Assessment of Serotonergic Function by Radioligands and Microdialysis

Visser, Anniek

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Focus on stress-related behaviour and antidepressant efficacy

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Contents

CHAPTER 1

General Introduction

Anniek K.D. Visser, Aren van Waarde, Fokko J. Bosker, Johan A. den Boer, Rudi A.J.O. Dierckx

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The serotonergic system as a target for positron emission tomography ligands, applications in affective disorders; Bruno P Guiard, Eliyahu Dremencov, Neurobiology of Mood disorders (Bentham eBooks), 2014

The serotonergic system

Serotonergic function in affective disorders

There have been many theories about the neurobiological underpinnings of major depression. A depressive state is considered to result from a combination of genetic, neuronal and environmental determinants or a reaction to an environmental event and a failure to adapt [1]. However, the many neurobiological theories have not yet led to a comprehensive explanatory framework for the different categories of affective disorders proposed by classification systems such as the DSM-IV. Depression consists of multiple symptoms involving cognitive, affective and somatic symptoms and there appears to be a considerable heterogeneity among patients.

This heterogeneity is expressed in the wide range of symptoms like depressed mood and anhedonia, insomnia or hypersomnia, loss of appetite, decreased libido, concentration problems and activity disturbances. Expression of symptoms can be triggered by genotype or different environmental circumstances like upbringing, while eventually being caused by disruptions of molecular signalling pathways and neurotransmission. The relationship between genetic aspects of susceptibility to mood disorders and possible defects at a neuronal level remains a topic of current research. 5-HT is the neurotransmitter most extensively associated with mood disorders such as depression.

Early studies revealed deficiencies in 5-HT concentrations and 5-HT turnover in depressive patients and relapse could partially be prevented by the administration of 5-HTP [2, 3]. Together with a large body of other studies, these data support the monoamine hypothesis of depression. In addition, several 5-HT receptor subtypes are related to the pathology of affective disorders and several of these receptors are involved in the actions of antidepressants and antipsychotics. Of most interest are the 5-HT_{1A} receptor, as it was hypothesized to be essential for antidepressant efficacy by influencing the firing rate of serotonergic neurons through a negative feedback loop [4], and the 5-HT_{2A} receptor, which is a target of most antipsychotics. More recent studies suggest involvement of the 5-HT_{2C} receptor, as 5-HT_{2C} antagonists appear capable of augmenting the effects of

antidepressants [5, 6]. These effects do probably not only involve the serotonergic system, but relate more to the interaction between 5-HT and, for example, the dopamine system. This implies that the modulatory effects of 5-HT may play an important role in the efficacy of antidepressants.

Serotonin synthesis

The serotonergic system is a neuromodulatory system, influencing many other neurotransmitter systems through neuronal cells originating in the dorsal (DRN) and median raphe (MRN) nuclei, projecting to almost every area of the brain. Synthesis of serotonin (5-HT) takes place within neurons and especially in serotonergic terminals, and this process includes two enzymatic steps (see Fig.1). The first step is the conversion of the precursor molecule, the amino acid tryptophan (Trp), to 5-hyroxytryptophan (5-HTP) by tryptophan hydroxylase (TPH) 1 or 2. The second step in the production of 5-HT involves the enzymatic action of aromatic amino acid decarboxylase (AADC) that has L-DOPA and 5-HTP as substrates. 5-HT is eventually degraded to 5-hydroxyindole acetic acid (5-HIAA) by monoamine oxidase (MAO).

Synthesized 5-HT is transported by the vesicular monoamine transporter (VMAT) and stored in vesicles at the neuronal presynaptic endings. When neurons fire, these vesicles fuse with the synaptic membrane and release 5-HT into the synaptic cleft. Released 5-HT can bind to many different receptors, located both postsynaptically and presynaptically, or be taken up by the serotonin reuptake transporter (SERT). There are at least fifteen different 5-HT receptors which are divided in seven distinct families ($5\text{-HT}_{1\text{-}7}$) [7]. An important role of 5-HT is the regulation of mood, and several 5-HT receptor subtypes are involved in the actions of antidepressants and antipsychotics. Serotonin synthesis may be of special interest because this process is controlled by 5-HT_{1A} receptors which are implied in the therapeutic efficacy of antidepressants [4].
5-HT influences many other neurotransmitter systems in an excitatory or inhibitory manner. One important key aspect that regulates serotonergic neurotransmission is the availability of the 5-HT precursor: the amino acid Trp.
In addition to conversion to serotonin, Trp is metabolized in the kynurenine-pathway and is used for protein synthesis.

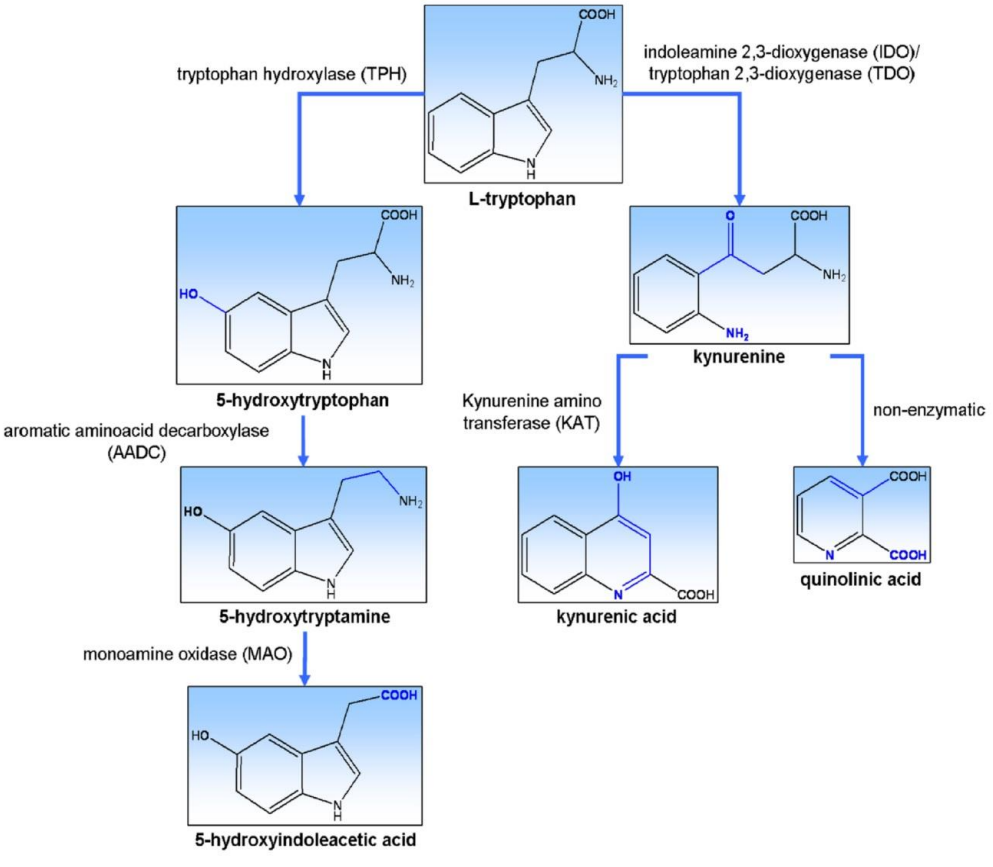

Fig. 1 Tryptophan metabolism

Tryptophan can either be broken down in two different pathways or incorporated in proteins The synthesis of 5-HT proceeds in two enzymatic steps: i) Conversion of Trp to 5-HTP by TPH ii) Catabolism of 5-HTP to 5-HT by AADC. Finally 5-HT is degraded to 5-HIAA by MAO. The amount of Trp entering the kynurenine pathway is increased under inflammatory conditions. Kynurenine is formed by the enzymes IDO and TDO and is eventually degraded to quinolinic acid.

The rate-limiting step in the kynurenine-pathway is the activity of indoleamine 2,3-dioxygenase (IDO) in the CNS and tryptophan 2,3-dioxygenase in peripheral organs. Both enzymes convert Trp to kynurenine. Activation of IDO within the central nervous system takes place under the influence of proinflammatory cytokines, mainly within microglial cells. Increased cytokines and IDO activity have been linked to major depression in depressed subjects and in patients with inflammatory somatic disorders [8]. Increased IDO activity under inflammatory

conditions may increase the amount of Trp used in the kynurenine pathway and consequently reduce the availability of Trp for 5-HT synthesis (see Fig. 1).

Serotonin-2 receptors

Postsynaptic receptor binding can be either inhibitory or excitatory, depending on which subtype is stimulated. The presynaptic receptors (5-HT_{1A}, located on the somatodendrites and 5-HT_{1B}, located on axon terminals) are autoreceptors that inhibit serotonergic neurotransmission, while all other 5-HT receptors are heteroreceptors and influence the release of neurotransmitters other than 5-HT [9]. Almost all 5-HT receptors are G-protein coupled (metabotropic), with exception of the 5-HT_3 subtype which is a ligand-gated ion channel [7]. Different subtypes of the 5-HT receptor are diversely expressed across different brain regions and mediate distinct physiological and behavioural functions. The 5-HT_2 receptors have been implicated in mood disorders and stress physiology.

Stimulation of 5-HT_2 receptors has a large variety of effects, e.g. on conditioned responses, which correlates to their location in the forebrain. All 5-HT_2 receptor subtypes have low affinity for 5-HT. In contrast to 5-HT_1 receptors, upon their activation they increase the accumulation of Ca^{+2} and reduce potassium conductance, leading to neuronal excitation. The 5-HT_{2A} receptor is very well characterized and is present throughout the brain, especially in forebrain regions. This subtype is most abundant in cortical areas, caudate nucleus, nucleus accumbens, olfactory tubercle, amygdala and hippocampus [10]. The 5-HT_{2A} receptor is of special interest because of its putative role in regulating gene transcription of brain-derived neurotrophic factor, which is involved in antidepressant action [11]. Depressed patients have an increased expression of 5-HT_{2A} receptors in post-mortem tissue of the pre-frontal cortex, while antidepressants can block 5-HT_{2A} binding [12]. Additionally, 5-HT_{2A} receptors are of interest because receptor stimulation can be hallucinogenic and therefore these receptors may be involved in regulating antipsychotic drug action [13-15].

Far less abundant is the 5-HT_{2B} receptor, which is particularly expressed in the dorsal hypothalamus, cerebellum, lateral septum and medial amygdala. There is evidence indicating a role for 5-HT_{2B} receptors in the regulation of anxiety, which corresponds to the location of this subtype in the medial amygdala [16].

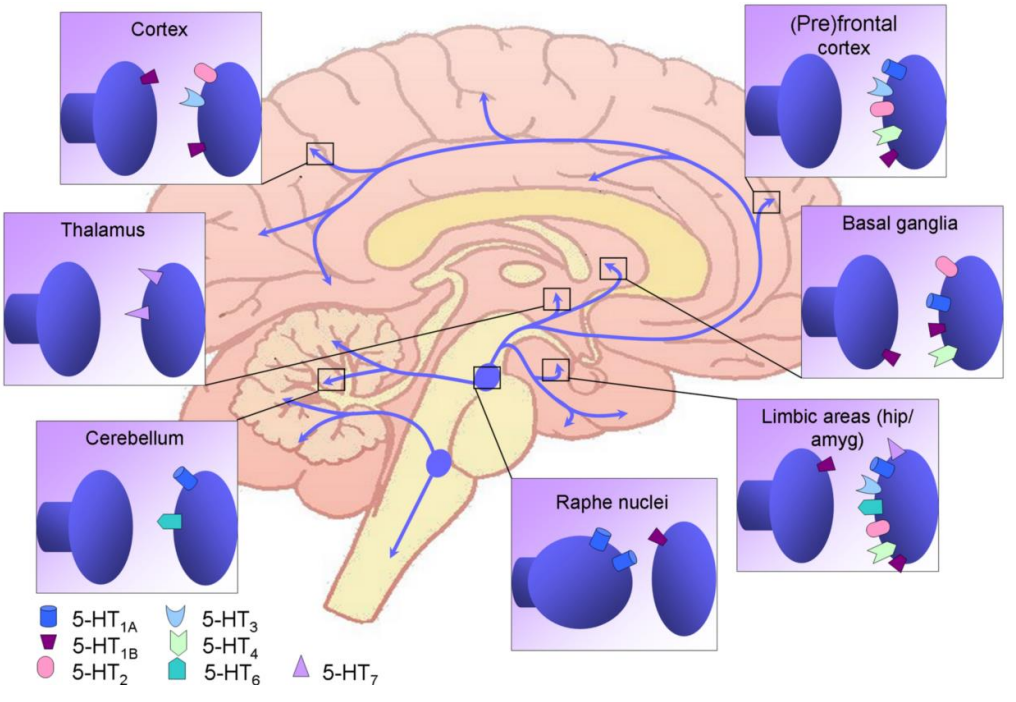

Fig. 2 The serotonergic system

The cell bodies of serotonergic neurons lay in the brainstem raphe nuclei. These neurons project to many brain areas like cortex, basal ganglia, cerebellum, thalamus, limbic areas like hippocampus and amygdala, and spinal cord. Different 5-HT receptor subtypes have a specific distribution in the brain. Autoreceptors in the raphe nuclei are depicted on neuronal cell bodies (5-HT_{1A}) or in presynaptic terminal areas (5-HT_{1B}). Other 5-HT receptor subtypes in terminal areas can either represent heteroreceptors or postsynaptic receptors on 5-HT neurons [18].

