Molecular mechanisms of early-life stress in 5-Htt deficient mice:

Gene x environment interactions and epigenetic programming

Molekulare Mechanismen von Entwicklungsstress

bei 5-Htt defizienten Mäusen:

Gen x Umwelt Interaktionen und epigenetische Programmierung

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Table of Contents

Preface

Understanding the molecular mechanisms underlying vulnerability and resilience to emotional disorders like depression and anxiety disorders is of great interest for psychiatrists, psychologists, neurobiologists and patients, as it helps to identify new routes to diagnose and treatment. For this purpose, mouse models of depression and anxiety have been generated. This thesis describes three different animal models that address the emerging concept of early-life programming and related gene x environment interactions. Some of the animals in my thesis were heterozygous for the serotonin transporter (5-Htt+/-) gene, a gene that is associated with depression and anxiety traits in humans and in all of the three animal models, mice were exposed to early-life stress. The main work of my thesis was to study the behavior of and to perform gene expression and DNA methylation screenings in prenatally stressed (PS) 5-Htt+/- female mice and wild-type control animals (see 3. Results).

Stress during development, especially when it comes along with a genetic predisposition, can have longlasting consequences including psychopathology in adulthood. At the basis of these gene x environment interactions lie epigenetic mechanisms, that pre- and posttranscriptionally alter mRNA production - sometimes in a stable fashion -, therefore programming the organism for later life. As the aim of my thesis was to study the molecular mechanisms that determine the outcome of early-life stress programming to be either negative (vulnerable offspring) or positive (resilient offspring), I looked at one candidate gene, the brain-derived neurotrophic factor (Bdnf), which is known for its prominent role in neuronal and synaptic plasticity and related cognitive and affective functions. I investigated the DNA methylation pattern (one epigenetic modification) of various *Bdnf* promoters in the hippocampus and cortex of PS and control mice by the help of EpiTYPER MALDI-TOF mass spectrometry and pyrosequencing (see appendix 5.2). Further, I applied bisulfite sequencing to study differences in the methylation pattern of *ADP-ribosylation factor guanine nucleotide-exchange factor (Arfgef) 1* between mice exposed to low and mice experienced high levels of maternal care (see appendix 5.3).

In particular, I thank Prof. Dr. K.-P. Lesch and Dr. DLA van den Hove for discussion and valuable comments on this work. Further, my thanks go to the DFG SFB TRR 58 and to EC 6th framework programme NewMood, who funded this project.

Sissi Jakob

Summary

Early-life stress has been shown to influence the development of the brain and to increase the risk for psychiatric disorders later in life. Furthermore, variation in the human serotonin transporter (5-HTT, SLC6A4) gene is suggested to exert a modulating effect on the association between early-life stress and the risk for depression. At the basis of these gene x environment (G x E) interactions, epigenetic mechanisms, such as DNA-methylation, seem to represent the primary biological processes mediating early-life programming for stress susceptibility or resilience, respectively. The exact molecular mechanisms however remain to be elucidated, though.

In the present study, we used two different stress paradigms to assess the molecular mechanisms mediating the relationship between early-life stress and disorders of emotion regulation later in life. First, a 5-Htt x prenatal stress (PS) paradigm was applied to investigate whether the effects of PS are dependent on the 5-Htt genotype. For this purpose, the effects of PS on cognition and anxiety- / depression-related behavior were examined using a maternal restraint stress paradigm of PS in C57BL/6 wild-type (WT) and heterozygous 5-Htt deficient (5-Htt+/-) mice. Additionally, in female offspring, a genome-wide hippocampal gene expression and DNA methylation profiling was performed using the Affymetrix GeneChip® Mouse Genome 430 2.0 Array and the AffymetrixGeneChip® Mouse Promoter 1.0R Array. Some of the resulting candidate genes were validated by quantitative real-time PCR. Further, the gene expression of these genes was measured in other brain regions of the PS animals as well as in the hippocampus of offspring of another, 5-Htt x perinatal stress (PeS) paradigm, in which pregnant and lactating females were stressed by an olfactory cue indicating infanticide. To assess resilience to PS and PeS, correlation studies between gene expression and behaviour were performed based on an initial performance-based LIMMA analysis of the gene expression microarray.

5-Htt+/- offspring of the PS paradigm showed enhanced memory performance and signs of reduced anxiety as compared to WT offspring. In contrast, exposure of 5-Htt+/- mice to PS was associated with increased depression-like behavior, an effect that tended to be more pronounced in female offspring. Further, 5-Htt genotype, PS and their interaction differentially affected the expression and DNA methylation of numerous genes and related pathways within the female hippocampus. Specifically, MAPK and neurotrophin signaling were regulated by both the 5-Htt+/- genotype and PS exposure, whereas cytokine and Wnt signaling were affected in a 5-Htt genotype x PS manner,

indicating a gene x environment interaction at the molecular level. The candidate genes of the expression array could be validated and their expression patterns were partly consistent in the prefrontal cortex and striatum. Furthermore, the genotype effect of XIAP associated factor 1 (Xaf1) was also detected in the mice of the PeS paradigm. Concerning resilience, we found that the expression of growth hormone (Gh), prolactin (Prl) and fos-induced growth factor (Figf) were downregulated in WTPS mice that performed well in the forced swim test (FST). At the same time, the results indicated that Gh and Prl expression correlated positively with adrenal weight, whereas Figf expression correlated positively with basal corticosteron concentration, indicating an intricate relationship between depression-like behavior, hippocampal gene expression and the hypothalamo-pituitary-adrenal (HPA) axis activity. Correlation studies in the PeS animals revealed a link between Gh / Prl expression and anxiety-like behavior. In conclusion, our data suggest that although the 5-Htt+/- genotype shows clear adaptive capacity, 5-Htt+/- mice, particularly females, appear to be more vulnerable to developmental stress exposure when compared to WT offspring. Moreover, hippocampal gene expression and DNA methylation profiles suggest that distinct epigenetic mechanisms at the molecular level mediate the behavioral effects of the 5-Htt genotype, PS exposure, and their interaction. Further, resilience to early-life stress might be conferred by genes whose expression is linked to HPA axis function.

Zusammenfassung

Zahlreiche Studien haben gezeigt, dass Stress während der Entwicklung die Gehirnentwicklung beinflusst und das Risiko an psychischen Störungen zu erkranken erhöht. Weiterhin wird vermutet, dass eine Variation im humanen Serotonintransportergen (5-HTT, SLC6A4) einen modulierenden Einfluss auf die Assoziation zwischen Entwicklungsstress und dem Risiko für Depression ausübt. Als Basis dieser Gene x Umwelt (GxE)-Interaktion scheinen epigenetische Mechanismen, wie DNA-Methylierung, die biologischen Prozesse darzustellen, die die Programmierung von Stressanfälligkeit oder Resilienz vermitteln. Die exakten molekularen Mechanismen sind jedoch noch unbekannt.

In dieser Studie wurden zwei verschiedene Stressparadigma verwendet um die molekularen Mechanismen zu klären, die Stress während der Entwicklung und emotionalen Störungen später im Leben zu Grunde liegen. Zuerst wurde ein 5-Htt x pränatales Stress (PS)-Paradigma verwendet um zu untersuchen, ob die Effekte von pränatalem Stress abhängig von dem 5-Htt Genotypen sind. Aus diesem Grund wurden die Effekte von PS auf Kognition, Angst- und Depressions-ähnliches Verhalten untersucht indem ein "maternal restraint stress"-Paradigma in C57BL/6-Wildtyp (WT) und heterozygoten 5-Htt defizienten (5-Htt+/-) Mäusen angewandt wurde. Zusätzlich wurde mit Hilfe des Affymetrix GeneChip® Mouse Genome 430 2.0 Arrays und des AffymetrixGeneChip® Mouse Promoter 1.0R Arrays bei den weiblichen Nachkommen ein Genexpressions- und DNA-Methylierungsprofil erstellt. Einige der daraus resultierenden Kandidatengene wurden mit quantitativer real-time PCR (qRT-PCR) validiert. Weiterhin wurde die Genexpression von diesen Genen auch in anderen Gehirnregionen der PS-Mäuse und im Hippocampus von Nachkommen aus einem perinatalem (PeS) Paradigma gemessen. In dem PeS-Paradigma wurden schwangere und stillende Weibchen durch einen olfaktorischen Stimulus, der Infantizid anzeigt, gestresst und die Nachkommen (WT und 5-Htt+/-) untersucht. Um PS- und PeS-Resilienz zu messen wurden Korrelationsstudien durchgeführt. Zuvor wurde eine LIMMA-Analyse, die auf dem Verhalten von den Mäusen im Forced swim-Test (FST) beruht, gerechnet.

Im Vergleich zu WT Nachkommen zeigten 5-Htt+/- Nachkommen des PS-Paradigmas verbesserte Gedächtnisleistung und Zeichen von reduzierter Angst. Im Gegensatz dazu war PS-Exposition von 5-Htt+/- Mäusen mit erhöhtem Depressions-ähnlichem Verhalten assoziiert, ein Effekt, der tendenziell eher in den weiblichen Nachkommen auffiel. Weiterhin beeinflussten der 5-Htt-Genotyp, PS und die Interaktion von beiden die Genexpression und DNA-Methylierung zahlreicher Gene und damit

verbundene Signalwege im weiblichen Hippocampus. Der MAPK- und Neurotrophin-Signalweg wurden zum Beispiel durch den 5-Htt-Genotyp und PS-Exposition reguliert, wohingegen der Zytokin- und Wnt-Signalweg in einer 5-Htt x PS Art beeinflusst wurden, was Gen x Umwelt-Interaktionen auf der molekularen Ebene andeutet. Die Kandidatengene konnten zumeist validiert werden und waren zum Teil auch im präfrontalen Kortex sowie im Striatum differentiell exprimiert. Weiterhin konnte der Genotypeffekt von XIAP associated factor 1 (Xaf1) in den Mäusen des PeS-Paradigmas nachgewiesen werden. Bezüglich der Resilienz konnten wir eine Herunterregulierung der Expression des Wachstumshormons (Gh), Prolaktins (Prl) und des fos-induzierten Wachstumsfaktors (Figf) in den WTPS-Mäusen detektieren, die eine gute Leistung im FST gezeigt haben. Gleichzeitig korrelierten die Gh- und Prl-Expression positiv mit dem Gewicht der Nebennieren, wohingegen die Figf-Expression mit dem basalen Kortikosteron-Konzentration positiv korrelierte, was eine komplizierte Beziehung zwischen Depressions-ähnlichem Verhalten, hippocampaler Genexpression und der Hypothalamus-Hypophysen-Nebennieren (HPA)-Achsenaktivität andeutet. Korrelationsstudien über die PeS-Tiere deckten einen Link zwischen der Gh- und Prl-Expression und Angst-ähnlichem Verhalten auf. Schließlich lassen unsere Daten den Schluss zu, dass, auch wenn der 5-Htt-Genotyp eine klare adaptive Kapazität aufweist, die 5-Htt+/- Mäuse, insbesondere die Weibchen im Vergleich zu den WT-Mäusen eine erhöhte Vulnerabilität für Entwicklungsstress zu zeigen scheinen. Weiterhin könnten die hippocampale Genexpressions- und DNA-Methylierungsprofile darauf schließen lassen, dass epigenetische Mechanismen auf der molekularen Ebene die Verhaltenseffekte des 5-Htt Genotyps, PS-Exposition und ihrer Interaktion vermitteln. Darüber hinaus könnte Resilienz zu Entwicklungsstress durch Gene reguliert werden, die mit der HPA-Achsen-Funktion assoziiert sind.

1. Introduction

1. Introduction

Goethe´s Faust seemed to be very depressed as he tried to kill himself with a poison (Faust I, V732-737).

„Hier ist ein Saft, der eilig trunken macht.

Mit brauner Flut erfüllt er deine Höhle.

Den ich bereite, den ich wähle,

Der letzte Trunk sei nun, mit ganzer Seele,

Als festlich hoher Gruß, dem Morgen zugebracht!

(Er setzt die Schale an den Mund)“

“Here is a juice, one's quickly drunk with it.

With its brown flood it fills thy ample bowl.

This I prepared, I choose this, high upborne;

Be this my last drink now, with all my soul,

A festal, lofty greeting pledged to morn!

(He puts the goblet to his lips.)“

Emotional disorders like depression can lead, in their severe forms, to suicide. According to the World Health Organisation (WHO) “depression is a common mental disorder that presents with depressed mood, loss of interest or pleasure, feelings of guilt or low self-worth, disturbed sleep or appetite, low energy, and poor concentration”. These symptoms can become chronic or cyclic and can hinder individuals in their daily life and responsibilities. Depression is a major reason for disability worldwide and has a lifetime prevalence of 16.6% (Kessler *et al.* 2005). Similarly, anxiety disorders can also have a strong emotional component. For example, being emotionally blunted, flat or numb or fear of loosing control or getting a heart attack. Anxiety disorders have a lifetime prevalence of 28.8% and the average age of onset is 11 years (Kessler *et al.* 2005). Although several lines of evidence suggest an important role for serotonin (5-hydroxytryptamine, 5-HT) in the pathophysiology

of depression and anxiety disorders, the exact underlying molecular mechanisms still remain to be elucidated.

Twin studies proposed that genetic factors may contribute to mood disorders like depression and anxiety (Sullivan *et al.* 2000). In this context, the serotonergic system, as a target for several antidepressants (see chapter 1.1), might play a pivotal role (Lesch and Heils 2000). A polymorphism in the 5-HT transporter (5HTT, SLC6A4) gene has been shown to be linked to depression- and anxiety-related personality traits (Lesch *et al.* 1996). To further study the underlying mechanisms of 5-HTT-related emotional vulnerability, 5-Htt deficient mice have been generated to model the human allelic variation in 5-HTT function (Bengel *et al.* 1998). Generally, these mice display elevated depressive- and anxiety-like behavior, altered stress coping abilities, and memory deficits like a disability in extinction recall (Holmes *et al.* 2002; Holmes *et al.* 2003; Carroll *et al.* 2007; Wellman *et al.* 2007; Heiming *et al.* 2009; Bartolomucci *et al.* 2010; Jansen *et al.* 2010).

As discussed in chapter 1.2, in addition to genetic vulnerability, a stressful early life environment, in particular during pregnancy, may also contribute to the development of psychopathology. For example, physical or emotional stress during gestation has been shown to influence the development of the fetal brain, thereby increasing the risk for neuropsychiatric disorders in adulthood, particularly disorders of emotion regulation such as depression (see review by Weinstock 2008). Likewise, prenatal stress (PS) exposure in rodents, particularly when exposure occurs during the last phase of pregnancy, is associated with a dysregulated hypothalamo–pituitary–adrenal (HPA) axis, concomitant with an increase in learning and memory deficits, as well as increased anxiety and depression-like behavior in adulthood (Pallares *et al.* 2007; Zuena *et al.* 2008; van den Hove *et al.* 2010; see also reviews by Weinstock 2001; Huizink *et al.* 2004). Nevertheless, the biological mechanisms by which PS exposure renders subjects susceptible to the development of neuropsychiatric disorders are as yet not fully understood.

By now, it has become increasingly clear that neither genetic nor environmental factors alone can account for the development of emotional disorders (see chapter 1.3). As such, variation in the 5-HTT gene was proposed to exert a modulating effect on the association between adverse experiences and the risk for anxiety disorders and depression (Collier *et al.* 1996; Lesch *et al.* 1996). Caspi and his colleagues (2003) tested maltreated children on depressive behavior in adulthood. They found that

only the short (s) variant of the 5-HTT gene-linked polymorphic region (5-HTTLPR) generated the depressive outcome of early life maltreatment.

The present study aimed to examine the effects of early-life stress on cognition, anxiety- and depression-like behavior using a maternal restraint stress paradigm of PS in C57BL/6 wild-type (WT) and 5-Htt+/- mice. More knowledge on the molecular basis of such a G x E interaction might help to identify novel targets for the diagnosis and treatment of disorders of cognition and emotion regulation. For that purpose, we performed a genome-wide expression and methylation profiling on the hippocampus - a brain region participating in learning and memory as well as in emotion regulation (Fanselow and Dong 2010)- derived from the female offspring, which showed most pronounced behavioral changes mediated by variation in 5-Htt genotype, PS, and their interaction.

1.1 Genetic variation of the serotonin transporter

Serotonin was first discovered by Vittoria Esparmer in the enterochromaffine cells of the gut in the early 1930ths. More than 10 years later, it was also found in the blood serum displaying vasoconstrictive features, for which it was called serotonin ("serum" and "tonus") from then on. In 1967, Coppen and colleagues for the first time discussed a decreased activity of serotonergic neurons in view of the pathogenesis of affective disorders (Coppen 1967; Coppen *et al.* 1967). In the same year, Schildkraut and Kety proposed the monoamine hypothesis of depression (Schildkraut and Kety 1967). 30 years later again, Lesch and colleagues identified an association between variation in 5-HTT genotype and anxiety-related traits comprising the personality dimension of "neuroticism" (Lesch *et al.* 1996).

1.1.1 Localization and impact of the serotonergic system

Neuronal 5-HT is located in the brain stem, especially in the pons and medulla oblongata. There, the serotonergic neurons form single groups of cells, which are described as the raphe-nuclei (B1-B9, see Fig.1-1). From there, two main complexes project into various areas of the brain and spinal cord: Neurons of the caudal raphe-complex (medulla oblongata and caudal pons; B1-4) descend to the motoric and autonomic system in the spinal cord. They activate motoric anterior horn cells and inhibit the dorsal horn of the spinal cord, which mediates nociception. Secondly, serotonergic groups of the rostral raphe-complex (mesencephalon and rostral pons; B5-B9) project mainly to the

diencephalon and the forebrain. There, the serotonergic fibers innervate various brain regions including the (prefrontal) cortex, striatum, hippocampus and amygdala, thereby contributing to the regulation of mood, cognition, sleep-wake cycle, thermoregulation, and eating and sexual behavior.

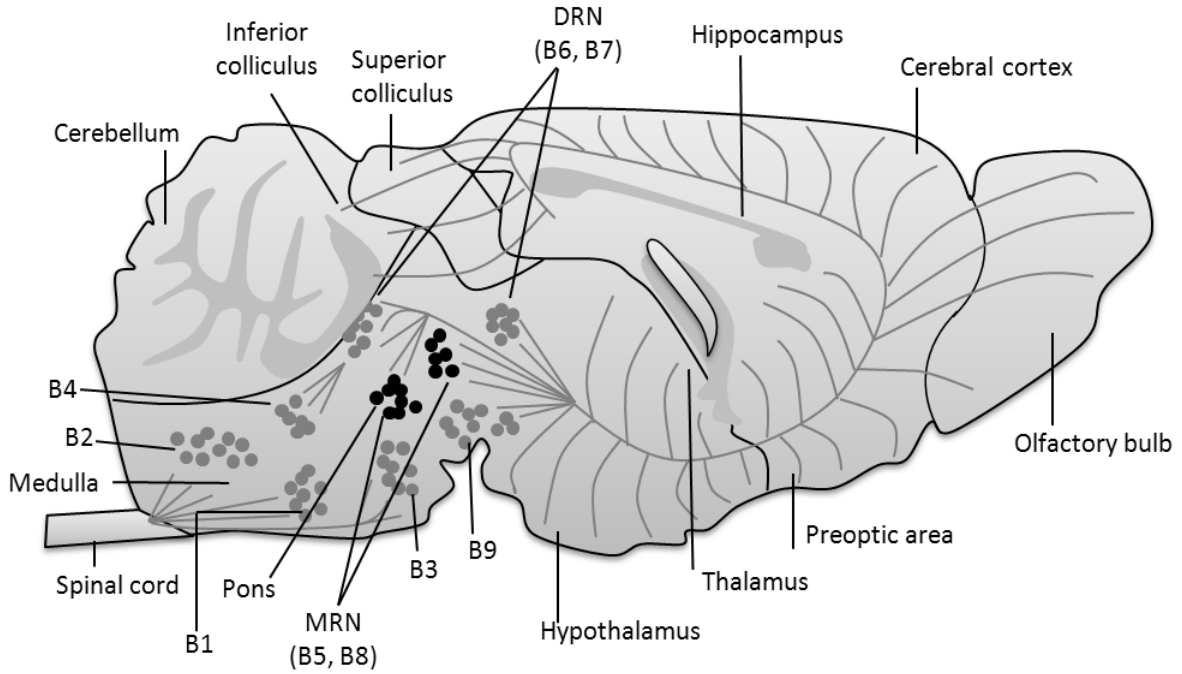


Figure 1-1. The murine central serotonergic system (Modified from Murphy and Lesch 2008). CNS 5-HT neuron cell-body groups in the nine raphe nuclei, B1–B9. The more caudal nuclei (B1–B3) in the medulla send axons to the spinal cord and the periphery. The more rostral raphe nuclei contain the principal dorsal raphe groups (B6 and B7; shown in grey) and the median raphe groups (B5 and B8; shown in black), which project to different brain areas.

1.1.2 Synthesis and metabolism of 5-HT

5-HT, as an indolamine, belongs to the monoamine neurotransmitters like the catecholamines adrenaline, noradrenaline and dopamine. Monoamines have an aminogroup and therefore closely resemble the amino acids from which they are produced. The synthesis of 5-HT occurs mainly in the soma, but to a lesser extent also in the dendrites and axons, in two distinct steps. In the first, rate-limiting step, the essential amino acid tryptophan (TP) is hydroxylated to 5-hydroxytryptophan (5-HTP). This reaction is catalysed by tryptophan hydroxylase (TPH). To date, two isoforms of TPH (TPH1 and TPH2) have been identified. Whereas TPH1 is expressed in the periphery and pineal gland, TPH2 is specific to the brain (Gutknecht *et al.* 2009). In the faster, second step of 5-HT synthesis, the carboxyl group of 5-HTP is removed by the aromatic L-aminoacid decarboxylase (AAAD) in order to yield 5-HT. AAAD is a soluble, ubiquitary enzyme, which is not specific for the decarboxylation of 5-HTP, but also contributes to the biosynthesis of the catecholamines. Decarboxylated monoamine

transmitters like 5-HT are not able to cross the blood-brain barrier. If a deficiency of transmitters is assumed, like in the 5-HT-deficiency hypothesis of depression, the 5-HT production can be enforced by increasing the availability of their amino acid precursors (L-tryptophan and L-5-HTP), which are able to pass the blood-brain barrier.

To guarantee a regulated release of transmitters, 5-HT is stored in intracellular vesicles (see Fig. 1-2). Neuronal 5-HT-containing vesicles are located in the cell body, along the axon and at the synapse. Upon stimulation by an action potential there is an influx of Ca^{2+} which activates the vesicles to move towards the presynaptic membrane where they merge with the membrane and release 5-HT into the synaptic cleft.

5-HT conveys its manifold physiological and pathophysiological effects through the activation of receptors, which are bound to the pre- and postsynaptic membrane (5-HT_1 – 5-HT_7). The presynaptic autoreceptors 5-HT_{1A}, 5-HT_{1B} and 5-HT_{1D} are responsible for negative feedback. In various depressive and anxiety disorders, a decreased 5-HT_{1A} receptor binding and 5-HT_{1A} density has been observed in different brain regions like the frontal cortex, hippocampus (where 5-HT_{1A} is situated at the postsynapse) and the raphe nuclei (Sargent *et al.* 2000; Lundberg *et al.* 2007).

The 5-HTT removes 5-HT out of the synaptic cleft back into the presynaptic neuron. Part of the 5-HT is transported back into the presynaptic cell and is packed into vesicles again, whereas the rest is degraded by the monoamine oxidase A (MAO-A) to 5-hydroxyindole-3-acetaldehyde (5-HIAL). Subsequently, in the second step 5-hydroxyindolacetic acid (5-HIAA) is formed upon oxidation by aldehyde dehydrogenase.

1.1.3 Serotonin transporter

The 5-HTT is located in the presynaptic membrane and is a monoamine transporter. It terminates serotonergic neurotransmission by actively transporting 5-HT back into the presynaptic neuron, thereby regulating the extracellular concentration of 5-HT and contributing to the maintenance of homeostasis (Torres *et al.* 2003). The 5-HTT is a target of several antidepressants like the selective 5-HT reuptake inhibitors (SSRIs, e.g. fluoxetine), tricyclic antidepressants (TCAs) and illicit drugs like cocaine and 3,4-methylenedioxymethamphetamine (MDMA or “ecstasy”), which underlines the

monoamine hypothesis of depression (Torres *et al.* 2003). In the brain, the 5-HTT is found exclusively in the raphe-complex and along the axons of serotonergic neurons (Chen *et al.* 1992; Blakely *et al.* 1994). The gene for the human 5-HTT is located at 17q.12.2, consists of 14 exons, is 35 kb long and has various 5' flanking regions and non-coding regulatory sequences (Lesch *et al.* 1994; Bengel *et al.* 1997). The transcriptional activity of the 5-HTT gene is regulated by a polymorphic repetitive element [5-HTT gene-linked polymorphic region (5-HTTLPR)] in the 5' untranslated region (Lesch *et al.* 1996), together with other allelic variations (Kilic *et al.* 2003; Prasad *et al.* 2005; Sutcliffe *et al.* 2005; Hu *et al.* 2006). The long (l) and s-alleles of the 5-HTTLPR are associated with high versus low 5-HTT protein expression, respectively. The rate of 5-HT reuptake is two times higher in cells which are homozygous for the l-variant when compared to cells which have one or two s-variants (Lesch *et al.* 1996). On average, the lower expressing s-allele carriers seem to be more at risk for anxiety, depressive and obsessive-compulsive disorders than l-allele carriers (Collier *et al.* 1996; Lesch *et al.* 1996; Hu *et al.* 2006). Besides some other deficits concerning emotionality, s-allele carriers excel in some cognitive functions, e.g. they show higher cognitive flexibility (for review see Homberg and Lesch 2011; Homberg 2012).

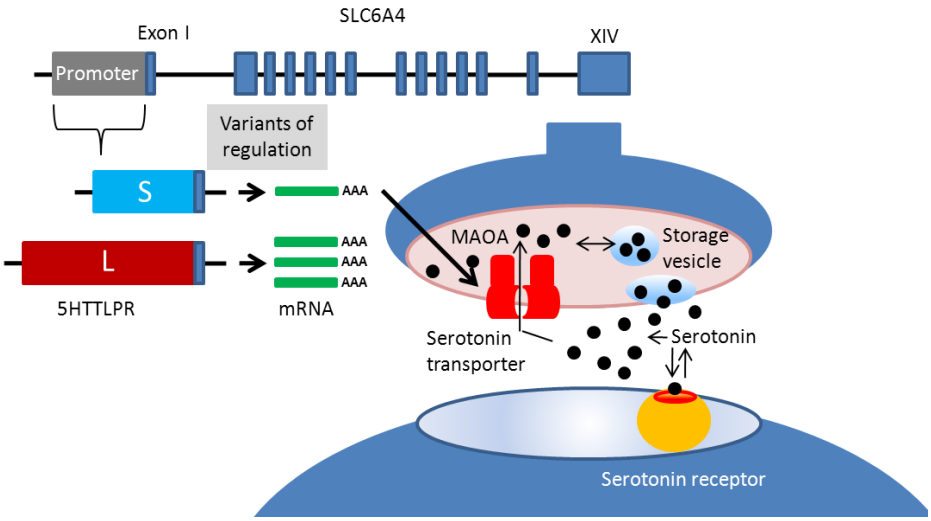


Figure 1-2. The serotonin transporter (5-HTT) and the associated 5-HT neurotransmission. Allelic variation of 5-HTT function in anxiety-related personality disorders, depression and other disorders of emotion regulation. The short (S) 5-HTTLPR variant (blue) of the 5-HTT gene (SLC6A4) produces significantly less 5-HTT mRNA and protein, as indicated by the green lines, than the long (L) variant (red), which results in higher levels of 5-HT in the synaptic cleft. The short variant is linked with anxiety-related personality traits such as neuroticism, which are risk factors for affective spectrum disorders. MAOA, monoamine oxidase A; (Modified from Canli and Lesch 2007)

To study the biological function of the 5-HTT in detail, an animal model with a targeted disruption (knockout; KO; -/-) of the 5-Htt was generated (Bengel *et al.* 1998). In 5-Htt-/- mice, the second exon of the 5-Htt gene has been eliminated by homologous recombination and therefore these mice

express a truncated, non-functional 5-Htt protein. Thus, 5-HT cannot be taken up by the transporter which has been shown to lead to a 7-13 fold increase of the extracellular concentration of 5-HT in the prefrontal cortex (PFC), striatum, caudate putamen and nucleus accumbens (Mathews *et al.* 2004; Shen *et al.* 2004). On the other hand, the extracellular 5-HT concentration in the hippocampus, frontal cortex and the striatum is decreased (Bengel *et al.* 1998), which may be caused by an altered 5-HT metabolism and/or compensatory alterations in 5-HT receptor levels. In the dorsal raphe nucleus (DRN), there is a decrease of 5-HT_{1a} receptor-density and response, while in the substantia nigra similar effects were observed for 5-HT_{1b} (Fabre *et al.* 2000). The reduction of $5HT_{1a}$ receptors is more pronounced in females (Li *et al.* 2000). Another compensatory mechanism in 5-Htt deficient mice might be the upregulation of a polyspecific organic cation transporter, OCT3, which transports 5-HT, albeit with low affinity, back into the cell (Schmitt *et al.* 2003). Moreover, Nietzer *et al*. revealed that 5-Htt-/- mice differ from controls when it comes to dendritic length, spine density and complexity of pyramidal neurons in the infralimbic cortex, and in the basolateral and lateral nucleus of the amygdala (Nietzer *et al.* 2011).

5-Htt-/- mice are more anxious than their WT littermates as measured in the light dark test and open field (Heiming *et al.* 2009). Furthermore 5-Htt-/- mice are less aggressive as tested in the resident-intruder test (Holmes *et al.* 2002) and in another social interaction test (Lewejohann *et al.* 2010). 5-Htt+/- mice, which show intermediate levels of 5-Htt, display no changes in daily behavior in regard of locomotion, socio-positive and aggressive behavior, compared to WT mice, whereas 5-Htt-/- show a decrease in locomotion and an elevation in socio-positive behavior (Lewejohann *et al.* 2010). Overall, it seems as the 5-HTT mediates the effects of stress. In this context, an interaction between stress and the serotonergic system has been proposed (Andrews and Matthews 2004).

As some of these changes observed in 5-Htt deficient mice resemble the human phenotype of s-allele carriers, the 5-Htt deficient mouse model has been recognized as a valuable animal model for investigating the molecular mechanisms of emotion dysregulation.

1.2 Prenatal Stress

Stress is the major environmental factor known to increase the susceptibility for emotional disorders. Depending on the stage of the development, stress is known to exert a different impact on

individuals. In general, the following rule applies: the earlier the stress is experienced, the more persistent the effects on the organism (Lupien *et al.* 2009). Pregnancy, for example, is a critical phase during which the programming of the HPA axis is extremely vulnerable to developmental stress exposure (Liu *et al.* 2001).

The idea that the maternal environment may be reflected in the child is not new. Leonardo Da Vinci wrote in his *Quaderni*: "The things desired by the mother are often found impressed on the child that the mother carries at the time of the desire… one and the same soul governs the two bodies, and the same body nourished both." According to the "developmental origins of disease concept" (Gillman 2005), the increased risk for endocrine, metabolic and emotional diseases in adulthood may in part be dependent on variations of the early environment. In turn, the "predictive adaptive response" hypothesis (Gluckman and Hanson 2004) proposes that, for an optimal survival, the developing child tries to anticipate the environmental conditions with which it is confronted later on and adapts its physiology accordingly. Recently, epigenetic mechanisms like DNA methylation have been shown to represent a molecular memory by which environmental adversity can program the DNA to change an individual's physiology and behavior throughout the lifespan (Tsankova *et al.* 2007).

As such, emotional or physical stress experienced by the mother during pregnancy may affect the developing fetus and might result in physical or mental disorders in the offspring´s later life. Although the long period between the adverse event and the onset of the disease makes it hard to estimate the exact number of people which are affected by PS, several retrospective studies document an increased incidence of pathological behavior in children exposed to maternal stress *in utero*. Stressful situations with longterm detrimental effects include familial and marital conflicts (Stott 1973), death of the husband (Huttunen and Niskanen 1978), threat related to wartime (Meijer 1985), an intense earthquake (Watson *et al.* 1999), and job loss (Schneiderman *et al.* 2005). Furthermore, depression and anxiety disorders during pregnancy represent stressful states as well. Importantly, women in their childbearing years are at an increased risk to develop these disorders (Kessler *et al.* 2005). Recent studies revealed that 10% of pregnant women suffer from depression and 18% show at least some symptoms (Heron *et al.* 2004; Marcus 2009), while anxiety affects approximately 13% of pregnant mothers (Heron *et al.* 2004).

Prenatal maternal stress has been associated with attention-deficit/hyperactivity disorder (Clements 1992), Gilles de la Tourette's syndrome (Pasamanick and Kawi 1956), schizophrenia, autism, reduced

cognitive ability, anxiety and depression (Stott 1973; Huttunen and Niskanen 1978; Meijer 1985; Ward 1991; van Os and Selten 1998; Watson *et al.* 1999; DiPietro 2006; Talge *et al.* 2007). In view of the mechanisms that underlie the relationship between PS exposure and adult psychopathology in humans, PS has been linked with an elevated incidence of congenital malformations (Hansen *et al.* 2000), lower birth weight, smaller head circumference (corrected for birth weight) and preterm delivery (Stein *et al.* 1987; Pagel *et al.* 1990; Hedegaard *et al.* 1993; Wadhwa *et al.* 1993; Lou *et al.* 1994; Copper *et al.* 1996; Hedegaard *et al.* 1996; Ruiz *et al.* 2002; Rondo *et al.* 2003; Torche 2011; Coussons-Read *et al.* 2012).

