, by the decreased expression of both MR and FKBP5 in this area. In contrast to 5-HTT+/- rats, this putatively disturbed hippocampal GR/MR balance would be predicted to lead to a relative increase of GR over MR function.

Overall, we observe a strong moderation by 5-HTT genotype of ELS-induced disbalances between GR and MR mRNA levels. We acknowledge that it remains to be empirically proven whether transcriptional GR/MR disbalances ultimately lead to functional GR/MR disbalance in our model, and, as the regulation of GR/MR/FKBP5 expression is highly dynamic, it remains to be determined how stable these transcriptional disbalances would be.

**ELS x 5-HTT genotype: possible functional consequences**

As said above, the interaction of ELS and 5-HTT genotype resulted in differential adult expression patterns of GR, MR, and FKBP5 mRNA levels in the mPFC and hippocampus of 5-HTT+/-, 5-HTT-/- and 5-HTT+ rats. From a genetic perspective, the 5-HTT+/- rats can be considered as the best model for human 5-HTTLPR S-allele carriers (Homberg et al., 2007). Only in these rats, the exposure to ELS led to an increase of MR mRNA levels in the dorsal as well as ventral hippocampus. The hippocampal MR has previously been shown to be involved in the stress-induced switching between learning/coping strategies (Oitzl and De Kloet, 1992; Schwabe et al., 2009). Therefore, the ELS-induced elevation of hippocampal MR expression in 5-HTT-/- rats could increase the use of habit-based learning strategies under stressful conditions (Schwabe et al., 2009). Furthermore, increased MR mRNA levels could increase the inhibitory regulation of the hippocampus on basal HPA activity (De Kloet et al., 1998). In 5-HTT-/- rats, ELS led to decreased GR mRNA levels in the dorsal mPFC and decreased FKBP5 mRNA levels in the ventral mPFC. These alterations could affect the feedback control exerted by the mPFC over HPA activity (McKeen et al., 2013). Furthermore, given the inhibitory regulation of FKBP5 on GR activity (Binder, 2009), GR function could be relatively decreased in the dorsal mPFC, but increased in the ventral mPFC of 5-HTT+/- rats exposed to ELS. This could result in an increased relative influence of stress-induced glucocorticoids on extinction vs. expression of fear memory (Gourley et al., 2009; Sotres-Bayon and Quirk, 2010). In contrast, ELS-exposed 5-HTT-/- rats showed a downregulation of FKBP5 mRNA in the dorsal mPFC and an up-regulation of FKBP5 mRNA in the ventral mPFC. Possibly, these changes also lead to an opposite effect in the relative impact of stress-induced glucocorticoids on emotional memory processing compared to ELS-exposed 5-HTT-/- rats. Interestingly, naïve 5-HTT-/- rodents already display impaired fear extinction recall (Wellman et al., 2007; Nonkes et al., 2012a).

**Translation of findings**

As ELS and ELS x 5-HTT genotype interaction are associated with depression, we expected to find similar changes in the transcription of GR, MR, and FKBP5 as reported in clinical studies. In major depression, decreased MR mRNA in the anterior cingulate cortex (ACC) has been documented recently (Q et al., 2013). On the basis of structure and function, the dorsal part of the ACC is thought to be homologous to the rodent prelimbic cortex (Jylings et al., 2003). In our study, a part of this cortical area was captured by the dorsal mPFC punches and we found indeed that ELS exposure led to decreased MR expression in these samples. However, 5-HTT deficiency was found to be associated with increased MR mRNA levels in this area. In the case of the hippocampus, depressed subjects have been reported to display decreased GR and MR mRNA levels (Webster et al., 2002; Klok et et al., 2011a). In addition, a recent study found decreased MR mRNA selectively in the anterior hippocampus (Medina et al., 2012), which corresponds to the rodent ventral hippocampus (Fanselow and Dong, 2010). In our study, we found that ELS was indeed associated with decreased GR mRNA and 5-HTT knockout with decreased MR mRNA in the ventral hippocampus. However, the interaction of ELS and 5-HTT genotype resulted in an increase of MR mRNA in the ventral hippocampus in 5-HTT-/- rats, which does not fit the increased risk for depression associated with the GxG interaction.

**Exposure to childhood abuse has been associated with reduced hippocampal GR expression** (McGowan et al., 2009), but this has not been reported (thus far) for childhood neglect. Indeed, childhood abuse is more frequently studied, in part due to the higher level of heterogeneity in cases of childhood neglect, although both types of maltreatment are convincingly associated with psychopathology (Teicher and Samson, 2013). We observed that the offspring that had been subjected to the maternal separation (MS) paradigm showed a reduction in GR mRNA levels in the ventral hippocampus. Whereas MS is an established model for ELS exposure, it however is likely a better approximation of the human situation of childhood neglect compared to that of childhood abuse. In the laboratory setting, the mother rat is observed to be frequently away from the nest for periods of 20-25 minutes (Jans and Woodside, 1990; Francis et al., 2002). In seminaturalistic conditions, subordinate mothers are often forced to build their nests far from nutritional sources, and this environmental challenge has been reported to lead to periods of separation for 2-3 h (Calhoun, 1962; Meany, 2001). Therefore, the daily 3 h separations are considered to be an ethologically relevant stressor for the offspring, which results in a deprivation of maternal care, and which has not been observed to lead to abusive behaviour of the mother rat. Consequently, our finding of that rats subjected to MS display reduced GR mRNA levels in the ventral hippocampus may indicate that this phenotype is not specific to childhood abuse, but may also apply to childhood neglect.

**Study limitations and comparison to rodent literature**

There are some limitations of the current study that should be mentioned. First, we examined here the expression of GR, MR, and FKBP5 at the mRNA level, but do not complement this with reporting the levels of the corresponding proteins, the functional end products of the genes. Secondly, we have used real-time PCR in combination with
biopsy punching as a quantitative approach instead of *in situ* hybridization. Due to this approach we had to make a selection of brain areas and lose anatomical resolution. We could therefore for instance not assess mRNA levels in separate cortical layers, or in the hippocampal subregions, e.g. the Cornu Ammonis (CA) areas and dentate gyrus (DG). Thirdly, to limit the amount of social stress, the rats were housed with their littermates after weaning. The rats were therefore housed in same-treatment groups (MS0/MS180), potentially constituting differential living environments on top of the early life treatment, which together may have led to the observed adaptations in gene expression.

MS has been shown before to lead to increased GR-immunoreactivity (GR-ir) in the CA1 region, but not the DG of the dorsal hippocampus of Sprague-Dawley rats (Biagini et al., 1998). In this rat strain, decreased total hippocampal GR mRNA levels have also been reported (Maniam and Morris, 2010). In Long-Evans rats, MS has been observed to lead to decreased GR mRNA and increased MR mRNA levels in the dorsal hippocampus (Ladd et al., 2004, 2005). Furthermore, Long-Evans rats that have experienced low quality of maternal care as pups (low levels of licking and grooming), a different but related model of early life adversity, display decreased hippocampal GR-ir (total hippocampus) and GR mRNA levels (dorsal hippocampus) (Liu et al., 1997; Weaver et al., 2004). In all of these studies, the ventral hippocampus has not been studied in isolation for the expression of GR and MR. Here, we have found that MS induces a decrease of GR mRNA levels in the ventral hippocampus of all 5-HTT genotypes. We observed that not 5-HTT+/+ rats, but the putatively more stress-sensitive 5-HTT+-/- and 5-HTT-/- rats displayed increased MR mRNA and decreased GR mRNA in the dorsal hippocampus, respectively. Our rats have a Wistar background, and given the present literature, it is possible that the genetic background of rat strains modulates the effects of ELS exposure (MS) on the expression of GR and MR (see also Ellenbroek and Cools, 2000). Previously, Wistar rats exposed to MS have been shown to exhibit decreased GR-ir of the total hippocampus (Aisa et al., 2007), while others did not find alterations of GR and MR mRNA levels (Wang et al., 2013), and unaltered GR-ir in the dorsal hippocampus (Renard et al., 2010; Vivinetto et al., 2013).

**Conclusions**

In conclusion, we report here that ELS and 5-HTT genotype interactively affect the expression of GR, MR, and FKBP5 in a brain area-specific way, with further distinction for dorsal vs. ventral subregions of the mPFC and hippocampus. These results could be a starting point for studies aiming to elucidate the role of altered extra-hypothalamic glucocorticoid signaling in the depressogenic interaction of ELS and 5-HTTLPR in humans.

**Acknowledgements**

We thank Anthonieke Middelman and Hussein Ghareh for their technical assistance.

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**Supplementary material: group sizes**

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Chapter 5

Early life adversity and serotonin transporter gene variation interact to affect DNA methylation of the corticotropin-releasing factor gene promoter region in the adult rat brain

Rick H.A. van der Doelen, Ilse A. Arnoldussen, Hussein Ghareh, Liselot van Och, Judith R. Homberg and Tamás Kozicz

CHAPTER 5 CRF AND DNA METHYLATION

Abstract

The interaction between childhood maltreatment and the serotonin transporter (5-HTT) gene-linked polymorphic region (5-HTTLPR) has been associated with increased risk to develop major depression. This gene x environment (GxE) interaction has furthermore been linked with increased levels of anxiety and glucocorticoid release upon exposure to stress. Both endophenotypes are regulated by the neuropeptide corticotropin-releasing factor or hormone (CRF/CRH), which is expressed by the paraventricular nucleus of the hypothalamus (PVN), bed nucleus of the stria terminalis (BNST) and the central amygdala (CeA). Therefore, we hypothesized that altered regulation of the expression of CRF in these areas represents a major neurobiological mechanism underlying the interaction of early life stress (ELS) and 5-HTT gene variation. The programming of gene transcription by GxE interactions has been proposed to involve epigenetic mechanisms such as DNA methylation. In this study, we report that ELS and 5-HTT genotype interact to affect DNA methylation of the Crf gene promoter in CeA of adult male rats. Furthermore, we found that DNA methylation of a specific site in the Crf promoter significantly correlated with CRF mRNA levels in the CeA. Moreover, CeA CRF mRNA levels correlated with stress coping behaviour in a learned helplessness paradigm. Together, our findings warrant further investigation of the link of Crf promoter methylation and CRF expression in the CeA with behavioural changes that are relevant for psychopathology.

Introduction

The risk to develop depression is largely determined by both genetic and environmental factors, particularly adverse childhood events (Heim et al., 2004). A common polymorphism (5-HTTLPR) in the serotonin transporter (5-HTT, SLC6A4) gene has been shown to moderate the impact of stressful life events on the occurrence of major depression (Caspi et al., 2003; Karg et al., 2011, but also see Risch et al., 2009). Individuals with the short (S) allele of the 5-HTTLPR polymorphism seem to be especially sensitive to the experience of early life stress (ELS) (Karg et al., 2011). Importantly, the increased sensitivity of S-allele carriers to the environment is not restricted to adverse events, as S-allele carriers show increased benefit from positive, supportive environments (Kaufman et al., 2004; Pluess et al., 2010; Van Ijzendoorn et al., 2012). Thereby, the 5-HTTLPR polymorphism seems to be consistent with the differential susceptibility model, which postulates that gene (5-HTTLPR) x environment interactions are for better and worse (Belsky et al., 2007; Ellis et al., 2011; Homberg and Lesch, 2011).

The depressogenic ELS x 5-HTTLPR interaction is likely influenced by additional interactions with other genetic and environmental factors (Heiming and Sachser, 2010; Uher, 2010; Van der Doelen et al., 2013). One example is the epistasis between the 5-HTTLPR and polymorphisms in the CRHR1 gene, which influences the impact of childhood maltreatment on depressive symptoms (Ressler et al., 2010; Cicchetti et al., 2011). CRHR1 is one of two genes encoding a receptor for corticotropin-releasing factor (CRF), or hormone (CRH).

CRF/CRH is a 41 amino acid peptide, which has an initiating and coordinating role in the physiological and behavioural stress response (Vale et al., 1981; Sutton et al., 1982). The activity of CRF neurons in the paraventricular nucleus of the hypothalamus (PVN) is important in the neurobiology of depression, as these neurons drive the activity of the hypothalamic-pituitary-adrenal (HPA) axis. In depression, a subset of patients exhibit hyperactivity of the HPA axis, resulting in elevated levels of glucocorticoids, which is thought to be driven by the increased activity of the CRF neurons in the PVN (Arboleya et al., 1999). Furthermore, postmortem studies have found an increased number of CRF neurons and CRF mRNA content in the PVN of depressed subjects (Radda et al., 1994; Radda et al., 1998; Wang et al., 2008). In addition, these patients display increased levels of CRF in their cerebrospinal fluid, which can be reduced by treatment with antidepressants or electroconvulsive shocks (Nemeroff et al., 1984; Nemeroff et al., 1991; Heuser et al., 1998). Notably, the intracerebroventricular administration of CRF in experimental animals produces responses reminiscent of symptoms of major depression, anhedonia, anxiety, decreased food intake, decreased sexual activity and disturbed sleep and locomotion (Bale and Vale, 2004; Binder and Nemeroff, 2010).

We have previously shown in rats that ELS and 5-HTT genotype interact to program the adult HPA-axis, but we did not find altered CRF mRNA levels in the PVN (Van der Doelen et al., 2014a). However, CRF is not only found in the PVN. It is also expressed in...
higher order brain regions, including the amygdala and the bed nucleus of the stria terminals (BNST). Both regulate the activity of the HPA-axis, as well as autonomic and behavioural responses to stress (Ulrich-Lai and Herman, 2009; Walker et al., 2009). The central amygdaloïd nucleus (CeA) and the oval subdivision of the BNST (BNSTov) contain the major populations of CRF neurons in the rat amygdala and BNST, respectively (Merchenthaler et al., 1982; Morin et al., 1999; Sterrenburg et al., 2012). Modulation of CRF expression in the CeA affects basal and stress-induced anxiety-like behaviour in rodents (Regev et al., 2011; Flandreau et al., 2012; Regev et al., 2012; Callahan et al., 2013). Furthermore, CRF overexpression in the CeA has been reported to lead to increased CRF mRNA in the PVN and increased stress-induced HPA activity (Koen-Rhinehart et al., 2009; Flandreau et al., 2012; Callahan et al., 2013). In the BNSTov, modulation of CRF expression has been shown to affect conditioned anxiety, as well as depression-like behaviour, but not HPA activity (Regev et al., 2011; Sink et al., 2013). Yet, the CRF populations in the CeA and BNSTov seem to have complementary functions in fear conditioning (Walker et al., 2009), and are responsive to exposure to acute and chronic stress paradigms (Rouwette et al., 2011; Sterrenburg et al., 2011).

During a stress response, CRF mRNA levels in the PVN, CeA and BNSTov are frequently found to be up-regulated (Kovács and Sawchenko, 1996; Ma et al., 1997; Makino et al., 1999; Rouwette et al., 2011). Prominent examples of transcription factors involved in CRF expression are AP-1, nerve growth factor induced gene B (NGFI-B), and cyclic AMP (cAMP) response element binding protein (CREB) (Ito et al., 1996; Kovács and Sawchenko, 1996; Murphy and Conneely, 1997; Yao et al., 2007). The proximal promoter region of Crf contains a CAMP response element (CRE), as well as a putative AP-1 binding site (Yao and Denver, 2007).

DNA methylation is an epigenetic mechanism that occurs at cytosine-phosphate-guanine (CpG) sites and is associated with the repression of gene transcription (Moore et al., 2013). Importantly, the epigenetic programming of gene transcription is considered to be a key interface for gene X environment (GxE) interactions (Bale et al., 2010; Meaney, 2010; Van IJzendoorn et al., 2010; Bogdan et al., 2013). Indeed, exposure to ELS has been shown to regulate DNA methylation and transcription of the glucocorticoid receptor gene in the hippocampus of rats (Weaver et al., 2004) and humans (McCowan et al., 2009), and the Crf and arginine vasopressin genes in the PVN of mice (Mueller and Bale, 2008; Murgatroyd et al., 2009; Chen et al., 2012).

A frequently used rodent model of ELS is the prolonged and repeated separation of newborn pups from their mother (maternal separation) (Pryce et al., 2005; Macrì and Würbel, 2006). Maternal separation has been reported to affect the basal and stress-induced expression of CRF in the PVN, CeA and BNSTov (Ladd et al., 2005; Plotisky et al., 2005; Aisa et al., 2007; Desbonnet et al., 2008; Veenema et al., 2008; Bravo et al., 2010; Chen et al., 2012; Pierce et al., 2014). The brain’s 5-HT system seems to have a significant impact on the regulation of the Crf gene as well. The modulation of neuronal 5-HT content for instance affects CRF mRNA levels in the PVN (Jørgensen et al., 2002), while the administration of selective serotonin re-uptake inhibitors can reverse stress-induced elevations of Crf transcription (Stout et al., 2002; Pan et al., 2013).

Based on the literature described above, we hypothesized that ELS and 5-HTT gene variation interact to affect DNA methylation of the Crf promoter region in the adult rat brain. As basal and stress-induced expression levels of CRF are expected to be programmed by Crf promoter methylation, this could represent an interesting target for intervening in ELS x 5-HTTLPR-related psychopathology. To test our hypothesis, we used an animal model of ELS x 5-HTTLPR interaction. Specifically, we performed a Crf promoter DNA methylation assay on PVN, CeA and BNSTov biopsies of adult heterozygous and homozygous 5-HTT knockout rats (5-HTT+/-, 5-HTT-/-), and their wild-type (5-HTT+/+) littermates exposed to ELS (maternal separation paradigm) or a control treatment. We have performed these analyses for a cohort of rats that was not exposed to any additional stressors (adult naïve cohort) and a cohort which was examined for stress coping behaviour in the learned helplessness (LH) paradigm (adult stress cohort). Furthermore, to study Crf expression, we performed quantitative real-time PCR analyses for both cohorts, and applied Crf immunohistochemistry to brains derived from the naïve cohort.

**Materials and Methods**

**Animals**

The experimental procedures were approved by the Committee for Animal Experiments of the Radboud University Nijmegen, The Netherlands, and all efforts were made to minimize animal suffering and to reduce the number of animals used. Serotonin transporter knockout rats (Slc6a4-/-) were generated by N-ethyl-N-nitrosourea (ENU)-induced mutagenesis (Smits et al., 2006). Experimental animals (5-HTT+/-, 5-HTT+/- and 5-HTT-/-) were derived from crossing 3 month old 5-HTT-/- rats that were outcrossed for at least twelve generations with commercial (Harlan, Ter Horst, The Netherlands) wild-type Wistar rats. The pregnant dams were housed in standard polycarbonate cages (40 x 20 x 18 cm) with sawdust bedding and ad libitum access to water and rodent chow (Sniff Spezialdiäten, Soest, Germany) in a temperature (21 ± 1 °C) and humidity-controlled room (45-60% relative humidity), with a 12 : 12 h light : dark cycle (lights on at 07:00 a.m.). The dams were inspected daily for delivery of pups at 5:00 p.m. and day of birth was designated as postnatal day (PND) 0. At PND 0, two paper towels (22.5 x 24.5 cm) were supplied to the mother for nest construction. Further, the litters were culled to a maximum of 10 pups, with gender ratios in favor of a male majority to maximally 7 : 3.

**Early life stress**

We used repeated and prolonged maternal separation as a model for ELS. Litters were randomly allocated to one of two rearing conditions (from PND 2 to 14): maternal separation for 180 min (MS180) or a control treatment with immediate reunion of mother and pups.
Adult stress
For this study, we have made use of two cohorts of male 5-HTT\textsuperscript{+/-}, 5-HTT\textsuperscript{+/-}, and 5-HTT\textsuperscript{+/-} rats that underwent ELS exposure. Cohort 1 was left undisturbed until sacrifice (PND 85-95) and was therefore naive in terms of adult stress exposure. Cohort 2 was a group of rats (PND 77-116) that underwent inescapable foot shocks for 2d, followed 24 h later by a shuttle box escape test (LH paradigm). The exposure to inescapable foot shocks induces a deficit in active coping behaviour in a subset of vulnerable rats, as measured by escape latencies in the shuttle box escape test (Vollmayr and Henn, 2001). Previously, we reported that the LH paradigm differentially affects coping behaviour in the current model of ELS x 5-HTT genotype interaction. Specifically, we found that 5-HTT\textsuperscript{+/-} rats were especially sensitive to show successful predictive adaptation to a putative match between early and adult life environmental conditions, which was expressed as a reduced latency to escape stress (Van der Doelen et al., 2013).

Tissue collection and preparation
Cohort 1 was split into a group of rats that was sacrificed by decapitation and a group of rats that was sacrificed by perfusion. Of cohort 2, all rats were sacrificed by decapitation.

For decapitations, rats were taken from their home cage into a separate room and decapitated within 10 s. Immediately after decapitation, the brains were isolated, frozen in aluminum foil on dry ice and stored at -80 °C. For perfusions, rats were taken from the home cage into a separate room and the mother was removed from the home cage and placed into an identical cage. The cage was placed on a heat pad, which was set to maintain a temperature of 31-33 °C for PND 2-7 and 29-31 °C for PND 8-14. At the end of the separation period, litters were returned to their home cage by first rolling them in the home cage bedding material and then placing them in the nests. This was followed by reunion with the mothers. During PND 0-22, half of the bedding material of the home cages was refreshed every week. At PND 14, ear punches were taken of the pups for identification and genotyping, which was performed by kbiosciences (Hoddesdon, United Kingdom). The procedure of genotyping has been described previously (Homberg et al., 2007). At PND 22, the pups were weaned and housed in groups of 2-3 littersmates of the same sex and rearing, under the same conditions as mentioned above.

DNA methylation assay
The DNA methylation assay was performed as described previously (Sterrenburg et al., 2011). Briefly, genomic DNA was isolated from punches of the BNSTov, PVN and CeA by use of the DNeasy blood & tissue kit (Qiagen, Valencia, CA, USA) and according to the manufacturer’s instructions. Bisulfite conversion of the DNA samples and pyrosequencing of the promoter region and the initial part of exon 1 of the Crf gene were performed by EpigenDX (Hopkinton, MA, USA) (Kim et al., 2007). The Crf promoter region contains important CpG-containing regulatory sites, such as binding sites for transcription factors like CREB and AP-1 (Yao et al., 2007).

Crf expression
The expression of Crf (mRNA, protein) was studied by the use of quantitative real-time PCR (brain punches, cohort 1 +2) and immunohistochemistry (fixated brain sections, cohort 1). A detailed description of both techniques is available in the supplementary material.

Statistical analysis
All statistical tests have been carried out using SPSS (version 20, IBM corporation, Armonk, NY, USA). The results are presented as the mean with the standard error of the mean (SEM). The RT-PCR data have been expressed as a ratio compared to the average of the MS0-wild-type group. All data have been examined with factorial ANOVA. If a significant main effect of genotype or interaction (“genotype x early life stress”) was found, post-hoc testing was performed. For the correlational analysis between Crf mRNA and DNA methylation at the twelve CpG sites as well as the average CpG methylation, Bonferroni correction was applied for every brain area (p < 0.05 divided by 13 = p < 0.003846). Furthermore, one-tailed Spearman correlation was performed because of the consensus with a Miltex 1.0 mm biopsy puncher (Integra Miltex, York, PA, USA), collected in sterile vials, immediately placed on dry ice and stored at -80 °C. Half of the punches were used for RNA isolation, the other half for the isolation of genomic DNA. The punches were distributed by systematic randomization, accounting for lateralization and Bregma position.

Prior to transcardial perfusion, rats received an intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight). The perfusion was performed with phosphate-buffered saline (PBS, pH 7.4) and a clamp on the abdominal aorta, followed by fixation of the brain with 4% paraformaldehyde (PFA) in PBS. The fixed brains were immediately isolated, postfixed in fresh 4%-PFA, transferred to a 30% sucrose solution in PBS. The sucrose solution was refreshed every two days until the brains were completely submerged. Then, the brains were frozen by use of dry ice and were cut in 25 µm-thick coronal slices on a freezing microtome (Microm, Walldorf, Germany). The sections were stored in a sterile antifreeze solution (0.05 M PBS, 30% ethylene glycol, 20% glycerol) at -20 °C.

CHAPTER 5 CRF AND DNA METHYLATION

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of a negative relationship between DNA methylation and transcriptional activity (Moore et al., 2013) and because the measure of DNA methylation is derived from an ordinal, non-linear scale (percentage of methylated versus unmethylated PCR clones). Statistical significance was set at $p < 0.05$. Group sizes are available in the supplementary material.

## Results

### DNA methylation of the CRF gene promoter region

We examined the DNA methylation of the promoter region of the $Crf$ gene, which contains important sites for the regulation of transcription. All but one of the examined CpGs are found upstream of the transcription start site of the gene, indicated by a minus sign (Supplementary Figure 1). CpG$_{-226}$ resides in the middle of a CRE site, while CpG$_{-79}$ is located in a putative AP-1 binding site (Yao and Denver, 2007). Furthermore, CpG$_{-36}$, CpG$_{-33}$ and CpG$_{-15}$ surround the gene’s TATA box, a sequence 24-30 bp from the $Crf$ transcription start site (TSS), which recruits RNA polymerase II and transcription factors (Lee and Young, 2000) (Supplementary Figure 1).

Overall, the DNA methylation levels of the promoter region of the $Crf$ gene were found to be highly similar in the two cohorts and across the BNSTov, PVN and CeA (Figure 1-2). In all three brain areas, DNA methylation was consistently the highest for CpG$_{-232}$, CpG$_{-226}$ and CpG$_{-212}$ (60-80%) compared to the downstream CpGs (30-40%), with the exception of CpG$_{-15}$ (50-60%).

