Gene-gene-environment interaction in schizophrenia and depression

The $BDNF$ Val66Met polymorphism, the $5-HTT$ length polymorphism and environmental factors as risk factors

Miriam Butler, Stijn Kragt & Daniëlle Stolzenbach

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Abstract

Depression and schizophrenia are two diseases with an increase in frequency. At this moment only the outcomes of these disorders are understood. Both disorders are caused through a combination of specifically changed genes and different environmental factors. Hereby scientists think there is a pool of genes (ca. 300) where a schizophrenic or depressed patient has one till three mutated genes or risk variants of genes (in case of polymorphisms). There exist overlap between the pools of different brain disorders. The interaction between genes and environment, leading to depression or schizophrenia, is thought to happen through the epigenetics or polymorphisms of the genes involved. Here we review the gene-environment interaction of the genes brain-derived neurotrophic factor (BDNF) and the serotonin transporter (5-HTT) gene. The polymorphism in BDNF includes a valine or methionine at a specific position, whereby the presence of a methionine is a higher risk factor for depression. The polymorphism in 5-HTT includes a short and a long variant, whereby the short variant is associated with higher risks for developing depression.

There are some more mechanisms known, which increases the risk of getting a psychosis but a lot is still unknown. Also the interactions between two (or more) risk genes with environmental risk factors have not been investigated yet. In this article, we include a research proposal, in which the interaction between the polymorphisms of BDNF and 5-HTT and the environmental factor maternal care is investigated.

1 Introduction

According to the World Health Organization, depression is now a days affecting about 121 million people worldwide, which makes this disorder among the leading causes of disability worldwide. The prediction is that in 2020, depression will be the second leading cause of disability [WHO 2012]. Schizophrenia affects about 50 million people worldwide according to the World Health Organization [WHO 2012]. These numbers (among other things) make that a lot of scientists are trying to unravel the causes of depression and schizophrenia for decades. But still they haven’t been able to understand the pathogenesis of these disorders. One of the reasons for this is the complexity of depression and schizophrenia. These disorders are not caused by only one genetic factor or probably not even by a combination of two genetic factors. Still they found out that schizophrenia is a strongly inherited disorders with a heritability of 80% or more [Quednow et al. 2011].

After many research, scientists invented the hypothesis that there are not only genetic risk factors, but also environmental risk factors that might play a role in these disorders. An well researched example of such an environmental risk factor is childhood maltreatment (often in the form of abuse or neglect).

According to this hypothesis, environmental factors might change the epigenome by mechanisms as DNA methylation, histone (de-)acetylation, histone (de-)ubiquitination or histone methylation. Consequences of this might be changes in the expression of genes and in that way for example changes in neurotransmitter pathways that (in combination with genetic risk factors) might lead to schizophrenia or depression.

Now a common idea is that every schizophrenic or depressive patient has a combination of genetic risk factors and a combination of environmental factors that together, by a complex interaction, leads to the phenotype of schizophrenia or depression. But these interactions are at this moment not understood and yet not very well investigated.

Although it is clear that approximately 80% of the risk of developing schizophrenia can be explained by genetic factors, so far no susceptibility genes with moderate to high effect sizes have been found [van Haren et al. 2008].
But there are a lot of genetic factors found that might interact with each other or with environmental factors and in that way play a role in schizophrenia or depression. These genetic factors are often single nucleotide polymorphisms (SNP): changes of one nucleotide in the DNA. Two examples of SNPs are the brain-derived neurotrophic factor BDNF Val66Met polymorphism and the 5-HTT (serotonin transporter) polymorphism. Together with environmental risk factors (such as early life stress), they might create a higher risk for getting schizophrenia or depression. The question we want to speculate about in this article is: ‘What is the link between BDNF and 5-HTT and stressful life events concerning schizophrenia and depression?’.

2 Genetic risk factors

2.1 Pool of genes

The polymorphisms in BDNF and 5-HTT mentioned in the introduction are just two of the many examples of genes that can be involved in brain disorders. The estimation is that there are over 300 genes involved in brain disorders that can enhance the risk for a certain brain disorder when the gene has undergone a specific change. You can say that every brain disorder has its own pool of genes that can possibly be involved in developing that specific brain disorder. The different pools of the disorders overlap. This gives rise to a simplistic figure, where we took the pools of depression, bipolar disorder and schizophrenia.[Craddock et al. 2005; Knight et al. 2009; Green et al. 2010; Darrik et al. 2011] We placed only a couple of genes in the pools for illustration (figure 1).

The genes in the pool are mostly involved in cell growth and differentiation or are regulators in neurotransmitter pathways. It is not surprising that especially these genes are involved in brain disorders as these disorders are related to a decreased cortical volume compared to healthy people and misbalanced neurotransmitter regulation. Examples of neurotransmitters that are wrongly regulated in brain disorders are GABA, dopamine, glutamate and serotonin.

In this article we will focus on the pools of genes of schizophrenia and depression, because they seems to have the most overlap according to literature. This leaves us to the second figure in the right column (figure 2).[Craddock et al. 2005, Craddock and Forty 2006; Knight et al. 2009; Green et al.2010; Darrik et al. 2011]

In these genes a lot of possible polymor-
Different polymorphisms in TCF that are observed in schizophrenia patients. Some of them are highly significant, such as the rs17512836 polymorphism. The colors of the diamonds give information about the correlation coefficient. (broadinstitute 2012)

phisms or mutations has to be taken into account. For example the schizophrenia risk gene TCF4, located on chromosome 18, has many SNPs, that have been observed in schizophrenia patients (figure 3). (broadinstitute 2012) TCF4 is a gene that is associated with impaired sensory gating, which often occur in schizophrenia patients. Sensory gating includes the neurological processing of filtering unnecessary stimuli from the environment in the brain. (Quednow et al. 2012)

In this article we decided to focus on the genes BDNF and 5-HTT. Studies have indicated TCF4 as more significant for schizophrenia and depression than BDNF and 5-HTT. Nevertheless we have chosen to review BDNF and 5-HTT because they are related to both schizophrenia and depression. This gives us the possibility of providing examples for possible working mechanisms, which are representative for the kind of mechanisms involved in developing schizophrenia and depression related to gene-environment interaction.

2.2 BDNF Val66Met polymorphism

Brain derived neurotrophic factor (BDNF) is a protein that promotes growth, differentiation and survival of nerve cells. It is also important to maintain the plasticity of the nerve cells when they are fully developed. It is dominantly expressed in prefrontal cortex, hippocampus and amygdala in the brain. These brain areas are involved in cognitive and emotional functions and in memory and stress systems, respectively. Thus, changes in the BDNF level may represent a risk factor in psychotic disorders, such as schizophrenia and depression.

BDNF is located on chromosome 11p13. Furthermore BDNF binds to two receptors, namely a tyrosine kinase receptor (TrkB) and p75. TrkB has its function in phosphorylating tyrosine and activating intracellular cascades, like the calcium flux. In combination with p75 it mediates cell survival by activating on NF-κB, which controls the transcription of DNA. On its own p75 initiates cell death.