In animals, antenatal stress has been linked to a reduced birth weight and a greater risk to become ill or die before weaning (Cabrera *et al.* 1999). Moreover, PS is associated with hyperglycaemia and glucose intolerance (Lesage *et al.* 2004), an altered immune response (Coe and Lubach 2005), a disturbed HPA axis (see e.g. review by Weinstock 2005), deficits in learning and memory (Hayashi *et al.* 1998; Gue *et al.* 2004), elevated anxiety levels (Griffin *et al.* 2003; Dickerson *et al.* 2005; Estanislau and Morato 2005; Van den Hove *et al.* 2006; Heiming *et al.* 2009; Laloux *et al.* 2012) and increased depression–related behavior in later life (Secoli and Teixeira 1998; Morley-Fletcher *et al.* 2003; Morley-Fletcher *et al.* 2003; van den Hove *et al.* 2011).

The route by which PS regulates the development of the fetus and newborn is not entirely clear. In general, it has been proposed that PS results in a permanent hypersensitization of the brain to following stressful situations (Weinstock 1997; Weinstock 2005). Most of the studies placed reprogramming of the HPA axis into the spotlight as its regulation and response to stress is disturbed in offspring exposed to PS. Therefore, their reaction to adverse life events might be inappropriate. Next to changes in the HPA axis, a decreased nutrient and oxygen supply as a result of a (stress-dependent) reduced blood transfer through the placenta have been described (see review by Huizink *et al.* 2004).

1.2.1 HPA axis

The body aims to maintain a complex equilibrium (homeostasis), which is constantly disturbed by stressors (Habib *et al.* 2001). It is very important that an organism is able to react properly to these disruptions and adapts itself to its environment (McEwen 1999), a process which is also called allostasis (McEwen 2003). In mediating these adaptive responses, the HPA axis plays a major role.

In response to stress, higher brain regions like the hippocampus, the cortex and the amygdala activate two important stress systems (see Figure 1-3). Firstly, the very fast acting sympathetic-adrenal-medullary axis is activated, which has the catecholamines adrenalin and noradrenaline as its effectors. Secondly, HPA axis activation results in a release of corticotrophin releasing hormone (CRH) into the hypophyseal portal vessel by the hypothalamic paraventricular nucleus (PVN). CRH, together with the antidiuretic hormone arginine vasopressine (AVP) that is also released by the PVN, stimulate the production and release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary gland into the circulation (Whitnall 1989). From there, ACTH reaches the adrenal cortex. Along this road, CRH and ACTH control the production and secretion of glucocorticoids, i.e. cortisol in humans and nonhuman primates and corticosterone in rodents, from the adrenal cortex. Furthermore, CRH activates noradrenergic neurons in the locus coeruleus, which in turn may activate the hypophysial adrenal axis and therefore may stimulate the sympathetic nervous system (Valentino *et al.* 1983; Rassnick *et al.* 1994). Finally, the glucocorticoids and catecholamines lead to an elevation of hepatic glucose synthesis and secretion, as well as an elevation of cardiovascular muscle tone (Sapolsky and Meaney 1986; Herman *et al.* 1996) in order to meet the threatening situation. On the other hand, reproduction, growth and certain components of the immune system are inactivated (Munck *et al.* 1984; Sapolsky *et al.* 1986; Herman *et al.* 1996). In the short run, these adaptations in response to stress are generally positive. However, if the stress systems are overactive for too long this can lead to damage or a so called “wear and tear on the body and brain (allostatic load)”, which in turn may lead to psychopathology (for review see McEwen 2003).

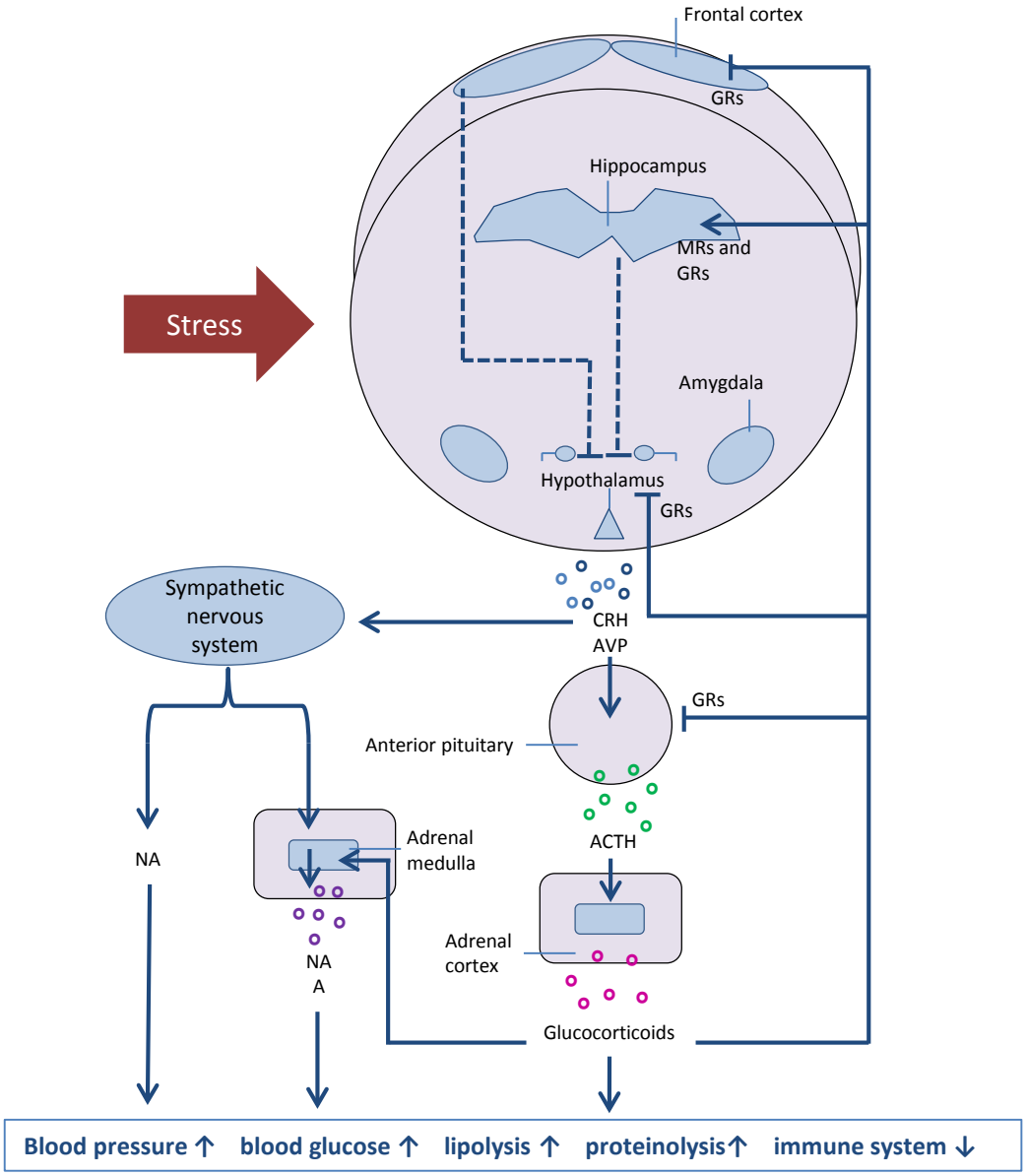


Figure 1-3 The two most important stress axes: the sympathico-adrenal-medullary axis and the hypothalamo-pituitary-adrenal (HPA) axis (modified from Lupien *et al.* 2009); After stress is perceived by higher brain regions, the sympathetic-adrenal-medullary axis and the HPA axis are activated. As a very fast response the sympathetic nervous system releases noradrenaline (NA) in the brain and activates the adrenal medulla to release NA and adrenaline (A). In addition, the slower but more perpetuate response from the HPA axes is initiated. The hypothalamus releases corticotrophin releasing hormone (CRH) and vasopressin (AVP). Subsequently, ACTH is secreted from the anterior pituitary to activate the release of glucocorticoids from the adrenal cortex. NA, A and glucocorticoids activate physiological responses and decrease immune system responses. As a negative feedback, glucocorticoids stimulate the glucocorticoid receptors (GR) at several levels, which, in turn inhibit the further production of glucocorticoids and therefore normalize the HPA axis.

For a proper regulation of the HPA axis, a negative feedback mechanism exists, which acts at several levels, namely at the hippocampus, hypothalamic PVN, and the pituitary gland. The feedback system

is mediated by the two corticosteroid receptors, i.e. the mineralocorticoid receptor (MR, type 1) and the glucocorticoid receptor (GR type 2) (Reul and de Kloet 1985). It is suggested that the high affinity-MR mainly acts at basal stress levels whereas the low-affinity GR acts when the organisms is stressed (De Kloet *et al.* 1998). An impaired negative feedback could stem from stress-induced damage to the hippocampus (McEwen 1999), or from a downregulation of GR in the PVN, pituitary gland and hippocampus (Prickaerts 2005).

1.2.2 Implication of the HPA axis activity in pregnancy

HPA axis regulation of pregnant women deviates slightly from non-pregnant women. Pregnant women have an ever increasing rate of plasma CRH concentration because the placenta releases CRH gradually into the maternal and fetal compartments across gestation (Petraglia *et al.* 1996; Weinstock 2005). In contrast to the brain, CRH release from the placenta is not inhibited but activated by cortisol in a positive feed-forward mechanism (Robinson *et al.* 1988; Majzoub and Karalis 1999). Thus, gestation can be considered as a situation of smooth and perpetuated hypercortisolism (McLean and Smith 1999). Importantly, the placenta acts like a barrier to secure the future child from pathologically high levels of CRH. It does so by means of binding and inactivating CRH through the CRH binding protein (CRH-BP). Towards the end of pregnancy the concentration of CRH-BP decreases thereby elevating free maternal CRH-BP (Perkins *et al.* 1995), which might initiate parturition (for review see Grammatopoulos 2008). Further, another protective mechanism concerning maternal cortisol/corticosterone exists. The placenta expresses the enzyme 11-β-hydroxysteroid dehydrogenase type 2 (11-β-HSD-2), which changes cortisol/corticosterone into bio-inactive 11-keto forms, i.e., cortisone and 11-dehydrocorticosterone (White *et al.* 1997). Despite its buffering capacity, there is still a linear relation between maternal and fetal cortisol levels (Gitau *et al.* 1998), which might be an issue in stressful situations (see below).

1.2.3 Reprogramming of the HPA axis by maternal stress exposure

Although the reactivity of pregnant mothers to acute stress seems to be mitigated, they display clear physiological reactions to stress, the extend of which differs between individuals (de Weerth and Buitelaar 2005). Generally, high levels of stress lead to increased CRH and ACTH levels in pregnant women (Wadhwa *et al.* 1996; Weinstock 2005) indicating that the suppressive mechanisms of HPA-feedback, which usually protect the organism from abnormal high levels of free CRH, may be impaired by prolonged stress. Though glucocorticoids are vital for developmental organizational

processes they have become major candidates when it comes to an impaired programming of the fetal HPA axis. For example, 10-year-old children of mothers with gestational anxiety showed a significant correlation between the degree of maternal anxiety and waking levels of plasma cortisol (O'Connor *et al.* 2005). In rat studies, antenatal stress leads to an increase of corticosterone levels in the mother and the fetus (Dauprat *et al.* 1984; Cadet *et al.* 1986). Furthermore maternal brain GR receptors may decline in sensitivity and number after chronic or repeated stress during pregnancy, which is supposed to lead to a dysfunction in maternal HPA axis regulation (see above). Of note, mothers that are determined to deliver preterm have higher CRH concentrations at mid-gestation than mothers that deliver at term (McLean *et al.* 1995). Abnormally high concentrations of maternal hormones can in turn reach the fetus and influence its development. Possibly, a hyperactive HPA axis of the mother may lead to fetal growth retardation, preterm delivery, and disturbed HPA axis function in the offspring (Weinstock 2001; Huizink *et al.* 2004; Weinstock 2005).

As already stated above, the future child is normally protected from high concentrations of CRH and glucocorticoids by factors like CRH-BP and 11-β-HSD-2. These protective mechanisms, however, might fail when they have to cope with high levels of stress-induced CRH and glucocorticoids. Furthermore, next to variability in placental 11-β-HSD-2 (Welberg *et al.* 2000) activity, PS has been shown to decrease placental 11-β-HSD-2, which might be another mechanism in fetal HPA axis programming (O'Donnell *et al.* 2012). In line with this, low 11-β-HSD-2 activity is linked with disturbed fetal growth (Benediktsson *et al.* 1997).

1.3 Gene x environment interactions

Emotional disorders may result from a complex interaction between an individual's genetic make-up and environmental factors. The hypothesis of gene x environment (G x E) interaction is based on the assumption that subtle genetic differences lead to different susceptibilities to environmental cues. This might be an explanation why some people are at a greater risk to develop a depression or an anxiety disorder after an environmental insult.

Research on G x E interactions in psychiatry has started with epidemiological studies using twins (e.g. (Cantor-Graae *et al.* 1994)). Although studies like these suggest that psychiatric diseases and normal behavior depend upon both environmental and genetic variation, they are not able to explore the

underlying molecular mechanisms (Caspi and Moffitt 2006). The first study that suggested that genotype moderates the outcome of an adverse environmental event was published in 2002 (Bennett *et al.* 2002). One year later, Caspi and his group reported an interaction between the 5-HTT polymorphism and childhood maltreatment on depressive outcome (Caspi *et al.* 2003). They revealed that early-life stress (between age 3 and 11) could predict adult depression (between age 21 and 26) but only in s-allele carriers and not in ll-carriers. Many other studies have tried to replicate this G x E interaction by the use of different populations and different study designs (Caspi *et al.* 2010), but this interaction could not be replicated in all investigations and some meta-analysis in fact described a negative effect (Munafo *et al.* 2008; Munafo *et al.* 2009; Risch *et al.* 2009). There are several reasons for these discrepancies, for example the fact that timing and type of stress (Karg *et al.* 2011) or the definition of the outcome variable may be different. Uher and colleagues (2011) reported that s-allele carriers exposed to early-life stress displayed an increased risk for (persistent) depression diagnosed at at least two time points, but not for single-episode depression. Further, differences in genetic backgrounds could contribute to the discrepancies as many other polymorphisms may also form the basis for this G x E interaction. For example, polymorphisms in the CRH receptor 1 (CRHR1) gene (Bradley *et al.* 2008), the GR gene (Bet *et al.* 2009), the hsp co-chaperone of the GR (FKBP5) gene (Luijk *et al.* 2010; Roy *et al.* 2010), the 5-HT_{3A} receptor and brain derived neurotrophic factor (BDNF) genes (Gatt *et al.* 2010), the dopamine D2 gene (Hayden *et al.* 2010) and the oxytocin receptor gene (Thompson *et al.* 2011) have been suggested to interact with early-life stress to predict depression.

Moreover, besides depression, the 5-HTTLPR x early-life stress interaction may also predict other stress-related traits. By the use of functional magnetic resonance imaging (fMRI), several studies revealed that different brain regions of s-allele carriers display altered neural activity and connectivity in response to emotional stimuli (Hariri *et al.* 2002; Canli *et al.* 2005; Heinz *et al.* 2005; Pezawas *et al.* 2005; Canli *et al.* 2006; Canli *et al.* 2008). For example, children that carry the s-allele display increased excitability in various brain regions while watching sad movies compared to l-allele carriers (Fortier *et al.* 2010).

Rhesus macaques posses a repeat length variation (rh-5-HTTLPR) which is in structure and function orthologous to the human 5-HTTLPR (Lesch *et al.* 1997). That makes them attractive to study 5-HTT x early adversity interactions. Champoux and colleagues (2002) separated infants (sl and ll) from their mothers at day 1-3 after birth and reared them in a nursery facility. Infants carrying the s-allele

displayed decreased orientation, lower attentional capabilities and a heightened affective response compared to ll-carriers. This increased behavioral stress-reactivity of the sl-infants was more pronounced in the maternal separation group than in the mother-reared group. Further, the endocrine response to stress was assessed in female and male rhesus infants, that were reared maternally or only in peer groups. ACTH and cortisol plasma levels were measured several times during the first 6 month of age at baseline and after a 30 min separation from the mother or peer group. Male infants with the s-variant of the rh5HTTLPR had higher ACTH levels when compared to monkeys homozygous for the l-variant. ACTH levels increased during separation and s-allele carriers that were reared among peers displayed higher ACTH levels during separation than the ll-monkeys. In addition, cortisol levels increased during separation and infants of the peer-only group showed diminished cortisol levels. Interestingly, especially female monkeys with the s-allele had lower cortisol levels in response to stress and increased ACTH responses to stress, but only in those females with a history of adversity. These sex differences may explain why women are more prone to stress and have a higher incidence of stress-related disorders (Barr *et al.* 2004). Overall, these findings resemble human studies, confirming that impaired function of the 5-HTT influences the HPA axis and that the effect of the rh5HTTLPR on hormonal responses to stress is moderated by adverse early life events in a sex dependent manner (O'Hara *et al.* 2007; Mannie *et al.* 2009; Wust *et al.* 2009).

Carroll and colleagues (2007) were the first who studied the interaction of early-life stress and the 5-HTT gene in mice. From postnatal day 7 on over a period of one week, mouse pups received three mild footshocks (150 s) per day. In adulthood, anxiety and depression-related behaviors were assessed using the elevated plus-maze, light/dark test, open field and FST. This type of stress was not able to alter the enhanced anxiety- and depression-like phenotype of the 5-Htt deficient mice. In another study, pregnant and lactating female mice, which were heterozygous for the 5-Htt, were exposed to an unfamiliar male bedding (Heiming *et al.* 2009). This olfactory cue was considered to be dangerous for the mothers as it indicates infanticide by the unfamiliar male. The offspring of mothers who lived in a threatening environment displayed elevated anxiety-like and diminished exploration behavior compared to controls. These effects were more pronounced in 5-Htt-/- mice. Another study examined the interaction of the 5-HTT gene and maternal neglect during the first weeks of life (Carola *et al.* 2008). Offspring suffering from low maternal care displayed deficient gamma-aminobutyric acid-A (GABA-A) receptor binding in the amygdala. Further, 5-Htt+/- mice were more anxious, showed more signs of depression-like behavior and diminished 5-HT turnover in the hippocampus and striatum compared to controls. Interestingly, Bdnf mRNA concentrations in the

hippocampus were only increased in 5-Htt+/- mice suffering from low maternal care. This may indicate that Bdnf is a common neuronal substrate of the 5-Htt x maternal neglect interaction.

Together with the findings in rhesus macaques it is tempting to speculate that the 5-HTT plays a pivotal role in modifying the long-term behavioral effects of the early life environment and that the underlying molecular mechanisms of this GxE interaction identified in animal models are important in the developmental programming of adult psychopathology (for review see Lesch 2011).

1.4 Resilience

Resilience is the ability to cope with stressful situations and not to succumb to adversity. Thus, resilient individuals show only few symptoms of psychiatric diseases like depression and anxiety disorders when compared to susceptible subjects. There are several psychosocial factors which allow resilient people to bounce back after stressful situations. For example, active coping strategies like problem solving and planning increase well-being. Also physical exercise can be considered as an active coping mechanism as it may lead to e.g. improved mood and dampened responses to stress (Southwick *et al.* 2005). Further, resilience requires optimism and high positive emotionality including humor, hope, joy, contentment, pride and love (Tugade and Fredrickson 2004; Ong *et al.* 2006). Such an attitude promotes adaptive coping, openness to social support (Ong *et al.* 2006), cognitive flexibility, exploration and a broadened focus of attention (Fredrickson 2001). Resilient individuals are characterized to have a sense of purpose in life, a moral compass, spirituality and the ability to find meaning in the midst of adverse events.

The underlying physiological, neurobiological and molecular mechanisms of resilience have just begun to be elucidated as the focus so far has been more on the negative effects of stress (i.e. vulnerability to psychopathology). For example, resilient individuals are thought to activate the stress response very fast but also terminate it efficiently. Therefore, a proper function of negative feedback of the HPA axis is needed, including optimal function and balance between MR and GR (Charney 2004; de Kloet *et al.* 2005; de Kloet *et al.* 2007). Further, dehydroepiandrosterone (DHEA; also secreted in response to stress) sulphate/cortisol levels might indicate an individual´s buffering capacity to stress. For example, soldiers who had higher DHEA levels during acute stress (rigorous survival training) displayed lower dissociative symptoms and performed better in the military when

compared to their companions (Morgan *et al.* 2004). Also male veterans with posttraumatic stress disorder (PTSD) display higher DHEA when symptoms improve (Yehuda *et al.* 2006). In this context neuropeptide Y (NPY) seems to play an important role as well. NPY shows anxiolytic-like effects and is supposed to increase cognition during stress exposure. Furthermore, it has opposing effects as compared to CRH when it comes to anxiety. Thus, resilient behavior might include a balanced NPY/CRH level during stress exposure (Sajdyk *et al.* 2004). An indication for that might be that soldiers with higher NPY levels performed better in the survival training (Morgan *et al.* 2000) and veterans without PTSD display higher plasma NPY levels as compared to those with PTSD (Yehuda *et al.* 2006).

Again, animal models help to provide insights into the underlying molecular mechanisms. Although not all psychological characteristics can be assessed in animals, they display traits which can be referred to as vulnerable or resilient responses to stress. For example, active coping or fight-or-flight responses like attempts to escape or aggression are considered as resilient. On the other hand, freezing and submission are passive reactions to stress reflecting a more vulnerable phenotype (Korte *et al.* 2005). A well-known mouse model for resilience is e.g. the social defeat stress paradigm. Krishnan and colleagues stressed C57BL/6 mice by introducing them into an area of a more aggressive CD1 mouse for 10 consecutive days (Krishnan *et al.* 2007). During 10 min of interaction with the aggressor the C57BL/6 mice were attacked and showed subordinate posturing. When tested on day 11, resilient mice displayed less social avoidance measured by the time spent in the interaction zone while a social target is present versus the time spent in the interaction zone without such a target. Interestingly, the group of Vialou found later, that ΔFosB is needed to show resilient behavior to social avoidance (Vialou *et al.* 2010). Furthermore, Schmidt and colleagues exposed mice to an unstable hierarchical situation during adolescence and the young adult period (Schmidt *et al.* 2010). Five weeks after the end of the stress exposure, basal morning corticosterone was measured. In comparison to vulnerable mice, resilient animals recovered faster from the stress (lower corticosterone levels). Further, they found that vulnerable subjects showed a higher number of AMPA receptor availability. Next to this, Shishkina and colleagues tested whether rats are resilient to the development of depression-like behavior in the FST. 40% of the animals showed resilience and had increased anti-apoptotic B cell lymphoma like X (Bcl-xl) to pro-apoptotic Bcl2-associated X (Bax) ratios in the hippocampus (Shishkina *et al.* 2010). Moreover, the work from Meaney and colleagues demonstrated that a positive environment can foster resilience. Offspring that experienced high licking-and-grooming and arched-back nursing displayed decreased anxiety and cared more about their own pups. Further, they showed dampened corticosterone responses to stress (for review see

(Meaney and Szyf 2005)). Another study used rough-and-tumble play, which elicits hedonic ultrasonic vocalizations (USVs) in rats, as a positive social environment (Burgdorf *et al.* 2010). They observed that this positive interaction was associated with an increase in insulin like-growth factor I (IgfI).

As indicated above, susceptible and resilient responses to stress can be very different in genetically identical rodents within a highly controlled environment. This makes it tempting to speculate that, genetic and environmental influences as well as their interactions are based on epigenetic mechanisms when it comes to variability in behavior.

1.5 DNA methylation and epigenetic programming

Epigenetic mechanisms represent heritable changes in gene expression caused by mechanisms other than variation in the underlying DNA sequence. These mechanisms are thought to play a crucial role in the interplay of genetic and environmental factors in determining a subject's phenotype. Many of those structural adaptations, which all act in concert to regulate gene transcription, have now been identified (e.g. DNA methylation, histone modifications and non-coding RNAs). Generally, gene transcription can occur if the chromatin (i.e. the combination of DNA, histones and other proteins associated with the DNA) is in an active, open state (euchromatin). An inactive, closed state (heterochromatin) does not allow gene transcription and results in gene silencing (for review see Tsankova *et al.* 2007).

DNA methylation is one of the best studied epigenetic modifications. The DNA is methylated at cytosine (C) residues (5-methylcytosine; 5mC) by DNA methyltransferases (Dnmt) (Yoder *et al.* 1997). Although not exclusively, DNA methylation takes place at loci where a C is followed by a guanine (G), i.e. so called CpG dinucleotides (Suzuki and Bird 2008; Lister *et al.* 2009). The frequency of CpG dinucleotides is unexpectedly low in mammalian genomes. This is due to the evolutionary loss of CpGs by *de novo* methylation of CpGs followed by spontaneous deamination of the C which transforms into a thymine (T) (Zemojtel *et al.* 2011). When a DNA sequence of at least 300 bp has a C+G content greater than 0.55, it is called a CpG island (Aerts *et al.* 2004). Generally, CpG islands are unmethylated and are associated with 75% of human genes (Ioshikhes and Zhang 2000). Recent studies showed that only approximately 50% of the CpG islands mark promoters of 5' regions of

protein-encoding genes, whereas the other CpG islands are found in gene bodies (in the transcribed regions), sometimes in promoter sequences of regulatory RNAs and in regions of unknown function (Illingworth *et al.* 2008). Usually, methylation of the promoter region leads to gene silencing (see Fig. 1-4) (Jaenisch and Bird 2003), whereas recent studies have shown that gene-body methylation results in an increase of gene expression (Hellman and Chess 2007; Backdahl *et al.* 2009). These effects are brought about by the recruitment of methyl-CpG-binding proteins (MBD such as MeCP2), histone modifying enzymes (e.g. histone deacetylases; HDAC) and microRNAs (see Fig. 1-4), which mediate chromatin remodeling and associated alterations in gene expression.

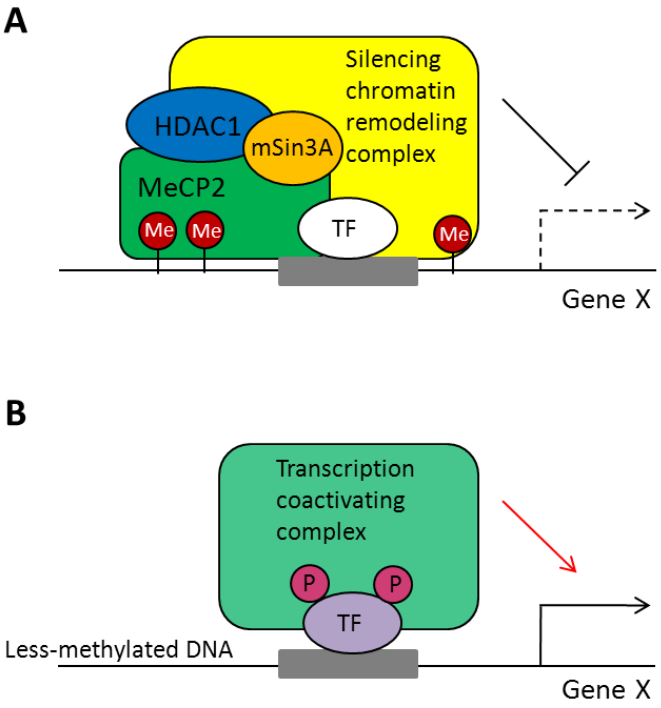


Figure 1-4. DNA methylation-mediated regulation of gene transcription A) DNA methylation is suggested to recruit methyl-CpG binding proteins (MBD) such as MeCP2, which in turn recruits histone deacetylases (HDACs) by the corepressor mSin3A to form a complex that mediates inactive chromatin remodelling. As such, transcription factors may not bind leading to gene silencing. B) When the DNA is less methylated, MeCP2 dissociates, so that transcription factors can bind and gene transcription is possible. (Modified from Martinowich *et al.* 2003)

Recently, several studies demonstrated that DNA methylation is negatively correlated with expression. For example, increased hippocampal GR expression in rats, which were exposed to high levels of maternal care, is associated with a reduced DNA methylation within the GR exon 1_7 promoter in the hippocampus, as well as elevated histone acetylation (Weaver *et al.* 2004). By these

epigenetic modifications, the binding of the transcriptional activator nerve growth factor 1 (NGF1A) to the GR promoter is eased (Weaver *et al.* 2007), which might be a plausible mechanism for the epigenetic programming of gene function by a caring mother early in life (Dudley *et al.* 2011). Interestingly, hippocampal GR mRNA was decreased and promoter DNA methylation was increased in suicide victims with a history of childhood abuse (McGowan *et al.* 2009). Although this is an indirect correlation, one can speculate that the epigenetic modifications involved in mediating stress vulnerability in rodents can be translated to humans. Another study shows that the elevated stress sensitivity of mice, that were maternally separated, is associated with increased expression of AVP in the hypothalamus and decreased levels of DNA methylation at particular CpG dinucleotides within an AVP enhancer region that is important for the binding of MeCP2 (Murgatroyd *et al.* 2009). Next to maternal care, also PS exposure seems to have an effect on epigenetic programming. For example, Mueller and Bale (2008) stressed pregnant mice during the first week of pregnancy and found elevated DNA methylation at GR and CRH promoter regions in the hippocampus of male offspring. However, less is known about the molecular mechanisms of early-life stress in 5-Htt+/- mice.

2. Materials and methods

2. Materials and Methods

This series of experiments consisted of two different mouse studies. In the first, 5-Htt x PS study, WT and 5-Htt+/- deficient mice were exposed to prenatal restraint stress in order to assess the behavioral effects of PS exposure in male and female offspring. Brains of these animals were used to screen for associated gene expression and DNA methylation changes. In a second study, brain material derived from another, 5Htt x *perinatal* stress (PeS) paradigm was used to screen for expression changes in candidate genes derived from the PS study.

2.1 Prenatal stress exposure

2.1.1 Animals and ethics

Animal handling and behavioral studies were performed in collaboration with Daniel van den Hove and his colleagues from the Maastricht University. This study was approved by the Animal Ethics Board of Maastricht University, The Netherlands (Permit number: OE 2007-109). All efforts were made to minimize suffering.

For breeding, acclimatized female 5-Htt+/- and male 5-Htt+/- deficient mice ([B6.129(Cg)-Slc6a4tm1Kpl/J] (Bengel *et al.* 1998)) were utilized. The mice were housed in individual cages. The temperature was controlled (21±1°C) and a 12 h light/12 h dark cycle (lights on from 7.00 h) was set. Standard rodent chow and water was available *ad libitum*. Pregnancy was determined by observation of vaginal plugs (embryonic day 0 – E0). Prenatal maternal stress (n=15) was performed daily during the last part of pregnancy (E13–E17). The dams were restraint in transparent 250 ml glass cylinders filled up to a height of 5 mm with water, whilst being exposed to bright light, 3 times daily (between 8.00 and 10.00 h, 12.00 and 14.00 h and 16.00 and 18.00 h), for 45 min per session (adapted from (Behan *et al.* 2011)). Control pregnant females (n = 14) were left undisturbed in their home cages and maternal weight was measured at E0, E12 and E17. To protect the litters for cannibalism, they were left undisturbed for 5 days after birth (P5). Only litters of 5 or more pups were included in the present study. Genotyping was performed by using polymerase chain reaction (PCR). DNA-fragments of either 225 bp refer to 5-Htt+/+, 272 bp to 5-Htt-/- or both to 5-Htt+/- mice. Offspring were individually housed in ventilated cages (TouchSLIMLine, Techniplast, Italy) after weaning (P25) under a reversed day-night cycle (12 h light/12 h dark cycle; lights on from 19.00 h). Pup mortality was monitored from P5 onwards. No more than two male and/or two female pups per litter were used to

prevent litter effects (Chapman and Stern 1979). Behavioral experiments were started when the offspring reached the age of 2 months (P60) (n=10-14/group). First, memory abilities were measured using the object recognition task (ORT). Next, anxiety- and depression-like behavior were assesed using the elevated zero maze (EZM) and forced swim task (FST), respectively. Tests were always performed in the dark phase (between 9.00 and 17.00 h for the ORT and between 9.00 and 13.00 h for the other tasks). Males and females were tested in all experiments separately. One week after behavioral tests, stress-induced plasma corticosterone (CORT) secretion was examined. One week later, the mice were sacrificed and brains removed. In addition, the adrenals were removed and weighted. Brains and blood samples were immediately placed on dry ice and stored at -80°C for future experiments.

2.1.2 Behavioral and physiological assessments

The behavioral studies were performed in collaboration with Daniel van den Hove and his colleagues at the Maastricht University.