For both cohorts, DNA methylation levels of the $Crf$ promoter in the CeA were significantly affected by the interaction between ELS and 5-HTT genotype (Figure 1-2). In cohort 1, methylation of CpG$_{-36}$ was found to be significantly affected by the GxE interaction ($F_{2,37} = 3.42, p < 0.05$) (Figure 1). Post-hoc analysis showed that 5-HTT$^+$ rats exhibit a higher degree of CpG$_{-36}$ DNA methylation compared to 5-HTT$^-$ rats ($p < 0.05$) and 5-HTT$^{++}$ ($p < 0.01$) rats under control conditions (MS0). With a history of ELS exposure, this 5-HTT genotype difference is no longer present, as this leads to a downregulation of CpG$_{-36}$ methylation in 5-HTT$^-$ rats ($p < 0.01$, Figure 1).

In cohort 2, the methylation of CpG$_{-226}$ ($F_{2,87} = 4.18, p < 0.05$) and CpG$_{-147}$ ($F_{2,87} = 3.60, p < 0.05$) were significantly affected by ELS x 5-HTT genotype interaction (Figure 2). Here, post-hoc analysis showed a significantly higher degree of CpG$_{-226}$ methylation in 5-HTT$^+$ rats compared to 5-HTT$^-$ rats ($p < 0.01$) under control conditions (MS0). With a history of ELS exposure, this 5-HTT genotype difference is no longer present, as this leads to an up-regulation of CpG$_{-226}$ methylation in 5-HTT$^+$ rats ($p < 0.05$). Furthermore, CpG$_{-147}$ methylation was found to be selectively decreased in 5-HTT$^+$ rats by exposure to ELS ($p < 0.01$, Figure 2).

In the BNSTov of cohort 1, DNA methylation levels were found to be affected at three different CpG sites: CpG$_{-226}$, CpG$_{-33}$ and CpG$_{-15}$ (Figure 1). In cohort 2 however, DNA methylation of the $Crf$ promoter in the BNSTov was not found to be significantly affected by early life stress (ELS) or 5-HTT genotype. Figure 1: Cohort 1: DNA methylation of the promoter region of the $Crf$ gene. DNA methylation levels of the $Crf$ promoter in the paraventricular nucleus (PVN), the oval subdivision of the bed nucleus of the stria terminalis (BNSTov) and the central amygdala (CeA) of serotonin transporter (5-HTT) homozygous knockout (5-HTT$^-$/), heterozygous knockout (5-HTT$^+$/-) and wild-type (5-HTT$^+$/+) rats exposed to daily 3 h separations (MS180) or a control treatment (MS0) from postnatal day 2 to 14. DNA methylation was assessed for eleven cytosine-phosphate-guanine (CpG) sites upstream of the transcription start site (e.g., -232, -226) and one CpG in exon 1 (+21) of the $Crf$ gene. In the CeA, methylation of CpG$_{-36}$ showed a significant regulation by 5-HTT genotype (G: $p < 0.05$). CpG$_{-226}$ methylation was found to be significantly affected by 5-HTT genotype x ELS interaction (GxE: $p < 0.05$). In the BNSTov, DNA methylation of the $Crf$ promoter region was not found to be significantly affected by either ELS or 5-HTT genotype.
CHAPTER 5 CRF AND DNA METHYLATION

(Figure 2). For cohort 1, factorial ANOVA revealed that DNA methylation at CpG<-226 was significantly affected by 5-HTT genotype ($F_{2,36} = 3.66, p < 0.05$), while CpG<-33 methylation was found to be decreased by ELS exposure ($MS0 > MS180, F_{1,36} = 5.04, p < 0.05$). DNA methylation at CpG<-15 was found to be significantly affected by ELS x 5-HTT genotype interaction ($F_{2,36} = 3.62, p < 0.05$). For CpG<-226, post-hoc analysis showed that 5-HTT<sup>-/-</sup> rats display significantly higher methylation levels compared to 5-HTT<sup>+/+</sup> rats ($p < 0.05$). For CpG<-33 and CpG<-15, post-hoc testing did not reveal any significant differences between the individual experimental groups.

For the PVN, DNA methylation of the Crf promoter region was not found to be significantly affected by ELS, 5-HTT genotype or their interaction (Figure 1-2).

CRF expression

Of cohort 1, we have previously published the qRT-PCR analysis of CRF mRNA levels in the PVN, for which we did not find any significant effect of ELS, 5-HTT genotype or their interaction (Van der Doelen et al., 2014a). Here, we report that CRF mRNA levels in the BNSTov and CeA of cohort 2 are also not significantly regulated by ELS, 5-HTT genotype or their interaction (Supplementary Figure 2-3).

In addition, we have performed immunohistochemistry to assess CRF expression at the protein level in cohort 1. In the BNSTov and CeA, the number and SSD of both CRF-ir neurons and CRF-ir boutons were not found to be significantly regulated by ELS, 5-HTT genotype or their interaction in the factorial ANOVA analysis (Supplementary Figure 4-5). For the PVN however, we found a significant effect of 5-HTT genotype on the number ($F_{2,21} = 6.06, p < 0.01$), as well as the SSD ($F_{2,21} = 3.79, p < 0.05$) of CRF-ir neurons. Post-hoc analysis indicated that 5-HTT<sup>-/-</sup> rats display an increased number of CRF-ir neurons in the PVN compared to 5-HTT<sup>+/+</sup> rats ($p < 0.01$). In contrast, 5-HTT<sup>-/-</sup> rats showed a lower SSD of CRF-ir neurons in the PVN compared to 5-HTT<sup>+/+</sup> rats, but according to post-hoc analysis this difference was not significant ($p = 0.07$). In contrast to 5-HTT genotype, ELS and ELS x 5-HTT genotype interaction were not found to have a significant impact on the number and SSD of CRF-ir neurons in the PVN (Supplementary Figure 6).

Correlational analysis: Crf promoter methylation and CRF mRNA levels

For both cohorts 1 and 2, the levels of CRF mRNA and DNA methylation of the promoter region of the Crf gene were measured in the same subset of animals, which enables an analysis of the correlation between transcription and DNA methylation of Crf. The analysis was performed by combining cohort 1 and 2 and with all the twelve individual CpGs in the Crf promoter region as well as the average methylation of the CpG sites. Bonferroni correction for multiple testing was applied ($p < 0.05$ divided by 13: $p < 0.0038$).

For the CeA, a negative correlation between CRF mRNA levels and DNA methylation at CpG<-36 was found to be highly significant ($r = -0.286, p = 0.00007$) (Figure 4). The
methylation of CpG_-33, CpG_-15, CpG_+21 and the average methylation of all CpGs also showed a negative correlation with CRF mRNA levels in the CeA (p < 0.05), but these correlations were not significant after Bonferroni correction.

For the PVN, negative correlations between CRF mRNA levels and DNA methylation at CpG_-95 (rs = -.298, p = 0.0003), CpG_-79 (rs = -.280, p = 0.0007) as well as the methylation of the individual CpG sites CpG_-95 (r_s = -0.298, p = 0.0003) and CpG_-79 (r_s = -0.280, p = 0.0007).

Figure 4 Correlations between DNA methylation of the Crf promoter and CRF mRNA levels in the central amygdala (CeA) and paraventricular nucleus of the hypothalamus (PVN). In the CeA, the negative relationship (r_s = -.286) between CRF mRNA and methylation of the cytosine-phosphate-guanine (CpG)_-36 site in the promoter region of the Crf gene was found to be significant (p = 0.0007). In the PVN, a significant negative relationship was found between CRF mRNA levels and the average (Avg) methylation of the Crf promoter (r_s = -.246, p = 0.0026) as well as the methylation of the individual CpG sites CpG_-95 (r_s = -0.298, p = 0.0003) and CpG_-79 (r_s = -0.280, p = 0.0007).

methylated of CpG_-33, CpG_-15, CpG_+21 and the average methylation of all CpGs also showed a negative correlation with CRF mRNA levels in the CeA (p < 0.05), but these correlations were not significant after Bonferroni correction.

For the PVN, negative correlations between CRF mRNA levels and DNA methylation at CpG_-95 (r_s = -0.298, p = 0.0003), CpG_-79 (r_s = -0.280, p = 0.0007) as well as the average methylation of all CpGs (r_s = -0.246, p = 0.0026) survived Bonferroni correction (Figure 4). One might consider that the extreme values of PVN CRF mRNA levels drive the significance of the correlations. Yet, these correlations remained significant with omission of the extremes of the CRF mRNA levels (p ≤ 0.031). In addition, methylation of CpG_-232, CpG_-212, CpG_-101, CpG_-55, CpG_-36 and CpG_+21 seemed to contribute (p < 0.05) to
Discussion

In this study, we report that ELS and 5-HTT genotype interact in the CeA to program DNA methylation of the Crf promoter region in two independent cohorts. Furthermore, methylation of Cpgs_-36, which immediately presides an essential transcription factor binding site (TATA box), was found to show a highly significant negative correlation with CRF mRNA levels in the CeA. However, we did not find ELS x 5-HTT genotype alterations in the expression of CRF (mRNA, protein), suggesting the involvement of additional (epigenetic) mechanisms in the regulation of CRF expression. Interestingly, we found correlative evidence suggesting that CeA CRF neurons could be involved in stress coping behaviour in our ELS x 5-HTT genotype model. Specifically, CeA CRF mRNA levels were found to negatively correlate with escape latencies in rats that were subjected to the LH paradigm, which has high face and predictive validity for the translation to clinical depression (Hammack et al., 2012; Pryce et al., 2011).

By applying the LH paradigm to our ELS x 5-HTT genotype model, we have previously reported that exposure to ELS is associated with increased expression of adaptive, active coping behaviour (Van der Doelen et al., 2013). The LH paradigm consisted of exposure to inescapable shock (IS) stress for two days, and an escape test on the third day in the same context where rats could now escape the foot shocks. As IS exposure induces an escape deficit (passive coping) in vulnerable rats, active vs. passive coping behaviour is quantified by measuring escape latencies in the escape test. Strikingly, when stratifying for 5-HTT genotype, we found that the adaptive effect of ELS exposure was only significant for 5-HTT+/- rats (Van der Doelen et al., 2013). These behavioural findings support the recently postulated match/mismatch hypothesis, which states that exposure to ELS is not necessarily pathological, but can be used to adaptively respond to future stress exposure (Gluckman et al., 2007; Champagne et al., 2009; Nederhof and Schmidt, 2012). The increased sensitivity of 5-HTT+/- rats to benefit from a positive, adaptive match between the early and adult life environment is also in support of the differential susceptibility hypothesis (Belsky et al., 2009; Ellis et al., 2011). In humans, 5-HTTLPR S-allele carriers have been shown to exhibit increased vulnerability to develop psychopathology following exposure to adverse environments (Caspai et al., 2003; Karg et al., 2011). In addition, S-allele carriers show increased sensitivity to negative environments compared to LL homozygotes by displaying stronger physiological responses to stress (Gotlib et al., 2008; Alexander et al., 2009), increased attentional bias to negatively valenced stimuli (Pergamin-Hight et al., 2012) and higher levels of neuroticism and negative emotionality (Lesch et al., 1996; Stein et al., 2008; Pluess et al., 2011). Whereas the diathesis-stress hypothesis focusses solely on the adverse consequences of genetic predispositions, the differential susceptibility hypothesis provides a theoretical framework to explain the evolutionary survival of ‘risk’ genes by emphasizing them as ‘plasticity’ genes with greater sensitivity to adverse as well as positive environmental events (Belsky et al., 2007; Bakermans-Kranenburg and Van...
Uzendoorn, Ellis et al., 2011). Indeed, the 5-HTTLPR S-allele is very common, with an allele frequency of approximately 40% in Caucasians (Lesch et al., 1996; Noskova et al., 2008; Eisenberg and Hayes, 2010), and S-allele carriers have repeatedly been shown to display increased benefit from positive environments in comparison to LL homozygotes (Kaufman et al., 2004; Pluess et al., 2010; Van Uzendoorn et al., 2012).

In the current study, we have found a neural correlate (CeA CRF mRNA levels) of coping behaviour and thus, as pointed out above, differential susceptibility. Firstly, we found that ELS and 5-HTT genotype interact to affect DNA methylation of the CRF promoter specifically in the CeA. Of the investigated CpG sites, CpG_-36 showed a highly significant negative correlation with CeA CRF mRNA levels, which itself showed a correlation with coping behaviour (escape latencies) in the LH paradigm.

Passive coping responses across a variety of rodent stress models have been associated with increased activity of 5-HT neurons in the dorsal raphe nucleus (DRN) (Valentino et al., 2010; Hammad et al., 2012, but also see Wood et al., 2013). The DRN is densely innervated by CRF-containing fibers, which are in part derived from the CeA (Peyron et al., 1998; Lowry et al., 2000; Retson and Van Bockstaele, 2013). Interestingly, pharmacological manipulations have shown that CRF can both stimulate and inhibit the activity of DRN 5-HT neurons, via corticotropin-releasing factor receptor 1 (CRF1R) and corticotropin-releasing factor receptor 2 (CRF2R), respectively (Waselus et al., 2009; Valentino et al., 2010). CRF has high affinity for CRF1R, but only low affinity for CRF2R, in contrast to the CRF peptide family members urocortin 1, 2 and 3, which have therefore been proposed to be the endogenous ligands for CRF2R (Vaughan et al., 1995; Hsu and Hsueh, 2001; Lewis et al., 2001; Reyes et al., 2001). Given the correlation we identified here of CeA CRF mRNA levels with increased active coping (decreased escape latencies), we hypothesize that increased CeA-CRF input to the DRN could strengthen CRF1R over CRF2R activation associated with lower DRN 5-HT activity and increased active coping responses. There is likely an optimum to increased CeA-CRF input though, as increasingly higher doses of CRF would start to activate CRF2R (Hammack et al., 2003a, 2003b). Furthermore, the consequences of increased DRN 5-HT neuron activity depend on the recruited forebrain projections, as CeA-stimulated 5-HT release in the medial prefrontal cortex has actually been associated with reduced passive coping behaviour (Forster et al., 2006, 2008).

It should be pointed out that although DNA methylation (CpG_-36) correlated significantly with CRF mRNA levels in the CeA, only the adult naive cohort showed GxE regulation of CpG_-36, while the adult stress cohort showed GxE regulation of CpG_-226 and CpG_-147. Furthermore, CeA CRF mRNA levels were not significantly affected by the interaction of ELS and 5-HTT genotype, and CpG_-36 methylation did not show a significant correlation with escape latencies unlike CeA CRF mRNA levels. Therefore, whereas our findings are suggestive of an ELS x 5-HTT genotype effect on CeA CRF promoter methylation that moreover could possibly be linked to stress coping behaviour, it is clear that our results also warrant replication as well as further experimentation to connect the dots between CRF promoter methylation, expression of CRF and behavioural changes that are relevant for psychopathology.

In contrast to the CeA, DNA methylation of the CRF promoter region was not consistently found to be affected by ELS x 5-HTT genotype interaction in the BNSTov and PVN. This is not entirely unexpected, as previous studies have shown that chronic stress exposure (early/adult life) can differentially affect DNA methylation of the CRF promoter in the BNSTov, PVN and CeA (Mueller and Bale, 2008; Sterrenburg et al., 2011). A recent study suggests that the use of more prolonged maternal separations could have induced ELS x 5-HTT genotype alterations of CRF promoter methylation in the PVN (Chen et al., 2012). In the BNSTov of the adult naive cohort, we observed higher methylation of CpG_-226 in 5-HTT” rats compared to 5-HTT” rats (5-HTT genotype effect), which however was not present in cohort 2. This is most likely explained because of a general downregulation of CpG_-226 methylation by the repeated exposure to foot shock stress, as chronic stress has previously been shown to lead to a decrease in CRF CpG_-226 methylation in the BNSTov (Sterrenburg et al., 2011). In line with the absence of consistent alterations of CRF promoter methylation in the BNSTov and PVN, we did not find significant effects of ELS x 5-HTT genotype interaction on CRF expression in these areas, although DNA methylation of the CRF promoter methylation did show a significant negative correlation with CRF mRNA levels in the PVN.

From this study, together with previous studies that have employed a similar maternal separation paradigm, the picture emerges that the genetic background (strain) of rats modulates the effects of ELS exposure on CRF expression in the BNSTov, PVN and CeA, supporting the importance of considering GxE interactions in psychopathology. For Long-Evans rats, it has been reported that ELS induces increased CRF expression in the BNST, PVN and CeA (Francis et al., 2002; Hsueh et al., 2004; Plotkey et al., 2005, but also see Ladd et al., 2005). In Sprague-Dawley and Wistar rats however, CRF expression in the BNST/PVN/CeA is unaltered or even lower following exposure to ELS (this study, as well as Desbonnet et al., 2008; Bravo et al., 2010; Chen et al., 2012, but also see Aisa et al., 2007). Together, these studies suggest that the effects of ELS (maternal separation) on the basal expression levels of CRF in the adult brain are moderated by the genomic differences between rat strains. However, it should be noted that this concerns only a limited number of studies of which none have directly compared rat strains for their sensitivity to ELS to induce alterations in CRF expression.

Technical note
It should be noted that in the current study, as well as in previous studies (Mueller and Bale, 2008; Elliott et al., 2010; Sterrenburg et al., 2011; Chen et al., 2012), DNA methylation of the CRF promoter has been examined in micropunches of brain areas. As these samples contain a large fraction of non-CRF expressing cells, the resulting DNA methylation
patterns are ‘condensed’ and the effects of ELS, 5-HTT genotype and their interaction are likely underestimated.

Conclusions
In conclusion, we report here that ELS and 5-HTT genotype interact to affect DNA methylation of the Crf promoter region in the CeA. Furthermore, methylation of CpG -36 was found to significantly correlate with CRF mRNA levels in the CeA, suggesting that there is a functional relationship between DNA methylation and Crf transcription. In addition, we found that CeA Crf mRNA levels correlated with behavioural resilience to inescapable stress. Therefore, our findings are suggestive of an ELS x 5-HTT genotype effect on CeA Crf promoter methylation, which is possibly linked to stress coping behaviour. As discussed above, our results warrant replication, as well as further experimentation to connect the dots between Crf promoter methylation, expression of CRF and behavioural changes that are relevant for psychopathology.

Acknowledgements
We thank Anthonieke Middelman, Ron Engels, Harm van Blijderveen and Mark de Leeuw for their technical assistance.

Supplementary Methods
RNA isolation and cDNA synthesis
From the micropunches of the BNStov, PVN and CeA, total RNA was isolated by a single step of guanidinium isothiocyanate/phenol extraction using PureZol RNA isolation reagent (Bio-Rad, Hercules, CA, USA) according to manufacturer’s instructions. RNA concentrations were measured and RNA purity checked (A260/280 ratio between 1.8 and 2.0) with a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Samples were treated with DNase to avoid DNA contamination. First strand cDNA synthesis was performed by incubating 40 ng of RNA dissolved in 12 µl of RNase-free water containing 0.25 mU random hexamer primers (Roche Applied Science, Penzberg, Germany) at 70 °C for 10 min, followed by double-strand synthesis in 1st strand buffer with 10 mM DTT, 100 U Superscript II (Life Technologies), 0.5 mM dNTPs (Roche Applied Science) and 20 U of rRNasin (Promega Corp., Fitchburg, WI, USA) at 37 °C for 75 min. The cDNA samples were stored at -20 °C.

Quantitative real-time PCR
Quantitative real-time PCR (qRT-PCR) was performed with the ABI Prism® 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). For the reactions a total volume of 25 µl of buffer solution was used containing 5 µl of template cDNA, 12.5 µl Power SYBR Green Master mix (Applied Biosystems), 1.5 µl RNase-free DEPC and 0.6 µM of each primer. Prior to analysis of the relative expression of Crf, it was evaluated whether Rn18S, Gapdh or Hprt1 would be the best internal control gene and all primer pairs were tested for reaction efficiency. Primers and probe sequences were purchased from Biolegio B.V. (Nijmegen, The Netherlands). The following primer sequences were used: Crf-Fw: 5’-CCGGGCAGAGCAGTTAGC-3’, Crf-Rev: 5’-CACACATTTCATTCCGGATAATCTC-3’, Gapdh-Fw: 5’-GCTGTTGACAGGATGTTGC-3’, Gapdh-Rev: 5’-CTGGAAGATGGTGATGGGTT-3’, Rn18S-Fw: 5’-GTACCCGGTTGACCCCCATT-3’, Rn18S-Rev: 5’-CCATCCAAATCGTGATAGCCG-3’, Hprt1-Fw: 5’-CAGACCCGGTTCCGGCC-3’, Hprt1-Rev: 5’-CCCCCCTTCAACGACACAGAG-3’. The cycling protocol started with 10 min at 95 °C, followed by 39 reaction cycles with 15 s at 95 °C and 1 min at 60 °C. For each reaction, the cycle threshold (Ct) was determined, i.e. the number of cycles needed to detect fluorescence above the arbitrary threshold. The procedure was concluded with a melting curve protocol, from 65 °C to 95 °C, measuring fluorescence every 0.5 °C, to control for product specificity. All RT-PCR analyses were carried out in triplicate, with newly synthesized cDNA. Relative target gene expression was calculated by the 2^-ΔCt method (Schmittgen and Livak, 2008).

Immunohistochemistry
Immunohistochemistry targeted at CRF was carried out on free-floating sections of the BNStov, PVN and CeA with the combined diaminobenzidine (DAB) and avidin-biotin-com-
plex (ABC) method. The sections were rinsed in PBS, treated with 0.5% Triton X-100 (Sigma-Aldrich, Zwijndrecht, The Netherlands) in PBS for 30 min to enhance antibody penetration, 1% H₂O₂ in PBS for 45 min to quench endogenous peroxidase activity, and pre-incubated in PBS with 2% normal donkey serum (NDS) for 1 h. Incubation with the primary antiserum, rabbit polyclonal anti-CRF (1:2000, kindly provided by the late Dr. Wylie Vale, Salk Institute, La Jolla, CA, USA) was performed with 2% NDS in PBS for 16 h. The high specificity of the CRF antiserum has been described on the basis of preabsorption with the homologous synthetic peptide (Rivier et al., 1983; Sawchenko et al., 1984). Following rinses in PBS, the sections were incubated for 2 h with the secondary antiserum, biotinylated donkey anti-rabbit IgG (1:200, Jackson Immunoresearch, West Grove, PA, USA) and 2% NDS in PBS. Following rinses in PBS, the sections were incubated for 1 h with ABC of the Vectastain Elite ABC kit (1:200, Vector Laboratories, Burlingame, CA, USA) in PBS, after which they were again rinsed with PBS. The immunostaining was visualized by incubating the sections with 0.5% DAB (Sigma-Aldrich) and 0.005% H₂O₂ in Tris-HCl buffer (pH 7.4) for 11 min. Following rinses in Tris-HCl buffer, the sections were mounted on gelatin-coated glasses, left to dry overnight at 37 °C, dehydrated by an ethanol-xylene sequence and coverslipped with Entellan (Merck, Darmstadt, Germany).

**Image analysis**

For each immunohistochemical reaction, image analysis was performed bilaterally in 3 sections of the BNSTov, PVN and CeA, interspaced by 125 µm. Digital images of the brain sections were taken at a resolution of 2592 x 1944 pixels with a DMBRE microscope equipped with a DC 500 digital camera (Leica microsystems, Wetzlar, Germany). The images were analyzed with ImageJ software (NIH, Bethesda, MD, USA), which was used to count CRF-immunoreactive (CRF-ir) neurons and boutons, as well as to quantify the specific signal density (SSD) of the CRF immunostaining of neurons and boutons. The analyses were performed by using the Analyze Particles-tool of ImageJ, which enables the selection of neurons and boutons by setting thresholds in the pixel size of particles. The measures were averaged, providing for each parameter and brain nucleus one value per animal.

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**Figure S1** Cytosine-phosphate-guanine (CpG) sites in the proximal promoter of the rat Crf gene. The cyclic AMP-response element (CRE) has been shown to be involved in the stress-induced elevation of CRF expression (Yao and Denver, 2007; Kovács, 2013) and is located upstream (–229 to –221) of the transcription start site (TSS). In addition, CpG_–79 is located in a putative binding site for activator protein 1 (AP-1). Furthermore, 24–30 base pairs upstream of the TSS a TATA box is located, surrounded by several CpGs (Yao and Denver, 2007). The TATA box sequence is a core promoter sequence involved in the recruitment of RNA polymerase II (Lee and Young, 2000). Furthermore, DNA methylation of CpGs that surround TATA box sequences has been linked to the regulation of gene transcription (Kitazawa and Kitazawa, 2007; Matsumoto et al., 2013).

**Figure S2** Cohort 1: Corticotropin-releasing factor (CRF) mRNA levels. CRF mRNA levels in the oval subdivision of the bed nucleus of the stria terminalis (BNSTov) and the central amygdala (CeA) of serotonin transporter (5-HTT) homozygous knockout (5-HTT_+/−), heterozygous knockout (5-HTT_−/−) and wild-type (5-HTT_+/+) rats exposed to daily 3 h separations (MS180) or a control treatment (MS0) from postnatal day 2 to 14. CRF mRNA levels were not found to be significantly affected by early life stress, 5-HTT genotype or their interaction. Data were normalized to the average of the MS0-5-HTT_+/+ group.
Figure S3 Cohort 2: Corticotropin-releasing factor (CRF) mRNA levels. CRF mRNA levels in the oval subdivision of the bed nucleus of the stria terminalis (BNSTov), the paraventricular nucleus of the hypothalamus (PVN) and the central amygdala (CeA) of serotonin transporter (5-HTT) homozygous knockout (5-HTT-/-), heterozygous knockout (5-HTT+/-) and wild-type (5-HTT+/+) rats exposed to daily 3 h separations (MS180) or a control treatment (MS0) from postnatal day 2 to 14. In addition, this cohort of rats was exposed on three consecutive days to footshocks in a learned helplessness paradigm (Van der Doelen et al., 2013). CRF mRNA levels were not found to be significantly affected by early life stress, 5-HTT genotype or their interaction. Data were normalized to the average of the MS0-5-HTT+/+ group.