The more widely distributed 5-HT_{2C} receptor is highly expressed in the choroid plexus and additionally present in cortex, limbic areas and basal ganglia [7]. Corresponding with its widespread distribution, this subtype is involved in the regulation of many aspects of behaviour like anxiety, food intake and sleep. Interestingly, besides a function in neuronal excitation 5-HT_{2C} receptors exert a tonic inhibitory effect on mesocorticolimbic dopamine and noradrenalin neurons,

probably through stimulating GABA release, resulting in increased release of dopamine and noradrenaline after administration of a 5-HT_{2C} antagonist. Combined therapy of a 5-HT_{2C} antagonist and an SSRI could result in augmentation of antidepressant efficacy [17].
The distribution of the different 5-HT receptors and the projection areas of the 5-HT system are shown in fig. 2.

The modulatory function of the serotonergic system and SSRI augmentation

Through serotonergic receptor stimulation several other neurotransmitter systems are influenced. In this way, 5-HT_2 receptors can influence dopaminergic (DA) neurotransmission. The mesolimbic DA system consists of neurons projecting from the ventral tegmental area (VTA) to the nucleus accumbens (NAcc). It was demonstrated that dopaminergic neurons in the VTA are under tonic inhibition by 5-HT through stimulation of 5-HT2C receptors (5-HT_{2C}R) positioned on GABA-ergic or dopaminergic neurons [19-21]. The reduction of DA neuronal activity is probably related to the interaction between 5-HT and DA in the mesolimbic system, through 5-HT_{2C}R [22]. This is especially interesting, considering that the increase in 5-HT after the application of an SSRI might reduce DA neuronal activity [23-25]. As DA is involved in motivation (which depressive patients often lack), one may assume that this reduction in DA activity will influence the efficacy of antidepressants. Indeed, a few studies are indicating that this 5-HT/ DA interaction is crucial for a rapid response to SSRI's [26, 27]. So ideally an antidepressant should increase both extracellular 5-HT and DA without inducing major side-effects.

Previous studies have shown an augmentation of the effects of an SSRI on extracellular 5-HT by a 5-HT_{2C}R antagonist [17]. This effect has been related to a reduction of GABA release following 5-HT_{2C}R antagonism [28, 29]. Augmentation, in this context, is an increase in the therapeutic effect of an antidepressant by an additional drug. In clinical studies, this augmentation effect could be demonstrated by combining an SSRI with different antipsychotic drugs that have 5-HT_{2C}R antagonistic properties.

Microdialysis studies have shown that combined administration of fluoxetine and olanzepine enhances extracellular brain levels of dopamine and norepinephrine more, than fluoxetine does alone [30, 31]. This may be attributed to the prominent 5-HT_{2C}R antagonistic properties of olanzepine, since blockade of this 5-HT receptor subtype has been reported to increase extracellular dopamine and norepinephrine in the brain. Interestingly, this combination did not augment extracellular serotonin, which may be due to the concurrent blockade by olanzepine of α-adrenoceptors in the raphe nuclei.

Recent studies in patients with treatment resistant depression show promising results. After one week of treatment, patients receiving the combined treatment scored a greater improvement than depressed subjects treated with either of the drugs alone [32, 33]. The safety profile of combined fluoxetine and olanzepine was comparable to mono-therapy with either fluoxetine or olanzepine [34]. Addition of risperidone, which has 5-HT_{2C}R antagonistic properties, to the SSRI fluvoxamine has also produced promising preliminary results [35].
Since decades of research have not resulted in widely applicable, efficacious antidepressants, clinicians may need to take a new road and combine general antidepressants with specifically targeted compounds, in order to increase the physiological response or to reduce side-effects.

Techniques to measure serotonin neurotransmission

All the above mentioned aspects of the serotonergic system may act in concert to enable the organism to function properly. The question is how we can obtain a reliable view of ongoing serotonergic processes in the living brain and what the contribution is of different receptor-subtypes and determinants of 5-HT release and its synthesis, considering the multitude of receptors, enzymes and transport systems. The different, widespread distribution of the receptor subtypes contributes to the large variety of natural behaviours which involve the serotonergic system, and the different pathologies where 5-HT is implied to play a major role.
Two techniques that allow us to measure components of neuronal functioning within a living animal are: positron emission tomography and microdialysis.

Positron emission tomography and kinetic modeling

Positron Emission Tomography (PET) is a non-invasive technique that enables quantification of physiologic processes by measuring tracer kinetics. PET can reveal the dynamics of biological processes like 5-HT neurotransmission. The great advantage of PET is that in principle every physiological process in the body can be monitored by labelling a compound with a radioactive isotope (tracer). These isotopes emit positrons that, when colliding with an electron in the surrounding tissue, emit 2 photons in exactly opposite (180°) directions. This process is called annihilation. The PET scanner can detect these photons and draw a line of response between these photons. Reconstructing these lines results in the estimation of the area where the annihilation took place and the amount of activity in that area. Especially PET tracers for targets in the brain need extensive kinetic modelling in order to estimate the parameters that describe the physiological process of interest. These kinetic models largely resemble the ones used in pharmacology for estimating drug kinetics; however, the essential properties of tracers and drugs are somewhat different.

In general, there are three different kinds of tracers: those that only enter and exit the brain without binding to a target; those that bind to a receptor or a transporter; and those that are metabolized by enzymatic action. Data of such tracers need to be analyzed with a kinetic model that best suits their kinetic properties. An overview and more extensive explanation of pharmacokinetic models can be found in [36].

For PET tracers that only enter and exit the brain without binding, the 1-tissue compartment model (1TCM) can be used. In this model, the tissue concentration c_t can be described by the tracer concentration in plasma c_a, the influx constant K_1, and the efflux constant k_2. Tracers like [^{15}O]water that are freely diffusible over the blood-brain barrier (BBB) and have a high permeability surface area product (meaning that they are easily extracted from plasma to tissue), can be used to measure cerebral blood flow [37].

Kinetic data of tracers that enter the brain and bind to a target, like a receptor or transporter, can be analysed with a 2-tissue compartment model (2TCM), where

the first compartment is the free concentration of the tracer C_f, and the second is the bound concentration of the tracer C_b. The model is usually simplified by ignoring the non-specific binding of the tracer, although such binding can contribute to the PET signal. Similar to the 1TCM, in the 2TCM K_1 and k_2 describe influx and efflux of the tracer across the BBB. In addition, the constants k_3 and k_4 describe the exchange between free and specifically bound tracer (Fig. 4). Binding potentials (BP), reflecting affinity of the tracer for its binding site and the amount of receptors, can be estimated according to an equation defined by Mintun *et al.* (1984) for *in vitro* binding studies [38]: $BP = \frac{B_{\max}}{K_d}$.

Where B_{max} is the maximal amount of receptors available for binding, and the dissociation constant, K_d, is the concentration of free radioligand resulting in 50% of the maximal binding. The BP can generally also be expressed as k_3/k_4. For explanations about *in vitro* and *in vivo* differences and the generally accepted nomenclature of the different parameters that can be measured with PET, see Innis *et al.* (2007) [39].

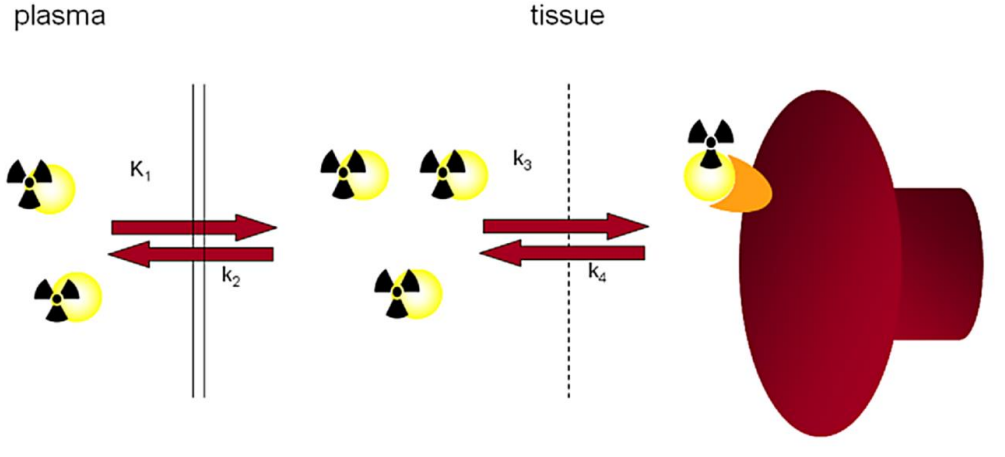

Fig. 3 Kinetics of receptor tracers.

Tracers are injected intravenously, whereafter they need to be transported over the BBB. Within tissue, there is free (unbound) tracer and tracer bound to the receptor. The tracer equilibrates between these compartments with the rate constants depicted in the figure. K_1 and k_2 describe the kinetics of exchange between plasma and tissue and k_3 and k_4 describe the kinetics of exchange between free and specifically bound tracer.

Every compound that has chemically favourable characteristics for labelling with a radioactive isotope could in principle be used as a tracer, although properties of the tracer should favour transport over the BBB and the labelled substrate should be specific for the target. On the other hand, the affinity for the target cannot be too high, as the parameters of the model (including k_4) should be measured under equilibrium conditions within the time span of the PET scan of about 60-90 min. As k_4 resembles k_{off} of the bound tracer from the target, high affinity of the tracer to the target prevents the measurement of BP within the time span of a PET scan.
Tracers that bind to targets in the brain can be used for a broad variety of applications, like measuring affinity, expression and occupancy of the targets. Also new chemical entities can be labelled with radioactive isotopes to investigate their distribution in the body and excretion route, although properties of a drug do not always favour its use as a tracer. Probably the most valuable asset of PET in pharmacology is its ability to calculate occupancy of a receptor (or other binding site) by novel or established drugs. By studying competition of the non-radioactive drug and tracer for binding to the same target receptor, occupancy of the receptor by the test drug can be measured in a non-invasive way. Through this method, the minimum dose of the drug required to acquire a certain receptor occupancy can be measured, and be related to the desired therapeutic effect or to unwanted side effects [40, 41].

Tracers that are substrates for enzymes, and therefore are metabolized in a similar way as the endogenous substrate of the enzyme, can be used to measure the activity of that enzyme by kinetic analysis with a 2TCM with irreversible tracer trapping. The difference compared to the above mentioned 2TCM used for calculating BP, is that there is no k_4 in this model. Instead, a constant resembling a metabolic rate can be calculated from the formula: $K_i = \frac{k_3 * K_1}{k_3 + k_2}$.

The best known tracer in this category is [^{18}F]2-fluoro-2-deoxy-D-glucose (FDG), which is probably the most frequently used PET tracer at present. Another example of such a tracer is [^{11}C]5-HTP, which is supposed to measure 5-HT synthesis rates. Similar to tracers that bind to receptors, tracers that are enzyme substrates need to cross the BBB, and of course need to be specific for the target

enzyme. In addition, the radioactive products should not leave the brain within the time frame of PET scanning, as this will lead to an underestimation of enzymatic activities.

The above mentioned models are the “gold standards”, but these models can be simplified to make the measurement more robust and less prone to variability in individual rate constants. One way is by using the radioactivity from a reference region, devoid of specific binding of the tracer, instead of the plasma radioactivity to calculate the input function. This method has the additional advantage that no arterial blood sampling is needed, making the method less invasive.

There are several PET tracers available that can measure components of the serotonergic system. These tracers include receptor ligands and tracers that measure serotonin synthesis rates.

Microdialysis

While for PET animals either have to be restrained or anesthetized during the scan, which can induce stress and physiological changes, microdialysis can be performed in the awake animal. The first reports on intracerebral microdialysis in animals stem from the early eighties of the previous century [42]. Microdialysis was developed to circumvent the tissue damage associated with its membrane-less push-pull forebear, which could pressurize brain tissue when the push and pull pumps were not perfectly aligned. Microdialysis does not involve exchange of fluid with brain tissue and owing to the membrane it also provides cleaner samples, which can often be injected without purification into a high performance liquid chromatograph (HPLC). A disadvantage of microdialysis is its modest recovery of the analyzed compounds at practicable flow rates, which in the early years challenged the analytical capabilities of many laboratories. For a long time it was even necessary to boost serotonin levels by including a serotonin reuptake inhibitor in the perfusion fluid. It is obvious that such measures have an impact on (local) neurochemistry in the brain, for instance by influencing local and global feedback mechanisms. It must also be noted that insertion of a microdialysis probe into the brain is an invasive procedure that will provoke cellular reactions in its direct environment [43]. These effects were somewhat lessened when the

relatively crude U-shaped probes from the initial studies were replaced by more sophisticated transversal and Y-shaped probes.