BDNF has an important role in synaptic transmission with effects on excitatory and inhibitory neurotransmitters. The role of BDNF on the GABAergic system is explained in the figure below (figure 4). BDNF has not only an important role on the GABAergic system, but also in dopaminergic neurons. The neurotrophin reduces the loss of tyrosine hydroxylase, which is a marker for dopaminergic neurons. Also it protects dopaminergic neurons from neurotoxic agents. In these ways BDNF improves survival of dopamine neurons and
regulates dopamine receptor expression and protein expression. A lack of BDNF causes reduced synaptic efficiency, while addition of BDNF causes improved synaptic efficiency. In this way BDNF controls the plasticity of nerve cells.[Favalli et al. 2011] Later on in this article the relationship between the gene BDNF and psychosis will be discussed (section 3.4).

Since BDNF has important effects on the GABAergic and dopaminergic systems, it could be an important gene involved in psychoses. An interesting SNP in BDNF, the Val66Met polymorphism, be related to psychosis. The SNP on chromosome 11p14.1, called rs6265, has been found at codon 66 in the N-terminal of the BDNF gene. Only in humans it is found that an exchange from valine to methionine is possible at this codon. The occurrence of the Met allele is about 20 to 30 percent, so the Met allele is called low expressed. The occurrence of individuals with homozygote 66Met is even much less (about 2 to 3 percent).

The relation of this polymorphism with depression is widely studied. According to these studies the 66Met allele of BDNF is associated with more occurrence of depression and anxiety disorder above the Val66 allele. Present results suggest that it is the occurrence of not one but two 66Met alleles that is associated with high trait anxiety.[Montag et al. 2010] The 66Met allele of BDNF is associated with lower hippocampal N-acetyl-aspartate levels, which is present in neuronal cell bodies, where it acts as a neuronal marker. Lower N-acetyl-aspartate levels are thus associated with lower hippocampal volumes. Studies on this polymorphism in humans revealed an 11 percent reduction of hippocampal volumes in healthy heterozygote Val66Met carriers. Furthermore the 66Met allele is associated with fewer dendritic arbors, a reduce of activity-dependent secretion of BDNF itself by 30 percent in knock-in mice studies and episodic memory loss.[Montag et al. 2010; Grabe et al. 2011] It is also found that carriers of the 66Met allele respond with stronger amygdala activity to emotional stimuli. The Val66Met polymorphism seems not to play a significant role in white matter integrity of healthy people, but there is evidence that it does for patients with late-onset depression.[Montag et al. 2010]

There is not an overall effect of the BDNF Val66Met polymorphism on depression confirmed, while it is for anxiety disorder. There are two studies that suggest an association of this polymorphism with depression, but three studies could not link the polymorphism with depression.[Montag et al. 2010] Some meta-analyses even concluded that the Val variant of the BDNF is linked with anxiety and is unrelated to depression.[Frustaci et al. 2008; Verhagen et al. 2010] It seems that the determination of the risk factor of developing a psychosis is more complex than just a gene-
environment interaction, but that the genetic risk factor of developing psychosis may depend on more than just one gene.

### 2.3 5-HTT length polymorphism

The serotonin transporter polymorphism is a functional three-base pair repeat polymorphism in the promoter region of a serotonin transporter (5-HTT) gene called SLC6A4. The serotonin transporter (5-HTT) is a regulator of 5-HT (serotonin) availability: it removes serotonin released into synaptic clefts [Caspi et al. 2010, Canli and Lesch 2007]. The reason why scientists looked at this gene, is because of the expected dysfunction of the serotonergic neurotransmission in the etiology of depression [Vergne and Nemeroff 2006]. This was based on (i) a reduced plasma tryptophan concentration in depressed patients, (ii) decreased concentrations of serotonin and its major metabolite (5-hydroxyindole acetic acid) in cerebrospinal fluid and in postmortem brain tissue of suicide victims, (iii) increased 5-HT2 receptor binding in postmortem brain tissue studies of depressed patients and/or suicide victims and (iv) effectiveness of drugs that increase serotonergic neurotransmission in depression (for example the selective serotonin reuptake inhibitors, tricyclic antidepressants and monoamine oxidase inhibitors) [Ressler and Nemeroff 2000].

The serotonin transporter polymorphism in the promoter region will be shortened from now on as 5-HTTLPR. In humans, it is located on chromosome 17. There is a short (‘s’) and a longer (‘l’) variant known of the 5-HTT gene. The shorter variant of the allele is associated with a lower transcription efficiency of the SLC6A4 gene and a reduced serotonin uptake compared with the longer allele. Although a direct relation of the short allele and depression is not yet proven [Vergne and Nemeroff 2006].

Yet there are a few indications for a potential relation between the 5-HTT gene and depression. Mice with disrupted 5-HTT (5-HTT−/− and 5-HTT+/−) show more fearful behavior and a larger increase of the stress hormone adrenocorticotropic hormone as a response to stress, in comparison with mice without a disturbed 5-HTT (5-HTT+/+). As a control experiment, they compared the mice when there was no stress present and they found out that there is no difference between the mice without stress. [Caspi et al. 2003]

Other experiments show also differences in the 5-HTT genes in non-primates. An experiment with Rhesus macaques showed that the rhesus s allele is associated with decreased transcriptional efficiency, just like the situation in humans [Caspi et al. 2010]. Besides they found out that there is a difference in the function of the serotonergic pathway in monkeys with the s allele in comparison with the l allele. Rhesus macaques with one or two s allele(s) showed a decrease of the serotonergic response when these animals were bred under stressful conditions in comparison with animals that were bred in ‘normal’ conditions. [Caspi et al. 2003]

Human neuroimaging studies suggest that the stress response is mediated by variations in the 5-HTTLPR. In one study, humans with different alleles of the 5-HTT gene got a fearful stimuli. Humans with one or two s allele(s) show a greater amygdala neuronal activity in comparison with humans that have one or two longer alleles. [Caspi et al. 2003]

In short, there is evidence for a potential relation between variations of the 5-HTT gene and psychological consequences in reaction to stressful experiences.

### 3 Gene-environment interactions

#### 3.1 Environmental risk factors

Next to the genes that are responsible for different brain dysfunction disorders, environmental factors play an important role in developing a brain dysfunction disorder such as schizophrenia and depression. You can have the risk genes for a brain disease without getting this certain disorder, but you can also have the risk genes for a brain disorder and get this disease indeed. The reasons for this are the environ-
mental factors an unborn baby suffers until getting older and being an adolescent. There are many different environmental factors increasing the possibility of getting schizophrenia or depression, which can be oversimplify as stressful life events. Some of them will be mentioned later on in this article.

If you look at the risk factors for developing different brain dysfunction diseases, you can see that they can be categorized in three different categories depending on the age of the person (figure 5):

- The environmental factors a baby suffers during pregnancy and birth
- The environmental factors a child suffers during the early life
- The environmental factors an adolescent suffers during the later life until the brain is fully developed

In connection with the first category one can refer to the mother’s stress, if the mother has diabetes or not, malnutrition during the first period during pregnancy or loss of birth weight.

The following two categories (2 en 3) contain in principle the same factors. The difference of the second and third category of stressful life events are not the stressful life events itself, but the strength in which they can influence the developing of a brain dysfunction disease in the different phases of life. In most cases the environmental factors a child suffers during the early life have more impact of developing schizophrenia or depression.