Object recognition task (ORT)

Object recognition memory with mice was performed as described elsewhere (Sik *et al.* 2003). The apparatus consisted of a circular arena, 43 cm in diameter. The test was performed with a constant illumination of approximately 20 lux. Two objects were placed symmetrically 5 cm away from the wall. Four objects were used: (1) a cone made of brass (maximal diameter 6 cm and total height 3.8 cm), (2) a transparent glass bottle (diameter 2.7 cm, height 8.5 cm) filled with sand, (3) a metal cube (2.5 cm× 5 cm× 7.5 cm) with two holes (diameter 1.5 cm), and (4) an aluminium cube with a tapering top (4.5 cm × 4.5 cm × 8.5 cm). Each object was available in triplicate. In the first week, the animals were handled daily and were allowed to explore the arena, twice for 5 min each day, without any objects. Next, the mice were assessed until they showed a good discrimination performance. A testing session comprised of two trials. The duration of each trial was 5 min. During the first trial (T1) the apparatus contained two identical objects. A mouse was always placed in the apparatus facing the wall at the middle of the front (transparent) segment. After the first exploration period, the mouse was put back in its home cage. Subsequently, after a predetermined delay interval (2, 3 or 4 h), the mouse was put back in the apparatus for the second trial (T2). However, now there were two dissimilar objects, a familiar one and a new one. The times spent exploring each object during T1 and T2 were recorded manually using a personal computer. Exploration was defined as follows: directing

the nose to the object at a distance of no more than 2 cm and/or touching the object with the nose. Sitting on the object was not considered as exploratory behavior. In order to avoid the presence of olfactory cues the objects were always thoroughly cleaned with 70% ethanol before each trial. All combinations and locations of objects were used in a balanced manner to reduce potential biases due to preferences for particular locations or objects. Each delay interval was tested once in each animal. At least a two-day period was in between any delay test sessions with a particular animal. The testing order was determined randomly. The relative discrimination index (RDI) ([time spent on new object in the second trial T2 - time spent on familiar object in T2]/total exploration time during T2) was determined for all mice.

Elevated zero maze (EZM)

The EZM is a task to measure anxiety-like behavior (Shepherd *et al.* 1994). The test was conducted on a maze constructed of black plastic, transparent for infrared light. The circular runway was 50 cm in diameter, with a pathway width of 5 cm placed 10 cm above floor level. The maze was equally separated in 2 opposite open and 2 opposite closed parts enclosed by 50 cm high side walls. To prevent falls, a 5 mm high rim lined the open parts. A mouse was placed into the middle of one of the open parts, facing the outside of the maze. The mouse was allowed to explore the arena for a 5 min period. The distance travelled and % of time spent in the open parts of the maze was determined under low light conditions (20 lux) by use of an infrared video tracking system (Ethovision Pro, Noldus, Wageningen, The Netherlands; van Donkelaar *et al.* 2010). In order to avoid the presence of olfactory cues the arena was always thoroughly cleaned with 70% ethanol before each trial.

FST

The FST is commonly utilized to score behavioral despair in rodents (Borsini and Meli 1988; van Donkelaar *et al.* 2010). Animals were individually placed in a transparent perspex cylinder (40 cm tall; 19 cm in diameter; filled to a height of 15 cm with water of 31°C; (van Donkelaar *et al.* 2010)). Distance moved, as an indicator of mobility of the mice was measured in a 5 min session using a computerized system (Ethovision Pro, Noldus, The Netherlands).

CORT response

When the offspring used in the behavioral testing reached an age of 3 months (P90) a blood sample was taken from the saphenous vein (basal CORT level). Subsequently, these mice were subjected to 20 min of restraint stress by a procedure identical to the PS procedure applied to the dams. Immediately following restraint stress, a second blood sample was taken (stress-induced CORT level). The mice were then returned to their home-cage for a 40 min recovery period, after which a third and final blood sample was taken ('recovery' CORT level). Blood collection, sample preparation and determination of plasma CORT levels were done as described in detail previously (Van den Hove *et al.* 2006). All blood samples were taken between 10:30 -13:00 h.

2.1.3 Expression study

RNA isolation and microarray

For this purpose, the left half of the hippocampus of females and males was used. The tissue was homogenized using 500 µl PegGOLD RNAPure (Peglab, Erlangen, Germany) and metal beads for 3 min at 20Hz in a Tissue Lyser (Qiagen, Hilden, Germany)). Subsequently, 100 µl chloroform was added and samples were centrifuged for 5 min at 4° C and 12.000 x g. The water phase was then mixed with 250 µl ethanol and from that point on the protocol of the RNeasy Mini kit (Qiagen, Hilden, Germany) was followed. RNA-quality was checked by the use of Experion (Bio-Rad, Munich, Germany). Afterwards, the RNA of female samples was pooled according to performance in the FST, creating 3 pools per group. Prior to hybridization, RNA integrity and comparability were tested by a BioAnalyzer (Agilent Technologies, Palo Alto, CA). RNA integrity numbers (RIN) of all RNAs was between 8.3 and 8.6. cDNA synthesis, labelling and the actual microarray analysis were performed by the Interdisciplinary Centre for Clinical Research (IZKF) at the University of Wuerzburg. Generation of double-stranded cDNA, preparation and labelling of cRNA, hybridization to GeneChip® Mouse Genome 430 2.0 Arrays (Affymetrix, Santa Clara, CA) and washing were performed according to the standard Affymetrix protocol. The arrays were scanned using a GeneChip® Scanner 3000 (Affymetrix Santa Clara, CA). Data analysis was performed using different R packages from the Bioconductor project (www.bioconductor.org). Probe sets were summarized using the PLIER algorithm. Resulting signal intensities (signal intensity from a specific probeset is referred to as the expression of the associated gene from here onwards) were normalized by variance stabilization normalization (VSN) (Huber *et al.* 2002). Quality and comparability of all data sets were tested by density plot, RNA degradation plot and correspondence analysis. All data is MIAME compliant and the raw data has been deposited in the Gene Expression Omnibus (GEO) (accession number: GSE26025).

Enriched pathway analysis

The Database for Annotation, Visualization and Integrated Discovery (DAVID) 2007 Functional Annotation Clustering was utilized to search the database of the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Dennis *et al.* 2003; Huang da *et al.* 2009) in order to identify significantly over-represented pathways in the subset of differentially expressed genes. More specifically, the latter is a curated pathway database comprising biological signaling pathways that are based on current knowledge of molecular interactions involved in various cellular processes. Settings used were: Count (2); EASE (0.1); $P<0.05$.

Quantitative real-time PCR

The validity of the microarray results was subsequently tested via quantitative real-time PCR (qRT-PCR) using the Bio-Rad CFX384 Real-Time PCR Detection System (in technical triplicates). For validation we selected 8 genes which showed a fold change (FC) > 1.5 (or 2.5) in the microarray (see below). The same RNA as for the microarray was utilized for cDNA synthesis which was performed by the use of the iScript™ kit (Bio-Rad, Munich, Germany) according to the manufacturer´s instructions. Mean efficiencies were calculated by LinReg (Ruijter *et al.* 2009). Reference genes for normalization were selected from the microarray and tested for stability using geNorm (Vandesompele *et al.* 2002). For normalization *CCCTC-binding factor (Ctcf), guanosine diphosphate (GDP) dissociation inhibitor 2 (Gdi2)* and *gap junction protein, alpha 1 (Gja1)* genes were used. Relative expression data were calculated with the normalization factors obtained from geNorm and the mean efficiencies from LinReg.

2.1.4 DNA methylation study

Methylated DNA immunoprecipitation followed by tiling array

For this study, the right part of the hippocampus of 16 5-Htt+/- and 14 WT female mice was utilized. Genomic DNA was extracted from the tissue using phenol/chloroform/isoamyl alcohol extraction. 300 µl 0.5%-SDS extraction buffer were added to the frozen tissue. The tissue was subsequently homogenized at 4°C in the Tissue Lyser (Qiagen, Hilden, Germany) using a metallic bead (30 sec – 1 min, 25 Hz, 4°C). After adding 200 µl 0.5% SDS extraction buffer and 50 µl proteinase K with a concentration of either 10 or 20 mg/µl , samples were incubated at 55°C over night/or 3 h. Following this, samples were incubated with 50 µl RNAse A with a concentration of 10mg/µl for 1 h at 37°C and then mixed with 700 µl of phenol/chloroform/isoamyl alcohol mixture (25:24:1). Phases were

separated using MaXtract high density tubes (Qiagen, Hilden, Germany) by centrifuging the samples at 14000 rpm for 5 min at RT. The aqueous phase was then mixed with 700 µl phenol isoamyl alcohol (24:1) and phases were again separated by centrifuging the samples in MaXtract high density tubes as described above. DNA was precipitated with 50 µl sodium acetate and 1000 µl cold absolute ethanol (incubation 10 min at -20°C, centrifugation 20 min at 4°C, 14 000 rpm). The resulting pellet was washed with 500 µl cold 80%-ethanol using the same conditions as for the precipitation, dried at RT for 5 to 30 min and resuspended in 50 µl 1xTE. DNAs of single animals were then pooled, creating 3 pools per group. To gain fragments of 300 +/- 200 bp size, DNA was sheared by the BiorupterTM UCD-200 (Diagenode, Liège, Belgium). Conditions were 20 KHz, 30 sec ON alternated by 30 sec OFF, for 3 x 5 min at low power. Efficiency of the shearing was controlled with the Bioanalyzer 2100 (Agilent, Santa Clara, USA). Methyl DNA immunoprecipitation (MeDIP, Diagenode, Liège, Belgium) was performed as described in the Diagenode manual. IP samples were performed in duplicate, input sample once. 1000 ng of DNA were used for each IP and 100 ng for the input. DNA was then purified using IPure (Diagenode, Liège, Belgium) according to the manufacturer's manual and the resulting IP DNA samples belonging together were pooled. Pooled IP DNA and input DNA was then amplified using two-step Whole Genome Amplification (GenomePlex Kit, Sigma, St. Louis, USA). Amplification, labeling, hybridization, and the actual tiling analysis were performed by the Interdisciplinary Centre for Clinical Research (IZKF) at the University of Wuerzburg. 7.5 µg DNA from the second amplification round were fragmented and labeled using the GeneChip 10K Xba Assay kit (Affymetrix, Santa Clara, USA). The size of the obtained biotinylated cDNA fragments was controlled with the Bioanalyzer (Agilent, Santa Clara, USA) and was in between 25 and 250 bp (peak at 100 bp). Hybridization to GeneChip ® Mouse Promoter 1.0R Arrays and washing using the GeneChip® Expression Wash, Stain and Scan Kit (Affymetrix, Santa Clara, USA) were performed as described in the Affymetrix manuals. Arrays were scanned with the GeneChip® Scanner 3000 (Affymetrix, Santa Clara, USA). The promoter tiling array used in this study is comprised of over 4.6 million 25-mer probes tiled to interrogate over 28,000 mouse promoter regions. Each promoter region covers approximately 6 kb upstream through 2.5 kb downstream of 5' transcription start sites. The probes are tiled at an average resolution of 35 bp, leaving a gap of approximately 10 bp between probes.

Affymetrix quality metrics and visual inspection of overall microarray signals confirmed high-quality readout from the hybridized samples. Genomic locations of microarray probes were adjusted to the *Mus musculus* NCBI assembly version 37.1 (MMv37 thereafter). Probe signals from corresponding MeDIP and input samples were subjected to within-sample pairwise loess normalization and calculation of MeDIP-input signal $\log_2$ ratios (SLRs). Quantiles normalization was employed to ensure a common signal distribution between samples. To decrease the noise in the experiment readout, a

sliding-window approach was applied to determine SLR medians in successive genomic regions of 300 bp width. The SLRs were then found to be free from biases introduced by varying probe GC content and particular probe sequence compositions (data not shown), thus confirming successful data normalization. A correspondence analysis further confirmed the absence of extreme outliers in samples and/or SLRs.

2.1.5 Statistical analyses

Percentage of maternal weight increase over the last week of gestation was compared using a one-way ANOVA (condition). EZM and FST data were explored by three-way ANOVAs (genotype x condition x sex). Data on the ORT were analyzed using a repeated measures ANOVA, as well as by a separate analysis at the distinct time-points. Furthermore, for the ORT the RDI from every group was compared to an RDI of 0 (no discrimination) as described previously (Sik *et al.* 2003). CORT data were ln-transformed prior to ANOVA and were analyzed using a repeated measures ANOVA, as well as by a separate analysis at the distinct time-points. Overall interaction effects were examined in more detail using Least Significant Difference (LSD) tests. In the absence of an interaction, main effects of genotype and condition were analyzed by an additional stratified analysis – i.e. stratified per genotype and sex in case of a condition effect, in order to test whether overall effects were specific to, or more pronounced in, a particular genotype or sex. This was expected, since the 5-HTT genotype is known to have specific effects on various behavioral phenotypes and may selectively affect the interaction with stressful life events (see e.g. Homberg and Lesch 2011), whereas, in addition, PS is known for its sex-specific effects on offspring outcome (e.g. Behan *et al.* 2011). The failure to detect significant interactions using the three-way ANOVA approach may be explained by the relative conservative nature of F-tests in general, in combination with the intricate logistical experimental design (with its associated breeding restrictions), resulting in a relative lack of statistical power. Perinatal and post-weaning mortality were examined using a one-sided Fisher´s exact test.

Statistical analysis to choose differentially expressed genes was performed using the Linear Models for Microarray Analysis (LIMMA) package (Smyth and Speed 2003; Smyth *et al.* 2005). LIMMA is a library for the analysis of gene expression microarray data, especially concerning the use of linear models for analyzing designed experiments and the assessment of differential gene expression profiles. As an output a table of the top-ranked genes from the linear model fit including a gene list, ratio on the log2 scale, average gene intensities, moderated t-statistic, adjusted P-value (false discovery rate) and log odds were created. By using LIMMA we calculated the following differences:

between 5-Htt+/- (HET) and WT mice [(HETPS+HETC)-(WTPS+WTC)] (G effect), between PS and control (C) animals [(WTPS+HETPS)-(WTC+HETC)] (E effect) and the interaction of G and E [(HETPS-HETC)-(WTPS-WTC)] (G x E effects). Genes were identified as differentially expressed if they showed a nominal P-value less than 0.01. There was no "cut off" for the linear FC concerning the microarray data (FC of 1 indicates no change, while a FC of 2 equals a double amount of cRNA).

Results of the gene expression microarray were validated by means of qRT-PCR. For the G and E effects we considered only genes with a FC > 1.5 to achieve reliable validation. We further restricted the selection by only choosing genes with an annotation grade (see affymetrix.com) of A or B into account. For an exact validation, we used only those genes for which we were able to amplify the same sequence as recognized by the microarray. These criteria encouraged us to pick *FBJ osteosarcoma oncogene* (*Fos*) and *paired-like homeobox 2a* (*Phox2a*) out of the 15 genes altered >1.5 fold by PS. Of the 29 genes altered >1.5 fold by the 5-Htt+/- genotype we selected *XIAP associated factor 1* (*Xaf1*), *zinc finger*, *ZZ-type with EF hand domain 1* (*Zzef1*), *protein phosphatase 1, regulatory (inhibitor*) *subunit 1B* (*Ppp1r1b*), *Kv channel-interacting protein 2* (*Kcnip2*) and *myelin basic protein* (*Mbp*). In addition, we validated the G x E effect of the *thyrotropin releasing hormone receptor* (*Trhr*), as the expression of this gene between WTC and WTPS differed 2.5 fold. Gene expression data using qRT-PCR were analyzed by two-way ANOVA (genotype x condition) for males and females separately because we wanted to validate the microarray which was performed only with females. The level of statistical significance was assumed to exist at $P<0.05$ in all tests. Except for microarray data analysis, all statistical analyses were performed using the SPSS 20.0 software package.

In each sample of the DNA methylation array, genomic regions enriched by the MeDIP procedure were detected by the CMARRT algorithm (Kuan *et al.* 2008). Briefly, this does not apply a fixed threshold to all SLRs, but rather tests for increased signal content correcting for the signal autocorrelation in considered genomic regions, resulting in higher sensitivity and specificity of the detected enriched regions. For CMARRT modeling, the typical fragment length after DNA sonication was assumed to be 300 bp; enriched regions were required to cover at least five consecutive array probes and to display an enrichment statistic with a false discovery rate (FDR) less than 0.05. Of all detected regions, only those were consistently retained in the analysis that were found within each analysis group. With the present (=1) and absent (=0) calls for MeDIP enrichment, effect directions were determined as previously described (van den Hove *et al.* 2011). Briefly, genotype (G) effect directions (d) were calculated by G_d = ((HETC + HETPS) – (WTC + WTPS)) * 0.5, environment (E) effect

directions by E_d = ((WTPS + HETPS) – (WTC + HETC)) * 0.5 and interaction (GxE) effect directions by GxE_d = ((HETPS – HETC) - (WTPS – WTC)) * 0.5.

Preprocessing and analysis of Affymetrix tiling arrays were performed with R v2.15 along with the Bioconductor package Starr (Zacher *et al.* 2010). The package ChIPpeakAnno (Zhu *et al.* 2010) was used for annotation of enriched regions.

2.2 Perinatal stress exposure

2.2.1 Animals

The brains of animals used for this study were obtained from Dr. Rebecca Heiming and Prof. Dr. Norbert Sachser at the University of Münster. Animals, general housing conditions, olfactory stimulation of the mothers and the effects on the offspring are described in detail previously (Heiming *et al.* 2009). Briefly, pregnant and lactating 5-Htt+/- and WT mice (for this study the null mutant 5-Htt-/- mice were omitted) were treated with neutral bedding or bedding that stemmed from the cages of unfamiliar adult males. The mothers were treated every 2-3 days in the morning, starting at experimental day 8. The dams received five treatments during the gestational period and two to five treatments during lactation. We analyzed brains of the following number of offspring: 6 male control WT mice, 9 female control WT mice, 9 male control 5-Htt+/- mice, 9 female control 5-Htt+/- mice, 9 male WT mice with PeS, 8 female WT mice with PeS, 10 male 5-Htt+/- mice with PeS and 6 female 5-Htt+/- mice with PeS.

2.2.2 Statistical analysis

qRT-PCR data were analyzed by two-way ANOVA for females and males separately to match the analyses for the PS exposure.

2.3 Resilience to stress exposure

For the microarrays, statistical analysis to select differentially expressed genes was performed using the LIMMA package (Smyth and Speed 2003; Smyth *et al.* 2005). As an output a table of the top-ranked genes from the linear model fit including a gene list, ratio on the log2 scale, average gene

intensities, moderated t-statistic, adjusted P-value (false discovery rate) and log odds were created. By using LIMMA we calculated the changes in expression between all animals of the HETC group and the good or poor performers in the FST of the HETPS group as a representative comparison identifying specific molecular changes in resilient and vulnerable HETPS offspring, respectively. Further, we compared all WTC animals with the good or poor performers of the WTPS group in order to identify molecular mechanisms specific to resilient and vulnerable WTPS offspring, respectively (see Figure 2-1). Genes were identified as differentially expressed if they showed a nominal P-value of less than 0.01.

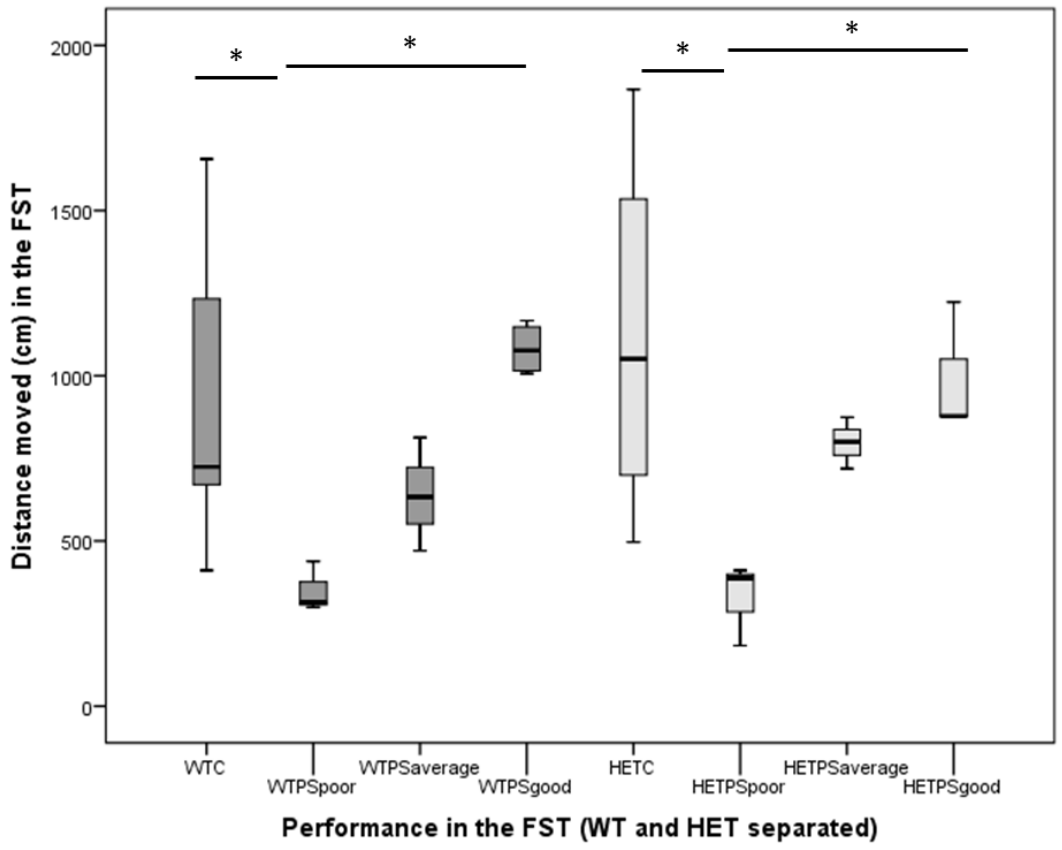


Figure 2-1. Performance in the forced swim test (FST). Wild-type (WT) and heterozygous (HET) prenatally stressed (PS) animals that performed poorly in the FST differ significantly from the respective controls (C). Further, poor and good performers of the respective group differ significantly (Mann-Whitney-U test without Bonferroni correction; n=3-10). * $p<0.05$

There was no "cut off" for the linear FC concerning the microarray data. From the microarray we picked following genes and performed qRT-PCR as explained above: *Growth hormone (Gh), prolactin (Prl), c-fos induced growth factor (Figf), synaptotagmin V (Syt 5)* and *calcium/calmodulin-dependent protein kinase II alpha (Camk2a)*. These genes were selected, because they showed a FC > 1.5 in WTPS or HETPS good performers. *Galanin receptor 3 (Galr3)* was chosen as the gene expression data obtained from the microarray correlated strongly with those of *Gh* and *Prl* (data not shown).

Additionally, we selected *protein kinase, cAMP dependent regulatory, type II beta (Prkar2b)*, because it was the only gene which was upregulated in the good performers and downregulated in the bad performers of the HETPS group. In general, we only picked genes which had an annotation grade (see affymetrix.com) of at least A or B and the ability to amplify the same sequence as recognized by the microarray.

Performance-oriented LIMMA analysis on the mRNA expression microarray data was followed by subsequent Spearman correlation analysis linking the individual mRNA expression data as obtained by qRT-PCR to behavioral performance as well as to neuroendocrinological measures. More specifically, the performance-based LIMMA analysis offers the opportunity to identify molecular mechanisms specific to either vulnerability or resilience to PS. The level of statistical significance was assumed to exist at $P<0.05$ in all tests. Except for microarray data analysis, all statistical analyzes were performed using the SPSS 20.0 software package.

3. Results

3. Results

3.1 Behavior

Dam weights during gestation and litter sizes

Increase of dam weight during pregnancy is depicted in Table 3-1. No differences in weight increase during the first two weeks of gestation between stressed versus unstressed dams were observed. Over the last week of pregnancy stressed dams gained significantly less weight when compared to control animals ($F_{1,25}$=25.024; P<0.001). Further, no differences were found in the litter sizes of stressed and control dams.

Table 3-1. Dam weight during pregnancy and litter size.

Condition	% Weight increase (E0-E12)	% Weight increase (E12-E17)	Litter size
C	33.78 ± 2.41	30.04 ± 1.16	7.08 ± 0.72
PS	33.44 ± 2.13	17.52 ± 1.94***	7.25 ± 0.66

During the last part of pregnancy (E12-E17), stressed (PS) dams gained significantly less weight compared to control (C) animals (***P<0.001). Data represent means ± S.E.M. N=12-15 litters/condition

Pre- and postweaning mortality

Preweaning mortality did not differ between the various groups (data not shown). A significantly higher postweaning mortality (P=0.04) was observed in PS offspring (7 out of 51 [13.7%] in PS animals vs. 1 out of 44 [2.3%] in controls; data not shown). Genotype had no effect on postweaning mortality.

Offspring cognition, anxiety, and depression-like behavior

Memory performance in the ORT is depicted in Table 3-2. Interval duration and recognition scores were negatively correlated ($F_{6,58}$=21.993; P<0.001). Further, an interval x genotype ($F_{6,58}$=24.851; P=0.027) and a condition x sex interaction ($F_{7,58}$=4.273; P=0.043) were observed, the latter of which indicated that PS particularly impaired memory performance in female offspring. When looking at the individual intervals, at the 2-hour interval, all animals were able to distinguish the old from the new object. Further, a significant condition x sex interaction was seen ($F_{7,60}$=6.518; P=0.013). Specifically, PS was associated with impaired memory performance in female offspring as post-hoc

analysis showed a significant decrease in memory performance in PS versus control females (P=0.049). At the 3-hour interval, among males, only WT controls were able to remember the old object. Further, all 5-Htt+/- groups still displayed intact memory performance. In line with this, a significant overall effect of genotype was observed at this interval ($F_{7,62}$=4.501; P=0.038), with 5-Htt+/- mice showing improved memory function as compared to WT animals. At the 4-hour interval, all groups displayed impaired memory performance. Of note, no differences in exploration times between groups were observed at any interval.

Table 3-2. Memory performance as assessed in the Object Recognition Test (ORT).

Group			2h	3h	4h	Exploration Time (sec)
WT	M	C	**0.286 ± 0.092**	**0.296 ± 0.066**	0.081 ± 0.074	18.16 ± 0.84
		PS	**0.452 ± 0.080**	0.195 ± 0.103	0.148 ± 0.066	17.83 ± 0.73
	F	C	**0.358 ± 0.078**	0.096 ± 0.065	0.225 ± 0.086	18.37 ± 0.83
		PS	**0.319 ± 0.082**	0.116 ± 0.102	-0.132 ± 0.123	17.98 ± 0.95
5Htt +/-	M	C	**0.355 ± 0.067**	**0.248 ± 0.070**	0.054 ± 0.085	18.58 ± 0.67
		PS	**0.436 ± 0.075**	**0.393 ± 0.056**	-0.014 ± 0.066	17.91 ± 0.74
	F	C	**0.509 ± 0.087**	**0.281 ± 0.062**	0.001 ± 0.099	18.13 ± 1.40
		PS	**0.217 ± 0.071**	**0.266 ± 0.098**	0.056 ± 0.093	17.66 ± 0.76

Bold data indicate intact memory performance, i.e., when animals were able to distinguish the old from the new object. At the 2-hour interval, a significant condition × sex interaction was observed (P=0.013). At the 3-hour interval, a significant overall effect of 5-Htt genotype was observed (P=0.038). See results section for more details. Data in the first three columns represent mean relative discrimination index (RDI) ± S.E.M. The last column shows the average exploration times (the average time (seconds) spent exploring each object during T1 and T2, averaged over the 3 intervals), which did not differ between groups. Abbreviations: WT, wild-type; M, males; F, females; C, control offspring; PS, prenatally stressed offspring. N=7-10 mice/group.

In Figure 3-1, the effects of PS on anxiety-like behavior in the EZM are shown. Time spent in the open arms of the EZM was significantly enhanced in 5-Htt+/- versus WT animals ($F_{7,64}$=4.466; P=0.038), indicating lower levels of anxiety in 5-Htt+/- offspring. In addition, females spent less time in the open arms of the EZM ($F_{7,64}$=20.091; P<0.001), indicating higher levels of anxiety in this sex. Distance covered within the EZM was decreased by PS ($F_{7,64}$=10.314; P=0.002). When stratifying the analysis per genotype, the observed PS effect was only significant in WT, but not in 5-Htt+/- offspring ($F_{3,29}$=8.343; P=0.007, versus $F_{3,30}$=2.493; P=0.123, respectively). Similarly, when stratifying for sex, the PS effect was only significant for males and not for females ($F_{3,34}$=7.199; P=0.011, versus $F_{3,30}$=3.527; P=0.070, respectively).

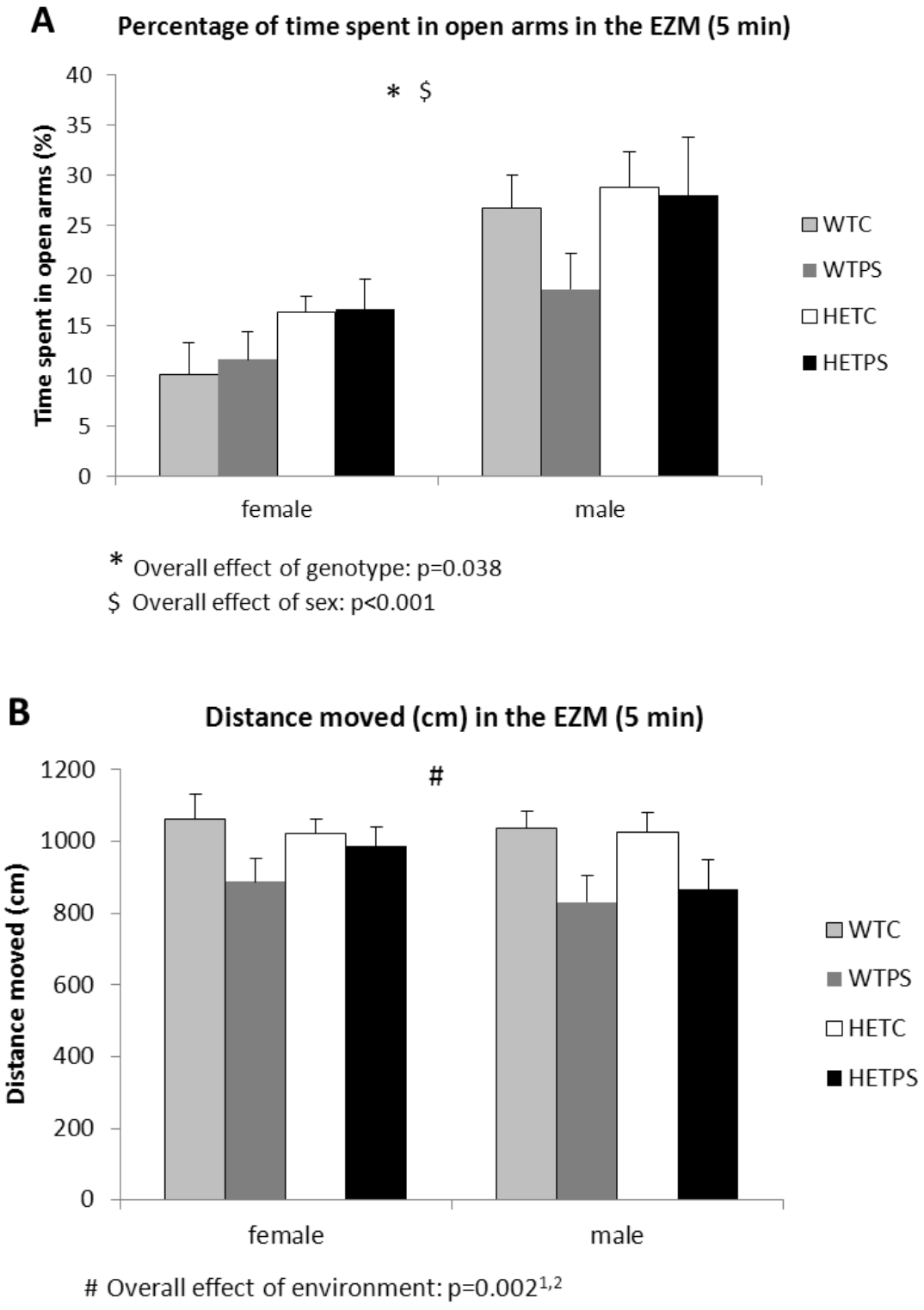


Figure 3-1. Performance in the Elevated Zero Maze (EZM). A) Time spent in the open arms of the EZM was significantly elevated in 5-Htt+/- versus wild-type (WT) animals (P=0.038). In addition, females (F) spent less time in the open arms of the EZM when compared to male (M) offspring (P<0.001). B) Distance covered within the EZM was decreased by prenatal stress (PS; P=0.002). Data represent mean + S.E.M. Abbreviation: C, control offspring. N=7-10 mice/group. [1]Only significant in WT offspring when stratified for genotype; [2]Only significant in male offspring when stratified for sex (see results section for more details).

Depression-like behavior in the FST is shown in Figure 3-2. Overall, a significant effect of condition was observed, indicating that PS animals exhibit more depression-like behavior ($F_{7,67}$=4.544; P=0.037). When stratifying the FST analysis per genotype, the observed PS effect was only significant in 5-Htt+/-, but not in WT offspring ($F_{3,36}$=6.869; P=0.013, versus $F_{3,31}$=0.395; P=0.534, respectively). Similarly, when stratifying for sex, the PS effect was only significant for females ($F_{3,35}$=0.980; P=0.329 versus $F_{3,32}$=5.494; P=0.025, for males and females, respectively). Of note, these observations also propose that the effect in the FST was independent from the lower mobility as observed in the EZM, the latter of which was primarily seen in WT male offspring (Figure 3-1B).