Basically, a microdialysis probe consists of an inlet and an outlet tube connected by a membrane. Performance of the membrane in terms of recovery and responsiveness greatly depends on its physical (molecular weight cut-off) and chemical (hydrophobicity) properties. The most popular Y-shaped microdialysis probe is a concentric design with an outer diameter of approximately 300 µm. Probes are inserted into the brains of laboratory animals at coordinates derived from a dedicated atlas (for instance Paxinos and Watson, 1986) using a stereotaxic instrument. In rodents surgery takes place under anesthesia and the animals are allowed to recover from surgery for at least 24 hours before the microdialysis experiments commence.

A microdialysis experiment begins by connecting the inlet of the probe via tubing to a high performance perfusion pump carrying a syringe filled with Ringer solution (artificial cerebrospinal fluid) to be perfused through the probe at a constant flow-rate mostly in the range of 1-2 µl/min. It is important that membrane and tubing are essentially inert to minimize sticking of endogenous or exogenous compounds. It is common practice to perfuse the probe for two hours prior to the actual microdialysis experiment to obtain a stable baseline. Samples can be collected in vials using a fraction collector for later analysis.

Theoretically, microdialysis does not involve exchange of fluid with brain tissue, but only exchange of endogenous compounds (anterograde microdialysis) and exogenous compounds (retrograde microdialysis). These compounds are able to diffuse through the membrane driven by the concentration gradients between the extracellular fluid in the brain and the perfusion fluid pumped through the microdialysis probe (see figure 4). It is thus possible to directly measure the effects of pharmacological interventions on the release of neurotransmitters [44]. At the end of the experiments the animals are sacrificed and the location of the probe is verified histologically. Monoamines can be analyzed either with HPLC and electrochemical detection or liquid chromatography with mass spectrometry (LC-MS). The latter method is more expensive but also more accurate and sensitive and it allows the measurement of several neurotransmitters in a single run.

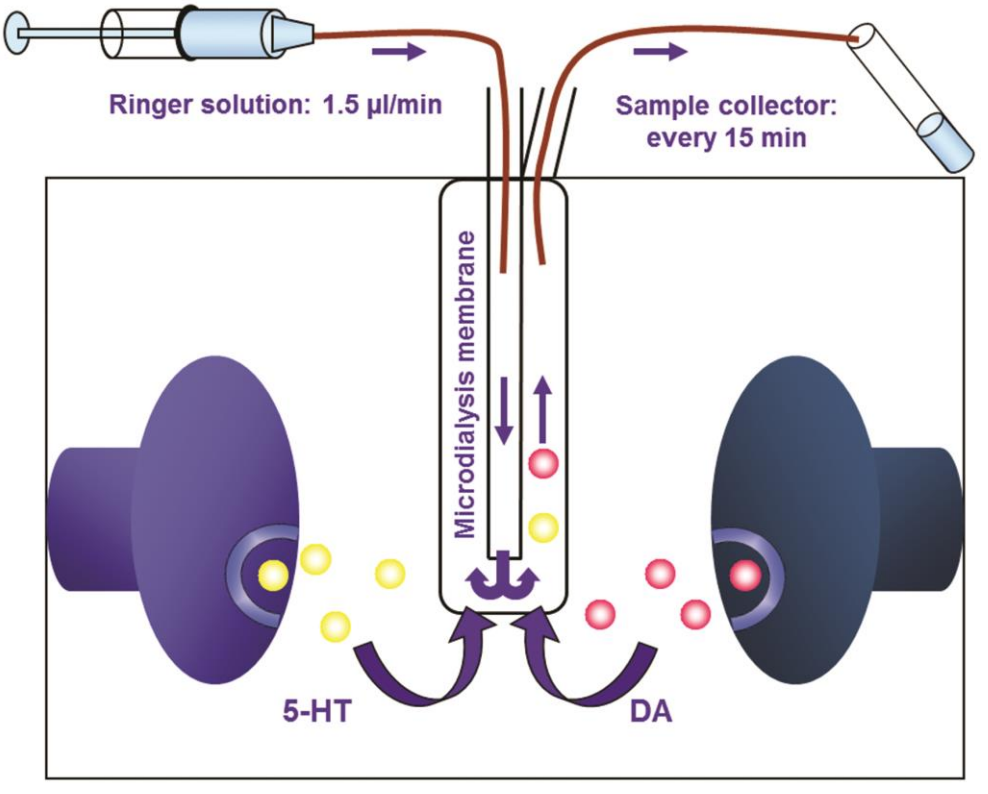

Fig 4 Principle of microdialysis
Artificial cerebrospinal fluid (Ringer solution) is pumped through the microdialysis probe, which was stereotactically implanted in a specific brain area. The tip of the probe consists of a membrane which filters the extracellular fluid within the brain area. Filtering occurs through an osmotic process. By the continuous flow of fluid through the probe, every 15 min a sample is collected. Because of the small pore size, relatively clean samples containing serotonin (5-HT) and dopamine (DA) can be obtained.

Measuring 5-HT_{2A} receptors with positron emission tomography

Microdialysis can give information about extracellular concentrations of neurotransmitters, while PET imaging with suitable radioligands gives a quantitative estimate of receptor distribution and density or affinity of the receptor in different brain areas. Kinetic analysis of tracer binding renders the BP as shown in equation 1. Although several $5HT_{2A}$ tracers exist, not all have the ideal properties to provide a quantitative estimate of receptor density and affinity.

The first radioligand that reached clinical application was [^{18}F]setoperone [45]. However, reliable measurements of 5-HT_{2A} receptor density were only possible in cortex, because this tracer additionally labels dopamine D_2 receptors in the striatum. A more selective ligand which does not bind to D_2 receptors and may be more suitable for clinical assessment of 5-HT_{2A} receptor density is [^{18}F]altanserin [46]. However, a disadvantage of altanserin is that lipophilic metabolites pass the BBB and contribute to non-specific uptake of radioactivity within the brain, reducing signal to noise ratios. The most promising PET tracer for measuring 5-HT_{2A} receptor availability is [^{11}C]MDL-100907, a highly selective antagonistic ligand with a high neocortex to cerebellum ratio [47]. This tracer appears to be insensitive to competition by endogenous 5-HT and cannot detect changes in extracellular 5-HT, thus tracer binding only reflects receptor density and affinity [48]. A disadvantage of [^{11}C]MDL-100907 is the rapid decay of carbon-11 (half-life of 20.4 min). Therefore Herth *et al* (2009) searched for MDL-100907 derivatives which can be labelled with fluorine-18 [49, 50]. They produced the promising radioligand [^{18}F]MH.MZ with comparable characteristics as MDL-100907. However, [^{18}F]MH.MZ binds with lower affinity to the 5-HT_{2A} receptor than [^{11}C]MDL-100907 and its washout from the brain is very slow, making it more difficult to estimate BP values. Clinical data for [^{18}F]MH.MZ have not yet been reported. The development of 5-HT_{2A} agonistic tracers is on-going, and has resulted in the tracer Cimbi-36 [51]. Such compounds may be more sensitive for competition with endogenous 5-HT. Although [^{11}C]MDL-100907 has been used in human studies, it has never been properly validated for use in rodents. Species differences may exist, thus validation in rodents is required before a tracer can be applied in animal models of disease.

Measuring serotonin synthesis rates with Positron Emission Tomography

In the pathway for 5-HT synthesis, availability of Trp determines the rate of 5-HT formation, because the K_m values of TPH and AADC are greater than the physiological Trp concentrations, thus the enzymes are not saturated [52]. This means that analogues of Trp and 5-HTP can be used for measuring 5-HT synthesis rates. The first attempts at imaging 5-HT synthesis were conducted by labelling natural Trp with tritium. Some disadvantages were noted, like the incorporation of Trp into proteins, which reduces tracer availability to the 5-HT synthesis

pathway [53, 54]. Therefore, other tracers have been developed with more favourable characteristics, such as α-[^{11}C]methyl-tryptophan ([^{11}C]AMT, Trp analogue) and 5-hydroxy-L-[β-^{11}C]tryptophan ([^{11}C]5-HTP, radiolabelled 5-HTP).
As Trp turned out to be unsuitable as a tracer, a radiolabelled analogue of Trp was introduced for measurement of 5-HT synthesis, α-methyltryptophan (AMT). This compound is a substrate of TPH and will eventually be converted to α-methylserotonin. Because α-methylserotonin is not degraded by MAO and cannot cross the BBB, it remains trapped for a long period in the brain [55].

However, there are some contradictory results concerning the efficiency and reliability of radiolabelled AMT. The major problem is that labelled AMT can enter the kynurenine pathway, since it is an analogue of Trp and activity of this pathway will increase the amount of radioactivity which is trapped in the brain [56]. Therefore, Chugani and colleagues refer to the constant reflecting AMT conversion, K_{acc}, as a reflection of the *capacity* for 5-HT synthesis, rather than the synthesis *rate* [57].

While under healthy conditions [^{11}C]AMT may provide estimates of 5-HT synthesis, a recent human PET study confirmed that this tracer can actually enter the kynurenine pathway. It was shown that brain tumours show differences in IDO (the enzyme converting tryptophan to kynurenine) expression and that this expression was related to the amount of AMT taken up by the tumour [58].
Tracer conversion to kynurenine can be prevented by labelling the direct precursor of 5-HT, which is only metabolized in the pathway for 5-HT synthesis. Injection of 5-HTP labelled in the β-position can provide insight in endogenously synthesized 5-HT, since 5-HTP is the substrate of the last enzyme involved in the production of 5-HT. [^{11}C]5-HTP will undergo the same conversions as 5-HTP and will eventually end up as [^{11}C]5-HIAA. Because of the difficulty of labelling 5-HTP in the β-position with carbon-11, a procedure which involves rapid enzymatic steps, this radiotracer has only been synthesized in a few imaging institutions [59, 60].
To the best of our knowledge, the first PET study with [^{11}C]5-HTP in the human brain was performed in 1991 [61]. Patients suffering from major depression showed a reduced uptake of the tracer in their brains. A recent clinical study reported a relationship between [^{11}C]5-HTP trapping and mood states [62]. A significant, negative correlation was observed between the cardinal symptoms of

premenstrual dysphoria in women, like irritability and depressed mood, and tracer trapping in the entire brain, prefrontal regions and some regions of the striatum. The opposite mood states, feelings of happiness and mental energy, showed a strong positive correlation with tracer trapping.
These studies indicate a prominent role for PET imaging in psychiatry, as this technique is capable of revealing pathophysiological mechanisms, which can otherwise only be detected with invasive techniques.

Eventually a tracer should have the ability to visualize physiological processes in humans, in order to clarify the pathophysiology of disease and to be employed in clinical routine. Clinical studies with [^{11}C]AMT and [^{11}C]5-HTP provided insight on psychiatry-related pathologies (see reviews by [63, 64]). However, initial pharmacological research and studies focusing on underlying mechanisms of disease are usually performed in experimental animals. [^{11}C]AMT has been used in such studies, in contrast to [^{11}C]5-HTP. Therefore we aimed to validate [^{11}C]5-HTP in rats, to enable use of this tracer in research on mechanisms underlying stress and the pharmacological effects of antidepressant therapy.

Aims of this thesis

General

- In the initial chapters of this thesis, several PET tracers for the serotonergic system are validated for application in rodents, to enable the use of these tracers in further preclinical studies.
- In other chapters, these PET tracers are applied to study the role of 5-HT in stress and stress sensitivity.
- Finally, interactions between 5-HT and dopamine have been examined, since interaction between various neurotransmitter systems may be even more important than the action of a single system.

Chapter 2
In this chapter we review the potential of [^{11}C]5-HTP PET to measure 5-HT synthesis in preclinical and clinical research. Other methods to measure 5-HT synthesis are compared to [^{11}C]5-HTP PET.

Chapter 3
Validation of a tracer in preclinical models is important before that specific tracer is used to investigate a biological process. Because of species differences in physiology and genetics, radiotracers may behave differently in rodents and humans. Therefore we have tested [^{11}C]5-HTP in rodents.

Chapter 4
An important part of tracer validation is the determination of the appropriate kinetic model to analyse the PET data. If a reference tissue (a tissue without specific binding) can be used, longitudinal studies can be performed as there is then no need for invasive blood sampling. In chapter 4, we have validated the 5-HT_{2A} ligand [^{11}C]MDL100907 for measurent of 5-HT_{2A} receptor binding potential in rats, using tracer-kinetic modelling.