Figure S4 Cohort 1: Oval subdivision of the bed nucleus of the stria terminalis (BNSTov): immunohistochemistry of corticotropin-releasing factor (CRF). The BNSTov contains CRF-immunoreactive (CRF-ir) neurons and boutons (A). In the magnification-inset B (white box in A) an arrow indicates a CRF-ir neuron, while arrowheads point out CRF-ir boutons. The number (#) and specific staining density (SSD) of CRF-ir neurons (C, D) and boutons (E, F) were quantified for serotonin transporter (5-HTT) homozygous knockout (5-HTT-/-), heterozygous knockout (5-HTT+/-) and wild-type (5-HTT+/+) rats exposed to daily 3 h separations (MS180) or a control treatment (MS0) from postnatal day 2 to 14. The number and SSD of CRF-ir neurons and boutons were not found to be significantly affected by early life stress, 5-HTT genotype or their interaction. Scale bar = 50 µm.
Cohort 1: Central amygdala (CeA): immunohistochemistry of corticotropin-releasing factor (CRF). The CeA contains CRF-immunoreactive (CRF-ir) neurons and boutons (A). In the magnification-inset B (white box in A) an arrow indicates a CRF-ir neuron, while arrowheads point out CRF-ir boutons. The number (E) and specific staining density (SSD) of CRF-ir neurons (C, D) and boutons (E, F) were quantified for serotonin transporter (5-HTT) homozygous knockout (5-HTT⁻/⁻), heterozygous knockout (5-HTT⁺/⁻) and wild-type (5-HTT⁺⁺) rats exposed to daily 3 h separations (MS180) or a control treatment (MS0) from postnatal day 2 to 14. The number and SSD of CRF-ir neurons and boutons were not found to be significantly affected by early life stress, 5-HTT genotype or their interaction. Scale bar = 50 µm.
Figure S5 Continued.

Figure S6 Continued.

Figure S6 Cohort 1: Paraventricular nucleus (PVN): immunohistochemistry of corticotropin-releasing factor (CRF). The PVN contains CRF-immunoreactive (CRF-ir) neurons (A), of which the number (B) and specific staining density (SSD) were quantified for serotonin transporter (5-HTT) homozygous knock out (5-HTT−/−), heterozygous knock out (5-HTT+/−) and wild-type (5-HTT+/+) rats exposed to daily 3 h separations (MS180) or a control treatment (MS0) from postnatal day 2 to 14. The number of CRF-ir neurons in the PVN (B) was found to be significantly affected by 5-HTT genotype (G: p < 0.01), with 5-HTT−/− rats showing a higher number of CRF-ir neurons in the PVN compared to 5-HTT+/+ rats. The SSD of CRF-ir neurons in the PVN (C) was also found to be significantly influenced by 5-HTT genotype (G: p < 0.05), and in an opposite way as the number of CRF-ir neurons. However, post-hoc analysis did not reveal significant intergroup differences. Scale bar = 50 µm, *: p < 0.05.
Supplementary material: group sizes

### Group sizes of analyzed variables: cohort 1

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### Group sizes of analyzed variables: cohort 2

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| CRF mRNA | 13 | 21 | 16 | 14 | 20 | 14 |
| DNA methylation | 13 | 18 | 16 | 14 | 16 | 14 |

|        | CeA    |        |        |        |        |        |
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|        | M50    | MS180  |        |        |        |        |
|        | 5-HTT<sup>+/+</sup> | 5-HTT<sup>+/−</sup> | 5-HTT<sup>/−</sup> | 5-HTT<sup>+/+</sup> | 5-HTT<sup>+/−</sup> | 5-HTT<sup>/−</sup> |
| CRF mRNA | 13 | 22 | 16 | 15 | 20 | 14 |
| DNA methylation | 12 | 20 | 15 | 14 | 19 | 13 |
Chapter 6

The reciprocal relationship between serotonin and urocortin 1 in the dorsal raphe and Edinger-Westphal nuclei after early life stress in serotonin transporter knockout rats

Rick H.A. van der Doelen, Berit Robroch, Ilse A. Arnoldussen, Maya Schulpen, Judith R. Homberg and Tamás Kozicz

Submitted
Abstract

The interaction of early life stress (ELS) and the serotonin transporter (5-HTT) gene-linked polymorphic region (5-HTTLPR) has been associated with increased risk to develop depression in later life. We have used the maternal separation paradigm as a model for ELS exposure of homozygous and heterozygous 5-HTT knockout rats. Previously, we have reported altered stress coping behaviour and activity of the hypothalamo-pituitary-adrenal (HPA) axis in this ELS x 5-HTT genotype model. Next to corticotropin-releasing factor (CRF), the related peptide urocortin 1 (Ucn1) has been implicated in the behavioural and HPA response to stress. Ucn1 is abundantly expressed by neurons of the centrally projecting Edinger-Westphal (EWcp) nucleus, which are known to project to the dorsal raphe (DR) nucleus. The DR is involved in the behavioural and physiological stress response and is a major source of forebrain-projecting 5-HT neurons. Interestingly, the EWcp displays a dense innervation of 5-HT fibers as well. Therefore, it is hypothesized that the reciprocal relationship of EWcp-Ucn1 and DR-5HT neurons is of interest for stress-related psychiatric disorders. In this study, we found that ELS and 5-HTT genotype increased the number of 5-HT neurons in specific DR subdivisions, and that 5-HTT knockout rats showed decreased 5-HT innervation of EWcp-Ucn1 neurons. Furthermore, ELS was associated with increased DNA methylation of the promoter region of the Ucn1 gene and increased expression of 5-HT receptor 1A in the EWcp. In contrast, 5-HTT deficiency was associated with site-specific increases and decreases of DNA methylation of the Ucn1 promoter, and heterozygous 5-HTT knockout rats showed decreased expression of CRF receptor 1 in the EWcp. Together, our findings reveal a reciprocal relationship between EWcp-Ucn1 and DR-5HT neurons, which were found to be affected by exposure to ELS and 5-HTT genotype. These findings at the molecular level are hypothesized to contribute to ELS x 5-HTTLPR-linked psychopathology.

Introduction

Vulnerability to stress-related psychiatric disease is largely determined by a complex interplay between genetic and environmental factors. A common polymorphism in the serotonin (5-hydroxytryptamine; 5-HT) transporter (5-HTT, SLC6A4) gene has been shown to moderate the impact of stressful life events on the occurrence of major depression (Caspi et al., 2003, Karg et al., 2011, but also see Risch et al., 2009). Individuals with the short (S) allele of the of the 5-HTT-linked polymorphic region (5-HTTLPR) seem to be especially sensitive to the experience of early life stress (ELS) (Karg et al., 2011), but importantly, also to positive environments (Van Ijzendoorn et al., 2012). To understand the (neuro)physiological underpinnings of the interaction of 5-HTTLPR with psychopathogenic environmental factors such as ELS is pivotal for progression towards rationale-based therapy.

Corticotropin-releasing factor (CRF) is a key neuropeptide involved in the stress response due to its control of the hypothalamo-pituitary-adrenal (HPA) axis and its initiating and coordinating role of HPA, autonomic and behavioural responses to stress (Bale and Vale, 2004; Joëls and Baram, 2009). We have shown before that ELS and 5-HTT genotype interact to program the adult HPA-axis (Van der Doelen et al., 2014a) as well as DNA methylation of the Crf promoter region in the amygdala (Van der Doelen et al., 2014b). Over the last decade, evidence has accumulated suggesting that other members of the CRF family of neuropeptides, certainly urocortin 1 (Ucn1), but also urocortin 2 and urocortin 3, complement the actions of CRF in the regulation of the stress response (Kuperman and Chen, 2008; Kozicz et al., 2011a; Ryabinin et al., 2012). These complementary functions are facilitated by differences in anatomical distribution and receptor pharmacology (Janssen and Kozicz, 2013). The actions of CRF and the urocortins are mediated by two G-protein coupled receptors, corticotropin-releasing factor receptor 1 and 2 (CRF-R, CRF-R). Ucn1 binds both receptors with high affinity, while CRF has low affinity for CRF-R, and Ucn2 and Ucn3 selectively bind to CRF-R (Vaughan et al., 1995; Hsu and Hsueh, 2001; Lewis et al., 2001; Reyes et al., 2001).

Ucn1 is a 40 amino acid-peptide, that is highly conserved across species (Vaughan et al., 1995; Donaldson et al., 1996; Zhao et al., 1998; Cepoi et al., 1999; Hauger et al., 2006). In the mammalian brain, Ucn1 is most abundantly expressed by neurons of the centrally projecting Edinger-Westphal nucleus (EWcp) in the midbrain (Vaughan et al., 1995; Kozicz et al., 1998, 2011b; Bittencourt et al., 1999; tino et al., 1999). The EWcp-Ucn1 neurons have been shown to be recruited by various but selective stressors (Weninger et al., 2000; Gaszner et al., 2004; Korosi et al., 2005; Rouwette et al., 2011) with activity (measured by expression of the immediate early gene cFos) in some cases lasting up to 18 hours (Kozicz et al., 2001). Furthermore, in contrast to the HPA-axis, EWcp-Ucn1 neurons do not show habituation upon chronic stress exposure (Yiau and Sawchenko, 2002; Korosi et al., 2005; Xu et al., 2010). Interestingly, Ucn1 expression is increased in CRF-knockout mice (Weninger et al., 2000) and decreased in mice overexpressing neuronal CRF (Kozicz et al., 2004). Based
on these findings and the differential activation of PVN-CRF and EWcp-Ucn1 neurons in the stress response, it has been proposed that the dynamics of Ucn1 are important for the adaptive phase of the central stress response (Kozicz et al., 2011a; Ryabinin et al., 2012).

The EWcp-Ucn1 neurons have been shown to innervate the lateral septum in the forebrain, but predominantly project to the brainstem and spinal cord (Kozicz et al., 1998; Bittencourt et al., 1999; Weitemier et al., 2005). In the brainstem, a major site of innervation by Ucn1-positive fibers is the dorsal raphe (DR) nucleus nucleus (Bittencourt et al., 1999; Bachtei et al., 2004; Weitemier et al., 2005), where lesions of the EWcp have been shown to lead to a significant reduction of Ucn1-positive fibers (Bachtei et al., 2004). The EWcp on the other hand exhibits a dense innervation of 5-HT-positive fibers, and the DR has been shown to send projections to the EWcp (Clements et al., 1985; Morin and Meyer-Bernstein, 1999). Therefore, EWcp-Ucn1 neurons are hypothesized to contribute to the regulation of DR 5-HT activity, and vice versa (Kozicz et al., 2011a).

The DR and median raphe (MR) nuclei contain the majority of forebrain-projecting 5-HT neurons. The DR especially has been implicated in the behavioural and physiological stress response (Hale et al., 2012), and receives denser Ucn1-positive innervation compared to the MR (Bittencourt et al., 1999; Weitemier et al., 2005). The DR is topographically organized in subregions encompassing the rostral, dorsal, ventral, lateral, interfascicular and caudal portions. Interestingly, 5-HT interneurons from the lateral wings of the DR (DRW) innervate the dorsal and ventral parts of the DR (DRD, DRV), and have been proposed, based on a collection of evidence, to have a tonic inhibitory control over the DRD and DRV via postsynaptic 5-HT$_{1A}$ receptors (Jasinska et al., 2012). In addition, 5-HT$_{1A}$ autoreceptors are crucially involved in the inhibitory regulation of DR 5-HT neuron activity (Savitz et al., 2009). In depressed subjects, 5-HT$_{1A}$ binding in the DR (postmortem emission tomography) has been reported to be both elevated and reduced (Drevets et al., 1999; Parsley et al., 2006a), with postmortem binding studies suggesting that this discrepancy might be explained by considering the rostrocaudal extent of the DR (Boldrini et al., 2008).

The activity of DR 5-HT neurons can furthermore be regulated by CRF and urocortins. Activation of CRF$_R$ has been associated with repression, while CRF$_R$ mediates excitation of DR 5-HT neurons (Lukkes et al., 2008). Exposure to stress influences the relative contributions of CRF$_R$ and CRF$_R$ by affecting internalization and plasma membrane recruitment (Waselus et al., 2009; Wood et al., 2013). It has been postulated that CRF$_R$-mediated inhibition of 5-HT release in DR projection areas is associated with active coping responses, and CRF$_R$-mediated stimulation with passive stress coping (Valentino et al., 2010). However, 5-HT$_{1A}$-linked behavioural responses may depend on the involved projection area(s), as well as the type and duration of the stressor (Forster et al., 2006; Wood et al., 2013).

The neurotransmission of 5-HT is not only affected by the regulation of the activity of 5-HT neurons, but also by the functioning of the 5-HT transporter (5-HTT), which serves to re-uptake 5-HT from the extracellular space. The expression of 5-HTT by DR 5-HT neurons has been shown to be stimulated by glucocorticoid hormones through the glucocorticoid receptor (GR) (Zhang et al., 2012; Lau et al., 2013). Furthermore, behavioural responses to stress have been shown to be significantly affected by GR signaling in DR 5-HT neurons (Espallergues et al., 2012; Vincent and Jacobson, 2014).

Rodents with a knockout of the 5-HTT transporter (5-HTT$^{-/-}$) display increased extracellular 5-HT levels across the brain, decreased excitability of DR 5-HT neurons, decreased 5-HT tissue levels, and an almost complete absence of 5-HT re-uptake (Bengel et al., 1998; Gobbi et al., 2001; Lira et al., 2003; Mathews et al., 2004; Kim et al., 2005; Homberg et al., 2007). Furthermore, 5-HTT$^{-/-}$ mice and rats have been shown to exhibit decreased pre- and postsynaptic 5-HT$_{1A}$ expression and functioning (Li et al., 1999; 2000; Fabre et al., 2000; Homberg et al., 2008a; Olivier et al., 2008b).

From experiments with the maternal separation (MS) paradigm, the perspective has risen that the genetic background of rat strains can have strong modulatory effects on the phenotypic outcome of exposure to ELS. The expression of 5-HT$_{1A}$ and 5-HTT have for instance been reported to be decreased in Sprague-Dawley rats (Lee et al., 2007; Bravo et al., 2014), but unaltered or increased (5-HTT mRNA) in Lister hooded and Long-Evans rats that were subjected to MS (Neumaier et al., 2002; Gartside et al., 2003; Arborelius et al., 2004; Gardner et al., 2009). For Ucn1 expression in the EWcp, exposure to ELS has been shown not to affect wildtype (5-HTT$^{+/+}$) Wistar rats (Gaszner et al., 2009), while 5-HTT$^{-/-}$ mice display decreased Ucn1 expression in the EWcp (Fabre et al., 2011). In contrast, a human postmortem study reported that male (but not female) suicide victims with major depression exhibited highly increased Ucn1 mRNA levels in the EWcp (Kozicz et al., 2008).

For the present study, we hypothesized that ELS and 5-HTT gene variation would interact to affect the reciprocal relationship between the EWcp-Ucn1 and DR-5-HT populations. Specifically, we exposed heterozygous (5-HTT$^{+/}$) and homozygous (5-HTT$^{-/-}$) 5-HTT knockout rats, and their wild-type (5-HTT$^{+/+}$) littermates to ELS, i.e. maternal separation, and examined the EWcp-Ucn1 neurons at the epigenetic, mRNA and protein level. Furthermore, we assessed the reciprocal innervation of Ucn1 and 5-HT, as well as the expression levels of 5-HT$_{1A}$, CRF$_R$, CRF$_R$ and GR in the EWcp and DR. Previously, we have shown differential stress coping behaviour and HPA-axis activity in our ELS x 5-HTT genotype model (Van der Doelen et al., 2013, 2014a).

Materials and Methods

Animals

The experimental procedures were approved by the Committee for Animal Experiments of the Radboud University Nijmegen, The Netherlands, and all efforts were made to minimize animal suffering and to reduce the number of animals used. Serotonin transporter knockout rats (Slc6a4$^{-/-}$) were generated by N-ethyl-N-nitrosourea (ENU)-induced mutagenesis (Smits et al., 2014).
Experimental animals (5-HTT+/+, 5-HTT+/- and 5-HTT−/− rats) were derived from crossing 3 month old 5-HTT+/- rats that were outcrossed for at least twelve generations with commercial (Harlan, Ter Horst, The Netherlands) wild-type Wistar rats. The pregnant dams were housed in standard polypropylene cages (40 x 20 x 18 cm) with sawdust bedding and ad libitum access to water and rodent chow (Sniff Spezialdiäten, Soest, Germany) in a temperature (21 ± 1 °C) and humidity-controlled room (45-60% relative humidity), with a 12:12 h light:dark cycle (lights on at 07:00 a.m.). The dams were inspected daily at 5:00 p.m. for delivery of pups and day of birth was designated as postnatal day (PND) 0. At PND1, two paper towels (22.5 x 24.5 cm) were supplied to the mother for nest construction. Further, the litters were culled to a maximum of 10 pups, with gender ratios in favor of a male majority to maximally 7:3.

Early life stress
We used repeated and prolonged maternal separation as a model for ELS. Litters were randomly allocated to one of two rearing conditions (from PND 2 to 14): maternal separation for 180 min (MS180) or a control treatment with immediate reunion of mother and pups (MS0). MS180/MS0 was started daily between 08:30 and 09:00 a.m., and was performed as follows: The mother was removed from the home cage and placed into an identical cage until the end of the separation period. Pups were then removed from the nest as complete litters and placed into a cage (24 x 15 x 14 cm) with sawdust bedding, after which they were transferred to an adjacent room. The cages were placed on heat pads, which were set to maintain a bedding temperature of 31-33 °C for PND 2-7 and 29-31 °C for PND 8-14. At the end of the separation period, litters were returned to their home cage by first rolling them in the home cage bedding material and then placing them in the nests. This was followed by reunion with the mothers. During PND 0-22, half of the bedding material of the home cages was refreshed every week. At PND 14, ear punches were taken of the pups for identification and genotyping, which was performed as described previously (Homberg et al., 2007). At PND 22, the pups were weaned and housed in groups of 2-3 littermates of the same sex and rearing, under the same conditions as mentioned above.

Tissue collection and preparation
For the collection of tissues only adult (PND85-95) male rats were used. At this point, the sample was split into a group of rats that was sacrificed by perfusion and a group of rats that was sacrificed by decapitation. Of every litter, where possible, a single rat was selected of all three genotypes. Prior to transcardial perfusion, rats received an intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight). The perfusion was performed with phosphate-buffered saline (PBS, pH 7.4) and a clamp on the abdominal aorta, followed by fixation of the brain with ice-cold 4% paraformaldehyde (PFA) in PBS. The fixedated brains were immediately isolated, postfixed in fresh 4% PFA and transferred to a 30% sucrose solution (PBS). The sucrose solution was refreshed every two days until the brains were completely submerged. Then, the brains were frozen by use of dry ice and were cut in 25 µm-thick coronal slices on a freezing microtome (Microm, Walldorf, Germany). The sections were stored in a sterile antifreeze solution (0.05 M PBS, 30% ethylene glycol, 20% glycerol) at -20 °C.

For decapitations, rats were taken from their home cage into a separate room and decapitated within 10 s. Immediately after decapitation, the brains were isolated, frozen in aluminum foil on dry ice and stored at -80 °C. In a cryostat (-15 °C), the brains were prepared in 420 µm-thick coronal slices in order to obtain punches from the EWcp (Bregma -5.28 and -5.70 mm, coordinates based on Paxinos and Watson, 2007) and DR (Bregma -7.00 and -7.50 mm). The brain areas were punched out with a Mitex 1.5 mm biopsy puncher (Integra Mitex, York, PA, USA), collected in sterile vials, immediately placed on dry ice and stored at -80 °C. Half of the punches were used for RNA isolation, the other half for the isolation of genomic DNA. The punches were distributed by systematic randomization, accounting for lateralization and Bregma position.

Immunohistochemistry
For dual immunofluorescence labelling of Ucn1 and 5-HT (n = 6-7), free-floating sections containing the EWcp and DR were used. The EWcp sections were rinsed in PBS, incubated for 30 min with 0.5% Triton X-100 (Sigma-Aldrich, Zwijndrecht, The Netherlands) in PBS to enhance antibody penetration, and pre-incubated in PBS with 2% normal donkey serum (NDS) for 1 h. Incubation with the primary antisera, polyclonal rabbit anti-Ucn1 (1:2000, kindly provided by the late Dr. Wylie Vale, Saik Institute, La Jolla, CA, USA) and monoclonal mouse anti-5-HT (1:20,000, kindly provided by Dr. Lucienne Léger, Université Claude Bernard, Lyon, France), was performed with 2% NDS in PBS for 16 h. The high specificities of rabbit anti-Ucn1 (Bittencourt et al., 1999; Turnbull et al., 1999) and mouse anti-5-HT (Léger et al., 1998, 2001) have been reported previously. Following rinses in PBS, the sections were incubated for 3 h with the secondary antisera, cyanine3-conjugated donkey anti-rabbit IgG and cyanine5-conjugated donkey anti-mouse IgG (both 1:100, Jackson Immunoresearch, West Grove, PA, USA), with 2% NDS in PBS.

The DR sections were treated in a similar fashion, but with the use of tyramide signal amplification (TSA fluorescence system kit, PerkinElmer, Boston, MA, USA) for the visualization of Ucn1-immunoreactivity. The DR sections were rinsed in PBS and were incubated for 30 min with 0.5% Triton X-100 (Sigma-Aldrich, Zwijndrecht, The Netherlands) in PBS to enhance antibody penetration. Next, the sections underwent heat-induced epitope retrieval by incubation for 10 min in 0.01 M sodium citrate buffer at 85 °C, were rinsed with PBS, and incubated with 1% H2O2 in PBS for 45 min to quench endogenous peroxidase activity. Following rinses with PBS, the sections were pre-incubated in PBS-BSA.
with 2% NDS for 1 h. Incubation with the primary antisera, rabbit anti-Ucn1 (1:50 000) and mouse anti-5-HT (1:2000), was performed with 2% NDS in PBS-BTSA for 16 h. With in-between rinses in PBS, the sections were then incubated for 1 h with biotinylated donkey anti-rabbit IgG (Jackson Immunoresearch) in PBS-BTSA, for 30 min with a conjugate of streptavidine and horseradish peroxidise (1:100, TSA kit) in PBS-BTSA, and for 1 h with cyanine3-tyramide (1:200, TSA kit) and AlexaFluor488-conjugated donkey anti-mouse IgG (Jackson Immunoresearch) in amplification diluent (TSA kit).

The EWcp and DR sections were mounted on gelatin-coated glasses, air-dried and coverslipped with Fluorsave (Calbiochem, San Diego, CA, USA). Digital images of the sections (1024 x 1024 dpi) were obtained with the use of a Leica DM IRE2 confocal laser scanning microscope (TCS SP2 AOBS system, Leica Microsystems, Leica, Wetzlar, Germany) and 488 nm Argon and 561 nm yellow diode lasers.

Image analysis
The images were analyzed with the use of ImageJ software (NIH, Bethesda, MD, USA) and by observers that were blind for experimental grouping. In the EWcp, the number and mean staining intensity of Ucn1-immunoreactive (Ucn1-ir) neurons were quantified by manually encircling Ucn1-ir neurons in three serial sections, interspaced by 125 µm. The analysis of 5-HT-immunoreactive (5-HT-ir) fibers in the EWcp was automated by the use of the Analyze Particles-tool of ImageJ, which enables the selection of particles by pixel size thresholding. The mean staining intensities were corrected with a measurement of the background intensity in the same sections, and expressed as specific signal density (SSD, arbitrary units).

For the quantification of the number and SSD of 5-HT-ir neurons in the DR, a distinction was made on the basis of the topographical organization of the DR (Lowry, 2002; Hafe et al., 2012). Firstly, three images were selected of every animal that contained the DRD, DRV and DRLW. The rostrocaudal extent of the selected DR images was in correspondence to the micropunches of the DR (approximately -7.08 to -7.92 mm from Bregma). Each image was subdivided by placing straight lines over the image, based on the extent of the cellular groups and specific landmarks (Roche et al., 2003). Next, the number of 5-HT-ir neurons was counted manually per section, and the mean SSD was obtained as described above for the EWcp images.

In addition, overlay images were created from the Ucn1-ir and 5-HT-ir signals of each EWcp and DR section (Photoshop, Adobe, San José, CA, USA), which were used to manually count the number of 5-HT-ir fibers that were juxtaposed to Ucn1-ir neurons in the EWcp and the number of Ucn1-ir fibers juxtaposed to 5-HT neurons in the different subdivisions of the DR. The total number and SSD of Ucn1-ir fibers in the DR subdivisions were not quantified. All obtained measures were averaged, providing for each parameter one value per brain area and animal.