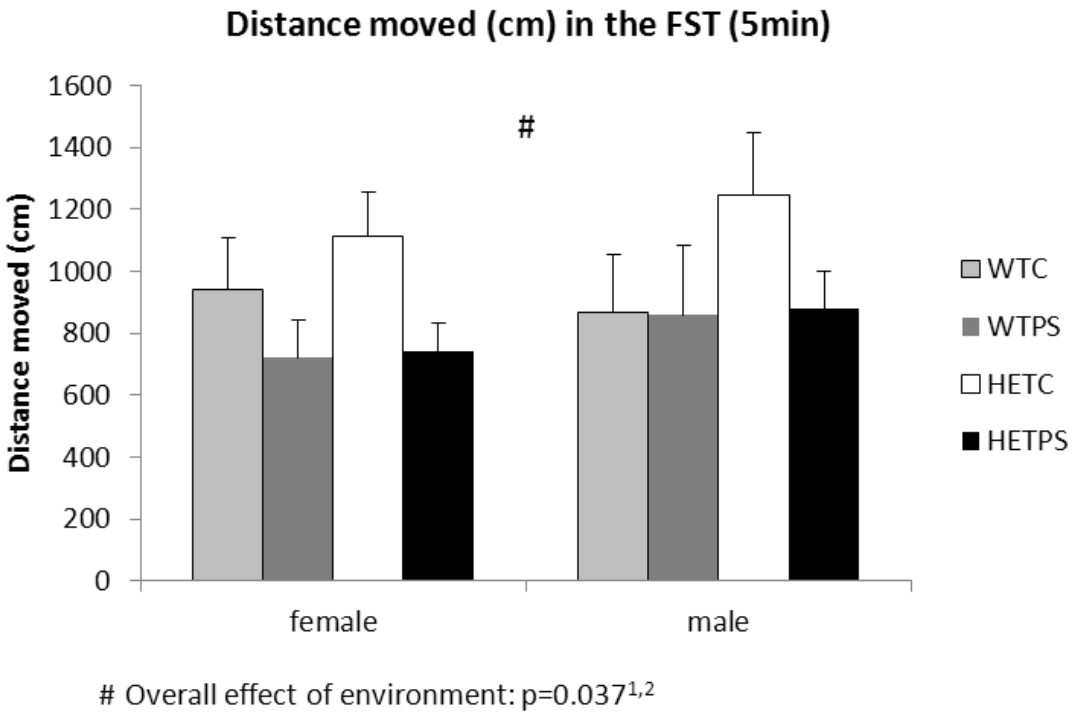


Figure 3-2. Performance in the Forced Swim Test (FST). Distance swum in the FST (5-minute period) was significantly decreased in prenatally stressed (PS) versus control (C) animals (P=0.037). Data represent mean + S.E.M. Abbreviation: WT, wild-type. N=7-10 mice/group. [1]Only significant in 5-Htt+/- offspring when stratified for genotype; [2]Only significant in female offspring when stratified for sex (see results section for more details).

Stress-induced plasma corticosterone (CORT) secretion

Data on stress-induced plasma CORT secretion of the offspring are depicted in Figure 3-3. Overall, a significant effect of time was observed ($F_{6,63}$=263.413; P<0.001). Furthermore, a time x genotype interaction was seen ($F_{6,63}$=3.594; P=0.030). In addition, over all 3 time points, significant effects of genotype and sex ($F_{7,64}$=4.000; P=0.050 and $F_{7,64}$=92.908; P<0.001, respectively) were observed, indicating lower CORT levels in 5-Htt+/- versus WT mice and higher levels in female versus male offspring, respectively. When looking at the individual time points, female offspring had higher CORT

levels when compared to male offspring at all time points (overall sex effect; F>37.187; P<0.001 in all cases). Further, at baseline, an overall genotype effect ($F_{7,65}$=6.476; P=0.013) was seen, indicating that basal CORT levels were lower in 5-Htt+/- as compared to WT mice, an effect that tended to be more profound in male offspring (when stratified for sex; $F_{3,33}$=6.678; P=0.014, versus $F_{3,32}$=1.444; P=0.238, in males versus females; see Figure 3).

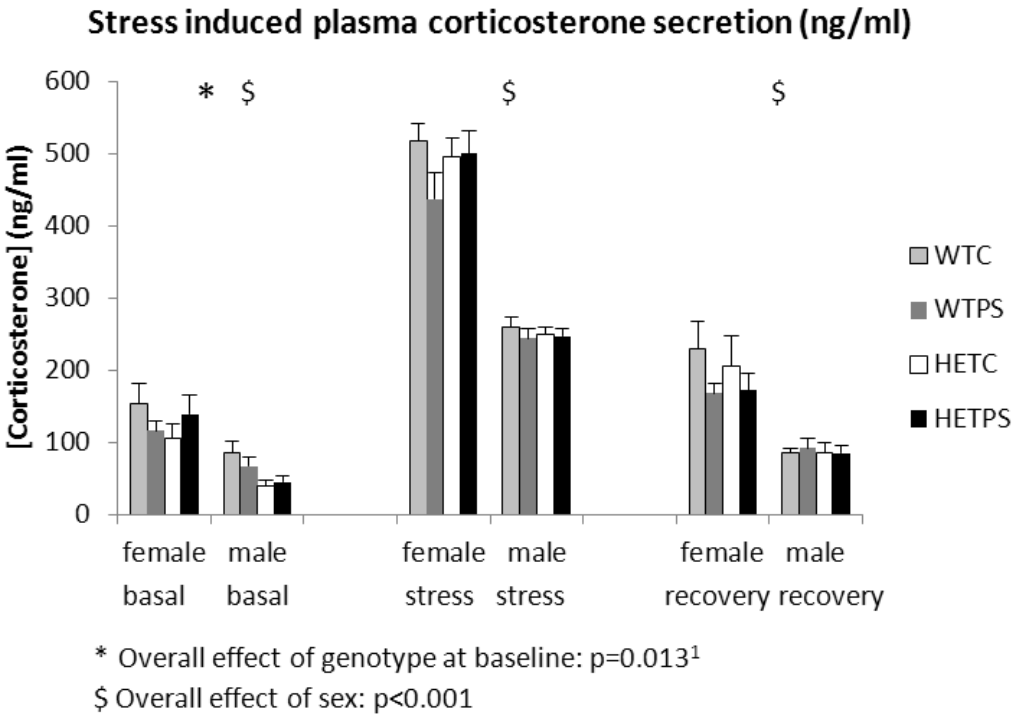


Figure 3-3. Stress-induced plasma corticosterone (CORT) secretion. At all time points, female (F) offspring had higher CORT levels when compared to male (M) offspring (overall sex effect; P<0.001 in all cases). At baseline, a significant genotype effect was observed (P=0.013). Abbreviations: WT, wild-type; C, control offspring. Data represent mean + S.E.M. N=7-10 mice/group. [1]Only significant in male offspring when stratified for sex (see results section for more details).

Adrenal weight

Adrenal weight was higher in female versus male offspring ($F_{7,56}$=166.817; P<0.001; Figure 3-4). In addition, overall, adrenal weight was heavier in 5-Htt+/- offspring when compared to WT animals ($F_{7,56}$=5.524; P=0.022), an effect that seemed to be particularly present in females ($F_{3,30}$=5.902; P=0.021, versus $F_{3,26}$=0.517; P=0.478, for females and males, respectively; see Figure 3-4 for more details).

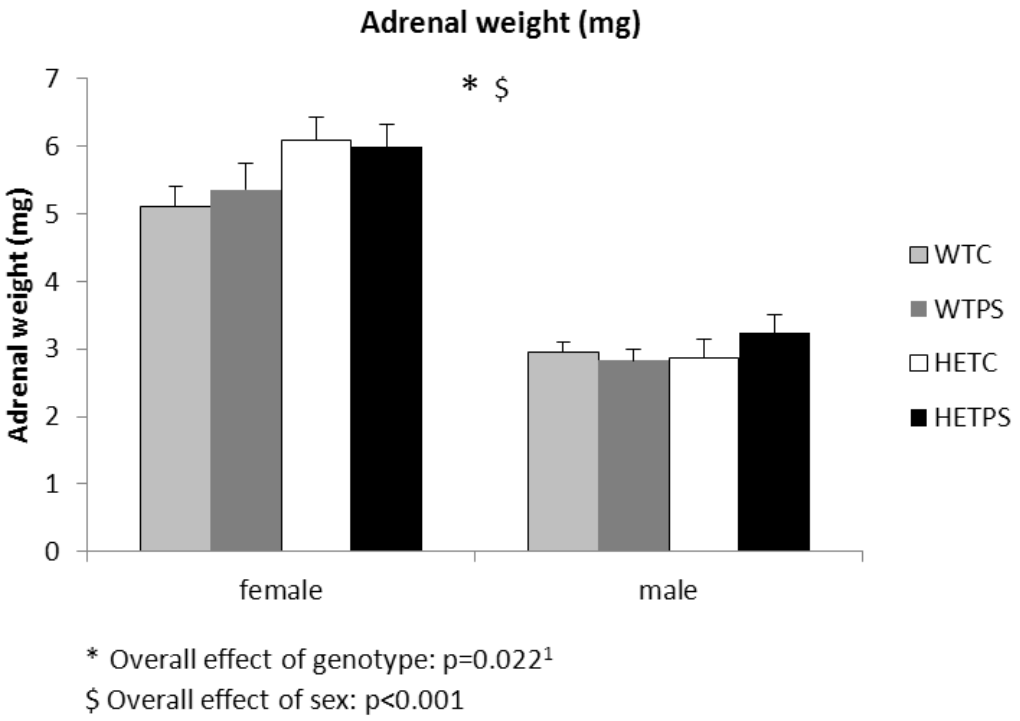


Figure 3-4. Adrenal weight. Adrenal weight was higher in female (F) versus male (M) offspring (P<0.001). Adrenal weight was elevated in 5-Htt+/- offspring when compared to wild-type (WT) animals (P=0.022). Data represent mean + S.E.M. Abbreviations: C, control offspring; PS, prenatally stressed offspring. N=7-10 mice/group. [1]Only significant in female offspring when stratified for sex (see results section for more details).

3.2 Gene expression study

3.2.1 Gene expression microarray

To further study the molecular mechanisms underlying the behavioral observations in female offspring – which showed most pronounced behavioral changes mediated by variation in 5-Htt genotype, PS, and their interaction –, we conducted a microarray-based expression profiling on the female hippocampus, a brain region participating in learning and memory as well as in emotion regulation (Fanselow and Dong 2010). For microarray analysis we focused on three different comparisons. We measured the changes between 5-Htt+/- and WT mice (G effect), in the differences between PS and control mice (E effect), and, moreover, in the interaction between G and E (G x E effects), i.e. indicating those genes of which the effect of PS exposure is depended upon the 5-Htt genotype. In brief, the 5-Htt+/- genotype and PS exposure changed the expression of 773 and 960 genes, respectively (Figure 3-5; also see Supplemental material 5.1.1 and 5.1.2 for a complete overview of all genes significantly affected by G and E, respectively). Furthermore, 651 genes were affected in a G x E manner (Supplemental material 5.1.3; also see Figure 3-5). In addition, G and E

showed overlap in the expression of 110 genes. Of those, 22 genes were upregulated, and 77 were downregulated by both. 11 genes were affected by G and E in an opposite direction (see Figure 3-5) whereas the expression of 3 genes was altered by G, E and in a G x E manner. To functionally categorize the differentially expressed genes we performed a pathway analysis using DAVID. We found 10 KEGG pathways affected by G, 9 by E and 10 by G x E (Table 3-3).

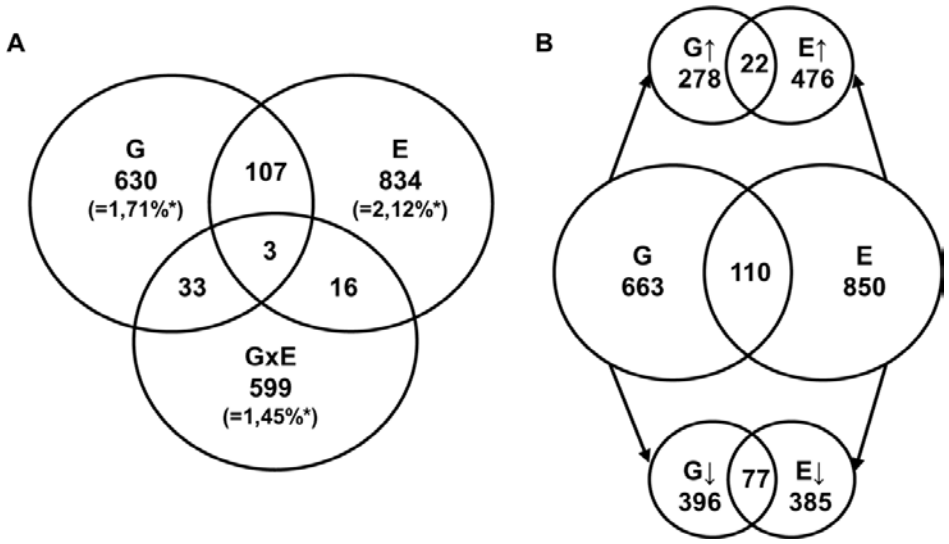


Figure 3-5. Gene expression analysis. A) Venn diagram illustrating the number of genes altered by the genotype (G; i.e. 5-Htt+/- versus wild type, 773 genes), the environment (E; i.e. prenatal stress versus control, 960 genes), both (110 genes), or in an interactive manner (G x E; i.e. indicating those genes of which the effect of the environment depends upon the genotype, 651 genes). B) Venn diagram illustrating the number of genes regulated by G, E or both, including the corresponding direction in which the 5-Htt+/- genotype and PS regulated their expression. Eleven genes were regulated in an opposite direction by G and E (not shown); *Percentage of probesets examined. (van den Hove *et al.* 2011)

Table 3-3. Significant KEGG pathways

FACTOR	DAVID ID	NAME	# GENES	P-VALUE
Genotype (G)	mmu04010	MAPK signaling pathway	26	1.79E-10
	mmu05200	Pathways in cancer	20	0.011
	mmu05412	Arrhythmogenic right ventricular cardiomyopathy	8	0.012
	mmu04810	Regulation of actin cytoskeleton	15	0.013
	mmu05410	Hypertrophic cardiomyopathy (HCM)	8	0.021
	mmu04722	Neurotrophin signaling pathway	10	0.029
	mmu05218	Melanoma	7	0.030
	mmu05414	Dilated cardiomyopathy	8	0.033
	mmu04114	Oocyte meiosis	9	0.037
	mmu04142	Lysosome	9	0.044
Environment (E)	mmu04010	MAPK signaling pathway	24	5.24E-11

	mmu04510	Focal adhesion	18	0.003
	mmu04012	ErbB signaling pathway	10	0.009
	mmu04722	Neurotrophin signaling pathway	12	0.018
	mmu04150	mTOR signaling pathway	7	0.023
	mmu00052	Galactose metabolism	5	0.024
	mmu05219	Bladder cancer	6	0.028
	mmu04520	Adherens junction	8	0.037
	mmu04114	Oocyte meiosis	10	0.047
G x E	mmu04060	Cytokine-cytokine receptor interaction	12	0.009
	mmu04310	Wnt signaling pathway	9	0.009
	mmu04672	Intestinal immune network for IgA production	5	0.022
	mmu04640	Hematopoietic cell lineage	6	0.023
	mmu05310	Asthma	4	0.027
	mmu05330	Allograft rejection	5	0.028
	mmu04012	ErbB signaling pathway	6	0.029
	mmu04514	Cell adhesion molecules (CAM)	8	0.033
	mmu04510	Focal adhesion	9	0.043
	mmu04360	Axon guidance	7	0.045

Significant KEGG pathways affected by genotype (G; i.e. 5-Htt+/- versus wild type), the environment (E; i.e. prenatal stress versus control) or in an interactive manner (GxE; i.e. indicating those genes of which the effect of the environment depends upon the genotype). Acquired using DAVID analysis.

Gene expression changes accompanying variations in 5-Htt genotype

Of the 773 genes affected by the 5-Htt+/- genotype, 300 genes were upregulated and 473 downregulated (Figure 3-5; also see supplemental material 5.1.1 for a complete overview of all genes regulated by 5-Htt genotype). Amongst others, DAVID analysis revealed the mitogen-activated protein kinase (MAPK) signaling pathway and neurotrophin signaling as significantly overrepresented pathways affected by G (see Table 3-3 for a complete overview of the functionally enriched pathways). In the MAPK signaling pathway, 73% of the 26 genes were downregulated, such as *fibroblast growth factor 1 (Fgf1), calcium channel voltage dependent, L type, alpha 1D subunit (Cacna1d)* and *mitogen-activated protein kinase 8 interacting protein 3* (*Mapk8ip3*; see Table 3-4A for an overview of all genes affected in the MAPK signaling pathway). In the neurotrophin signaling pathway, 10 genes were significantly affected, among which was the *neurotrophic tyrosine kinase, receptor type 2 (Ntrk2*; also known as *TrkB receptor*) and *transformation related protein 53 (Trp53*; see Table 3-4B for a complete overview). Interestingly, the MAPK and neurotrophin signaling KEGG pathway partially overlap. One gene, namely the *v-crk sarcoma virus CT10 oncogene homolog (avian; Crk)*, was affected in both KEGG pathways. Of note, the expression of the *solute carrier family 6, member 4* (*Slc6a4*, encoding for the 5-Htt) was upregulated in 5Htt +/- mice 10.6 fold indicating an upregulation of the allele leading to a truncated protein (see Ravary *et al.* 2001).

Table 3-4. Genes regulated by the 5-Htt+/- genotype

A. MAPK signaling pathway

EFFECT	AFFY ID	DAVID GENENAME	ENTREZ SYMBOL	# (%) GENES	FC	P-VALUE
up	1432647_at	*epidermal growth factor receptor*	*Egfr*		1.2	0.007
	1444344_at	*fibroblast growth factor 10*	*Fgf10*		1.2	0.007
	1460296_a	*fibroblast growth factor 22*	*Fgf22*		1.2	0.005
	1421473_at	*interleukin 1 alpha*	*Il1a*		1.1	0.005
	1452383_at	*ribosomal protein S6 kinase polypeptide 3*	*Rps6ka3*		1.2	0.009
	1449901_a	*mitogen-activated protein kinase kinase kinase 6*	*Map3k6*		1.2	0.007
	1460176_at	*v-crk sarcoma virus CT10 oncogene homolog (avian)*	*Crk*	**7 (27%)**	1.3	4.68E-04
down	1438031_at	*RAS, guanyl releasing protein 3*	*Rasgrp3*		1.2	0.003
	1421297_a	*calcium channel, voltage-dependent, L type, alpha 1C*	*Cacna1c*		1.1	0.008
	1428051_a	*calcium channel, voltage-dependent, L type, alpha 1D*	*Cacna1d*		1.2	0.004
	1425812_a	*calcium channel, voltage-dependent, N type, alpha 1B*	*Cacna1b*		1.3	0.004
	1450520_at	*calcium channel, voltage-dependent, gamma subunit 3*	*Cacng3*		1.3	0.001
	1450869_at	*fibroblast growth factor 1*	*Fgf1*		1.1	0.004
	1418498_at	*fibroblast growth factor 13*	*Fgf13*		1.3	0.009
	1425911_a	*fibroblast growth factor receptor 1*	*Fgfr1*		1.3	0.003
	1427776_a	*fibroblast growth factor receptor 4*	*Fgfr4*		1.2	0.003
	1426677_at	*filamin, alpha*	*Flna*		1.1	0.009
	1450097_s_	*guanine nucleotide binding protein, alpha 12*	*Gna12*		1.2	0.002
	1417885_at	*microtubule-associated protein tau*	*Mapt*		1.2	0.001
	1425679_a	*mitogen-activated protein kinase 8 interacting protein 1*	*MAPK8ip*		1.1	0.008
	1416437_a	*mitogen-activated protein kinase 8 interacting protein 3*	*MAPK8ip*		1.3	0.003
	1421446_at	*protein kinase C, gamma*	*Prkcc*		1.3	6.73E-04
	1424287_at	*protein kinase, X-linked*	*Prkx*		1.3	0.004
	1450368_a	*protein phosphatase 3, regulatory subunit B, alpha*	*Ppp3r1*		1.3	0.007
	1427739_a	*transformation related protein 53*	*Trp53*		1.2	0.010
	1420837_at	*neurotrophic tyrosine kinase, receptor, type 2*	*Ntrk2*	**19**	1.5	2.93E-04
Total				**26**		

B. Neurotrophin signaling pathway

EFFECT	AFFY ID	DAVID GENENAME	ENTREZ SYMBOL	# (%) GENES	FC	P-VALUE
up	1437122_at	*predicted gene 3655; B-cell leukemia/lymphoma 2*	*Bcl2*		1.16	0.008
	1454378_at	*predicted gene, EG546165; predicted gene 2423; hypothetical protein LOC674211; tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, theta polypeptide*	*Ywhaq*		1.78	0.008
	1452383_at	*ribosomal protein S6 kinase polypeptide 3*	*Rps6ka3*		1.21	0.009
	1439005_x_at	*tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide; predicted gene 4202*	*Ywhaz**		1.26	0.009

	1460176_at	*v-crk sarcoma virus CT10 oncogene homolog (avian)*	*Crk*	**5 (45%)**	1.39	4.68E-04
down	1448668_a_at	*interleukin-1 receptor-associated kinase 1*	*Irak1*		1.20	0.005
	1420837_at	*neurotrophic tyrosine kinase, receptor, type 2*	*Ntrk2*		1.57	2.93E-04
	1425070_at	*neurotrophic tyrosine kinase, receptor, type 3; similar to neurotrophic tyrosine kinase, receptor, type 3*	*Ntrk3*		1.23	0.008
	1427739_a_at	*transformation related protein 53*	*Trp53*		1.28	0.010
	1452325_at	*transformation related protein 73*	*Trp73*		1.21	0.008
	1448218_s_at	*tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptid; predicted gene 4202*	*Ywhaz**	**6 (55%)**	1.24	0.004
Total				**11**		

Differentially expressed genes within the mitogen-activated protein kinase (MAPK) signaling (A) and neurotrophin signaling (B) pathways (5-Htt+/- versus wild type). Abbreviation: FC, fold change. * Probesets recognize different transcripts.

Gene expression changes induced by PS exposure

The expression of 960 genes was altered by PS exposure, of which 462 were upregulated and 498 downregulated (Figure 3-5; also see supplemental material 5.1.2 for a complete overview of all genes regulated by PS exposure). Moreover, 9 KEGG pathways with significant P-values were detected by DAVID analysis (see Table 3-3). As the 5Htt +/- genotype, PS affected the MAPK and the neurotrophin signaling pathway (Table 3-5; see Table 3-3 for a complete overview of the functionally enriched pathways). The *calcium channel, voltage-dependent, gamma subunit 3 (Cacng3)* and *protein kinase C, gamma (PrKcc)* are examples of genes negatively influenced by both G and E.

Table 3-5. Genes regulated by prenatal stress

A. MAPK signaling pathway

EFFECT	AFFY ID	DAVID GENENAME	ENTREZ SYMBOL	# (%) GENES	FC	P-VALUE
up	1444199_at	*ELK4, member of ETS oncogene family*	*Elk4*		1.16	0.007
	1423100_at	*FBJ osteosarcoma oncogene*	*Fos*		1.76	0.001
	1417409_at	*Jun oncogene*	*Jun*		1.22	0.005
	1438992_x_at	*activating transcription factor 4*	*Atf4*		1.30	1.16E-04
	1447511_at	*calcium channel, voltage-dependent, N type, alpha 1B subunit*	*Cacna1b*		1.28	0.006
	1420287_at	*calcium channel, voltage-dependent, P/Q type, alpha 1A subunit*	*Cacna1a*		1.32	7.17E-04
	1449773_s_at	*growth arrest and DNA-damage-inducible 45 beta*	*Gadd45b*		1.19	0.003
	1452318_a_at	*heat shock protein 1B*	*Hspa1b*		1.35	0.001
	1448950_at	*interleukin 1 receptor, type I*	*Il1r1*		1.20	0.006

	1443540_at	*mitogen-activated protein kinase kinase kinase 1*	*Map3k1*		1.24	0.009
	1438908_at	*mitogen-activated protein kinase kinase kinase 12*	*Map3k12*		1.19	0.003
	1447667_x_at	*mitogen-activated protein kinase kinase kinase 4*	*Map3k4*		1.14	0.010
	1456467_s_at	*nemo like kinase*	*Nlk*		1.15	0.007
	1421416_at	*predicted gene 14378; similar to transforming growth factor, beta receptor III (betaglycan, 300kDa); mitogen-activated protein kinase kinase 7*	*Map2k7*	**14 (58%)**	1.18	0.004
down	1447941_x_at	*Braf transforming gene*	*Braf*		1.21	0.006
	1450520_at	*calcium channel, voltage-dependent, gamma subunit 3*	*Cacng3*		1.34	3.88E-04
	1424932_at	*epidermal growth factor receptor*	*Egfr*		1.13	0.007
	1425911_a_at	*fibroblast growth factor receptor 1*	*Fgfr1*		1.27	0.006
	1426677_at	*filamin, alpha*	*Flna*		1.19	0.001
	1453712_a_at	*mitogen-activated protein kinase kinase 5*	*Map2k5*		1.22	0.010
	1455441_at	*mitogen-activated protein kinase kinase kinase 7; predicted gene 8188*	*Map3k7*		1.23	0.005
	1421446_at	*protein kinase C, gamma*	*Prkcc*		1.31	0.001
	1451943_a_at	*protein phosphatase 1A, magnesium dependent, alpha isoform*	*Ppm1a*		1.18	0.006
	1429759_at	*ribosomal protein S6 kinase polypeptide 6*	*Rps6ka6*	**10 (42%)**	1.31	1.48E-04
Total				**24**		

B. Neurotrophin signaling pathway

EFFECT	AFFY ID	DAVID GENENAME	ENTREZ SYMBOL	# (%) GENES	FC	P-VALUE
up	1417409_at	*Jun oncogene*	*Jun*		1.21	0.005
	1438992_x_at	*activating transcription factor 4*	*Atf4*		1.29	1.16E-04
	1443540_at	*mitogen-activated protein kinase kinase*	*Map3k1*		1.24	0.009
	1425514_at	*phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 1 (p85 alpha)*	*Pik3r1*		1.18	0.005
	1421416_at	*predicted gene 14378; similar to transforming growth factor, beta receptor III (betaglycan, 300kDa); mitogen-activated protein kinase kinase 7*	*Map2k7*		1.18	0.004
	1454378_at	*predicted gene, EG546165; predicted gene*	*Ywhaq*		1.79	0.004
	1439005_x_at	*tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide*	*Ywhaz*		1.36	5.72E-04
	1436981_a_at	*tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide*	*Ywhaz*	**8 (62%)**	1.28	0.005
down	1447941_x_at	*Braf transforming gene*	*Braf*		1.21	0.006

	1455869_at	*calcium/calmodulin-dependent protein kinase II, beta*	*Camk2b*		1.77	9.13E-04
	1453712_a_at	*mitogen-activated protein kinase kinase 5*	*Map2k5*		1.22	0.010
	1429759_at	*ribosomal protein S6 kinase polypeptide 6*	*Rps6ka6*		1.31	1.48E-04
	1438839_a_at	*tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, epsilon polypeptide*	*Ywhae*	**5 (38%)**	1.19	0.008
Total				**13**		

Differentially expressed genes within the mitogen-activated protein kinase (MAPK) signaling (A) and neurotrophin signaling (B) pathways (PS versus control). Abbreviation: FC, fold change.

Gene expression profiles indicating a gene x environment interaction

At the G × E level, 651 genes displayed a significant expression pattern (Figure 3-5; see Supplemental material 5.1.3 for a complete overview of all genes regulated in a G x E fashion). DAVID pathway analysis revealed that G x E significantly enriched 10 pathways (Table 3-3). The most significantly enriched biological process was cytokine-cytokine receptor interactions (Table 3-6; see Table 3-3 for a complete overview of the functionally enriched pathways). Examples of genes affected within this pathway are *interleukin 4 (Il4), interleukin 12a (Il12a)* and *tumor necrosis factor receptor superfamily, member 1a (Tnfrsf1a)*. Another pathway significantly influenced in a G x E manner was the Wnt signaling pathway amongst which the *RAS-related C3 botulinum substrate 2 (Rac2), Rho-associated coiled-coil containing protein kinase 1 (Rock1), calcium/calmodulin-dependent protein kinase II, delta (Camk2d)* and *presenilin 1(Psen1)* were differentially expressed.

Table 3-6. Genes regulated in a gene x environment manner

A. Cytokine-cytokine receptor interactions

AFFY ID	DAVID GENENAME	ENTREZ SYMBOL	P-VALUE
1437382_at	*activin receptor IIA*	*Acvr2a*	0.004
1421188_at	*chemokine (C-C motif) receptor 2*	*Ccr2*	0.003
1421843_at	*interleukin 1 receptor accessory protein*	*Il1rap*	0.005
1425454_a_at	*interleukin 12a*	*Il12a*	0.004
1422397_a_at	*interleukin 15 receptor, alpha chain*	*Il15ra*	0.008
1449864_at	*interleukin 4*	*Il4*	0.009
1415855_at	*kit ligand*	*Kitl*	0.006
1450272_at	*tumor necrosis factor (ligand) superfamily, member 8*	*Tnfsf8*	0.009
1430259_at	*tumor necrosis factor receptor superfamily, member 11a*	*Tnfrsf11a*	0.003

1427600_at	*tumor necrosis factor receptor superfamily, member 19*	*Tnfrsf19*	0.005
1417291_at	*tumor necrosis factor receptor superfamily, member 1a*	*Tnfrsf1a*	0.006
1448951_at	*tumor necrosis factor receptor superfamily, member 1b*	*Tnfrsf1b*	0.007

B. Wnt signaling pathway

AFFY ID	DAVID GENENAME	ENTREZ SYMBOL	P-VALUE
1417620_at	*RAS-related C3 botulinum substrate 2*	*Rac2*	4.86E-04
1441162_at	*Rho-associated coiled-coil containing protein kinase 1*	*Rock1*	0.007
1422659_at	*calcium/calmodulin-dependent protein kinase II, delta*	*Camk2d*	0.009
1449730_s_at	*frizzled homolog 3 (Drosophila)*	*Fzd3*	0.007
1458002_at	*mitogen-activated protein kinase 10*	*Mapk10*	0.002
1434275_at	*naked cuticle 2 homolog (Drosophila)*	*Nkd2*	0.007
1425549_at	*presenilin 1*	*Psen1*	0.005
1443270_at	*prickle-like 2 (Drosophila)*	*Prickle2*	0.005

Differentially expressed genes within the cytokine-cytokine receptor interactions (A) and Wnt signaling (B) pathways involving genes regulated in a genotype (G) x environment (E) manner, i.e. indicating those genes of which the effect of the (prenatal) environment depends upon the 5-Htt genotype).

3.2.2 Validation of the expression microarray

Using qRT-PCR we tried to confirm the microarray data. Of the 8 genes, 6 were replicated in terms of the overall G, E or G x E effects, such as *Fos* ($F_{3,32}$=7.076; P=0.012), *Mbp* (G effect: $F_{3,32}$=9.421; P=0.004; E effect: $F_{3,32}$=12.152; P=0.001; G x E effect: $F_{3,32}$=4.897; P=0.034), *Ppp1r1b* ($F_{3,32}$=9.055; P=0.005*), Trhr* (E effect: $F_{3,32}$=5.087; P=0.031; G x E effect: $F_{3,32}$=15.599; P<0.001), *Xaf1* ($F_{3,32}$=102.615; P<0.001) and *Zzef1* ($F_{3,32}$=14.128; P=0.001). We could not replicate the overall effect of 5-Htt genotype for *Kcnip2* (G effect: $F_{3,32}$=2.048; P=0.162; G x E effect: $F_{3,32}$=5.612; P=0.024) and the overall effect of PS for *Phox2a* ($F_{3,30}$=2.871; P=0.101) (see table 3-7 for more details).