Chapter 5
As 5-HT seems to play a crucial role in stress and depression, PET is a nice technique to investigate time-dependent changes in the serotonergic system. We have investigated the effect of social stress on 5-HT_{2A} receptors in rats by two different methods: PET and binding assays.

Chapter 6
Even in laboratory animals there are differences in physiology, although minimized by breeding. In nature, such differences are bigger and therefore individual differences within an animal species can influence the response of mammals to stress. Although we could not show significant effects of stress on 5-HT_{2A} binding in socially defeated rats, there may be differences between animals in receptor sensitivity or receptor expression related to their individual coping styles (way to cope with their environment).

Chapter 7
When investigating a small piece of a big puzzle, it is easy to overlook the larger picture. Investigating interactions between different neurotransmitter systems is equal to looking at a greater part of the puzzle. Especially in depression, the interaction between 5-HT and dopamine is crucial as both systems are involved in symptoms of this disease. Therefore, treatment should also act on both systems.

In the final chapter we investigate whether 5-HT and dopamine levels in the brain can both be increased by applying a combination of a 5-HT_{2C} inhibitor and an SSRI.

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CHAPTER 2

Measuring Serotonin Synthesis: From Conventional Methods to PET Tracers and their (Pre)clinical Implications

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Abstract

The serotonergic system of the brain is complex, with an extensive innervation pattern covering all brain regions and endowed with at least 15 different receptors (each with their particular distribution patterns), specific reuptake mechanisms and synthetic processes. Many aspects of the functioning of the serotonergic system are still unclear, partially because of the difficulty of measuring physiological processes in the living brain. In this review we give an overview of the conventional methods of measuring serotonin synthesis and methods using positron emission tomography (PET) tracers, more specifically with respect to serotonergic function in affective disorders.

Conventional methods are invasive and do not directly measure synthesis rates. Although they may give insight in turn-over rates, a more direct measurement may be preferred. PET is a non-invasive technique which can trace metabolic processes, like serotonin synthesis. Tracers developed for this purpose are α-[^{11}C]methyl-tryptophan ([^{11}C]AMT) and 5-hydroxy-L-[β-^{11}C]-tryptophan ([^{11}C]5-HTP). Both tracers have advantages and disadvantages.

[^{11}C]AMT can enter the kynurenine pathway under inflammatory conditions (and thus provide a false signal), but this tracer has been used in many studies leading to novel insights regarding antidepressant action. [^{11}C]5-HTP is difficult to produce, but trapping of this compound may better represent serotonin synthesis. AMT and 5-HTP kinetics are differently affected by tryptophan depletion and changes of mood. This may indicate that both tracers are associated with different enzymatic processes.
In conclusion, PET with radiolabelled substrates for the serotonergic pathway is the only direct way to detect changes of serotonin synthesis in the living brain.

Keywords: Serotonin, Positon Emission Tomography, [^{11}C]5-HTP, [^{11}C]AMT, depression

Introduction

Serotonergic innervations are widely spread throughout the brain with cell bodies of origin lying in the dorsal (DRN) or median (MRN) raphe nucleus, and a column of raphe nuclei in lower brainstem regions, projecting to basically all divisions of the brain and spinal cord (Fig 1). Synthesis of serotonin (5-HT) takes place within neurons and especially in serotonergic terminals, and this process includes two enzymatic steps. The first step is the conversion of the precursor molecule, the amino acid tryptophan (Trp), to 5-hyroxytryptophan (5-HTP) by tryptophan hydroxylase (TPH) 1 or 2. The second step in the production of 5-HT involves the enzymatic action of aromatic amino acid decarboxylase (AADC) that has L-DOPA and 5-HTP as a substrate. 5-HT is eventually degraded to 5-hydroxyindole acetic acid (5-HIAA) by monoamine oxidase (MAO).

After synthesis, 5-HT is transported by the vesicular monoamine transporter and stored in vesicles at the neuronal presynaptic endings. When neurons fire, these vesicles fuse with the synaptic membrane and release 5-HT into the synaptic cleft. Released 5-HT can bind to many different receptors, both postsynaptic and presynaptic or be taken up by the serotonergic reuptake transporter (SERT). There are at least fifteen different 5-HT receptors which are divided in seven distinct families ($5\text{-HT}_{1\text{-}7}$) [1]. Postsynaptic receptor binding can be either inhibitory or excitatory, depending on which subtype is stimulated. The presynaptic receptors (5-HT_{1A}, located somatodendritic and 5-HT_{1B}, located on terminals) are autoreceptors that inhibit serotonergic neurotransmission, while heteroreceptors influence the release of neurotransmitters other than 5-HT [2]. Almost all 5-HT receptors are G-protein coupled (metabotropic), with exception of the 5-HT_3 subtype which is a ligand-gated ion channel [1].

Different subtypes of the 5-HT receptor are located in different brain regions and probably regulate different behavioural functions. An important role of 5-HT is the regulation of mood, and several 5-HT receptor subtypes are involved in the actions of antidepressants and antipsychotics. Serotonin synthesis may be of special interest because this process is controlled by 5-HT_{1A} receptors which are implied in the therapeutic efficacy of antidepressants [3].

It is clear that 5-HT influences many other neurotransmitter systems in an excitatory or inhibitory manner. One important key aspect that regulates serotonergic neurotransmission is the availability of the 5-HT precursor: the amino acid tryptophan.

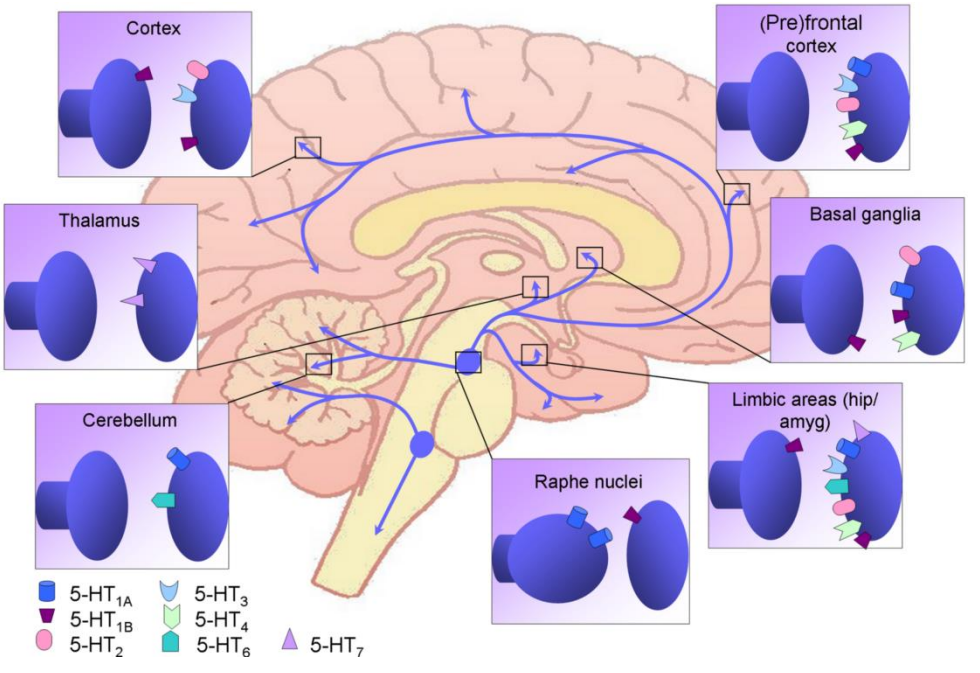

Fig 1 The serotonergic system

The cell bodies of serotonergic neurons lay in the brainstem raphe nuclei. These neurons project to many brain areas like the cortex, basal ganglia, cerebellum, thalamus, limbic areas like hippocampus and amygdala, and spinal cord. Different 5-HT receptor subtypes have a specific distribution in the brain. In the figure autoreceptors in the raphe nuclei are depicted on neuronal cell bodies ($5\text{-}HT_{1A}$) or in terminal areas and raphe nuclei on the presynaps ($5\text{-}HT_{1B}$). The depiction of other 5-HT receptor subtypes in terminal areas can either represent heteroreceptors or postsynaptic receptors on 5-HT neurons.

In addition to conversion to serotonin, Trp is metabolized in the kynurenine-pathway and being used for protein synthesis. The rate-limiting step in the kynurenine-pathway is the activity of indoleamine 2,3-dioxygenase (IDO) in the CNS and tryptophan 2,3-dioxygenase in peripheral organs. Both enzymes convert Trp to kynurenine. Activation of IDO within the central nervous system takes place

under the influence of proinflammatory cytokines mainly within microglial cells. Increased cytokines and IDO activity have been linked to major depression in depressed subjects and in patients with inflammatory somatic disorders [4]. Increased IDO activity under inflammatory conditions may increase the amount of Trp used in the kynurenine pathway and consequently reduce the availability of Trp for 5-HT synthesis.

All the above mentioned aspects of the serotonergic system may act in concert to enable the organism to function properly. The question is how we can obtain a reliable view of ongoing serotonergic processes in the living brain and what the contribution is of different receptor-subtypes and determinants of 5-HT release (like it's synthesis) considering the multitude of receptors, enzymatic activity and transport systems. PET can quantify these processes in a non-invasive manner. In table 1, the most often used radiotracers to measure aspects of the serotonin system are listed [5-25]. Such tracers are reviewed elsewhere in greater detail [26,27]. As there are no single photon emission computed tomography (SPECT) tracers to measure serotonin synthesis, we mention only PET tracers.
In the present review we will mainly focus on the quantification of serotonin synthesis and its pre-clinical and clinical application using conventional and PET imaging techniques.

Conventional methods: Measuring 5-HT and its metabolites in platelets and CSF

In early studies of experimental animals, concentrations of 5-HT and its metabolites in tissue after inhibition of AADC or MAO were used as an estimate of 5-HT turnover. Inhibiting MAO results in a decrease of the conversion of 5-HT to 5-HIAA. By measuring either the reduction of 5-HIAA or the accumulation of 5-HT, turnover rates of 5-HT can be estimated. A similar approach is inhibition of the transport of 5-HIAA over the BBB, from brain to the circulation. Inhibition of this transport by probenecid results in 5-HIAA accumulation within the brain and the rate of this accumulation is related to the turnover rate of 5-HT.

Table 1 PET tracers used for research on serotonergic neurotransmission

Serotonergic component	Function	Radioligand	Literature
5-HT$_{1A}$	Autoreceptor on cell bodies in DRN/ inhibitory postsynaptic receptor	[^{11}C]NAD-195 [^{18}F]MPPF [*carbonyl*-^{11}C]WAY-100635 [*carbonyl*-^{11}C]*desmethyl*-WAY-100635 [^{18}F]FCWAY [^{18}F]MEFWAY [^{11}C]RWAY [^{11}C]CUMI-101	Sandell et al., 1999 [22] Shiue et al., 1997 [23] Pike et al., 1996 [19] Pike et al., 1998 [20] Lang et al., 1999 [13] Saigal et al., 2006 [114] Yasuno et al., 2006 [25] Kumar et al., 2007 [12]
5-HT$_{1B}$	Autoreceptor on nerve terminals/ inhibitory heteroreceptor	[^{11}C]AZ10419369 [^{11}C]P943	Pierson et al., 2008 [18] Gallezot et al., 2009 [8]
5-HT$_{2A}$	Excitatory receptor (e.g. regulation gene transcription)	[^{18}F]setoperone [^{18}F]altanserin [^{11}C]MDL-100907 [^{18}F]MH.MZ	Blin et al., 1988 [6] Lemaire et al., 1991 [14] Lundkvist et al., 1996 [15] Herth et al., 2009 [10]
5-HT$_4$	Excitatory receptor	[^{11}C]SB207145	Marner et al., 2009 [17]
SERT	Reuptake transporter (e.g. target SSRI)	[^{11}C]McN5652 [^{11}C]DASB [^{11}C]MADAM [^{18}F]ADAM	Suehiro et al., 1993 [24] Houle et al., 2000 [11] Halldin et al., 2005 [9] Ma et al., 2009 [16]
tryptophan	Precursor 5-HTP and substrate TPH	α-[^{11}C]methyl-tryptophan	Diksic et al., 1990 [7]
5-HTP	Precursor 5-HT and substrate AADC	5-hydroxy-L-[β-^{11}C]tryptophan	Bjurling et al., 1989 [5]

The accumulation of 5-HTP in the brain after AADC inhibition with NSD 1015 can be used as a measure for 5-HT synthesis. Assays of serotonin and its metabolites can be performed by analysis of tissue homogenates, by microdialysis or by analysis of body fluids (blood, urine or cerebrospinal fluid (CSF)) [28-30]. Such methods have four major disadvantages: i) it is not certain that the target enzymes are fully inhibited under the conditions of the study, ii) the inhibitors may influence other physiological processes (for example 5-HT synthesis) iii) the measurements in plasma and urine include peripheral processes and iv) these invasive techniques cannot be applied in humans.