RNA isolation and cDNA synthesis
From the micropunches of the EWcp and DR, total RNA was isolated by use of the Nucleospin® RNA II kit (Macherey-Nagel, Düren, Germany) according to manufacturer’s instructions. Samples were treated with DNase to avoid DNA contamination. RNA concentrations were measured and RNA purity checked (A260/280 ratio between 1.8 and 2.0) with a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). First strand cDNA synthesis was performed by incubating 40 ng of RNA dissolved in 12 µl of RNase-free water containing 0.25 µl random hexamer primers (Roche Applied Science, Penzberg, Germany) at 70 °C for 10 min, followed by double-strand synthesis in 1st strand buffer with 10 mM DTT, 100 U Superscript II (Life Technologies), 0.5 mM dNTPs (Roche Applied Science) and 20 U of rNasin (Promega Corp, Fitchburg, WI, USA) at 37 °C for 75 min. The cDNA samples were stored at −20 °C.

Quantitative real-time PCR
Quantitative real-time PCR (qRT-PCR, n = 5-8) was performed with the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). For the reactions a total volume of 25 µl of buffer solution was used containing 5 µl of template cDNA, 12.5 µl Power SYBR Green master mix (Applied Biosystems, Foster City, CA, USA), 1.5 µl RNase-free DEPC and 0.6 µM of each primer. Primers were designed using NCBI Primer-Blast (www.ncbi.nlm.nih.gov/tools/primer-blast/) and synthesized by Biolegio BV (Nijmegen, The Netherlands). With standard curve analysis all primer pairs were confirmed to have reaction efficiencies of > 1.8. Prior to analysis of the relative expression of the genes of interest (GOI), for each tissue it was evaluated whether Rn18S, Gapdh or Hprt1 would be the best internal control gene. The following primer sequences were used; Cntrl-Fw: 5’-TCCAGAGATTCTCAACGAA-3’, Cntrl-Rev: 5’-AAAGCGGAGATGAGTTCAG-3’, Cntr1-Rev: 5’-TCTCCGCATGCACAGAAG-3’, Gapdh-Fw: 5’-GGTGTAAGCGATTGTG-3’, Gapdh-Rev: 5’-CTGGAAGATGGTGATGGGTTG-3’, Hprt1-Fw: 5’-CAGACCGCTGCTCCCGC-3’, Hprt1-Rev: 5’-CCCTTCAGCACACAGAAG-3’, Htr1a-Fw: 5’-TTCTTCATCCATCCACGCC-3’, Htr1a-Rev: 5’-GCTCTGCTCCCCCTTTCATC-3’, Nkx2.1-Fw: 5’-TGTCAAAAGGGAAGGGAACT-3’, Nkx2.1-Rev: 5’-TGGGAAAGTAGTTAGGAGAAGA-3’, Rn18S-Fw: 5’-GGTACCGTTGACAACCATT-3’, Rn18S-Rev: 5’-CCATCCAATCCAGTAGTACGCC-3’, Slc6a4-Fw: 5’-CCTTCTGTGTTCTTCCTCCTAC-3’, Slc6a4-Rev: 5’-TAGCCCAAGACGATCTACCTC-3’, Ucn-Fw: 5’-ACTGGGCAACACTCCCTGACA-3’, Ucn-Rev: 5’-TCCACTGCGTCATCCCG-3’. The cycling protocol started with 10 min at 95 °C, followed by 39 reaction cycles with 15 s at 95 °C and 1 min at 60 °C. For each reaction, the cycle threshold (Ct) was determined, i.e. the number of cycles needed to detect fluorescence above the arbitrary threshold. Relative expression of the GOI was calculated by the 2^-ΔΔCt method (Schmittgen and Livak, 2008). The procedure was concluded with a melting curve protocol, from 65 °C to 95 °C, measuring fluorescence every 0.5 °C, to control for product specificity. All qRT-PCR analyses were carried out in triplicate, with newly synthesized cDNA.
DNA methylation assay
DNA methylation is an epigenetic mechanism that occurs at cytosine-phosphate-guanine (CpG) sites and is associated with the repression of gene transcription (Moore et al., 2013). The DNA methylation assay (n = 5-8) was performed as described previously (Sterrenburg et al., 2011). Briefly, genomic DNA was isolated from punches of the EWcp and DR by use of the DNeasy blood & tissue kit (Qiagen, Valencia, CA, USA) and according to the manufacturer’s instructions. Bisulfite conversion of the DNA samples and pyrosequencing of the promoter region of the Ucn gene were performed by EpigenDX (Hopkinton, MA, USA) (Kim et al., 2007). The promoter region of the Ucn gene contains important sites for the regulation of transcription, such as a cyclic-AMP response element (CRE), and putative binding sites for transcription factors such as GATA (Vaughan et al., 1995; Zhao et al., 1998). All but one of the examined CpG sites are found upstream of the transcription start site of the gene, indicated by a minus sign (Figure 1).

Figure 1 Cytosine-phosphate-guanine (CpG) sites in the proximal promoter of the rat Ucn gene. The cyclic AMP-response element (CRE) has been functionally characterized for the mouse and human (Ucn, UCN) genes (Zhao et al., 1998). Furthermore, 77-83 base pairs upstream of the transcription start site (TSS) a TATA-like sequence (TATATAA) is located, surrounded by several CpGs. There are multiple putative binding sites for transcription factors in the 5’-flanking region, but these remain to be experimentally followed up on (Zhao et al., 1998).

Statistical analysis
All statistical tests have been carried out using SPSS (version 20, IBM corporation, Armonk, NY, USA). The results are presented as the mean with the standard error of the mean (SEM). The qRT-PCR 2-ΔΔCt data have been expressed as a ratio compared to the average of the MS0-wild-type group. All data have been examined with factorial ANOVA. If a significant main effect (“genotype”, “early life stress”) or interaction (“genotype x early life stress”) was found, post-hoc testing was performed. For the number of Ucn-ir neurons in the EWcp, the mRNA levels of CRF₁R, CRF₂R, Ucn1, 5-HT₁A in the EWcp and CRF₁R mRNA in the DR, a log-transformation was applied to achieve a normal distribution. For the SSD of 5-HT-ir fibers in the EWcp, the DNA methylation measurements and DR 5-HTT mRNA levels, log-transformation did not achieve a normal distribution. For these parameters, bootstrap analysis was applied to confirm if the results from the factorial ANOVA were robust, and non-parametric post-hoc testing was used. For correlational analysis, Bonferroni correction was applied to correct for multiple comparisons. Furthermore, Pearson or Spearman correlations were performed in the case of parametric or non-parametric variables, respectively. Statistical significance was set at p < 0.05.

Results
Immunohistochemistry of Ucn1 and 5-HT in the EWcp
In the EWcp, immunohistochemistry with the Ucn1 and 5-HT antisera revealed the presence of Ucn1-ir somas, as well as a dense network of 5-HT-ir fibers (Figure 2A, B). We quantified the number and SSD of both Ucn1-ir neurons and 5-HT-ir fibers using ImageJ (Figure 2). Furthermore, overlay images were created (Figure 2C) to quantify the number of Ucn-ir neurons that were found to be juxtaposed with 5-HT-ir, as well as the average number of juxtaposed 5-HT-ir fibers per Ucn-ir neuron.

The number and SSD of Ucn-ir neurons were not found to be significantly affected by ELS, 5-HTT genotype or their interaction. In contrast, 5-HTT deficiency, but not ELS or ELS x 5-HTT genotype interaction, was found to significantly affect the number (F2,34 = 10.09, p < 0.001) and SSD (F2,34 = 7.82, p = 0.001) of 5-HT-ir fibers in the EWcp. Post-hoc testing indicated that 5-HTT-/- rats display a significantly lower number and SSD of 5-HT-ir fibers in the EWcp compared to 5-HTT+/+ rats (p ≤ 0.01) and 5-HTT +/- rats (p ≤ 0.001). Moreover, the number of Ucn1-ir neurons that were juxtaposed to 5-HT-ir fibers was also found to be significantly affected by 5-HTT genotype (F2,34 = 6.65, p < 0.01), with 5-HTT-/- rats showing a decreased number of Ucn1-ir neurons with 5-HT contacts compared to both 5-HTT +/+ rats (p < 0.01) and 5-HTT +/- rats (p < 0.05). Interestingly, the average number of 5-HT-ir contacts per single Ucn1-ir neuron was not found to be significantly affected by 5-HTT genotype, nor by ELS or the interaction between ELS and 5-HTT genotype (Figure 2).

Gene expression in the EWcp
The mRNA levels of Ucn1, CRF₁R and GR in the EWcp were found not to be significantly affected by ELS, 5-HTT genotype or their interaction. In contrast, CRF₁R mRNA levels were found to be significantly affected by 5-HTT genotype (F2,35 = 4.03, p < 0.05). Post-hoc analysis indicated that CRF₁R mRNA levels in the EWcp were significantly lower for 5-HTT+/− rats compared to 5-HTT+/+ rats (p < 0.05). Moreover, the number of Ucn1-ir neurons that were juxtaposed to 5-HT-ir fibers was also found to be significantly affected by 5-HTT genotype (F2,34 = 6.65, p < 0.01), with 5-HTT +/- rats showing a decreased number of Ucn1-ir neurons with 5-HT contacts compared to both 5-HTT -/+ rats (p < 0.01) and 5-HTT +/− rats (p < 0.05). Interestingly, the average number of 5-HT-ir contacts per single Ucn1-ir neuron was not found to be significantly affected by 5-HTT genotype, nor by ELS or the interaction between ELS and 5-HTT genotype (Figure 2).
Figure 2: Edinger-Westphal (EWcp) nucleus immunohistochemistry of urocortin 1 (Ucn1, A) and serotonin (5-HT, B). The number (#) and specific staining density (SSD) of Ucn-ir neurons and 5-HT-ir fibers were quantified for serotonin transporter (5-HTT) homozygous knockout (5-HTT-/-), heterozygous knockout (5-HTT+/-) and wild-type (5-HTT +/+ ) rats exposed to daily 3 h separations (MS180) or a control treatment (MS0). The number and SSD of Ucn-ir neurons were not found to be significantly affected by ELS, 5-HTT genotype or their interaction. In contrast, the number and SSD of 5-HT-ir fibers were found to be significantly lower in the EWcp of 5-HTT-/- rats compared to 5-HTT+/- and 5-HTT+/+ rats (G: p < 0.01). In addition, overlay images were created to quantify the number of Ucn-ir neurons that were found to be juxtaposed with 5-HT-ir, as well as the average number of juxtaposed 5-HT-ir fibers per Ucn-ir neuron. An example overlay image is included (C), including magnifications of Ucn1-ir neurons without (white lining) and with juxtaposed 5-HT-ir fibers (yellow, orange lining). The number of Ucn1-ir neurons that were juxtaposed to 5-HT-ir fibers was found to be significantly decreased in 5-HTT-/- rats compared to 5-HTT+/- and 5-HTT+/+ rats (G: p < 0.05). Scale bar = 100 µm.
DNA methylation of the Ucn1 gene promoter region in the EWcp

Overall, the DNA methylation levels of the promoter region of the Ucn gene in the EWcp were found to be remarkably low (Figure 4). Across the promoter region, only at CpG_-171 and CpG_+27 the methylation levels were found to be above 5%, while at CpG_-89 there was a complete absence of methylation.

Factorial ANOVA revealed that DNA methylation of CpG_-171 (F2,35 = 4.55, p < 0.05) and CpG_-24 (F2,35 = 4.46, p < 0.05) was significantly affected by 5-HTT genotype (Figure 4). Post-hoc analysis indicated that CpG_-171 methylation was significantly higher in 5-HTT-/- rats compared to 5-HTT+/- rats (p < 0.05) and 5-HTT+/+ rats (p < 0.01). Furthermore, CpG_-24 methylation was completely absent in 5-HTT -/- rats, resulting in a significant difference from 5-HTT+/- rats (p < 0.05) (Figure 5). In addition, DNA methylation of CpG_-156 (F1,35 = 7.01, p < 0.05) and CpG_-49 (F 1,35 = 9.08, p < 0.01) was found to be significantly affected by ELS, with the MS180 group displaying significantly higher methylation levels at these CpG sites compared to the MS0 group (Figure 4).

DNA methylation of the other CpG sites was not found to be significantly regulated by ELS, 5-HTT genotype or their interaction. Furthermore, we found no significant correlations between Ucn1 mRNA levels and the methylation of individual CpG sites or the average methylation of the Ucn promotor region.

Immunohistochemistry of 5-HT and Ucn1 in the DR

In the DR, immunohistochemistry with the Ucn1 and 5-HT antisera revealed the abundant presence of 5-HT-ir somas in the different subdivisions of the DR, but only a sparse innervation by Ucn1-ir fibers. The number and SSD of 5-HT-ir neurons was quantified using ImageJ (Figure 6A), as well as the number of contacts (juxtapositions) between 5-HT-ir neurons and Ucn1-ir fibers (Figure 6B, C).
The number of 5-HT-ir neurons in the DRLW were found to be significantly affected by ELS ($F_{1,35} = 8.40, p < 0.01$) with the MS180 group displaying a higher number of 5-HT-ir neurons in the DRLW compared to the MS0 group (Figure 6). In addition, in the DRV the number of 5-HT-ir neurons were found to be significantly affected by 5-HTT genotype ($F_{2,35} = 3.52, p < 0.05$). Post-hoc analysis indicated that 5-HTT -/- rats exhibit a significantly higher number of 5-HT-ir neurons in the DRV compared to 5-HTT+/- and 5-HTT+/+ rats (both $p < 0.05$) (Figure 6). For the DRD, no effect of ELS, 5-HTT genotype or their interaction was found regarding the number of 5-HT-ir neurons (Figure 6). The SSD of 5-HT-ir neurons in all three subdivisions was not found to be significantly affected by ELS, 5-HTT genotype or their interaction (Figure 6).

Furthermore, the number of juxtaposed Ucn1-ir fibers and 5-HT-ir neurons in the studied subregions of the DR was also not found to be affected by ELS, 5-HTT genotype or their interaction (Figure 6).

**Gene expression in the DR**

As for the EWCP, the mRNA levels of Ucn1, S-HTT, CRF1R, CRF2R and GR were assessed in punches of the DR (Figure 7). Furthermore, we took the opportunity to include an analysis of Slc6a4 expression to validate the impact of heterozygous and homozygous 5-HTT knockout on 5-HTT mRNA levels. Indeed, we found that 5-HTT mRNA levels in the DR were affected by 5-HTT genotype ($F_{2,35} = 23.43, p = 0.000$), with highly significant differences between S-HTT-/- rats and the other genotypes; S-HTT+/- and S-HTT+/+ rats ($p = 0.000$). Furthermore, as expected, S-HTT-/- rats showed a significant, approximate 50% reduction in DR S-HTT mRNA levels compared to S-HTT+/+ rats ($p < 0.01$) (Figure 7).
Figure 6  Dorsal raphe (DR) nucleus: immunohistochemistry of serotonin (5-HT, A) and urocortin 1 (Ucn1) in the dorsal (DRD) and ventral (DRV) parts, as well as the lateral wings (LW) of the DR. The number (#) and specific staining density (SSD) of 5-HT-ir neurons were quantified in the different subdivisions of the DR for serotonin transporter (5-HTT) homozygous knockout (5-HTT-/-), heterozygous knockout (5-HTT+/-) and wild-type (5-HTT+/+) rats exposed to daily 3 h separations (MS180) or a control treatment (MS0). In the LW, the number of 5-HT-ir neurons was found to be increased in the MS180 compared to the MS0 group (E: p < 0.05). In the DRV, the number of 5-HT-ir neurons was found to be significantly higher in 5-HTT-/- rats compared to 5-HTT+/- and 5-HTT+/+ rats (G: p < 0.05). In addition, overlay images were created to quantify the number Ucn1-ir fibers that were juxtaposed (B) or co-localized (C) with 5-HT-ir neurons, which was not found to be significantly affected. Scale bar = 150 µm.

Figure 7  Dorsal raphe nucleus: mRNA levels of serotonin transporter (5-HTT), urocortin 1 (Ucn1), corticotropin-releasing factor receptor 1 (CRF1R) and 2 (CRF2R), serotonin receptor 1A (5-HT1A) and the glucocorticoid receptor (GR). Quantitative real-time PCR was used to analyze these mRNA levels in serotonin transporter homozygous knockout (5-HTT-/-), heterozygous knockout (5-HTT+/-) and wild-type (5-HTT+/+) rats exposed to daily 3 h separations (MS180) or a control treatment (MS0). 5-HTT mRNA levels were found to be significantly different between 5-HTT-/-, 5-HTT+/- and 5-HTT+/+ rats (G: p < 0.01). Data were normalized to the average of the MS0-5-HTT+/+ group.
Although no other significant effects were found of ELS, 5-HTT genotype or their interaction on gene expression in the DR, there were a number of correlations that remained significant after Bonferroni correction for multiple comparisons. Interestingly, DR Ucn1 mRNA levels showed highly significant negative correlations with the mRNA levels of both 5-HT1A ($r_s = -0.60, p = 0.000$) and CRF2R ($r_s = -0.78, p = 0.000$). As would consequently be expected, the mRNA levels of 5-HT1A and CRF2R showed a highly significant positive correlation ($r_s = 0.72, p = 0.000$) (Figure 8).

**Discussion**

In this study, ELS x 5-HTT genotype interaction was not found to significantly affect the EWcp-Ucn1 and DR-S-HT populations in terms of neuronal expression or reciprocal innervation. Yet, DNA methylation of the Ucn promoter region in the EWcp was found to be altered at specific CpG sites by main effects of ELS and 5-HTT genotype. Furthermore, exposure to ELS was associated with increased EWcp 5-HT1A mRNA levels and an increased number of 5-HT neurons in the DRLW. In addition, 5-HTT$^{+/+}$ rats showed significantly decreased CRF1R mRNA levels in the EWcp, while 5-HTT$^{-/-}$ rats displayed a significant reduction of 5-HT innervation of EWcp-Ucn1 neurons, as well as an increased number of 5-HT neurons in the DRV. Altogether, we identified a number of potentially interesting effects of ELS exposure and 5-HTT genotype at the molecular level, which are hypothesized to contribute to ELS x 5-HTTLPR behavioural findings, as discussed below. In addition, we obtained correlational evidence suggesting the importance of 1) balanced CRF1R/GR signaling in the EWcp and 2) Ucn1 in the modulation of 5-HT neuron activity, via the balanced expression of 5-HT1A and CRF2R in the DR.

**EWcp-Ucn1 neurons**

In the EWcp, DNA methylation of the Ucn promoter region was found to be increased by ELS exposure at CpG$_{-156}$ and CpG$_{-49}$ and by 5-HTT deficiency at CpG$_{-171}$, while 5-HTT$^{+/+}$ rats exclusively showed an absence of CpG$_{-24}$ methylation. Remarkably, DNA methylation of the Ucn promoter region overall was very low, in stark contrast with the high methylation that is observed in the Crf promoter region in the PVN and the extended amygdala (Elliot et al., 2010; Sterrenburg et al., 2011, Van der Doelen et al., 2014b). It is tempting to speculate that these strikingly different promoter methylation patterns, together with the complementary dynamics of PVN-CRF and EWcp-Ucn1 neurons, could be related to their proposed roles in controlling the stress response (Kozicz et al., 2011a; Janssen and Kozicz, 2013). It should be noted that the Ucn promotor methylation differences did not translate into altered expression levels of Ucn1 (mRNA, protein) in the EWcp. Further (in vitro) studies are therefore needed to directly assess the functionality of DNA methylation of the Ucn promotor region. Previously, in line with the current study,
maternal separation (MS) of Wistar 5-HTT+/- rats was shown not to lead to alterations in Ucn1 expression in the EWcp (Gaszner et al., 2009). In contrast to our study, 5-HTT-/- mice have been shown to display decreased Ucn1 expression in the EWcp (Fabre et al., 2011). Possibly, this discrepancy could indicate a species difference for the consequences of 5-HTT knockout, which is for instance also known for the sensitivity for MDMA (3,4-methylenedioxymethamphetamine or ‘ecstasy’) (Trigo et al., 2007; Oakly et al., 2014).

Furthermore, we observed that 5-HTT-/- rats displayed significantly reduced co-localization (juxtaposition) of 5-HT fibers and Ucn1 neurons and decreased overall 5-HT innervation of the EWcp, consistent with the reduced 5-HT tissue levels across the brain of 5-HTT-/- mice and rats (Bengel et al., 1998; Kim et al., 2005; Homberg et al., 2007). At this point, it is not yet known how 5-HT contributes to the regulation of EWcp-Ucn1 neurons. The actions of 5-HT in the EWcp could be mediated by one of the many 5-HT receptors (Hoyer et al., 2002). One of them is likely the 5-HT1A receptor, which we could reliably detect with real-time PCR in the EWcp. As 5-HT1A couples to inhibitory Gαi proteins (Barnes and Sharp, 1999; Albert et al., 2014), stimulation of 5-HT1A would be expected to inhibit the activity of EWcp-Ucn1 neurons. Previously, we found that ELS exposure was associated with reduced stress-induced activation (Cfos expression) of Ucn1 neurons in the EWcp (Gaszner et al., 2009). Interestingly, the present study shows that ELS exposure was associated with increased 5-HT1A mRNA levels in the EWcp. Therefore, increased 5-HT1A expression could be involved in the ELS-induced decreased recruitment of EWcp-Ucn1 neurons by acute stress (Gaszner et al., 2009). Together, the decreased 5-HT innervation of EWcp-Ucn1 neurons displayed by 5-HTT-/- rats and the increased 5-HT1A expression in the EWcp of ELS-exposed rats, could constitute an ELS x 5-HTT genotype interaction at the level of stress-induced activity of EWcp-Ucn1 neurons. We hypothesize that 5-HTT-/- rats and 5-HTT+/- rats display decreased activity of EWcp-Ucn1 neurons (increased 5-HT1A expression) upon acute stress exposure, while this effect of ELS exposure is diminished or absent in 5-HTT+/- rats (increased 5-HT1A expression, but decreased 5-HT innervation).

Next to Ucn1 and 5-HT1A, the expression of CRF, β, CRFR1, and GR was examined at the mRNA level in the EWcp. We found a highly significant correlation between CRF, β mRNA and GR mRNA levels, indicative for the importance of balanced CRF, β/GR signal transduction in EWcp-Ucn1 neurons. This is not surprising given the complementary homeostatic functions in which these receptors are involved. While CRF, β activation is central to the initiation of HPA activity and the behavioural fight-flight response, GR activation is crucial for the termination of HPA responses and the facilitation of recovery from the stress response (De Kloet et al., 2005; Joëls and Baram, 2009). In 5-HTT-/- rats, we found that EWcp CRF, β mRNA levels were significantly decreased. Stimulation of CRF, β leads to the activation of CAMP response element binding protein (Arzt and Holsboer, 2006), which has the capacity to stimulate Ucn1 expression via its binding (CRE) site in the Ucn gene. Therefore, the decreased expression of CRF, β in 5-HTT-/- rats might compromise CRF-induced Ucn1 expression in the EWcp, which could lead to an imbalance in the actions of CRF and Ucn1 during responses to stress (Kozić et al., 2011a). As we did not find a significant correlation of CRF, β and Ucn1 mRNA levels in the EWcp, the decreased CRF, β mRNA levels in 5-HTT-/- rats might only have functional consequences when the animals would be exposed to additional stress later in life (De Kloet, 2008; Gaszner et al., 2009). In this regard, the altered DNA methylation of the Ucn promoter could modulate stress-induced Ucn1 expression.

**DR-5-HT neurons**

In the DR, we found that ELS exposure was associated with an increased number of 5-HT neurons in the DRLW, while 5-HTT-/- rats displayed an increased number of 5-HT neurons specifically in the DRV. Interestingly, based on a collection of evidence, it has recently been proposed that DRLW-5-HT neurons have an inhibitory influence on the 5-HT projection neurons in the DRD and DRV (Jasinska et al., 2012). Therefore, the ELS-induced increase in DRLW-5-HT neurons is expected to increase inhibitory influence on 5-HT projection neurons. The inhibition of 5-HT release in DR projection areas has been linked with active coping behaviour and resilience to learned helplessness. (LH) (Valentino et al., 2010; Hammack et al., 2012; Warden et al., 2012). We have previously applied a LH paradigm to our ELS x 5-HTT genotype model. The LH paradigm induces an escape deficit (passive coping) in vulnerable rats by pre-exposure to inescapable shock stress. We found that exposure to ELS was associated with increased expression of active coping behaviour and thus resilience to inescapable stress (Van der Doelen et al., 2013). These behavioural findings support the recently postulated match/mismatch hypothesis, which states that exposure to ELS is not necessarily pathological, but can be used to adaptively respond to future stress exposure (Gluckman et al., 2007; Champagne et al., 2009; Nederhof and Schmidt, 2012). Importantly, it remains to be elucidated which specific combinations of early and later life stressful events have adaptive and maladaptive consequences. Here, we suggest that the ELS-induced increase in DRLW-5-HT neurons may contribute to the ELS-induced increase in active coping behaviour in the LH paradigm (Van der Doelen et al., 2013).