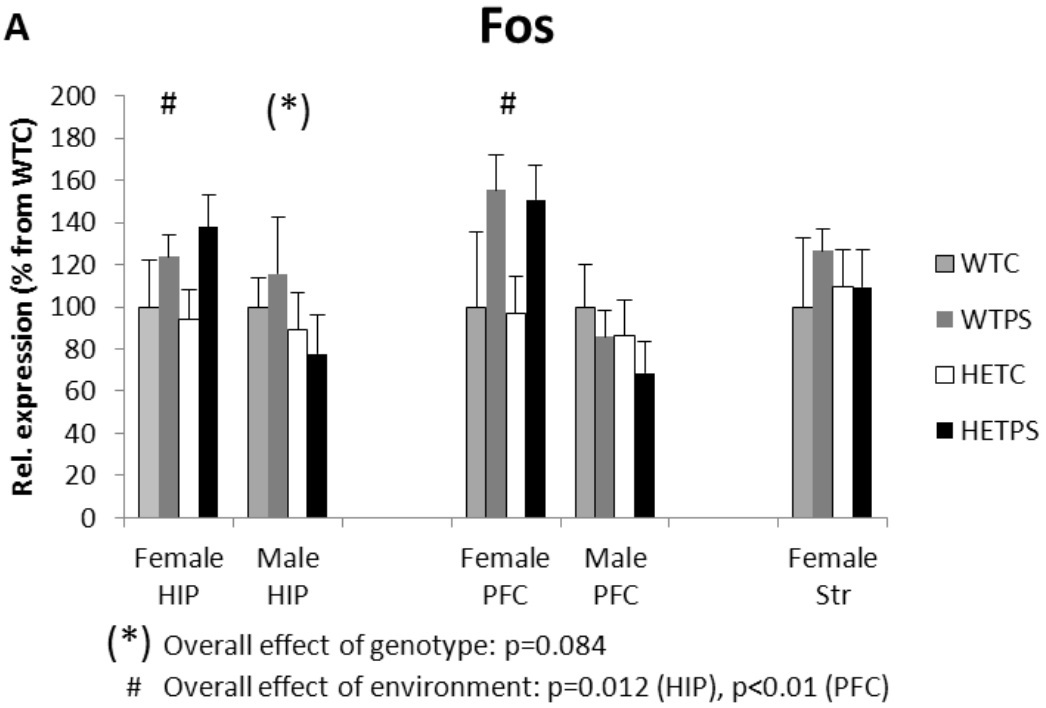
Table 3-7. Genes validated by qRT-PCR

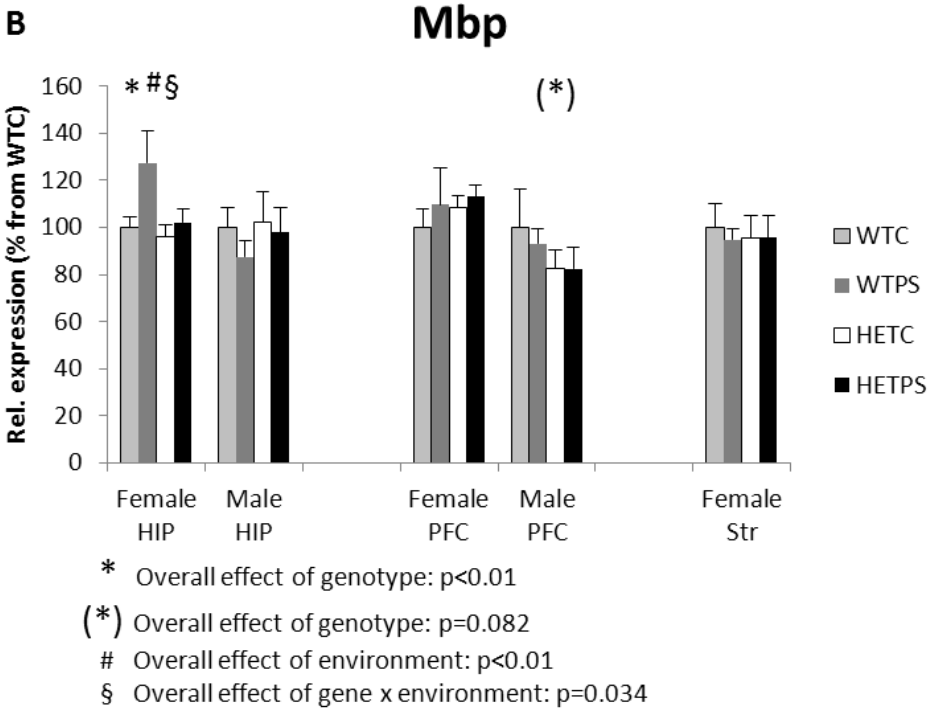
Gene	WTFC	WTFPS	5-Htt+/- FC	5-Htt+/- FPS	Main effect(s) Microarray	Main effect(s) qRT-PCR
Fos	100 ± 22.2	123.9 ± 10.5	94.3 ± 14.0	137.7 ± 15.6	E (↑)	E (↑)
Kcnip2	100 ± 5.3	97.6 ± 6.8	86.1 ± 5.4	101.0 ± 3.8	G (↓)	G x E
Mbp	100 ± 4.7	127.4 ± 7.7	95.9 ± 4.9	102.0 ± 5.8	G (↓)	G (↓), E (↑), G x E
Phox2a	100 ± 20.0	98.6 ± 9.2	77.4 ± 11.6	118.6 ± 19.7	E (↑)	-

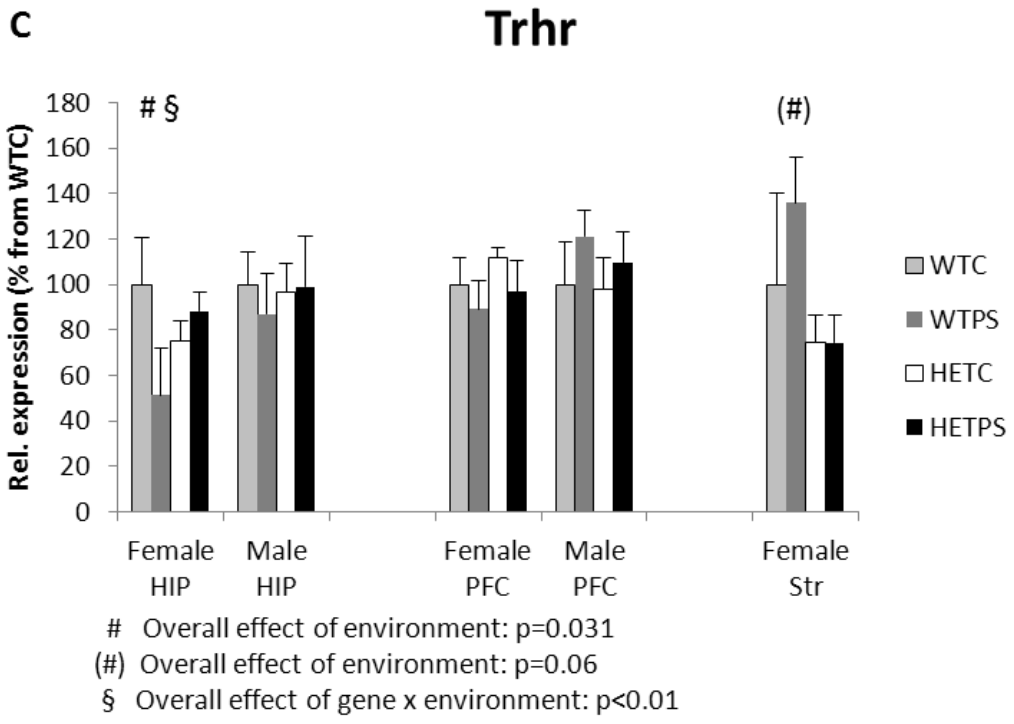
Ppp1r1b	100 ± 14.3	118.4 ± 13.6	89.1 ± 8.2	83.6 ± 2.9	G (↓)	G (↓)
Trhr	100 ± 20.4	51.2 ± 20.8	74.9 ± 9.5	88.2 ± 8.3	G x E	E (↓), G x E
Xaf1	100 ± 14.3	478.0 ± 117.9	2582.7 ± 8.3	2599.3 ± 9.4	G (↑)	G (↑)
Zzef1	100 ± 3.5	102.7 ± 3.7	86.6 ± 5.2	91.3 ± 6.1	G (↓)	G (↓)

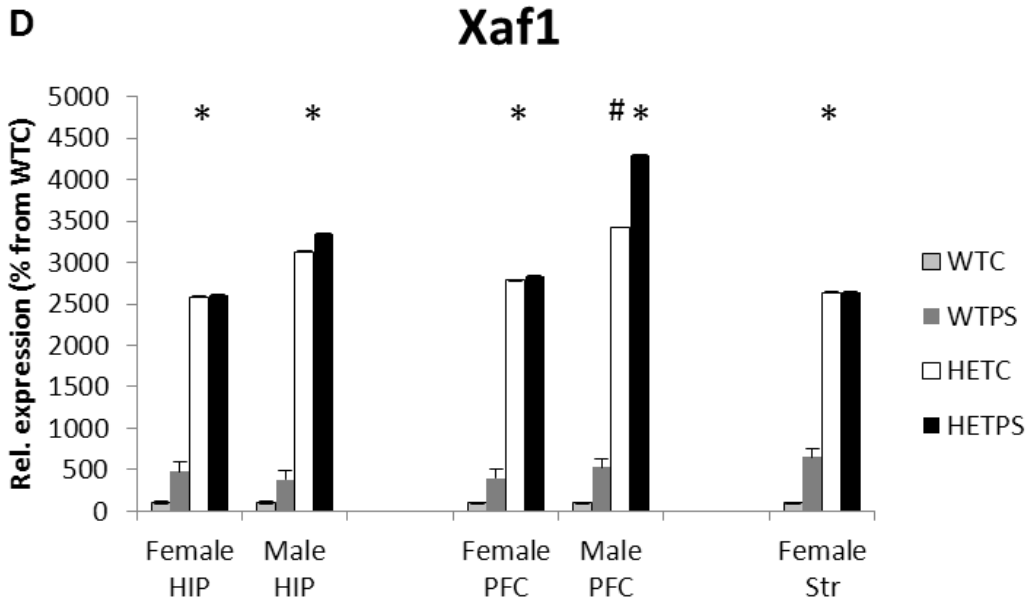
Genes validated by qRT-PCR. Values indicate average expression as percentage of wild type female control offspring. Data represent mean ± S.E.M. Abbreviations: WT, wild type; F, females; C, control offspring; PS, prenatally stressed offspring.

Furthermore, of these 8 genes differentially expressed within the female hippocampus, we examined the expression patterns in both the cortex and striatum of the PS males and females, as well as in the hippocampus of the animals of the 5-Htt x PeS paradigm. Of the 8 genes, the observed effects of 5 were also detected in other brain regions or the other sex of the PS model, such as *Fos* ($_{F3,32}$=9.399; P=0.004), *Mbp* ($_{F3,34}$=3.218; P=0.082), *Trhr* ($_{F3,32}$=3.807; P=0.06; different direction), *Xaf1* (male HIP: $F_{3,35}$=112.041; P<0.01; female PFC: $F_{3,32}$=230.247; P<0.01; male PFC: $F_{3,34}$=163.184; P<0.01; female Str: $F_{3,32}$=78.742; P<0.01) and *Zzef1* ($F_{3,32}$=6.054; P=0.019). Next to this, in the hippocampus of the PeS mice, *Xaf1* was differentially expressed between 5-Htt+/- and WT offspring (female: $F_{3,28}$=2750.193; P<0.01; male: $F_{3,30}$=155.341; P<0.01).



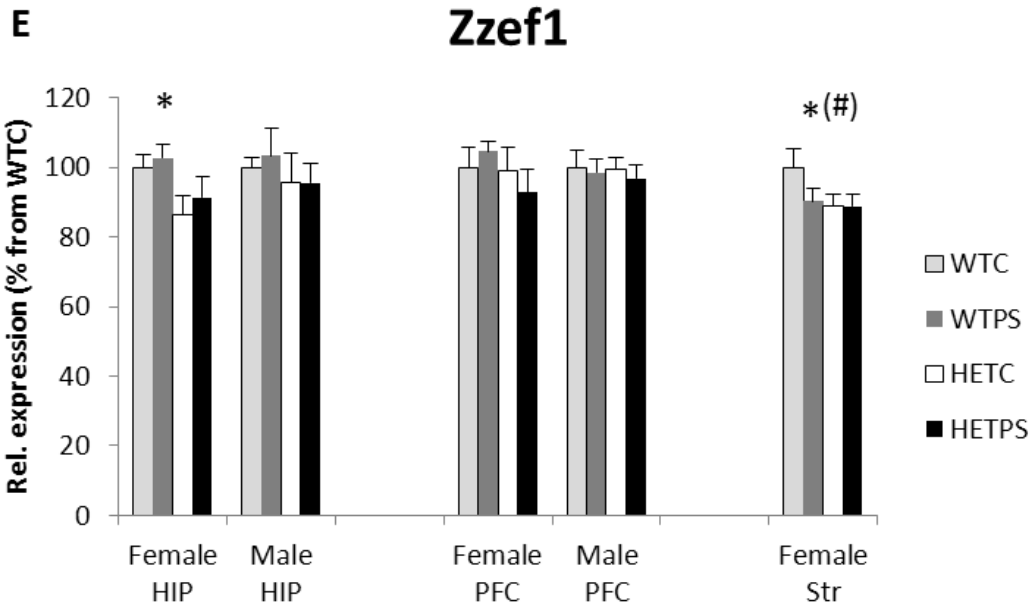

B
Mbp
Rel. expression (% from WTC)
160
140
120
100
80
60
40
20
0
*#§
(*)
Female HIP
Male HIP
Female PFC
Male PFC
Female Str
WTC
WTPS
HETC
HETPS
* Overall effect of genotype: p<0.01
(*) Overall effect of genotype: p=0.082
Overall effect of environment: p<0.01
§ Overall effect of gene x environment: p=0.034


C
Trhr
Rel. expression (% from WTC)
180
160
140
120
100
80
60
40
20
0
§
(#)
Female HIP
Male HIP
Female PFC
Male PFC
Female Str
WTC
WTPS
HETC
HETPS
Overall effect of environment: p=0.031
(#) Overall effect of environment: p=0.06
§ Overall effect of gene x environment: p<0.01



* Overall effect of genotype: p<0.01

\# Overall effect of environment: p=0.026



* Overall effect of genotype: p<0.01 (HIP), p=0.019 (Str)

(#) Overall effect of environment: p=0.061

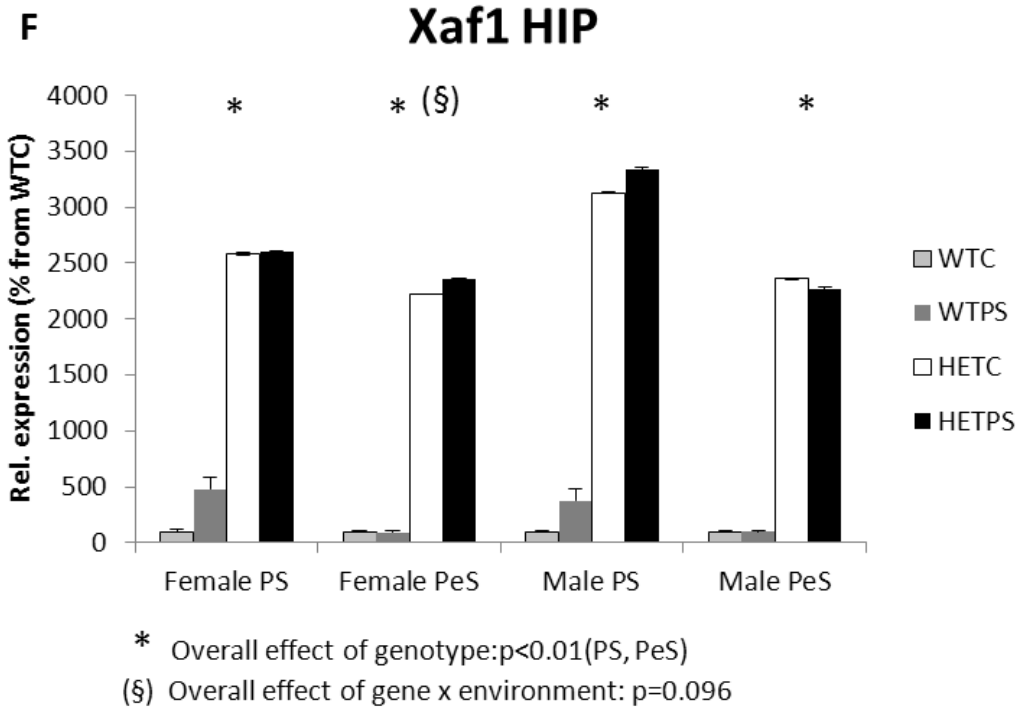


Figure 3-6. Validation of gene expression differences in distinct brain regions of male and female prenatal (PS; A-E) and perinatal (PeS; F) animals. Abbreviations: WT, wild-type; C, control offspring.; HET, 5-Htt+/- offspring. Data represent mean + S.E.M. N=7-10 mice/group. ( ): 0.05<p<0.1 (tendency)

3.3 DNA methylation study

3.3.1 DNA methylation microarray

To further investigate the molecular mechanisms underlying the behavior of the female offspring and to see which genes may be regulated by DNA methylation we conducted a microarray-based methylation profiling on the female hippocampus. As in the gene expression profiling, we concentrated on three different comparisons. We measured the changes between 5-Htt+/- genotype and WT mice (G effect), as well as the differences between PS and control mice (E effect), and the interaction of G and E (GxE effects). In brief, the 5-Htt+/- genotype and PS exposure altered the methylation of 279 and 400 genes, respectively (Figure 3-7; see also Supplements 5.1.4 and 5.1.5 for a complete overview of all genes significantly affected by G and E, respectively). Further, 268 genes were affected in a GxE manner (see Supplements 5.1.6 and Figure 3-7). In addition, G and E showed overlap in the methylation of 10 differentially methylated regions (DMRs; one gene can be composed of several regions which can be differentially regulated). Of those, 6 DMRs were upregulated and one – *titin (Ttn)* - downregulated by both. Moreover, the methylation of 3 DMRs – *Ttn, phospholipase A2, group IIA (Pla2g2a) and Pla2g V (Pla2g5)* - was affected in a G, E and GxE manner.

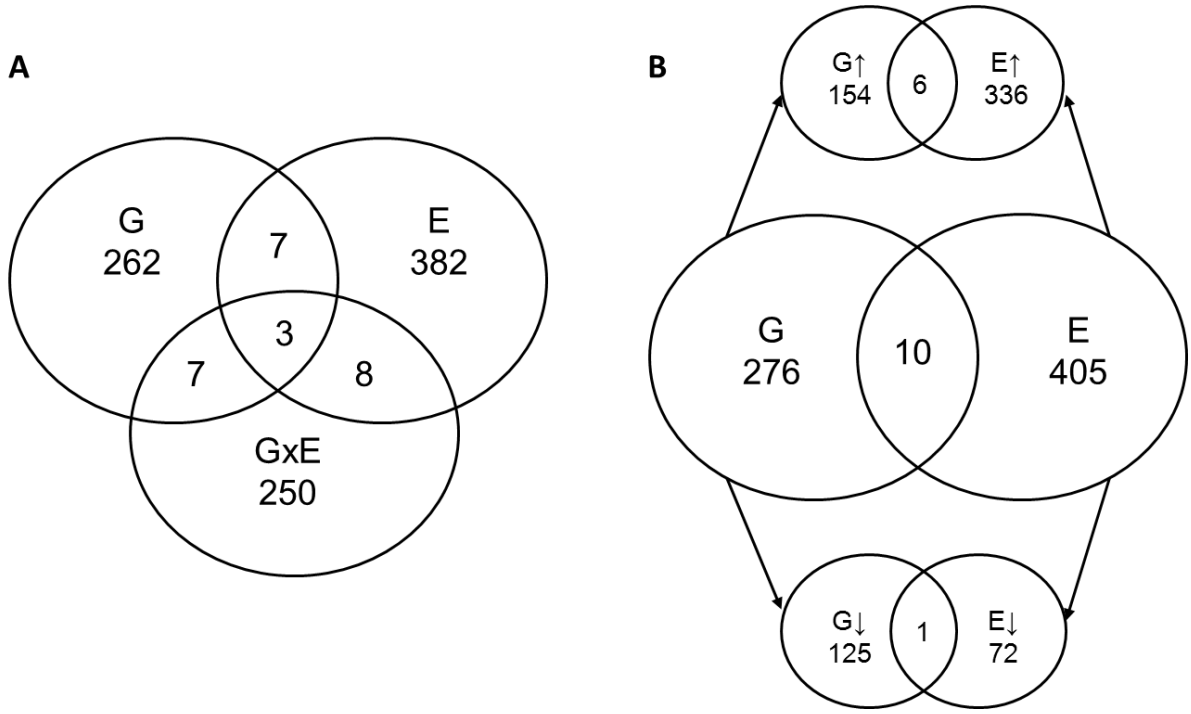


Figure 3-7. Methylation - number of altered genes. A) Number of differentially methylated genes regulated by either the 5-Htt+/- genotype (G), the environment (E) or in a GxE fashion. B) Number of differentially methylated regions (DMRs) which are either up or downregulated.

Additionally, 28 (G), 37 (E) and 24 (GxE) genes showed an overlap between methylation and gene expression (see Figure 3-8). One gene - *Pla2g5* - was affected by both, G and GxE, whereas around half of those genes showed classical methylation, indicating upregulated methylation and downregulated expression or vice versa (see Table 3-8).

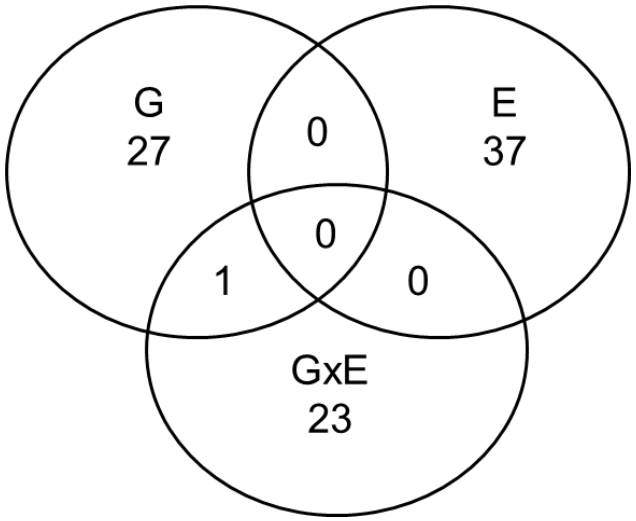


Figure 3-8. Overlap of differentially methylated and expressed genes. Number of genes that were differentially methylated and differentially expressed by the 5-Htt+/- genotype (G), the environment (E) or GxE interaction.

Tab. 3-8 A. Genes affected by gene expression and methylation through 5-Htt genotype

#	SYMBOL	GENENAME	Effect exp	Effect meth	FC exp
1	0610040J01Rik	RIKEN cDNA 0610040J01 gene	-	-	1.12
2	Akap6	A kinase (PRKA) anchor protein 6	+	-	1.14
3	Bbx	bobby sox homolog (Drosophila)	+	+	1.26
4	BC030307	cDNA sequence BC030307	+	+	1.15
5	Bcl2l11	BCL2-like 11 (apoptosis facilitator)	-	+	1.11
6	Bmpr1b	bone morphogenetic protein receptor, type 1B	-	+	1.19
7	C030046E11Rik	RIKEN cDNA C030046E11 gene	+	-	1.14
8	Cybrd1	cytochrome b reductase 1	+	-	1.13
9	Dhx32	DEAH (Asp-Glu-Ala-His) box polypeptide 32	-	-	1.19
10	E2f3	E2F transcription factor 3	+	-	1.14
11	Fabp6	fatty acid binding protein 6, ileal (gastrotropin)	-	+	1.27
12	Fank1	fibronectin type 3 and ankyrin repeat domains 1	-	-	1.17
13	Fgfr4	fibroblast growth factor receptor 4	-	+	1.26
14	Kcnj5	potassium inwardly-rectifying channel, subfamily J, member 5	+	+	1.18
15	Kif13a	kinesin family member 13A	-	-	1.12
16	Krt23	keratin 23	-	-	1.21
17	Ldlrad3	low density lipoprotein receptor class A domain containing 3	-	+	1.12
18	Lgr5	leucine rich repeat containing G protein coupled receptor 5	+	+	1.13
19	Mllt6	myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to, 6	-	-	1.23
20	Mttp	microsomal triglyceride transfer protein	+	+	1.25
21	Nr5a1	nuclear receptor subfamily 5, group A, member 1	-	-	1.17
22	Pdlim4	PDZ and LIM domain 4	-	+	1.21
23	Pla2g5	phospholipase A2, group V	-	+	1.60
24	Smad7	MAD homolog 7 (Drosophila)	-	+	1.12
25	Spsb1	splA/ryanodine receptor domain and SOCS box containing 1	-	+	1.19
26	Tcea3	transcription elongation factor A (SII), 3	-	-	1.30
27	Tmem100	transmembrane protein 100	+	-	1.22
28	Ttn	titin	+	-	1.21

Tab. 3-8 B. Genes affected by gene expression and methylation through PS exposure

#	SYMBOL	GENENAME	Effect exp	Effect meth	FC exp
1	Akap13	A kinase (PRKA) anchor protein 13	-	+	1.10
2	BC051628	cDNA sequence BC051628	+	-	1.12
3	Bcl10	B-cell leukemia/lymphoma 10	+	-	1.18
4	Cdc73	cell division cycle 73, Paf1/RNA polymerase II complex component, homolog (S. cerevisiae)	+	-	1.15
5	Chaf1b	chromatin assembly factor 1, subunit B (p60)	+	+	1.24
6	Cldn18	claudin 18	+	+	1.19
7	Clstn2	calsyntenin 2	-	+	1.19
8	Ddx46	DEAD (Asp-Glu-Ala-Asp) box polypeptide 46	-	+	1.16
9	Dna2	DNA replication helicase 2 homolog (yeast)	+	+	1.25
10	Fjx1	four jointed box 1 (Drosophila)	-	+	1.20
11	Gja1	gap junction protein, alpha 1	-	+	1.42
12	Kat2a	K(lysine) acetyltransferase 2A	-	+	1.15

13	Kcnh3	potassium voltage-gated channel, subfamily H (eag-related), member 3	-	+	1.11
14	Kdm6b	KDM1 lysine (K)-specific demethylase 6B	+	+	1.14
15	Map3k1	mitogen-activated protein kinase kinase kinase 1	+	+	1.24
16	Mast4	microtubule associated serine/threonine kinase family member 4	+	-	1.14
17	Mcm3	minichromosome maintenance deficient 3 (S. cerevisiae)	+	+	1.18
18	Mettl7a1	methyltransferase like 7A1	+	+	1.13
19	Msi1	Musashi homolog 1(Drosophila)	+	+	1.24
20	Nos1	nitric oxide synthase 1, neuronal	-	+	1.20
21	Nr2c1	nuclear receptor subfamily 2, group C, member 1	-	+	1.19
22	Pnlip	pancreatic lipase	+	+	1.25
23	Prkab1	protein kinase, AMP-activated, beta 1 non-catalytic subunit	-	+	1.13
24	Prok2	prokineticin 2	+	+	1.15
25	Prss23	protease, serine, 23	-	-	1.17
26	Ptpn21	protein tyrosine phosphatase, non-receptor type 21	+	+	1.15
27	Rad9b	RAD9 homolog B (S. cerevisiae)	-	+	1.18
28	Ropn1l	ropporin 1-like	-	+	1.15
29	Slc30a1	solute carrier family 30 (zinc transporter), member 1	-	+	1.15
30	Sox6	SRY-box containing gene 6	+	-	1.14
31	Stt3b	STT3, subunit of the oligosaccharyltransferase complex, homolog B (S. cerevisiae)	+	+	1.22
32	Tex2	testis expressed gene 2	+	+	1.16
33	Tlr12	toll-like receptor 12	-	+	1.10
34	Tmtc2	transmembrane and tetratricopeptide repeat containing 2	+	-	1.14
35	Xpo7	exportin 7	+	+	1.15
36	Xpot	exportin, tRNA (nuclear export receptor for tRNAs)	+	+	1.14
37	Zfp64	zinc finger protein 64	+	+	1.21

Tab.3-8 C. Genes affected by gene expression and methylation in a gene x environment fashion

#	SYMBOL	GENENAME
1	2010001K21Rik	RIKEN cDNA 2010001K21 gene
2	5430421N21Rik	RIKEN cDNA 5430421N21 gene
3	Ank3	ankyrin 3, epithelial
4	Atp10b	ATPase, class V, type 10B
5	Cabin1	calcineurin binding protein 1
6	Cage1	cancer antigen 1
7	Chrna9	cholinergic receptor, nicotinic, alpha polypeptide 9
8	Clpb	ClpB caseinolytic peptidase B homolog (E. coli)
9	Dppa3	developmental pluripotency-associated 3
10	F10	coagulation factor X
11	Fam135a	family with sequence similarity 135, member A
12	Fam170a	family with sequence similarity 170, member A
13	Foxj1	forkhead box J1
14	Glra1	glycine receptor, alpha 1 subunit
15	Heatr1	HEAT repeat containing 1
16	Mbp	myelin basic protein
17	Parp14	poly (ADP-ribose) polymerase family, member 14
18	Pcdhb5	protocadherin beta 5

19	Pim1	proviral integration site 1
20	Pla2g5	phospholipase A2, group V
21	Psd3	pleckstrin and Sec7 domain containing 3
22	Tcof1	Treacher Collins Franceschetti syndrome 1, homolog
23	Uevld	UEV and lactate/malate dehyrogenase domains
24	Wdr66	WD repeat domain 66

Affected genes by gene expression and methylation through the 5-Htt genotype, PS exposure and gene x environment interactions. Abbreviations: exp, expression; FC, Fold change; meth, methylation

3.4 Resilience to stress exposure

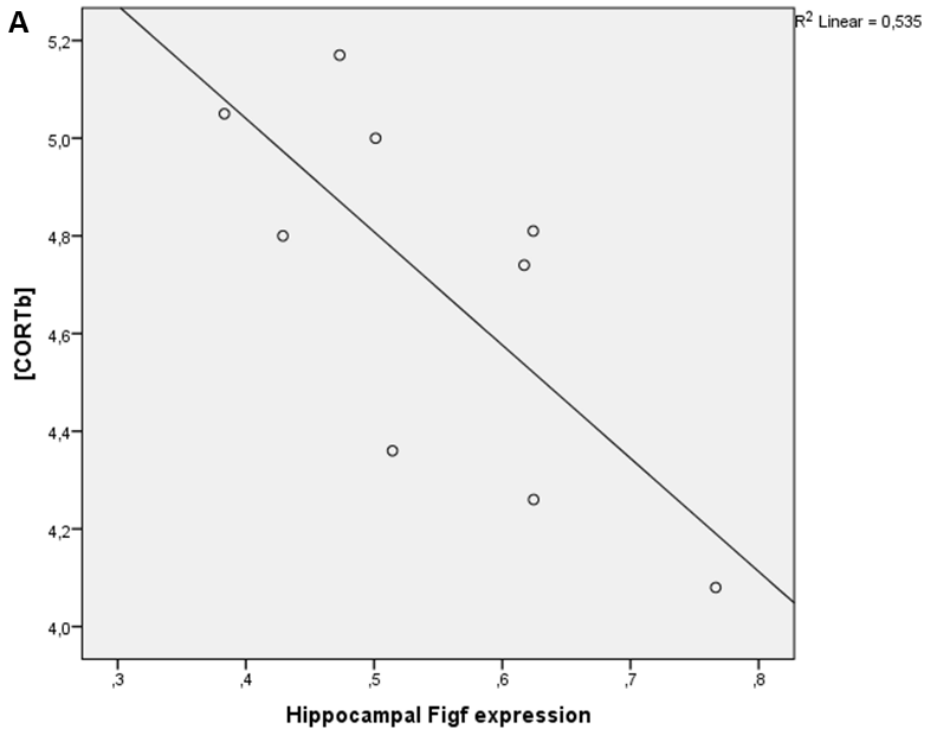
3.4.1 Prenatal stress exposure

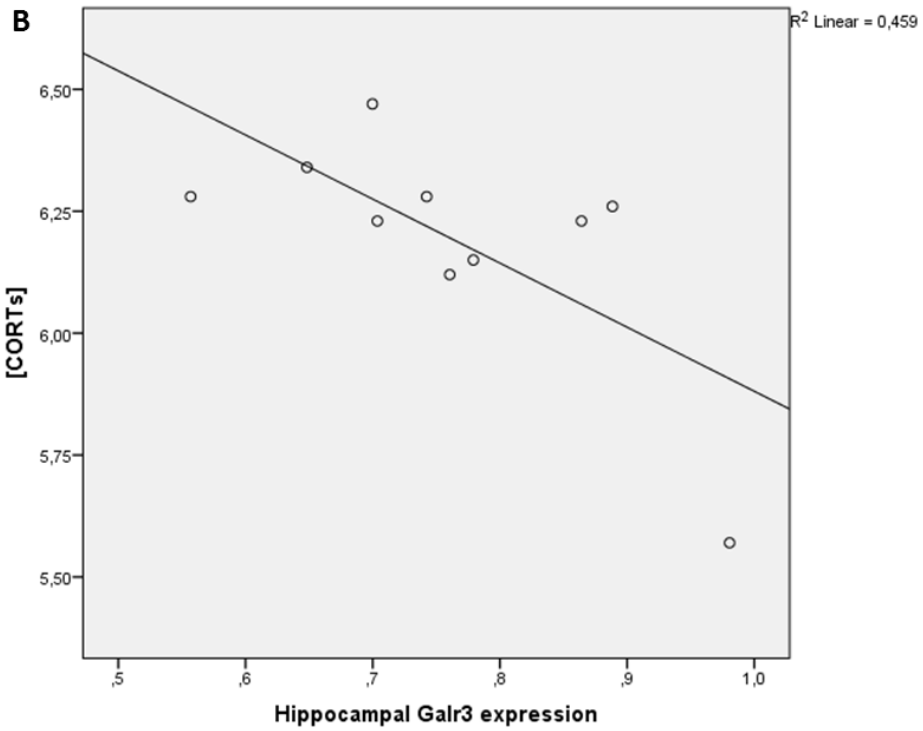
All of the 7 selected resilience genes from the microarray (see Supplement 5.1.7 and 5.1.8) showed several correlations with distinct behaviors in the PS group (see Tab. 3-9). For example, in female WTPS mice, *Figf* correlated with basal corticosterone (CORTb) concentration (r=-0.767; P= 0.016). Further, *Galr3* showed a significant negative correlation with stressed CORT levels in female 5-Htt+/- PS mice (CORTs; r=-0.713; P=0.021). As depicted in Figure 3-10 C and D, *Gh* (r=-0.714; p= 0.071) and *Prl* (r=-0.714; p= 0.071) tended to correlate positively with adrenal weights.