Turnover rates of 5-HT in humans are usually assessed by measuring 5-HT content of blood platelets or by analysis of samples of CSF which are acquired through lumbar puncture, an uncomfortable and invasive procedure. Usually the ratio of 5-HIAA and 5-HT is measured and sometimes only 5-HIAA concentrations are used as an index of 5-HT turnover (because 5-HT concentrations are negligible compared to 5-HIAA concentrations) [31]. Assays of platelet 5-HT content are of questionable value, since peripheral processes may not be an accurate reflection of the corresponding processes in the central nervous system. In research focusing on this question contradictory results were obtained.

Some studies indicate a close relationship between 5-HT turnover in brain and platelets. There are similarities between neurons and platelets regarding the mechanisms of 5-HT transport and the presence of certain binding sites including the 5-HT2 receptor [32,33]. For example, rats show decreased levels of 5-HT both in platelet-rich plasma and in brain homogenates after the forced swim test (FST), used to assess antidepressant efficacy. This decrease is reduced after acute treatment of animals with an SSRI (fluoxetine) and in naive rats, fluoxetine causes an increase in 5-HT [34]. The 5-HT concentration in brain homogenates after chronic (12 days) treatment of rats with an SSRI was comparable to the amount found in platelet-rich plasma. The 5-HT concentration in isolated platelets returned to control levels at day 12, which may reflect comparable changes in neurons.

In contrast to these positive results, there is also evidence indicating that 5-HT in platelets and in brain may not always be changed in parallel. In 5-HT_{1A} receptor

knock-out mice, 5-HT concentrations in platelets and in brain show similar decreases until 2 weeks after birth. After 2 weeks, however, the 5-HT content of platelets is increased compared to wild type mice, whereas brain 5-HT concentrations are normalized [35]. In addition, no correlation was observed between BP of the 5-HT_{2A} ligand [^{18}F]-setoperone in the brain and binding of [^{3}H]-LSD in blood platelets of healthy volunteers [36]. This indicates that extrapolation of measurements in blood platelets to 5-HT neurotransmission in the brain is difficult. Such extrapolations must be performed with caution and direct measurements of 5-HT in the brain should be preferred.

Another alternative for directly measuring brain concentrations is measurement of 5-HT and its metabolites in samples of CSF acquired by lumbar puncture. Because the levels of 5-HT in CSF are very low (less than 10 pg/ml), measurements of 5-HT concentration cannot be used for determination of 5-HT turnover rates [37]. Another option is measuring 5-HIAA concentrations in CSF, because 5-HIAA is present in much greater quantities. Increases of 5-HIAA after inhibition of MAO or of 5-HIAA transport by probenecid should correlate to the formation rate of 5-HT. However, this method has also many drawbacks [31]:

- A lumbar puncture is invasive and often experienced as unpleasant.
- Measurements of 5-HIAA concentrations will partly represent the rate of transport of 5-HIAA into the CSF.
- Because of the high concentrations of 5-HIAA compared to 5-HT, changes in 5-HIAA are only detectable after a delay of several hours.
- 5-HT concentrations in lumbar CSF are not an accurate reflection of cerebral 5-HT synthesis, since they partially reflect synthesis of 5-HT within the spinal cord. There is a gradient from cisterna magna to spinal subarachnoid as more 5-HT is synthesized in the brain than in the spinal cord.
- 5-HIAA is transported from brain and CSF, back into the blood stream.

The last process can be inhibited by administration of probenecid, which blocks the active transport of acidic metabolites out of the brain and CSF. Measurements of 5-HIAA in CSF and the “probenecid test” were frequently used by Van Praag and Korf [38]. Concentrations of 5-HIAA were measured in the CSF at baseline and after administration of probenecid. By using this method they were one of the

pioneers linking serotonin deficiency to depressive symptoms and proposed the "predisposition hypothesis" which is partially maintained even today. The increase of 5-HIAA concentrations after probenecid was lower in depressive patients compared to the control group. This indicates involvement of 5-HT in depression. The predisposition hypothesis was based on different findings. A higher frequency of depression was observed in patients with 5-HT deficiency and this deficiency in 5-HT persisted even after a depressive episode. Additionally, the use of 5-HTP as a prophylactic agent reduced the rates of relapse in depressed patients [39,40].

A recent study reported that 5-HIAA in the blood of patients with major depression, using a jugular vein catheter, were actually increased, suggesting increased 5-HT turnover. This increase in 5-HIAA was reduced by SSRI treatment and dependent on the s- and l-allele polymorphisms of SERT [41]. This result conflicts with assumptions that 5-HT synthesis is decreased in depressed patients and that antidepressants cause an increase in 5-HT signal transduction. A possibility is that SSRIs influence 5-HT synthesis differently under acute and chronic circumstances, but they could also indirectly influence breakdown of 5-HT by MAO resulting in decreased turnover. SSRIs may increase extracellular 5-HT concentrations and concomitantly reduce 5-HT storage and breakdown because of the decreased reuptake of 5-HT.
Later it appeared that 5-HT deficiency is related to other behavioural dysfunctions like aggression and impulsivity, while not solely deficiencies in 5-HT neurotransmission underlie depressive symptoms. This lead to the denosologisation hypothesis implying that serotonergic dysfunction may be related to dimensions of behaviour cutting across diagnostic boundaries, and thus not necessarily show correlations with diagnostic entities [42]. This approach was probably for the first time systematically applied in imaging studies by the Gent group (head R.A. Dierckx) through transnosological research of impulsivity using SPECT activation studies and 5-HT_{2A} receptor imaging in suicidality, eating disorders and personality disorders (in men and dogs) [43-46].
Depression is a multi-symptom pathology and may probably be caused by flaws in several neurotransmitter systems and molecular signalling pathways. Yet, the serotonergic system may play an important role as it is a modulatory system, influencing the activity of many other neurotransmitter pathways throughout the brain.

Recent technologies: Radiopharmaceuticals for measuring serotonin synthesis

Recent technologies allow research in living animals and humans. PET is such a non-invasive technique that enables quantification of physiologic processes by measuring tracer kinetics. PET can reveal the dynamics of biological processes like 5-HT neurotransmission. In the pathway for 5-HT synthesis, the availability of Trp determines the rate of 5-HT formation, because the K_m values of TPH and AADC are greater than the physiological Trp concentrations, the enzymes are not saturated [47,48]. This means that both Trp and 5-HTP analogues can be used for measuring 5-HT synthesis rates. The first attempts at imaging 5-HT synthesis were conducted by labelling natural Trp with tritium. Some disadvantages were noted, like the incorporation of Trp into proteins which reduces tracer availability [49,50]. Therefore, other tracers have been developed with more favourable characteristics, such as α-[^{11}C]methyl-tryptophan ([^{11}C]AMT, Trp analogue) and 5-hydroxy-L-[β-^{11}C]tryptophan ([^{11}C]5-HTP, radiolabelled 5-HTP).

α-[^{11}C]methyl-tryptophan

As Trp turned out to be unsuitable as a tracer, a radiolabelled analogue of Trp was introduced for measurement of 5-HT synthesis, α-methyltryptophan (AMT). This compound is a substrate of TPH and will eventually be converted to α-methylserotonin. Because α-methylserotonin is not degraded by MAO and cannot cross the BBB, it is trapped for a long period in the brain [51].

Pre-clinical data

<u>Kinetic modelling and validation</u>

The first studies employed AMT labelled with ^{3}H and ^{14}C to perform autoradiography in rats. A kinetic model for measuring [^{14}C]-AMT uptake was developed using a three-compartment model (or two-tissue compartment model) with irreversible tracer trapping, the compartments being plasma, brain and irreversibly trapped tracer [7,52]. The slope of the linear function depicting distribution volume (DV) plotted against time under steady-state conditions

represents the unidirectional trapping of the tracer indicated by the constant K^{α}. Subsequent studies used AMT labelled with carbon-11 for PET scanning in monkeys and dogs to measure individual rate constants and to enable Patlak analysis.

In this model, the K^{α} (or K-complex) describes a trapping constant that takes all individual rate constants into account according to the following formula:

$$K^{\alpha} = \frac{K_1^{\alpha} k_3^{\alpha}}{\left(k_2^{\alpha} + k_3^{\alpha}\right)} \qquad \text{Equation 1}$$

In equation 1, K_1 resembles tracer influx into the brain; k_2 is the efflux constant and k_3 the irreversible trapping constant (Fig 2).

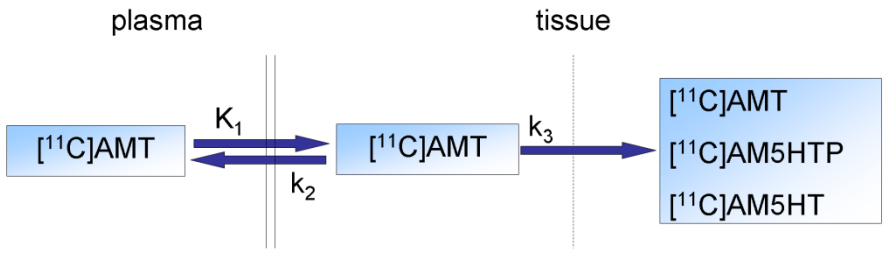

Fig 2 Kinetic model of irreversible tracer trapping

Three-compartment model, or two-tissue compartment model, with irreversible tracer trapping. [^{11}C]AMT in plasma is transported over the BBB into the brain, where it can be irreversibly trapped, mainly as [^{11}C]AMT but also as [^{11}C]AM5HTP or [^{11}C]AM5HT. The three compartments are plasma, precursor pool and irreversible trapping compartment.

To estimate physiological rates of 5-HT synthesis, K^{α} must be divided by a lumped constant (LC) to correct for difference in affinity of AMT and Trp for TPH and the different amounts of both compounds entering the kynurenine pathway. The LC is on average 0.42 in rat brain [53,54]. In this way, a K^{T} value can be obtained which is further converted to 5-HT synthesis rates by multiplication with free-tryptophan concentrations in plasma (Cp^{Trp}). Thus, reliable in vivo 5-HT synthesis rates (R) may be estimated [55]:

$$R = \left(K^{\alpha} / LC\right) * \left(Cp^{Tryp}\right) \qquad \text{Equation 2}$$

K^{α} can also be measured with a graphical method like the Patlak plot [56]. This graphical method is not constrained by individual rate constants, but based on macro-system parameters, usually resulting in less variability. The slope of the Patlak plot represents K^{α}.

However, there are some contradictory results concerning the efficiency and reliability of radiolabelled AMT. In the first 60 minutes after injection, only a small fraction of labelled AMT is converted to labelled AM5HT in the rat brain [57]. Different research groups have obtained significantly different results in calculating the percentage of radioactivity corresponding to [^{11}C]AM5HT in the DRN, ranging from 2-4 % after 90 minutes in monkeys [58] to 31% after 60 minutes in rats [7]. It has been suggested that AMT-PET measures Trp uptake in the brain rather than rates of 5-HT synthesis [58], although Diksic and colleagues argue that the significantly better fit of a 3-compartment model compared to a 2-compartment model suggests irreversible tracer trapping and not only the presence of AMT in the brain [59]. The slow kinetics resulted in the lack of a linear portion of the Patlak plot at the moment of tracer equilibrium between reversible compartments and plasma [58,60]. Gharib and colleagues correctly pointed out that AMT does not meet all the assumptions made in the Patlak model [57]. The transfer of unmetabolized tracer between brain and plasma is not fully reversible. Another problem is that labelled AMT can enter the kynurenine pathway since it is an analogue of Trp and the activity of this pathway will increase the amount of radioactivity which is trapped in the brain. Therefore, Chugani and colleagues refer to the measured K^{α} as a reflection of the *capacity* of 5-HT synthesis, rather than the synthesis *rate* [60].

Although a kinetic analysis of AMT uptake may not provide true synthesis rates, labelled AMT is sensitive enough to detect physiological changes and may provide more information about serotonergic neurotransmission. Neurons labelled with 5-HT or hydroxytryptophan colocalized with neurons taking up [^{3}H]-AMT in the rat brain and [^{3}H]-AMT5HT was released from serotonergic cell bodies in the raphe nucleus and serotonergic terminals in projection areas like the hippocampus and striatum. This release was increased after depolarization by 50 mM KCl, as compared to baseline [61]. Studies using autoradiography revealed that the half-life of the precursor pool in rats is approximately 20 minutes and treatment with

lithium results in a 52% increase of 5-HT synthesis rates in the parietal cortex and a 47% increase in the caudate nucleus [7,52]. This indicates the ability of AMT to detect changes in serotonergic neurotransmission.

