

# Diplomarbeit

## **The influence of genetic variants on serotonin transporter binding under SSRI treatment in Major Depressive Disorder**

zur Erlangung des akademischen Grades

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# *Abstract*

Major Depressive Disorder (MDD) is a multicausal disease with genetic variation making up for probably 15-60 % of its genesis. According to the most common hypothesis MDD is caused mainly by a dysfunction of the serotonergic system. Many studies have shown the influence of single nucleotide polymorphisms (SNP) of serotonin-associated genes on both the risk of developing MDD and treatment response to antidepressants. However, for very few SNPs molecular neuroimaging studies have been carried out and some results have been contradictory. Hence more research is needed using PET and SPECT to clarify the previous findings.

Therefore, I investigated the influence of 6 SNPs involved in the serotonergic system on the serotonin transporter (5-HTT) binding potential. SNPs analyzed include 2 SNPs for HTR1A gene (rs878567, rs1364043) and one SNP each for HTR1B gene (rs6296), HTR2A gene (rs6311), TPH1 gene (rsrs1800532) and VMAT2 gene (rs363399). As main focus of my study I analyzed the effect of these SNPs on 5-HTT binding potential under antidepressant treatment. Binding potential was measured via PET using [<sup>11</sup>C]DASB, a highly specific radioligand binding to the 5-HTT.

Data analysis included 29 subjects, 14 patients with MDD and 15 healthy controls measured by PET and the radioligand [<sup>11</sup>C]DASB. The patient group was measured three times: drug naive, 2 weeks as well as approximately 2 month after start of treatment. Blood samples were collected before the PET analysis and the DNA was extracted using a QIAamp DNA Mini Kit. Genotyping was performed using SEQUENOM®.

All of the investigated SNPs have not been analyzed using PET neuroimaging of the 5-HTT before. While 5 of the SNPs did not show any significant effects after Bonferroni correction, rs1364043 of the HTR1A gene was associated with the 5-HTT binding potential. My study therefore indicates the relevance of this SNP in MDD and encourages further investigations with higher statistical power.

# Zusammenfassung

Major Depression (MDD) zeigt eine Lebenszeitprävalenz von 15-20% und zählt damit zu den größten Herausforderungen in der Psychiatrie. Nichtsdestotrotz sind die ätiologischen Faktoren dieser Erkrankung nach wie vor nicht genügend ergründet.

Die Serotonin-Hypothese, welche einen veränderten Serotoninhaushalt als hauptsächliches Substrat von MDD ansieht, gilt zurzeit als anerkanntes Erklärungsmodell. Des Weiteren ist schon länger bekannt, dass MDD zu einem beträchtlichen Prozentsatz erblich bedingt ist. Vor allem in den letzten Jahren wurden etliche Polymorphismen in Genen des serotonergen Systems mit MDD assoziiert, allerdings mit teilweise widersprüchlichen Ergebnissen.

Als junge Disziplin bietet „Molecular Neuroimaging“ die Möglichkeit *in vivo* Effekte von Polymorphismen auf das serotonerge System zu analysieren. In dieser Studie untersuche ich den Einfluss von 6 Single Nucleotid Polymorphismen (SNP) auf das Serotonin-Transporter (5-HTT) Bindungspotential (rs1364043 und rs878567 des HTR1A Gens, rs6296 des HTR1B Gens, rs6311 des HTR2A Gens, rs1800532 des TPH1 Gens sowie rs363399 des VMAT2 Gens).

Dazu wurden insgesamt 29 Studienteilnehmer, nämlich 14 Patienten mit MDD und 15 gesunde Kontrollprobanden mit dem Radioliganden [<sup>11</sup>C] DASB im PET Scan gemessen. Der Fokus dieser Studie liegt hierbei auf dem Effekt der SNPs auf das Serotonin-Transporter Bindungspotentials vor und unter antidepressiver Therapie. Jeder Patient wurde dreimal mit PET gemessen, bevor Therapiebeginn sowie 2 Wochen und ca. 2 Monate danach.

Während bei 5 der SNPs nach Bonferroni-Korrektur für multiples Testen keine signifikanten Effekte nachgewiesen werden konnte, zeigte der Polymorphismus rs1364043 des HTR1A Gens einen signifikanten Zusammenhang mit dem Serotonin-Transporter Bindungspotential. Dies ist die bisher erste PET Studie des 5-HTT bei MDD, welche diesen SNP untersucht hat, und meine Ergebnisse legen weitere Studien nahe.

# *Background & Current State of Research*

## **I. Depression**

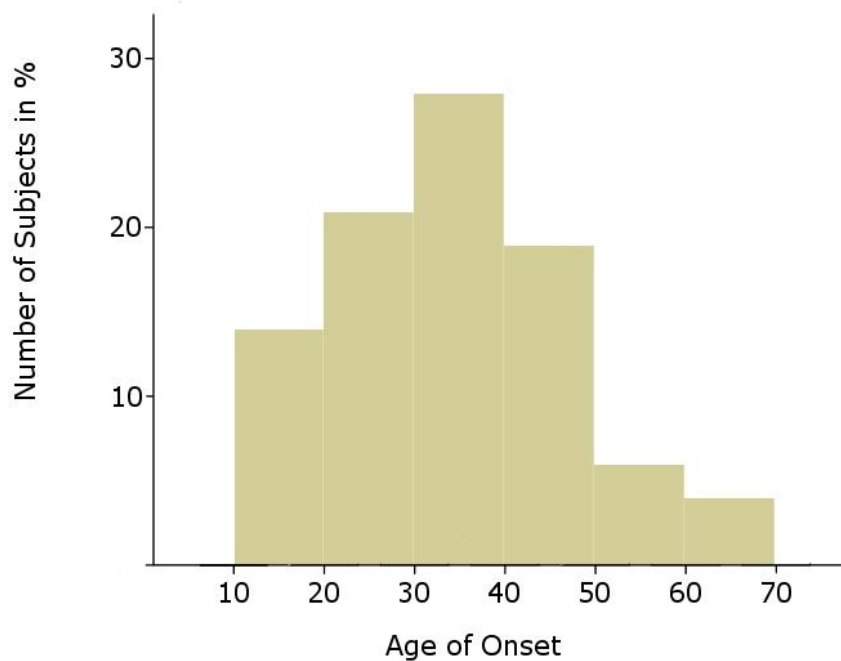
### **Public Health, Symptoms, Classification**

#### **➤ Statistics and Public Health**

With a life-time prevalence to suffer from at least one depressive episode of 15-20% depression is a decisive public health problem [2]. After incurring a depressive episode with an average lasting time of about 6 month around 15% of the patients sustain chronic recurrence [3, 4]. The quota of those who experience at least one more depressive episode is even expected to be 80% [5]. Thereby the onset time of a major depressive disorder (MDD) varies heavily. The disease can manifest from childhood to the old age and varies in signs and symptoms [6]. The peak lies between the third and fifth decade as seen in Figure 1 while some studies detected an additional peak between 50 and 60 [4, 6, 7].

It currently affects 7-11 % of the general population and often leads to a lifelong burden [8]. Thus it was accounted for 4.5% of disability adjusted life years and 12% of years lived in disability in 2000 [9]. Depression is even expected to be the 2nd largest disease burden leading to loss of years of healthy life to death and impairment until 2020 [10].

The mortality of people suffering from MDD is significantly higher than that of the general population due to depressed being prone to suicide and more severe course of comorbidities [11, 12]. In which way antidepressants and adequate treatment of comorbidities reduce this risk currently remains unclear [13].



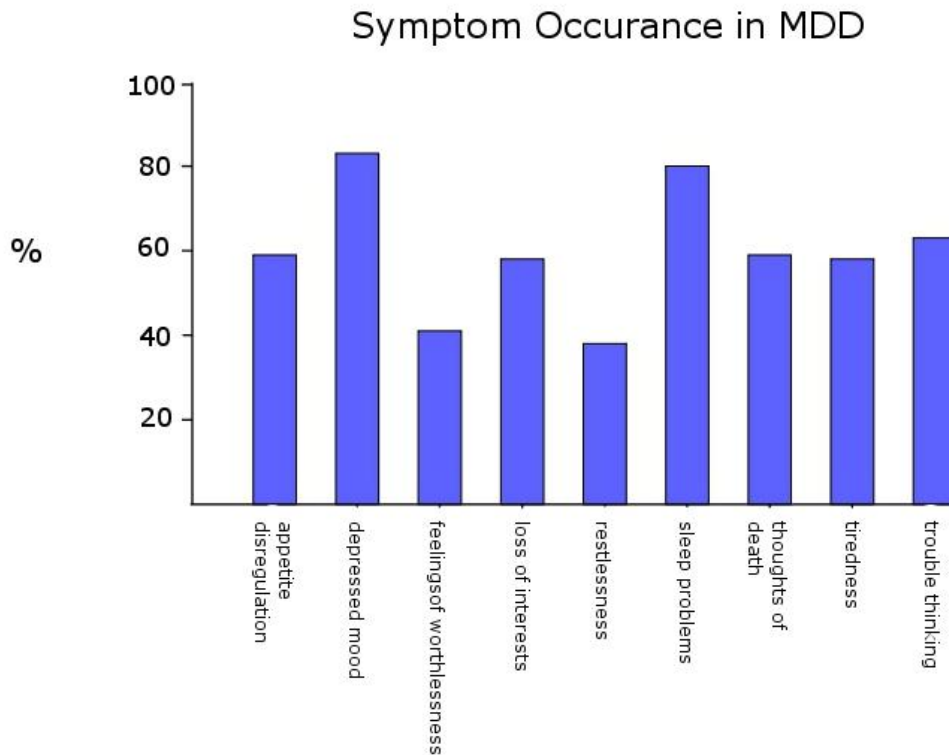
**Figure 1:** Distribution of MDD onset time based on the findings by Eaton [4].

It shows that MDD can manifest at nearly every age. The first depressive episode usually occurs between the third and fifth decade of life.

### ➤ Symptoms

Depression is primarily characterized by a lowered mood and impetus that affects peoples' ability to work, eat, sleep, interact with others and enjoy activities that once pleased them. Symptoms include feelings of sadness, guilt and despair, lack of motivation for daily life tasks, sleeplessness and tiredness and sometimes fear and psychotic symptoms. Furthermore, depression leads to impaired cognitive functioning affecting memory and vigilance.





**Figure 2:** Symptom groups and their frequencies in patients with major depressive disorder. The figure shows the wide range and high diversity in MDD symptoms. Based on data from a study by Chen et al [6].

Another issue of depression is the susceptibility to other chronic sicknesses like diabetes, metabolic syndrome and cardiovascular disease as well as suicidal behavior [14, 15]. Furthermore coexisting depression leads to more severe symptoms of both MDD and the other illnesses and makes it difficult to adapt to the particular medical condition [16]. Subsequently medical costs are much higher in patients having depression than in those who do not have co-existing depression. Hence accurate treatment of depression is important to improve the outcome of the other mentioned illnesses [17].

On the other hand suicide is one of the main causes for the lower life expectancies in depressed. Mood disorders have been attributed to 20-fold increase the life time risk of committing a suicide attempt compared to the general population [18]. According to Barlow in “Abnormal Psychology” 3.4% of MDD Patients commit suicide leading to a quota of 60% of suicides being attributed to depressed people [19].

## *Depression: Hardship of the Old*

Depression is an important health issue especially in elderly [20, 21]. In this person group MDD is often associated with predominant cognitive symptoms as forgetfulness and deceleration of physical and psychic actions and therefore depressive episodes sometimes resemble a beginning Alzheimer's disease [22]. In addition it has been shown that long time depression is probably a risk factor for Alzheimer's disease [23]. Furthermore, in old patients MDD usually coexists with physical disorders as chronic obstructive pulmonary disease or cardiovascular diseases like stroke [24, 25]. Other neuropsychiatric illnesses as Parkinson's disease and Alzheimer's disease are common as well. These chronic diseases are on their part increasing the risk of developing MDD [26, 27].

Also the risk of suicide is especially distinct in elderly [28]. However, even in disregard of suicidal tendency MDD is a severe mortality risk [29].

### ➤ **Classification: ICD 10 & DCM IV**

The American Psychiatric Association's revised fourth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV-TR) and the World Health Organization's International Statistical Classification of Diseases and Related Health Problems (ICD-10) provide the current state of the art criteria for diagnosing depressive disorders and episodes.

The ICD-10 is used in mainly in Europe while the DSM-IV is used in the US and most non-European countries. Even though they exhibit some slight differences there is a tendency to align one system to the other by both fractions.

Both classification systems use a similar diagnosis model.

### *ICD-10*

The ICD-10 demands at least 2 of 3 defined depressive symptoms for the diagnosis of depressive disorder. Possible main symptoms are depressed mood, anhedonia and reduced energy.

Depression is categorized among the group V F00-99 „psychic or behavioral diseases“. It belongs to the sub-group „affective disorders“ F30-39. Depending on the number of episodes that occur a division is made between depressive episodes and recurrent depressive disorder, ranked F32 and F33. Both diagnostic branches are further divided in

groups from .1-.9 taking in account current disease states and additional symptoms. Depressive disorders are typically classified mild, moderate or severe.

### *DCM-IV*

On the other hand DSM-IV only features the first two symptoms listed in the ICD-10. The presence of one of these is sufficient for diagnosis. Thereby the term major depressive episode is used and major depression is assigned to mood disorders. A distinction between mood episodes and depressive disorders is made, assimilable to the ICD 10. The recurrent form is called Major Depressive Disorder. Like in the ICD-10 special qualifiers are used to further highlight the characteristics of the major depression and comorbidities.

If a manifestation does not meet either criteria the category “Depressive Disorder Not Otherwise Specified” is diagnosed.

In this work the term major depressive disorder as described in the DCM-IV is used.

### *Evaluating Depression*

In order to evaluate the severity and exact condition of major depression several scores have been developed. The most used depression scale is the Hamilton Rating Scale of Depression (HRSD, more commonly abbreviated HAM-D) which was designed by Hamilton in 1960 and has been repeatedly updated since then [30, 31]. The HAM-D is a questionnaire consisting of at least 17 questions with 3-5 possible responses. They are assessed by a doctor who interviews the patient. The HAM-D provides useful information about symptom occurrence and severity. However, it has to be cautiously used as a diagnostic tool [32].

Among many other scales the Montgomery-Åsberg Depression Rating Scale (MADRS) or the Beck Depression Inventory (BDI) are popular as well. All of these scales are comparable in their design and are used alternatively or combined.

Most clinical studies on MDD and its genetic background use the HAM-D score to evaluate treatment response and disease severity. Studies of this design relevant for this work are discussed later on.

## II. Treatment

First-line treatment of depression relies mostly on antidepressants and psychotherapy.

Psychotherapy should always be undergone by patients under 18 due to the fact that in children and young adults a higher risk of both suicidal ideations and suicidal behavior if treated with SSRIs has been witnessed while less of a risk was found in patients elder than 24.

Regardless of this advantage psychotherapy has been shown to be effective with or without combination of antidepressants. Cognitive behavioral therapy (CBT) is currently the state of the art psychotherapy in MDD [33, 34].

There are several other, less used treatment approaches. Most notable are probably electroconvulsive therapy (ECT) and transcutaneous electrical nerve stimulation( TENS).

ECT means to induce a seizure in patients under general anesthesia via pulses of electricity sent through the brain via an electrode on each temple respectively. Usually ECT is only applied as a last-ditch effort to patients that did not show sufficient response to other treatment forms or in very acute and severe forms of depression, e.g. catatonic depression.

### ➤ Antidepressants

Antidepressants are without doubt the most important treatment arm. They show generally far superior effects than psychotherapy in moderate to severe cases of chronic major depression. Mild forms of depression can probably not be treated that well with antidepressants. It is not clear whether antidepressants are apt of alleviating mild depression; some studies propose antidepressant effectiveness not different from placebo while others approve their use [35, 36]. Psychotherapy seems to be more efficient in these patients [37].

Typically a gap of several days to a few weeks occurs between initiation of AD treatment and amelioration of symptoms. Treatment must then be maintained for usually six to eight

weeks or longer until the depressive episode is overcome [38]. Nevertheless medication often has to be continued for several months up to a year in order to prevent the patients from relapsing in depressive conditions [39]. Cases of severe chronic depression are often reliant on antidepressants for their lifetime.

However, the use of a single antidepressant even in high dosage is sometimes without effect. In major studies on treatment success that the first prescribed antidepressants proved to be ineffective in 25-50% of cases. Even when additional antidepressants of different groups are administered the quota of therapy refractory patients is estimated to stay at 30%. In these cases where patients do not adequately respond to at least two antidepressants the terms "refractory depression" and "treatment-resistant depression" are used [40].

The phenomenon of refractory depression is not completely understood yet. It has been shown in numerous studies that remission can possibly be predicted by genotype. On the other hand the influence of common co-disease on MDD therapy is probably strong as well.

To further clarify question whether and to which extent genetic variants control therapy efficacy is one of the main goals of this study. Detailed analysis of the current data basis and my approach are described farther below.

### *Types of Antidepressants*

Several groups of antidepressants have been developed all of which affect the serotonergic system and directly or indirectly impact the serotonin (5-HT) level.

Selective serotonin reuptake inhibitors (SSRIs) are the most used first line medication for depression due to their effectiveness, mild side effects and little toxic effects even in overdose [41]. They unfold their effects primarily through blockage of the serotonin transporter (5-HTT) and subsequently increase the level of extracellular serotonin. The SSRIs Sertraline, Fluoxetine and Escitalopram are currently the three most frequently prescribed antidepressants in the US.

The group of the serotonin-norepinephrine reuptake inhibitors (SNRIs) differs in mechanism of action due to the additional boosting effect on noradrenaline level. They may be modestly more effective than SSRIs, however, feature more severe side effects as well and have not

proved to be explicitly superior to SSRIs yet [42, 43]. The most used substance of this group is venlafaxine.

Other options are the tricyclic antidepressants which were the first known medication against depression. However, they feature many negative side effects owing to their wide-ranging effects disregarding the main mechanism of blocking the serotonin transporter and norepinephrine transporter. Therefore they fell into desuetude except for some definite indications [44, 45].

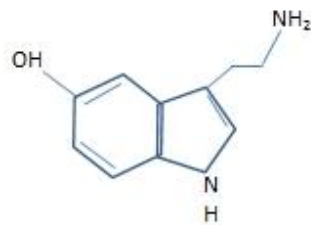
In addition to antidepressants exerting their main effect through blockage of the serotonin transporter substances targeting the monoamine oxidase A enzyme (MAO A inhibitors), the norepinephrine transporter (norepinephrine reuptake inhibitors, NRIs) or  $\alpha_2$ -adrenergic as well as 5-HT<sub>2A</sub>, 5-HT<sub>2C</sub> and 5-HT<sub>3</sub> receptors (Noradrenergic and specific serotonergic antidepressant, NaSSA) [46]. Furthermore, the atypical antidepressant bupropion can be used [47].