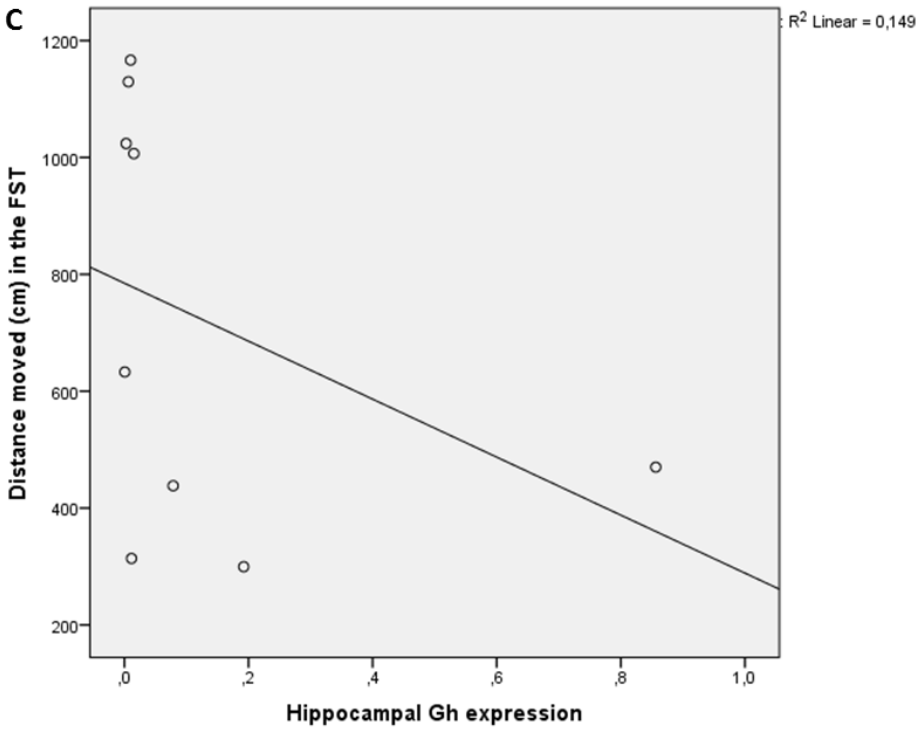
Table 3-9. Correlation of resilience genes with behavior

Gene	Female		Male	
	Spearman Correlation		Spearman Correlation	
	WTPS (n=7-9)	HETPS (n=9-10)	WTPS (n=8-9)	HETPS (n=6-9)
Gh	FST: P=0.099; r=-0.583 Adr: P=0.071; r=0.714	EZMdm: P=0.099; r=0.583	EZMdm: P=0.099; r=-0.583	
Prl	FST: P=0.058; r=-0.65 Adr: P=0.071; r=0.714	EZMdm: P=0.099; r=0.583	EZMoA: P=0.088; r=-0.6	Adr: P=0.024; r=-0.870
Camk2a			CORTb: P=0.016; r=-0.767	
Figf	EZMdm: P=0.067; r=-0.633 CORTb: P=0.016; r=-0.767		EZMoA: P=0.067; r=0.633 CORTr: P<0.01; r=-0.933	CORTb: P=0.067; r=0.633 AdrBW: P= 0.02; r=0.75
Galr3	EZMdm: P=0.088; r=-0.6	CORTs: P=0.021, r=-0.713	CORTb: P=0.067; r=-0.633 Adr: P<0.01; r=-0.838	
Syt5	EZMdm: P=0.088; r=-0.6		CORTb: P=0.088; r=-0.6 CORTr: P=0.025; r=-0.733 Adr: P<0.01; r=-0.922	
Prkar2b		CORTr: P=0.056; r=0.62	CORTb: P=0.025; r=-0.733	

Correlations of resilience genes with behavior. Abbreviations: Adr, Adrenal weight; AdrBW; Adr corrected for body weight; CORTln, logarithm of the corticosterone concentration (B, basal; R, recovery; S, stress); EZMdm, distance moved in the elevated zero maze; EZMoA, time spent in open arms of the elevated zero maze; FST, forced swim test




B
R² Linear = 0,459
6,50
6,25
6,00
5,75
5,50
[CORTs]
,5
,6
,7
,8
,9
1,0
Hippocampal Galr3 expression


C
R² Linear = 0,149
1200
1000
800
600
400
200
Distance moved (cm) in the FST
,0
,2
,4
,6
,8
1,0
Hippocampal Gh expression

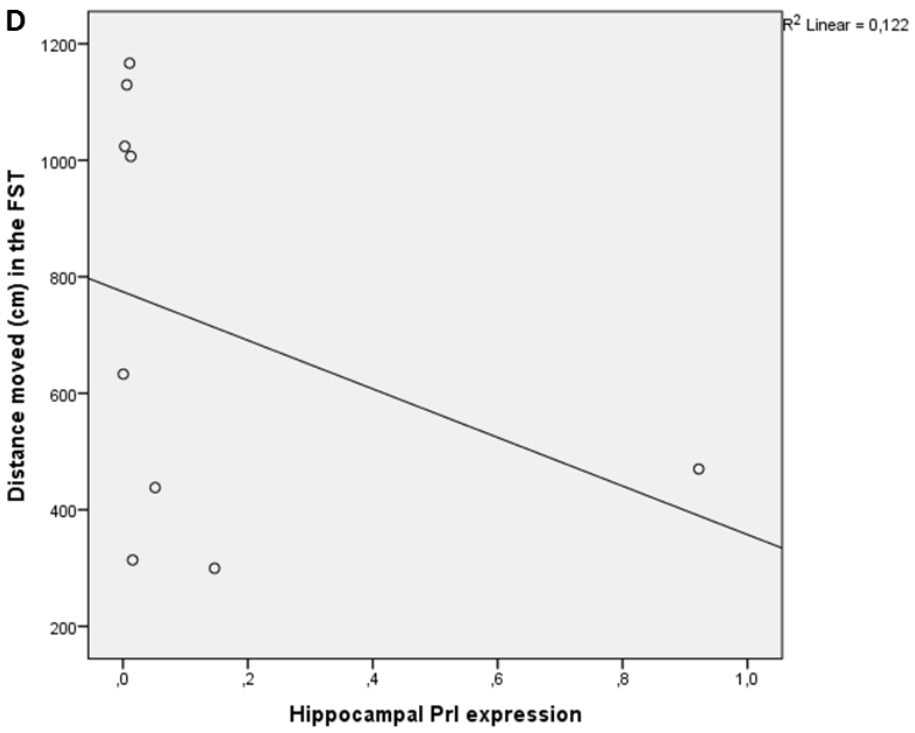


Figure 3-9 A-D. Scatterplot of correlation of resilience genes with behavior in the prenatal stress paradigm. A) *Fos-induced growth factor (Figf)* correlated negatively with basal corticosteron levels (CORTb). B) *Galanin receptor 3 (Galr3)* is negatively correlated with CORT stress (CORTs) concentrations. C.) *Growth hormone (Gh)* tended to correlate posetively with adrenal weights as did *prolactin (Prl*; D)

3.4.2 Perinatal stress exposure

We tested, whether *Gh* and *Prl* also correlated with behavior in the 5-Htt x PeS paradigm. Accordingly, *Gh* expression tended to show a negative correlation with the percentage of time spent on the open arms of the elevated plus maze (EPM; r=-0.69, P=0.058) in WTPeS animals. Further, *Prl* correlated positively with the percentage of time spent in the open arms of the EPM (r=0.812, P=0.05; see Figure 3-11) in the HETPeS group.

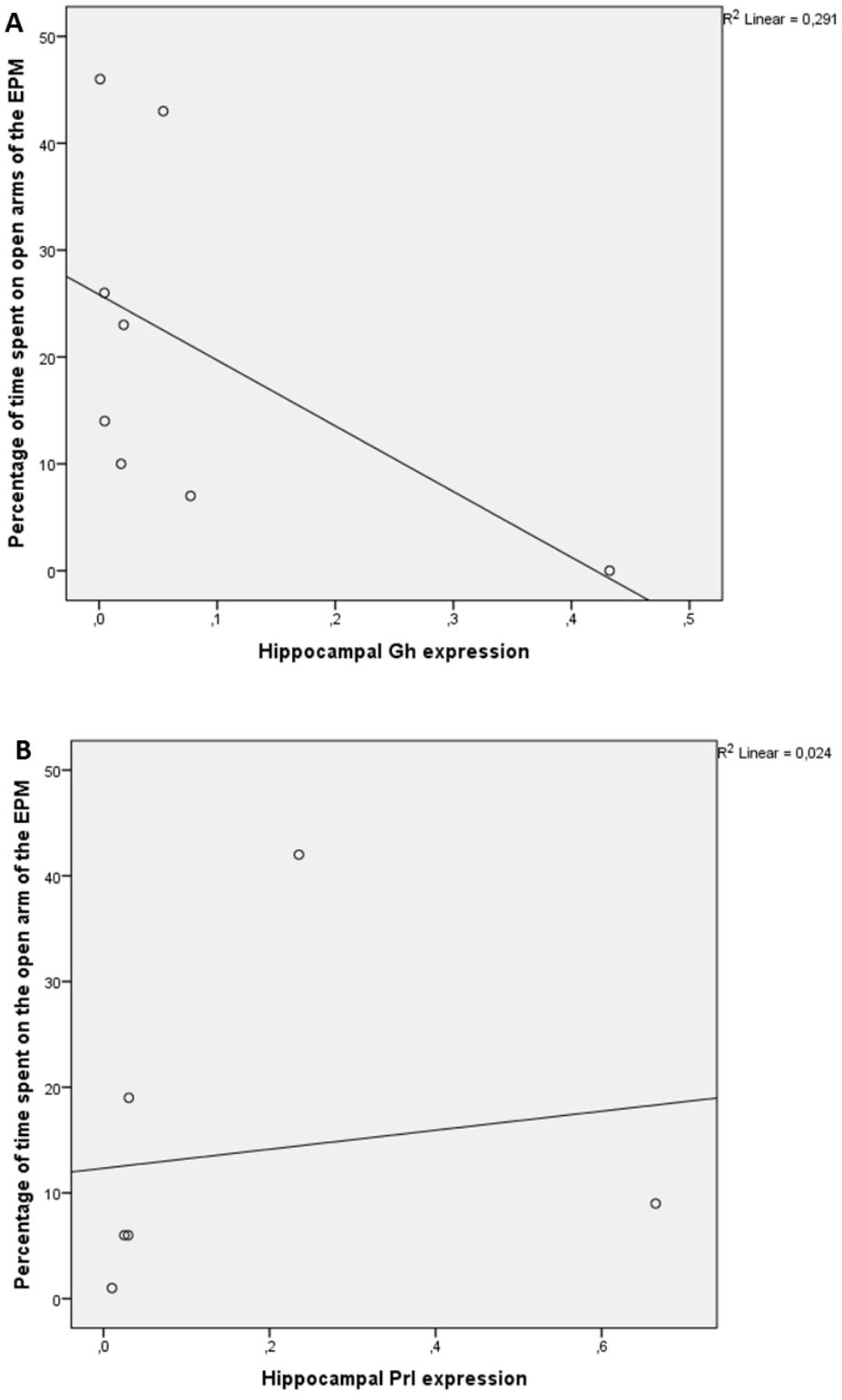


Figure 3-11 A-B. Scatterplot of correlation of resilience genes with behavior in the perinatal stress paradigm. A) Growth hormone (Gh) expression tended to correlate negatively with the percentage of time spent on open arms of the eleveated plus maze (EPM). Spearman correlation, 0.05<P<0.1 B) Prolactin (Prl) displayed a significant, positive correlation with the percentage of time spent on open arms of the EPM. Spearman correlation, P<0.05

4. Discussion

4. Discussion

The present study demonstrates that exposure of 5-Htt+/- mice to prenatal maternal stress is associated with increased depression-like behavior, an effect that appeared to be more profound in female offspring. Conversely, adult 5-Htt+/- mice displayed increased memory performance as well as signs of reduced anxiety in comparison to WT offspring. Further, female 5-Htt genotype, PS and their interaction were associated with distinct hippocampal gene expression and DNA methylation profiles, the implications of which are discussed in more detail below. In addition, this study indicated an intricate relationship between PS/PeS exposure, emotional behavior and the HPA axis.

Combined behavioral effects of offspring 5-Htt genotype and PS

This is the first study measuring the effects of developmental stress exposure on adult cognition, anxiety and depression-like behavior as well as HPA axis responsivity in both male and female 5-Htt+/- offspring. While, 5-Htt+/- mice showed improved memory performance in the ORT when compared to WT offspring, PS exposure appeared to impair object memory performance in the same task. Further, rather unexpectedly, 5-Htt+/- mice seemed to be less anxious in comparison to WT mice, as indicated by an increased time spent in the open arms of the EZM. While PS exposure did not affect this particular type of anxiety-like behavior, it did reduce exploratory behavior in the same task. This was indicated by a reduced distance moved, primarily in WT offspring. In addition, exposure to the FST was linked to an enhanced depression-like behavior in PS mice, an effect which seemed to be particularly pronounced in 5-Htt+/- female offspring. Furthermore, 5-Htt+/- mice showed reduced basal CORT levels when compared to WT offspring.

While, the 5-Htt+/- genotype showed beneficial effects in the ORT, PS exposure appeared to impair object memory performance, which is in line with previous observations (Behan *et al.* 2011). The 'protective' genotype effect is in contrast with a previous study by Olivier and coworkers (Olivier *et al.* 2008), which demonstrated that 5-Htt+/- rats have impaired object memory when using longer intervals in the ORT. There is limited information about the role of the human 5-HTT genotype in learning and memory. In line with our data, Roiser and colleagues (Roiser *et al.* 2007) found that individuals homozygous for the 5HTTLPR s-allele show improved memory and attention in comparison to ll-carriers. Recently, it has been proposed that the beneficial cognitive effects of the s-allele may explain why genetic variation resulting in low 5-HTT function has been maintained throughout evolution (Homberg and Lesch 2011). The exact role of the 5-HTT in cognition remains to

be elucidated, though. For example, it would be interesting to study the effects of 5-Htt genotype variation in spatial, hippocampus-dependent memory processing, by e.g. employing a spatial variant of the ORT, i.e., the object location test (OLT; see (Vanmierlo *et al.* 2011)).

Observations in the EZM revealed a significant genotype effect indicating that 5-Htt+/- mice were less anxious when compared to WT mice. A recent study by Heiming and colleagues (Heiming *et al.* 2009), which measured exposure of 5-Htt+/- females to olfactory cues of unfamiliar adult males during pregnancy and lactation did not detect any differences in anxiety between 5-Htt+/- and WT offspring. Another recent investigation by Jones and coworkers (Jones *et al.* 2010) indicated that offspring of both 5-Htt+/- and WT dams, which were stressed during pregnancy showed signs of reduced anxiety as compared to control offspring. Next to this, maternal stress exposure enhanced anxiety-like behavior in offspring from WT mothers only. Another study by Carola *et al.* (Carola *et al.* 2008) revealed clear signs of evidence for enhanced anxiety in male 5-Htt+/- mice when exposed to low levels of maternal care during early life. The observed discrepancy between the various investigations might be based on different study designs. First, diverse breeding schemes were employed. Second, maternal and/or paternal genotype varied. Third, there were different experimentally induced variations in the pre- and postnatal environment. Forth, the age differed at which the behavioral testing was performed. For example, in the study by Heiming and coworkers (Heiming *et al.* 2009), 5-Htt+/- dams were exposed to stress perinatally. Jones and colleagues (Jones *et al.* 2010), applied a different breeding design and utilized a chronic variable stress paradigm, starting from gestational day 6 and lasting until parturition. Further, offspring genotype was not taken into account in that study, which makes accurate comparison of the data difficult. In the study by Carola et al. (Carola *et al.* 2008), reciprocal inter-crossing between C57BL/6J and BALB/cByJ was applied, in order to measure the effect of variations in maternal care. Both strains carried different alleles of Tph2, thereby introducing additional genetic variation to the study design. In fact, the Tph2 genotype significantly affected the behavioral outcome, indicating a G x G x E effect. In addition, only WT mothers were utilized for breeding in that study. All in all, when it comes to adult anxiety-like behavior, exposure of 5-Htt+/- mice to developmental stress may have various, differential, complex programming effects, the nature of which is dependent on numerous factors.

Of interest, especially male 5-Htt+/- mice seemed to have lower basal CORT levels in comparison to WT offspring. At the same time, female 5-Htt+/- mice seemed to have larger adrenals compared to WT mice. Thus, one might speculate that the 5-Htt+/- genotype is linked with a sex-dependent

change in the set-point of the HPA axis, which, in turn, may be related to the different susceptibility of both sexes when it comes to e.g. developmental stress exposure. In this context, Wuest and colleagues demonstrated that male ss-allele carriers displayed the lowest cortisol awakening response (an indirect measurement of the basal cortisol secretion) when compared to sl- and ll-carriers, whereas female ss-carriers showed the highest (Wust *et al.* 2009). Whether the diminished basal CORT levels in 5-Htt+/- offspring contribute to the reduced levels of anxiety as seen in the EZM in 5-Htt+/- mice remains to be elucidated.

Adaptive capacity of variations in the 5-Htt genotype

All in all, the increased memory performance and decreased anxiety as seen in 5-Htt+/- mice underscore the adaptive capacity of this specific genetic variation (Homberg and Lesch 2011; Homberg 2012). In fact, when looking more closely at the FST data, control 5-Htt+/- offspring even appeared to show lower levels of depression-like behavior in comparison to WT mice. At the same time, only 5-Htt+/- mice exposed to PS showed enhanced levels of depression-like behavior. Taken together, these findings echo the perception that the classical deficit-oriented association of the 5-HTTLPR variants may be too simple. In effect, our current data propose that variation in the 5-Htt genotype acts in concert with variations in the prenatal environment, thereby determining (i.e. programming), in a sex-specific manner, whether a response to an acute environmental challenge in adulthood (e.g. cognitive and/or emotional) will turn out to be positive or negative.

Overall, the current data indicate that although the 5-Htt+/- genotype shows clear adaptive capacity, while it appears to enhance the susceptibility to developmental stress exposure, predominantly in female offspring.

Hippocampal gene expression profiles

Microarray analysis indicated various effects of female 5-Htt genotype, PS, and their interaction on hippocampal gene expression profiles, which may partly explain the distinct behavioral phenotypes observed among the different experimental groups. Below, the role of several relevant genes and biological pathways are discussed in more detail.

MAPK signaling

DAVID analysis indicated an overall negative effect of the 5-Htt+/- genotype and a neutral to positive effect of PS on the MAPK signaling pathway within the female hippocampus. MAPK signaling plays an important role in embryogenesis, cell differentiation, cell proliferation and cell death (Asaoka and Nishina 2010; Keshet and Seger 2010). Aberrant MAPK signaling has been implicated in the development and course of several psychiatric disorders (Pearson *et al.* 2001; Li *et al.* 2009). The MAPK pathway comprises three major signaling cascades, i.e. the extracellular signal-regulated kinases 1 and 2 (ERK 1/2) cascade, the c-Jun N-terminal kinases (JNK) cascade and the p38 pathway, all of which are regulated in a complex, interactive manner. Downstream signaling is mediated via a kinase phosphorylation cascade culminating in the activation of transcription factors and the expression of specific genes.

When scrutinizing the impact of the 5-Htt+/- genotype and PS exposure, we found that PS, but not the 5-Htt+/- genotype, exerts a strong positive effect on the JNK cascade, whereas the p38 cascade remains nearly unaffected. A similar effect was demonstrated by Liu *et al*. (Liu *et al.* 2004) and Meller *et al*. (Meller *et al.* 2003) after acute stress in both the mouse and rat hippocampus. We found three JNK-activating genes, the *interleukin 1 receptor, type 1 (Il1r1), mitogen-activated protein kinase kinase kinase 12 (Map3k12)* and *mitogen-activated protein kinase kinase 7 (Map2k7)* and one Jnk-substrate, *jun oncogene (Jun*, also known as *c-Jun*) being up-regulated by PS. Furthermore, both the 5Htt+/- genotype and PS exert an overall negative effect on the ERK1/2 cascade. Genes which were down-regulated by both factors are e.g. the *calcium channel, voltage-dependent, gamma subunit 3 (Cacng3), protein kinase C (Prkcc)* and the *fibroblast growth factor receptor 1 (Fgfr1)*. In addition, the 5Htt+/- genotype decreased the expression of *Fgf1*, the major ligand of *Fgfr1*. Interestingly, a dysfunction in FGF signaling has been proposed to play an important role in the etiology of mood disorders (Turner *et al.* 2006), which is underlined by the finding that patients with major depression disorder (MDD) show an increased *FGFR1* expression in various hippocampal subregions (Gaughran *et al.* 2006). Next to this, the expression of *Fos*, a transcription factor, which represents a critical downstream target of the ERK pathway, was increased after PS exposure (an effect which was validated by qRT-PCR).

Neurotrophin signaling pathway

The neurotrophin family consists of BDNF, nerve growth factor (NGF), neurotrophin 3 (NT-3), and neurotrophin 4 (NT-4), which bind to the tyrosine kinase (Trk) receptor family (including TrkA, TrkB and TrkC) as well as to the p75 neurotrophin receptor ($p75^{NTR}$). This leads to the activation of different downstream signaling cascades which modulate neuronal and synaptic plasticity. Thus, the neurotrophin signaling pathway is implicated in the etiology and therapy of depression ("neurotrophin hypothesis of depression";(Duman *et al.* 2000; Jacobs *et al.* 2000)). We demonstrated that the 5-Htt+/- genotype and PS exposure significantly affect neurotrophin signaling indicating that both genetic and environmental factors contribute to dynamic neuronal and synaptic plasticity in the hippocampus. More specifically, TrkB signaling was targeted by both the 5Htt+/- genotype and PS. For example, *Ntrk2 (TrkB)* receptor expression itself was diminished in 5-Htt+/- offspring. Besides its essential role in promoting long-term potentiation, hippocampal TrkB signaling is critical in cell survival (Lu *et al.* 2005; see below). The TrkB receptor is activated by Bdnf and controls three different major pathways, the P13 kinase cascade, the PLC-γ1 cascade and the ERK1/2 MAPK cascade (Reichardt 2006). The last one was primarily influenced by both 5-Htt+/- and PS. This cascade targets Creb1 whose nuclear activation is an important component of a general switch that converts short-term into long-term plasticity (Barco *et al.* 2002). Before inducing Creb1-dependent transcription, its transcriptional repressor Creb2 (or activating transcription factor 4; Atf4) has to be released. Consequently, it could be suggested that the observed decline in *Ntrk2* expression in 5-Htt+/- animals, in combination with the increase in *Creb2* expression, induced by PS exposure, contributed to impaired Bdnf signaling and related neuronal and synaptic plasticity, thereby eliciting depression-like behavior (Taliaz *et al.* 2010).

While both 5-Htt+/- genotype and PS appear to impair TrkB signaling in a similar manner, G and E seem to affect $p75^{NTR}$ signaling in an opposite way. The majority of genes influenced by the 5-Htt+/- genotype showed decreased expression patterns, among which are the *transformation-related protein 73 (Trp73), transformation-related protein 53 (Trp53), tyrosine 3-monooxygenase / tryptophan 5-monooxygenase activation protein, zeta polypeptide (Ywhaz),* and the *interleukin-1 receptor-associated kinase 1 (Irak1)*. On the other hand, the vast majority of genes affected by PS in this pathway displayed enhanced expression patterns, among which the *mitogen-activated protein kinase kinase kinase 1 (Map3k1*), *mitogen-activated protein kinase kinase 7 (Map2k7*) and *c-Jun* could be found. Activation of the $P75^{NTR}$ pathway is proposed to play a prodepressive role (Martinowich *et al.* 2007). In more detail the activation of the $P75^{NTR}$ pathway has been linked to an elevation in long

term depression (LTD) (Woo *et al.* 2005). Emerging evidence supports the idea that LTD may play a role in regulating stress- and depression-like behavior. For example, several studies reported a correlation between (behavioral) stress and the induction of LTD in adult rats (Xu *et al.* 1997; Holderbach *et al.* 2007). Moreover, treatment with antidepressants could reverse chronic mild stress–induced LTD (Holderbach *et al.* 2007). All in all, hypothetically, dysfunctional neurotrophin signaling, might mediate, at least in part, the altered depression-like behavior observed in PS 5-Htt+/- mice.

Cytokine-Cytokine receptor interaction

Although the 5-Htt+/- genotype and PS exposure often act on distinct specific molecular targets, there is extensive degree of overlap when considering the biological signaling pathways they affect *independently* from each other. When it comes to hippocampal gene expression profiles that indicate a G x E interaction, i.e. those genes of which the regulatory impact of PS is *dependent* upon the 5-Htt genotype, a different pattern is observed. In this respect, our data propose that cytokine-cytokine receptor interactions play a vital role when a dysfunctional 5-HT system and stress interact. This notion is supported by the study of Fredericks *et al.* (Fredericks *et al.* 2010) who found that healthy women homozygous for the s-allele of the 5-HTTLPR have enhanced pro-inflammatory cytokine levels and a higher IL-6/IL-10 ratio both at baseline and during stress, when compared to ll-individuals. Interestingly, the risk for developing a clinically relevant depression after cytokine therapy is elevated in people who carry the s-allele (Lotrich *et al.* 2009). Further, the pro-inflammatory cytokine level of patients suffering from major depression is higher in comparison to non-depressed individuals (Tsao *et al.* 2006; Capuron *et al.* 2008; Cizza *et al.* 2008; Dantzer *et al.* 2008). However, treatment with selective serotonin reuptake inhibitors (SSRIs) is able to reduce this enhancement (Kenis and Maes 2002; Dantzer *et al.* 2008). In addition, after experimental or therapeutic administration of pro-inflammatory cytokines, humans with originally no signs of depression, show depressive symptoms (Capuron *et al.* 2000; Reichenberg *et al.* 2001; Miller *et al.* 2009; Raison *et al.* 2009). For example, about half of all patients treated for a long period with interferon get depressed, and this state of mood can be meliorated by SSRI treatment (Capuron *et al.* 2002). Interestingly, it has been proposed that interferon-induced immune activation on depression may be explained in part by alterations in neurotrophin signaling capacity, reflected by decreases in serum BDNF following interferon treatment (Kenis *et al.* 2011). In the present study neurotrophin signaling was affected by both G and E. In this context, it has been suggested that pro-inflammatory cytokines like interferon-γ (INF-γ) and tumour necrosis factor α (TNFα) reduce the availability of

tryptophan, the precursor for 5-HT, by inducing indoleamine-2,3-dioxygenase (IDO) (Robinson *et al.* 2005; Schiepers *et al.* 2005; Leonard and Myint 2009). In addition, the expression of several members of the Tnf and Tnf receptor superfamily, such as *Tnfsf8, Tnfrsf11a, Tnfrsf1a, Tnfrsf1b*, was regulated in a G x E manner. Further evidence for a molecular interaction between 5-HT and cytokines is given by the observation that mice lacking the interleukin-15 receptor (Il15ra), the expression of which was also affected within our model in a G x E manner, showed increased depression-like behavior, whereas fluoxetine was able to reduce it. These Il15ra knockout mice displayed decreased hippocampal expression of 5-Ht_{1A} receptor, increased hippocampal expression of 5-Ht_{2C}, and region-specific alterations of 5Htt immunoreactivity (Wu *et al.* 2011). Furthermore, the lack of Il15ra resulted in decreased anxiety in these mice (Wu *et al.* 2010), which indicates a comparable behavioral phenotype as observed in the present study. All in all, it may be hypothesized that, when challenged by e.g. developmental stress exposure, a dysfunctional 5-HT system could lead to a disturbed cytokine balance thereby increasing the vulnerability to stress, eventually resulting in psychiatric conditions.

Wnt signaling pathway

Next to cytokine-cytokine receptor interactions, also Wnt signaling was significantly influenced in a G x E manner. Wnt proteins are required for basic developmental processes and act via at least 3 different Wnt pathways: the canonical pathway, the planar cell polarity (PCP) pathway and the Wnt/Ca^{2+} pathway (Staal *et al.* 2008). All 3 cascades are initiated via Wnt binding to frizzled (Fzd). Interestingly, the mRNA expression of *frizzled homolog 3 (Fzd3)* was altered in the present study in a G x E fashion, indicating that all three abovementioned cascades are affected in our model. Furthermore, our microarray analysis revealed 4 other affected genes in the PCP signaling pathway, among which were *Rac2* and *Rock1*. With respect to this pathway, binding to Fzd3 results in the activation of the small GTPases RhoA and Rac1, which activate the stress kinase Jnk (Jun N-terminal kinase) and Rock, which eventually induces remodelling of the cytoskeleton and associated changes in cell adhesion and motility. The canonical Wnt pathway and especially 2 of its key players, glycogen synthase kinase 3beta (GSK-3beta) and beta-catenin, have been highly implicated in the etiology of psychiatric disorders such as depression, schizophrenia and bipolar disorder (Emamian *et al.* 2004; Gould *et al.* 2004; Gould *et al.* 2008; Wada 2009). Moreover, diminished Gsk-3beta and enhanced beta-catenin levels in the mouse brain have been associated with a better performance in the FST (Gould *et al.* 2008). Although these two key players were not directly affected in terms of gene expression within our 5-Htt x PS model, the mRNA expression of *presenilin1 (psen1)*, whose protein

product is known to bind and stabilize beta-catenin (Chen and Schubert 2002), was regulated in a G x E fashion.

Xaf1, Mbp and Trhr

qRT-PCR validation revealed significant genotype effects for e.g. *Xaf1, Mbp* and *Trhr*. Xaf1 plays an important role in programmed cell death by inhibiting the anti-apoptotic functions of the X-linked inhibitor of apoptosis (XIAP) and of other members of the family of inhibitors of apoptosis (IAP), like survivin (Arora *et al.* 2007). Xiap has trophic effects on hippocampal neurons by enhancing *Bdnf* and *TrkB* activity (Kairisalo *et al.* 2009). Thus, the observed elevation in *Xaf1* and the decline in *TrkB* expression (see above) in adult 5-Htt+/- mice suggest a decreased resistance to apoptosis in these animals. Previous investigations by Ravary *et al.* (Ravary *et al.* 2001) and Persico *et al.* (Persico *et al.* 2003) demonstrated no signs of elevated neuronal apoptosis in 5-Htt+/- versus WT mice. Whether additional environmental stress exposure influences the levels of apoptosis in 5-Htt+/- animals remains to be elucidated. Next to *Xaf1*, the effects of e.g. *Mbp* and *Trhr* were also validated by qRT-PCR. Mbp is involved in myelinisation of the central nervous system and has recently been linked to schizophrenia (Martins-de-Souza *et al.* 2010). Trhr plays a role in the hormone system and neuromodulation. Interestingly, mice with a deficiency for the *Trhr1* show an anxiety- and depression-like behavior (Zeng *et al.* 2007). Evidently, the implications of the present findings await further research.

DNA methylation

Microarray analysis indicated that several genes that were differentially expressed were also differentially methylated. Below, the role of several relevant genes, which were both differentially expressed and methylated, is discussed in more detail.

The gene expression and DNA methylation screenings showed that the expression of Mbp is dependent on the interaction of 5-Htt+/- genotype and PS exposure, the regulation of which may be mediated by Mbp promoter methylation. Approximately 40% of the myelin in the CNS is composed of Mbp. In the rat brain, myelin formation is initiated at postnatal day 14 and is active during the next 7 days, leading to an accumulation of myelin in all brain areas. During the following month, myelinisation is decreased (Akiyama *et al.* 2002). Interestingly, early weaning, which is accompanied

by an increased anxiety-like behaviour in adulthood (Kikusui *et al.* 2004), resulted in an increase of Mbp expression in the third week and a decline in the fifth week when compared to normally weaned mice, proposing that early maternal deprivation induces precocious myelin formation (Kikusui *et al.* 2007). In humans, cortical postmortem tissue of bipolar and major depressive disorder patients showed a reduction in myelin (Ongur *et al.* 1998), indicating that Mbp might also play a role in affective disorders as it obviously does in schizophrenia (Hakak *et al.* 2001; Hof *et al.* 2003; Martins-de-Souza *et al.* 2010). Further, corticosterone metabolites led to a decrease in Mbp in oligodendrocytes (Melcangi *et al.* 1997). An independent study by our group in the hippocampus of C57BL/6 mice revealed a decreased Mbp protein level in WT female offspring when compared to 5-Htt+/- mice, which supprts the notion that Mbp plays an important role when it comes to developmental stress exposure (Föcking 2012, in preparation).

Our microarrays revealed that the expression of *bone morphogenetic protein receptor type 1B (Bmpr1b)* and *MAD homolog 7 (Drosophila; Smad7)* was downregulated, whereas the methylation was upregulated in 5-Htt+/- mice when compared to WT mice. BMPs belong to the TGFß superfamily and are secretory growth factors. They promote astrogliagenesis *in vivo* and *in vitro* (Gross *et al.* 1996; Bonaguidi *et al.* 2005) and neurogenesis in the cortex (Li *et al.* 1998; Mabie *et al.* 1999), whereas neurogegesis is inhibited by BMP2 and -4 in the embryonic and adult striatum (Gross *et al.* 1996; Lim *et al.* 2000). Inhibition of BMP signalling by HDACs or the downstream cytoplasmic factor Smad7 leads to increased neurogenesis (on the expense of astrogliagenesis) in the ganglionic eminence, whereas this has opposite effects in the embryonic cortex (Shaked *et al.* 2008). Evidently, BMPs play a major role in the switch from neurogenesis to astrogliagenesis in late gestation. Interestingly, mice that were constitutively deficient for *Bmpr1b* and conditionally deficient for *Bmpr1a*, the dentate gyrus was markedly diminished in size when compared to control mice and the stem cell niche that generates new neurons in the adult hippocampus was reduced. Furthermore, these double mutant mice responded less to fear- and anxiety-provoking stimuli (Caronia *et al.* 2010). Whether the reduced expression of *Bmpr1b* in 5-Htt+/- deficient mice leads to a decreased anxiety remaines to be clarified. Next to this, the expression of *potassium inwardly-rectifying channel, subfamily J, member 5 (Kcnj5*, also known as *Girk4*) appeared to be upregulated in the 5-Htt+/- mice. Kcnj5 could be inhibited by several antidepressants (Kobayashi *et al.* 2011). The methylation, was upregulated as well which crashes with the classical notion that an increased methylation leads to a decrease in expression. Recently, it was suggested that the classical point of view is oversimplified (for review see Suzuki and Bird 2008).