In 5-HTT-/- rats, we found an increased number of 5-HT neurons in the DR compared to both 5-HTT-/- and 5-HTT+/- rats. This molecular phenotype would be consistent with a behavioural phenotype of increased passive coping behaviour (Jasinska et al., 2012). Indeed, 5-HTT-/- mice and rats have been shown to express higher levels of passive coping behaviour in inescapable contexts, by displaying increased immobility in the forced swim test, as well as decreased exploration in tests for anxiety-like behaviour as the elevated plus maze and the open field (Holmes et al., 2002, 2003a; Lira et al., 2003; Olivier et al., 2008a). Yet, despite previous exposure to inescapable shocks, we have observed that 5-HTT-/- rats show higher levels of active coping behaviour in a context where stress has become escapable (Van der Doelen et al., 2013). Overall, these behavioural findings are
consistent with the hypervigilant phenotype of 5-HTT<sup>−/−</sup> rodents, as they display increased sensitivity for environmental factors (Homborg and Van den Hove, 2012). Further studies are needed to address how the role of the subdivisions of the DR contribute to stress coping behaviour with differential 5-HTT availability. Previously, the number of 5-HT neurons was found to be decreased in the DR of 5-HTT<sup>−/−</sup> mice (Liu et al., 2003) and unaltered in 5-HTT<sup>+/−</sup> rats (Olivier et al., 2008a). These studies did not distinguish the different subdivisions of the DR, which may explain the novelty of our present findings.

In addition, we have examined the mRNA levels of 5-HTT, 5-HT<sub>1A</sub>, GR, CRF<sub>1R</sub> and CRF<sub>2R</sub> in micropunches of the DR. We confirmed the approximate 50% reduction of 5-HTT expression in 5-HTT<sup>−/−</sup> rats (Homborg et al., 2007). The mRNA levels of 5-HT<sub>1A</sub>, GR, CRF<sub>1R</sub> and CRF<sub>2R</sub> were however not found to be significantly affected by 5-HTT genotype, nor by ELS exposure or ELS x 5-HTT genotype interaction. In Sprague-Dawley rats, ELS exposure has been found to lead to increased CRF<sub>1R</sub> and decreased S-HT<sub>1A</sub>, 5-HT<sub>1B</sub>, and CRF<sub>2R</sub> mRNA levels in the DR (Lee et al., 2007; Bravo et al., 2011, 2014). In Long-Evans and Lister-Hooded rats however, 5-HT<sub>1A</sub> mRNA was not affected by ELS exposure (Neumaier et al., 2002; Gartside et al., 2003; Arborelius et al., 2004), while 5-HT expression was reported to be unaltered or increased in the DR of Long-Evans rats (Arborelius et al., 2004; Gardner et al., 2009). It would be interesting to elucidate whether genomic differences between these rat strains may explain the differential effects of ELS exposure on gene expression in the DR.

In depressed subjects, exposure to childhood abuse has been associated with decreased 5-HTT binding in the midbrain (positron emission tomography) (Miller et al., 2009), and adolescent rhesus monkeys exposed to ELS have been shown to display decreased 5-HTT binding across the brain, including the raphe (Ichise et al., 2006). In contrast, 5-HT<sub>1A</sub> binding in the raphe was not found to be affected by ELS exposure in juvenile rhesus monkeys (Spinelli et al., 2010). Furthermore, depressed subjects have been reported to exhibit both elevated and reduced 5-HT<sub>1A</sub> in vivo binding in the DR (Drevets et al., 1999; Parsey et al., 2006b), while postmortem binding studies suggest that this discrepancy might be explained by considering the rostrocaudal extent of the DR (Baldini et al., 2008). A limitation of our current study is therefore that we have only collected micropunches of the mid-DR (approximately 7 to 8 mm caudal to Bregma) and could therefore not assess gene expression along the rostrocaudal axis of the DR.

An interesting observation is that both 5-HT<sub>1A</sub> and CRF<sub>2R</sub> mRNA levels in the DR were found to both show highly significant negative correlations with Ucn1 mRNA levels. These correlations suggest that locally translated Ucn1 might contribute to the regulation of 5-HT<sub>1A</sub> and CRF<sub>2R</sub> expression. Both receptors are expressed on 5-HT neurons (Day et al., 2004; Kyasova et al., 2013), and the activation of 5-HT<sub>1A</sub> in the DR is associated with auto-inhibition of 5-HT neurons, while stimulation of CRF<sub>2R</sub> is associated with excitation of 5-HT neurons in the DR (Valentino et al., 2010; Albert et al., 2014). Furthermore, CRF has only low affinity for CRF<sub>2R</sub> in contrast to the urocortins, which have therefore been proposed to be the endogenous ligands for CRF<sub>2R</sub> (Vaughan et al., 1995; Hsu and Hsueh, 2001; Lewis et al., 2001; Reyes et al., 2001; Kozicz, 2010). Interestingly, double knockout of Ucn1 and 2 was found to be associated with reduced anxiety-like behaviour and altered 5-HT activity in DR projection areas (Neufeld-Cohen et al., 2010). The current findings suggest that Ucn1 could be involved in the homeostatic regulation of 5-HT neurons by balancing the expression of 5-HT<sub>1A</sub> and CRF<sub>2R</sub>. Possibly, this effect is mediated directly by CRF<sub>2R</sub> activation, as direct protein-protein interactions have been shown to underlie the altered expression of 5-HT<sub>1A</sub> receptors after CRF<sub>2R</sub> stimulation in the medial prefrontal cortex (Magalhaes et al., 2010). It should be noted that Ucn1 mRNA levels and Ucn1 innervation (juxtaposition) of DR 5-HT neurons were not found to be affected in our ELS x 5-HTT genotype model. Possibly, as mentioned above, differential Ucn1 expression might only become apparent after exposure to a ‘third hit’, a stressful event in adolescence or adulthood (Daskalakis et al., 2013).

**Conclusions**

In conclusion, we report here that exposure to ELS is associated with increased 5-HT<sub>1A</sub> expression in the EWcp and an increased number of 5-HT neurons in specifically the DRLW. Furthermore, 5-HTT deficiency was found to be associated with decreased CRF<sub>2R</sub> expression in the EWcp and an increased number of 5-HT neurons in the DRV. Moreover, both ELS exposure and 5-HTT genotype had an impact on DNA methylation of the Ucn promoter in the EWcp, which potentially affects stress-induced expression of Ucn1. In addition, we found correlative evidence suggestive of the importance of balanced CRF<sub>1R</sub>/GR signaling in the EWcp and a role for Ucn1 in the regulation of DR 5-HT neuron activity. Together, these effects of ELS and 5-HTT genotype at the molecular level could constitute ELS x 5-HTT genotype interaction effects at the level of stress-induced activity of EWcp-Ucn1 and DR-5-HT neurons, which are linked to behavioural changes (Van der Doelen et al., 2013) that are relevant for human ELS x 5-HTTLPR effects at the epidemiological level (Caspi et al., 2003; Karg et al., 2011). Our results however foremost warrant further experimentation in order to elucidate the links between DNA methylation, gene expression, EWcp-DR functional connectivity and stress coping behaviour.

**Acknowledgements**

We thank Anthonieke Middelman for her technical assistance.
Exposure to early life stress regulates Bdnf expression in 5-HTT mutant rats in an anatomically selective fashion

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Abstract

Although the causes of psychiatric disorders are not fully understood, it is well established that mental illness originates from the interaction between genetic and environmental factors. In this regard, compelling evidence demonstrates that depression can be the consequence of altered, and often maladaptive, response to adversities during pre- and early post-natal life. In this study, we investigated the impact of chronic maternal separation on the expression of the neurotrophin brain-derived neurotrophic factor (BDNF) in serotonin transporter (S-HTT) knockout rats in the ventral and dorsal hippocampus as well as the ventral and dorsal medial prefrontal cortex (mPFC). We found that both S-HTT deletion and maternal separation led to an overall reduction of Bdnf expression in the ventral hippocampus and the ventral mPFC, whereas in the dorsal hippocampus and the dorsal mPFC we observed a significant increase in the neurotrophin gene expression after MS exposure, specifically in heterozygous S-HTT knockout rats. In summary, we show that the modulation of Bdnf expression in S-HTT mutant rats exposed to maternal separation reflects the complex functional consequences of this gene-environment interaction with a clear distinction between the ventral and the dorsal subfields of the hippocampus and the mPFC.

Introduction

The response to an adverse event is significantly affected by the genetic background, suggesting that gene-environment interaction represents a key feature in psychiatry (Caspi et al., 2010). One of the most investigated human polymorphisms in gene-environment interaction is the serotonin transporter (5-HTT/LINKED polymorphic region (5-HTTLPR), which comes in two variants, the long (L) and the short (S) allele (Lesch et al., 1996; Murphy and Lesch, 2008). Specifically, the S-allelic variant is directly associated with neuroticism (Lesch et al., 1996) and may enhance depression susceptibility following interaction with stressful events (Caspi et al., 2003; Munafo et al., 2009; Karg et al., 2011). Since this polymorphism is not found in rodents, S-HTT mutant animals have been generated in which the function and the expression of the gene has been altered (Caspi et al., 2010). We have shown that adult S-HTT knockout rats, characterized by depression- and anxiety-related behaviours (Olivier et al., 2008a), have reduced levels of neuroplastic molecules in brain regions involved in depression and anxiety-related disorders (Calabrese et al., 2010, 2013; Molteni et al., 2010; Guidotti et al., 2012). In particular, we demonstrated that the reduction of the expression of the neurotrophin brain-derived neurotrophic factor (BDNF) was specific to the ventral part of the hippocampus, whereas no change was observed in the dorsal hippocampus (Calabrese et al., 2013). This distinction is relevant, because the ventral hippocampus is involved in emotion regulation, and the dorsal hippocampus in spatial memory (Fanselow and Dong, 2010). Moreover, the impairment in neuronal plasticity that follows genetic inactivation of 5-HTT originates early in development and worsens during the first 2-3 weeks of postnatal life (Calabrese et al., 2013).

It has now been well-established that the effect of stress exposure on brain function depends on the timing and duration of the adverse experience. In particular, the negative influences exerted by early life events on brain development is relevant to later psychopathology since they will affect brain structures that are not yet mature (Fumagalli et al., 2007). Indeed, humans exposed to maltreatment early in life show an increased risk of developing psychiatric diseases (Heim et al., 2004; Fisher et al., 2014).

Among the animal models developed to study the effects of early perinatal adversities, the maternal separation (MS) model is of interest since it triggers anxiety and depression-related behaviours as well as deficits in cognitive functions in later life (Ladd et al., 2000; Sanchez et al., 2001; Gutman and Nemeroff, 2002; Newport et al., 2002). Furthermore, MS has been demonstrated to be associated with alterations in the functioning of the hypothalamic-pituitary-adrenal (HPA) axis (Weaver et al., 2004; Van der Doelen et al., 2014) and impaired function of neuroplastic mechanisms (Fumagalli et al., 2007; Lippmann et al., 2007; Calabrese et al., 2011). In this regard, previous studies conducted in our lab have demonstrated that single or repeated MS produces a significant reduction in the expression of BDNF in adulthood (Roceri et al., 2002, 2004).
On these bases, the purpose of this study was to investigate the impact of chronic maternal separation on the expression of Bdnf in the ventral and dorsal parts of the hippocampus, as well as the medial prefrontal cortex (mPFC) of animals with partial or total deletion of the 5-HTT gene. We aimed to evaluate if the anatomical selectivity observed under basal conditions is found also after stress exposure, or whether the differential basal deficits in neuroplasticity influence the response to an early life manipulation.

Materials and Methods

Animals
Serotonin transporter knockout Wistar rats (Slc6a41Hubr) were generated by ENU-induced mutagenesis (N-ethyl-N-nitrosourea) (Smits et al., 2006). Briefly, high-throughput resequencing of genomic target sequences in progeny from mutagenized rats revealed an ENU-induced premature stop codon in exon 3 of the 5-HTT gene in a female rat (Wistar/Crl background). The heterozygous mutant animal was outcrossed for at least six generations to a Wistar background to eliminate confounding effects from other mutations that may have been induced by the ENU mutagenesis. Under the used mutagenesis conditions the mean mutation frequency was roughly 1 in 1.2 million base pairs (about 1 cM). Although the chance for the occurrence of a strongly linked mutation with a phenotypic effect is very small, this possibility should be taken into account in the design and interpretation of experiments. To control for this possibility as much as possible, experimental animals were always generated by incrosses between outcrossed heterozygous 5-HTT knockout (5-HTT+/−) rats (Homberg et al., 2007). All subjects were bred and reared in the Central Animal Laboratory of the Radboud University Nijmegen Medical Centre in Nijmegen, The Netherlands. At the age of 14 days, ear cuts were taken for genotyping. Animals were supplied with food and water ad libitum and were kept on a 12 h: 12 h dark–light cycle (lights on at 6:00 a.m.).

For the collection of brain tissue, adult (postnatal day (PND) 85–95) male rats were used. Of every litter, where possible, a single rat was selected of all three genotypes. The rats were sacrificed between 9.00 a.m. and 2.00 p.m. by acute decapitation. Across this time period, the rats were randomized for their genotype and early life treatment. Immediately after decapitation, the brains were isolated, frozen in aluminum foil on dry ice and stored at −80 °C. In a cryostat (−15 °C), the brains were prepared in 420 μm-thick coronal slices to obtain punches from dorsal and ventral parts (prelimbic, infralimbic respectively) of the mPFC (Bregma +3.72 and +3.30 mm) and dorsal (Bregma −2.14 and −2.56 mm) and ventral hippocampus (Bregma −4.80 and −5.22 mm). The brain areas were bilaterally punched out with a Miltex 1.5 (hippocampal samples) or 1.0 mm (medial prefrontal cortex) biopsy puncher (Integra Miltex, York, PA, USA), collected in sterile vials, immediately placed on dry ice and stored at −80 °C.

RNA preparation and gene expression analysis by quantitative real time RT-PCR
Total RNA was isolated by a single step of guanidinium isothiocyanate/phenol extraction using PureZol RNA isolation reagent (Bio-Rad Laboratories s.r.l. Italia) according with the manufacturer’s instructions, and quantified by spectrophotometric analysis. Following total RNA extraction, the samples were processed for real time retrotranscriptase polymerase chain reaction (RT-PCR) to assess: total Bdnf long 3′-UTR Bdnf and Bdnf exon IV, and VI mRNA levels. An aliquot of each sample was treated with DNase to avoid DNA contamination. RNA was analyzed by TaqMan qRT-PCR instrument (CFX384 real time system, Bio-Rad Laboratories, Italy) using the iScriptTM one-step RT-PCR kit for probes (Bio-Rad Laboratories). Samples were run in 384 well formats in triplicate as multiplexed reactions with a normalizing internal control (36B4). Probe and primer sequences used (Table 1) were purchased from Eurofins-MWG–Operon (Germany) and Life Technologies.

Brain tissue collection
For the collection of brain tissue, adult (postnatal day (PND) 85–95) male rats were used. Of every litter, where possible, a single rat was selected of all three genotypes. The rats were sacrificed between 9.00 a.m. and 2.00 p.m. by acute decapitation. Across this time period, the rats were randomized for their genotype and early life treatment. Immediately after decapitation, the brains were isolated, frozen in aluminum foil on dry ice and stored at −80 °C. In a cryostat (−15 °C), the brains were prepared in 420 μm-thick coronal slices to obtain punches from dorsal and ventral parts (prelimbic, infralimbic respectively) of the mPFC (Bregma +3.72 and +3.30 mm) and dorsal (Bregma −2.14 and −2.56 mm) and ventral hippocampus (Bregma −4.80 and −5.22 mm). The brain areas were bilaterally punched out with a Miltex 1.5 (hippocampal samples) or 1.0 mm (medial prefrontal cortex) biopsy puncher (Integra Miltex, York, PA, USA), collected in sterile vials, immediately placed on dry ice and stored at −80 °C.

Thermal cycling was initiated with an incubation at 50°C for 10 min (RNA retrotranscription) and then at 95°C for 5 min (TaqMan polymerase activation). After this initial step, 39 cycles of PCR were performed. Each PCR cycle consisted of heating the samples at 95°C for 10 s to enable the melting process and then for 30 s at 60°C for the annealing and extension reactions. A comparative cycle threshold (Ct) method was used to calculate the relative target gene expression.

Statistical analysis
The effects of 5-HTT genotype and maternal separation were analyzed with a two-way analysis of variance (ANOVA) followed by Single Contrast Post Hoc Testing (SCPHT). Significance for all tests was assumed at p < 0.05. Data are presented as means ± standard error of the mean (SEM). For graphic clarity, results are presented as mean percent of 5-HTT+/−/No Stress rats.
In the ventral mPFC (Figure 1C), a region structurally and functionally linked to the ventral hippocampus, we found a significant modulation due to 5-HTT genotype ($F_{1,36} = 3.29, p < 0.05$) as well as exposure to MS ($F_{1,36} = 6.48, p < 0.05$). Indeed, total Bdnf expression was decreased in 5-HTT+/- (-39%, $p < 0.01$) and 5-HTT-/- rats (-33%, $p < 0.05$), compared to 5-HTT+/+ rats under control conditions. Following exposure to MS, total Bdnf mRNA levels were significantly reduced in 5-HTT+/+ animals (-46%, $p < 0.001$ vs 5-HTT+/+No Stress), whereas the decreased neurotrophin expression in 5-HTT+/- and 5-HTT-/- animals was not exacerbated by stress exposure.

Results

Modulation of total Bdnf expression in 5-HTT (SERT) mutant rats after exposure to maternal separation

We initially investigated total Bdnf mRNA levels in the hippocampus and mPFC (Figure 1) of 5-HTT+/-, 5-HTT+/- and 5-HTT-/- rats exposed (or not) to MS from PND2 to PND14.

In the ventral part of the hippocampus, which is involved in anxiety, fear and stress responses (Figure 1A), the total Bdnf mRNA levels were modulated by the genotype ($F_{1,33} = 4.91, p < 0.05$) as well as by the stress exposure ($F_{1,33} = 11.22, p < 0.01$). Indeed, the single contrast post hoc test revealed a statistically significant reduction of total Bdnf levels in 5-HTT-/- (-43%, $p < 0.05$ vs 5-HTT+/+No Stress). Similarly, exposure to MS significantly decreased Bdnf expression in 5-HTT+/- (-43%, $p < 0.05$ vs 5-HTT+/-/No Stress) as well as in 5-HTT+/- (-44%, $p < 0.05$ vs 5-HTT+/-/No Stress) animals. However, this did not occur in 5-HTT-/- rats since the reduction due to the genetic background was not exacerbated by the manipulation early in life.

Conversely, in the dorsal part of the hippocampus, which is mainly involved in spatial learning and memory (Figure 1B), we observed a significant effect of exposure to MS ($F_{1,37} = 8.96, p < 0.01$), whereas 5-HTT genotype did not affect the expression of the neurotrophin ($F_{1,37} = 2.19, p > 0.05$). Indeed, the exposure to MS led to an increase in total Bdnf gene expression in 5-HTT+/- animals (64%, $p < 0.05$ vs 5-HTT+/-/No Stress), without affecting its mRNA levels in 5-HTT+/- rats, nor significantly in 5-HTT-/- rats.

In the ventral mPFC (Figure 1C), a region structurally and functionally linked to the ventral hippocampus, we found a significant modulation due to 5-HTT genotype ($F_{1,36} = 3.29, p < 0.05$) as well as exposure to MS ($F_{1,36} = 6.48, p < 0.05$). Indeed, total Bdnf expression was decreased in 5-HTT+/- (-39%, $p < 0.01$) and 5-HTT-/- rats (-33%, $p < 0.05$), compared to 5-HTT+/- rats under control conditions. Following exposure to MS, total Bdnf mRNA levels were significantly reduced in 5-HTT+/- rats (-46%, $p < 0.001$ vs 5-HTT+/-/No Stress), whereas the decreased neurotrophin expression in 5-HTT+/- and 5-HTT-/- animals was not exacerbated by stress exposure.

Figure 1  Modulation of total Bdnf expression by maternal separation in serotonin transporter (5-HTT/SERT) wild-type (SERT+/+), heterozygous (SERT+/-) and homozygous (SERT-/-) knockout rats. Total Bdnf mRNA levels were measured in the ventral hippocampus (A), dorsal hippocampus (B), ventromedial prefrontal cortex (C) and dorsomedial prefrontal cortex (D). The data, expressed as a percentage of SERT+/+No Stress (set at 100%), reflect the mean ± SEM from at least 5 independent determinations. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs SERT+/-/No Stress; #$p < 0.05$ vs SERT-/-/No Stress.

Table 1  Sequences of forward and reverse primers used in Real-Time PCR analysis and purchased from Eurofins MWG-Operon (Germany).
Similarly to what was observed in the dorsal part of the hippocampus, in the dorsal mPFC (Figure 1D) we found a significant effect only of MS exposure ($F_{(1,19)} = 3.15, p < 0.05$). In this brain region, total Bdnf mRNA levels were up-regulated by the early life stress procedure specifically in 5-HTT+/+ animals (+33%, $p < 0.05$ vs 5-HTT+/+No Stress).

**Modulation of long 3'-UTR Bdnf expression in 5-HTT(+/SERT) mutant rats after exposure to maternal separation**

In the ventral hippocampus (Figure 2A), the levels of long 3'-UTR Bdnf mRNAs were significantly affected by MS ($F_{(1,19)} = 11.46, p < 0.01$) but not by 5-HTT genotype ($F_{(1,19)} = 2.8$, $p > 0.05$). It was found that MS only significantly decreased long 3'-UTR Bdnf mRNA levels in 5-HTT+/+ animals (-36%, $p < 0.01$ vs 5-HTT+/+No Stress). In contrast, in the dorsal hippocampus (Figure 2B), the expression of long 3'-UTR Bdnf was specifically increased by MS ($F_{(1,31)} = 7.22, p < 0.05$) in both 5-HTT+/+ (+30%, $p < 0.05$ vs 5-HTT+/+No Stress) and in 5-HTT-/- animals (+44%, $p < 0.05$ vs 5-HTT-/No Stress).

In the ventral mPFC (Figure 2C), long 3'-UTR Bdnf mRNA levels were significantly reduced in 5-HTT-/- animals under control conditions (-40%, $p < 0.05$ vs 5-HTT+/+No Stress), whereas MS decreased long 3'-UTR Bdnf mRNA levels only in 5-HTT+/+ rats (-29%, $p < 0.05$ vs 5-HTT+/+No Stress). Differently, in the dorsal mPFC (Figure 2D) the expression of the Bdnf mRNA levels were measured in the ventral hippocampus (A), dorsal hippocampus (B), ventromedial prefrontal cortex (C) and dorsomedial prefrontal cortex (D). The data, expressed as a percentage of SERT+/+No Stress (set at 100%), reflect the mean ± SEM from at least 5 independent determinations. *$p < 0.05$, vs SERT+/+No Stress; **$p < 0.01$, ##p < 0.001 vs SERT+/+No Stress; $\#p < 0.05$, ###p < 0.001 vs SERT-/No Stress.

**Figure 2** Modulation of long 3'-UTR Bdnf expression by maternal separation in serotonin transporter (5-HTT/SERT) wild-type (SERT+/+), heterozygous (SERT+/-) and homozygous (SERT-) knockout rats. Long 3'-UTR Bdnf mRNA levels were measured in the ventral hippocampus (A), dorsal hippocampus (B), ventromedial prefrontal cortex (C) and dorsomedial prefrontal cortex (D). The data, expressed as a percentage of SERT+/+No Stress (set at 100%), reflect the mean ± SEM from at least 5 independent determinations. *$p < 0.05$, vs SERT+/+No Stress; **$p < 0.01$, ##p < 0.001 vs SERT+/+No Stress; $\#p < 0.05$, ###p < 0.001 vs SERT-/No Stress.

**Figure 3** Modulation of Bdnf exon IV expression by maternal separation in serotonin transporter (5-HTT/SERT) wild-type (SERT+/+), heterozygous (SERT+/-) and homozygous (SERT-) knockout rats. Bdnf exon IV mRNA levels were measured in the ventral hippocampus (A), dorsal hippocampus (B), ventromedial prefrontal cortex (C) and dorsomedial prefrontal cortex (D). The data, expressed as a percentage of SERT+/+No Stress (set at 100%), reflect the mean ± SEM from at least 5 independent determinations. *$p < 0.05$, **p < 0.01, ***p < 0.001 vs SERT+/+No Stress; $\#p < 0.05$, ###p < 0.001 vs SERT-/No Stress.
long 3'-UTR Bdnf transcript was up-regulated significantly only in 5-HTT+/- rats (+33%, p < 0.05 vs 5-HTT+/+/No Stress) under control conditions, while exposure to MD was without effect.

Modulation of Bdnf exon IV and VI expression in 5-HTT(SERT) mutant rats after exposure to maternal separation

Next we investigated the expression levels of the main neurotrophin transcripts, namely exon IV (Figure 3) and VI (Figure 4). In the ventral hippocampus (Figure 3A) the expression levels of exon IV were influenced by 5-HTT genotype (F(1,35) = 6.18, p < 0.01) as well as by MS (F(1,35) = 11.00, p < 0.01). Indeed, we observed a significant reduction of exon IV mRNA levels in 5-HTT-/- animals under control conditions (-33%, p < 0.01 vs 5-HTT+/+/No Stress), while MS reduced its expression only significantly in 5-HTT-/- rats (-31%, p < 0.05 vs 5-HTT+/+/No Stress). Differently, in the dorsal part of the hippocampus (Figure 3B) we observed only an effect of 5-HTT genotype (F(1,34) = 5.02, p < 0.05) with an up-regulation of exon IV gene expression in 5-HTT+/-/No Stress rats (+38%, p = 0.05 vs 5-HTT+/+/No Stress).

In the ventral mPFC (Figure 3C), we found a significant effect of 5-HTT genotype (F(1,38) = 13.60, p < 0.001). Indeed, exon IV mRNA levels were significantly reduced in 5-HTT+/- and 5-HTT-/- rats compared to 5-HTT+/+ rats under control conditions (-47%, p < 0.001; -30%, p < 0.01, respectively). Moreover, MS influenced the expression of this transcript in 5-HTT+/- rats (-25%, p < 0.05 vs 5-HTT+/+/No Stress), without increasing the reduction already present in mutant animals.