It is very difficult to demonstrate specific environmental factors playing a role in different brain dysfunction diseases. Therefore long studies have to be done with detailed observations and analyses. In the following paragraphs will be discussed how some of the many different environmental factors will affect the development of schizophrenia or depression or both.

### 3.1.1 Pregnancy and birth complications

There are many studies investigating and correlation between complications during pregnancy and birth and schizophrenia or depression later on in life. There are many different complications responsible for an increasing risk of getting such a disease. Complications of pregnancy are for example bleeding or diabetes. A complication during birth and pregnancy are an abnormal fetal growth and development. In this connection loss of birth weight, congenital malformations and reduced head circumference have to be mentioned.[Cannon et al. 2002]

Also malnutrition plays a role in developing schizophrenia. This phenomenon was explored by a study which analyzed the prenatal exposure to the Dutch hunger winter of 1944 - 1945. The result of this study was that early prenatal malnutrition increases the risk of getting schizophrenia, but this was only observed in women.[Susser et al. 1992] Another risk factor for schizophrenia and depression is maternal smoking during pregnancy.
3.1.2 Early parental loss (EPL)

Another factor increasing the risk of getting schizophrenia or depression is the early parental loss. This relation was investigated through different studies and among others in a study where parental loss before the age of 17 of patients with schizophrenia, depression, bipolar disorder or healthy people was analyzed. Therefore EPL can occur when the parents died or when they were separated when the parent permanently leaving home. The reasons for this are for example divorce and working far away from the family. Early parental loss increases the risk of getting depression with a factor 4. Children without EPL have a chance of getting depression with 7.6%, while children with EPL have a chance of getting depression from 29.1%. The risk of getting schizophrenia also increases with a factor 3 due to EPL, whereby children without EPL have a chance of 7.9% of getting schizophrenia and children with EPL a chance of 22.4%.[Agid et al. 1999]

3.1.3 Place of birth

The risk of schizophrenia can also be associated with the season of birth and place of birth. The highest probability of getting schizophrenia was observed in persons born in February or March and the lowest in August or September. The possibility for getting schizophrenia is higher in urban areas than in rural areas. In connection with this there are different explanations. One of them is that persons living in cities have an increasing risk of getting infections. When pregnant women get infected, their unborn child suffer from stress during pregnancy, which increases the risk of getting schizophrenia.[Mortensen et al. 1999]

3.1.4 Drugs

The use of drugs such as cannabis and smoking also increase the risk of developing schizophrenia or depression: If you use such drugs you have an higher risk of getting one of these diseases.[Arseneault et al. 2002]

Thus, there are so many factors having an impact on the development of schizophrenia and depression. All in all you can say that these factors are all stressful life events during the life from unborn until the person’s brain is fully developed. Suffering such stressful life events increases the risk of getting depression, schizophrenia or other brain dysfunction diseases.

3.2 Sensitization

We have seen that different stressful life events may increase the risk of getting schizophrenia and depression. The question arises whether the number of stressful life events matter in determining the risk factor. To answer this question we have to introduce the concept of sensitization, which means the observation that individuals who are exposed repeatedly to an environmental risk factor may develop progressively greater responses over time. This would finally result in a lasting change in response amplitude. So a person who is exposed to more than one stressful life event in his or her early life (person C in figure 6) has a higher risk to develop, in this case, schizophrenia in his or her later life in comparison to a person with less exposure to stressful life events in his or her early life (person A in figure 6). Different studies have been done to investigate a correlation between a specified genetic factor and different environmental factors. In some of the studies there is also differentiated how much stressful life events a person had. Many of these population studies show that more people develop a psychosis and that it last longer when they have had severe environmental exposure in their early years of living than that they have less environmental circumstances.[Collip et al. 2008]

There are different mechanisms involving sensitization. The first is that an exposure to stressful life events during the early life causes negative thinking about yourself and others so that there is a negative approach to the world. Another thought is that exposure to severe stress increase the sensitivity to stresses in daily life which are normally not seen as stress. This suggests that effects of early stress may give rise to a lasting liability in the form of emotional and psychotic reactivity (behavioural sensitization).
Figure 6: Sensitization behavioral phenotype: Person A has normal developmental expression of subclinical psychotic experiences that are mild and transient. Person B has similar expression, but longer persistence due to additional but mild environmental exposure. Person C has prolonged persistence due to severe repeated environmental exposure and subsequent transition to clinical psychotic disorder. [Collip et al. 2008]

Some research revealed that dopamine is released in response to stress. Postnatal rearing conditions in animals lead to profound and lasting changes in the responsiveness of mesocorticolimbic dopamine. Also agonist drugs like cannabis may induce sensitization. Schizophrenia may also be associated with increased amphetamine-induced dopamine release. There is evidence that the mesocortical dopaminergic innervations of prefrontal cortex (PFC) may regulate the activity of mesolimbic subcortical dopamine innervations. Environmental risk factors may take the PFC ‘off-line’ and alter the response of subcortical dopaminergic innervations. Also excessive levels of catecholamine release during stress impair PFC cognitive function in intracellular signaling pathways. Mutations in DISC1 and RGS4 have weaker regulation of these intracellular stress pathways. The neurotransmitter sensitization may be associated with epigenetic mechanisms (e.g., DNA methylation, histones modification, changes in mRNA levels). [Collip et al. 2008]

The underlying mechanisms of sensitization could lie in polymorphisms, like the examples mentioned before or in epigenetic mechanisms which will be discussed later on in this article.

3.3 Epigenetics

Scientists are still wondering why some people become for example depressive when they have been in a stressful life event early in life and why others don’t become depressive. Some scientists say the differences between people in response to stress has to do with the genes someone has. They think that everyone has a potential risk to become depressive from the day of existence. In section 2 there is already a lot told about some genes that could form a potential risk for depression and/or schizophrenia.

The question is, if this is the only factor that plays a role in the potential risks someone has or if there is another ‘code’ that plays a role. Some scientists believe there is, next to these genes, another code that can influence the response to stress: epigenetics. These are changes in the function of genes without changing the DNA sequence. These changes can be induced by environmental factors. So the assumption that epigenetics play a role in diseases, implies that not only the DNA can be responsible but that the environmental factors can be relevant as well. There are different mechanisms of epigenetic modifications of DNA. All these mechanisms change the expression of genes and in that way also the function of genes. Examples of these mechanisms are: methylation, acetylation, ubiquitination and phosphorylation of the DNA.

3.3.1 Methylation of DNA

Methylation of the base cytosine in promoter areas can change accessibility of DNA changes without changing the DNA sequence. The methylation of cytosine is caused by the enzyme Cytosine-Methyltransferase Dnmts. If cytosine is methylated the transcription factors can not bind the DNA because there is
a methyl-group which avoid this binding. So the genes are off which means that there is no gene expression. If there is no methyl group on the cytosine the transcription factor can bind the DNA so the gene can be transcribed and is called on. But in some cases the other way around is also possible, which means that when the cytosine is methylated the gene is on and with no methylation the gene is off. This happens when the binding region of a repressor will be methylated. So the gene is only expressed if the repressor is off by methylation (figure 7).