Our microarrays indicated that the expression and the methylation of *prokineticin 2 (Prok2)* was upregulated in PS animals. Prok2 belongs to a pair of cysteine-rich secretory proteins and has recently been suggested to be a central output molecule of the suprachiasmatic nucleus (SCN) (Cheng *et al.* 2002; Cheng *et al.* 2005), the pacemaker that drives circadian rhythms (Moore 1997). Interestingly, a disruptive circadian rhythm is linked to several mood disorders including depression (McClung 2007; Turek 2007). For instance, mice that are deficient for Prok2 showed decreased anxiety-like and depression-like behaviors (Li *et al.* 2009), which suggests that the observed increase of Prok2 as seen in our study might play an important role in conveying depression-like behavior in the PS mice. Further, the microarrays revealed an upregulated methylation and a downregulated expression of *nitric oxide synthase 1, neuronal (Nos1)* in PS compared to control mice. Nos1 has been shown to be an important neurotransmitter in the nervous system. It is involved in the regulation of various cognitive and emotional processes involved in depression and anxiety (Wiley *et al.* 1995; Harkin *et al.* 1999). Selective inhibitors of Nos1 have antidepressant- and anxiolytic-like effects (Volke *et al.* 2003), Nos1 knockout mice however, did not show any differences in depression-like behaviour compared to WT mice (Wultsch *et al.* 2007). Merely, these knockout mice showed reduced anxiety-like behavior (Wultsch *et al.* 2007). Wether the reduced expression of Nos1 in the PS mice mediates the observed depression-like effects needs to be further investigated.

Vulnerability and resilience to PS

Performance-oriented LIMMA analysis on the mRNA expression microarray data followed by subsequent Spearman correlation analysis linking the individual mRNA expression data as obtained by qRT-PCR to behavioral performance as well as to neuroendocrinological measures revealed various significant correlations pinpointing specific molecular substrates that may, at least in part, explain the behavioral response to PS. More specifically, the performance-based LIMMA analysis offers the opportunity to identify molecular mechanisms specific to either vulnerabilty or resilience to PS. Accordingly, amongst PS WT animals, we observed a negative correlation between *Figf* expression and basal plasma CORT levels. Similarly, among PS 5-Htt+/- mice, *Galr3* expression was negatively correlated with stress-induced plasma CORT levels. Furthermore, in WT animals, *Gh* and *Prl* expression tended to correlate positively with adrenal weights.

Figf belongs to the family of vascular endothelial growth factors (Vegf) and is also known as Vegf-D. Within the vascular system, Figf plays a major role in the regulation of lymphangiogenesis (Joukov *et al.* 1996; Achen *et al.* 1998; Kranich *et al.* 2009). As other members of the Vegf family are well studied in the brain, our knowledge about Figf and its function in the brain is poor. Here, we

demonstrate that *Figf* is expressed within the hippocampus, the extent of which is correlated with basal corticosterone levels. A study of Kranich and colleagues has already shown that *figf* was expressed in different brain cells (Kranich *et al.* 2009), among which e.g. microglial, schwannoma and glial precursor cells (GPCs), whereas the expression of its receptor Vegfr-3 was restricted to GPCs. This suggests an autocrine and paracrine function of Figf in the brain. Next to this, Figf was able to increase cell proliferation and the migration of GPC. Overall, the authors hypothesized that figf might play an important role in neurogenesis, possibly after ischemic insults or during brain development. In the present study, *figf* showed a negative correlation to basal CORT levels. This is consistent with a study of Yano and colleagues who demonstrated that (synthetic) glucocorticoids decrease Vegf production directly via the GR (Yano *et al.* 2006). Interrestingly, in the view of the notion that those animals showing the lowest basal CORT levels in response to stress exposure could be regarded as resilient (Schmidt *et al.* 2010) our data suggest that an elevated hippocampal *figf* expression might play an important role in mediating the central aspects of resilience in this respect.

Galr3 is one of the three receptor subtypes of galanin (Galr1-3). Galr3 mRNA is localized in distinct brain regions such as locus coeruleus, DRN, caudate putamen, hypothalamus, pituitary, spinal cord, but also in the pancreas, liver, kidney, stomach and adrenal gland suggesting that Gal3 might play an important role in emotion, feeding, pituitary hormone release, nociception and metabolism (Mennicken *et al.* 2002). Its localization in the brain might indicate that Galr3 in particular mediates especially the mood-related function of galanin. Galanin itself is a neuropeptide and is involved in the modulation of acetylcholine release and associated memory functions, and, as such, has been shown to be implicated in Alzheimer's disease (Crawley 1996; McDonald and Crawley 1997). In addition, galanin is known to influence the serotonergic, noradrenergic and dopaminergic system and has therefore been suggested to be implicated in the regulation of e.g. feeding behavior (Leibowitz 1995), pain (Kask *et al.* 1997) and affective states (Bing *et al.* 1993; Fuxe *et al.* 1998; Weiss *et al.* 1998; Moller *et al.* 1999; Khoshbouei *et al.* 2002; Lu *et al.* 2005). When galanin is injected into the DRN it decreases extracellular 5-HT output in the hippocampus which can be partly reversed by a Galr3 antagonist. This antagonist has anxiolytic- and antidepressant-like effects and identifies Galr3 to take part in the inhibitory effect of galanin in anxious and depressive states (Swanson *et al.* 2005). Furthermore, when galanin is injected into the paraventricular nucleus of the hypothalamus, it inhibits corticosterone release (Tempel and Leibowitz 1990), whereas peripheral administration shows no effect (Dunning and Taborsky 1988), indicating that this inhibition is mediated centrally. In line with this observed negative interaction, the present data show that increasing *Galr3* levels are linked to lower stressed plasma CORT levels, thereby possibly conferring resilience to PS exposure.

The present study revealed that hippocampal *Gh* expression was markedly downregulated in animals that performed well in the FST, and that *Gh* expression tended to correlate positively to adrenal weight. Gh is a 19-21 kDa cytokine polypeptide and is primarily expressed in the anterior pituitary, where it mediates longitudinal growth in the postnatal period (Kopchick and Andry 2000). Recently, evidence for an endogenous hippocampal *Gh* expression emerged (Donahue *et al.* 2002; Sun *et al.* 2005). In the hippocampus, it possibly induces long term potentiation via Creb-1 and may be involved in synaptic plasticity (Zearfoss *et al.* 2008), which might explain its role in memory formation (van Dam and Aleman 2004; Sonntag *et al.* 2005). Further, an acute stressor was able to increase Gh production in the hippocampus, possibly indicating a higher degree of learning (Donahue *et al.* 2002; Donahue *et al.* 2006). On the other hand, Gh decelerates extinction in rats (Schneider-Rivas *et al.* 2007), possibly indicating that the resilient animals do not learn the stressful stimulus or forget it faster and therefore display less depression-like behavior compared to the poorly performing mice. Interestingly, in Gh-deficient rats, Gh prevents adrenal weight growth, which is normally seen in early experimental diabetes (Kunjara *et al.* 2012). Along similar lines, insulin-like growth factor, the downstream effector of Gh, increases adrenal growth (Jackson *et al.* 1991). If, and if so, how hippocampal *Gh* expression regulates adrenal functions and therefore may confer resilience remains to be investigated.

The present study revealed that hippocampal *Prl* expression is markedly downregulated in animals who performed well in the FST, whereas its expression tended to correlate positively to adrenal weight. Prl is a polypeptide hormone secreted by the anterior pituitary which might act directly on the adrenal gland. For example, it stimulates steroidogenesis synergisticly with ACTH (Ogle and Kitay 1979). Several studies delineated that Prl stimulates aldosterone-producing adenomas and adrenocortical carcinoma (Carroll *et al.* 1982; Bole-Feysot *et al.* 1998). Interestingly, hyperprolactinemia in rodents resulted in an absolute and relative enhancement of adrenal weight (Fang *et al.* 1974; Bartke *et al.* 1984; Phelps *et al.* 1987; Silva *et al.* 2004). When looking at the perinatal stress paradigm, Prl significantly correlated with the percentage of time spent on open arms of the EPM in a positive manner indicating an increased *Prl* expression in anxiety-resilient animals. Prl has recently been suggested to act as a neuropeptide and its receptors can be found in the brain, including the hippocampus (Clapp *et al.* 1994; Ben-Jonathan *et al.* 1996; Pi and Grattan 1998). Interestingly, the Prl receptors in the brain convey anxiolysis (Torner *et al.* 2001), which makes it tempting to speculate that the higher Prl level in unsusceptible compared to susceptible mice confers an anxiety-resilient state.

Study limitations

It should be noted that the present study has several limitations. For example, animals were housed individually from weaning onwards in order to prevent the establishment of a hierarchy. Although the cages were in close proximity to each other (enabling visual contact between neighbouring mice) and the home cage was enriched with paper tissues and a cardboard tube, the isolated housing conditions could represent an additional stressor to the animals. Further, it is likely that the behavioral testing paradigms exhibited an independent effect on hippocampal gene expression profiles. Although behavioral task exposure was identical for all groups, one cannot exclude that the animals' response to it was different among groups. Thus, behavioral testing may have left a permanent imprint on hippocampal gene expression patterns in a genotype- and/or condition-dependent manner. Evidently, investigating behavior and its underlying biological mechanisms in the same set of animals enables the possibility of linking both features in a more direct way. Similarly, prior behavioral testing may have had an influence on the basal and stress-induced CORT levels. Nevertheless, an acute effect can be excluded, since the animals were left undisturbed for a week in between behavioral test sessions and blood sampling. Another remarkable notion is the fact that PS was associated with a higher degree of mortality in the offspring. Although a similar effect has been reported previously (e.g. Patin *et al.* 2002), in the present study this effect was observed immediately after weaning, when the offspring were moved to IVC cages. Personal observations suggest that the affected mice were too small and weak to drink enough water out of the IVC sipper tubes. In light of previous work from our group, which has shown a direct correlation between low birth weight –as a consequence of restricted fetal growth associated with PS exposure– and, amongst others, adult depression-like behavior (van den Hove *et al.* 2010), the consequent loss of these mice in the behavioral comparison later in life may have even weakened some of the observed behavioral effects of PS. Concerning the expression array, the information on the array (from 2002) and the current information about the genes did not match in all cases leading to false or non-annotated genes. Furthermore, we did not control for the estrous cycle in the female animals. As Gh and Prl are regulated by estrogens (Donahue *et al.* 2006; Nogami *et al.* 2007) we cannot rule out that a different state in the estrous cycle can confer resilience or vulnerability. This might also explain the high variations in the *Gh* results (data not shown).

Conclusion

Taken together, the present data suggest that the 5-Htt+/- genotype is associated with improved object memory function as well as signs of reduced anxiety. In contrast, exposure of 5-Htt+/- mice to

PS was associated with increased depression-like behavior, an effect that tended to be more pronounced in female offspring. Furthermore, 5-Htt genotype, PS and their interaction differentially affected the expression of numerous genes and related pathways within the female hippocampus. Whereas MAPK and neurotrophin signaling were regulated by both the 5-Htt+/- genotype and PS exposure, cytokine and Wnt signaling were affected in a 5-Htt genotype x PS manner, indicating a G x E interaction at the molecular level. Thus, the present study indicates that the long-term stress- and depression-related behavioral effects of PS in C57BL/6 mice are partly dependent on the 5-Htt genotype. Moreover, our gene expression and DNA methylation findings provide evidence for a molecular basis of such a G x E interaction, which eventually might help to identify novel targets for the diagnostic assessment and treatment of disorders of emotion regulation.

Outlook

As the current study design was not primarily designed to study the concept of resilience, one may come up with a modified resilience-based study design with more animals to increase power. To investigate our candidate genes, an overexpression of the candidates in the hippocampus can be performed. Further, we may perform fluorescence or magnetic activated cell sorting (FACS or MACS) to analyze specifically neuronal cells.

Nevertheless, the investigated genes are promising candidates and need to be further examined to acquire more knowledge about the neurobiological basis of emotional disorders. This knowledge is essential for understanding the molecular mechanisms underlying the emergence of these disorders and for the development of new treatment strategies.

5. Appendix

5. Appendix

5.1 Gene lists

5.1.1 Gene expression affected by the genotype (5-Htt+/- versus wild-type)

See CD

5.1.2 Gene expression affected by the environment (prenatal stress versus control)

See CD

5.1.3 Gene expression regulated at the gene × environment level

See CD

5.1.4 DNA methylation affected by the genotype (5-Htt+/- versus wild-type)

See CD

5.1.5 DNA methylation affected by the environment (prenatal stress versus control)

See CD

5.1.6 DNA methylation regulated at the gene × environment level

See CD

5.1.7 WTPS good performers in the FST versus all WTC mice

See CD

5.1.8 HETPS good performers in the FST versus all HETC mice

See CD

5.2 DNA methylation patterns of various Bdnf promoters in PS mice

5.2.1 Introduction

Chronic and/or excessive stress during pregnancy is known to enhance the release of stress hormones like cortisol and corticotrophin-releasing hormone (CRH) into the maternal and fetal bloodstream, thereby impacting upon early brain development. As such, prenatal stress (PS) is suggested to increase the risk of developing depression and anxiety disorders in the offspring later in

life (for review see e.g. Weinstock 2008). Whereas emotional disorders are among the leading causes of disability worldwide, their underlying molecular mechanisms are still poorly understood. There is growing evidence that brain-derived neurotrophic factor (BDNF), which is known for regulating neurogenesis as well as synaptic plasticity (McAllister *et al.* 1999) and its associate involvement in learning and memory (Poo 2001; Lu 2003; Lu and Chang 2004), plays an important role in this respect (Pezet and Malcangio 2004). For example, chronic administration of antidepressants results in an increase in rodent hippocampal and cortical BDNF levels (Nibuya *et al.* 1995; Nibuya *et al.* 1996). In addition, individuals, who were treated with antidepressants at the time of suicidal death, display enhanced BDNF levels in the hippocampus and prefrontal cortex (Chen *et al.* 2001; Dwivedi *et al.* 2003; Karege *et al.* 2005). Next to this, effects of antidepressants can be imitated by infusions of BDNF protein into the hippocampus of rodents (Siuciak *et al.* 1997; Shirayama *et al.* 2002), supporting the so-called 'neurotrophin hypothesis of depression', which postulates that decreased BDNF levels in the hippocampus and prefrontal cortex may contribute to atrophy and cell loss in the respective brain area, as observed in some depressed subjects (Duman and Monteggia 2006). In line with this hypothesis, stress, be it acute or chronic, is able to downregulate BDNF expression (Nibuya *et al.* 1995; Smith *et al.* 1995; Schaaf *et al.* 1998; Pizarro *et al.* 2004), whereas chronic, but not acute administration of antidepressants is able to reverse the effects of stress on BDNF (Nibuya *et al.* 1995; Russo-Neustadt *et al.* 1999). Recently, it has been shown that epigenetic modifications may play an important role in this context (for overview, see Boulle *et al.* 2012). For example, social defeat stress was shown to enhance the repressive histone methylation on the Bdnf promoters of transcript III and IV, whereas chronic administration of imipramine reversed this effect by inducing permissive histone acetylation at the same promoter regions (Tsankova *et al.* 2006). However, less is known about the role of DNA methylation patterns of the *Bdnf* gene in this respect.

In the present study we used bisulfite treatment of DNA followed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analyzed by the sequenom-EpiTYPER platform in order to examine the effects of prenatal maternal stress exposure on the degree of methylation at various *Bdnf* promoters (I,IV and IX).

5.2.2 Materials and methods

Animals for the EpiTYPER MALDI-TOF mass spectrometry study

DNA of murine hippocampus and cortex was provided by Daniel van den Hove and his group (Behan *et al.* 2011). The mice were treated as already described elsewere (Behan *et al.* 2011). Briefly, the pregnant dams (C57BL/6) were exposed to PS by restraining them in 250 ml glass cylinders, filled with 5 mm water, whilst being exposed to bright light. This procedure was applied during the last week of pregnancy 3 times daily for 45 min. Control mice were left undisturbed in their home cages. After behavioral tests for cognition (ORT), anxiety (EZM) and depressive-related behavior (Sucrose intake and FST) the mice were killed and brains collected. DNA was extracted by the help of QIAamp® DNA Mini kit (Qiagen, Hilden, Germany) following the manufactor´s instructions.

Animals for the pyrosequencing study

See 2.1.1

EpiTYPER MALDI-TOF mass spectrometry

EpiTYPER is a bisulfite-treatment-based method for the detection and quantitation of DNA methylation. Non-methylated cytosine (C) is converted to uracil (U), while methylated Cs remain unchanged by bisulfite treatment of genomic DNA. The differences in methylation can be detected by the mass spectrometer because C and U bases differ in their weights.

The bisulfite treatment was followed by a modified protocol from Zymo Research EZ DNA Methylation Kit. First, the CT-conversion reagent was prepared. To the CT-conversion reagent, 750 µl ddH_2O (or DEPC-H_2O) and 210 µl M-Dilution Buffer were added. This was mixed by vortexing for 10 min. Afterwards, to 500 – 1000 ng of total DNA, 5 µl M-dilution buffer and ddH_20 to a total volume of 50 µl were added, well mixed and incubated at 37°C for 15 min. After incubation, 100 µl of the prepared CT-conversion reagent was added to each sample and lightly vortexed. The reaction mix was then incubated in the dark with the following protocol: 21 cycles (95°C, 30 sec; 50°C, 15 min). Subsequently, the samples were incubated on ice for 10 min. 400 µl M-binding buffer was added to the samples and mixed by pipetting up and down. After that, the samples were loaded into a Zymo-Spin I Column, which was placed in a 2 ml collection tube. Afterwards, it was centrifuged at 11200

rpm for 30 sec and the flow through was discarded. M-wash buffer was then added to the column and spinned for 30 sec at full speed. Subsequently, 200 µl of M-desulphonation buffer was added to the column and incubated at room temperature for 15 min. After that, it was centrifuged for 30 sec at full speed. Afterwards, it was washed with M-wash buffer for 2 times. At the end, 100 µl ddH_2O was added directly to the column matrix and briefly spinned at 3000 rpm to elute the DNA. The bisulfited DNA (Bi-DNA) was aliquoted and stored at -20°C if not directly used.

Following the bisulfite treatment, a PCR was performed. 2.22 µl ddH_2O, 0.5µl 10x Hot Start buffer (Qiagen), 0.04 µl dNTP Mix (25mM each), 1 µl forward + reverse primer with a T7 promoter (1 µM each), 0.2 µl $MgCl_2$ and 1 µl Bi-DNA were mixed and incubated: 94°C for 15 min; 45 cycles (94°C, 20 sec; 62°C (variable), 30 sec; 72°C, 1 min); 72°C, 1 min; 4°C, 5 min. During PCR amplification, a T7 promoter for *in vitro* transcription was introduced to the 5' end of one strand of each amplicon. After the PCR, the shrimp alkaline phosphatase (SAP) enzyme solution (1.7 µl RNase free water, 0.3 µl SAP) was added to each reaction to degrade any unincorporated nucleotides and incubated at 37°C for 20 min and then for 5 min at 85°C. Subsequently, the cleavage transcription/RNase A cocktail (3.21 µl RNase free water, 5xT7 polymerase buffer, 0.22 µl T-cleavage mix, 0.22 µl DTT (10 mM), 0.4 µl T7 R&DNA polymerase (50 U/µl) and 0.06 µl RNase A) was added to 2 µl of PCR/SAP solution and incubated for 3 hours at 37°C. In this step, RNase A cleaves specifically at every C. For cleaning up the reaction products, 20 µl ddH2O and 6 mg resin were added to each reaction and rotated for 10 min. Afterwards it was centrifuged at 3000 rpm. Then, using the MassArray® Nanodispenser S, 20 nl of the samples was transferred onto a 384 SpectroCHIP® and calibrant was transferred from the calibrant well (70 µl) onto the calibrant patches of the 384 SpectroCHIP®. Subsequently, the MALDI-TOF MS was run.

To validate the results, we aimed at replicating some of the differentially methylated CpG sites in a second experiment.

Pyrosequencing

For validation of the DNA methylation microarray, we established the pyrosequencing method by using a candidate gene which we did not pick from the microarray (Bdnf).

For pyrosequencing, the DNA methylation Gold kit and its protocol from Zymo Research (Irvine, USA) were used in a slightly modified manner. The input amount of DNA was 800 ng and the conversion took place at following conditions: 98°C for 10 minutes, 53°C for 4 hours.

The PCR protocol (with biotinylated primers), was as follows: 94°C, 5 min; 41 cycles (94°C, 20 sec; 54°C, 30 sec; 72°C, 1 min); 72°C, 3 min. To 10 µl of the PCR product, 2 µl of streptavidin sepharose HP beads, 40 µl of binding buffer and 28 µl of high purity water were added. After shaking the plate for 5 min at 1400 rpm, a vacuum head isolated the template. Then, templates were washed with 70% ethanol, denaturized with basic solution and washed with water. The template was added to 11.5 µl of annealing buffer and 0.5 µl pyroprimer (10mM), which was incubated for 2 minutes at 80°C.

Statistical analysis

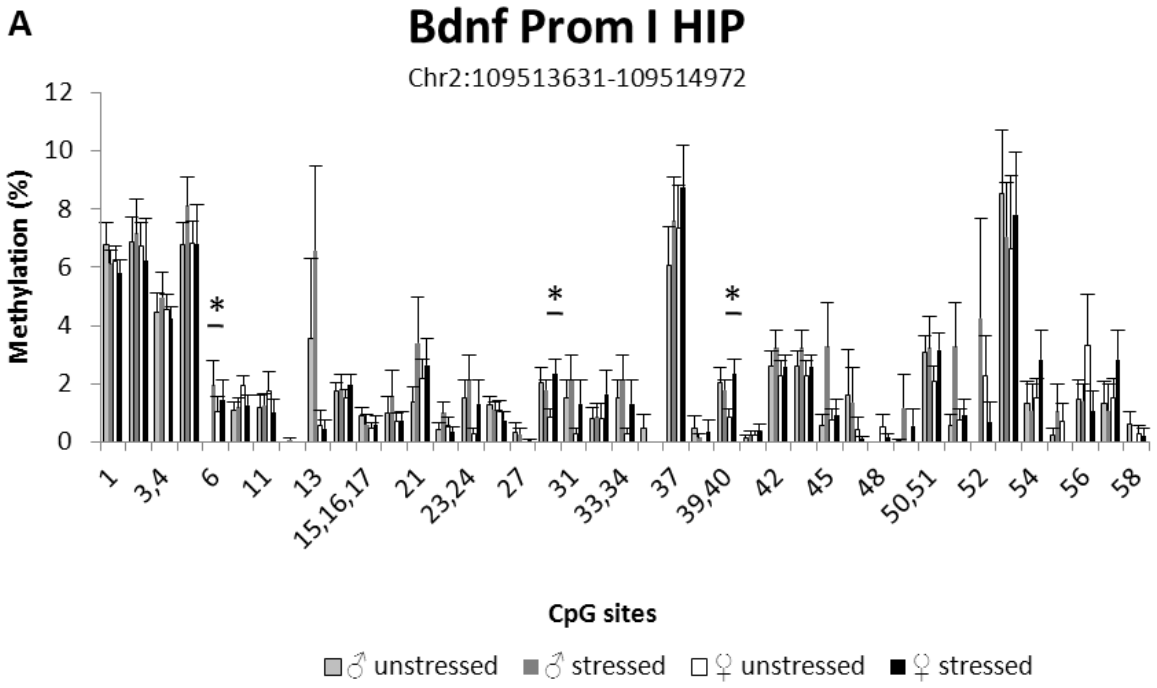
As we expected differences between females and males in the PS paradigm, we stratified for sex. Stress effects in males or in females were calculated by the help of non-parametric Mann-Whitney-U test. The significance level was set at $P < 0.05$ for every test. P-values between 0.05 and 0.1 were referred to as a trend. Differences in methylation of the Bdnf promoter IV assessed by pyrosequencing were analyzed by the help of two-way ANONVA. All analyses were carried out by the help of the SPSS software Version 20.0.

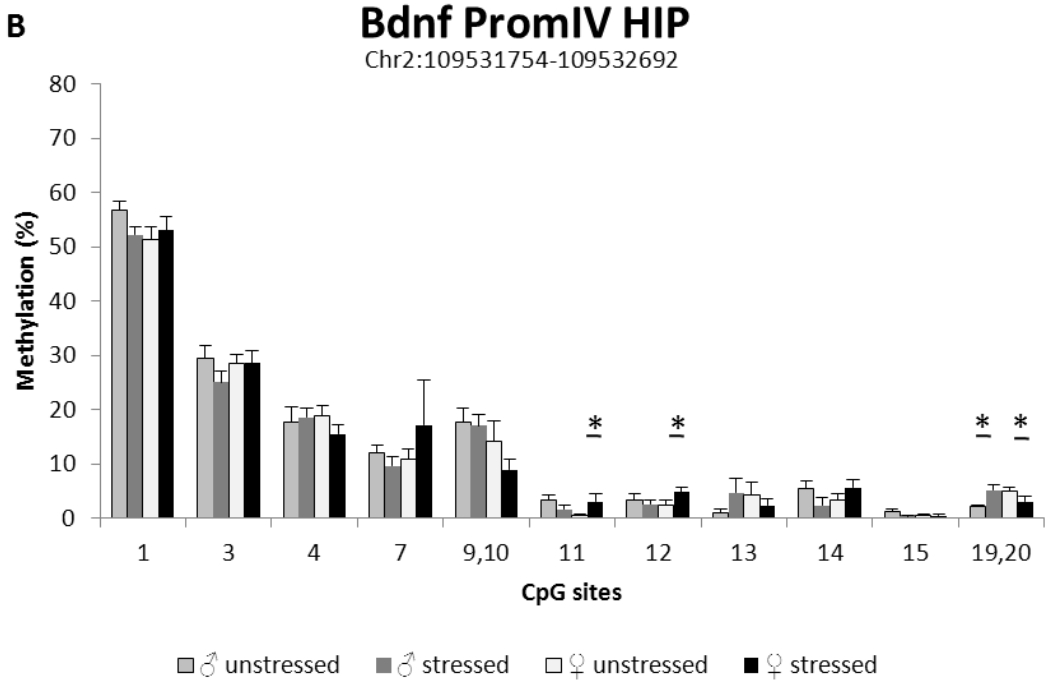
5.2.3 Results

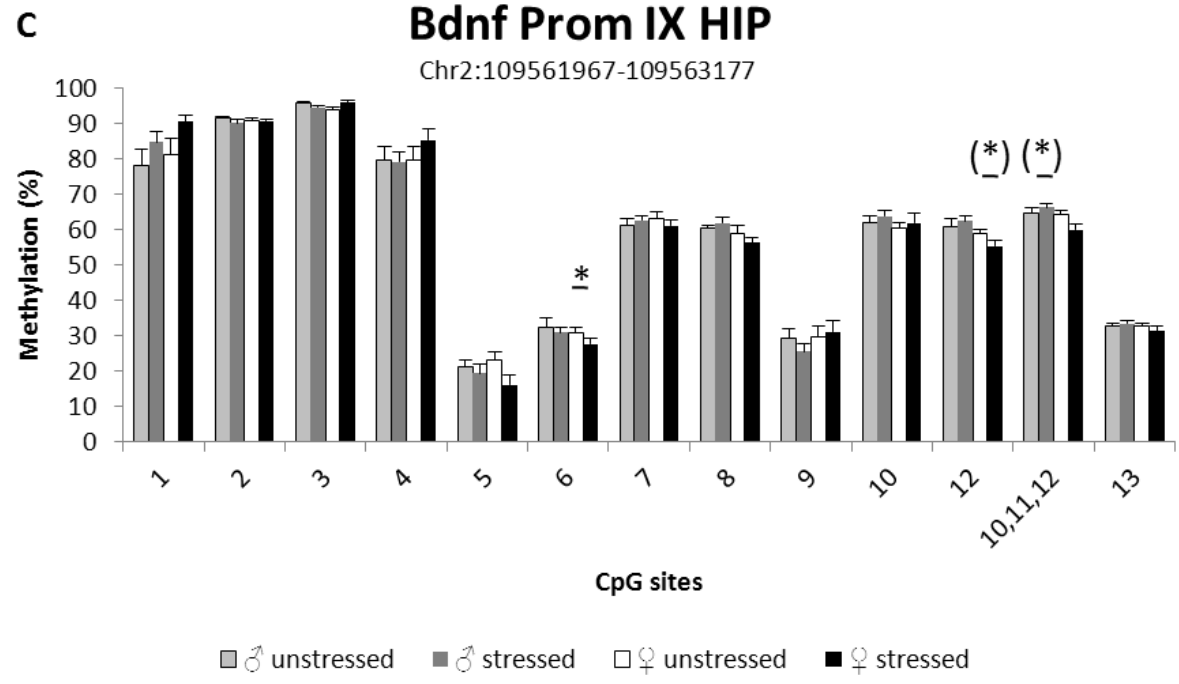
The percentage of DNA methylation within the promoter I of the Bdnf gene was very low in the hippocampus and in the cortex (see Figure 5-1A, 5-1D). In the hippocampus, we could detect significant differences between PS and control males at CpG site 6 (P=0.008) and between PS and control females at CpG site 28 and 39-40 (P=0.025, respectively). In the cortex, there were significant changes between PS and control females at CpG sites 28 (P=0.003), 37 (P=0.050) and 39-40 (P=0.003). At promoter IV, a descending pattern towards the transcription start side (between CpG site 15 and 19-20) could be measured in the hippocampus and in the cortex. In the hippocampus, several significant changes could be detected: At CpG sites 19-20 between control and PS males (P=0.004) and at CpG site 11 (P=0.026), 12 (P=0.049) and 19-20 (P=0.018) between control females and PS females. At promoter IX, in comparison to promoter IV and I, a high mehylation level was observed. In the hippocampus, we found a significant difference at CpG site 6 (P=0.028) between PS

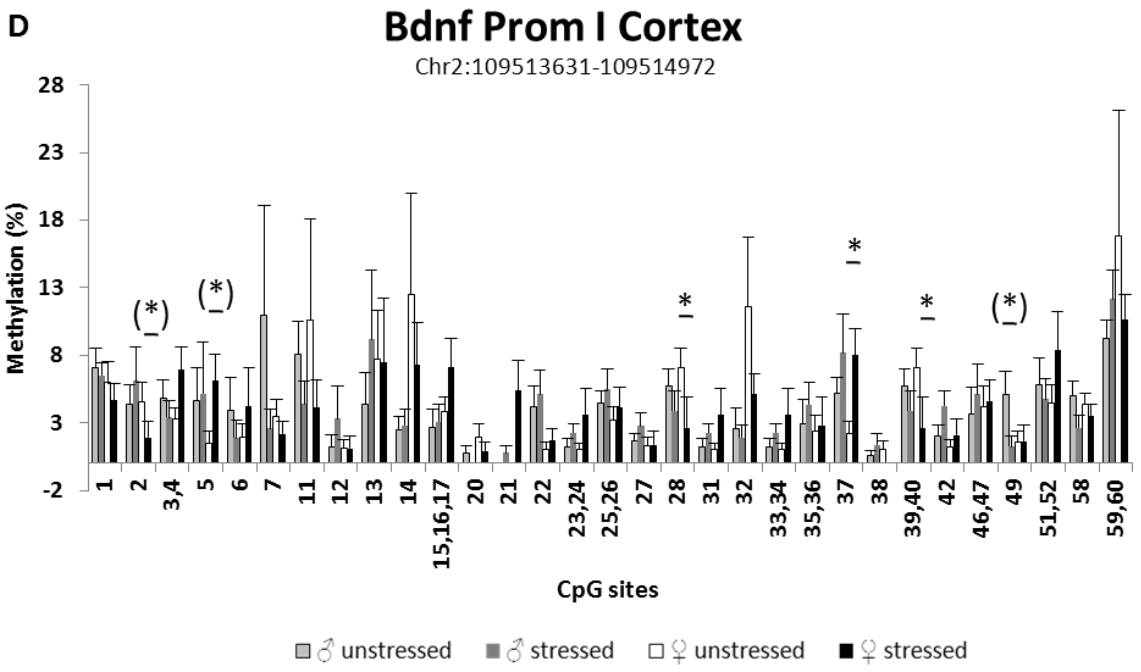
and control females. In the cortex, we detected significant differences between control males and PS males at CpG sites 6 (P=0.033), 7 (P=0.033), 9 (P=0.04), 10 (P=0.035) and 13 (P=0.04).