Effect of pharmacological challenges

Studies with ^{14}C labelled AMT in experimental animals using autoradiographic techniques after various interventions and brain lesions indicated that AMT could detect changes in the rate of 5-HT synthesis (see reviews by [31,55]). These pharmacological interventions revealed differences in the acute or chronic effect of SSRIs on serotonin synthesis rates [62,63], that could possibly be explained by autoreceptor stimulation.

This was also shown in a more recent study with the SSRI citalopram (10 mg/kg/day for 14 days) in olfactory bulbectomized (OBX) rats, a depression model. OBX rats showed an increase of 5-HT synthesis in terminal areas and reductions in the DRN. Chronic citalopram reduced 5-HT synthesis to the levels of sham operated rats receiving citalopram in the terminal areas, and marginally increased synthesis in the DRN. As citalopram treatment in sham operated rats also reduced 5-HT synthesis in some brain areas (DRN, hippocampus), the reduction of 5-HT synthesis in terminal areas of OBX rats may be explained by feedback inhibition through autoreceptors [64].

Autoreceptors located on serotonergic neurons are very important in the regulation of 5-HT synthesis and they play a crucial role in the therapeutic action of antidepressants. The 5-HT_{1A} (somatodendritic receptor on cell bodies) and 5-HT_{1B} subtypes (presynaptic receptor on nerve terminals), regulating the feedback inhibition of 5-HT release, deserve attention because of their role in the late onset of therapeutic effects of many antidepressants.

Compared to the above mentioned studies with antidepressants, similar effects were seen with the 5-HT_{1A} receptor agonist buspirone. Acute buspirone treatment of rats (10 mg/kg, subcutaneous) significantly decreased 5-HT synthesis rates, while chronic treatment (10 mg/kg/day for 14 days, subcutaneous) abolished this effect [65]. This finding is in accordance with previous results showing a reduction of serotonergic firing rate and reduced 5-HT in projection areas like the hippocampus [66,67].

Less is known about the role of 5-HT_{1B} receptors on the nerve terminals in projection areas. The non-selective, 5-HT_{1B} receptor agonists TFMPP and CGS12066B acutely decrease 5-HT synthesis rates in the DRN and MRN (probably caused by partial action on 5-HT_{1A} receptors) of rat brain [68]. Acute CGS12066B decreases 5-HT synthesis rates in brain areas known to contain solely 5-HT_{1B} receptors (e.g. the median of the nucleus caudatus and the nucleus accumbens) [69], while TFMPP decreases 5-HT synthesis in almost all terminal areas. Subchronic treatment (7 days) with both compounds decreases 5-HT synthesis in terminal areas.
The much more selective 5-HT_{1B} receptor agonist CP-93,129 when administered acutely (7 mg/kg, i.p.) decreased synthesis rates only in projection areas. This effect was abolished by chronic treatment (7 mg/kg/day for 14 days, subcutaneous) which is explicable because of the desensitization of the 5-HT_{1B} autoreceptors [70].
In conclusion, both 5-HT_{1A} and 5-HT_{1B} autoreceptors can reduce 5-HT synthesis rates in the brain, but the receptors desensitize in response to chronic stimulation, so that their inhibitory effects are transient.

These different effects of the pharmaceuticals are difficult to detect by simple measurements of 5-HT concentrations and made it clear that antidepressants have a regional specific effect on serotonin synthesis. Eventually effects on serotonin synthesis will influence the 5-HT availability for release and therefore may be a very important process in the efficacy of antidepressants. The described studies with AMT are an excellent example of how PET tracers can provide novel insights about physiological processes.

The most pronounced effects of pharmacological challenge are expected when the enzymes of the 5-HT synthesis pathway (AADC and TPH) are directly inhibited and this may provide information about the validity of the method. Indeed, the TPH inhibitor p-chlorophenylalanine (PCPA, 200 mg/kg for 3 days i.p.) and the inhibitor of TPH activation, AGN-2979 (10 mg/kg, i.p.), both reduced 5-HT synthesis rates [71,72]. Surprisingly, the AADC inhibitor NSD 1015 (100 mg/kg, i.p.) appeared to increase 5-HT synthesis [73]. This discrepancy may be explained by the additional inhibition of MAO by NSD1015 or by the ability of NSD1015 to increase levels of free Trp in plasma [74]. Therefore, results obtained with

NSD1015 should be interpreted with caution as they are probably not solely attributable to inhibition of AADC.

Preclinical PET studies

Although the above mentioned studies may provide important insights regarding physiological processes in animals, autoradiography does not take individual rate constants into account. Higher accuracy can be obtained by monitoring tracer kinetics in living animals and humans, using PET. The first study using ^{11}C labelled AMT for PET imaging was performed in dogs [75]. Both oxygen and Trp increased the trapping of [^{11}C]AMT in dog brain, which should be expected if [^{11}C]AMT trapping reflects 5-HT synthesis. Another experiment in dogs evaluated the time-dependent effect of 3,4-methyleendioxymethamfetamine (MDMA) infusion (2 mg/kg). After 1 hour, 5-HT synthesis was strongly increased (up to six times above baseline), though subsequently a decline in 5-HT synthesis rates was observed to 50% of baseline after 5 hours [76]. This is in accordance with the observation that MDMA first stimulates 5-HT release which leads to increased 5-HT synthesis, but finally destroys 5-HT terminals with a corresponding decrease of neurotransmitter formation [77].

Interestingly, 5-HT synthesis rates measured with [^{11}C]AMT PET in rhesus monkeys did not correlate with 5-HIAA concentrations in the CSF. Whether this is due to a lack of accuracy of the AMT-method or a difficulty of linking 5-HIAA in CSF to 5-HT synthesis within brain remains unclear [78], although in theory, during steady state there should be a close correlation between the conversion of 5-HT to 5-HIAA and the elimination of 5-HIAA from brain to CSF.
More concerns about the AMT-method were raised by the same research group as they showed that even after 3 hours in rhesus monkeys no equilibrium had been reached between tracer in plasma and tracer in reversible tissue compartments. Therefore, the Patlak plot showed no linear portion, which is necessary for calculation of influx rates [58].
However, the preclinical data contributed to the understanding of what the tracer is really measuring and whether the tracer is valid for clinical research, making it worthwhile to further investigate serotonin synthesis under clinical conditions.

Clinical data

Eventually a tracer should have the ability to visualize physiological processes in humans, in order to clarify the pathophysiology of disease and to be employed in clinical routine.

Human PET data of [^{11}C]AMT are modelled in approximately the same way as canine or monkey data (see above). However, in humans both a Patlak approach and a 2-tissue compartment model can be used, although the value of the LC in humans is unknown. While in animals the Patlak approach may not be valid, in humans a steady state appears to be reached which is accompanied by a linear portion of the Patlak plot justifying its use for quantification purposes [79]. By comparing different studies in humans as well as in monkeys it was found that there was a high correlation between [^{11}C]AMT trapping, [^{11}C]5-HTP accumulation and 5-HT concentrations determined post mortem [80].

A disadvantage of kinetic modelling is that an arterial cannula is required for blood sampling (determination of an arterial input function), which is a quite invasive procedure. The use of venous radioactivity as input causes a bias in the results with overestimation of the K^{α} values, but this may be acceptable if no arterial blood samples can be taken [81].

The first study using [^{11}C]AMT PET focused on gender differences and Trp depletion [79]. Both females and males showed much lower K^{α} values after acute Trp depletion through ingestion of a Trp-free amino acid mixture. The change was about 90% in males and 95% in females. Acute Trp depletion has been associated with lowered mood in vulnerable subgroups and with sensitivity to stress [82-84]. At baseline women had lower levels of free Trp in plasma then men. Possibly due to this difference in Trp levels, women showed lower rates of 5-HT synthesis than men at baseline, although the K^{α} did not differ between genders. The K^{α} should not be confused with rates of 5-HT synthesis which are also based on plasma levels of free Trp. Conflicting results were reported regarding gender differences. Where Chugani et al. [85] found an increase; Sakai et al. [21] described a decrease of the K^{α} in females. These conflicting findings could be due to the different protocols that were employed including a different nutritional and metabolic state of the subjects.

Later studies focused on the effect of age on 5-HT synthesis and on the examination of various pathologies using [^{11}C]AMT PET (see reviews by [31,55]). More recent research has focused on the effect of oxygen on 5-HT synthesis, as it is necessary for TPH activity. Even slight hypoxia affects the metabolism of Trp, probably because TPH has a low affinity for oxygen [86]. This is reflected in the K^{α} values measured under high and low oxygen concentrations (60% and 15 % oxygen, respectively). The increase in the measured rate of 5-HT synthesis at high oxygen concentrations is about 50 % [87], providing evidence that [^{11}C]AMT can be used for measuring changes of TPH activity.

When clinical applications for a tracer of 5-HT synthesis are considered, research on depressed patients is of great interest. Changes in Patlak K^{α} were detectable with [^{11}C]AMT PET in medication-free patients with major depression [88]. Most obvious was the reduction of Patlak K^{α} in the cingulate cortex (CC), bilaterally in women and in the left hemisphere in men. This brain area is involved in attention and emotion and shows abnormalities of cerebral blood flow and glucose metabolism in patients with major depression [89]. The CC receives large projections from the dorsal and median raphe nuclei and projects to orbitofrontal cortex (OFC) and amygdala, two areas hypothesized to show dysfunction in depression. Remarkably, no differences in 5-HT synthesis rate were found in the OFC or dorsolateral prefrontal cortex. This suggests that the difference in glucose metabolism observed in these regions may not be attributed to altered 5-HT synthesis. Surprisingly, K^{α} did not correlate with the severity of depression [88].

Treatment with the SSRI citalopram increased K^{α} in the CC and this increase is associated with elevated mood as assessed by Hamilton rating scores [90]. Other brain areas where citalopram increased 5-HT synthesis rates are the left and right prefrontal gyrus. These effects were not seen after 10 days, only after 24 days. This delay in the onset of therapeutic effects of an SSRI was probably caused by a feedback loop involving 5-HT_{1A} autoreceptors. It is known that blocking the 5-HT_{1A} receptor with pindolol can accelerate the therapeutic effects of antidepressants [91]. Indeed, at day 24 the increase in 5-HT synthesis rate induced by an SSRI was greater in patients who received pindolol at day 10 compared to placebo. Whether this increase in 5-HT synthesis is due to 5-HT_{1A} autoreceptor blocking remains questionable, because pindolol also excites dopaminergic and

noradrenergic neurons [92]. Most probably the total blockage of central beta-adrenoceptors by pindolol plays an important role [93].
In addition, the binding potential of [^{18}F]MPPF, a 5-HT$_{1A}$ receptor ligand, could not be correlated to 5-HT synthesis rates as measured with [^{11}C]AMT in the raphe nuclei [94]. However, in terminal areas of serotonergic neurons (like hippocampus, anterior cingulate cortex and anterior insula) a negative correlation was found, indicating that decreased binding of [^{18}F]-MPPF to 5-HT$_{1A}$ heteroreceptors increased 5-HT synthesis. These studies show that a combination of different tracers can lead to greater understanding of processes in the human brain.
While under healthy conditions [^{11}C]AMT may give estimates of 5-HT synthesis, a recent human PET study confirmed that this tracer can actually enter the kynurenine pathway. It was shown that brain tumours show differences in IDO (the enzyme converting tryptophan to kynurenine) expression and that this expression was related to the amount of AMT taken up by the tumour [95].

[^{11}C]5-HTP

Tracer conversion to kynurenine can be prevented by labelling the direct precursor of 5-HT, which is only metabolized in the pathway for 5-HT synthesis. Injection of 5-HTP labelled in the β-position can provide insight in endogenously synthesized 5-HT, since 5-HTP is the substrate of the last enzyme involved in the production of 5-HT. [^{11}C]5-HTP will undergo the same conversions as 5-HTP and will eventually end up as [^{11}C]5-HIAA (Fig 3). Because of the difficulty of labelling 5-HTP in the β-position with carbon-11, a procedure which involves rapid enzymatic steps, this radiotracer has only been synthesized in a few imaging institutions [5,96].

Neuroendocrine tumour imaging

[^{11}C]5-HTP was initially developed for the detection of neuroendocrine tumours and not for brain imaging. These tumours are usually slowly growing, highly differentiated and may have various characteristics, although active uptake and decarboxylation of monoamine precursors like DOPA and 5-HTP and overproduction of hormones are typical. Conventionally used metabolic PET tracers, like [^{18}F]FDG, appeared unsuitable for the detection of neuroendocrine

tumours, whereas detection of the uptake of monoamine precursors with [^{11}C]5-HTP/PET resulted in the visualization of lesions which were missed by FDG. Especially the diagnostic sensitivity of pancreatic islet cell tumours greatly benefits from [^{11}C]5-HTP/PET in combination with a CT-scan, while carcinoid tumours are better visualized with [^{18}F]DOPA, a radiolabelled analogue of the precursor of dopamine [97].