Exposure to MS significantly influenced the expression of exon IV also in the dorsal mPFC (Figure 3D) (F(1,37) = 7.57, p < 0.01). Here, the levels of exon IV were significantly reduced in 5-HTT+/+ rats (-30%, p < 0.05 vs 5-HTT+/+/No Stress) while an increase was detected in 5-HTT+/- rats (+43%, p < 0.01 vs 5-HTT+/+/No Stress).

For exon VI, we found that MS exposure (F(1,36) = 5.79, p < 0.05) only significantly decreased its expression in the ventral hippocampus (Figure 4A) of 5-HTT+/- rats (-32%, p < 0.01 vs 5-HTT+/+/No Stress). In the dorsal part of the hippocampus (Figure 4B), both 5-HTT genotype (F(1,36) = 3.41, p < 0.05) and exposure to MS significantly influenced exon VI mRNA levels (F(1,36) = 3.97, p < 0.05). Indeed, the levels of exon VI were significantly reduced in the 5-HTT+/- rats (-40%, p < 0.01 vs 5-HTT+/+/No Stress) under control conditions, whereas MS was associated with increased expression in these rats only (+90%, p < 0.01 vs 5-HTT+/+/No Stress).

In the ventral mPFC (Figure 4C) we found a significant effect of 5-HTT genotype (F(1,37) = 8.03, p < 0.01). Indeed, both 5-HTT+/- and 5-HTT-/- animals showed reduced exon VI mRNA levels compared to 5-HTT+/+ rats under control conditions (-45%, p < 0.01; -38%, p < 0.05, respectively). In the dorsal mPFC, no effects of 5-HTT genotype or exposure to MS were observed (Figure 4D).

Discussion

Our results demonstrate that early life stress affects the expression of Bdnf in an anatomically distinct manner as a function of 5-HTT genotype. Indeed, we found brain region specificity with marked differences between the ventral and the dorsal parts of the brain regions considered. Specifically, we found that both 5-HTT deletion and exposure to MS led to an overall reduction of Bdnf expression in the ventral hippocampus and in the ventral mPFC, whereas in the dorsal hippocampus and in the dorsal mPFC we observed a significant increase in the neurotrophin gene expression after MS exposure specifically in 5-HTT+/- rats.

We confirm previous results showing that the total form of Bdnf was reduced in the ventral hippocampus (Calabrese et al., 2013), as well as the ventral mPFC of 5-HTT+/- and 5-HTT-/- rats, compared to 5-HTT+/+ rats under control conditions, with the latter in line with previous observations with the whole prefrontal cortex (Molteni et al., 2010).
In addition to 5-HTT genotype, MS differentially affected Bdnf transcript levels in the ventral vs the dorsal parts of the hippocampus and mPFC. Indeed, in the ventral hippocampus as well as in the ventral mPFC, exposure to MS substantially reduced total Bdnf expression in a manner that corresponds to that exerted by 5-HTT deletion, without any MS x 5-HTT genotype interaction. Conversely, within the dorsal hippocampus as well as in the dorsal mPFC, we found a MS x 5-HTT genotype interaction; Bdnf gene expression was up-regulated selectively in 5-HTT+/- rats after exposure to MS.

Behavioural characterization of the 5-HTT+/- rat model has shown these animals to display anxiety- and depression-like behaviour in the elevated plus maze, open field, forced swim test and sucrose consumption tests (Olivier et al., 2008a). Yet, we have also demonstrated that 5-HTT+/- rats show an improved stress coping response in the learned forced swim test and sucrose consumption tests (Olivier et al., 2008a). These "for better and for worse" phenotypes are in line with the concept of the predictive adaptive response (PAR) (Gluckman et al., 2007). PAR entails that stressful experiences in the past can be useful for responding to a subsequent challenging situation (the performance is optimal when early and later life environment match). Moreover, recent findings have demonstrated that humans and nonhuman primates carrying the 5 variant of the 5-HTTLPR outperform subjects carrying the L allele in a wide array of cognitive tasks (Homberg and Leisch, 2011). It is tempting to speculate that the differential modulation of Bdnf in the dorsal and ventral hippocampus and mPFC underlies the "for better and for worse" phenotypes in 5-HTT mutant rats. Reduced Bdnf levels in the ventral regions due to MS or 5-HTT deficiency could contribute to the anxiety- and depression-related phenotypes ("for worse"). Conversely, increased Bdnf levels in dorsal regions after exposure of 5-HTT+/- rats could exert beneficial effects on cognition and may explain the "for better" phenotype shown by these rats at the behavioural level (Van der Doelen et al., 2013). Indeed, by applying the LH paradigm to MS x 5-HTT genotype experiments, we have previously reported increased expression of adaptive, active coping behaviour. When stratifying for 5-HTT genotype, we found that the adaptive effect of MS exposure was only significant for 5-HTT+/- rats (Van der Doelen et al., 2013). These behavioural findings support the postulated match/mismatch hypothesis, which states that exposure to ELS is not necessarily pathological, but can be used to adaptively respond to future stress exposure (Schmidt, 2011; Homberg, 2012).

The effects observed in 5-HTT mutant rats under control conditions, as well as in 5-HTT+/- rats exposed to MS, confirm our previous results that juvenile and adult 5-HTT mutants rats exhibit a decrease in Bdnf levels in the hippocampus and prefrontal cortex (Molteni et al., 2010; Calabrese et al., 2013), and match the finding that early life stress exposure leads to a reduction in BDNF levels in adult animals (Roceri et al., 2004). Dissecting the brain regions more precisely, we could reveal that early life stress has a specific impact on neuroplasticity in the ventral hippocampus and the ventral mPFC.

Conversely, considering the fact that gene–environment interactions increase the risk for psychiatric disorders, we found that MS did not worsen the neuroplastic alterations due to the putative ‘vulnerable’ 5-HTT genotype in the ventral and dorsal hippocampus and mPFC. This may be due to the fact that the MS paradigm used in our study is already sufficient to produce behavioural (Meaney, 2001; Fumagalli et al., 2007) as well as molecular alterations (reduction of Bdnf in wild-type animals) (Roceri et al., 2004), putatively leading to a floor effect in 5-HTT mutant rats, which would hinder the identification of a gene x environment (GxE) interaction.

Moreover, the reduction of neurotrophin levels in 5-HTT mutant rats occurs already between the first and second weeks of life (Calabrese et al., 2013), suggesting that this period may be critical for the correct development of neuroplastic mechanisms. If unaltered, changes in neurodevelopment may in turn underlie the "for better and for worse" phenotypes in adult 5-HTT mutant rats. Since MS was carried out exactly during this vulnerable developmental time window, it may be inferred that the alteration due to the manipulated genotype was maximal. Hence, any further effect of MS would be overshadowed by the developmental changes in 5-HTT mutant rats.

In an attempt to assess whether the mechanism underlying the modification of Bdnf expression induced by 5-HTT genotype and early life stress are comparable or distinctive, we measured the mRNA levels of the long 3'-UTR pool of transcripts, and of the type IV and VI 5'-exons. The Bdnf gene is characterized by the presence of two different polyadenylation sites at the 3'-UTR (Pruunsild et al., 2011) giving rise to a short and a long transcript form. While the short 3'-UTR mRNAs are restricted to the somata, the long 3'-UTR mRNAs are also localized in dendrites (An et al., 2008), suggesting that they may subserve different physiological roles within the cell (Lau et al., 2010; Waterhouse et al., 2012; Orefice et al., 2013). The effect observed in the dorsal hippocampus on total Bdnf gene expression was paralleled by a similar modulation of the long 3'-UTR Bdnf and exon VI, two subpopulations of Bdnf/mRNA that can be targeted to dendrites (An et al., 2008; Baj et al., 2013). Interestingly, in the ventral part of the hippocampus, a more comparable pattern between total Bdnf and the specific expression of exon IV was found. This would suggest that 5-HTT deletion and MS exposure not only affect the expression of Bdnf in an anatomy-specific manner, but also that the mechanisms through which this modulation is achieved are possibly specific for the considered brain region. Moreover, alterations in different pools of the neurotrophin may be translated in different modulation of downstream pathways. In summary, we show that the modulation of Bdnf expression in 5-HTT mutant rats exposed to MS reflects the complex functional consequences of this GxE interaction with a clear distinction between the ventral and the dorsal subfields of the hippocampus and mPFC. The further characterization of these mechanisms may provide novel cues for modulation neurotrophin function, which is dysregulated in several psychiatric conditions.

Acknowledgments
This publication was made possible by Grant from the Italian Ministry of University and Research to MAR (PRIN 20107MSMA4).
Chapter 8

General Discussion

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The risk to develop depression after exposure to early life stress (ELS) is significantly increased in individuals with the 5-hydroxytryptamine (5-HT) transporter (5-HTT)-linked polymorphic region (5-HTTLPR) S-allele (Caspi et al., 2003; Karg et al., 2011; Sharpley et al., 2014, but also see Risch et al., 2009). In this thesis, we have aimed to come to a further understanding of how this gene x environment (GxE) interaction affects stress coping behaviour (chapter 2), hypothalamic-pituitary-adrenal (HPA)-axis activity (chapter 3) and gene expression of related factors in the brain (chapters 4-7) in adulthood. In chapter 2, we found that rats that experienced repeated maternal separation (MS) during the early postnatal period (rodent model of ELS) do not necessarily develop a phenotype that is reminiscent of maladaptation or disease vulnerability. Rather, we found that ELS increased the degree of active coping behaviour as expressed by decreased escape latencies in the learned helplessness (LH) paradigm. As increased escape performance directly decreases the exposure to foot shocks in the third day of this paradigm, active coping can unequivocally be interpreted as an adaptive behavioural response in this specific context. Moreover, heterozygous 5-HTT knockout (5-HTT+/-) rats seemed to be especially sensitive to the effect of ELS, suggesting that 5-HTTLPR S-allele carriers do not necessarily develop vulnerability to stress-related psychiatric diseases like depression following ELS exposure, but that this is dependent on the congruence between the early and later life environment. In chapter 3, we presented another major finding: we confirmed the hypothesis that ELS and 5-HTT genotype interact to program the basal activity of the adult HPA-axis. Specifically, the data from postmortem gene expression and adrenal in vitro analyses suggest that this ELS x 5-HTT genotype effect is largely programmed at the level of adrenal gene expression, leading to differential adrenocorticotropic hormone (ACTH) sensitivity of the adrenals. In addition, in chapters 4 and 7 we identified a complex change in medial prefrontal cortex (mPFC) and hippocampal mRNA levels of the glucocorticoid receptor (GR), mineralocorticoid receptor (MR), FK506-binding protein 51 (FKBP5), and different transcripts of brain-derived neurotrophic factor (BDNF) in response to ELS and 5-HTT genotype. Finally, we found altered DNA methylation of the promoter regions of the genes encoding corticotropin-releasing factor (CRF) and urocortin 1 (Ucn1) as a function of ELS and 5-HTT genotype (chapter 5 and 6).

1. ELS, 5-HTT genotype and stress coping behaviour

In the literature, a dominant view has been that the exposure to (early life) stress predisposes individuals to increased vulnerability to subsequent periods of stress (cumulative stress hypothesis; CSH), and with that the adaptive value of coping with life’s challenges has been underrecognized (Bonanno and Mancini, 2008; Heim and Binder, 2012). Yet, many individuals show resilience in the face of chronic stress or traumatic events (Feder et al., 2009; Schmidt et al., 2010; Russo et al., 2012) and exposure to moderate, predictable stressors could even be beneficial (Parihar et al., 2011; Suo et al., 2013). Recently, the match/mismatch hypothesis has been advocated, which states that exposure to ELS (and stressors in other phases of life) may not necessarily have negative consequences for the individual. Moreover, this model postulates that the individual may use the experience of past stressors in order to improve his/her adaptive fitness and successfully cope with later life challenges (Champagne et al., 2009; Oitzl et al., 2010; Schmidt, 2011; Homberg, 2012; Nederhof and Schmidt, 2012; Daskalakis et al., 2013). In line with this hypothesis, in chapter 2 we found that ELS-exposed rats showed improved stress coping behaviour, as expressed by decreased escape latencies in a shuttle-box test, despite of exposure to inescapable foot shocks the days before. We do emphasize that replication of our findings (including those presented in the other chapters) is warranted, preferably by other laboratories.

1.1 The match/mismatch hypothesis (MMH)

In addition to our study, an increasing body of experimental data from animal models is present in the literature that is supportive of the MMH (e.g. Abraham and Gruss, 2010; Daskalakis et al., 2012; Ricon et al., 2012; Santarelli et al., 2014; Zalosnik et al., 2014). Experiments by Danielle Champagne, Harm Krugers and colleagues have been an important starting point for the experimental support for the MMH. In these studies, rats that were exposed to ELS were found to show decreased cognitive performance under basal conditions, but superior cognitive performance under stressful conditions, compared to control rats (Champagne et al., 2008; Oomen et al., 2010). This was parallelized with the finding that exposure of hippocampal slices to glucocorticoid (CORT) levels reminiscent of stress impaired long-term potentiation (LTP; synaptic correlate of learning) in control rats, but enhanced LTP in ELS rats (Champagne et al., 2008; Bagot et al., 2009), involving alterations in glutamate neurotransmission in the hippocampus (Bagot et al., 2012a, 2012b). The improved learning performance of ELS-exposed rats in the fear conditioning paradigm (stressful condition) was furthermore found to be associated with increased spontaneous recovery of the memory (Xiong et al., 2014).

Despite the evidence from animal studies, two recent studies with depressive subjects, which directly addressed the match/mismatch as well as the classical cumulative stress hypothesis (CSH), concluded that the observed interaction between stressors across
the life span was more in line with the CSH, instead of the MMH (Power et al., 2013; Vinkers et al., 2014). Yet, it has been proposed that the CSH and MMH models may co-apply in varying degrees, dependent on the individual programming sensitivity and the severity of stressful life events (Nederhof and Schmidt, 2012).

1.2 Context-dependence

In chapter 1, we already introduced a prominent example of experimental studies that support the MMH, the stress inoculation (SI) treatment of squirrel monkeys by David M. Lyons and co-workers. In summary, social separations as juveniles were found to be associated with resilience to a moderate stressor in adulthood (Parker et al., 2004; Lyons and Parker, 2007). The similarity between the juvenile and adult treatment (type and severity of stressors) likely underlies the reduced sensitivity to the adult stressor in the SI-treated monkeys. In the case of our study, it would be interesting to further examine which properties of the MS treatment and the LH paradigm are instrumental for their putative match, as these stressors are not very similar at first sight.

It is increasingly recognized that the outcome of the interaction between the environments of different (early postnatal, juvenile, pubertal, adolescent and adult) developmental time windows is context-dependent (Santarelli et al., 2014). This makes sense, as different types and durations of stressors require different responses of the individual (Myers et al., 2014). Therefore, if the early life environment would not lead to adequate predictive programming of behavioural and physiological coping mechanisms for later life challenges, maladaptation and disease vulnerability could arise if stressors are not efficiently dealt with. The context-dependence of phenotypic programming in response to certain environments has been illustrated by Igor Branchi and colleagues. In their study, mice were reared in a communal nesting (CN) condition, which is regarded as an enrichment of the early environment, or in standard animal facility rearing (AFR) conditions. It was confirmed that CN was associated with social enrichment, as increased interactions with the mother and peers were observed compared to the AFR condition. At adulthood, the mice were exposed for 4 weeks to the forced swim test (FST), a physical stressor, or a social stressor (social instability), and stress-induced anhedonia was assessed. Consistent with the hypothesis of context-dependence, the CN mice showed increased resilience to the social, but not the physical stressor, in comparison to the AFR mice (Branchi et al., 2013). Furthermore, another recent study showed that readouts of stress vulnerability can depend on the behavioural test that is used. In this study, adolescent S-HTT+/− mice and wildtype (S-HTT+/+) mice were exposed to nine sessions of exposure to electric shocks. Anxiety-like behaviour was assessed by re-exposure to the shock context (RESC) or the elevated plus maze (EPM). Significant interactions of adolescent stress (AS) and S-HTT genotype were found for both tests. In the RESC however, AS-S-HTT+/− mice showed increased freezing compared to control-S-HTT+/+ mice, which was not found for S-HTT+/+ mice, while in the EPM, AS was found to selectively decrease anxiety-like behaviour in S-HTT+/− mice (Spinelli et al., 2013). Therefore, the context was found to determine the level of anxiety-like behaviour displayed by S-HTT+/− mice after exposure to AS.

With regard to S-HTTLPR, the results of our study and Spinelli et al. (2013) suggest that carrying the S-allele is not invariably related to increased stress vulnerability. Rather, in the case of matching early and adult life environmental conditions, S-allele carriers may exhibit increased adaptive fitness compared to LL homozygotes. The increased sensitivity of S-HTT+/− rodents to benefit from a positive, adaptive match between the early and adult life environment is also in support of the differential susceptibility hypothesis (DSH) (Belsky et al., 2009; Ellis et al., 2011). The DSH suggests that common ‘vulnerability’ genes do not only confer increased sensitivity to adverse, but also positive environmental events. As such, the DSH provides a theoretical framework for the evolutionary survival for these ‘plasticity’ genes (Belsky et al., 2007; Ellis et al., 2011; Bakermans-Kranenburg and Van IJzendoorn, 2011).

2. Learned helplessness; uncontrollable vs inescapable stress

The labs of Steven Maier, Martin Seligman and Linda Watkins have shown that the potency of electric shock stress to affect subsequent escape behaviour - but also social interaction, fear conditioning and anxiety-like behaviour - involves the (lack of) perception of control over the situation. In their experiments, rats receive electric shocks to their tails while being restrained in boxes which have a wheel that can be turned to terminate the shock (controllable shocks). Coupled to these boxes are identical setups in which rats receive an identical amount of tail shocks, but cannot control the duration or onset of the shocks by wheel turning (yoked, uncontrollable shocks). In the controllable shock situation, the number of wheel turns required to terminate each shock is increased as the rats started to learn to use the wheel. With this setup, it could be shown that rats that were subjected to uncontrollable stress (US) subsequently failed to learn to escape foot shocks in a shuttle box, which was therefore termed as LH. In contrast, rats that received an equal amount of tail shocks but that learned to control the shocks by turning the wheel show an unaffected phenotype in comparison to unstressed controls. Hence, the experience of control seems to prevent the induction of LH by US. Furthermore, the effects of US were found to be ‘trans-situational’, as the tail shocks were administered in a different context than the shuttle box, where foot shocks were applied (Maier and Seligman, 1976; Maier and Watkins, 2005). In our design, we have not looked at controllability and we have therefore chosen to use the term inescapable stress (IS). Furthermore, the escape test and IS were both administered in the shuttle box, and we therefore, cannot speak of trans-situational effects of IS. Nevertheless, we observed in pilot studies that IS-exposed rats show a highly significant escape deficit (increased escape latencies and escape failures) in comparison to unstressed rats. The failure to escape shocks in two-thirds of the trials is generally
accepted as a criterion of what is termed LH, and the percentage (15-20%) of helpless
5-HTT⁻/⁻ rats is consistent with what is typically observed for wild-type rats (Vollmayr and
Herrn, 2001).

2.1 The biology of learned helplessness
In addition to their demonstration of the importance of the lack of controllability for the
behavioural consequences of IS exposure, the Maier lab and others have also provided
insights into the underlying neurobiology. Accordingly, the activity of the dorsal raphe
(DR) 5-HT neurons has been shown to inhibit active coping responses by projections to
the dorsolateral periaqueductal gray (PAG), while potentiating fear/anxiety via projections
to the amygdala. The exposure to IS was shown to lead to sensitization of DR 5-HT neurons
for subsequent stressor exposure and consequently leading to increased passive coping
responses to a variety of stressors (Maier and Watkins, 2005, 2010). Downstream of the DR
5-HT neurons, resilience to IS was found to be associated with activation of ΔFosB
(immediate early gene) in the ventrolateral PAG. In this study, the experimental setup was
similar to ours with IS exposure and escape testing both occurring in the shuttle box
(Berton et al., 2007). Interestingly in this regard that dorsolateral PAG activity has been linked
to unconditioned fear responses, while the ventrolateral PAG seems to be exclusively
involved in contextual fear conditioning (Vianna et al., 2001).

Upstream of DR 5-HT neurons, input from the mPFC has been shown to be instrumental
for the role of controllability. It is thought that control over the stressor is detected by (so
far unknown) cortical areas which convey this information to the mPFC. Then, glutamatergic
projections of the mPFC activate y-aminobutyric acid (GABA) interneurons in the DR that
in turn inhibit the DR 5-HT neurons. Via this mechanism, the mPFC can prevent the
sensitization of the DR 5-HT neurons to subsequent stress responses (Amat et al., 2005;
Maier and Watkins, 2010). By probing metabolic activity in the brain in vivo, it was recently
shown that the IS-induced escape deficit is associated with activity of a neurocircuitry that
extends beyond the mPFC, DR, PAG and amygdala. Interestingly, it appeared that the lateral
septum and the habenula were central nodes in this ‘LH-network’ (Morrone et al.,
2014). Indeed, the habenula has been shown to regulate stress coping behaviour by
modulation of DR neurons, by use of optogenetics, selective stimulation of habenula
input to the DR was shown to stimulate passive coping in the FST, while stimulation of
mPFC input to the DR was shown to increase active coping behaviour (Warden et al., 2012).

2.1.1. CRF and the regulation of DR 5-HT neurons
Next to the above discussed brain areas, the activity of DR 5-HT neurons is known to be
regulated by CRF and its related neuropeptides urocortin 1, 2 and 3 (Ucn1, Ucn2, Ucn3), via
the G-protein coupled CRF receptors 1 and 2 (CRF₁R, CRF₂R). Although it is not yet elucidated which of these neuropeptides are endogenously involved in the modulation
of CRF₁R and CRF₂R in the DR, the exogenous administration of agonists and antagonists
of CRF₁R and CRF₂R has shown that CRF₁R activation is associated with inhibition, and
CRF₂R activation with stimulation of DR 5-HT neuron activity (Valentino et al., 2010). As CRF
has high affinity for CRF₁R compared to CRF₂R, at first instance CRF would be expected to
inhibit DR 5-HT neuron activity. However, increasingly higher doses of CRF activate CRF₁R
in the DR (Hammack et al., 2003a, 2003b), and stressors have been found to induce CRF₁R
internalization paralleled with CRF₂R plasma membrane recruitment in the DR (Waselus et
al., 2009). In chapter 5, we found that CRF mRNA levels in the central amygdala (CeA)
show a significant positive correlation with escape performance in rats that had been
subjected to the LH paradigm. The DR is densely innervated by CRF-containing fibres,
which are in part derived from the CeA (Peyron et al., 1998; Lowry et al., 2000; Retson and
Van Bockstaele, 2013). Therefore, we hypothesize that increased CeA-CRF input to the DR
is associated with increased CRF₁R over CRF₂R activation that leads to lower DR 5-HT
neuron activity and increased active coping responses. Yet, it should be noted that although
ELS and 5-HTT genotype both affected escape latencies in the LH paradigm, CeA CRF
mRNA levels were not found to be significantly altered in our ELS x 5-HTTLPR model.

In addition to the dual CRF-mediated control via CRF₁R and CRF₂R, the regulation of
DR 5-HT neurotransmission is further complicated by the topographical organization of
the DR, which is subdivided in regions encompassing the rostral, dorsal, ventral, lateral,
interfascicular and caudal portions of the DR (Lowry, 2002; Fox and Lowry, 2013). The 5-HT
interneurons of the lateral wings of the DR (DRLW) innervate the dorsal and ventral parts
of the DR (DRII, DRV) and are thought to have a tonic inhibitory control over DRD and DRV
5-HT projection neurons (Jasinska et al., 2012). In chapter 6, we found that ELS exposure is
associated with an increased number of 5-HT neurons in the DRLW, while homozygous
5-HTT⁻/⁻ (5-HTT⁻/-) knockout rats displayed an increased number of 5-HT neurons in the DRV.
Therefore, ELS exposure may be associated with increased DRLW-inhibition of 5-HT
projection neurons, which may contribute to the ELS-induced increase in active coping
behaviour in the LH paradigm. The increased number of 5-HT neurons in the DRV of
5-HTT⁻/- rats would, according to the same model, be predicted to be associated with
increased escape latencies. Although 5-HTT⁻/- mice and rats indeed show higher levels of
active coping in inescapable contexts as the EPM and FST, the 5-HTT⁻/- rats showed a
better performance in the shuttle box escape test compared to 5-HTT⁺/+ and 5-HTT⁺/- rats,
whereas the 5-HTT⁻/- mice did not show a significant difference in shuttle box escape
coping compared to 5-HTT⁺/+ mice (Van Bockstaele et al., 2007). Overall, the behavioural findings
are consistent with a hypervigilant phenotype of 5-HTT⁻/- rodents (Honrubia and Van den
Hove, 2012). On the other hand, the inconsistency of increased active coping behaviour in
the escape test and increased DRV 5-HT neuron number as displayed by 5-HTT⁻/- rats
questions the validity of the DRLW-DRD/DRV model of 5-HT neurotransmission.