From the 30,000 to 40,000 human genes around 29,000 genes can be regulated by methylation because of having such an area in their promoter region. [Herrler et al. 2003]

### 3.3.2 Structure of chromatin as regulator

Next to the methylation of the DNA, the structure of the DNA itself can also function as a regulator. The DNA has not a linear structure, but a 3D structure (chromatin), so that a part of the sequence is open for transcription and a part is closed and not available for the transcription factors. We can say the same as by the methylation of DNA that the transcription is dependent on the accessibility of the DNA. Take up on highly alkaline proteins, the histones, is the first step from changing the linear DNA-structure to a 3D-structure. The complex from DNA and histones is called nucleosomes (figure 8).

So there are some DNA-sequences which are available for the transcription factors and some are not. The availability of the DNA sequences can be changed by moving a histone along the DNA so that other DNA sequences are available for transcription. This process is called chromatin remodeling (figure 9).

Histone proteins are organized into core octamer having histone-tails with lysine residues (figure 10). These residues can be modified by 150 different mechanisms such as acetylation, methylation, ubiquitination and phosphoryla-

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**Figure 7:** Methylation of the nucleotide Cytosin - epigentic control of gene activity. [Herrler et al. 2003]

**Figure 8:** Structure of chromatin [Griffiths et al. 2008]

**Figure 9:** Chromatin remodeling [Griffiths et al. 2008]
tion, which can alter the 3D-structure and regulates in this way the availability and transcription of the genes. [Griffiths et al. 2008]

By histone acetylation en deacetylation genes can be activated or inactivated. This process is reversible so that there is always a change that the genes can be expressed. The addition of acetyl groups caused by histone acetyltransferase changes the interaction between the DNA and a histone octamer in this way that the octamer slides along the DNA to a new position, so that the genes gets activated. Histone deacetylases are responsible for delete acetyl groups of the histone-tail, so that the histone becomes hypoacetylated which inactivates the genes. [Griffiths et al. 2008] Another mechanism which can influence the remodeling of the nucleosomes is histone methylation. Histones become methylated on arginine and lysine by the histone methyltransferase. This causes changes in the gene expression, but on different residues can this methylation act as a repressor or activator of the gene expression. [Zhang and Reinberg 2001] Histone ubiquitination and deubiquitination are also involved in regulating gene expression. Both mechanisms causes a transcriptional activation. [Zhang 2003]

3.4 BDNF-environment interaction

According to developing schizophrenia and depression there is an hypothesis that deals with the dopamine system. Long time was thought that there are abnormally high levels of dopamine transmission in schizophrenia patients, but this is not proven. There is more evidence that the dopamine system itself is normal, but that it is abnormally driven. Electrophysiological studies give evidence to the hypothesis that loss of an important class of GABAergic interneurons in schizophrenia patients may be responsible for increased activity of the hippocampus, which is then called hyper-active. This hyper-activity leads to an overdrive of dopamine neuron population activity and hyper-responsivity of dopamine receptors in the dopamine system in schizophrenia patients. The origin of this hippocampal dysfunction could lay in environmental factors, such as different kinds of stress. Early life stress could cause high levels of the stress hormone cortisol, which is associated with a smaller hippocampus in the first episode of schizophrenia. Also there is preliminary evidence that acute stress increases the transcription factor c-fos in ventral hippocampal neurons, while chronic stress increases excitability in amygdala, which lead to more excitatory sources to hippocampus. [Grace 2011]

As we have seen in section 2.2 BDNF has an important SNP that is a risk factor for schizophrenia, but this gene may also play as a risk factor in the role of an epigenetic mechanism. There has been found interesting results in schizophrenia patients, which has to deal with the mechanism between GADD45b and BDNF. Before the results will be discussed it is necessary to explain the mechanism of interest between these two genes.

GADD45b is an enzyme that is required for DNA demethylation of specific promoter regions and expression of the corresponding genes, which of one is the IXaabcd promoter region of BDNF. When there is a methylated cytosine at the promoter region, GADD45 binds to it, proximal to an acetylated histone. Then GADD45 recruits a deaminase, which convert the methylated cytosine (5-methylcytosine) into an thymine. This induces a mismatch between a thymine and a guanine, so a glycolase is recruited to solve this problem. The glycolase
removes the thymine and replaces it by an un-methylated cytosine (figure 11). [Gavin et al. 2011]

A growth arrest and DNA demethylation involving GADD45b has been found as a contributing factor to psychotic disorders. Increased GADD45b mRNA and protein has been found in psychotic patients and parallel to that a reduced BDNF IXabcd mRNA expression (figure 12).

![Figure 12: Results of levels of GADD45 and of some promoter regions, including among others BDNF IXabcd. NPS stands for non-psychotic subjects, while PP stands for psychotic patients. Levels GADD45b and BDNF IXabcd are significantly different in psychotic patients (p=0.034 and p=0.016 respectively. Age, sex, pH or RIN (RNA integrity number) and use of antipsychotics are not significantly correlated with these findings. [Gavin et al. 2011]

Other important findings according to psychotic patients are an increase in cells containing GADD45b in prefrontal cortical layers II, III and IV in psychotic patients referred to non-psychotic subjects (p=0.007, 0.01 and 0.02 respectively). Also there is found less binding of GADD45b to BDNF IXabcd promoter region in psychotic patients compared with controls (p=0.013), associated with an increase in 5-methylcytosine and 5-hydroxymethylcytosine at the promoter compared to controls (p=0.044 and 0.040 respectively). All these results are not significant correlated to age, sex, pH or use of antipsychotics. These results suggest the epigenetic modulation in the expression of the BDNF gene. It seems contradictory that there is an increased GADD45b level, but less binding of GADD45b to BDNF IXabcd promoter. For this there is an hypothesis that act on a proposal of a restricted chromatin state in psychotic patients compared with non-psychotic subjects, which prevents GADD45b binding to specific promoters and in that way DNA de-methylation. To compensate this, there could probably be a higher level of expression of GADD45b. This hypothesis could explain these seemingly contradicting results, but further studies have to be done to determine whether this hypothesis is plausible or not. If it is, chromatin ‘opening’ drugs would be expected to increase GADD45b access to gene promoters and potentially result in favorable gene expression changes. Anyway, the mentioned results show that complexity in methylation, gene expression and disease risk of psychosis may be a consequence of a dynamic equilibrium between methylation and demethylation. [Gavin et al. 2011]

3.5 5-HTT-environment interaction

In the earlier paragraph about the pool of genes, there is already a bit told about the serotonin transporter polymorphism. Although there are a lot of studies done that suggest a relation between the s allele and depression, there are individuals that do have the s allele but don’t become depressive in their life. Next to this genetic factor, there are more risk factors that could increase the susceptibility of getting a depression. Very often researched examples of these risk factors in general are environmental factors. And in the topic depression, childhood maltreatment in the form of abuse or neglecting is very well investigated. Two studies have shown that childhood maltreatment is associated with a 50% increase in the odds of depression in adult life [Uher et al. 2011]. But again there are individuals who experienced childhood maltreatment and don’t get depression later in life. One of the first experiments that is done to
Figure 11: Proposed mechanism of activity-dependent DNA demethylation. Following depolarization GADD45 protein binds to a methylated promoter region proximal to an acetylated histone (a). GADD45 recruits a deaminase (DA), which converts 5-methylcytosine (5MC) to thymine leading to a T:G mismatch (b). GADD45 recruits a DNA glycosylase (GLY), which removes thymine from the T:G mismatch. Thymine is later replaced with an unmethylated cytosine (c). [Gavin et al. 2011]

test the influence of the polymorphism on early life stress, is a study with Rhesus macaques. Researchers tested the differences between infant monkeys with and without the s allele when they were taken away from their mother and bred with other infant monkeys (so they were raised by another mother), compared with monkeys that were bred by their own mother. Outcome of the research was that monkeys that had the s allele and were separated from their mother display greater anxiety, agitation, stereotypies, an exaggerated HPA axis response and a higher ACTH response to stress for homozygous monkeys. [Caspi et al. 2010]

Because rodents don’t have an orthologue of the 5-HTTLPR, mice and rats have been genetically engineered with loss-of-function mutations in the 5-HTT gene to study what the consequences of these mutations were for behavior and brain function. Mice with no function 5-HTT (targeted mutation or chemical mutagenesis) show more anxiety-like behavior, impaired fear extinction and exaggerated HPA-axis responses to acute stress.