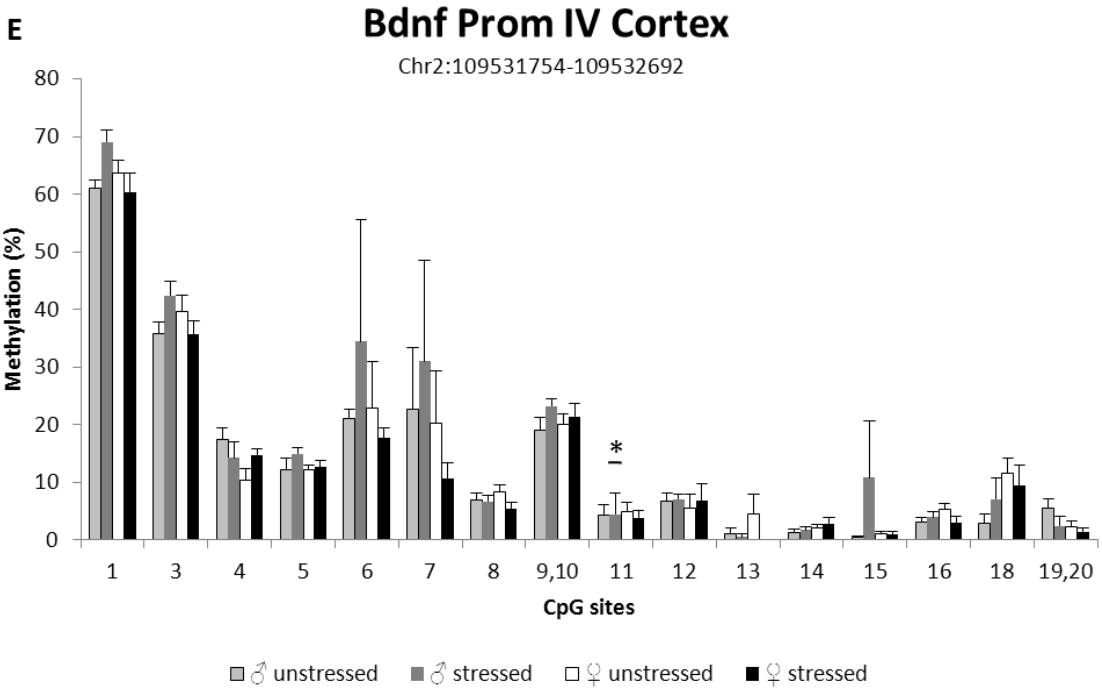
In a second control experiment we tried to replicate some of these significant changes. Although the overall degrees of methylation were similar at the various CpG sites, the specific effects did not reach significance anymore (data not shown).




B
Bdnf PromIV HIP
Chr2:109531754-109532692
Methylation (%)
80
70
60
50
40
30
20
10
0
1
3
4
7
9,10
11
12
13
14
15
19,20
CpG sites
♂ unstressed
♂ stressed
♀ unstressed
♀ stressed


C
Bdnf Prom IX HIP
Chr2:109561967-109563177
Methylation (%)
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
7
8
9
10
12
10,11,12
13
CpG sites
♂ unstressed
♂ stressed
♀ unstressed
♀ stressed


D
Bdnf Prom I Cortex
Chr2:109513631-109514972
Methylation (%)
28
23
18
13
8
3
-2
(*)
(*)
*
*
*
(*)
1
2
3,4
5
6
7
11
12
13
14
15,16,17
20
21
22
23,24
25,26
27
28
31
32
33,34
35,36
37
38
39,40
42
46,47
49
51,52
58
59,60
CpG sites
♂ unstressed
♂ stressed
♀ unstressed
♀ stressed


E
Bdnf Prom IV Cortex
Chr2:109531754-109532692
Methylation (%)
80
70
60
50
40
30
20
10
0
*
1
3
4
5
6
7
8
9,10
11
12
13
14
15
16
18
19,20
CpG sites
♂ unstressed
♂ stressed
♀ unstressed
♀ stressed

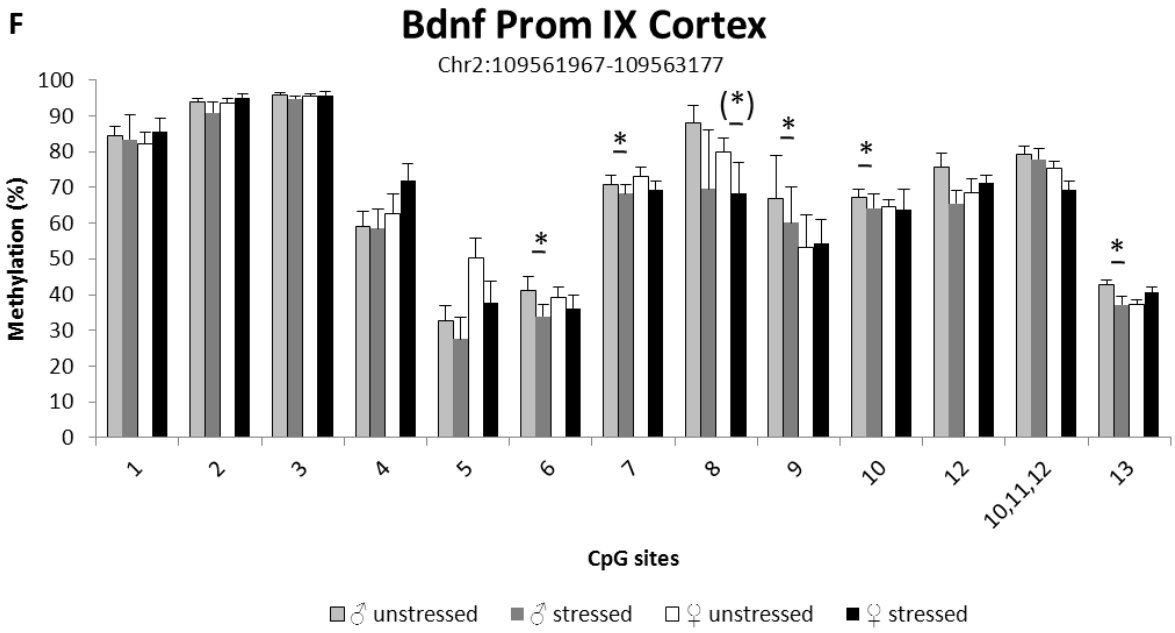


Figure 5-1 A-F. DNA Methylation patterns of various brain-derived neurotrophic factor (Bdnf) promoters (Prom) in the hippocampus (HIP) and cortex. At Prom I (A, D), the average degree of methylation was relatively low in the cortex and in hippocampus. At Prom IV (B, E), a decrease towards the transcription start side (between 15 and 19,20) was observed. Prom IX (C, F) was relatively highly methylated in comparison to Prom I and IV. Data represent mean + SEM.

The methylation status of three CpG sites of the Bdnf promoter IV (CpG 1,2,3; see Figure 5-2) was assessed by the use of pyrosequencing. At these distinct CpG sites no differences between control and PS mice or 5-Htt+/- and WT mice could be detected.

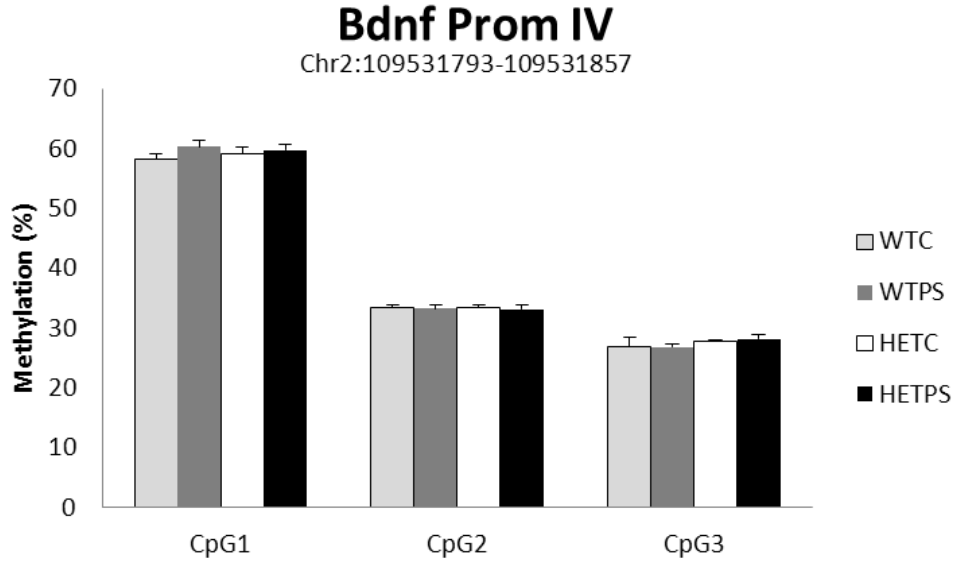


Figure 5-2. Methylation status of Bdnf Promoter IV. Three CpG sites of the Bdnf Promoter IV were measured but no signififcant differences between prenatal stress (PS) and control (C) or 5-Htt deficient (HET) mice and wildtype (WT) mice could be detected. Data represent mean + S.E.M. N=7-10 mice/group.

5.2.4 Discussion

Although the results at single CpG sites (indicating various sex-dependent effects of prenatal stress) could not be replicated, the overall methylation patterns of the various CpG sites at the different promoters were confirmed. The low methylation state at promoter I points to an increase of Bdnf exon I expression in the hippocampus and cortex. Overall, information about the methylation state at promoter I in the brain is poor. Interestingly, Munoz and coworkers revealed a correlation between performance in the novel object recognition task and Bdnf promoter I methylation (Munoz *et al.* 2010). Further, Bredy and colleagues report an upregulation of Bdnf exon I expression after extinction of conditioned fear in the prefrontal cortex, which came in parallele with a trend of histone (H) 4 acetylation around the respective promoter I (Bredy *et al.* 2007). Another study on bipolar disorder (BD) I and II demonstrated a downregulation of BDNF expression in BD II patients but not in BD I patients in comparison to control individuals (D'Addario *et al.* 2012). Accordingly, the BDNF promoter I was hypermethylated in BD II patients. Interestingly, BDNF promoter I was specifically hypermethylated in BD patients who took mood stabilizers plus antidepressants when compared to those who only took mood stabilizers. Furthermore, lithium and valproate, the latter of which is known to inhibit HDAC, were linked with a decreased promoter I methylation in comparison to other drugs.

Our data suggest that there is a decline in methylation of promoter IV towards the transcription start site. Furthermore, the binding site for calcium/cyclic adenosine monophosphate (cAMP)–responsive element binding protein (CREB) at CpG 13 is only minimally methylated in all four groups indicating a binding oportunity for CREB. This pattern is similar when examining promoter IV (former promoter III) methylation in murine E18 + 5 days *in vitro* neuronal cells cultured with 10 % calf serum (amongst other cultures) (see supplements of Chen *et al.* 2003). Additionaly, a study by Mueller and colleagues revealed a relatively low degree of promoter IV methylation state near the CREB binding site, whereas the CpG site before the CREB binding site (CpG 14) showed an equal percentage of methylation like in our study (Mueller et al 2008). Interestingly, Mueller and coworkers, who investigated the effects of PS exposure during the first week of pregnancy, could not detect any differences at the examined CpG sites between stressed and control mice, as well.

Using pyrosequencing, we assessed the methylation status of 3 CpG sites of the Bdnf Promoter IV and could not detect any significant differences. As already stated above, this is in line with another study by Müller and coworkers which revealed no significant changes in the Bdnf Promoter IV methylation between PS and control animals (Mueller and Bale 2008). Interestingly, Tsankova and her colleagues found that defeat stress was able to increase histone methylation near promoter IV, an epigenetic modification which is thought to repress gene expression (Tsankova *et al.* 2006). Chronic treatment with imipramine could reverse this effect by inducing permissive histone acetylation. Further studies have to investigate if histone modifications also play a role in PS exposure on a 5-Htt deficient background.

In contrast to promoter I and IV, we found very high degrees of methylation at promoter IX, which is in line with another study by Zajac and coworkers, which also revealed high methylation at promoter IX (Zajac *et al.* 2010).

Limitations

The Sequenom EpiTYPER Mass Array MALDI-TOF analysis has a sensitivity of 5 %. That possibly could be the reason for the non-replication as we detected only small differences. Another drawback of this method is that it cannot distinguish between fragments of the same mass which leads to overlapping fragments. Furthermore, if two or more CpG sites are located on one fragment, the software is not able to calculate different rates for each CpG site. In general, bisulfite treatment causes a loss of 95% of DNA material and might therefore lead to variability of the results (Grunau *et al.* 2001).

Conclusions

In conclusion, the EpiTYPER Mass Array MALDI-TOF technique is not very sensitive and has further drawbacks. In general, the bisulfite-treated DNA should be aliquoted and frozen at -20°C and a thaw and freezing cycle should be avoided.

5.3 DNA methylation analysis of Arfgef1 in a maternal care paradigm

This study was performed at the European Molecular Biology Laboratory (EMBL) in Monterotondo (Italy) in colaboration with Dr. Cornelius Gross and his colleagues.

5.3.1 Introduction

Early-life adversity can have severe consequences for adult psychopathology. One of such an adverse situation is bad parenting. As far as humans are concerned, studies on the influence of early family experiences are primarily based on cases of severe adversity like childhood maltreatment or parental loss, which have been associated with enhanced ACTH (for review see (Tarullo and Gunnar 2006)) or cortisol responses (Luecken 1998; Luecken and Appelhans 2006) after psychosocial stress. However, even milder variations in parenting behavior have shown to be relevant. For example, Engert as well as Pruessner and colleagues reported that young adults and elderly who experienced low parental care display enhanced cortisol responses (Engert *et al.* 2010) and dopamine release in the ventral striatum (Pruessner *et al.* 2004) in reaction to psychosocial stress. Furthermore, a parenting style with low levels of structure (i.e. organization and consistency) in middle childhood predicts awakening cortisol responses as well as cortisol responses to the "Trier Social Stress Test" in the offspring at around 16 years of age (Ellenbogen and Hodgins 2009).

Animal studies indicate that in rodents, maternal behavior has longlasting consequences on anxiety-like behavior of the offspring. For example, maternal separation for several hours a day during the early postnatal period leads to enhanced anxiety-like behaviors and enhanced hormonal reactivity to stress (Kalinichev *et al.* 2002).Similarly, pups raised by low licking-and-grooming mothers show increased levels of anxiety compared to pups raised by mothers that display high licking-and-grooming (Caldji *et al.* 1998). Cross-fostering studies revealed that these influences are primarily environmental and cross-fostering offspring of low licking-and-grooming mothers to high licking-and-grooming mothers is able to convey low anxiety to the offspring (Liu *et al.* 2000). These findings indicate that variations in maternal behavior serve as a mechanism for the nongenomic transmission of individual differences in stress reactivity across generations. There are several indications of the underlying molecular mechanisms in adult animals exposed to different rearing environments pointing to persistent changes in gene expression. For example, decreased expression and function of the glucocorticoid receptor (GR) has been linked to low maternal care (for review see (Seckl and Meaney 2004)). Additionally, the DNA degree of methylation at the promoter region of the GR gene

was increased whereas histone H3-K9 acetylation was decreased in the hippocampus of these animals (Weaver *et al.* 2004). Treatment of these rats in adulthood with the histone deacetylase (HDAC) inhibitor tricostatin A reversed the effects of low licking-and-grooming on GR expression. McGowan and colleagues were able to translate these findings to the human by a study in which they examined the hippocampus of suicide victims with a history of childhood abuse and found a decreased GR expression combined with an increased GR promoter methylation in suicide victims compared to controls (McGowan *et al.* 2009).

5.3.2 Materials and methods

Animals

The hippocampal material for this study was provided by Dr. Cornelius Cross and his colleagues. Briefly, for this study, BALB/cByJIco (later called BC) and C57BL/ 6JIco (later called B6) strains were used to bred for two different F1 hybrids, B6xBC (B6 mother, BC father) and BCxB6 (C mother and B6 father). These two groups of offspring were genetically identical except for the sex chromosomes of the male mice. Further, they differ in the rearing environment during the first weeks of life as BC mothers display lower maternal care when compared to B6 mothers. At 2 months of age the right hippocampus from 6-8 male F1 hybrid mice was collected.

DNA extraction

For the actual probes, the DNA was extracted by Dr. Enrica Audero according to the following protocol: 200 µl lysis buffer and 10µl proteinkinase (20mg/ml) was added to the brain tissue, which was then incubated at 56°C over night. Afterwards it was centrifuged for 5 min at room temperature and the supernatant was kept. 250 µl (1 Vol) phenol-chloroform-isoamylalcohol (PCI; 25:24:1) was added. Then, it was again centrifuged at 4000 rpm at 4°C and the upper layer was kept. To that, 200 µl (1 Vol) PCI was added. After that, it was centrifuged at 14.000 rpm at 4°C for 5 min. To the upper phase was then added 2.5 Vol ethanol (100%) and 0.1 Vol 3M NaOH. It was again centrifuged for 5 min at 14.000 rpm (4°C). Afterwards the pellet was washed (2 times) with 70% cold ethanol, air-dried for one hour and solved in 100 µl TE (1x).

Genome-wide methylation profiling with SOLiD™ System Mate-Paired Libraries

To examine the genome-wide methylation in F1 BCxB6 and B6xBC mice, Dr. Enrica Audero prepared Mate-Paired Libraries in collaboration with Timothy Bestor. Briefly, each DNA sample was split into two compartments. To isolate unmethylated DNA, the DNA was digested with a methylation-specific restriction enzyme (McrBC; leading to the M-signal). This enzyme is an endonuclease which cleaves DNA containing methylcytosine on one or both strands. To isolate methylated domains, the DNA was digested with methylation-sensitive restriction enzymes (AciI, BstUI, HhaI, HpaLL, HpyCH4IV; leading to the R-signal). These enzymes are endonucleases whose activity is blocked by cytosine methylation. Afterwards the DNA ends were repaired to blunt-ended DNA. Then, the genomic DNA was methylated by EcoP15I, which is a restriction enzyme that is able to methylate the 5th base in its binding site CAGCAG in the presence of a methyl-group donor and the absence of ATP. Afterwards, the methylated DNA is ligated with EcoP15I CAP (CA-overhang; unmethylated binding site of EcoP15I). Next, DNA fragments were separated according to their size by the use of gel fractionation, because small fragments are preferred to circulate in the next step. The fragments were then circulated by ligating them with a biotin-dT oligo (TG/GT-overhang). Afterwards the circulated DNA fragment was cleaved again by digestion with EcoP15I, which cleaves at two unmethylated, inversely oriented recognition sides which results in 90 bp Mate-paired genomic DNA fragments. To amplify all fragments with the same primers, the fragments are ligated with 2 primer adaptors at their ends, resulting in 156 Mate-paired genomic DNA fragments. To purify the library from side products, streptavidin beads were added, which bind specifically to biotin labeled internal adaptors in the library molecules. After that, the library was amplified and in the last step the libraries were purified by PAGE electrophoresis of the PCR products and excision of the 156 bp library band. The resulting DNA was measured by a Bioanalyzer. The actual genome-wide methylation profiling by Methyl-MAPS (methylation mapping analysis by paired-end sequencing) was performed by Bestor and colleagues (see (Rollins *et al.* 2006))

Bisulfite sequencing

To analyze the methylation pattern of the genome-wide screening resulted candidate genes, I established the bisulfite sequencing method: First, bisulfite conversion was performed according to the protocol of the EpiTect bisulfite kit from Qiagen (Cat. no.: 59104). Afterwards a PCR was performed: 45 µl Platinum PCR SuperMix (High fidelity), Invitrogen, 0.125 µl forward + 0.125 reverse primer (100 µM), 0.75 µl H_2O and 4 µl bisulfited DNA. The PCR protocol was as follows: 1 cycle 5 min, 94°C; 40 cycles (1 min, 94°C; 30 sec, 52°C [individual for each primer]; 45 sec, 72°C); 1 cycle 7 min,

72°C. The PCR product was then purified by a gel extraction according to QIAquick gel extraction kit, Qiagen. Next, A-overhangs were added to the PCR product for ligation afterwards: 26 µl PCR product, 1 µl dATP (10 mM), 5 µl 10x PCR-Buffer II (Applied Biosystems) and 1.2 µl ampliTaq DNA Polymerase (5 U/µl) (Applied Biosystems). Then, this was incubated for 30 min at 72°C. For ligation 4 µl PCR product, 1 µl salt solution and 1 µl TOPO vector were incubated for 5 min at room temperature. Transformation and cloning was performed according to the TOPO TA cloning kit dual promoter, (with Top10F cells; Invitrogen). For minipreparation, the picked clones were incubated in 3 ml LB-medium (+ampicilline) over night at 37°C. Plasmid purification was performed according to the QIAprep Spin Miniprep kit protocol, Qiagen. For sequencing the probes were prepared as follows: A sequencing PCR was performed with 10 ng for 1000 bp DNA, 1.3 µl (of 2.5 µM) M13-F Primer, 8 µl Big Dye and to the final volume of 20 µl water was added. The PCR protocol was as follows: 95°C, 5 min; 25 cycles (95°C, 15 sec; 52°C, 15 sec; 60°C, 3 min). Afterwards a precipitation followed. To the PCR product 64 µl ethanol (100%) and 16 µl water was added. Then, it was centrifuged at 14.000 rpm for 20 min at room temperature. The supernatant was removed and the pellet was washed with 50 µl ethanol (70%). It was again centrifuged for 5 min at room temperature (14.000 rpm). The supernatant was removed completely and the pellet was air-dried. Afterwards the pellet was dissolved in 10 µl water. To 3 µl of this solution 12 µl of formamide was added and boiled at 95°C for 2 min. At the end, the solution was put on ice for 2 min. The actual sequencing was performed by the sequencing service at the EMBL in Monterotondo. BiQ Analyzer was used to analyze the data (Bock *et al.* 2005).

5.3.3 Results

Genome-wide methylation profiling revealed that *ADP-ribosylation factor guanine nucleotide-exchange factor (Arfgef) 1* may be differentially methylated in BC versus B6 mice as indicated in the unmethylated-signal (M) at the BC section where there are no signals at the B6 and BC (R) sections (see Fig. 5-2). To further evaluate the meaning of the signals, bisulfite sequencing was performed. When applying this method for one sample of the BC group, we found that most of the CpG sites were unmethylated. CpG sites 4, 8 and 9 showed a methylation status of 25% (see Fig. 5-3).

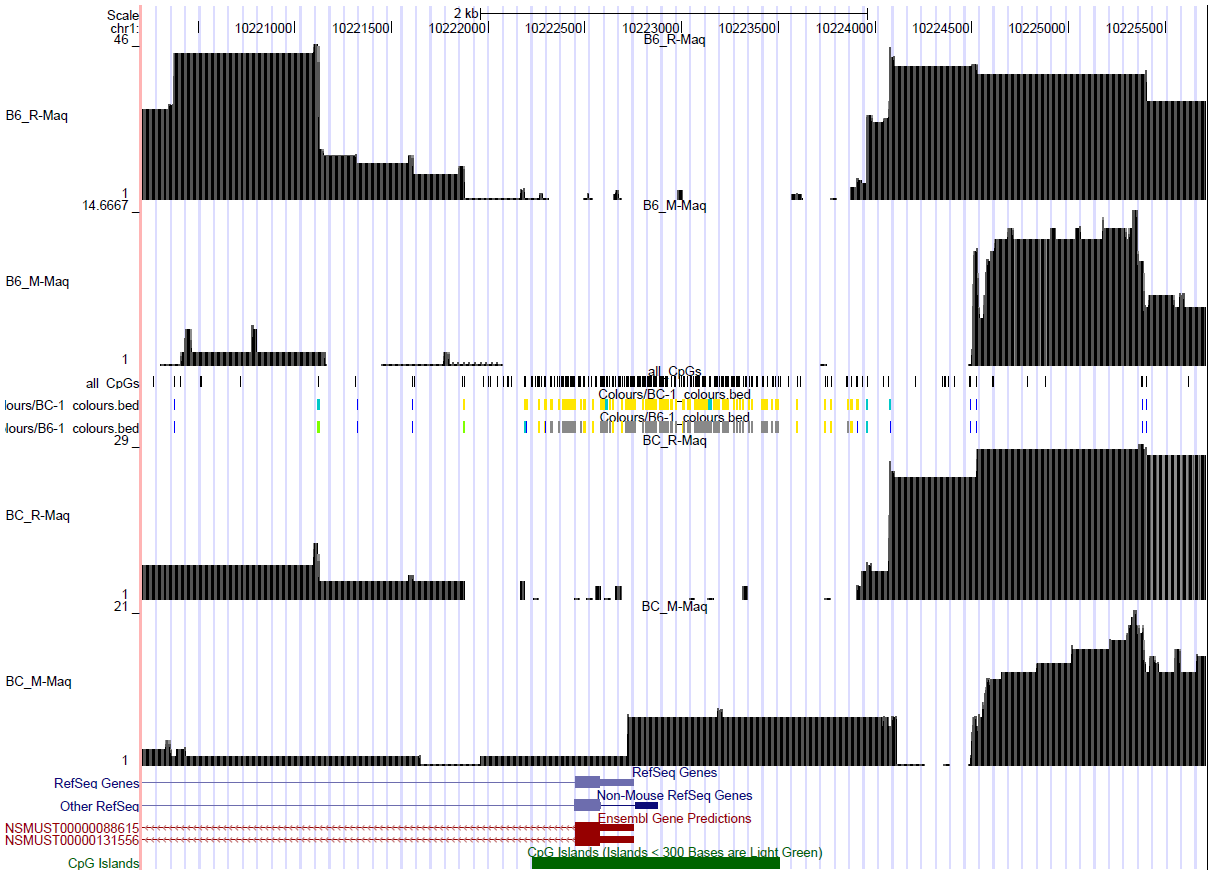


Figure 5-2. Methylation signals for ADP-ribosylation factor guanine nucleotide-exchange factor (Arfgef) 1. The M-signal (unmethylated) at the Balb/C (BC) section where there are no signals at the other sections might indicate a different methylation status between BC and C54BL/6 (B6) mice.

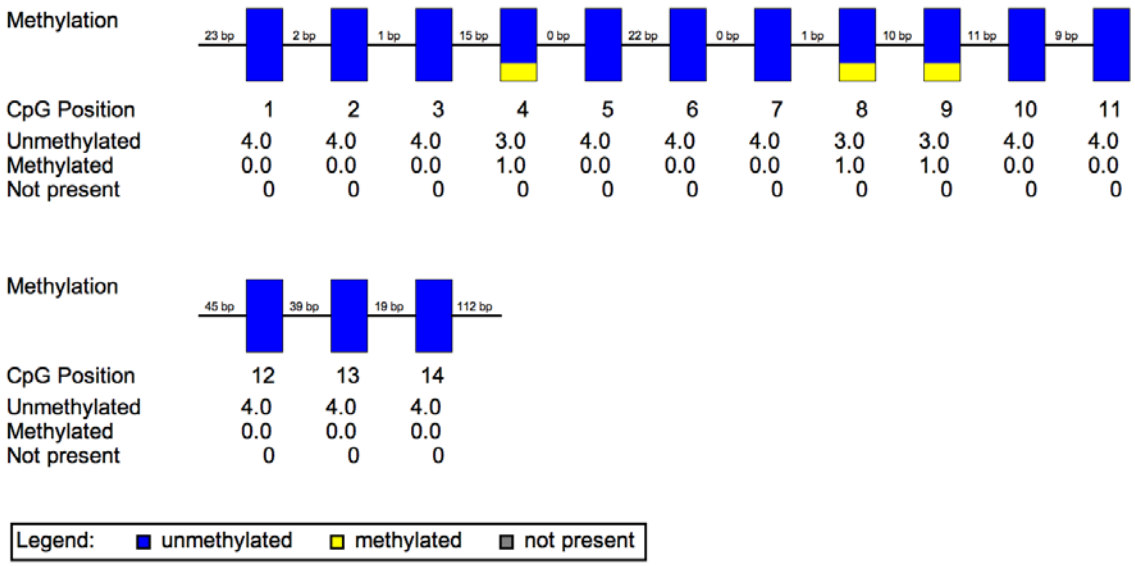


Figure 5-3 Methylation profile of some CpG sites in the Arfgef1 promoter (Chr1: 10222025-10223730). Most of the CpG sites were unmethylated whereas CpG site 4, 8 and 9 were 25 % methylated.

5.3.4 Discussion

Genome-wide methylation profiling revealed that Arfgef1 may be differentially methylated in BC versus B6 mice. Bisulfite sequencing revealed that the M-signal of the BC mice is really an unmethylated signal. Arfgef (also called BIG1) is a brefeldin A-inhibited guanine nucleotide-exchange protein, which activates class I ADP-ribosylation factors (ARF1-3) by catalyzing the replacement of bound GDP with GTP, an action critical for the regulation of protein transport in eukaryotic cells (Padilla *et al.* 2008). Additionally, Arfgef1 is known to scaffold and interact with proteins in other cellular compartments. In mammalian cells, its dysregulation may affect the structure and function of the Golgi apparatus (Pfeffer 1992). Recently, it was reported that Arfgef1 promoter is hypermethylated in breast cancer cells (Kim *et al.* 2011). Furthermore, it is known to be important in the glycosylation of beta1 integrin by Golgi enzymes which shows its important role in development and other vital processes. Therefore, a different regulation of Arfgef1 expression by DNA methylation through developmental stress might account for the differences in anxiety behavior seen when there are variations in maternal care (Calatayud and Belzung 2001). These data suggest that Arfgef1 may play an important biological role which, however, needs further validation.

5.4 References

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5.5 Abbreviations

5-HT	Serotonin
5-HTT	Serotonin transporter
5-Htt+/-	heterozygous for the serotonin transporter
5-HTTLPR	5-HTT linked polymorphic region
ACTH	Adrenocorticotrophic hormone
Arfgef1	ADP-ribosylation factor guanine nucleotide-axchange factor 1
AVP	Vasopressin
B6	C57BL/6
BC	Balb/C
Bdnf	Brain derived neurotrophic factor
Bmpr1b	bone morphogenetic protein receptor type 1B
bp	base pairs
C	Cytosine
C	Control
Cacna1d	calcium channel voltage dependent, L type, alpha 1D subunit
Cacng3	calcium channel, voltage dependent, gamma subunit 3
Cacng3	calcium channel, voltage-dependent, gamma subunit 3
Camk2a	calcium/calmodulin-dependent protein kinase II alpha
Camk2d	calcium/calmodulin-dependent protein kinase II, delta
CNS	Central nervous system
CORT	Corticosterone
Crh	Corticotrophin releasing hormone
Crk	v-crk sarcoma virus CT10 oncogen homolog
Ctcf	CCCTC-binding factor
DAVID	Database for Annotation, Visualization and Integrated Discovery
Dnmt1	DNA methyltransferase
DRN	Dorsal raphe nucleus
EZM	Elevated zero maze
Fgf1	fibroblast growth factor 1
Fgfr1	Fibroblast growth factro receptor 1
Figf	Fos-induced growth factor
Fos	FBJ osteosarcoma oncogene
FST	Forced swim test
Fzd3	frizzled homolog 3
G	Guanine

Gal	Galanin
Galr3	galanin receptor 3
Gdi2	dissociation inhibitor 2
GDP	guanosine diphosphate
Gh	Growth hormone
Gja	gap junction protein, alpha 1
GPC	glial precursor cell
GR	Glucocorticoid receptor
HDAC	Histone-deacetylase
HET	5-Htt+/-
HPA	Hypothalamus-pituitary-adrenal
Il12a	interleukin 12a
Il1r1	interleukin 1 receptor, type 1
Il4	interleukin4
Jun	jun oncogen
Kcnip2	Kv channel-interacting protein 2
Kcnj5	potassium inwardly-rectifying channel, subfamiliy j, member 5
kDa	kilo Dalton
KEGG	Kyoto Encyclopedia of Genes and Genomes
LIMMA	Linera models for Microarray analysis
l	long
LSD	Least significant difference
MALDI-TOF	Matrix assisted laser disorption ionisation time of flight
Map2k7	mitogen-activated protein kinase kinase 7
Map3k12	mitogen-activated protein kinase kinase kinase 12
Mapk8ip3	mitogen-activated protein kinase 8 interacting protein
Mbp	Myelin based protein
MDD	major depression disorder
min	Minute
NGF	nerve growth factor
Nos1	nitrit oxide synthase 1, neuronal
NT	Neurotrophin
Ntrk2	neurotrophic tyrosine kinase, receptor type 2
ORT	Object regocnition task

PeS	Perinatal stress
Phox2a	paired-like homeobox 2a
Pla2g5	Phospholipase As, group 5
Ppp1r1b	protein phosphatase 1, regulatory (inhibitor) subunit 1B
Prkar2b	protein kinase, cAMP dependent regulatory, type II beta
Prkcc	protein kinase C, gamma
Prl	Prolactin
Prok2	prokineticin
PS	Prenatal stress
Psen1	Presenilin 1
qRT-PCR	quantitative real-time polymerase chain reaction
Rac2	RAS-related C3 botulinum substrate 2
RDI	relative discrimination index
Rock1	Rho-associated coiled-coil containing protein kinase 1
s	short
sec	second
Slc6a4	solute carrier family 6, member 4
Smad7	MAD homolog 7
Syt5	synaptotagmin V
Tnfrsf1a	tumor necrosis factor receptor subfamily, member 1a
Trhr	thyrotropin releasing hormone receptor
Trp53	transformation related protein 53
Ttn	Titin
Vegf	vascular endothelial growth factor
Xaf1	XIAP associated factor 1
Zzef1	zinc finger, ZZ-type with EF hand domain 1

5.7 Publications

- Van den Hove DLA* and **Jakob SB***, Schraut KG, Kenis G, Schmitt AG, Kneitz S, Scholz CJ, Wieschollеck V, Ortega G, Prickaerts J, Steinbusch H, Lesch KP. Differential effects of prenatal stress in 5-Htt deficient mice: towards molecular mechanisms of gene x environment interactions. Plos one, 2011, 6(8):e22715. Epub 2011 Aug 12.

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Affidavit

I hereby declare that my thesis entitled “Molecular mechanisms of early-life stress in 5-Htt deficient mice: Gene x environment interactions and epigenetic programming” is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

Furthermore, I verify that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

Würzburg……

Date Signature

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation „Molekulare Mechanismen von Entwicklungsstress bei 5-Htt defizienten Mäusen: Gen x Umweltinteraktionen und epigenetische Programmierung“ eigenständig, d.h. insbesondere selbstständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

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Datum Unterschrift