2.1.2. The contribution of contextual fear
Along this line, it has been observed that raphe 5-HT lesions do not prevent escape
deficits if IS and escape testing occur in the same context (Soubrie et al., 1986; Siegel and

Brown, 1988; Maier and Watkins, 2005), which was the case in our experimental setup. While this does not rule out a role for DR 5-HT neurons in the modulation of escape behaviour in our experiments, it is suggestive of the importance of additional mechanisms, which are likely related to contextual fear memory. The conditioning of contextual fear is the learning of the association of an adverse stimulus with certain environmental (contextual) features; rats that receive foot shocks in a certain environment will later show increased levels of freezing in that environment compared to unshocked rats (Maren et al., 2013). Contextual fear, but also other stress-related memories, are modulated by stress-induced release of CORT, and this modulation consists of immediate and delayed effects on various aspects of memory. Post-training application of CORT or GR agonists promotes the consolidation of information, while retrieval of memory and working memory are negatively affected by increased CORT levels. Importantly, the effects of CORT on learning and memory are a matter of timing, and furthermore depend on the degree of CORT release, and the level of arousal, as activation of the noradrenergic system interacts with central CORT effects in the formation of emotional memory (Het et al., 2005; De Quervain et al., 2009; Rozendaal et al., 2009; Hendens et al., 2011; Joels et al., 2011; Rozendaal and McGaugh, 2011; Schilling et al., 2013; Van Ast et al., 2013). Administration of CORT immediately after fear conditioning increases freezing 24 h later, while the same dose administered 1 h before the test decreases freezing 24 h later. The application of GR agonists and blockade of CORT synthesis by pre-administration of metyrapone have been associated with the opposite effects (Roozendaal et al., 1996; Cordero and Sandi, 1998; Cordero et al., 1998; Abrari et al., 2009; Atsak et al., 2012). With regard to the LH paradigm (with IS exposure and escape testing in the same context), administration of metyrapone before IS exposure has been shown to decrease the IS-induced escape deficit. In contrast, adrenalectomy has been reported to have no effect, or to potentiate the IS-induced escape deficit. An explanation that has been proposed for this discrepancy is that bilateral adrenalectomy induces compensatory changes different from those associated with acute blockade of CORT synthesis (Edwards et al., 1990; Báez et al., 1996; Kademian et al., 2005). We hypothesize that increased levels of IS-induced CORT release partially mediate the IS-induced escape deficit by enhancing contextual fear. Experiments targeted at this hypothesis may also reveal the possible contribution of IS-induced CORT levels to the differential escape performance displayed by ELS-exposed S-HTT+/- and S-HTT++ rats. From our study of the HPA-axis in chapter 3, we would suggest that after ELS exposure S-HTT+/- rats show greater IS-induced CORT release compared to S-HTT++ rats. This may explain why S-HTT++ rats seem to display a less pronounced improvement of escape performance associated with ELS in comparison to S-HTT++ rats. Given the association of ELS with improved fear conditioning (Champagne et al., 2008; Oomen et al., 2010) as well as improved escape performance (chapter 2), however, it remains to be seen whether increased contextual fear actually would be associated with increased passive coping behaviour during escape testing (electric shock exposure), as well as in response to context alone. Therefore, in subsequent experiments it would be instrumental to assess freezing behaviour during the intertrial interval of the escape test, which was unfortunately not possible retrospectively because of the technical limitations of the video camera setup.

3. ELS, S-HTT genotype and physiology

Next to the assessment of stress coping behaviour, the main aim of this thesis has been to elucidate the physiological changes that are associated with ELS x S-HTT genotype interactions. Specifically, we have studied the adult HPA-axis and central expression of GR, MR and FKBP5, the epigenetic (DNA methylation) regulation of the expression of the related neuropeptides CRF and Ucn1 as well as the expression of the neurotransphin BDNF in the brain. Before discussing these findings in further detail, we would like to point out that one could expect that the depressogenic interaction of ELS and S-HTTLPR first described at the epidemiological level (Caspi et al., 2003; Karg et al., 2011; Sharples et al., 2014), at the physiological/molecular level would not necessarily only constitute of ELS x S-HTT genotype effects, but also various physiological changes that are (statistically) independent effects of ELS and S-HTT genotype. These together, however, could give rise to a significant interaction of ELS and S-HTT genotype at the behavioural (epidemiologically) level. In all of the chapters of the thesis, we indeed found main effects of ELS and S-HTT genotypes on several parameters, next to interaction effects.

3.1 ELS x S-HTT genotype: the HPA-axis

In the case of the HPA axis (chapter 3), we found that basal plasma levels of CORT were affected by a significant interaction of ELS and S-HTT genotype. This GxE interaction comprised an opposite effect of S-HTT genotype depending on the early life treatment; S-HTT++ rats displayed the highest CORT levels in the control group, but not after exposure to ELS. In contrast, S-HTT++ rats showed an up-regulation of plasma CORT levels due to ELS, such that the S-HTT++ rats showed the highest plasma CORT levels in the ELS group. S-HTT++ rats, in contrast to their stress coping behaviour (chapter 2), were not affected by ELS exposure in their basal HPA activity. Therefore, for the HPA-axis the S-HTT++ rats may represent a better model of the S-HTTLPR S-allele than the S-HTT++ rats, which is associated with increased basal CORT levels in healthy controls (O’Hara et al., 2007; Wüst et al., 2009; Wankerl et al., 2010). Furthermore, our results resonate with the finding that S-HTTLPR S/S individuals displayed the highest basal CORT levels with a low-risk for depression group (family history and environment), while within a high-risk group S/S subjects showed the lowest and L/L individuals the highest CORT levels (Jabbi et al., 2007).

In addition, the HPA axis has also been studied for the interaction of S-HTTLPR with stressful life events (SLE) across the life span. Firstly, Alexander et al. (2009) reported that basal CORT levels were unaffected, while Mueller et al. (2011) unfortunately did not analyze
basal activity of the HPA-axis (correspondence with Dr. Anett Mueller). Both studies, however, did report that the CORT response following public speaking was significantly affected by S-HTTLPR x SLE interaction. Furthermore, both studies indicate that an increasing number of SLEs leads to increased HPA reactivity in S-allele carriers, but decreased CORT responses to stress in L/L individuals. The study by Mueller et al. (2011) also reported that only SLEs up to 5 years of age interact with S-HTTLPR, and that this GxE interaction only manifests itself in young adults. These studies do not seem to directly fit our findings on the influence of ELS x 5-HTT genotype on basal HPA activity, which we hypothesize to be predictive of HPA reactivity to stress. In this regard, it should be considered that in our study ELS was likely applied in an earlier developmental stage. Furthermore, the studies of Alexander, Mueller and colleagues included a multitude of SLEs, which were not evaluated for their possible interactive effects on the programming of the HPA-axis. Other studies have indicated that the effects of ELS on adult HPA activity are highly dependent on the later life environment (Essex et al., 2011; Goldman-Mellor et al., 2012; Jaffee et al., 2014).

From the perspective of pathophysiology, altered basal HPA activity seems to be an endophenotype that is relevant to a number of psychiatric diseases, with lower CORT levels consistently reported in atypical depression and post-traumatic stress disorder, and elevated plasma CORT levels in melancholic depression (Checkley, 1996; Gold and Chrousos, 2002; Yehuda, 2009). As discussed above, animal studies suggest that the adaptive- or maladaptiveness of altered HPA activity is highly dependent on the specific demands and context of a given stressor (Dittl et al., 2010; Myers et al., 2014). Hence, successful programming of physiology and behaviour is dependent on the prediction of later life environmental challenges (Belsky and Pluess, 2009; Heiming and Sachser, 2010; Schmidt, 2011). Therefore, it would be highly relevant to further characterize our animal model of ELS x 5-HTTLPR interaction by assessing behaviour in conjunction with HPA responses to different types of acute and chronic stressors. In addition, it would be highly recommended to extend the study of basal HPA activity by considering circadian and ultradian rhythmicity.

### 3.2 Adrenal ELS x 5-HTT genotype programming

Beyond the assessment of HPA activity under various conditions and variables, it would be interesting to further explore the regulation of adrenocortical gene expression. The starting point here was the observation that ACTH levels were not found to be significantly affected, while plasma CORT levels were associated with ELS x 5-HTT genotype interaction, suggesting altered adrenal sensitivity for ACTH. Indeed, we found an equivalent and highly significant ELS x 5-HTT genotype programming of adrenal mRNA levels of the ACTH receptor and a number of factors involved in the synthesis of CORT (chapter 3). To rule out an overall altered activity of the adrenal glands we measured plasma adrenalin, adrenal tyroxine hydroxylase mRNA levels and adrenal weight, which were all found to be unaffected, suggesting that the identified ELS x 5-HTT genotype interaction selectively affects the adrenal cortex. Moreover, in an in vitro study we found that 5-HTT genotype was associated with altered adrenal ACTH sensitivity, at least in animals reared in AFR conditions. For future experiments, an ELS x 5-HTT genotype experiment with an in vitro adrenal assay would be envisaged, which could furthermore address the involvement of epigenetic mechanisms in the regulation of gene expression. An advantage here is that the adrenal cortex and medulla likely provide higher tissue homogeneity compared to the brain. Nonetheless, the adrenal gland comprises many cell types, sympathetic nerve endings and many intra-adrenal systems that regulate steroidogenesis (Nussdorfer, 1996; Ehnhart-Bornstein et al., 1998). The chromaffin cells of the rat medulla contain for instance 5-HT, which potently stimulates the release of aldosterone and CORT (Contesse et al., 1998). We were notified by Dr. Hervé Lefebvre that next to 5-HT receptor 4 (5-HT4) (chapter 3), we should also have considered 5-HT receptor 7 (5-HT7) to subserve this function in the rat adrenal, based on two studies that we had not yet identified in the literature (Lenglet et al., 2002; Garcia-Iglesias et al., 2013). At the mRNA level, both receptors were however not found to be affected by ELS, 5-HTT genotype or their interaction (data not shown). To further assess the intra-adrenal 5-HT system, pharmacological manipulations (including 5-HT, and 5-HT, agonists/antagonists) of the in vitro adrenal assay could be most helpful at this point.

### 3.3 GR, MR, FKBP5

Despite the marked ELS x 5-HTT genotype programming of adrenocortical activity, we found little to no changes in gene expression at the level of the pituitary and paraventricular nucleus (PVN) of the hypothalamus. Numerous studies have shown that areas as the mPFC, hippocampus and extended amygdala are involved in the regulation of HPA activity (Ullrich-Lai and Herman, 2009). Therefore, we hypothesized that the extrahypothalamic expression of GR, MR and their co-chaperone FKBP5 would be affected by ELS, 5-HTT genotype or their interaction. Indeed, we found a complex adaptation, including ELS x 5-HTT genotype effects, of GR, MR and FKBP5 mRNA levels in the mPFC and hippocampus (chapter 4). The altered expression of GR and MR, in conjunction with FKBP5, could potentially lead to a functional GR/MR disbalance in the mPFC and hippocampus, which has been proposed to contribute to increased vulnerability to stress-related psychopathology (De Kloet et al., 1998, 2005). It has to be acknowledged that the stability of the identified transcriptional GR/MR disbalances remains to be further examined. In chapter 4, we have furthermore discussed in detail the possible functional consequences (regulation of the HPA-axis, stress-related behavioural and mnemonic functioning) of the differences we observed in GR, MR and FKBP5 mRNA levels between S-HTT⁺⁺, S-HTT⁺⁻ and S-HTT⁻⁻ rats that received a control treatment or were exposed to ELS.
3.4 CRF and Ucn1
The CRF family of neuropeptides (CRF, urocortins) is involved in the regulation of HPA activity, as well as the behavioural stress response (Binder and Nemeroff, 2010). In the central amygdala (CeA) and the oval subdivision of the bed nucleus of the stria terminalis (BNSTov), and the centrally-projecting Edinger-Westphal nucleus (EWcp), we found that ELS x 5-HTT genotype affected the DNA methylation of the promoter regions of the genes encoding CRF, and Ucn1, respectively (chapter 5 and 6). Moreover, DNA methylation of the Crf promoter was found to show highly significant negative correlations with CRF mRNA levels in the PVN and the CeA. Furthermore, despite ELS x 5-HTT genotype interaction effects on DNA methylation of the promoter regions of these genes, the mRNA and protein levels of CRF in the PVN, BNSTov and CeA, and Ucn1 in the EWcp, were comparable between experimental groups. Therefore, additional epigenetic mechanisms (e.g. miRNAs, histone modifications) should be considered to be involved in the regulation of Crf and Ucn expression. In addition, we hypothesize that the effects of ELS x 5-HTT genotype on DNA methylation of these genes may represent an early programming that would regulate stress-induced expression of CRF and Ucn1 in response to later adverse life events. Therefore, the inclusion of exposure to acute or chronic stressors in adolescence/adulthood in our ELS x 5-HTTLPR model (as proposed above) could represent a ‘third hit’ (De Kloet, 2008; Daskalakis et al., 2013), which may reveal functional consequences of the altered DNA methylation of the Crf and Ucn promoter regions.

Interestingly, the levels of DNA methylation of Crf, which were consistent with previous reports (Elliott et al., 2010; Sterrenburg et al., 2011), were far higher than those observed for Ucn (chapter 5 and 6). We hypothesize that these differential promoter region DNA methylation patterns may be associated with the complementary dynamics of PVN (BNSTov, CeA)-CRF and EWcp-Ucn1 neurons (Kozicz et al., 2011a; Janssen and Kozicz, 2013). Furthermore, we obtained correlative evidence in chapter 6 suggestive of a role of EWcp-Ucn1 neurons in the regulation of DR 5-HT neurons. Specifically, Ucn1 mRNA levels in the DR showed highly significant negative correlations with mRNA levels of CRF-R and the inhibitory autoreceptor 5-HT receptor 1A (5-HT1A). As CRF-R (stimulation) and 5-HT1A (inhibition) have opposite roles in the regulation of DR 5-HT neurons, locally translated Ucn1 might contribute to the balanced expression of CRF-R and 5-HT1A. It could be highly relevant to further address the role of Ucn1 in the regulation of DR 5-HT neuronal activity, since the dual CRF/R/Crf-R regulation is associated with potent effects on stress coping behaviour (Valentino et al., 2010; Wood et al., 2013).

3.5 BDNF
Besides the role of 5-HTT in the HPA-axis, 5-HTT has also been under scrutiny because of its tight connection with BDNF at the molecular level (Martinowich and Lu, 2008; Homberg et al., 2014). Indeed, epidemiological studies suggest the relation between ELS x 5-HTTLPR interaction and stress-related depression is moderated by the Val66Met polymorphism of the BDNF gene (Kaufman et al., 2006; Wichers et al., 2008). The BDNF_Val66Met Polymorphism impairs the dendritic trafficking, synaptic localization and secretion of BDNF (Egan et al., 2003). Therefore, we expected and indeed found that ELS and 5-HTT genotype interact to affect central expression levels of BDNF (chapter 7). Similar to GR, MR and FKBP5 mRNA levels, the effect of ELS x 5-HTT genotype interaction on the expression of different Bdnf transcripts in the mPFC and hippocampus was found to depend on the dorsoventral subdivision. Specifically, ELS and 5-HTT genotype were both associated with decreased BDNF mRNA levels in the ventral mPFC and hippocampus, while ELS was observed to selectively increase Bdnf expression in the dorsal mPFC and hippocampus of 5-HTT<sup>−/−</sup> rats. Moreover, by assessing differential exon usage (the main Bdnf transcripts include exon IV versus V) and polyadenylation of the 3′-untranslated region (UTR) (An et al., 2008; Lubin et al., 2008; Molteni et al., 2009; Orefice et al., 2013), our study indicates that the differential effects of ELS x 5-HTT genotype on total BDNF mRNA levels in the dorsal versus ventral areas include effects on specific, functionally different (cellular localization), Bdnf transcripts (chapter 7). Our investigation of altered expression of Bdnf has been preceded by a study with an alternative ELS x 5-HTT genotype model. In this study by Carola et al. (2008), a model of ELS was obtained by intercrossing two inbred mouse strains, C57BL/6J (B6), 5-HTT<sup>−/−</sup> and BALB/cByJ (C), that exhibit large differences in maternal care (MC). Females derived from these crosses – B6 x C and C x B6 – are genetically identical but provide high and low levels of maternal care (licking and grooming, L/G, respectively. A significant ELS x 5-HTT genotype interaction was found, with increased dorsal hippocampal BDNF mRNA levels as well as anxiety-like behaviour displayed by low L/G-5-HTT<sup>−/−</sup> mice compared to the 5-HTT<sup>−/−</sup> and high L/G groups. Furthermore, the BDNF mRNA levels showed a nearly significant positive correlation with anxiety-like behaviour in the open field test (Carola et al., 2008). Our data suggest that this ELS-induced upregulation of BDNF mRNA levels in the dorsal hippocampus of 5-HTT<sup>−/−</sup> rodents constitutes a long 3′-UTR and exon VI Bdnf transcript (chapter 7).

4. Possible future avenues for ELS x 5-HTT genotype rodent studies
In the above sections we have already discussed important research questions that have arisen as a consequence of the findings presented in this thesis. In addition, we would like to propose three broader avenues for further studies with our ELS x 5-HTTLPR model. The first would be to study the effects of MS across different developmental stages (prenatal, juvenile, pubertal, adolescence, senescence) in addition to adulthood. With this approach, it could be elucidated at which age certain features arise and how stable they are across development. For instance, in rat pups, repeated MS as well as 24 h deprivation of maternal contact have been shown to induce increased adrenal expression of the ACTH receptor (Schmidt et al., 2004; Daskalakis et al., 2011); therefore, it would be interesting to assess the
Second, the use of different types of ELS exposure could be considered. As touched upon in chapter 1, the MS model may actually represent a model of human prenatal stress, with regard to maturation of the neocortex and the HPA-axis. From an ethological (roden) perspective however, the MS model would evidently be a postnatal model, which furthermore seems to best approximate the human situation of early life neglect. In the laboratory setting, the mother rat is observed to be frequently away from the nest for periods of 2-3 h (Calhoun, 1962; Meaney, 2001). Therefore, the daily 3 h separations are considered to be an ethologically relevant stressor for the offspring, which results in a deprivation of maternal care. Furthermore, in chapter 3 we found that in our MS experiments, the mother rat seemed to compensate for nutritional deprivation (physical care), but did not show an increase in licking and grooming (emotional care) of the pups because of the separation period. To further characterize the immediate effects of MS, peer interaction (Branchi, 2009; Branchi and Cirulli, 2014) and within-litter variation of maternal care (Claessens et al., 2011; Van Hasselt et al., 2012) could be considered.

From systematic analyses of human studies, it seems that emotional maltreatment is associated with increased risk for depression compared to physical or sexual abuse, which may predispose more to other psychiatric disorders (Boudewyn and Lien, 1995; Gibb et al., 2001; Hankin, 2005; Shapiro et al., 2014). A recent study even reported that emotional abuse and not physical/sexual abuse moderated the relationship between later life stress and depressive episodes (Shapiro et al., 2014). The vulnerability to depression conferred by emotional abuse and neglect is thought to be mediated by an insecure attachment style, negative cognitive styles, and additional adverse life events (Barnet et al., 2005; Hankin, 2005; Van Harmelen et al., 2010). Although the literature is thus far only indicative on this matter, we propose that the interaction of S-HTTLPR with different types of ELS exposure in human as well as animal studies could be key in understanding/explaining how a common gene variant is associated with depression (Barnett et al., 1993; Manly et al., 2001).

A third major possibility of follow-up could be to study sex differences in ELS x S-HTT genotype interactions, as in our studies thus far, only male rats have been used for phenotypic analyses. Sex differences actually are a major issue, because females appear to have twofold higher risk compared to men to develop depression (Kendler et al., 2002; Holden, 2005; Wittchen and Jacobi, 2005). Indeed, a number of studies have also indicated that the phenotypic correlates of S-HTTLPR and ELS x S-HTTLPR interaction are modulated by sex (Sjöberg et al., 2006; Brummett et al., 2008; Everaerd et al., 2012). As the heightened female vulnerability is limited to the period of postpuberty up to menopause, much attention has been paid to the role of estrogen (Krishnan and Nestler, 2010). Furthermore, animal studies confirm that a sexual dichotomy exists for many aspects of the stress response (Galea et al., 1997; Wood and Shors, 1998; Carvalho-Nietto et al., 2011; Dalla et al., 2011), and exposure to ELS has been described to have different effects in male and female rodents (Glover and Hill, 2012; Kundakovic et al., 2013; Davis and Pfaff, 2014; Fuentes et al., 2014). Therefore, sex is a highly relevant factor and the underlying mechanisms of ELS x S-HTTLPR interaction may exhibit important differences between males and females.

Although the primary objective of this thesis has been to provide mechanistic insights into the relation of S-HTTLPR with major depression (Caspi et al., 2003; Karg et al., 2011; Sharpley et al., 2014), this common genetic variant has also been associated with post-traumatic stress disorder (Xie et al., 2009; Gressier et al., 2013), anxiety disorders (Stein et al., 2008; Reinelt et al., 2013), and substance abuse (Fein et al., 2005; Gerra et al., 2007). It is highly likely that this divergence is in part due to the modulation of ELS x S-HTTLPR interaction by other genomic variants. In addition, we hypothesize that the systematic examination of ELS x S-HTTLPR interaction by the study of sex differences, different types of ELS, and different developmental stages for phenotyping and additional (third hit) stressors, may explain how a common genetic variant such as S-HTTLPR is associated with such diverse psychopathology, and at the same time, could perhaps alleviate the controversy that has existed over the relation between S-HTTLPR and psychiatric disease (Risch et al., 2009; Munafò et al., 2009; Margoob and Mushtaq, 2011; Navarro-Mateu et al., 2014).

5. Conclusions of this thesis

Overall, with this thesis we have come to a further understanding as well as additional questions about ELS x S-HTTLPR interactions. With regard to physiology, we found that the effect of ELS x S-HTTLPR interaction on activity of the HPA-axis (Jabbi et al., 2007; Alexander et al., 2009; Muller et al., 2011) is likely to involve programming of the sensitivity of the adrenals to circulating levels of ACTH (chapter 3), which was associated with a complex adaptation of central expression levels of GR, MR and FKBP5 (chapter 4), CRF (chapter 5), and Ucn1 (chapter 6). For BDNF specifically, we extended the study by Carola et al. (2008) that indicated the involvement of altered dorsal hippocampal BDNF expression in increased anxiety/stress sensitivity of individuals with low S-HTT availability, by showing that this altered BDNF expression likely constitutes the long 3’-UTR and exon VI BDNF transcript (chapter 7). With regard to behaviour, the findings presented in chapter 2 suggest that 1) vulnerability to psychopathology is not necessarily increased after exposure to ELS, but is determined by the interaction between the early and later life...
environment (match/mismatch hypothesis) (Champagne et al., 2009; Nederhof and Schmidt, 2012), and 2) these data best support the view that the 5-HTTLPR S-allele is associated with a plasticity, rather than a vulnerability phenotype (differential susceptibility hypothesis), namely environmental sensitivity (Kaufman et al., 2004; Belsky et al., 2007, Homberg and Van den Hove, 2012; Van Ijzendoorn and Bakermans-Kranenburg, 2012).
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The aim of the research presented in this thesis has been to increase the understanding of the etiology of stress-related psychiatric disorders. Major depressive disorder, commonly referred to as (unipolar) depression, is a psychiatric disorder that inflicts a huge personal and societal burden, and, according to the World Health Organization, is among the leading causes of disability worldwide. The heritability of depression has been estimated to contribute to 30-50% of the risk to develop depression, with the remaining liability accounted for by non-genetic factors as stress and traumatic events, but also viral infections and even stochastic processes during brain development. Over the past decade it has become increasingly clear that vulnerability to depression consists of a complex interplay of genome and environment.

Perhaps the most illustrative and well-known example of gene x environment interaction in psychiatry involves the serotonin (5-hydroxytryptamine; 5-HT) transporter gene. The 5-HT transporter (5-HTT) plays a critical role in the termination of 5-HT neurotransmission by the re-uptake of 5-HT into the presynaptic neuron, and is the target of the most widely prescribed class of antidepressants (selective serotonin reuptake inhibitors). The 5-HTT-linked polymorphic region (5-HTTLPR) is associated with two genetic variants of the 5-HTT gene: a short (S) and a long (L) allele. In 2003, Caspi and co-workers reported that the increased risk to develop depression after exposure to stress was 2-3 times higher in individuals with the S-allele compared to individuals without the S-allele. Further studies showed that this effect of the S-allele versus the L-allele is particularly evident for early life exposure to stress, and that, on the molecular level, the S-allele is associated with reduced expression of the 5-HTT gene.