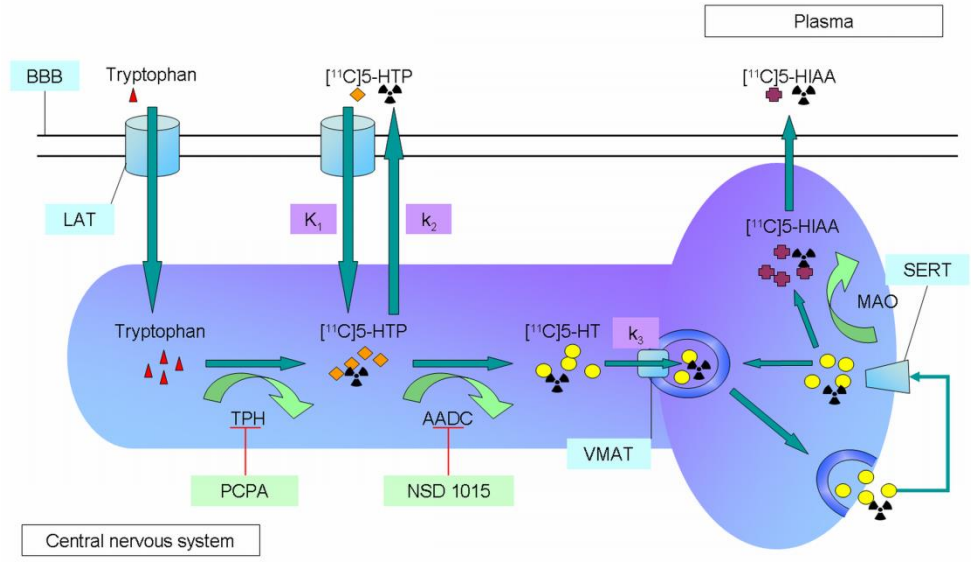

Fig 3 Metabolism of [^{11}C]5-HTP

Most of 5-HT synthesis takes place in the terminal areas. Tryptophan is acquired through the diet and is transported across the blood-brain barrier (BBB) by the large amino acid transporter (LAT). Within neurons Trp is catabolised by tryptophan hydroxylase (TPH) to 5-HTP. Subsequently, 5-HTP is converted to 5-HT by AADC. 5-HT is taken up and stored in vesicles by the vesicular monoamine transporter (VMAT). When neurons fire, the vesicles fuse with the synaptic membrane whereafter 5-HT is released within the synaptic cleft. The serotonin transporter (SERT) causes reuptake of 5-HT that can either be restored into vesicles or be broken down by monoamine oxidase (MAO) to 5-HIAA. Eventually, 5-HIAA is released into the blood stream and excreted by the kidneys. A similar process takes place in peripheral organs. Radiolabeled 5-HTP undergoes the same conversions as endogenous 5-HTP and is therefore a suitable tracer for 5-HT synthesis. A two-tissue compartment model with irreversible tracer trapping can be used for modelling [^{11}C]5-HTP kinetics. The rate constant for transport from plasma to brain is indicated by K_1, k_2 represents efflux of the tracer back into the blood stream and k_3 is the irreversible trapping constant.

However, a problem in this detection method is the high urinary concentration of carbon-11, caused by excretion of radiolabelled 5-HIAA. Inhibition of peripheral decarboxylase activity by administering the AADC inhibitor carbidopa reduces the excretion of carbon-11 and increases tracer uptake in the tumours [98,99]. The effects of carbidopa on tracer uptake have also been investigated in a xenograft model of neuroendocrine pancreatic tumours by Neels and colleagues [100]. Carbidopa improved tumour imaging also in this animal model, probably by inhibiting peripheral AADC activity and increasing availability of the tracer.

Pre-clinical data

In 1992, an initial preclinical study with [^{11}C]5-HTP for measuring cerebral 5-HT synthesis was performed in Rhesus monkeys [101]. The authors used a reference area in the brain for modelling the time-activity curves of other brain areas, in order to analyze tracer kinetics. In this model the rate constant k_3 represents irreversible tracer trapping (Fig 2&3).

Since blocking of specific enzymatic steps in the metabolic pathway had the expected effects, [^{11}C]5-HTP appeared to be a valid tracer for measurement of the rate of decarboxylation of 5-HTP to 5-HT. Blocking central AADC with NSD 1015 resulted in a decrease of the rate constant k_3 in both monkeys and rats. This constant reflects 5-HTP decarboxylation and mirrors 5-HT synthesis. The nonspecific blocking of MAO with pargyline (2 days 2 x 4 mg/kg) or the selective blocking of MAO-A with clorgyline (0.2 mg/kg) did not change the rate constant indicating that radiolabelled 5-HIAA does not readily leave the brain [101,102]. Especially in the striatum, levels of radioactivity were high and the value of k_3 was influenced by the concentration of pyridoxine or vitamin B6, the co-factor of AADC [103].

Not only 5-HTP is a substrate of AADC, but also L-DOPA, the precursor of dopamine. The affinity of AADC for 5-HTP is probably higher than for L-DOPA [104]. When unlabelled substrates were administered to increase the size of the endogenous pools, the measured value of k_3 was decreased. This indicates a limited capacity of the enzyme for substrate conversion and saturation of the decarboxylation reaction [104]. The detriment of [^{11}C]5-HTP is that AADC is not only present in serotonergic but also in dopaminergic and nor-adrenergic neurons, possibly trapping the tracer in these neurons as well [104,105].

The only experiments with [^{11}C]5-HTP in rodents were performed by Lindner and colleagues [102]. PET imaging was not performed in this study, but animals were sacrificed 40 min after tracer injection and high performance liquid chromatographic (HPLC) was used to separate [^{11}C]5-HTP from its metabolites in brain extracts. At 40 min after injection, 95% of the radioactivity within the brain originated from [^{11}C]5-HTP, [^{11}C]5-HT and [^{11}C]5-HIAA, the latter compound comprising 75% of total brain radioactivity. These data indicated an extensive metabolism of [^{11}C]5-HTP in the 5-HT synthesis pathway. Less than 5% of the cerebral radioactivity was related to other metabolites. By blocking the enzyme MAO, the fraction of 5-HT in the striatum was increased, which could be expected if MAO degrades 5-HT. Blocking of central AADC by NSD 1015 decreased the conversion of 5-HTP to 5-HT and 5-HIAA, while the blocking of peripheral AADC with carbidopa increased the brain uptake of 5-HTP, although it decreased the formation of 5-HIAA. Surprisingly, carbidopa increased k_3 in the striatum indicating increased turnover of the tracer, but it lowered k_3 in the cerebellum. The underlying mechanism is unclear.

Most of the above mentioned research was performed with a reference tissue analysis or with HPLC rather than PET. HPLC can be used in preclinical research, but PET offers opportunities to visualize the living brain in humans. The most accurate way of determining tracer uptake in tissue is to relate this to plasma input, instead of using a reference tissue. An input function derived from arterial blood samples can be used to model time-activity curves in brain to characterize the cerebral kinetics of the tracer. The most suitable model for analysis of the kinetics of [^{11}C]5-HTP is a 2-tissue compartment model with irreversible tracer trapping (Fig 3). This model is approximately the same as for [^{11}C]AMT. The individual rate constants for tracer uptake (K_1), tracer efflux (k_2) and irreversible tracer trapping (k_3) can be used for calculating the accumulation constant K_{acc} (see equation 1).

This model appears to be valid in the Rhesus monkey, as it could detect changes in AADC activity after pharmacological manipulation, and elimination of [^{11}C]5-HIAA was negligible within a scan time of 60 minutes [106].
In another study [107], the authors compared the ability of the PET tracers [^{11}C]5-HTP and [^{11}C]AMT to measure AADC activity in the monkey brain. It appeared that

these tracers had different rate constants and accumulation rates. While [^{11}C]AMT showed higher uptake of radioactivity in the brain, which is not surprising because less [^{11}C]5-HTP than [^{11}C]AMT is available in plasma, the values of K_1, k_3 and K_{acc} in striatum and thalamus were lower. The reason for a lower availability of [^{11}C]5-HTP could be extensive decarboxylation of this tracer by AADC in peripheral organs. Remarkable is the fact that although 5-HT concentrations differ highly between different brain areas, the trapping of [^{11}C]AMT is rather uniform throughout the brain, while this is not the case for [^{11}C]5-HTP [107].

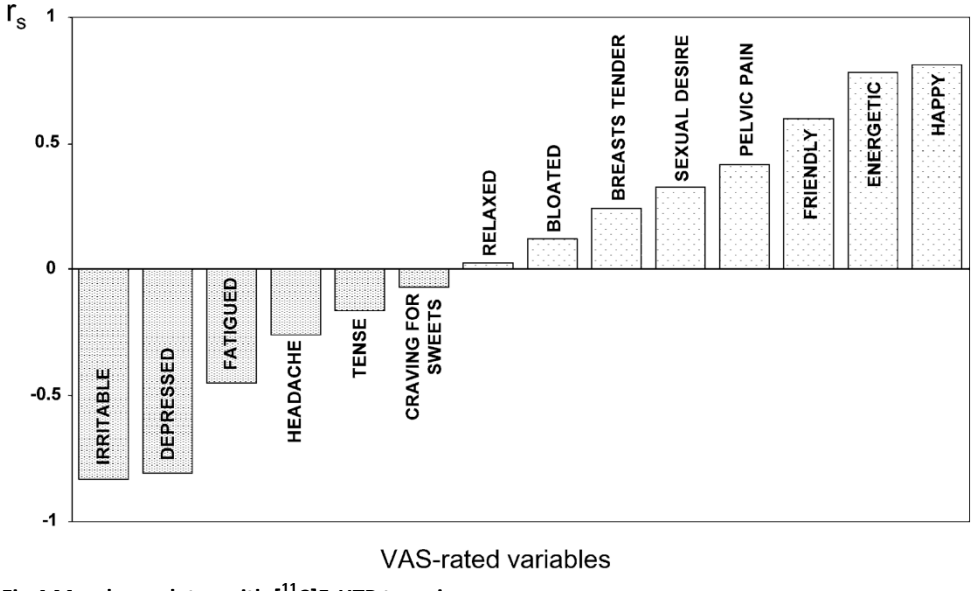

Fig 4 Mood correlates with [^{11}C]5-HTP trapping

Both positive and negative mood states are related to the amount of tracer trapping in the brain of women with premenstrual dysphoria. Especially irritability, depression, energy and happiness show strong correlations with r_s. VAS, Visual Analogue Scale; r_s, rate for [^{11}C]5-HTP irreversible trapping. Reprinted from [109], with permission from Elsevier.

Clinical data

To the best of our knowledge, the first PET study with [^{11}C]5-HTP in the human brain was performed in 1991 [108]. Patients suffering from major depression showed a reduced uptake of the tracer in their brains. A recent clinical study reported a relationship between [^{11}C]5-HTP trapping and mood states [109]. A clear negative correlation was observed between the cardinal symptoms of premenstrual dysphoria in women, like irritability and depressed mood, and

changes in tracer trapping in the entire brain (Fig 4), prefrontal regions and some regions of the striatum. The opposite mood states, feelings of happiness and mental energy, showed a strong positive correlation with tracer trapping. The same 2-tissue compartment model as was used for monkeys has been employed for PET studies of 5-HT synthesis in the human brain [110]. Tracer influx should never be rate limiting or it will lower the k_3. Hagberg and colleagues found a distribution volume above zero, indicating considerable tracer uptake in the brain. The constant that takes the distribution volume into account is the net accumulation rate constant K_{acc}, which is referred to as K^{α} in [^{11}C]AMT/PET.

There are only a few published reports on the use of [^{11}C]5-HTP for imaging 5-HT synthesis and thus, there are many opportunities for clinical studies with this tracer. The correlation of tracer trapping with mood indicates that this method may be useful for assessing the therapeutic efficacy of antidepressants. Other pathological conditions may be elucidated using [^{11}C]5-HTP/PET, such as the role of serotonergic dysfunction in eating disorders.

Discussion

The serotonergic system is complex, influencing many other neurotransmitter systems and behavioural functions. Monitoring 5-HT synthesis or other elements of serotonergic neurotransmission *in vivo* with PET gives insight into what is going on in the living brain. Research reviewed here shows the possibilities of this technique to elucidate processes otherwise not fully understood. However, refinement is necessary to increase resolution and increase target to background ratios. In addition, many elements of the 5-HT system have not yet been visualized, making the picture incomplete. The most elegant studies are studies where multiple tracers could be used, visualizing different aspects of serotonergic neurotransmission like receptor binding potential and 5-HT synthesis.