Caspi and his colleagues (2003) did research about the vulnerability for depression in human. They found a number of interesting observations, which has been a breakthrough in the research on the role of environmental factors in diseases, depression in particular. These researchers used 5-HTT to measure the genetic vulnerability for depression and tested if 5-HTT gen variation moderates the influence of stressful life events on depression. Caspi and his colleges defined stressful life events as events that involve threat, loss, humiliation or defeat. Their research was based on 146 individuals with the s/s genotype (among them 43, 37, 28, 15 and 23 individuals experienced respectively zero, one, two, three and four or more stressful life events), 435 individuals with the s/l genotype (among them 141, 101, 76, 49 and 68 experienced respectively zero, one, two, three and four or more stressful life events) and 264 individuals with the l/l genotype (among them 79, 73, 57, 26 and 29 experienced respectively zero, one, two, three and four or more stressful life events). Caspi and colleges found a positive correlation between the number of stressful life events and the patients self-report of depressive symptoms (figure 13A). They also reported that individuals with the s/s or s/l genotype reported more severe depressive symptoms in response to stressful life events. Besides they observed an association between the s/s and s/l genotypes and the probability of major depressive episodes and suicidal ideation or attempts (figure 13B and 13C).

In addition they found that those individuals that were exposed to childhood maltreatment who possessed the s/s genotype had the highest probability of developing a major depressive episode, followed by the s/l genotype. Individuals with two l alleles seem to be ‘protected’ against the effects of stressful life events and had the lowest probability of developing a major depressive episode [Caspi et al. 2003].
Figure 13: Regression analyses estimating the association between the number of stressful life events (age 21-26) and depression outcomes at age 26 as a function of the 5-HTT genotype. **A**: Self-reports of depression symptoms. The main effect of 5-HTTLPR (an effect not conditional on other variables) was marginally significant (P = 0.06), the main effect of stressful life events was significant (P = 0.001), and the interaction between 5-HTTLPR and life events was in the predicted direction (P = 0.02). The interaction showed that the effect of life events on self-reports of depression symptoms was stronger among individuals carrying an s allele (P = 0.001 among s/s homozygotes, and P = 0.001 among s/l heterozygotes) than among l/l homozygotes (P = 0.08).

**B**: Probability of major depressive episode. The main effect of 5-HTTLPR was not significant (P = 0.29), the main effect of life events was significant (P = 0.001), and the gene-environment interaction was in the predicted direction (P = 0.056). Life events predicted a diagnosis of major depression among s carriers (P = 0.001 among s/s homozygotes, and P = 0.001 among s/l heterozygotes) but not among l/l homozygotes (P = 0.24).

**C**: Probability of suicide ideation or attempt. The main effect of 5-HTTLPR was not significant (P = 0.99), the main effect of life events was significant (P = 0.001), and the gene-environment interaction was in the predicted direction (P = 0.051). Life events predicted suicide ideation or attempt among s carriers (P = 0.09 among s/s homozygotes, and P = 0.001 among s/l heterozygotes) but not among l/l homozygotes (P = 0.62).

**D**: Informant reports of depression. The main effect of 5-HTTLPR was not significant (P = 0.33), the main effect of life events was significant (P = 0.001), and the G x E was in the predicted direction (P = 0.01). The effect of life events on depression was stronger among s carriers (P = 0.001 among s/s homozygotes, and P = 0.001 among s/l heterozygotes) than among l/l homozygotes (P = 0.01). [Caspi et al. 2003]

Uher and his collegues did research to the difference in influence of childhood maltreatment on chronic or recurrent depression and single-episode depression diagnosed at a single time point. They used two cohorts from two different studies as data source. The people were evaluated on having depression on different ages and they were investigated on having the s/s, s/l or l/l genotype. The researchers classified recurrent depression as individuals were diagnosed with depression on two or more of the four assessment occasions and that were 255 individuals of both cohorts. Single-episode depression was classified as no
or one time diagnosed with depression of the four assessment occasions and that were 379 individuals in both cohorts. Uher and colleges did statistical tests on the data and found that having the s allele and recurrent depression were not consistent associated. But childhood maltreatment and recurrent depression were strongly associated in both cohorts ($p=0.0002$ and $p<0.0001$). During childhood maltreated individuals were more likely to get recurrent depression. They also tested if single-episode depression and the s allele were associated and they found a negative outcome. The relationship between single-episode depression and maltreatment during childhood was weaker and inconsistent across cohorts (smallest $p=0.099$). There was no significant relation between the gene and environment in individuals with single-episode depression.

So Uher and his colleges found positive results for gene-environment interactions for recurrent depression, but not for single-episode depression. Individuals with two s alleles and childhood maltreatment had elevated risk of recurrent depression but not of single-episode depression.[Uher et al. 2011]

Many researchers are wondering what biological consequences stress in early life has combined with the s/s genotype. So scientists started looking at differences in brain structures between individuals with the l/l, s/l and s/s genotype. Two independent research groups have demonstrated that the amygdala is overactive in individuals that have the s allele. So research groups tried to find a reason for this difference and found out that cerebrocortical brain regions help to regulate the activity of the amygdala when a stressful stimulus is processed. Functional imaging studies with depressed patients with one or two s allele(s) have indicated a malfunction in the connectivity between regions of the frontal cortex and the amygdala. So theoretically dysfunction of frontal inhibitory structures could lead to amygdala hyperactivity and stress/depressive symptoms.[Vergne et al. 2006]

Recent thoughts are that the variations of 5-HTT may not be only limited to the effects of 5-HT availability or even to the 5-HTT pathways like researchers have thought for decades. This new idea has come after experiments with 5-HTT knockout mice. They showed an abnormally high density of excitatory dendritic spines on amygdala neurons and an increase in dendritic arborization of prefrontal cortex neurons. These findings together with findings of many other experiments have led to a recently new hypothesis, namely that 5-HTT variation may in part modulate the capacity to cope with stress by shaping the early life development of corticolimbic circuitry and thereby the effects of 5-HTT knockouts are developmentally driven.[Caspi et al. 2010]

As a conclusion on the gene-environment researches that have been done with the serotonin transporter polymorphism, you could say that there are a lot of indications that the vulnerability to get depression increases if you have two s alleles and are maltreated during childhood. It is evident from research with multiple species that variation of 5-HTT modifies organisms’ stress responses to their environments. But it is still unclear if this could be the (only) inducement for depressed humans and if that is the case, for how many of the patients this is the cause of their depression.