The antidepressant put on the market most recently is Agomelatine which is in clinical use since 2005. It is a melatonergic agonist on the MT1 and MT2 receptors and 5-HT<sub>2C</sub> antagonist. It putatively does neither alter monoamine uptake nor shows affinity for adrenergic, histaminergic, cholinergic or dopaminergic receptors. Therefore agomelatine differs significantly from the other antidepressants. Agomelatine is not yet available in the US due to ambiguity regarding its efficacy, however, it has been put on the Austrian market in 2009 [48-50].

### III. Etiology

Still much is unknown about the etiology and molecular mechanisms of MDD. Currently among the theories in discussion the monoaminergic theory is the most supported hypothesis. According to this thesis a dysregulation of the serotonergic system is the main substrate of depression and other psychiatric disorders.

## ➤ Serotonin



**Figure 3:** The monoamine neurotransmitter serotonin is synthesized from the essential amino acid tryptophan. The highest concentration of 5-HT and about 90% of average 5-HT expression can be found in the enterochromaffine cells of the guts, however, serotonin acts as an important CNS transmitter as well.

In the brain serotonin unfolds its effects through a variety of specific receptors which are further highlighted below. In summary serotonin is affecting many cognitive functions as feelings of well-being and happiness, regulation of mood, appetite and sleep as well as memory and learning.

## ➤ The Monoamine Hypothesis

The most accepted understanding of MDD is based on the serotonergic hypothesis proposing that an imbalance of serotonin is the main molecular substrate of the disease. The monoamine hypothesis was first shaped in 1960s by Schildkraut et al who was put on track by the great success of imipramine and Iproniazid in MDD therapy [51]. Iproniazid was actually developed as a tuberculostaticum and its antidepressant effect was found by chance in the 50s [52, 53]. The substance unfolds its effects through blockage of the monoamine oxidase.

Iproniazid on the other hand was the first antidepressant of the class of tricyclica [54].

Both substances share the boosting effect on the extracellular monoamine level. Hence a lowered monoamine level is believed to be the main substrate of MDD.

Half a century after Schildkraut hypothesized the monoamine theory it is still the most plausible model for depression etiology. It has been affirmed by many observations and the prominent role of serotonin was further highlighted. Most of these supportive findings are pharmacological phenomena:

SSRIs disseminate their therapeutic effects mainly through blockage of the serotonin transporter [55]. Therefore, they increase the serotonin level in the synaptic cleft.

On the other hand substances like reserpine, which empty the monoaminergic presynaptic vesicles, have been reported to trigger depressive episodes and cause relapses of remitted depressive patients.

Another source of evidence is brains of suicide victims who are to a high percentage suffering from depressive disorder as described above. The 5-HT levels in these brains were often found significantly lowered.

Most recently imaging studies have proven the involvement of the serotonergic system in affective disorders. PET studies in depressed using specific radioligands for the serotonin transporter and 5-HT receptors have shown an altered binding potential. Most commonly reduced 5-HT<sub>1A</sub> as well as 5-HTT receptor binding has been reported in patients suffering from mood disorders [56, 57]. However, PET studies on 5-HTT density in depressed patients led to contradictory results, sometimes demonstrating 5-HTT elevation during MDD episodes [58, 59].

On the other hand fMRI studies detected brain gray matter loss and abnormal activation patterns leading to increased metabolism in regions important for monoaminergic transmission such as the anterior cingulate cortex, the amygdala, hippocampus and the orbitofrontal cortex. [60-66]

### *The Role of the HPA axis*

Another frequent finding in depressed people is a dysregulation of the HPA axis. This results in an inadequate reaction to stress with high excretion of ACTH, CRH and finally cortisol [67]. However, cortisol levels have been found elevated only in 40-60 % of depressed patients [68].

It has been shown that serotonin acts as a suppressor of cortisol secretion due to negative feedback on the CRH receptors. Whether the dysregulation of the HPA axis is an effect of the lower serotonin level in depressed or a genuine part of MDD etiology is therefore not clear. Some studies reported a positive effect on depression course if cortisol was lowered while



others observed the contrary and proposed a high cortisol level due to insufficient cortisol binding and effects.

However, cortisol at high levels has a negative effect on the neuro-regeneration of the hippocampus and maybe other regions which is suspected to be part of the MDD etiology.

Furthermore, a connection between HPA axis, serotonin transporter and vulnerability to develop MDD after adverse life events has been shown by Gotlib et al in 2007 [69]. Carriers of the s allele of the 5HTTLPR length polymorphism in the serotonin transporter gene showed higher cortisol levels after an average stress stimulus.

Thus the current state of research indicates that this vulnerability to stress in depressed is caused by the impaired serotonergic transmission in limbic and cortical brain areas. Thus genetic polymorphisms of the serotonergic system lead to a higher risk of being unable to cope with environmental challenges. According to this theory MDD is based on a gene environment interaction. Fittingly some genetic variants like the already mentioned length polymorphism in the serotonin transporter have been associated with altered ability to cope with stressors like adverse childhood events and traumatic experiences [70].

### *Other neurotransmitters involved?*

There are still some inconsistencies with the monoaminergic theory. Most of all the therapeutic effect does not unfold simultaneously with the biochemical effect of antidepressants. Even though the serotonin level is altered within hours, it usually takes several weeks before a therapeutic effect can be observed. In addition some substances like tricyclic antidepressants bind equally to several receptors. Distinguishing which blockage leads to a certain effect is often impossible.

In the last decades growing evidence for the importance of glutamate as well as GABA has emerged [71].

In summary the predominant substrate of depression is believed to be serotonin with many other neurotransmitter systems interacting.

### *Thus the questions arise:*

How much influence do genes actually exert on the development of major depressive disorder? Are some people predestinated to suffer from this disease because of their genes?

Can MDD be excluded for others after a genome analysis? Could the thought of customized pharmacotherapy that will put an end to non-responders and side effects finally become reality?

# *From Twin Studies to Genome Wide Association and further*

## **1. Psychiatric disorders as multicausal diseases**

The idea of depression being a hereditary disease has already been documented in the first high cultures. It has been observed for centuries that children born into families with a history of depression and other psychic disease show a high risk of developing these diseases too. More intriguing studies on this have been performed in the last decades. Usually twin and adoption models are applied. Children of depressed parents that have been adopted and hence separated from the milieu of depression have shown an increased risk of MDD nonetheless. Twins that have been separated after their birth feature a high concordance of developing MDD. Studies performed by many teams range in the percentage of estimated heritability from 20 to 70% [72-76]. These figures are a good model for the genetic background of MDD and lead to the conclusion that probably more than 50% of the causing agents are genetic.

Therefore scientists have tried to identify risk genes. Their effort however led to mixed results. While several genes have been associated with MDD, the effect of a single polymorphism or even gene is estimated very low [77]. As a complex, multicausal disease MDD is influenced by dozens of genes featuring hundreds of possible functional polymorphisms. Hence MDD is caused by the interplay of all these polymorphisms with each other and environmental influences that causes serotonergic dysregulation and susceptibility to stress.

The human genome consists of millions of genetic variants. Polymorphisms that only include a single base pair are called single nucleotide polymorphisms while such variants spanning over larger regions from a few up to thousands of base pairs are named copy number variations.

## 2. SNP (single nucleotide polymorphism) assessment

Not until recently the potential of association analysis has gained a strong boost due to rapid technical advancement at decreasing costs. Association studies using DNA microarrays technology has become the state of the art and genome wide studies on many SNPs have become a viable path for researchers. In consequence many SNPs of most genes involved in the serotonergic system have been connected to major depressive disorder in association studies comparing genotype in patients and controls.

Following previous data suggesting the importance of a genetic variant other studies featuring clinical aspects are performed. For example frequently applied models for MDD research are comparison of depression scores in view of genotype or treatment response to antidepressants in dependency of SNPs.

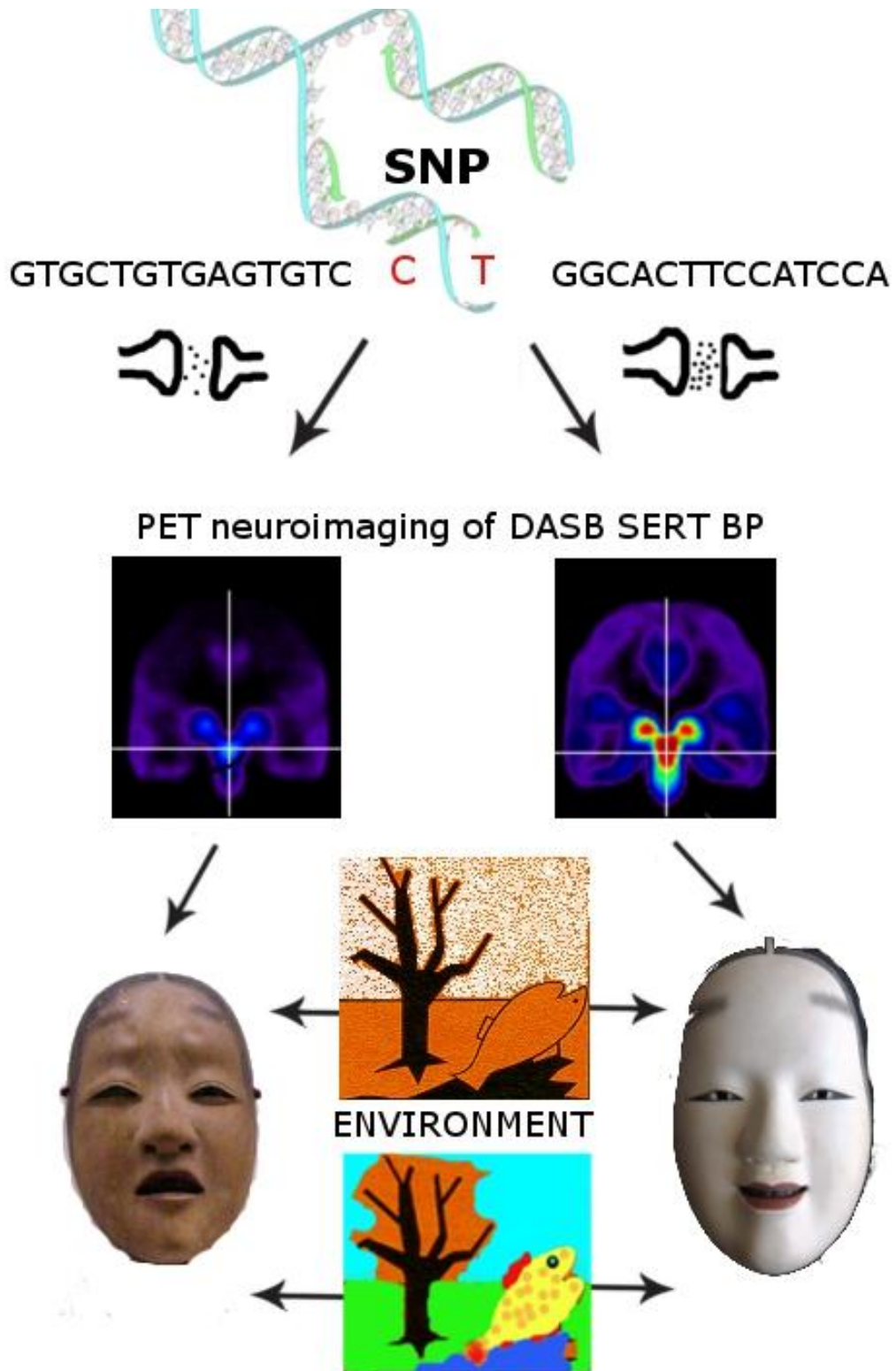
Such studies provide a more intriguing approach, however, often lead to contradictory results. Unfortunately they are hindered by many obstacles. Different populations differ in allele frequency and it is most likely that many SNPs have different effects according to ethnic groups. Furthermore, it is impossible to determine if the analyzed SNPs are actually the ones exerting the observed effect or if they are just in linkage disequilibrium with unknown functional SNPs. Due to the fact that the influence of a single SNP is estimated very low another challenge are the large groups of patients that would be required to gain definite results. The probably greatest handicap is however the lack of information on the direct impact of a genetic variant on the serotonin level in the brain.

## 3. Neuroimaging – the solution?

In the last years a new field of research has emerged which raised great expectations in psychiatry and neuroscience. Molecular neuroimaging genetics means to assess the serotonergic activity in the brain via positron emission tomography (PET) in the view of genotype [78, 79]. This has become possible with the development of highly specific traceable radioligands that are binding to their appendant receptors in the brain. Such radioligands contain a radioactive part that can be viewed in PET scanners. Thereby the binding potential (BP) can be measured using the formula

$$BP = \frac{f \times B}{K}$$

whereas  $f$  means the fraction of free radioligand,  $B$  the receptors available to ligand binding and  $K$  the radioligand dissociation constant.  $B$  is assumed to be constant in some receptor interaction models. BP measurement is achieved by comparison of the regions of interest with a reference tissue where no or hardly any binding can be expected. For neuroimaging of the serotonin transporter binding the cerebellum is used as reference tissue.



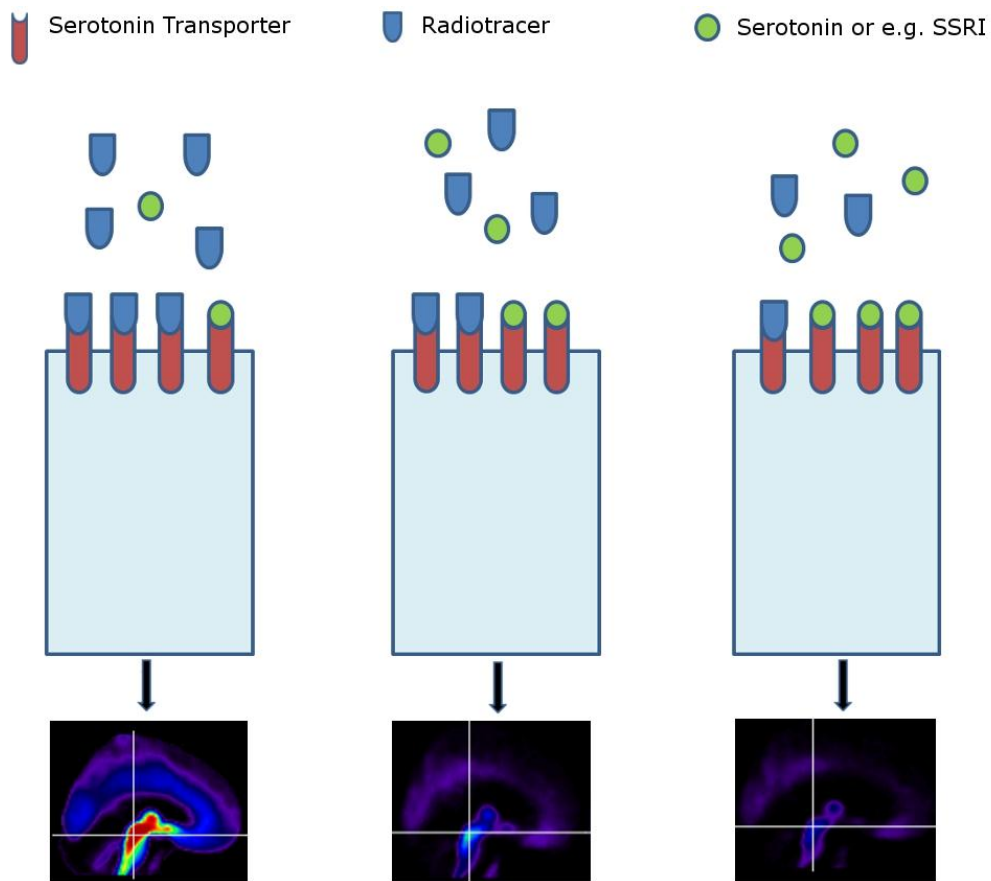
**Figure 4.** The basic idea of Imaging Genetics:

Certain genetic variants as SNPs alter molecular serotonin expression, e.g. the activity of the serotonin transporter SERT. Thus they exert influence on neural pathways that are shown via radioligand BP measurement (e.g., 5-HTT binding potential compared between genotypes, portrayed schematically in this figure). Subsequently SNPs determine how individuals cope with environmental stimuli and their risk to develop mood disorders as MDD.

Hence PET neuroimaging provides highly informative data by giving an *in vivo* display of the neurotransmitter binding in the brain. However, with some restraint.

The limitations of PET neuroimaging are to a great extent the limitations of radiotracer development. Initially this new technique was applied to studies on e.g. dopamine. Highly specific agonistic tracers as [<sup>11</sup>C]raclopride or [<sup>123</sup>I]IBZM binding to the dopamine receptor have been disposable since the 80s and enabled very informative studies on genetic configuration of the dopaminergic system. Dopamine PET neuroimaging studies have exceedingly shaped the understanding of diseases as schizophrenia or Parkinson and dopamine activity in general [80, 81].

As a model for the competitive binding of serotonin as the endogenous agonist and the radiotracer as exogenous agonist the conveniently simplistic competition or occupancy model can be applied (Figure 5). It is based on endogenous displacement and proposes a direct and self-sufficient interplay between radioligands and neurotransmitters. However, even for the ideal requirements as in dopamine neuroimaging soon critic on this model arose [82].



## THE COMPETITION MODEL

**Figure 5.** The competition model is the simplest assumption of receptor / ligand interaction [83]. The number of available receptors  $B_{avail}$  is constant. The radioligand competes against the neurotransmitter for receptor binding. Thus BP of the tracer will increase after depletion of the endogenous agonist and decrease after neurotransmitter stimulation. This change in occupancy is visualized in the PET images.

### *Expanding to other Neurotransmitter Systems*

A similar tracer for the serotonin transporter has not yet been established. The first described tracer  $[^{123}\text{I}]\beta\text{-Cit}$  had the disadvantage that it shows high affinity to dopamine transporters too. Hence no reliable conclusions can be drawn from BP observed in regions that feature both transporters e.g. the midbrain. Similar problems arise with the use of  $[^{11}\text{C}](+)\text{McN5652}$  which weakly binds to monoamine receptors as well as dopamine receptors besides the serotonin transporter.