For the current thesis, we have studied the biological and behavioural consequences of the interaction between 5-HTT gene variation and early life stress (ELS) exposure. To perform these studies, we have subjected 5-HTT homozygous and heterozygous knockout rats during the early postnatal life to repeated and prolonged separations from their mothers. In chapter 2, the adult male offspring was assessed for their stress vulnerability by use of the learned helplessness paradigm. Remarkably, we found that the ELS exposure rendered the rats more stress resilient compared to control rats, as they displayed increased adaptive coping behaviour. These experimental findings have challenged the prevailing theory that postulates that ELS exposure invariably leads to adverse consequences. Rather, our results suggest that ELS exposure can lead to adaptive responses, depending on the challenges in later life. In addition, we found that the adaptive effect of ELS exposure was the strongest for heterozygous 5-HTT knockout rats (the S-allele model). This suggests that the S-allele does not only confer increased sensitivity to negative environmental programming, but that it is associated with differential susceptibility; an increased sensitivity for both adverse and beneficial environmental effects.
In chapter 3-4, we explored how the interaction of ELS and 5-HTT gene variation would affect the stressor-responsive hypothalamo-pituitary-adrenal (HPA) axis and related gene expression in the brain. The HPA-axis functions to regulate the cortex of the adrenal glands with regard to the synthesis and release of glucocorticoids in the bloodstream. In response to stress, glucocorticoids, mainly cortisol in primates and corticosterone in rodents (collectively referred to as CORT), mobilize energy resources by elevating circulating glucose levels, increasing heart rate and blood pressure, and decreasing the activity of the immune and digestive systems. Furthermore, as part of the stress response, CORT acts on the brain to affect neural plasticity, cognition and behaviour. The effects of CORT are mediated via the glucocorticoid and mineralocorticoid receptors (GR, MR), and GR-mediated feedback is instrumental for the termination of stress-induced CORT elevations. In depression, about 50% of patients display altered basal HPA activity and GR-mediated negative feedback. Altogether, the HPA-axis is an important regulatory system in the stress response, and we hypothesized that the interaction of ELS and 5-HTT gene variation could affect vulnerability to depression via altered activity and regulation of the HPA-axis. In chapter 3, we indeed found that ELS x 5-HTT genotype interaction significantly affected basal plasma CORT levels. Furthermore, we found evidence that strongly suggests that this effect was, at least in part, mediated by alterations of gene expression in the adrenal gland. In chapter 4, we found that ELS x 5-HTT genotype interaction also significantly affected the expression of GR and MR in the hippocampus and medial prefrontal cortex, brain areas known to be involved in stress coping behaviour and regulation of the HPA-axis.

In chapter 5, we found that the mRNA levels of corticotropin-releasing factor (CRF) in the central amygdala showed a significant correlation with the levels of active coping behaviour displayed in the learned helplessness paradigm. Moreover, the interaction of ELS and 5-HTT gene variation was found to affect DNA methylation of the Crf gene promoter in the central amygdala, and the methylation levels of a specific site in the Crf promoter also significantly correlated with the CRF mRNA levels. As the selection of active stress coping responses has been associated with reduced activity of 5-HT neurons in the dorsal raphe nucleus, we hypothesize that increased CRF input to the dorsal raphe leads to lower 5-HT neuron activity via stimulation of CRF receptor 1, and therefore increased levels of active coping behaviour. At this point, further investigation is first needed to confirm the link of Crf promoter methylation with stress coping behaviour, which should for instance include the analysis of CRF protein levels.

As referred to above, the dorsal raphe nucleus is known to be involved in a wide array of physiological and behavioural stress responses, via its serotonergic (5-HT) projections to other brain areas. And for behavioural responses the activity of 5-HT neurons is regulated by CRF receptors 1 and 2. Besides CRF itself, a number of related proteins, urocortins 1-3, are also endogenous ligands for CRF receptor 1 and 2. In chapter 6, we have examined the principal site of urocortin 1 expression in the brain, the centrally projecting Edinger-Westphal (EWcp) nucleus. We found that ELS was associated with increased DNA methylation of the urocortin 1 gene, and increased expression of 5-HT receptor 1A in the EWcp. In addition, urocortin 1 mRNA levels significantly correlated with CRF receptor 2 and 5-HT (auto)receptor 1A in the dorsal raphe. These findings suggest it could be worthwhile to investigate the functional relationship between EWcp urocortin 1 and dorsal raphe 5-HT neurons in the pathophysiology of depression as a function of ELS and 5-HTT gene variation.

In chapter 7, we investigated the expression of different transcripts of the gene encoding brain-derived neurotrophic factor (BDNF). The relation between ELS and depression has been shown to be modulated by a number of genetic variants other than the 5-HTT polymorphism. Moreover, it is thought that the pathophysiology of depression arises from the interplay of a multitude of genetic and environmental factors, although evidence is still scarce. Yet, a polymorphism of the BDNF gene has been shown to interact with ELS and 5-HTT genotype to moderate the risk to develop depression. Therefore, it was perhaps not surprising to find that the interaction of ELS and 5-HTT genotype significantly affected the expression of BDNF in the hippocampus and medial prefrontal cortex. By assessing functionally different BDNF transcripts, our findings in chapter 7 suggest that this BDNF transcript is constituted of a long 3'-untranslated region and the inclusion of exon VI.

In chapter 8, we have provided an extensive discussion of the research findings summarized above and we furthermore propose that follow-up studies are needed to address the questions that have arisen from our work. To our opinion, the major conclusions of this thesis are 1) the S-allele can also be linked to stress resilience in the context of predictive adaptive programming, and 2) ELS and 5-HTT gene variation interact at the level of the adrenal gland to program HPA-axis activity. At the moment, further experiments are planned to elucidate the role of altered HPA-axis activity in stress network activation and behavioural stress responses, after ELS x 5-HTT genotype interaction. Next, the investigation of ELS x 5-HTT interactions could be advanced by including the evaluation of different developmental stages after ELS exposure, the use of different types of ELS exposure, and the evaluation of sex differences. Overall, we think that the research described in this thesis has contributed to a further understanding of the interaction of ELS and 5-HTT gene variation, and has provided new avenues for research aimed at elucidating the biological mechanisms involved in the etiology of depression.
Samenvatting

Het onderzoek dat in dit proefschrift is beschreven had het doel om het ontstaan van stress-gerelateerde, psychiatrische stoornissen beter te leren begrijpen. Depressie is een psychiatrische stoornis die een enorme persoonlijke en maatschappelijke last met zich meebrengt. Het risico om depressie te ontwikkelen heeft volgens (wetenschappelijke) schattingen een grote erfelijke component (zo’n 30 tot 50%), met daarnaast een belangrijke rol voor omgevingsfactoren als stress en traumatische gebeurtenissen, maar ook virale infecties en zelfs random gebeurtenissen tijdens de ontwikkeling van de hersenen. Gedurende het afgelopen decennium is het steeds duidelijker geworden dat het ontstaan van depressie het gevolg is van een complexe wisselwerking tussen genetische varianten en omgevingsfactoren.

Misschien wel het bekendste voorbeeld van zo’n gen x omgevingsinteractie in de psychiatrie is die van het serotonine transporter polymorfisme. De serotonine transporter is betrokken bij het beëindigen van de communicatie tussen hersencellen via de neurotransmitter serotonine. Dit doet de serotonine transporter door serotonine terug de cel in te transporteren nadat het ter overbrenging van het signaal is afgegeven in de ruimte tussen een contactpunt van hersencellen; de zogenaamde synaps. De serotonine transporter is een bekend eiwit, omdat hierop de bekendste klasse van antidepressiva op inwerken; de SSRIs (serotonin transporter reuptake inhibitors). Het serotonine transporter polymorfisme bestaat uit twee varianten: een kort (short: S) en lang (L) allel. In 2003 werd een epidemiologisch onderzoek gepresenteerd waaruit bleek dat het risico om depressie te ontwikkelen na blootstelling aan bepaalde stressvolle gebeurtenissen twee tot drie keer zo groot was voor individuen met het S allel in vergelijking met individuen met het L allel. Studies die hierop volgden toonden aan dat dit effect van het S allel versus het L allel vooral erg groot was bij blootstelling aan stress in de vroege jeugd, en dat op moleculair niveau het S allel geassocieerd is met een lagere expressie van het serotonine transporter gen.

In dit proefschrift hebben we de consequenties van de interactie tussen genetische variatie van het serotonine transporter gen, serotonine transporter genotype, en de vroege blootstelling aan stress onderzocht. Om zulk onderzoek tot op moleculair niveau te kunnen doen hebben we gebruik gemaakt van ratten waarbij de serotonine transporter volledig ontbrak, of maar voor de helft van het normale niveau aanwezig was. Deze ratten hebben we als pups vervolgens regelmatig gescheiden van hun moeders als een model voor vroege blootstelling aan stress. In hoofdstuk 2 hebben we vervolgens gekeken hoe goed de ratten met stress om konden gaan toen ze volwassen waren geworden. Opvallend genoeg bleken de op jonge leeftijd gestresste ratten minder kwetsbaar te zijn voor blootstelling aan stress in het volwassen leven in vergelijking met ratten die als pups enkel een controlebehandeling hadden gekregen. Deze experimentele bevindingen strokken niet met de heersende theorie dat vroege blootstelling aan stress enkel negatieve
consequences zou kunnen hebben. Onze resultaten suggereren juist dat dit ook geassocieerd kan zijn met adaptatie van het individu, en dat dit een voordeel t.o.v. anderen met zich mee kan brengen, afhankelijk van de uitdagingen die het latere leven biedt.

In hoofdstuk 3 en 4 hebben we bestudeerd hoe de serotonine transporter genotype en vroege blootstelling aan stress de stressgevolgen hypothalamus-hypofysie-bijnier (HHB) as zouden kunnen beïnvloeden. De functie van de HHB-as is om de bijnierschors aan te sturen in diens productie en afgifte van glucocorticoïden aan de bloedbaan. Tijdens een stress respons zorgen glucocorticoïden ervoor dat energie beschikbaar komt, o.a. door stijging van de suikerspiegel en toename van de hartslag en bloeddruk. Daarnaast hebben glucocorticoïden belangrijke effecten in de hersenen, zoals op cognitie, geheugen en gedrag. Bij veel depressieve patiënten is er een afdwijn in de activiteit en regulatie van de HHB-as, en daarom hadden wij de hypothese dat de effecten van serotonine transporter genotype en vroege blootstelling aan stress op het risico om depressie te ontwikkelen o.a. door veranderingen in de HHB-as zouden kunnen komen. In hoofdstuk 3 toonden wij aan dat de activiteit van de HHB-as inderdaad beïnvloed werd door zowel serotonine transporter genotype als door vroege blootstelling aan stress, en dat deze effecten waarschijnlijk voor een groot deel gemedieerd worden door veranderingen in genexpressie in de bijniere. In hoofdstuk 4 toonden we daarnaast aan dat de expresie van de receptoren voor glucocorticoïden in het brein ook beïnvloed werden door zowel serotonine transporter genotype als door vroege blootstelling aan stress, en dat deze effecten bovendien sterk verschillen waren voor verschillende hersengebieden.

In hoofdstuk 5 vonden we dat de mRNA niveaus van corticotropin-releasing factor (CRF) in de centrale amygdaal een significante correlatie vertoonden met het gedrag dat bestudeerd was in hoofdstuk 2. Daarnaast bleek dat zowel de vroege blootstelling aan stress als het serotonine transporter genotype een effect hadden op de methylatie van het CRF-gen in de centrale amygdaal, en dat deze methylatieniveaus op hun beurt een significante correlatie vertoonden met de mRNA niveaus van CRF. Het specifieke gedrag dat in hoofdstuk 2 is bestudeerd is eerder al gelinkt aan de activiteit van serotonine neuronen in de dorsale raphe kern. Daarnaast weten we dat de activiteit van de serotonine neuronen gereguleerd kan worden door de receptoren voor CRF in de dorsale raphe. Onze hypothese is dat verhoogde input van CRF vanuit de centrale amygdaal naar de dorsale raphe leidt tot lagere activiteit van serotonine neuronen, en daarmee een actiervene manier van omgaan met stress. Op dit moment is nog verder onderzoek nodig om deze functionele connecties in ons onderzoeksmode te bevestigen.

In hoofdstuk 6 hebben we de dorsale raphe kern verder bestudeerd, net als de hersenkern die de grootste populatie neuronen herbergt die het eiwit urocortine 1 tot expresie brengen, de Edinger-Westphal (EW) kern. Urocortine 1 is een eiwit dat gerelateerd is aan CRF, en waarvan ook is aangetoond dat het de activiteit van serotonine neuronen in de dorsale raphe kan reguleren. We vonden dat de methylatie van het gen dat codeert voor urocortine 1 lager is na vroege blootstelling aan stress, en dat urocortine 1 mRNA niveaus correleerden met de expressie van de CRF receptor in de dorsale raphe. Dit zijn interessante aanknopingspunten om de relatie tussen de EW en de dorsale raphe verder te bestuderen, om zo te zien of urocortine 1 in onze experimenten ook daadwerkelijk een functione rol heeft, die bovendien beïnvloed wordt door serotonine transporter genotype en vroege blootstelling aan stress.

In hoofdstuk 7 zijn verschillende molecule transcripten van het gen dat codeert voor brain-derived neurotrophic factor (BDNF) onderzocht. Van de effecten van vroege blootstelling aan stress op het latere risico op depressie is namelijk aangetoond dat deze tegelijkertijd door polymorfismes van zowel het serotonine transporter als het BDNF gen wordt beïnvloed. De verwachting was daarom dat er ook een interactie op moleculair niveau zou zijn, en we vonden inderdaad dat de expressie van BDNF afhankelijk was van serotonine transporter genotype en vroege blootstelling aan stress. In een vergelijkbare studie was ook een verband aannemelijk gemaakt tussen angstgedrag en de expressie van BDNF in een bepaald hersengebied, de hippocampus. In hoofdstuk 7 vonden wij dat dit waarschijnlijk een specifiec transcript van het BDNF gen betreft.

In hoofdstuk 8 hebben we alle resultaten uitvoerig bediscussieerd en nog eens kritisch onder de loep genomen. Daarnaast hebben we ook voorstellen tot vervolgonderzoek gedaan. Naar onze mening zijn de belangrijkste inzichten n.a.v. dit proefschrift dat 1) het polymorfisme van het serotonine transporter gen niet alleen geassocieerd is met een toegenomen gevoeligheid voor negatieve, maar juist ook voor positieve omgevingsfactoren, en dat 2) serotonine transporter genotype de effecten van de vroege blootstelling aan stress op de activiteit van de HHB-as beïnvloedt via de regulatie van genexpressie in de bijniere. Alles tezamen denken wij dat dit proefschrift heeft bijgedragen aan een verbeterd inzicht in de gen x omgevingsinteractie van serotonine transporter genotype en vroege blootstelling aan stress, en dat het aanleiding biedt voor nieuw onderzoek naar de biologische mechanismen die betrokken zijn bij het ontstaan van depressie.
Dankwoord | Acknowledgements

Een PhD project doet niemand alleen, en ik ben daarop zeker geen uitzondering. Ik grijp graag de gelegenheid aan om hier mijn dankbaarheid uit te spreken aan iedereen die mij geholpen heeft met het tot stand komen van dit proefschrift, en dankzij wie ik vier ontzettend leuke jaren heb gehad.

Op de eerste plaats zijn daar Judith en Tamás. Ik ben jullie erg dankbaar voor het vertrouwen, jullie begeleiding, feedback, ideeën, advies en steun. Ik had mij geen betere mentoren kunnen wensen, en ik waardeer het erg dat jullie mij altijd de ruimte hebben gegeven in onze discussies. Ik zou ook in de toekomst graag nog af en toe eens bij jullie binnenlopen!

Benno, Marian en Mathias. Ik ben zeer vereerd dat jullie zitting wilden nemen in de manuscriptcommissie. Benno, erg bedankt dat je met ons mee hebt willen denken over de vervolgexperimenten, die dit boekje helaas niet gehaald hebben. Marian en Mathias, de interactie met jullie op meerdere congressen inspireerde me, en motiveerde mij om nog beter en kritischer na te denken over mijn data.

Anthonieke, Bram en Ron. Ik ben erg blij en trots dat jullie mijn paranimfen wilden zijn. Jullie zijn ontzettend lief en aardige mensen, en hebben een grote bijdrage geleverd aan dit proefschrift. Anthonieke, jij staat altijd klaar voor iedereen, en je bent ontzettend belangrijk voor onze experimenten. Bedankt voor al het meedenken met de planning, en niet te vergeten de genotyperingen! Bram, bedankt dat je altijd bereid was mij te helpen, met het meedenken over antilichamen, de qPCRs, en de verhuizing naar Anatomie. Ron, dankzij jou was het bijna echt leuk om in de weekenden te werken. Erg bedankt voor je hulp met de experimenten, en ook voor die paar keer dat je mij uit het CDL hebt geholpen, toen ik mijzelf daar had ingesloten…

Ik wil ook graag een aantal van jullie collega-analisten bedanken. Debbie, bedankt voor je hulp met het moleculaire werk, en voor alle begrip en hulp tijdens onze verhuizing naar Anatomie (ik bedenk me nu, heb ik alles al wel uit jullie –80 gehaald?). Frouwke, bedankt voor je tolerantie voor onze grote kleuringen op je lab, en je tips bij de moeilijkere immunohistochimie. Peter, al tijdens mijn stage bij Bruce heb je mij heel veel geleerd op het lab. Bedankt ook voor de hulp bij de ELISAs. Jos, bedankt voor alle goede zorgen, bovenal met de cryostat. Dankzij jou heeft Hussein er een custom-handleiding voor kunnen maken. Liesbeth, bedankt dat je me telkens weer wilde uitleggen hoe ik ‘dat’ ook alweer kon doen op de confocale microscoop, en dat je altijd een oogje in het zeil wilde houden als een nieuwe student aan de slag ging.
En dat waren er een flink aantal. Ik prijs mezelf gelukkig dat ik met zoveel studenten heb mogen werken. Claudia, you were the first one, after only a few months into my PhD project. Thank you for your understanding that I did not always have the answer either, for your enthusiasm, and for your invaluable help with the maternal care observations. William, your orderly way of working and qPCR analyses have been instrumental for chapter 3. Besides having talent for doing research you were also a great guy to have around, which I'm sure a lot of colleagues will agree with. Deborah en Charlotte, jullie waren slechts korte tijd op ons lab, omdat het jullie BSc stages betrof. Toch hebben jullie er beiden uitgehaald wat erin zat, al was het ene project succesvoller dan het andere. Uiteindelijk denk ik (hoop ik) dat jullie er allebei toch veel van geleerd hebben. Ilse, het zegt eigenlijk al genoeg dat je bij twee hoofdstukken tweede auteur bent. Je hebt in je stage erg veel werk verzet, en er daardoor voor gezorgd dat ik mij volledig op het tweede experiment kon richten, zonder dat de productie (analyses) uit het eerste experiment hieronder te lijden had. Later zijn we ook collega's geworden op Anatomie. Een fijnere student of collega dan jullie zou niemand zich kunnen wensen. Harm, je hebt mij en Ron geassisteerd bij de uitvoering van het tweede experiment en daarnaast ook nog zelfstandig immunohistochemie gedaan. Je ijver en scherpe vragen vielen op, dat gaat je vast nog van pas komen in je verdere carrière. Mark, tijdens je stage heb je het niet makkelijk gehad. Het heeft ons beiden moeite gekost om er op een gegeven moment nog wat van te maken. Je gaf de moed echter niet op, en ik hoop dat je er net als ik ook veel van hebt geleerd. Berit, jij had het bij momenten af en toe ook lastig met je stage, en gaf dat ook heel eerlijk aan. Ik heb dat zeer gewaardeerd, en hoe jij het vervolgens op hebt gepakt al leuksam. Hopelijk kunnen we hoofdstuk 6 binnenkort publiceren! Maya, jij staat terecht ook bij de auteurs voor hoofdstuk 6. Je stelde soms hele goede vragen, schroom vooral niet om die in de toekomst vaker te stellen! Hussein, it was great fun to have you at the lab. Admittedly, on a few occasions you drove me somewhat mad. But then again, you were quite a hero to be able to work with that antique cryostat of us, cutting countless sections in vitro. Admittedly, on a few occasions you drove me somewhat mad. But then again, you were quite a hero to be able to work with that antique cryostat of us, cutting countless sections in vitro. You were not only a great guy to have around, but also a real source of inspiration! Hussein, you later became colleagues again at Anatomy. I've really appreciated our occasional talks. Diane, Miyuki, Kari, Saskia, Freek, Rik, Cor en Gé, jullie ook erg bedankt voor de leuke tijd!

Op de afdeling Cognitive Neuroscience was ik niet zo vaak aanwezig als bij Cellulaire Dierfysiologie en Anatomie, waar ik natuurlijk mijn kantoorplekje had. Maar, ik kwam er heel graag, en dan in het bijzonder natuurlijk om met mijn collega's van de groep van Judith bij te kletsen. Lourens, bedankt voor alle technische oplossingen op het CDL, die jij drie keer zo snel paraat had, en voor je proefschrift wat ik vaak als voorbeeld heb doorgenomen. Pien, toen Lourens vertrok kon ik gelukkig altijd bij jou aankloppen voor hulp met de spullen van Med Associates, en ik hoop dat jij net als ik ook veel gehad hebt aan onze gezamenlijke discussies met Judith en Tamás. Velt en Noortje, het was super leuk om met jullie een kleine week door West USA te toeren! Peter, bedankt voor de Game of Thrones-downloads. Omdat ik daaraan verslingerd raakte ben ik de boeken ook gaan lezen, en die hebben me heel veel wachten op het CDL heel geholpen! Michel, Stephanie, Deborah, en alle andere collega's en studenten van CNS, bedankt voor de mooie tijd! Dirk en Nael, jullie nog erg bedankt voor het gebruik van jullie labs ter voorbereiding (en uitvoering) van het in vitro experiment met de bijniemen! Erno en Floris, de Stress meetings waren een echte stimulans, en ik wil jullie in het bijzonder bedanken voor de feedback!

Toen het zover was dat Cellulaire Dierfysiologie ophield te bestaan kwam er een nieuwe leerstoel en afdeling Neurofysiologie. Tansu, I would like to thank you for ensuring me that...
I would always be able to do my work, whether it would have been at your department or somewhere else. Armaz, it was great fun to share the office for some time, as it also always was to run into you and to hear how you were doing. Aron en Nikië, ook voordat we samen een tijdje op hetzelfde lab zaten voelden jullie eigenlijk al als collega’s, erg leuk om het een tijdje ook echt te zijn!

Bij Neurofysiologie ben ik niet gebleven, het laatste jaar tot anderhalf jaar heb ik op de afdeling Anatomie door mogen brengen. Ook al was het jammer om andere collega’s achter te laten, ik vond het heel leuk om de mensen voor terug te krijgen! Amanda, met dankzij jou heb ik me erg welkom gevoeld bij Anatomie en ik ben erg blij dat ik tweemaal jullie zomeroefenavond heb mogen meemaken! Carola, Max, Valerio, Ilse, Diane, Tim en Maartje, ik vond het super om met jullie het kantoor (en lab) te delen, erg gezellig en erg veel gelachen! Ook de andere collega’s van Anatomie wil ik van harte bedanken!

Al mijn resultaten begonnen met experimenten op het CDL en ik bedank graag het CDL-personeel, en in het bijzonder Janneke, Michael en Karin, voor hun hulp. Sjef, ook erg bedankt voor de adviezen!

Danielle Champagne en Barbara Vollmayr, you may both not remember that I visited your labs for a single day. I want to thank you both very much though for so generously taking the time to share your expertise with me.

Marco, Francesca and Gianluigi, thank you very much for our fruitful collaboration!

Dóra and Zsuzsa, thank you very much for all the efforts for the in vitro adrenal assay!

Balázs, thank you for checking all those ELISA results for us with the ACTH and CORT RIAs!

Familie, vrienden, (ex-)collega’s, oud-studiegenoten en eenieder die ik onverhoopt vergeten ben, bedankt voor de steun en interesse!

Pap en Mam, ontzettend bedankt voor jullie steun, dat jullie er altijd voor me zijn, voor jullie trots, en het vertrouwen in mij.

Lieve Loes, Roos, Jan, Elsebep, Jolies, Meryl, Jeroen et Els. Bedankt dat jullie zo vaak aan me vroegen hoe het nou eigenlijk ging met mijn onderzoek, het was erg leuk en fijn om jullie daar regelmatig wat over te mogen vertellen.

Nanda, lieve schat. Voor jou schrijf ik een apart dankwoord. Ik hoop dat je het nog heel lang wilt blijven lezen.

Rick Hendrikus Adrianus van der Doelen (September 14th, 1986) obtained his pre-university degree (VWO) at Gymnasium Bernrode in Heeswijk-Dinther. Subsequently, he obtained his Master’s degree in Molecular Life Sciences at Radboud University (RU) in Nijmegen (with honour). As part of his Bachelor and Master studies, he performed multiple research internships. At the Laboratory of Pediatrics and Neurology of the RU Medical Center, the potential use of aminoglycosides in Alzheimer’s diseases was explored under the supervision of Dr. Ilona B. Bruinsma and Dr. Marcel M. Verbeek. At the RU department of Cellular Animal Physiology, he worked under the guidance of Dr. Bruce G. Jenks to show that purines are involved in the regulation of melanotrope cells of the pituitary gland. Following these internships, he left Nijmegen to study the Pro15Ser polymorphism of the 5-ht1A receptor under the supervision of Dr. Nicholas G. Irving at the department of Molecular Pharmacology of the Schering Plough Research Institute in Newhouse, Scotland. For his final internship, he worked with Prof. Dr. Andries Kalisbeek at the Netherlands Institute for Neuroscience (Amsterdam) to study the central action of hypothalamic thyrotropin-releasing hormone in the regulation of glucose homeostasis.

His PhD research was conducted at the departments of Cellular Animal Physiology, Cognitive Neuroscience and Anatomy in Nijmegen, under the guidance of Dr. Judith R. Homberg and Prof. Dr. Tamás Kozicz. The aim of the research has been to increase the understanding of the interaction of early life stress exposure and serotonin transporter gene variation in the etiology of major depression. The findings of these studies are described in this thesis, and have been presented at (inter)national conferences as the Donders Discussions, the Dutch Neuroscience Meeting, the International Congress of Comparative Endocrinology, and the meetings of the Dutch Pharmacological Society, the LARC Neuroscience Network, the Society for Neuroscience, the International Society of Psychoneuroendocrinology, and the European Brain and Behaviour Society. As of September the 1st, 2014, he is a resident in Clinical Chemistry at the VieCuri Medical Centre Noord-Limburg.
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