4 Gene-gene-environment interactions

As we have seen in section 2.2 there is not a consistent result concerning the highest risk variant of the Val66Met polymorphism. Some studies indicate that the Met allele of this polymorphism is associated with higher risk for developing depression, while others disagree. To investigate an explanation for these contradictory results, researches started to think about a more complex network that determines the risk factor for developing depression. This network includes more than one gene in combination with an environmental factor.

The expression of BDNF is partly mediated via the second messenger cAMP, which responds to 5-HT-induced intracellular signal-
ing and BDNF itself promotes development and function of serotonergic neurons. Because of this there could exists a gene-gene-environment interaction between the Val66Met polymorphism of BDNF, the 5-HTT polymorphism and environmental factors. [Pezawas et al 2008]

Indeed several studies found such a three-way interaction, but the results are not consistent according to the effect of this interaction to the risk of developing depression. There are studies which results suggest that carriers of the Met allele of BDNF and the s allele of 5-HTTLPR have the highest risk of developing depression. [Kaufman et al, 2006; Kim et al, 2007; Wichers et al 2008]. Other studies found a protective effect of the Met allele of BDNF against the effects of the s allele of 5-HTTLPR. According to their findings a combination of these genes brings a lower risk factor for developing depression to an individual, while exposed to stressful life events. [Pezawas et al 2008; Grabe et al 2011]. They even found that the Val/Val genotype of BDNF in combination with s/s genotype of 5-HTTLPR increases the risk for developing depression.

The last result seems to be contradictory to first expectations, since there is more evidence that the Met allele of BDNF is linked to higher risk for depression above the Val allele. But the interaction between the two investigated genes could give an explanation for these results. These genes influence each other in the following way: serotonin up-regulates BDNF mRNA expression, while BDNF activate Tyrosine kinase B (TrkB) receptors on serotonergic neurons, which induces activation of serotonin uptake and transcription factors. It is indeed found that a reduced expression of BDNF is associated with reduced serotonin reuptake and thus higher extracellular serotonin levels. In short, the genetic background of BDNF moderates the differential reactivity to environmental factors caused by 5-HTTLPR.

Pezawas and his colleagues (2008) found a highly significant reduction of volume anterior cingulated cortex in individuals with the Val/Val genotype in BDNF and at least one s allele in 5-HTTLPR (p<0.001), but no effect in individuals with Met alleles in combination with at least one 5-HTTLPR s allele. It was allowed to measure the volume of anterior cingulated cortex, because it has a covariance with amygdala volume. To remind, a decreased amygdala volume is associated with depression, parallel to the hippocampus (section 3.2). This group has the hypothesis that the BDNF Met allele is less sensitive to stimuli that induce BDNF secretion above the BDNF Val allele. In this way the Met allele lessens the developmental impact of the 5-HTTLPR s allele on the volume of the amygdala.

As a genetic control Pezawas and his colleagues (2008) had chosen the COMT Val(108/158)Met genotype, which encodes catechol-O-methyl transferase. This degrades catecholamines such as dopamine. This gene is a binding control, because its associations with cognitive functions, brain activation, cortical regulation of emotional networks and mood-related measures. It is present in almost the same brain areas as 5-HTTLPR and BDNF. There was found no epistatic effect with the COMT gene.

Unless this result of Pezawas and colleagues it is not unlikely that the network of genes in combination with stressful life events is more complex than just two genes. The contradictory results on the mentioned gene-gene-environment interaction underlines this suggestion. It may include three genes or even more. Such studies, where the interaction of three genes with stressful life event are very difficult, and are as far as we could find not yet done. But with advances in imaging genetics and the availability of large human samples may allow such a study in the future. It is possible that these studies bring researchers a step closer to understanding the underlying mechanisms and risk factors of psychosis.

5 Discussion and conclusion

Concerning psychiatric diseases a lot a questions are still unanswered, but there is evidence for interactions between genes and environment to cause depression and schizophrenia. So it is not surprising that researches keep
trying to untie what these interactions include and what they change in the intracellular or neuronal pathways. There are some hypothesis that could give an answer to the question why gene-environment interactions are taking place. Here you have to think about the concept of sensitization and its probable underlying mechanisms as the exposure to severe stress, which could misbalance a neurotransmitter pathway. This could for example be done by influencing the release of the neurotransmitter in the way of responsiveness to stress.

Also SNPs or epigenetic changes, like DNA methylation and histone modification, of certain genes may change the responsiveness to stressful life events. One of the biggest challenges in this field of work is to figure out what comes first: the environmental risk factors or the epigenetic or genetic factors. Is it the environment, that induces polymorphisms or epigenetic changes (e.g. a mother drinks alcohol during pregnancy, which causes a methylation of a certain gene in the fetus and this gene causes the higher risk of becoming depressed in later life)? Or is it the other way around and do epigenetic changes, make an individual more sensitive to a certain environmental risk factor (e.g. an epigenetic or genetic change makes an individual feel more sensitive to start smoking and in this way causes the higher risk of getting depressed later in life)? It could be a part of both or specified for different cases. It is really hard to do studies on this, because those studies would be long-term studies, you would need a lot of experimental participants. Besides you would need to know every individuals genetic make-up and every individual exposures to environmental risk factor. You may think about periodic mapping of genotypes of subject, while keeping up with a journal of every individual to evaluate the environmental risk factors. It is imaginable that this is tough work.

Without understanding exactly why some individuals develop a psychosis and some do not, it is also very difficult to develop medicine against psychosis. Nowadays there are antipsychotics that block dopamine-receptors or serotonin-receptors, because researchers know that these neurotransmitters play an important role in depression and schizophrenia have to deal with these neurotransmitters. In this way the chance of success is present, but this chance could be much bigger when knowledge increases. If you know for example how genes and environment interact you could improve the existing treatment cures for depression and schizophrenia, which includes besides specific developed medicine also a matching psychiatric treatment.

It is found that genes are playing an important role in developing psychoses, so a question that may arise is: why don’t develop medicines that change those genes in a way, which decreases the risk of a psychoses? The answer lies in the concept of liability threshold. This includes that the liability for a psychotic disease increases, as the number of genes that causes high risk increases. So probably more genes are present in an individual that could cause in combination with a certain environmental factor high risk for a psychosis. Of course you could be lucky and modify the gene of action, but probably the network of interacting genes is immense complex, that the change for this is small.

Researchers are trying firmly to understand how multiple genes interact in the presence of stressful life events. Because the point of view that multiple genes may interact whether in combination with a stressful life event is relatively new, there are not so many studies done to investigate those multiple-way interactions. To contribute to this big challenge in this research field we set up a research proposal, which can be found in the next section.
6 Research proposal

Gene-gene-environment interaction between \textit{BDNF} and \textit{5-HTT} polymorphisms and maternal care in rodents

6.1 Abstract

In psychotic disorders, gene-environment interactions are thought to play an important role. These interactions may involve more than just one gene, but so far not that much studies on these multiple-way interactions have been performed. Here we present a research proposal in which the interaction between the \textit{Val66Met BDNF} polymorphism and the \textit{5-HTT} length polymorphism as genetic risk factors and maternal care as an environmental risk factor is investigated. For this purpose we use genetically modified mice of which a part will be separated from their mother. We test the mice on behaviour as well as neurochemical changes. With this research we hope to contribute to a better understanding of molecular pathways in gene-gene-environment interactions that may be involved in psychotic disorders, and provide targets for diagnoses and therapies.