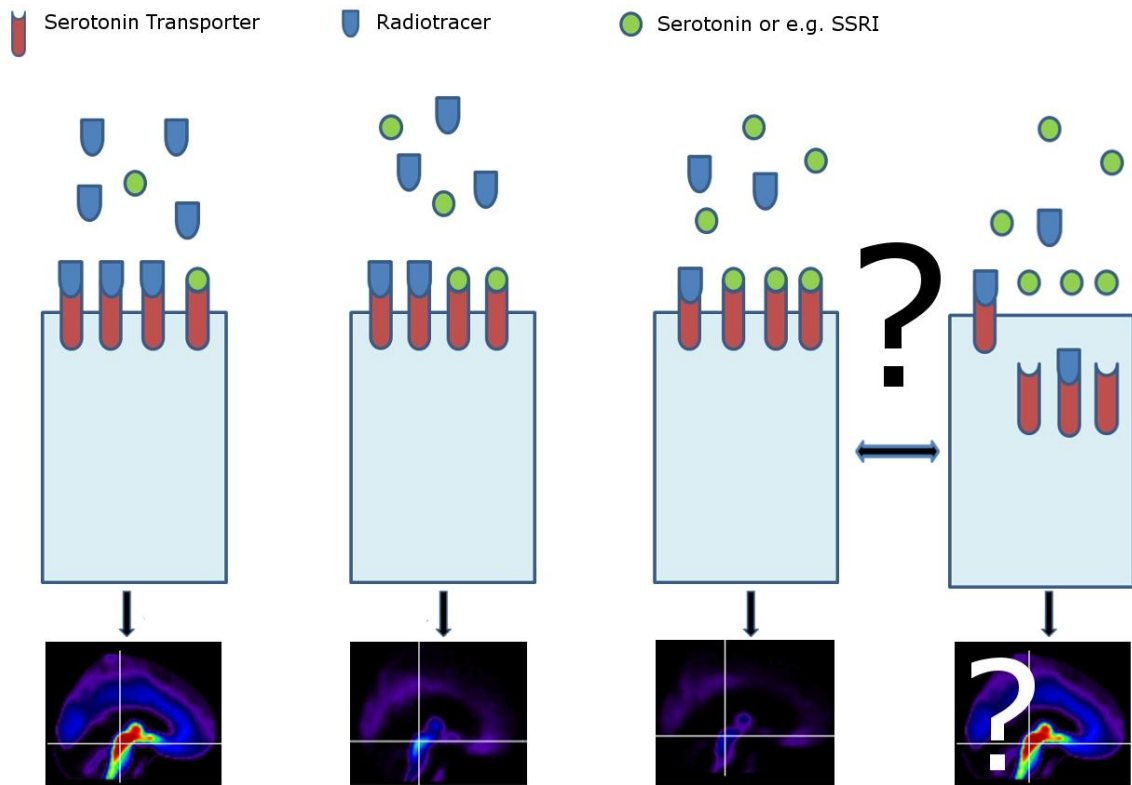


With the development of [<sup>11</sup>C]DASB more than a decade ago the first reliable tracer for 5-HTT has been found. DASB is an antagonistic tracer that has very low between-subject variability in terms of 5-HTT BP and specific-nonspecific binding fraction. DASB can be used to evaluate all brain regions that are of interest for MDD. It is still the best available radioligand binding specifically to the 5-HTT. The younger [<sup>123</sup>I]ADAM tracer has comparable features as DASB but proved to be overall of less power.

Therefore, DASB is the most commonly used radioligand for 5-HTT. It shows similarities to the group of SSRI. In full substance name it is called 3-amino-4-[2-[(di(methyl)amino)methyl]phenyl]-Sulfanylbzenzotrile.

### *DASB & the Internalization Model*

The only definite disadvantage of DASB lies within its effect as antagonist. Obviously an antagonist deviates more from the endogenous agonist than agonistic tracer. Especially in regard of G-protein coupled receptors antagonists putatively have access to receptors that antagonists cannot bind to. The simple competition model has to be abandoned in favor of the complicated and not yet fully understood internalization model (Figure 6). In this model an increase in radioligand BP does not incontrovertibly indicate lower concentration of the endogenous agonist. Receptor internalization and partition in compartments play a role as well. It has to be proposed that the antagonist and endogenous agonist do not have the same ability to reach these differently located receptors. Furthermore, the intracellular milieu affects radiotracers and alters their binding behavior.



## THE INTERNALISATION MODEL

**Figure 6:** The internalization model does not feature a static  $B_{avail}$  and is therefore more realistic than the competition level. For most radiotracers their binding behavior to internalized receptors is not sufficiently understood.

Not much is known about the detailed affinities of DASB or any other tracers to internalized receptors according to the internalization model. However, recent studies have shown that DASB binding is to a high percentage of 95% displaceable in animal models [84]. It has further been shown that physiological changes in endogenous serotonin do not or only very little affect DASB BP. Therefore DASB does not seem to be prone to unmanageable stimuli other than requested in the study model and is a reliable and well established radio tracer for serotonin transporter density in the human brain.

# *Study Protocol*

## *Study Aim & SNP selection*

The aim of this study is to further investigate putative risk genes for major depressive disorder via PET neuroimaging technique. Six SNPs in 5 genes responsible for serotonin synthesis, transport and receptor binding as well as receptor efficacy have been selected. Polymorphisms were chosen following the current state of research. I chose SNPs that have repeatedly been associated with MDD and/or treatment response to antidepressants in genome wide association studies and clinical studies. Detailed analysis of the featured SNPs and the genes that they are located at can be found in the next chapter. To my knowledge this is the first study of this design for all of the SNPs I selected.

## *Material & Methods*

Blood samples were included from 14 patients diagnosed with MDD and 15 healthy controls. In order to be included in my study patients had to be drug naïve or drug free for at least 3 months. Next DNA was extracted from the collected blood manually using a DNA mini kit. The DNA was further analyzed via Affymetrix and Illumina SNP genotyping chips.

Furthermore, baseline PET images were acquired from all probands using the state of the art radioligand [<sup>11</sup>C]DASB. The patient group was subsequently put in antidepressant therapy with SSRI. A second image was gained within a span of two weeks and 6 hours after treatment initiation of each patient respectively. Finally a third PET image was acquired 2 month after the first SSRI dose.

Detailed information on study subjects and material & methods can be found in the synonymous chapter.

## *Study hypothesis*

My study hypothesis is that 5-HTT binding potential will be significantly altered by genotype of the SNPs rs1800532 of the TPH1 gene, rs1364043 and rs878567 of the HTR1A gene, rs6296 of the HTR1B gene, rs6311 of the HTR2A gene and rs363399 of the VMAT2 gene.

### *Experiment & Statistics*

To test this hypothesis I compared BP in the baseline measurement between genotypes to investigate the general effect of the genetic variants on SERT expression.

My main focus however is the comparison of 5-HTT binding potential changes over time in the patient group to detect effects of the seven SNPs on treatment response to SSRI.

The data I collected was then analyzed using SPSS. I applied an analysis of variance (ANOVA) and t-tests for the baseline hypothesis and repeated measures ANOVA for the treatment hypothesis.

The results can be found in the synonymous chapter below and will be argued about in the discussion part.

# *Genes of the Serotonergic System*

The serotonergic system is regarded as one of the most complicated neurotransmitter systems. It is influenced by many genes and their coded proteins, among them the serotonin transporter, the serotonin receptors, serotonin synthesis, transport and degradation enzymes as well as other neurotransmitters and enzymes as cannabinoids, dopamine, brain derived neurotropic factor and angiotensin converting enzyme. Furthermore, G-protein related genes as GNB3 and other genes that influence pathways as FAAH or mtDNA are associated with the serotonergic system.

The genes and their specific SNPs that are featured in this study will be discussed in detail in this chapter:

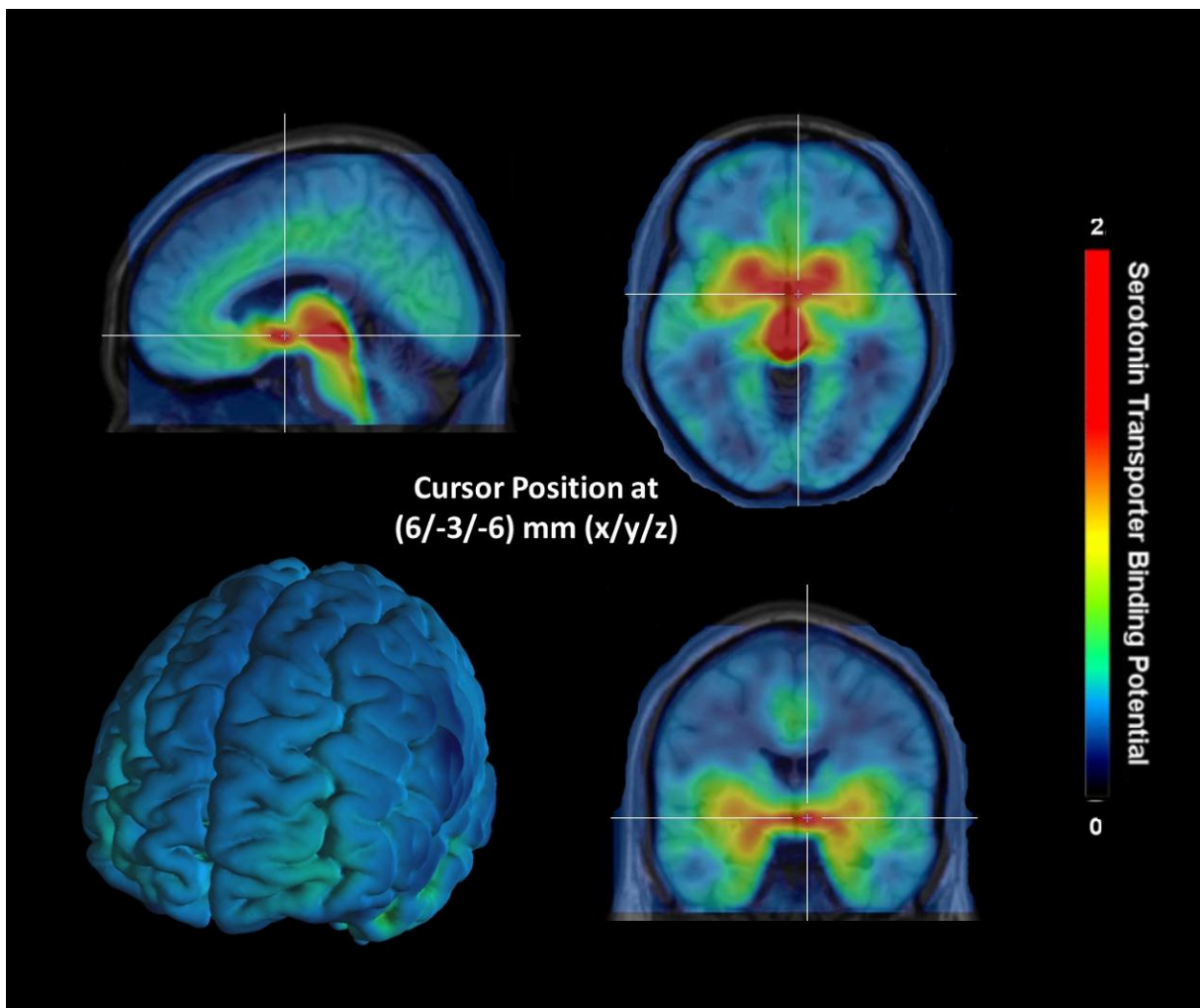
## **I. Serotonin Transport**

### **1. The Serotonin Transporter (5-HTT)**

Present research has concentrated on the serotonin transporter as the most obvious target. Being located on chromosome 17q11.2 the SLC6A4 or 5-HTT gene encodes a transmembrane protein of 630 amino acids, the serotonin transporter. It belongs to the sodium neurotransmitter transporter family and is located predominantly on the synaptic cleft. There it foraminates the presynaptic cell membrane with 12 transmembrane domains responsible for eliminating 5-HT from the cleft where it unfolds its effect. Thereby a cotransport of sodium and chloride is providing the necessary energy for taking up serotonin into the presynaptic bouton against the concentration gradient. Back in the cell serotonin can be reused.

The serotonin transporter is expressed both in the central nervous system and the periphery. The latter subtype can be found in platelets and triggers vasoconstrictive effects.

More importantly the serotonin transporter is wide spread in the brain where it regulates the serotonin neurotransmitter level. Areas of high serotonin transporter density are the raphe nuclei and the hypothalamus. However, also thalamus, amygdala, putamen, hippocampus and the prefrontal cortex show high serotonin transporter expression. Furthermore, the cerebellum is of special interest since it is an area that is putatively serotonin transporter free. Therefore, it proved to be especially useful for serotonin transporter imaging techniques as reference region as described later.



**Figure 7:** Distribution of the serotonin transporter in the brain shown via [<sup>11</sup>C] DASB binding measured in PET. Highest binding potential can be found in raphe nuclei, thalamus, hypothalamus and striatum. Pictures were created by myself, based on a study by Saulin et al and measurements on 16 healthy subjects performed by our study group [85].

Hence higher activity of the serotonin transporter leads to a decrease in serotonin level which is expected to be an important substrate for MDD. Therefore many studies have described its role in the pathogenesis of MDD, however, with no definite result.

Most recently a study conducted by Selvaraj reported decreased SERT binding of DASB in depressed patients compared to healthy controls [56]. In contradiction to these findings Cannon reported increased 5-HTT binding potential in both MDD and bipolar disorders compared to healthy controls in 2007 [58]. Boileau published similar results in 2008 in a group of depressed patients suffering from Parkinson's disease [86].

Nevertheless the prevalent etiological model for MDD proposes decreased SERT as a major risk factor for MDD. Convincing argument for this model lies in the 5HTTLPR:

### **The 5-HTT linked polymorphic region (HTTLPR)**

Among the known variants of the serotonin transporter especially a length polymorphism in the 5-HTT linked polymorphic region exerts a strong influence on the serotonergic system. Heils et al first described this polymorphism in 1995 [87, 88]. It was then associated with affective disorders in 1996 by Collier et al and has been featured in several hundred studies since [89]. Over the years the serotonin transporter polymorphic region has become one of the best known genetic variants in regard of psychiatric diseases.

The HTTLPR is a copy number variation located at the promoter region of SLCA4. It is a copy number variation (CNV) showing either 14 or 16 copies of a sequence of base pairs and is therefore named long/short polymorphism of 5HTTLPR.

Since then lots of studies have been performed making this polymorphism the probably best known genetic factor of MDD. The "l" version has been shown to exert an enhancing effect on the serotonin level and seems to make childhood adverse events more tolerable for the bearer. The "s" polymorphism is associated with a risk increase for MDD of about 5%. Later on a single nucleotide polymorphism named rs2552 was found within the span of the CNV. Hence the genotype can be extended from biallelic to triallelic: short, long and long'. With rs2552 only existing in the long version "l'" is the combination of "l" and the serotonin enhancing allele of the SNP rs2552. "l'" is now believed to show less efficacy than l', however, it is probably less a risk factor for MDD than the short version.

Based on the current data on MDD the serotonin transporter is of paramount relevance for this disease. However, imaging studies on the 5HTTLPR using DASB and PET technology have shown ambivalent results as well. A recent study on a comparatively large set of probands (n=68) could not detect any effect of the bi- or triallelic length polymorphism on 5-HTT binding potential [90]. On the other hand many studies achieved to verify the proposed effect of 5-HTTLPR in imaging studies and reported higher SERT availability in carriers of the “l” allele [91, 92].

Therefore despite being of great importance the influence of the genetic variants located directly in the serotonin transporter gene accounts only partly for the etiology of MDD. All other players in the serotonergic system exert influence on MDD through serotonin level. Furthermore, interactions between serotonin transporter polymorphisms and serotonin activity on 5-HT receptors have been described in several studies [93, 94]

Therefore the 5-HTT binding potential measured by DASB is regarded as an adequate model for changes in the serotonergic system caused by various genetic factors.

## **2. The Vesicular Monoamine Transporter (VMAT)**

The idea of a transporter responsible for filling the presynaptic vesicles first arose due to pharmacological observations. Reserpine which blocks the filling mechanism can lead to lower mood and depression like phenotype. It also antagonizes the effect of antidepressants because no serotonin can be set free. Together with the vesicular acetylcholine transporter (VACHT) the two forms of vesicular monoamine transporters VMAT1 and VMAT2 are members of the solute carrier family 18. Both subtypes are permanently attached to the cell membrane and are responsible for transporting monoamines as serotonin, dopamine, adrenaline, noradrenaline and histamine from the cytosol into the presynaptic vesicles.

The main difference between VMAT 1 and 2 is their distribution. While VMAT 1 is supposedly expressed only in the periphery VMAT 2 is active in the brain. However, as described further below many studies associated VMAT1 SNPs with neuropsychiatric diseases. Nevertheless until now the focus of research has been on VMAT2 [95].



The group of Lin et al described several putatively functional polymorphisms of VMAT2 in the promoter region. Subsequent studies by this group however focused on VMAT interaction with alcoholism [96].

### *Genetic Variation*

The SLC18A2 (solute carrier 18 member 2) gene which is encoding the VMAT is located on chromosome 10 at position 10q24.-3-q25.1. Few studies on its relevance for MDD have been performed yet. In 2007 a Japanese research group reported that VMAT2 heterozygous mutant mice show a depression like behavior analyzed via forced swim test and shock escape tests [97]. Nonetheless information on polymorphisms and their influence is very scarce so far.

#### *i. rs363399*

One of the few SNPs that were investigated in regard of MDD is rs363399. This polymorphism is located in the promoter region of VMAT2 at position 69813335 of Chromosome 10. It features either a T or a C allele with the latter being the minor allele (MAF C=0.25). Christiansen et al observed a significant effect of rs363399 in 684 elderly Danish twins that were assessed in symptoms and depression scorers over years. Rs363399 was analyzed haplotype-wise with rs4752045 and Christiansen observed the strongest effect in T<sub>rs363399</sub>/C<sub>rs4752045</sub> haplotype. Thus T-allele carriers showed a significantly lower risk of depression, however, this finding was only valid for the male subgroup [98].

Another study by Crowley et al on VMAT2 polymorphisms and MDD did not find any associations. Crowley compared treatment outcome after citalopram application in view of genotype [99].

Hence the data for this gene and its polymorphism is insufficient to convey VMAT's importance for MDD and other diseases. The PET approach might further illuminate this ambiguity.

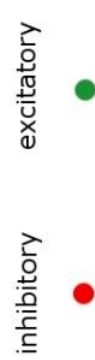
## II. The Serotonin Receptor Family

The 5-HT receptors are a large group of receptors coupled with heterotrimeric guanine nucleotide-binding proteins (G-proteins). Such proteins are composed of three subunits named alpha, beta and gamma. After binding to serotonin the 5-HT receptors trigger a type-dependent intracellular second messenger. That is, either the cAMP signal pathway or the phosphatidylinositol signal pathway as shown in figure 8.

5-HT receptors are ordered numerically from 5-HT<sub>1</sub> to 5-HT<sub>7</sub> and some have excitatory and others inhibitory effects. Each type is divided further into alphabetic subtypes. The accordant genes are located on different chromosomes.

Especially the 5-HT<sub>1A</sub>, 5-HT<sub>1B</sub> and 5-HT<sub>2A</sub> receptors have been intensively studied in regards of affective disorders.

Type	coupled with..	Pathway	expressed mainly in..
<b>5-HT1</b>	G <sub>i</sub> /G <sub>o</sub> -protein coupled	↓ intracellular CAMP	cerebral cortex, hippocampus, septum, amygdala, raphe nucleus, basal ganglia, thalamus, striatum
<b>5-HT2</b>	G <sub>q11</sub> -protein coupled	↑ intracellular IP3 & DAG	septum, hypothalamus, amygdala, claustrum, basal ganglia
<b>5-HT3</b>	Ligand-gated Na <sup>+</sup> /K <sup>+</sup> channel	depolarizing plasmamembrane	central and peripheric presynaptic nerve terminals
<b>5-HT4</b>	G <sub>s</sub> -protein coupled	↑ intracellular CAMP	putamen, caudate nucleus, nucleus accumbens, globus pallidus, substantia nigra
<b>5-HT5</b>	G <sub>i</sub> /G <sub>o</sub> -protein coupled	↓ intracellular CAMP	
<b>5-HT6</b>	G <sub>s</sub> -protein coupled	↑ intracellular CAMP	limbic and extrapyramidal cerebral zones
<b>5-HT7</b>	G <sub>s</sub> -protein coupled	↑ intracellular CAMP	



**Figure 8:** Types of serotonin receptors with appendant G-proteins, pathways and coarse receptor distribution in the central nervous system. Based on data from Pytliak et al [1].

## a. The 5-HT<sub>1</sub> Receptors

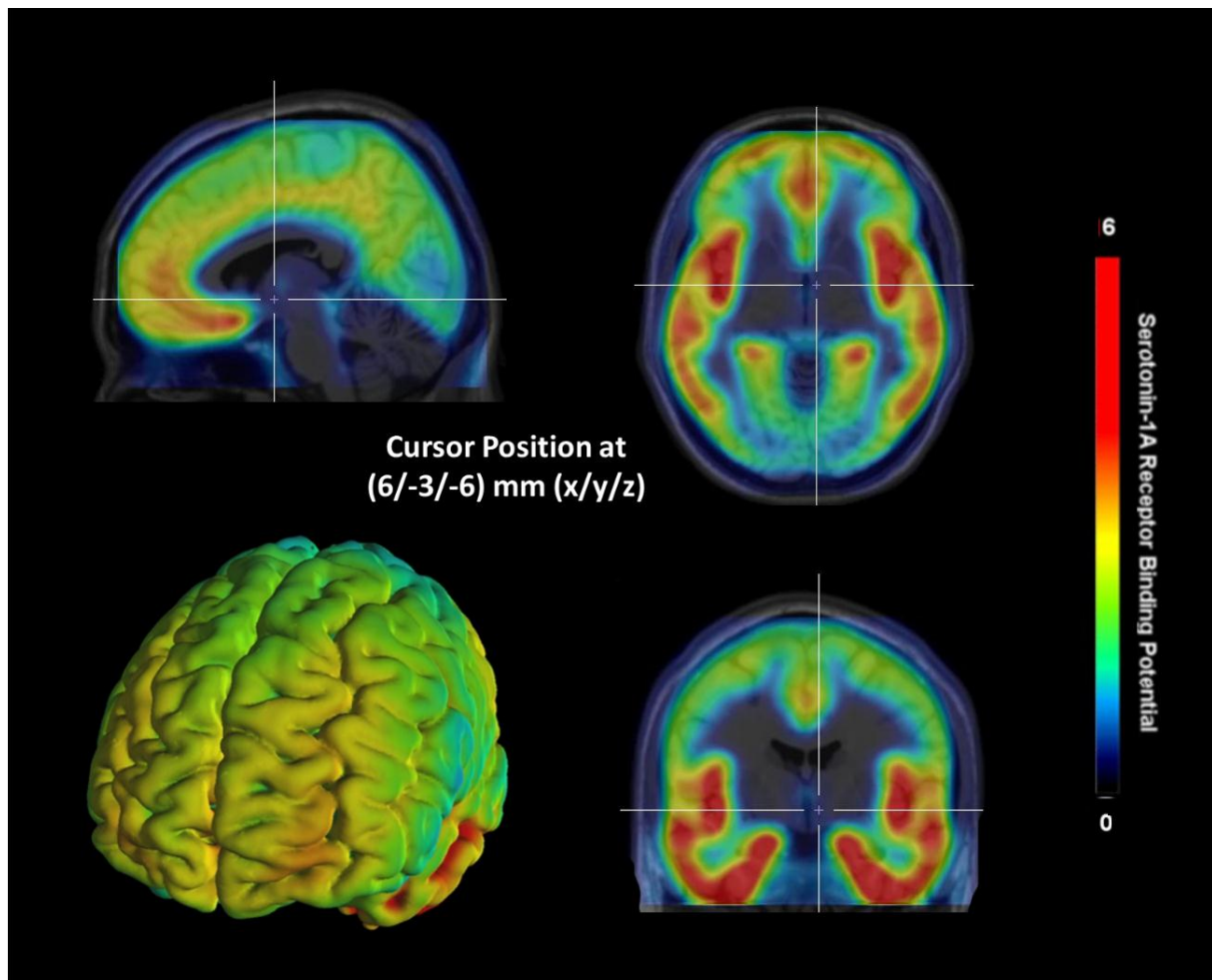
### 3. 5-HT<sub>1A</sub> Receptor

The 5-HT<sub>1A</sub> receptor is the main inhibitory serotonergic receptor subtype. It is a G<sub>i</sub>/G<sub>o</sub>-coupled receptor consisting of 422 amino acids and the according HTR1A gene is located on chromosome 5 spanning 63.29 - 63.29 Mb.

The 5-HT<sub>1A</sub> receptor is regarded as the most extensively distributed serotonin receptor. It can be found in the myenteric plexus and the whole gastrointestinal tract and is widespread in the neural system. HTR1A is expressed in the cerebral cortex, hippocampus, septum, amygdala, raphe nuclei and in less extent in the basal ganglia and thalamus. The cerebellum features only marginal 5-HT<sub>1A</sub> receptor distribution.

However, it has been shown that there are differences among receptors located pre- and postsynaptically. The presynaptic receptors are inhibitory autoreceptors which are prevalent in the raphe nuclei. There they inhibit the firing of serotonergic neurons and diminish serotonin release. Postsynaptic receptor activation triggers all serotonergic effects and putatively the anxiolytic and anti-depressive actions of SSRIs and other antidepressants.

5-HT<sub>1A</sub> receptor activation exerts influence on a wide spectrum of cognitive and behavioral functions including appetite, mood, social behavior, impulsivity and aggression, memory, sleep, anxiety as well as sexuality and addiction. In addition 5-HT<sub>1A</sub> receptor activation enforces or inhibits the secretion of several hormones including ACTH,  $\beta$ -endorphin and oxytocin. These effects have been studied in regard of their relevance in affective disorders and antidepressants with no definite result.



**Figure 10:** Distribution of 5-HT<sub>1A</sub> receptors in the human brain. Measurements have been performed with the radioligand [Carbonyl-<sup>11</sup>C]-WAY100635 which is highly selectively binding to the 5-HT<sub>1A</sub> as an antagonist. HTR1A is expressed in the cerebral cortex, hippocampus, septum, amygdala, raphe nuclei and in less extent in the basal ganglia and thalamus. The Pictures have been created by myself and are based on PET measurements on 36 healthy subjects [85].

Many drugs used in psychiatry like buspiron or pindolol are partial agonists on the 5-HT<sub>1A</sub> receptor and can boost the therapeutic effects of SSRIs. Due to these observations HTR1A's relevance for affective disorders is well-known, however, not without ambiguity. The two different effects caused by presynaptic and postsynaptic receptors are usually triggered simultaneously and are therefore difficult to investigate. Currently it is believed that the anxiolytic and anti-depressive action of 5-HT<sub>1A</sub> agonists is based on the postsynaptic receptor activation and simultaneous desensitization of the autoreceptors. Buspiron for example is a partial agonist on the postsynaptic receptors and a full agonist on autoreceptors. Accordingly

the latter first have to be bypassed or down regulated before an antidepressant effect can be established. This can be achieved through other mechanisms as well, e.g. a high serotonin level induced by blockage of the serotonin transporter or 5-HT degrading enzymes as MAO or COMT. This 5-HT<sub>1A</sub> differentiation is a possible explanation for the gap between start of medication and treatment effect.

### *Genetic Variation of 5-HT<sub>1A</sub> Receptor*

The 5-HT<sub>1A</sub> receptor and genetic variations altering its effect are considered important substrates of MDD and anxiety disorders. A PET study using [Carbonyl-<sup>11</sup>C] WAY reported lowered availability of 5-HT<sub>1A</sub> receptors in depressed, especially in the raphe [100]. Furthermore, HTR1A knockout mice were shown to be prone to anxiety and depression related phenotypes [101, 102]. These studies thus support the important role of this receptor for mood disorders.

Since the mid-nineties many polymorphisms of HTR1A have been discovered. However, only one SNP has been investigated via PET analysis so far [64].

I included two SNPs previously not featured in a PET neuroimaging analysis for this study:

#### *i. rs878567*

Located on the position 63255991 of chromosome 5 this SNP features either a T or C allele and a MAF of T=0.5 in CEU. It was first analyzed by Serretti et al. This study compared HTR1A and HTR2C genotypes of German and Italian suicide completers and controls. However, only faint associations could be detected. rs878567 among other HTR1A SNPs was linked to higher scores of anger and aggressive behavior [103].

The importance of rs878567 for mood disorders has not been implicated until recently. In 2009 rs878567 was first associated with MDD in the Japanese population by Kishi et al [104]. Nonetheless this result has to be regarded with caution because the association did not remain after Bonferroni correction which gives the analysis a more exploratory character. Later on another study by the same group on HTR1A and psychosis endorsed the relevance of rs878567 in psychiatric disease however.

Further investigations of this SNP reported its influence on mood disorders through gene-environment interaction and poor resilience to childhood physical abuse in C allele

homozygotes. Additionally the C allele was linked to memory deficits mediated by the hippocampus. This was proposed by Brezo et al in a report of findings in 1200 Canadians who were monitored over 22 years in order to study the serotonergic diathesis of mood disorders and suicide [105].

Most recently Mekli et al discovered a correlation between rs878567 T allele and a higher risk to develop major depression after undergoing negative life events in sample of 1300 Caucasians [106]. Accordingly rs878567 probably exerts its influence through epigenetic interactions.

On the other hand a follow up study by Serretti using the same approach as in 2007 failed again to find other than marginal associations of rs878567 [107].

Although the role of rs878567 is still unclear yet it was suggested by the affirmative studies that this SNP may have a functional effect in the brain:

Brezo et al and Mekli et al recommended functional analysis of rs878567 in their studies mentioned above. Hence the rs878567 polymorphism appears to be a promising topic for imaging genetics.

#### ii. rs1364043

Another auspicious SNP is rs1364043 which is also located at the position 63250851 of chromosome 5. It features both a G or T allele and a MAF of G=0.276. Rs1364043 has been analyzed further in two recent studies. In both cases rs1364043 has been shown to interact with antidepressant treatment. In 2009 Kato et al performed a study on treatment response to SSRI and SNRI in 137 Japanese patients. This analysis comprised some SNPs that had not been investigated before, among them rs1364043 6.7 kb downstream from HTR1A. Kato et al described an increased response in T-allele carriers [108].

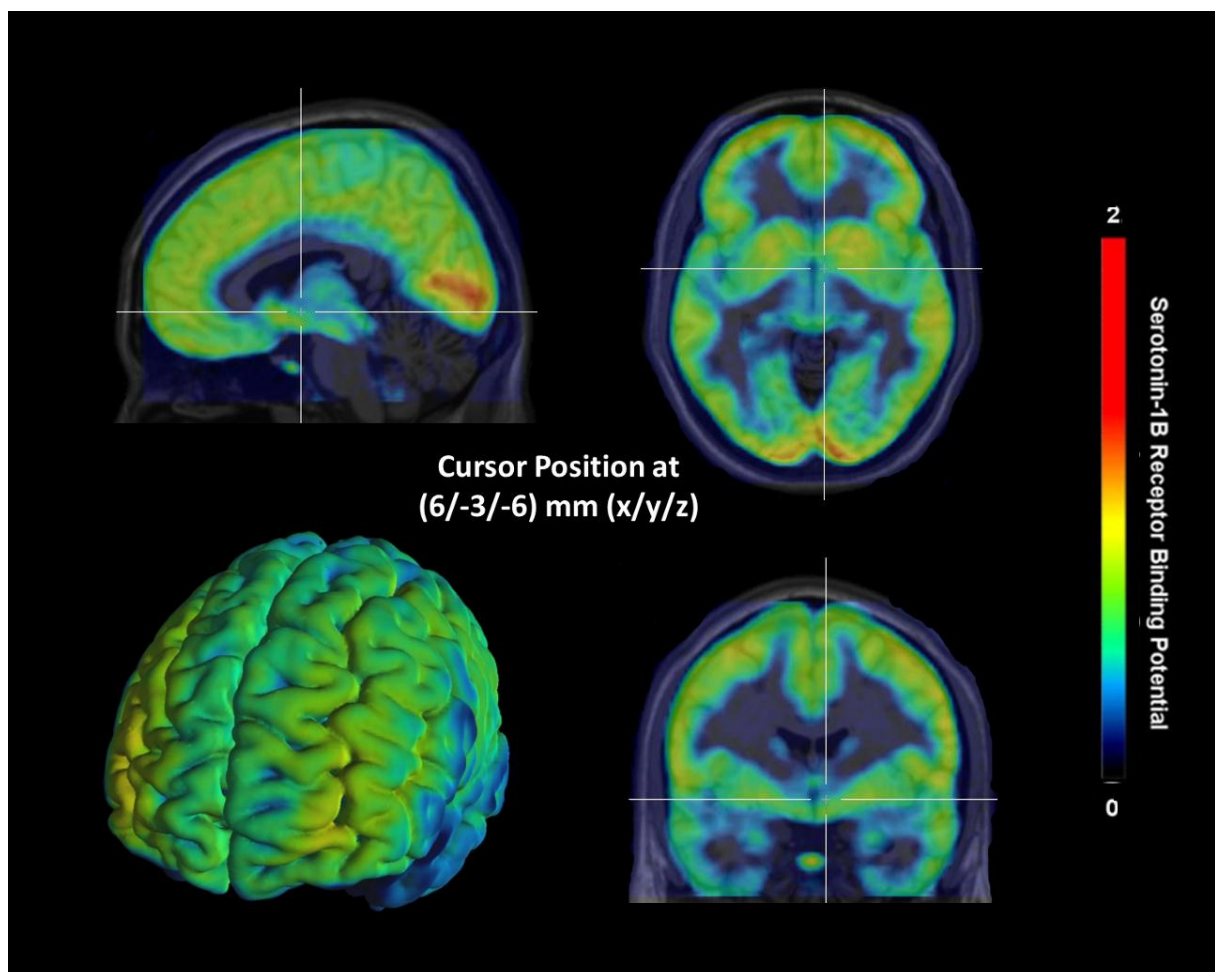
The same year Villafuerte et al. observed a better response to SSRI citalopram in patients featuring the G allele [109]. 153 mainly Caucasian patients suffering from MDD have been monitored for 12 weeks under treatment. This finding however is contradictory to the previous report of Kato.

A possible explanation is the difference in population used for analysis. Kato et al assessed a MAF of T=0.32. That frequency is in concordance with other findings in Japanese however strongly deviates from those in people with European ancestry.

PET analysis showing the effect of rs1364043 on 5-HTT binding potential *in vivo* which will be covered in this study will help to clarify the SNPs role in pharmacogenetics.

#### 4. The 5-HT<sub>1B</sub> Receptor

The 5-HT<sub>1B</sub> receptor gene HTR1B is located on chromosome 6 spanning from 78.23 to 78.23 Mb. The 5-HT<sub>1B</sub> receptor that it encodes is widely distributed in the brain, however mostly expressed in striatum, basal ganglia and frontal cortex. According to a recent study using the new radiotracer [<sup>11</sup>C]AZ10419369 the highest concentrations of 5-HT<sub>1B</sub> receptors can be found in the pallidum, the ventral striatum and the occipital cortex while the cerebellum shows very little binding again.



**Figure 11:** Distribution of 5-HT<sub>1B</sub> receptors measured in PET via [<sup>11</sup>C]P943. The pictures were created by myself, following measurements in 10 healthy subjects performed by our study group [85]. Highest concentration is found in pallidum, striatum and the occipital cortex.



On the other hand HTR1B also operates in the outside the central nervous system. Vascular effects and change of bone mass and osteoblast number have been reported.

Its main effect in the central nervous system and especially the basal ganglia is presynaptic auto inhibition. Other effects as inhibition of dopamine release and interaction with glutamate and norepinephrine have been reported [1].

A study on HTR1B knockout mice showed that such mutants are prone to inadequate aggression and addiction to alcohol [110]. Thus HTR1B influences sociability, anxiety, mood and aggressive as well as addictive behavior. Furthermore, it seems to be responsible for memory and learning as well as sexual behavior.

Its role in several psychiatric diseases was investigated in the last decades:

### *Genetic Variants of the 5-HT<sub>1B</sub> Receptor*

#### i. rs6296

A frequently reported polymorphism is rs6296 that is also known as G861C. Two studies from 1995 mapped the HTR1B gene and its polymorphisms and first mentioned the SNP at locus G861C in the coding region [111, 112]. Later on a couple of studies proposed its functionality or at least strong linkage disequilibrium with another highly functional SNP [113, 114].

It has first been featured in a clinical study on migraine treatment by the team of Maassen Van Den Brink in 1998 [115]. Hereupon several association studies have been performed and over the years rs6296 has been linked to many diseases. Besides migraine and depression especially substance dependence as alcoholism as well as aggression behavior and OCD have commonly been studied. A couple of studies have been performed on rs6296 in concern of MDD and depression related suicide behavior:

Huang et al first associated the C-allele of rs6296 with a higher risk of having a history of depression and substance abuse in 490 participants [116].