6.2 Introduction

In the past years, a lot of genetic risk factors for schizophrenia and depression have been investigated. But thus far none of these factors appeared to be a susceptibility gene with moderate to high effect sizes [Van Haren et al. 2007]. So a general hypothesis is that the already found (and probably even more) genetic risk factors might interact in a special way, whereby these factors in combination with environmental factors can cause schizophrenia or depression. But there are not yet many researches done to study the interaction between multiple genetic factors and especially not in combination with environmental risk factors. So we tried to make a proposal to study the interaction between genetic risk factors and one environmental risk factor. These studies are very complicated, especially when multiple genetic risk factors are involved. That’s why we have chosen to pick only two genetic risk factors: the brain derived neurotropic factor (\textit{BDNF}) \textit{Val66Met} polymorphism and the serotonin transporter (\textit{5-HTT}) length polymorphism. We think the best way to study the interaction is by making genetically modified mice of different variations of the polymorphisms. We decided to do a mice study, because a study with humans is much more difficult. When you use human subject you can only use those subjects with the specific genetic risk factors that you need for the study. Finding subjects with the genetic make-up that you are looking for, would cost lots of time and money. Next to this genetic make-up you also need to find individuals that are exposed to the specific environmental risk factor that you want to study. Moreover it is very difficult to characterize these risk factors and also every individual probably experiences the environmental risk factor in a different way (abuse for example has many different forms). Furthermore in human studies, one has to believe the subjects (and maybe their family if they want and are able to cooperate) about what they have experienced in early life (which can be difficult with schizophrenic or depressed patients). Besides, in humans one cannot analyze the brain in molecular and cellular detail.

Next to the two genetic risk factors, we also want to study the interaction of these genes in combination with one environmental risk factor. In mice low maternal care is associated with high anxiety-related behavior and exaggerated stress response in adulthood.[Carola et al. 2008] Also other studies have already shown that maternal care in the early postnatal period is very important for rats and mice.[Millstein and Holmes 2007] Separation of pups from the nest in the first two postnatal weeks has been found to produce increased anxiety-like and depression-related behaviours, and exaggerated hypothalamic-pituitary-adrenal (HPA)-axis response to stress in adulthood.[Millstein and Holmes 2007] So we decided to make two categories for the environmental risk factor: one group of mice with ’normal’ maternal care and one group where the pups get separated from the nest. In rodents there seems to exist a special period of time after birth of the pups that they are vulnerable for environmental alternations.
Table 1: Genotypes and environmental risk factor of the various groups of mice

<table>
<thead>
<tr>
<th>Group of mice</th>
<th>BDNF polymorphism variation</th>
<th>5-HTT polymorphism variation</th>
<th>Environmental risk factor: maternal care?</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>Val/Val</td>
<td>s/s</td>
<td>Maternal separation</td>
</tr>
<tr>
<td>A2</td>
<td>Val/Val</td>
<td>s/s</td>
<td>Normal maternal care</td>
</tr>
<tr>
<td>B1</td>
<td>Val/Val</td>
<td>l/l</td>
<td>Maternal separation</td>
</tr>
<tr>
<td>B2</td>
<td>Val/Val</td>
<td>l/l</td>
<td>Normal maternal care</td>
</tr>
<tr>
<td>C1</td>
<td>Met/Met</td>
<td>s/s</td>
<td>Maternal separation</td>
</tr>
<tr>
<td>C2</td>
<td>Met/Met</td>
<td>s/s</td>
<td>Normal maternal care</td>
</tr>
<tr>
<td>D1</td>
<td>Met/Met</td>
<td>l/l</td>
<td>Maternal separation</td>
</tr>
<tr>
<td>D2</td>
<td>Met/Met</td>
<td>l/l</td>
<td>Normal maternal care</td>
</tr>
</tbody>
</table>

The effects of the variations of the BDNF polymorphism is unclear, because studies found different results about this.

If we would combine all these different variations, there would be too many groups. So we decided to confine the groups by using four groups: A, B, C and D. Groups A and B with the same variation of the BDNF polymorphism, namely Val/Val, and groups C and D with the same variation of the BDNF polymorphism, namely Met/Met. Groups A and group C will have the same variation of the 5-HTT polymorphism, namely s/s, and groups B and D will also have the same variation of the 5-HTT polymorphism, l/l.

To study the effect of the environmental risk factor on the gene-gene-interaction, there has to be an extra group for every genetic make-up (every letter) group. Groups A-D will therefore be divided into two groups: group 1 will be the group where the pups are separated from their mother and nest, while group 2 include the pups with normal maternal care. This experimental design is summarized in table 1.

6.2.1 Aim of the proposal

Goal of this research will be to study what the effect is of the interaction between the BDNF Val/Met polymorphism, the 5-HTT length polymorphism and maternal separation on depression in mice. More knowledge about the interactions of multiple genes in combi-
nation with environmental risk factors, might help in understanding the molecular pathways that are involved in depression and maybe also in other mood disorders. And in this way this study might help to identify novel targets for the diagnosis and therapy.

6.3 Experimental procedures

For the experiments we need mice with specific genotypes (table ??). The humanized BDNF<sup>Met/Met</sup> knock-in transgenic mouse is available and can be obtained on a collaborative basis. The 5-HTT<sup>s/s</sup> transgenic mice have to be generated according to standard procedures. Briefly, first of all the knock-in alleles has to be designed and created as a vector, namely BDNF<sup>Met/Met</sup> en 5-HTT<sup>s/s</sup>. The knock-in mice will be generated by targeted insertion of the transgene at the selected locus (5-HTT). Then embryonic cells from a mouse embryo in the early blastula stadium have to be retrieved, where the genetic modification, a change from 5-HTT<sup>l/l</sup> in 5-HTT<sup>s/s</sup> has occurred. These genetically modified cells must be transferred to another mouse embryo in the early blastula stage. This genetically modified chimeric mouse has 'normal' cells (BDNF<sup>Val/Val</sup> and 5-HTT<sup>l/l</sup>) and cells with changed genotype (BDNF<sup>Met/Met</sup> and 5-HTT<sup>s/s</sup>).[Chen et al. 2006] For generating the mice with the genotypes that are needed a number of crossings have to be performed. The first is a crossing from the chimeric mouse with a 'normal' mouse. Hereby mice with the genotype BDNF<sup>Val/Val</sup> and 5-HTT<sup>l/l</sup> and with the genotype BDNF<sup>Met/Met</sup> and 5-HTT<sup>s/s</sup> will be generated.