In 2011 Mekli et al replicated the previous positive findings in CC-allele carriers. They showed higher scores of both anxiety and depression than heterozygotes or GG-carriers [106]. Nevertheless in a study by Villafuerte et al from 2009 the CC-allele carriers showed better response to antidepressants [109].

In contrast the already mentioned study by Christiansen in 2007 however could not find any significant influence of rs6296 [98].

Susceptibility to suicide has often been linked to rs6296 too. With suicide being a common aspect of MDD this research further frames the picture of HTR1B polymorphisms and depression. Following a long history of sometimes contradictory results the most recent study was performed by Murphy et al in 2011 [117-119]. The heterozygote frequency was found significantly higher in suicide attempters compared to controls whereat both groups comprised depressed patients.

Other SNPs have been described, among them probably most noteworthy those analyzed in the already mentioned study by Villafuerte (rs130058, rs11568817) [109]. However, these findings are not shared by two other studies on HTR1B by Zhang and Mekli [106, 120]. Therefore, rs6296 is probably a highly interesting SNP of this gene for an approach via neuroimaging and I decided to include it in my study.

## 5. Other 5-HT<sub>1</sub> Receptors

The remaining 5-HT<sub>1</sub> receptors <sub>D-F</sub> are presumably of less importance for depression. Furthermore, genotyping was restricted to other SNPs due to research budget reasons.

## b. The 5-HT<sub>2</sub> Receptors

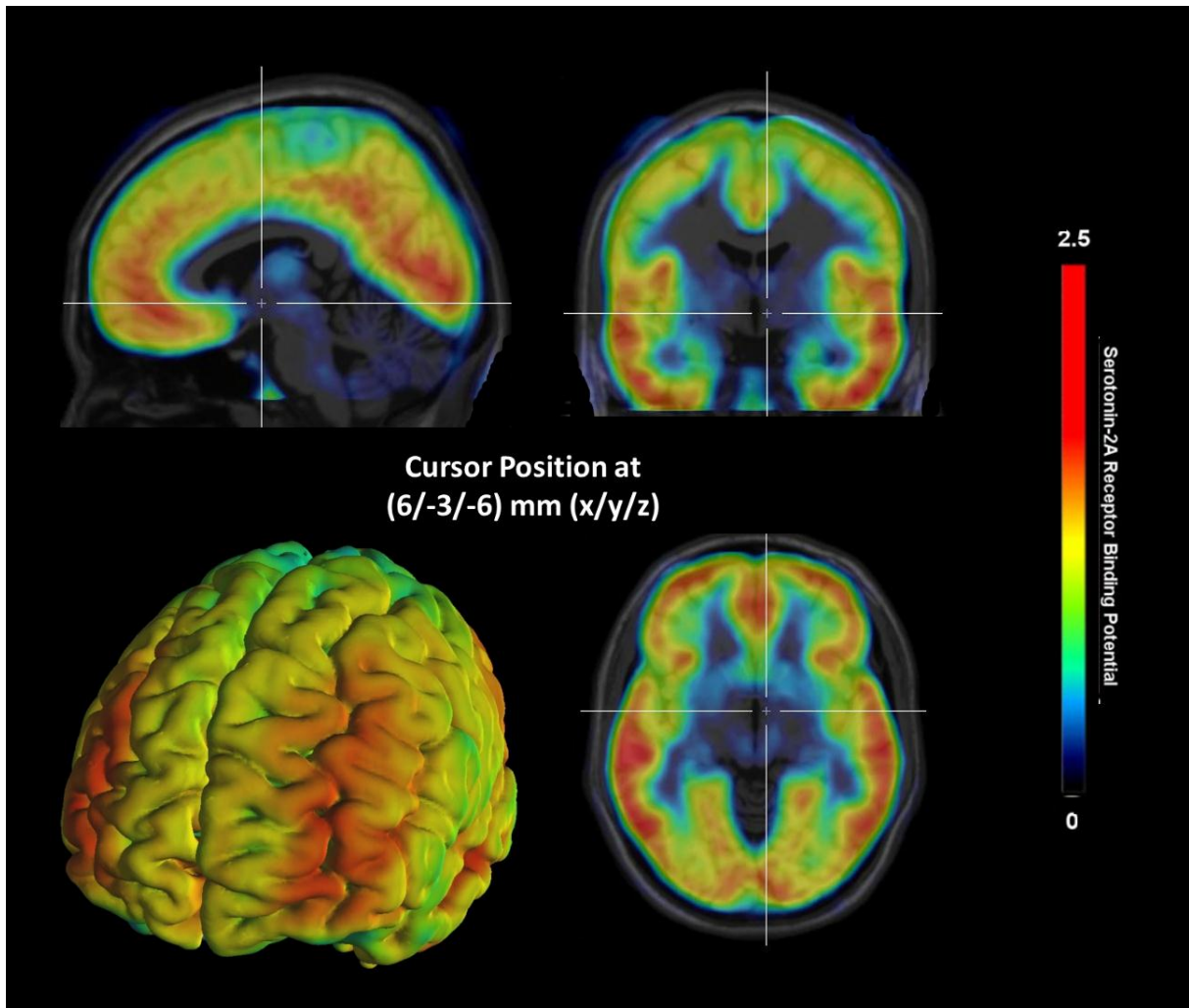
This group consists of three types, the 5-HT<sub>2A</sub>, 5-HT<sub>2B</sub> and 5-HT<sub>2C</sub> receptor. They feature a structural homology of 46-50 % and all of them are coupled with G<sub>q</sub>/G<sub>11</sub> proteins and bind serotonin with an excitatory effect.

### 6. The 5-HT<sub>2A</sub> receptor

Among the 5-HT<sub>2</sub> receptors the A type is without doubt the best known concerning depression. The coding gene HTR2A is located on chromosome 13 spanning from 47.41 to 47.47 Mb. HTR2A is expressed in many regions of the brain. High concentrations can be found in claustrum, basal ganglia, septum, hypothalamus and amygdala. It is the main excitatory receptor in the serotonergic system and unfolds its effect through the PLC pathway leading to Ca<sup>2+</sup> release.

Activation of 5-HT<sub>2A</sub> receptor leads to stimulation of secretion of ACTH, oxytocin, renin, and prolactin [121, 122]

In addition 5-HT<sub>2A</sub> activation has peripheral effects. It is involved in platelet aggregation and smooth muscle contraction and has a vasoactive component.



**Figure 12:** Distribution of 5-HT<sub>2A</sub> receptor in the central nervous system measured via PET using the radio-tracer [<sup>18</sup>F]altanserin. Highest concentrations can be found in cortical areas as well as the amygdala and the limbic system. Pictures were created by myself, based on previous work of our study group measuring 17 healthy subjects as described above [85].

### *Genetic Variants of the HTR2A gene*

#### i. rs6311

The HTR2A polymorphisms have been analyzed since the mid-1990s [123, 124]. Since then many association studies have been performed. Among the known SNPs, rs6311 and rs6313 seem to be most interesting.

rs6311 or -1438A/G is located upstream of the core promoter in the regulatory region [125]. The functional effects of rs6311 have been affirmed [126]. There is evidence that the

1438A/G promoter polymorphism rs6311 effects transcription factor binding and promoter methylation and therefore directly influences the 5-HT<sub>2A</sub> activity [127].

The first association with mood disorders was found by Enoch et al who described the relevance of rs6311 in seasonal affective disorder in 1999 [128].

Two Swedish studies reported depressed mood and a higher score of MDD symptoms for A-allele carriers. In 2003 Jansson et al analyzed a sample of 1590 elderly Swedish patients [129]. Association was only found in the male subgroup however. In consequence Christiansen et al replicated these findings in 680 patients in the study mentioned above [98].

In 2004 Peters et al linked a group of SNPs containing rs6311 with treatment response to SSRI without further distinguishing between the effects of sole SNPs [130].

In consequence a Korean research group around Choi published an association as well as a clinical study and proposed the modulatory effect of rs6311 on treatment response to SSRI as well as a general risk for MDD in A-allele carriers [131, 132].

One of the first clinical studies was undergone by a Japanese research group in 2002. They could not affirm their thesis that fluvoxamine effect is dependent on genotype [133]. However, another study conducted by Kato et al in Japanese showed a dependency of treatment response to SSRI on a HTR2A haplotype including rs6311. The G-allele was associated with better treatment response [134]. Succeeding this initial research in Japanese patients Kishi et al tried to replicate the previous findings in 2010. The association of rs6311 to treatment response found in this study did not overcome correction after multiple testing which makes it more an exploratory result [135].

Another analysis by Lin et al showed remote association of rs6311 to short-time treatment response through gene-gene interaction [136].

Most recently in 2011 Viikki et al described an important combined effect of HTR2A genotype featuring rs6311 and gender. That interaction significantly influences change in Montgomery and Åsberg Depression Rating Scale after SSRI treatment [137].

In conclusion the role of the -1438A/G polymorphism is currently not clear. Many studies have shown interactions with psychiatric disease but the results are not consistent. A recent meta-analysis of Japanese and Italian collaboration named -1438A/G as one of the most promising SNPs [138]. Therefore I believe that neuroimaging is an essential new step to understanding rs6311 in its role for MDD.

## ii. Other SNPs

Some other SNPs that are not analyzed in this study have been associated with MDD. Most noteworthy is the already mentioned rs6313 which has been associated with depression in several studies. An overview is provided by the meta-analysis by Kato and Serretti mentioned above [138].

# III. Serotonin Metabolism

Even though the receptors have obviously strong impact on the serotonergic system other factors are relevant as well. Especially the enzymes involved in the synthesis, transport and degradation of 5-HT are of interest.

## 1. Tryptophan Hydroxylase

The synthesis of serotonin features two enzymes. These are the tryptophan hydroxylase (TPH) and amino acid decarboxylase (AAD). The decisive protein however is TPH which catalyzes the reaction  $L\text{-tryptophan} \rightleftharpoons 5\text{-Hydroxytryptophan}$ . The latter is subsequently metabolized to 5-HT by AAD.

TPH is member of the aromatic amino acid hydroxylases and the rate-limiting enzyme in the serotonin synthesis. Only recently in 2003 a dimorphism of the TPH enzyme was discovered by Walther et al which led to a distinction between TPH1 and TPH2 [139, 140]. The two forms share approximately 70% of their sequence and have probably similar functions. The TPH1 gene coding for the synonymous enzyme is expressed mainly in the periphery. The current data whether or in which extent TPH1 is active in the brain as well is not satisfactory. In contrast TPH2 is expressed only in the central nervous system.

### *Genetic Variants of TPH1*

As mentioned above TPH2 is probably the more important form in the CNS. Nonetheless the primary focus of MDD related research has been on TPH1 and there is even evidence that TPH2 is of less relevance for MDD than the other subtype [141, 142]. This enzyme is coded by a synonymous gene located on chromosome 11 at position p15.3-p14.

#### *i. rs1800532*

A couple of polymorphisms have been described however rs1800532 is the only noteworthy. The 218A/C polymorphism is the only one that has been repeatedly linked to MDD over the years.

Nielsen et al described the polymorphism in 1997 as located in intron seven and within a GATA transcription factor binding site [143]. The same year Jonsson et al proposed a direct effect of TPH1 polymorphisms on the serotonin metabolism by measuring monoamine metabolites in the cerebrospinal fluid [144]. An investigation on the effect of 218A/C on tryptophan was undergone by Porter et al [145]. The group suggested that TPH1 A218C effects on the brain are mediated through the peripheral tryptophan level which was significantly altered by 218C phenotype in their study.

Subsequently many studies have been performed on rs1800532, however, with primary focus on suicide, anger and alcoholism [146]. The first study on MDD was undergone in 1999 by Frisch et al and could not detect any association [147].

The relevance of the 218A/C polymorphism for MDD was described contemporaneously in 2001 by Du et al and two related studies by Serretti et al [148-150]. Du et al associated the A-allele with somatic anxiety in depressed patient but failed to detect differences of allele frequencies between controls and depressed.

The studies by Serretti et al analyzed response of A218C to fluvoxamine and paroxetine, respectively. In both investigations C-allele carriers showed better response. These findings are in accordance with the results of Ham et al who linked the C-allele of rs1800532 to better response to citalopram [151].

A longitudinal study by Jokela et al monitored healthy Finns over four years to investigate the influence of A218C on the risk of developing depressive symptoms [152]. The researchers proposed that the A-allele may be responsible for a reduced ability to cope with social stressors that in consequence lead more often to depression in A-allele carriers.

In addition to these clinical studies Anttila et al performed another association study [153]. They described an association between TPH1 and GNB3 and a higher risk of being in the patient group for C-allele carriers. Furthermore, exclusively for females the T-allele of GNB3 rs5344 in combination with rs1800532 CC genotype is associated with poor remission rates after ECT. A second study on patients under ECT treatment affirmed Anttila's results in 2010 [154]. These results appear contradictory to the previous data indicating a protective aspect of the C-allele. However, a similar mechanism of ambiguity in concern of general risk and treatment effect as described for GNB3 is possible.

On the other hand a recent study linked the A-allele of rs1800532 to MDD in Taiwanese patients [155]. However, in this study Wang et al implicate that TPH1 is not related to treatment response, a finding that is contradictory to earlier data but shared with a study by Kato et al (2007) [156]. Viikki et al. related the C-allele to both severe forms of MDD and low remission rates.

The A218C polymorphism was recently featured in a MRI neuroimaging study in 26 female MDD patients conducted by Lee et al [157]. They observed an altered activation pattern of the amygdala to negative facial stimuli in A-allele carriers and thus proposed a modulatory role of 218A on amygdala activity. To my knowledge that is the only neuroimaging study on TPH1 so far and its results are encouraging to perform a PET analysis to further clarify the role of rs1800532.

## 2. Monoamine Oxidase (MAO)

The enzyme responsible for serotonin degradation is Monoamine Oxidase A (MAOA) [158]. The coding gene MAO-A is located on chromosome X at position 43.4 - 43.49. MAO-A uses the neurotransmitters serotonin, norepinephrine and dopamine as substrate. Thereby serotonin is degraded to 5-hydroxyindoleacetic acid (5-HIAA) which is an inactive metabolite. Therefore MAO-A is relevant for MDD and especially its therapy. It is suppressed by the antidepressant group of the MAO inhibitors which are commonly used against atypical depression. Furthermore, it has been implied that MAO polymorphisms are responsible for aggression related behavior and a variety of psychiatric diseases.

I will not further highlight the MAO gene however, because it is not featured in this study.



# *Material & Methods*

## **I. Probands**

22 medication-free patients with major depression established by a structured clinical interview (SCID, DSM-IV diagnosis) have been included [159]. Furthermore, 15 healthy controls have been included. Measures have not been completed by 3 patients and 5 patients dropped out due to genotyping issues. Hence I performed the analysis on the final number of 14 patients and 15 controls.

### *Inclusion Criteria*

My inclusion criteria for patients comprised an age between 18 and 55 years and a DSM-IV diagnosis of major depression. The latter was confirmed in a structured clinical interview and evaluated per 17 item HAM-D score. Patients had to show a value of 16 or higher to be included. Furthermore, female patients had to pass a urine pregnancy test prior to each PET measurement.

I did not use gender as an inclusion criterion and all patients fulfilling the mentioned criteria had to sign an informed consent form after the objective and procedures of the study were explained to them.

The Study has been approved by the ethics commission of the the medical university of Vienna (EC number: 578/2006).

### *Exclusion Criteria*

All patients that showed a concomitant neurological disease or another severe medical issue as major illness or clinically relevant abnormalities were excluded from the study. These criteria were tested in a physical examination including ECG and blood pressure and a routine laboratory screening profiling haematology and the general clinical chemistry as well as neuropsychological tests.

Furthermore, patients with a history of substance abuse or that have used drugs with affinity to the serotonin transporter as SSRI at least 3 month prior to the study were excluded. Finally an exposition to radiation within the last 6 month led to exclusion.

Healthy controls only had to meet the exclusion criteria. In addition they must not feature a history of depression.

## II. Treatment

Patients with major depression received treatment with citalopram or escitalopram. After the screening visit, each patient has been randomly assigned to one of two medication groups using one of the following medications:

- Escitalopram 10 mg/day (11 patients), Cipralex<sup>®</sup>, H. Lundbeck A/S
- Citalopram 20 mg/day (11 patients), Seropram<sup>®</sup>, H. Lundbeck A/S

The two medications are expected to have a similar treatment effect.

Treatment was initiated 6 hours prior to the second PET scan. The last PET image was acquired after a treatment period of at least 22 days.

## III. Blood Sampling & Genotyping

### *Blood Sampling for Genotyping*

After meeting all required criteria and giving the informed consent blood samples of 20 ml were collected from all probands. 4ml of the blood were then extracted into Leucosep tubes and centrifuged in order to select specifically peripheral blood monocyctic cells (PBMC). Subsequently the PBMC was manually processed using the QIAamp DNA Mini Kit by Quiagen. In several steps following the protocol of the kit the DNA is purified ([Protocol at quiagen.com](http://www.qiagen.com)).

Due to initial DNA genotyping problems further steps in genotyping have been performed at the “Ludwig-Maximilians-University” in Munich.

In Munich genotyping is performed using polymerase-chain-reaction and SEQUENOM®.

### *Blood Sampling during PET*

Blood sampling for the measurement of plasma concentrations of S- and R-enantiomers (performed by Lundbeck) were done 10 minutes prior to the start of the 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> PET scan, during the 2<sup>nd</sup> and 3<sup>rd</sup> scan, and within 10 minutes after the 2<sup>nd</sup> and 3<sup>rd</sup> scan which makes altogether 7 blood samples in each patient. PET scans started six hours after the last drug dose according to the ADAM SPECT data [160].

## **IV. PET Image Acquisition & Quantification**

Each patient underwent three PET scans. The controls only completed the first PET scan.

The GE Advance PET (GE Medical Systems, Wukesha, WI) at the Medical University of Vienna was used for obtaining BP measures. Simultaneously with the intravenous bolus injection of [<sup>11</sup>C]DASB scans were initiated using a standard protocol implemented at the PET center [161, 162].

### *Dynamic PET Scans in 3D Mode*

In 90 minutes of acquisition time optimized to the modeling considerations a series of 30 successive time frames (15\*1min, 15\*5min) was collected [163, 164]. After scatter correction of the emission data 35 contiguous slices of a 128\*128 matrix and thickness of 4.25mm each were reconstructed. Thus I finally obtained a reconstructed volume with the spatial resolution of 4.36mm full-width-half-maximum (FWHM) at the center of the field of view (FOV).

### *Preparation of [<sup>11</sup>C]DASB*

[<sup>11</sup>C]DASB was synthesized as previously described by Solbach et al [165]. Desmethyl-DASB is dissolved in dimethylsulfoxide and then reacted with [<sup>11</sup>C]methyl iodide. After being heated and diluted the composition is transferred to a semi-preparative HPLC system. The resulting compound is subsequently purified following the protocol of Frankle et al [166]. After reduction of contents of residual solvents the mixture is formulated with saline and phosphate buffer and filtrated under a laminar air flow hot cell. The whole preparation is set-up in a lead shielded hot cell and runs fully automated.

The preparation of 2-6 GBq [<sup>11</sup>C] DASB takes usually about 40 minutes and is directly followed by quality control. Thereby radiochemical and chemical purity, pH, isotonicity, purity and residual solvents are assessed. Later on sterility and endotoxines are controlled following the rules for radiopharmaceutical preparations described in the European Pharmacopoeia.

### *Quantification of SERT BP*

Quantitative tracer kinetic modeling was performed using reference tissue compartmental models and the kinetic modeling tools implemented in the biomedical image quantification software PMOD 2.7 (<http://www.pmod.com>) [167]. The reference tissue model features good test-retest reproducibility in [<sup>11</sup>C] DASB PET imaging [163, 168, 169]. ROI-based statistical parametric mapping (SPM) was used for computing regional quantitative values of [<sup>11</sup>C] DASB binding. As reference region I used the cerebellum [162]. In order to minimize bias for multiple testing I chose a set of 3-9 SNP-specific regions of interest based on previous findings and the current literature. In sum the following ROIs were comprised in this study: anterior cingulate cortex, orbitofrontal cortex, temporal cortex, occipital cortex, rectus, amygdala, hippocampus, thalamus, midbrain, striatum, nucleus accumbens as well as medial raphe nuclei. These regions are believed to be the most relevant in the serotonergic system.

### *Statistical Analysis*

I used the SPSS version 18.0 program for data evaluation. The two different hypotheses are tested using ANOVA, t-tests and repeated measures ANOVA respectively.

My first hypothesis on baseline BP being altered by genotype was tested with ANOVA using gender and genotype with 3 labels (AA, AB, BB alleles) as main effect. For those SNPs and subgroups that had to be merged t-tests were applied. ROIs of all baseline PET images have been used for this testing.

The main hypothesis on BP being altered by genotype during SSRI treatment over 2 month was tested with repeated measures ANOVA. Thereby genotype with two or three labels was used as between subject variables. "Time", i.e. the three PET images acquired over 3 weeks, was labeled as within subject variable. "Time" and genotype were analyzed as main effects. Furthermore, interactions between "time" & genotype were accounted for.

P values below 0.05 are considered significant, values between 0.05 and 0.09 are referred to as trend in this study. Correction of multiple testing was applied for all SNP in the main analysis and is further debated in the results and discussion part. The corrected p-value was  $p=0,00125$ .

## Results

A set of regions of interest was chosen for every SNP regarding the expected areas of high protein expression and clinical relevance (table 2).

In total 12 regions of interest (ROI) have been analyzed:

Anterior cingulate cortex (ACC), orbitofrontal cortex (OFC), rectus (REC), hippocampus (HPC), amygdala (AMY), thalamus (THL), striatum (STR), nucleus accumbens (ACCU), temporal cortex (TC), occipital cortex (OCC), midbrain (MDB) and medial raphe nuclei (MRN).

The two SNPs for the 5-HT<sub>1A</sub> receptor rs13604043 and rs878567 have been tested for the anterior cingulate cortex, rectus, orbitofrontal cortex, hippocampus, amygdala, thalamus, striatum, nucleus accumbens and midbrain.

The 5-HT<sub>1B</sub> SNP rs6296 was analyzed for orbitofrontal cortex, anterior cingulate cortex, striatum, hippocampus, thalamus and occipital cortex.

Being located on the HTR2A gene rs6311 was tested in the anterior cingulate cortex, orbitofrontal cortex, temporal cortex, thalamus, midbrain and amygdala.

The rs363399 SNP of the VMAT2 gene that is involved in 5-HT transportation has been tested for the medial raphe nuclei, striatum and thalamus. Previous studies on VMAT2 expression in the brain using specific radioligands suggested these regions among others as the main foci of VMAT2 expression [170, 171]. I excluded regions that seem to reflect the importance of VMAT2 for other neurotransmitters as the locus ceruleus for norepinephrinergic and dopaminergic regions. However, less information on the actual VMAT expression in humans can be provided since most studies investigated brains of rodents.

For rs1800532 of TPH1 6 regions were chosen to further clarify the SNP's role in the central nervous serotonergic system. I selected the anterior cingulate cortex, orbitofrontal cortex, thalamus, striatum, midbrain and accumbens.

## I. Baseline Analysis (before Treatment)

My first study hypothesis that allele expression of all SNPs investigated would significantly alter 5-HTT binding potential in the baseline PET images was tested on 29 subjects. 14 of these were treatment-naïve MDD patients. The remaining 15 subjects were healthy controls.

I performed a multivariate ANOVA testing the effects of genotype, i.e. allele expression of a single SNP, on the 5-HTT binding potential in a specific set of regions. Three of my SNPs featured too little counts of one allele form. Therefore I merged the smallest group with the group of heterozygotes. Subsequently for those SNPs (rs13604043, rs363399 and rs6311) a t-test for independent groups was performed.

Furthermore, I performed further testing on the subgroups of patients (n=14) and healthy controls (n=15) on all SNPs. Merging was necessary for these investigations as well, details can be found in the (Table1) showing allele distributions for each SNP and subgroups.

## **Allele Distribution of each SNP in Consideration of Sub Groups & Merging**

<b>rs1364043</b>	<b>GG</b>	<b>GT</b>	<b>TT</b>	<b>rs6296</b>	<b>CC</b>	<b>CG</b>	<b>GG</b>
All	17	11	1	All	6	13	10
Patients	10	3	1	Patients	2	6	6
Healthy Controls	7	8	0	Healthy Controls	4	7	4
<b>rs1800532</b>	<b>AA</b>	<b>AC</b>	<b>CC</b>	<b>rs878567</b>	<b>AA</b>	<b>AG</b>	<b>GG</b>
All	11	14	4	All	5	15	9
Patients	8	4	2	Patients	1	6	7
Healthy Controls	3	10	2	Healthy Controls	4	9	2
<b>rs363399</b>	<b>CC</b>	<b>CT</b>	<b>TT</b>	<b>rs6311</b>	<b>AA</b>	<b>AG</b>	<b>GG</b>
All	1	11	17	All	2	17	10
Patients	0	7	7	Patients	1	6	7
Healthy Controls	1	4	10	Healthy Controls	1	11	3

**Table1:** SNP allele distributions divided by subgroups. Allele groups highlighted with blue color have been merged for the baseline analysis before treatment. SNPs that required merging were analyzed via t-test. The other SNPs, highlighted with yellow color, have been tested via ANOVA.

Red numbers in patient the group indicate that merging was performed for repeated measures ANOVA analysis.



# 1. Baseline Results (before Treatment)

		HTR1A	HTR1A	HTR1B	HTR2A	VMAT	TPH1
ROI		rs1364043	rs878567	rs6296	rs6311	rs363399	rs1800532
ACC	PAT+HC	>0,05	>0,05	>0,05	>0,05		>0,05
	Control	>0,05	>0,05	>0,05	>0,05		>0,05
	Patients	0,017	>0,05	>0,05	>0,05		>0,05
OFC	PAT+HC	>0,05	>0,05	>0,05	>0,05		>0,05
	Control	>0,05	>0,05	>0,05	>0,05		>0,05
	Patients	>0,05	>0,05	>0,05	>0,05		>0,05
OCC	PAT+HC			>0,05			
	Control			>0,05			
	Patients			>0,05			
TC	PAT+HC				>0,05		
	Control				>0,05		
	Patients				>0,05		
REC	PAT+HC	>0,05	>0,05				
	Control	>0,05	>0,05				
	Patients	>0,05	>0,05				
THL	PAT+HC	0,043	>0,05	>0,05		>0,05	>0,05
	Control	>0,05	<b>0,001</b>	>0,05		>0,05	>0,05
	Patients	>0,05	>0,05	>0,05		>0,05	0,046
STR	PAT+HC			>0,05		>0,05	>0,05
	Control			>0,05		>0,05	>0,05
	Patients			>0,05		>0,05	>0,05
MDB	PAT+HC	>0,05	>0,05		>0,05		>0,05
	Control	0,028	<b>0,038</b>		>0,05		>0,05
	Patients	0,029	>0,05		>0,05		>0,05
AMY	PAT+HC	>0,05	>0,05		>0,05		
	Control	>0,05	>0,05		>0,05		
	Patients	>0,05	>0,05		>0,05		
HPC	PAT+HC	>0,05	>0,05	>0,05			
	Control	>0,05	>0,05	>0,05			
	Patients	>0,05	>0,05	>0,05			
ACCU	PAT+HC	>0,05	>0,05				>0,05
	Control	0,035	>0,05				>0,05
	Patients	>0,05	>0,05				>0,05
MRN	PAT+HC					>0,05	
	Control					>0,05	
	Patients					>0,05	

**Table 2:** Each SNP has been tested with ANOVA or t-test for a specific set of regions of interest. The observed effects of genotype on 5-HTT binding potential are shown in this table. Those results that were obtained via ANOVA are highlighted with blue color. Bold letters indicate that the findings remain significant if the corrected p-value threshold ( $p=0,00125$ ) is applied. The 2<sup>nd</sup> and 3<sup>rd</sup> lines show the results for the investigation of subgroups.

i. rs1364043

Baseline testing for the rs1364043 SNP was undergone via a t-test after the groups of GT- (n=11) and TT-carriers (n=1) had been merged. The analysis did not result in any significant findings.

ii. rs878567

The 5-HT1A SNP rs878567 did not show significant results in the baseline ANOVA analysis. The three allele groups compared consisted of 5 AA-carriers, 15 heterozygotes and 9 GG-carriers.

However, the ANOVA performed in the subgroups of controls led to a significant genotype effect in the thalamus ( $p=0,001$ ) that remained significant after Bonferroni correction.

iii. rs6296

For the baseline testing of rs6296 an ANOVA was performed. The three allele groups compared consisted of CC-carriers (n=6), heterozygotes (n=13) and GG-carriers (n=10).

No effects of genotype on baseline 5-HTT binding potential in the regions of interest could be detected.

iv. rs6311

In the baseline t-test analysis for rs6311 I did not detect any significant influence of genotype. AA-carriers (n=2) and AG-carriers (n=17) have been merged and compared to GG-carriers (n=10).

v. rs363399

The baseline testing for rs363399 was performed via a t-test comparing the merged groups of CC- (n=1) and CT-carriers (n=11) with the TT-carriers (n=17). I did not find any significant effects.

vi. rs1800532

An ANOVA analysis on genotype, consistent of AA- (n=11), AC- (n=14) and CC-carriers (n=4), was performed for the rs1800532 SNP. No significant effects could be shown.

## II. Repeated Measures ANOVA

All SNPs have been investigated in a repeated measures ANOVA analysis. PET images of 14 patients have been acquired before treatment and 2 weeks as well as 2 months afterwards.

The main effects of genotype and PET-image time point as well as their interaction have been tested for the SNP-specific set of regions of interest as described in the baseline analysis section.

Except for rs363399 all SNPs featured insufficient numbers in one of the allele groups and required merging.

Furthermore, ANOVA or t-tests have been used on all SNPs investigating the effect of genotype at the time points of the second and third PET image. These tests provide additional information in genotype and treatment effect on 5-HTT binding potential.

## 1. RMA Results

### **Repeated Measures ANOVA Results**

**Table 8:** Each SNP has been tested with repeated measures ANOVA for their specific regions of interest. The significant p-values of the main effect genotype, i.e. allele-carrier status for the SNP tested, are demonstrated for all regions in this table. Bold values indicate significant findings using the corrected threshold for the p-value ( $p < 0,00125$ ).

	HTR1A	HTR1A	HTR1B	HTR2A	VMAT	TPH1	GNB3
<b>ROI</b>	rs1364043	rs878567	rs6296	rs6311	rs363399	rs1800532	rs5433
<b>ACC</b>	<b>0,0001</b>	>0,05	0,017	>0,05		>0,05	
<b>OFC</b>	<b>0,0003</b>	>0,05	>0,05	0,045		>0,05	
<b>OCC</b>			0,029				
<b>TC</b>				>0,05			
<b>REC</b>	<b>0,0009</b>	>0,05					
<b>THL</b>	0,043	>0,05	0,025		>0,05	0,037	
<b>STR</b>	<b>0,0009</b>	>0,05	>0,05		>0,05	>0,05	
<b>MDB</b>	0,011	>0,05		>0,05		>0,05	
<b>AMY</b>	0,034	>0,05		>0,05			
<b>HPC</b>	0,011	>0,05	0,008				
<b>ACCU</b>	0,004	>0,05				0,046	
<b>MRN</b>					0,012		

## t-test Analysis of the 3 Time Points

**Table 9:** Each SNP has been t-tested for a specific set of regions of interest. In this table effects of genotype on 5-HTT binding potential in the patient group measured at three time points (drug-naïve, 2 weeks & 2 month after treatment onset) are shown. None of the findings shown below remain significant if the corrected p-value threshold ( $p < 0,00125$ ) is applied.

		HTR1A	HTR1A	HTR1B	HTR2A	VMAT	TPH1
<b>ROI</b>		rs1364043	rs878567	rs6296	rs6311	rs363399	rs1800532
<b>ACC</b>	<b>B.T.</b>	0,017	>0,05	>0,05	>0,05		>0,05
	<b>&lt; 2 weeks</b>	0,021	>0,05	>0,05	>0,05		>0,05
	<b>&lt; 2 month</b>	0,022	>0,05	>0,05	>0,05		>0,05
<b>OFC</b>	<b>B.T.</b>	>0,05	>0,05	>0,05	>0,05		>0,05
	<b>&lt; 2 weeks</b>	0,012	>0,05	>0,05	>0,05		>0,05
	<b>&lt; 2 month</b>	0,025	>0,05	>0,05	>0,05		>0,05
<b>OCC</b>	<b>B.T.</b>			>0,05			
	<b>&lt; 2 weeks</b>			>0,05			
	<b>&lt; 2 month</b>			>0,05			
<b>TC</b>	<b>B.T.</b>				>0,05		
	<b>&lt; 2 weeks</b>				>0,05		
	<b>&lt; 2 month</b>				>0,05		
<b>REC</b>	<b>B.T.</b>	>0,05	>0,05				
	<b>&lt; 2 weeks</b>	0,020	>0,05				
	<b>&lt; 2 month</b>	0,008	>0,05				
<b>THL</b>	<b>B.T.</b>	>0,05	>0,05	>0,05		>0,05	0,046
	<b>&lt; 2 weeks</b>	>0,05	>0,05	>0,05		>0,05	>0,05
	<b>&lt; 2 month</b>	>0,05	>0,05	>0,05		>0,05	>0,05
<b>STR</b>	<b>B.T.</b>	>0,05		>0,05		>0,05	>0,05
	<b>&lt; 2 weeks</b>	0,008		>0,05		>0,05	>0,05
	<b>&lt; 2 month</b>	0,044		>0,05		>0,05	>0,05
<b>MDB</b>	<b>B.T.</b>	0,029	>0,05		>0,05		>0,05
	<b>&lt; 2 weeks</b>	0,05	>0,05		>0,05		>0,05
	<b>&lt; 2 month</b>	>0,05	>0,05		>0,05		>0,05
<b>AMY</b>	<b>B.T.</b>	>0,05	>0,05		>0,05		
	<b>&lt; 2 weeks</b>	>0,05	>0,05		>0,05		
	<b>&lt; 2 month</b>	>0,05	>0,05		>0,05		
<b>HPC</b>	<b>B.T.</b>	>0,05	>0,05	>0,05			
	<b>&lt; 2 weeks</b>	0,019	>0,05	>0,05			
	<b>&lt; 2 month</b>	>0,05	>0,05	>0,05			
<b>ACCU</b>	<b>B.T.</b>	>0,05	>0,05				>0,05
	<b>&lt; 2 weeks</b>	>0,05	>0,05				>0,05
	<b>&lt; 2 month</b>	>0,05	>0,05				>0,05
<b>MRN</b>	<b>B.T.</b>					>0,05	
	<b>&lt; 2 weeks</b>					0,035	
	<b>&lt; 2 month</b>					>0,05	

i. rs1364043 (HTR1A)

Repeated measures ANOVA was performed on rs1364043 after merging of the groups as described in the baseline section.

Main effects of genotype could be detected in all analyzed regions, e.s. the anterior cingulate cortex ( $p=0.0001$ ), rectus ( $p=0.0009$ ), orbitofrontal cortex ( $p=0.0003$ ), striatum ( $p=0.0009$ ), midbrain ( $p=0.011$ ), accumbens ( $p=0.004$ ), amygdala ( $p=0.034$ ), hippocampus ( $p=0.011$ ) and thalamus ( $p=0.041$ ).