Next to noticing these symptoms, the proposal contains experimental tests to see whether the mice show depressed behaviour under stressful conditions. This can be done by the application of stressors to the adult rodents. The most commonly used tests are the 'forced swim test' (FST) and the 'tail suspension test' (TST). In the FST mice are placed in individual water containing cylinders for six minutes. At the start of the experiment, the mice try to escape. But after about two minutes, the mice become immobile and are only making passive movements necessary to remain afloat.[Lucki 2007] In the TST mice are suspended by the tail from a horizontal bar or platform for six minutes. Mice usually show escape-like limb and body movements immediate after the start of the experiment. After a few minutes, they will stop moving and become immobile.[Lucki 2007]
The FST and TST can be used to measure which group of mice is 'the most depressed' and which group is 'the least depressed' by measuring the time of 'trying to escape' and immobility. Important for both experiments is that the test conditions are the same for all mice; for example water temperature, water depth and cylinder diameter should be kept constant for all mice.

### 6.3.2 Neurochemical analysis

Cellular and molecular research on the brains after genetically modifying the mice, could give some interesting results regarding the molecules and molecular pathways involved in depression. For example, a study of Carola and colleagues in 2008 demonstrated that heterozygous 5-HTT knock-out mice showed decreased 5-HT turnover in the hippocampus and regression analysis revealed a significant negative correlation between 5-HT turnover and anxiety-related measures.[Carola et al. 2008] Also the levels of BDNF mRNA are selectively elevated in the hippocampus of heterozygous 5-HTT knock-out mice exposed to low maternal care.[Carola et al. 2008] According to Carola et al., previous studies have revealed that over-expression of BDNF selectively in the postnatal mouse forebrain causes increased anxiety-related behavior and levels of BDNF protein in the dorsal hippocampus of mice is positively correlated with anxiety-related behavior. Results of their study argue that increased BDNF expression in the hippocampus is associated with increased hippocampal-dependent anxiety-related behavior.[Carola et al. 2008]

Also functional neuroimaging studies with humans have shown that baseline neural activity in the amygdala and hippocampus is positively correlated with the number of self-reported life stress events in s variant carriers of the 5-HTT length polymorphism, but negatively in l variant carriers of the 5-HTT length polymorphism.[Canli et al. 2006] To study the hippocampus and amygdala, we suggest a quantitative autoradiographic study of the 5-HTT protein expression levels with the selective radioligand 125I-IDAM, following the protocol described by Carola and colleagues.[Carola et al. 2008] To compare the BDNF mRNA between the different mice, we suggest an in situ hybridization with frozen 16 µm coronal sections from the brain, following the protocol of

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**Figure 14:** Comparison of human symptoms of depression with behaviour in rodents.[Cryan et al. 2004]
6.4 Analysis
All data will be analyzed by analysis of variance (ANOVA) to measure the statistical significance. If the ANOVA test gives a P < 0.01, data should be considered as significant.

6.5 Hypothesis
It is still unclear in literature which combination of variations of the two polymorphisms has the highest risk of getting depression. Some studies suggest that the Met allele and the s allele have the highest risk (so that would be C in our study), while other studies suggest that the Met allele in combination with the s allele has a ‘protective’ effect for developing depression and they suggest that Val/Val in combination with s/s increases the risk of developing depression (so that would be group A in our study) [Pezawas et al. 2008; Grabe et al. 2011]. According to these studies, we expect group A or group C to have higher risk to develop depression in comparison with group B and D. But we cannot predict if group A or group C has the highest risk, because literature search is unclear about this.

Applying this on the above-mentioned tests, mice from group A or C will show more of the symptoms enumerated in figure 14. Our hypothesis for the FST and TST is that ‘the most depressed mice’ (group A or C) have the longest ‘immobility-period’ and therefore trying the shortest period to escape.

The above mentioned-hypothesis of the most depressive mice is mostly based on human research. According to Urani and colleagues, the BDNF genotype of the mice might not have any effect at all. [Urani et al. 2005] They came to this conclusion by reading a lot of studies about BDNF knock-out mice and saw that BDNF heterozygous knock-out mice did not differ from wildtype control mice in different behavioural tests. What they find out, is that BDNF heterozygous knock-out mice show about 50% reduced levels of BDNF mRNA and proteins, but this is probably not sufficient to induce strong behavioural effects and Urani and colleagues think compensatory mechanisms may additionally have occurred during development. [Urani et al. 2005]

After reading the above-mentioned paper of Urani and colleagues, we don’t know anymore if the variations of the BDNF polymorphism will have any effects on the behavioural tests. Especially because the variations of the BDNF polymorphism might have even less effect on the phenotype than a knock-out of one allele of the gene.

According to the literature, we think mice that are maternal separated have a greater risk of getting depressed in adulthood than mice that are raised normally. So we think that group A1 for example has a greater risk of getting depressed than group A2. But we don’t know if this maternal separation has an effect on the above mentioned hypothesis for the highest risk of developing depression. It might be that maternal separation has more effect on for example group C than group B and in that way there is a chance that the overall risk of getting depression in adulthood is bigger for group C than group B. But we don’t know what the interaction of the two genetic risk factor is on the effect of the environmental risk factor (maternal separation), because we haven’t found literature about this. We can say that we expect maternal separated mice to have a higher risk on getting depressed, but we can’t say with certainty if maternal separation has an effect on the combination of the two polymorphisms, and so we don’t know if the environmental risk factor has an effect on the above hypothesized order of groups.

What the result will be of the neurochemical analysis is probably the most unclear aspect of our proposal: for instance, Carola and colleagues have used knock-out mice while we want to use genetically modified mice (where the polymorphism is inserted) and also they have made knock-out mice for only one gene (5-HTT) and we want to combine two genetic risk factors [Carola et al. 2008]. Nevertheless, if we apply their findings to our research proposal, we would expect the mice with s/s variation of the 5-HTT length polymorphism
(groups A and C) to have a decreased $5$-HT turnover in the hippocampus and selectively elevated levels of $BDNF$ mRNA. And therefore we would expect groups A and C to show a more depressive neurochemical analysis of the hippocampus in comparison to the mice of groups B and D.

But according to two earlier discussed studies [Montag et al. 2010; Grabe et al. 2011] the Met allele of $BDNF$ is associated with a reduction in activity-dependent secretion of $BDNF$ itself. The hypothesis is that the Met allele of $BDNF$ might be associated with lower levels of mRNA of $BDNF$. For our research proposal, this means that mice with Met/Met alleles might have lower levels of $BDNF$. Assuming this will be the case, groups C and D will have lower levels of $BDNF$.

Taken the above mentioned two expectations together, we hypothesize that group D has the lowest amounts of $BDNF$ in the hippocampus and thus will have 'the least depressive' neurochemical analysis of the hippocampus. The neurochemical analysis of the hippocampus of group C will be a sort of surprise, because Carola and colleagues results are contradictory with the results of Montage et al and Grabe et al. The hypothesis for group A will be that these hippocampi will have 'the most depressive' neurochemical analysis, because they will have the highest levels of BDNF.

6.5.1 Time table

The experiments described in this proposal will be performed by a PhD student during a period of four years (or three years by a post-doc). Before the start of the project, the genetically modified mice will be ordered from a commercial supplier; generation of the mice will take at least six months.

- Months 1-24: behavioural tests.

(change to three years for post-doc)

6.5.2 Budget

- Personnel: 220k euro
- Consumables: 80k euro
- Animals (generation and maintenance of genetically modified mice): 120k euro
- Other (e.g. travel expenses): 10k euro
- Total amount requested: 430k euro
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