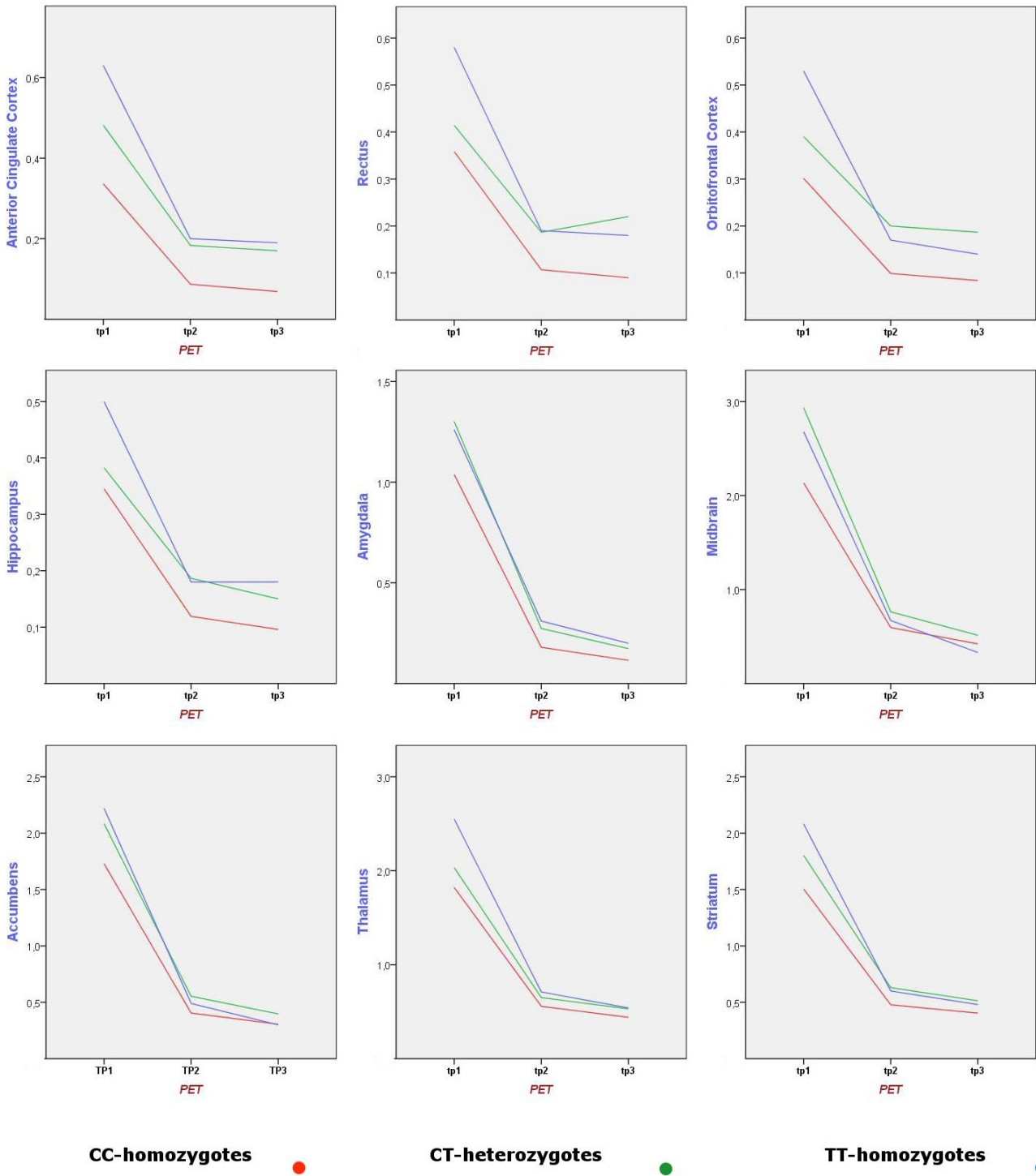
The genotype effects observed in anterior cingulate cortex, orbitofrontal cortex, rectus and striatum remained highly significant after correction for multiple testing.

Further analysis of the 2<sup>nd</sup> time point showed stronger genotype effect than the baseline analysis. Significant results were found in anterior cingulate cortex ( $p=0,021$ ), insula ( $p=0.032$ ), orbitofrontal cortex ( $p=0.012$ ), caudate ( $p=0.004$ ), putamen ( $p=0.009$ ), midbrain ( $p=0.050$ ) and accumbens ( $p=0.007$ ).

Finally the 3<sup>rd</sup> time point images were investigated. Significant effects of genotype were detected in the anterior cingulate cortex ( $p=0.022$ ) and orbitofrontal cortex ( $p=0.025$ ).

Nevertheless none of these effects remain if corrected for multiple testing.

## Line Chart rs1364043



**Figure 13:** Line chart diagram showing the mean 5-HTT binding potential changes over time in every ROI by genotype of rs1364043. For this diagram no merging was undergone. Significant effects of merged genotype were detected for all ROIs. The TT-homozygote scored overall higher in 5-HTT binding potential and declined the most after SSRI treatment was conducted. This treatment effect could not be detected in the repeated measures ANOVA analysis, most likely due to the fact that only one TT-carrier was comprised in the analysis and therefore had to be merged with heterozygotes.



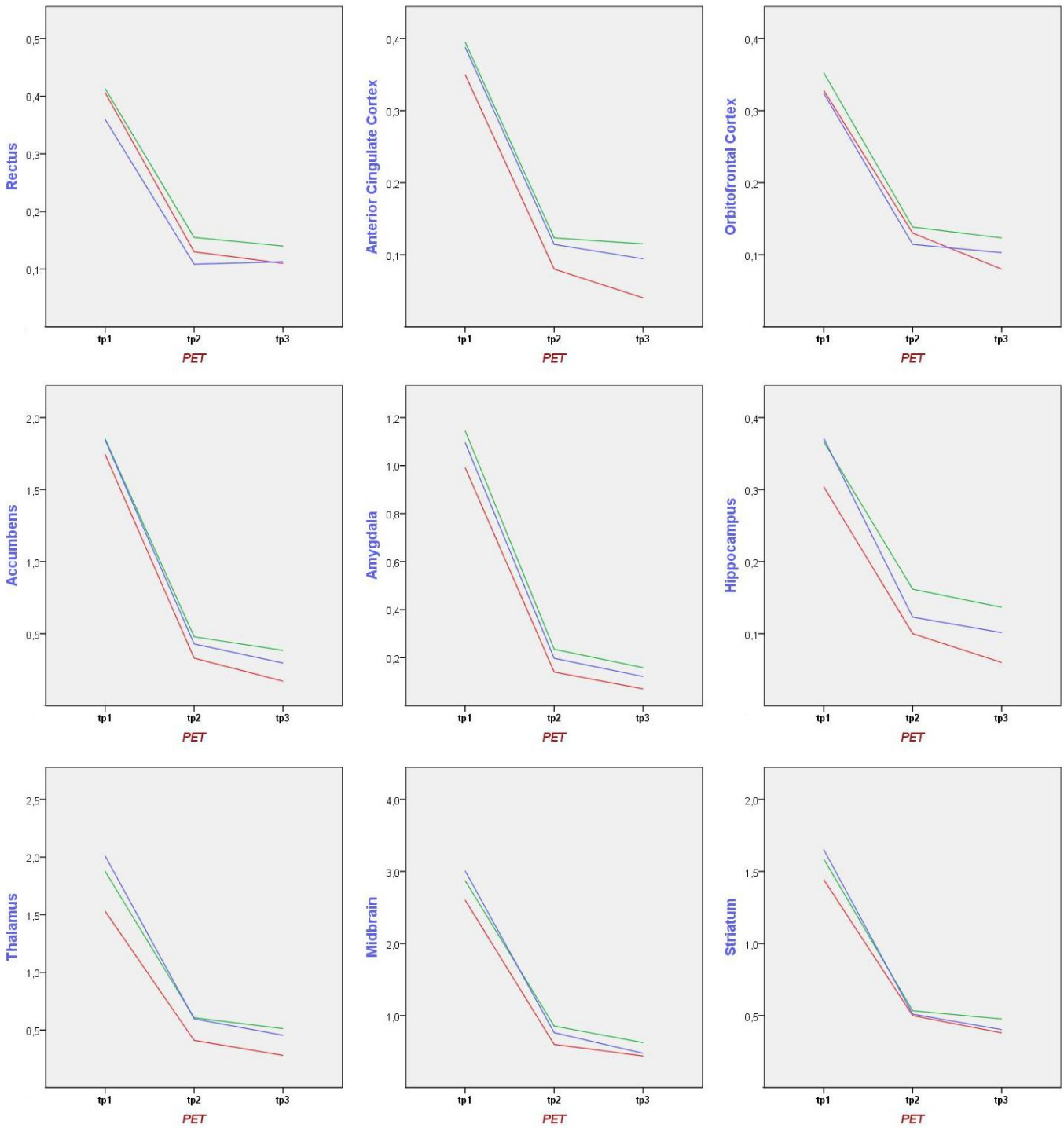
ii. rs878567 (HTR1A)

Repeated measures ANOVA was performed on rs878567 after merging of the groups was conducted as described in the baseline section.

The main effect of PET image time point was highly significant; however, no main effect of genotype could be detected. Nor did I find any interactions of genotype and time point.

No significant effects could be described in the t-tests performed on the data collected in the 2<sup>nd</sup> and 3<sup>rd</sup> PET images.

## Line Chart rs878567



**CC-homzygotes** ●

**CT-heterozygotes** ●

**TT-homozygotes** ●

**Figure 14:** Line chart diagram for rs878567 showing baseline 5-HTT binding potential and its alteration along time. All ROI analyzed in the repeated measures ANOVA are depicted for all three allele-carrier groups. TT-carriers show the least binding potential at all time-points without exhibiting significantly different characteristics in the statistical analysis.

iii. rs6296 (HTR1B)

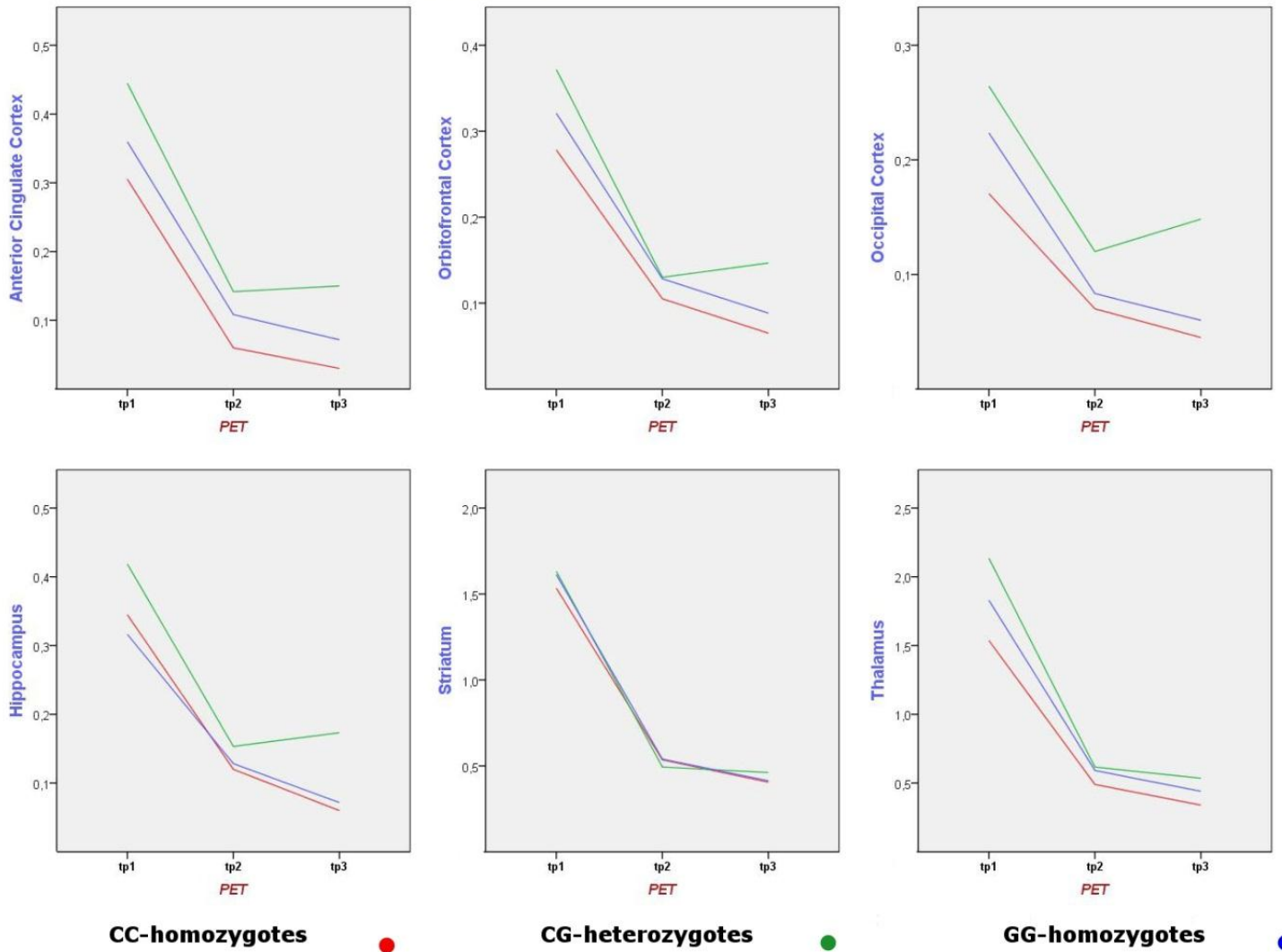
Repeated measures ANOVA was performed on rs6296 after merging of the groups as described in the baseline section.

Only one genotype effect in the hippocampus ( $p=0.048$ ) was demonstrated. Correction for multiple testing nullified this finding.

Subsequently I performed a t-test on genotype effects for the other time points. No significant influence of allele-carrier status could be shown.

However, as seen in figure 15 the group of heterozygotes actually shows much higher binding potential than the homozygote groups. Even though this is an unusual finding I decided to perform another set of statistical tests. After having merged the homozygote groups I performed another repeated measures ANOVA that showed genotype effects in hippocampus ( $p=0.008$ ), thalamus ( $p=0.025$ ) and anterior cingulate cortex (0.017). However, none of these values are below the threshold for correction for multiple testing.

## Line Chart rs6296



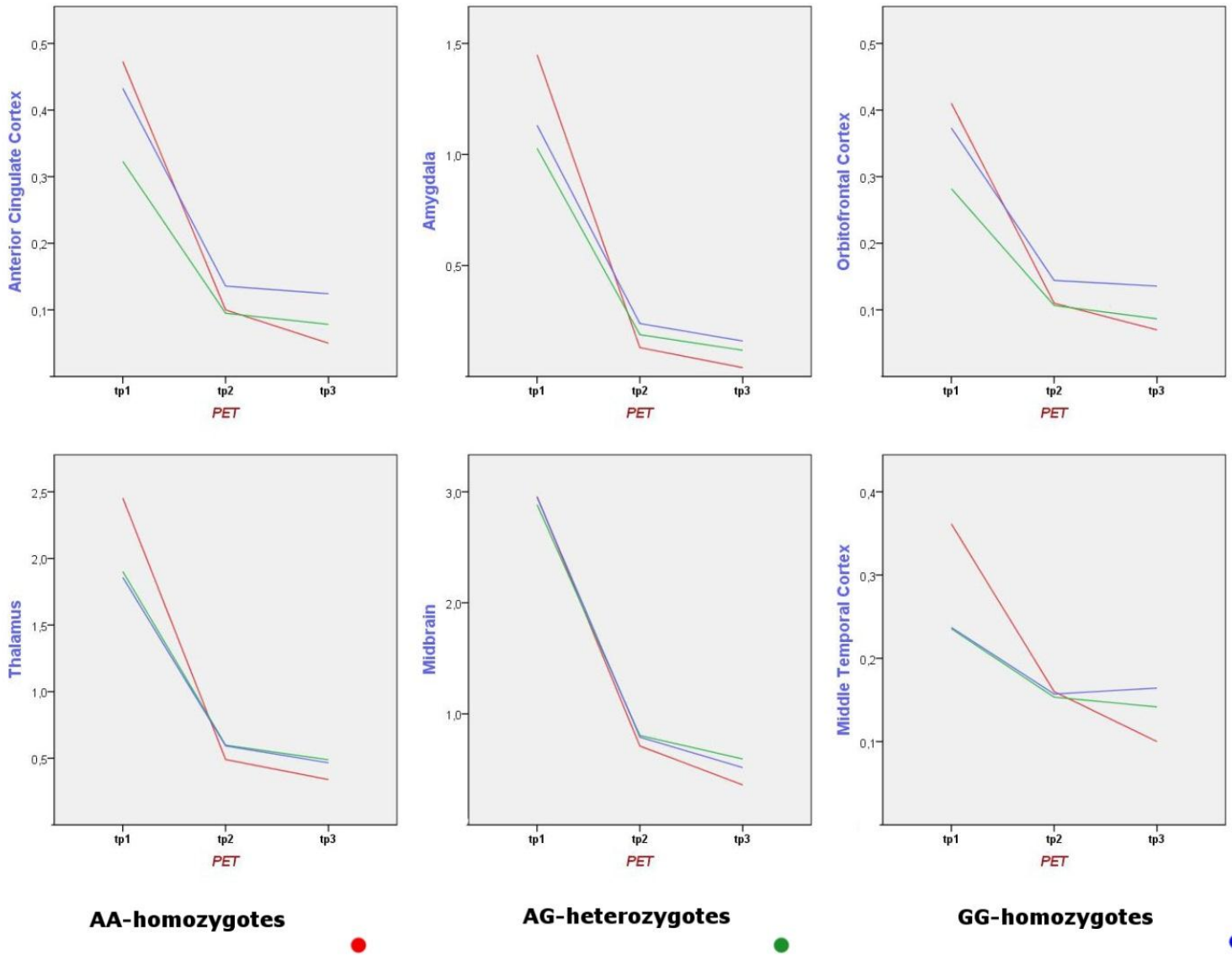
**Figure 15:** Line chart diagram for rs6296 showing the alteration of 5-HTT binding potential over time in all ROI. CC-carriers mostly show least 5-HTT binding potential at all times. GG-carriers manifest slightly higher overall values but show similar decline after SSRI treatment as CC-homozygote. The heterozygote however exhibit highest 5-HTT binding potential in all regions except striatum. Furthermore, the show inclining values after the first 2 weeks of treatment which implies accelerated treatment resistance in these individuals. However, no statistical significant genotype effects could be detected in the corrected results of the repeated measures ANOVA.

iv. rs6311 (HTR2A)

Repeated measures ANOVA was performed on rs6311 after merging of the groups was undergone as described in the baseline section.

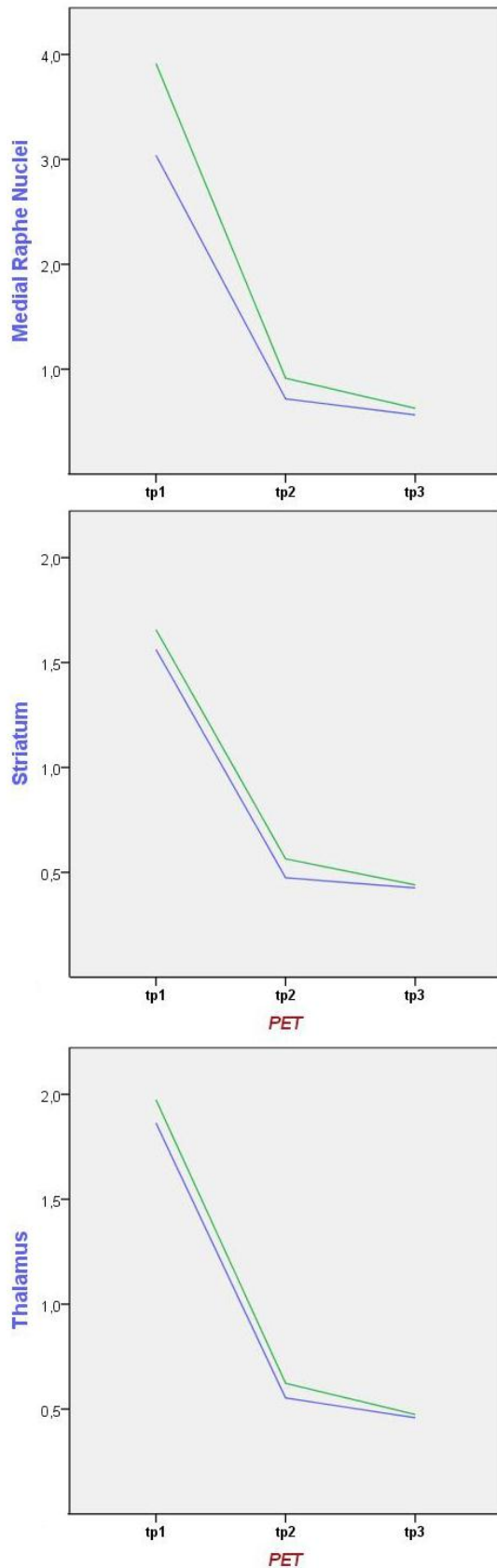
An effect of genotype was detected in the orbitofrontal cortex ( $p=0.045$ ), however, it does not remain after correction for multiple testing requiring a  $p$ -value below 0.00125.

# Line Chart rs6311



**Figure 16:** Line chart diagram showing the mean 5-HTT binding potential changes over time in every ROI by genotype of rs6311. No merging has been performed for this diagram. The AA-carrier showed overall higher 5-HTT binding potential when drug-naïve, however, tended to show the least binding potential after medication. Nevertheless repeated measures ANOVA did not detect any significant genotype effects.

## Line Chart rs363399



CT-heterozygotes



TT-homozygotes



v. rs363399 (VMAT2)

No merging was necessary for the repeated measures ANOVA analysis of the rs3623399 SNP since I unfortunately lacked any AA-carriers in my investigation.

The RMA analysis led to an effect in the medial raphe nuclei ( $p=0,012$ ) that does not remain after correction for multiple testings.

Studying the data from the 2<sup>nd</sup> pet images led to a similar result in the medial raphe nuclei ( $p=0,035$ ). However, the analysis of the 3<sup>rd</sup> time point did not repeat these findings.

**Figure 17:** Line chart diagram showing the mean 5-HTT binding potential changes over time in every ROI by genotype of rs363399. Effects can be seen in the medial raphe nuclei where heterozygotes showed higher 5-HTT binding potential at all time-points. However, repeated measures ANOVA does only show significant effects without correction for multiple testing.

vi. rs1800532 (TPH1)

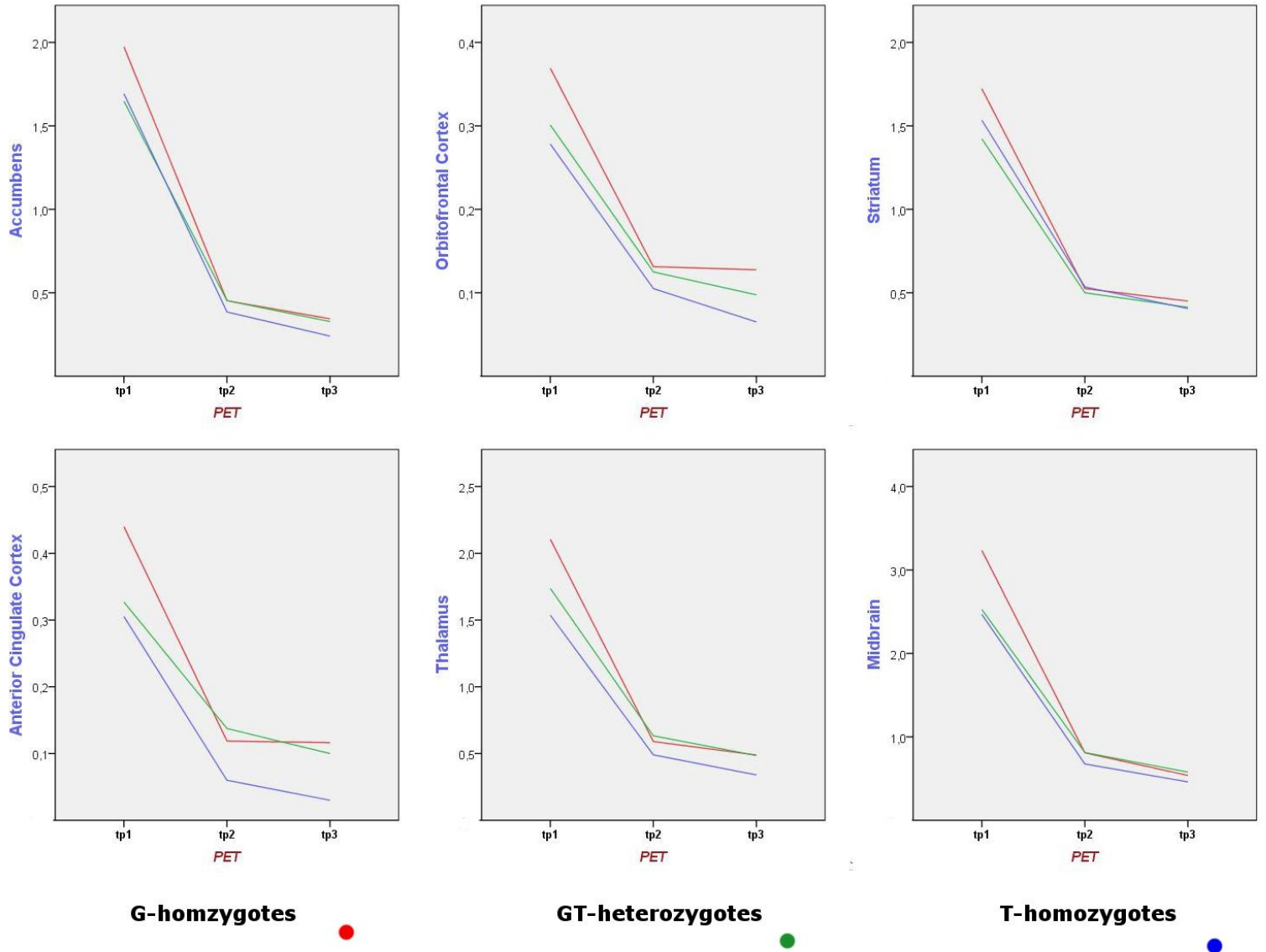
Repeated measures ANOVA was performed on rs1800532 after merging of the groups was conducted as described in the baseline section.

I could demonstrate uncorrected genotype effects in the accumbens ( $p=0.046$ ), thalamus ( $p=0.037$ ) and caudate ( $p=0.031$ ). No effects remained after correction for multiple testings.

The analysis of the images acquired at the 2<sup>nd</sup> and 3<sup>rd</sup> time point did not show any genotype effects.



## Line Chart for rs1800532



**Figure 18:** Line chart diagram showing the mean 5-HTT binding potential changes over time in every ROI by genotype of rs1800532. G-homozygotes showed overall the highest 5-HTT binding potential and sometimes less decrease of 5-HTT binding potential after medication. However, in the repeated measures ANOVA significant effects did not remain after correction for multiple testing.

## *Discussion*

In this study the effect of allele carrier status in 6 SNPs on 5-HTT binding potential measured via [<sup>11</sup>C]DASB PET neuroimaging has been analyzed.

On one hand I investigated the influence of SNP allele-carrier status on 5-HTT binding potential in mixed group of drug naïve MDD patients and healthy controls with a total count of 29. For each SNP an ANOVA was performed with genotype as main effect. Those SNPs that did not feature all allele carrier groups or showed too little counts in one group were investigated via t-test after merging of the heterozygote group with the insufficient homozygote group.

On the other hand as main focus of this study I searched for genotype and genotype-treatment effects in the patients group consisting of 14 people analyzing the effect of each SNP on 5-HTT binding potential over time. Therefore 3 PET images were acquired, before treatment onset, within 2 weeks after the first SSRI dose and 2 month afterwards. A repeated measures ANOVA analysis was performed for each SNP with genotype as main effect and consideration of interactions between genotype and timepoint.

The results of each SNP investigated are discussed below:

i. rs1364043 (HTR1A)

### **Baseline**

The baseline analysis showed significant results only in the two subgroups. Since the group of controls did not feature any T-homozygotes I expected weaker genotype effects than in the patient group. Heterozygotes showed higher BP in the thalamus and midbrain.

I detected significant effects in the anterior cingulate cortex, striatum, midbrain and accumbens in the patient group.

Even though none of these findings remained significant after Bonferroni correction I believe that my findings support previous studies proposing a relevant gene-dose effect of rs1364043 on the serotonergic system, even more so when considering the results of my main investigation for this SNP:

### **Repeated Measures ANOVA**

The main focus of my analysis of rs1364043 was treatment interaction with genotype. Performing the repeated measures ANOVA I could not find any significant interactions of time point and genotype while the main effect “genotype” was significant in all areas analyzed and remained significant in several ROI after Bonferroni correction.

T-allele carrier status led to significant increase in 5-HTT binding potential in my study. Due to the fact that only one TT-carrier was comprised in my analyses I had to compare T-allele carriers with CC-carriers and still found relevant effects.

Even though I did not obtain statistical relevant treatment-genotype interactions, I still suggest an influence of the rs1364043 SNP on treatment effect and remission.

In my study I have shown a highly significant influence of HTR1A rs1364043 allele carrier status on 5-HTT binding potential in the ACC, OFC, REC and STR. Hence my study is in accordance with previous positive findings regarding rs1364043. However, being the first PET neuroimaging study on this SNP it provides informative new data.

Furthermore, studies by Kato et al and Villafuerte et al indicate that rs1364043 allele carrier status affects treatment response to SSRI [108][109].

Regarding these previous findings in combination with the highly significant rs1364043 genotype effects on 5-HTT binding potential demonstrated in my study I believe that also genotype-time point interactions could be significant if a greater collective with more T-homozygotes was analyzed. Thus my study strongly recommends further investigations of rs1364043 using a study design with greater power.

ii. rs878567 (HTR1A)

### **Baseline**

According to the results of my analysis I cannot confirm the previous findings implicating a noteworthy relevance of the rs878567 polymorphism on the serotonergic system.

The baseline analysis had sufficient counts in all three carrier groups. I did not find any adverse effects in CC-carriers as I would have expected following the data mentioned above. Neither did my results confirm the observations of Mekli et al since TT-carriers did not differ in 5-HTT binding potential binding either [106].

In contrast to these results I could demonstrate an effect in CC-carriers in the subset of healthy controls. The four CC-carriers comprised in this analysis showed significantly lower 5-HTT binding potential in the thalamus region. However, this effect cannot be interpreted as cogent since it only appeared in the subgroup of healthy controls.

### **Repeated Measures ANOVA**

The repeated measures ANOVA analysis did not show any effects of genotype or interactions of allele-carrier status and treatment effect.

Nonetheless my results have to be interpreted with some caution since I could not arrange a group CC-carriers for the patient group and subsequently the repeated measures ANOVA. Thus a genotype effect of treatment cannot be ruled out by this study. However, regarding the fact that the one CC-carrier did not differ much from the mean 5-HTT binding potential it seems unlikely. Furthermore, to my knowledge there is no study indicating treatment effect dependency on rs878567 genotype.

Hence the results of my study support the work by Serretti et al who failed in finding significant influence of rs878567 on MDD [107]. However, even though my results picture strong effects unlikely additional studies on the rs878567 SNP will be necessary since I lacked sufficient CC-carriers in my main analysis and found a strong effect in one subgroup that is impossible to interpret in the setting of this study.

### iii. rs6296 (HTR1B)

## **Baseline**

The rs6296 SNP has been linked to MDD in several studies suggesting a higher risk for MDD in CC-Carriers. I therefore assumed that 5-HTT binding potential would be significantly altered in this group. The baseline analysis using ANOVA did not lead to any effects of genotype.

## **Repeated Measures ANOVA**

However, some significant results were found in the main analysis using repeated measures ANOVA. Merging of the groups was necessary due to very few CC-carriers. Eventually I decided to merge the homozygote groups and compare them to the heterozygotes.

This decision was based on the diagrams derived from the unmerged groups that show akin 5-HTT binding potential curves in CC- and GG-carriers but intriguingly a deviation in the heterozygote group.

## **Molecular Heterosis**

There has been evidence of strongest effects in the heterozygote groups in many studies on genetic influence in general and psychiatric diseases specifically [172]. The phenomenon is known as molecular heterosis and described best by Comings et al in their review from 2000.

While the assumption that homozygosis of one allele shows the strongest, heterozygosis an intermediate and homozygosis of the other allele the least effect is obvious it has in fact been suggested that the biggest effect amplitude is often manifested in heterozygotes. Such observations have been made for a wide range of genes including HTR2A, TPH and SLC6A of

the serotonergic system. In fact molecular heterosis can be found in up to 50% of association studies according to Comings et al.

Several explanations have been presented. On one hand heterozygotes show a broader spectrum of genetic information. On the other hand an U-shaped effect curve has been suggested with homozygotes showing the optimal expression level for an effect. Finally a hidden additional factor associated with one or more allele-groups could lead to molecular heterosis.

## **Conclusion**

My results point toward molecular heterosis in the rs6296 SNP. Even though no effects could be observed if corrected for multiple testing I recommend further analysis if the rs6296 SNP of HTR1B. As shown in the repeated measures ANOVA CG-carriers differed significantly in 5-HTT binding potential in the anterior cingulate cortex ( $p=0.017$ ), hippocampus ( $p=0.008$ ), thalamus ( $p=0.025$ ) and insula ( $p=0.035$ ). The heterozygotes always showed higher drug-naïve 5-HTT binding potential. Furthermore, hardly any 5-HTT binding potential decrease can be seen after the first 2 weeks of treatment. Actually 5-HTT binding potential started to incline between the 2<sup>nd</sup> and 3<sup>rd</sup> pet images for most regions analyzed. On the other hand no interaction of time point and genotype could be detected and none of the described effects remained if corrected for multiple testing. Therefore I concluded that the effect indicated in the diagrams is too weak to be detected by my study due to limited power.

### iv. rs6311 (HTR2A)

## ***Baseline***

Based on the results of the previous studies on rs6311 I expected altered 5-HTT binding potential in AA-carriers. Due to the fact that I did recruit only two AA-carriers I could only compare AA- and AG-carriers to GG-carriers.

No effects of genotype could be demonstrated in the baseline analysis. Accordingly my study does confirm the negative findings of Sato rather than the affirmative work [133].

## ***Repeated Measures ANOVA***

Following the studies previously conducted I primarily expected a main effect of genotype on treatment effect. However, significant results could only be obtained in the orbitofrontal cortex and did not survive Bonferroni correction. Furthermore, no interaction between genotype and time point could be detected. These findings lead to the conclusion that rs6311 allele-carrier status does not affect response to SSRI as described by Peters, Choi and others [130, 131].

However, as with SNPs discussed above, the group of the most promising allele-carriers was too small and had to be merged. Hence I cannot rule out an effect in AA-carriers.

## **Conclusion**

Intriguingly the diagrams that are based on the original groups (without merging) show that AA-carriers always have the highest 5-HTT binding potential when drug naïve. Two month after treatment they show the lowest binding rates in all investigated regions.

Even though these characteristics did not lead to significant results in the statistical tests I performed, they suggest that there might be a genotype effect on treatment that cannot be detected with the limited patient count of this study. I therefore recommend further studies with greater power on the HTR2A rs6311 SNP which is still a promising target for research.

### v. rs363399 (VMAT2)

## **Baseline**

Since information on the rs363399 SNP is scarce I expected binding potential to be altered by genotype without allele-specific predictions.

The baseline 5-HTT binding potential was only significantly affected by genotype in the subgroup of patients. I report higher 5-HTT binding potential in the medial raphe nuclei in heterozygotes compared to T-homozygotes. Unfortunately I could only comprise one CC-

carrier who is part of the healthy control subgroup. In future studies stronger effects would most likely be obtained in comparison of the homozygote groups.

## **Repeated Measures ANOVA**

I detected a genotype effect in the medial raphe nuclei in the repeated measures ANOVA that did not remain after Bonferroni correction.

## **Conclusion**

Heterozygotes scored higher in 5-HTT binding potential than TT-carriers, however, no genotype interaction with time point was found. This leads to the conclusion that the VMAT2 SNP rs363399 could exert influence on serotonin activity in the brain predominantly in the raphe nuclei. However, these possible effects could only be detected by a study with higher power.

vi. rs1800532 (TPH1)

## **Baseline**

The tryptophan-hydroxylase SNP rs1800532 has been featured in many studies over the past decade and results have been hard to interpret or sometimes even contradictory. Based on the majority of previous findings I expected AA-carriers to show altered 5-HTT binding potential in the baseline analysis.

No effects were observed in the general analysis, however, in the subgroup of patients AA-carriers showed significantly higher 5-HTT binding potential in thalamus and tended to higher levels in caudate and midbrain. All of these findings did not overcome correction for multiple testing.

## **Repeated Measures ANOVA**

Several studies suggested an effect of rs1800532 allele-carrier status on treatment effect with SSRIs. Especially the CC-carriers have shown higher remission rates in previous investigations which is why I expected CC-carriers to have altered 5-HTT binding potential over treatment time.



I could not demonstrate any treatment effect since no interactions between genotype and time point were found. As in the baseline analysis AA-carriers showed higher 5-HTT binding potential. Especially the thalamus and accumbens showed higher binding potential in A-homozygotes. None of these findings remained significant after Bonferroni correction.

## **Conclusion**

Therefore my study cannot support the idea of a genotype effect of the rs1800532 SNP as described by Ham, Du, Serretti et al [148-151].

However, I detected genotype effects in the uncorrected analysis that further indicate the relevance of TPH1 in the central nervous system and imply that more research on rs1800532 could be useful.

## *Conclusion*

The baseline approach on drug naïve patients and controls did not result in significant genotype effects. While some effects could be detected in the uncorrected sample, none of those remained significant after Bonferroni correction.

The main focus of this study, however, is the repeated measures ANOVA performed on the patient group and taking into account data acquired from 3 PET measurements over 2 month of SSRI therapy. 5-HTT binding was significantly altered by allele carrier status of one of the polymorphisms analyzed, the rs1364043 SNP of HTR1A. In four ROI these effects feature a p-value below 0.00125 and therefore remain significant after Bonferroni correction.

Due to the fact that this study was hindered by limited patient counts that required merging of allele-carrier groups and resulted in little power the negative findings concerning the 5 other SNPs do not rule out a genotype effect. On the contrary some of these findings encourage performing further studies with higher power.

Most importantly the positive findings endorse theories proposing relevant influence of the HTR1A SNP rs1364043 on the serotonergic system.

Having performed the first neuroimaging study on rs1364043 I believe that the described findings will help to emphasize the important role of this SNP for the serotonergic system.

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