A dynamic process such as 5-HT synthesis cannot reliably be assessed by single time point measurements of 5-HT and its metabolites in CSF or blood platelets. Since 5-HT synthesis is one of the initial processes in serotonergic neurotransmission and a crucial determinant of 5-HT-mediated signal

transduction, quantification of this process by PET is of great interest. Two tracers, [^{11}C]5-HTP and [^{11}C]AMT have been developed for this purpose, which have distinct advantages and disadvantages.

A specific tracer that measures 5-HT synthesis rates may answer some of the questions about changes of 5-HT synthesis in different physiological or pathological conditions. Most important results show the effects of antidepressants on 5-HT synthesis through activation of autoreceptors, which may indicate a crucial role for 5-HT synthesis in the efficacy of antidepressants. This should be elucidated in future research.
As outlined above, more research has been done with [^{11}C]AMT than with [^{11}C]5-HTP, probably because producing [^{11}C]5-HTP is difficult, requiring several enzymatic steps [111]. At the moment it is only produced in 4-5 centres all over the world.
The most striking difference between the results of AMT and HTP studies concerns the effect of Trp depletion and its correlation with mood states. While [^{11}C]AMT detects a large decrease in 5-HT synthesis rates after acute Trp depletion, [^{11}C]5-HTP does not [79,112]. The opposite accounts for mood states; no correlation was found between [^{11}C]AMT radioactivity in the brain and Hamilton scores, whereas the brain uptake of [^{11}C]5-HTP is correlated with different mood states [88,109]. The different results obtained with [^{11}C]5-HTP and [^{11}C]AMT may be due to the fact that 5-HTP and AMT are substrates for different enzymes, AADC and TPH respectively. The tracers may measure different aspects of Trp metabolism and 5-HT synthesis. There are some reasons why [^{11}C]5-HTP may be preferred over [^{11}C]AMT:

- [^{11}C]AMT kinetics is very slow resulting in a low production of [^{11}C]AM5HT and a high fraction of trapped tracer representing [^{11}C]AMT (parent) [57].
- AMT is an analogue of Trp which behaves differently than the natural amino acid (Fig 5).
- In rats and monkeys, equilibrium between irreversible compartments and plasma is not reached within a PET time scale. As a consequence of this, Patlak modelling produces erroneous results [57,58].

- In contrast to [^{11}C]5-HTP, [^{11}C]AMT can enter the kynurenine pathway since it is an analogue of Trp. This route becomes important under inflammatory conditions and it may cause difficulties in the interpretation of [^{11}C]AMT scan data [95].

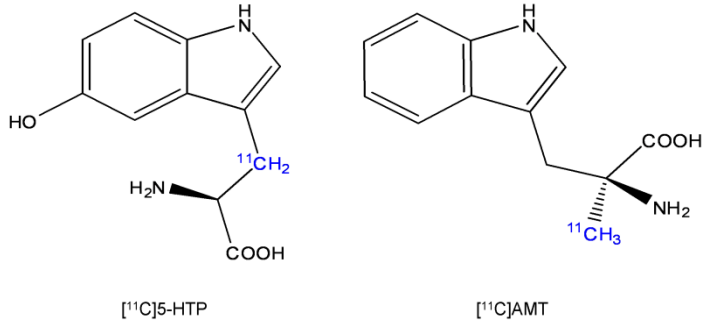

Fig 5 Chemical structures of [^{11}C]5-HTP and [^{11}C]AMT
The radionuclide ^{11}C (indicated in blue) is incorporated in the β-position of the carbon skeleton of 5-HTP, but in the methyl group of AMT.

Results obtained with [^{11}C]AMT under pathological conditions may reflect activation of the kynurenine pathway rather than 5-HT synthesis. Because 5-HTP is the endogenous direct precursor of 5-HT its metabolic fate is much less complex, even though AADC is also present in dopaminergic neurons to convert L-DOPA into dopamine. After oral administration of 5-HTP in rats, the immunoreactivity of 5-HT and 5-HTP co-localized in the raphe nuclei, but also in the dopaminergic neurons in the substantia nigra pars compacta. This suggests that [^{11}C]5-HTP could also be converted to ectopic [^{11}C]5-HT in dopaminergic neurons [113].
Thus, based upon these considerations we would prefer [^{11}C]5-HTP PET for the study of alterations of 5-HT synthesis in different pathologic conditions.
However, some prerequisites of the model used for calculating 5-HT synthesis rates with [^{11}C]5-HTP should be mentioned. Erroneous data can be obtained if the biological system does not meet the following conditions:

- AADC must operate far from saturation, so that changes in the rate of 5-HT formation can be measured. This condition is probably met, because the tissue concentration of 5-HTP is below the Michaelis-Menten constant

of AADC. However, the enzyme may approach saturation under conditions where 5-HT synthesis is strongly increased [104].

- 5-HIAA should not leave the brain within the time span of the scan. This metabolite is finally excreted, but MAO inhibition does not affect k3 indicating that within a 60 minute scan the loss of radiolabelled 5-HIAA from brain tissue is negligible [101].
- 5-HIAA from the blood should not contribute to measured radioactivity in the brain. 5-HTP is converted to 5-HT and 5-HIAA in peripheral organs. Although 5-HT cannot be transported across the BBB, 5-HIAA can. However, plasma concentrations of 5-HIAA are only large at the end of the scanning period and MAO inhibition does not change the k3. Therefore, the contribution of 5-HIAA in the circulation to cerebral radioactivity is probably minor.
- Enough tracer should enter the brain as the amount of tracer should not be rate limiting. Therefore the cerebral distribution volume must be above zero, as indicated by [110]. The delivery of [^{11}C]5-HTP to the brain could be facilitated by intraperitoneal administration of carbidopa [100].
- Synaptic transport of Trp and 5-HTP should be limited to 5-HT neurons and AADC should be specific for 5-HTP. Although L-DOPA is also a substrate of AADC, it seems to influences [^{11}C]5-HTP trapping to a lesser extent than cold 5-HTP, indicating that 5-HTP may be predominantly used by serotonergic neurons [104].

Most of these prerequisites have been investigated in humans and monkeys and the conditions for modelling [^{11}C]5-HTP kinetics seem to be met in these species, but tracer validation for microPET studies in rodents has not yet been performed. [^{11}C]5-HTP scans in rodents could be used in preclinical testing of the effects of antidepressants and provide new insight in the pathophysiology of disease. Future research should indicate whether [^{11}C]5-HTP and [^{11}C]AMT measure enzymatic activity (TPH, AADC, IDO) or the true rates of 5-HT synthesis.

The above named prerequisites of measuring 5-HT synthesis with [^{11}C]5-HTP and the fact that [^{11}C]AMT is not an ideal tracer for this purpose, emphasize the complexity of measuring 5-HT synthesis. Although most properties of [^{11}C]5-HTP seem appropriate, the difficult production of this radiopharmaceutical limits

its widespread application. Future research should concentrate on elucidating what [^{11}C]5-HTP is exactly measuring and improving tracer properties. Attempts to develop a novel tracer with improved properties should focus on: (i) specific uptake of the tracer by serotonergic neurons, (ii) chemical modification of the radiopharmaceutical so that it is no longer converted to a 5-HIAA analogue and (iii) a simplified production process.

Conclusion

We have reviewed several techniques for the evaluation of serotonin synthesis. PET can directly visualize this physiological process, whereas other techniques can only provide an indirect measurement, making it a valuable tool in clinical research. Especially because results indicate that serotonin synthesis seems to play a role in depression and antidepressant action. Although, widespread application of [^{11}C]5-HTP and [^{11}C]AMT in clinical research is not possible yet.
A unified theory of affective disorders can only be achieved if we consider different imaging methods and also take into account both animal and human histological data. In the future it may be worthwhile to develop the tools to study both receptor density and 5-HT synthesis, and this will hopefully yield a better and more complete understanding of the processes involved in the pathophysiology of affective disorders.

Conflict of interest

The authors declare that they have no conflict of interest.

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CHAPTER 3

[11C]5-HTP and microPET are not suitable for pharmacodynamic studies in the rodent brain

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Abstract

The PET tracer [^{11}C]5-hydroxytryptophan ([^{11}C]5-HTP), which is converted to [^{11}C]5-hydroxytryptamine ([^{11}C]5-HT) by aromatic amino acid decarboxylase (AADC), is thought to measure 5-HT synthesis rates. But can we measure these synthesis rates by kinetic modeling of [^{11}C]5-HTP in rat?

Male rats were scanned with [^{11}C]5-HTP (60-min) after different treatments. Scans included arterial blood sampling and metabolite analysis. 5-HT synthesis rates were calculated by a two-tissue compartment model (2TCM) with irreversible tracer trapping or Patlak analysis.
Carbidopa (inhibitor peripheral AADC) dose-dependently increased [^{11}C]5-HTP brain uptake, but did not influence 2TCM parameters. Therefore, 10 mg/kg carbidopa was applied in all subsequent study groups. These groups included treatment with NSD 1015 (general AADC inhibitor) or p-chlorophenylalanine (PCPA, inhibitor of tryptophan hydroxylase, TPH). In addition, the effect of a low-tryptophan (Trp) diet was investigated. NSD 1015 or Trp depletion did not affect any model parameters, but PCPA reduced [^{11}C]5-HTP uptake, and the k3.

This was unexpected as NSD 1015 directly inhibits the enzyme converting [^{11}C]5-HTP to [^{11}C]5-HT, suggesting that trapping of radioactivity does not distinguish between parent tracer and its metabolites. Since different results have been acquired in monkeys and humans, [^{11}C]5-HTP-PET may be suitable for measuring 5-HT synthesis in primates, but not in rodents.

Keywords: [^{11}C]5-HTP, carbidopa, kinetic modeling, PET, serotonin synthesis

Introduction

The neurotransmitter serotonin, or 5-hydroxytryptamine (5-HT), is implied in several functions of the CNS, such as regulation of mood. Antidepressants, like selective serotonin reuptake inhibitors (SSRIs), increase the levels of serotonin in the synaptic cleft by blocking the serotonin transporter (SERT) or by occupying the serotonin receptors. 5-HT synthesis may be an important aspect in the efficacy of antidepressants, as enough 5-HT should be produced for replenishing 5-HT stocks [1-3].

Serotonin is produced by neurons, of which the cell bodies lie in the raphe nuclei and project to almost every region of the brain. Serotonin is synthesized from the amino acid tryptophan (Trp), mainly in synaptic endings. This process takes place in two enzymatic steps. First, Trp is converted to 5-hydroxytryptophan (5-HTP) by the enzyme tryptophan hydroxylase (TPH). Second, 5-HTP is converted to 5-HT by aromatic amino acid decarboxylase (AADC). 5-HT is transported into vesicles by the vesicular monoamine transporter (VMAT). When serotonergic neurons fire, such vesicles fuse with the synaptic membrane to release 5-HT into the synaptic cleft. Released serotonin can be pumped back into the nerve endings by SERT, and can either be stored in vesicles or be degraded by monoamine oxidase (MAO) to 5-hydroxyindoleacetic acid (5-HIAA), which leaves the brain through the cerebrospinal fluid (CSF).

Besides activity of the enzymes involved in 5-HT synthesis, several other processes may influence the production of 5-HT. The availability of Trp for 5-HT production is the rate-limiting step in this metabolic pathway [4, 5]. Trp is transported over the blood-brain barrier (BBB) by the large neutral amino acid transporter (LAT). Transported Trp is not only used for 5-HT synthesis, but also incorporated into proteins and used for the production of kynurenine. The rate-limiting step in kynurenine production is the activity of the enzyme indoleamine 2,3-deoxygenase (IDO). Increased activity of IDO may result in reduced availability of Trp for serotonin synthesis [6].
Since direct measurement of serotonin synthesis is difficult, indirect estimates are commonly employed. 5-HT turnover rates can be measured by determining 5-

HIAA concentrations or the 5-HTP/5-HIAA ratio in CSF [7]. However, this is an invasive method, as a lumbar puncture is necessary to obtain CSF. Another method is measurement of the levels of 5-HT in blood platelets, as 5-HT is synthesized within these cells in a way similar to neurons. The validity of this procedure is questionable, for peripheral levels of 5-HT may not reflect corresponding levels in the brain [8-10].

The only direct and non-invasive methods for measuring metabolic processes in the living brain are positron emission tomography (PET) and single-photon emission computed tomography (SPECT). Several PET tracers have been produced for this purpose [7]. Radiolabeled Trp is not optimal, as Trp is also incorporated into proteins and used in the kynurenine pathway, rather than being used for 5-HT synthesis [11]. Tracer incorporation into proteins can be avoided by using a radiolabeled analog of Trp. Diksic and colleague's labeled α-methyltryptophan (AMT) with carbon-11 [12-17]. However, AMT can still enter the kynurenine pathway [18, 19]. Consequently, [^{11}C]AMT uptake reflects both 5-HT and kynurenine synthesis. Although some studies support the idea that [^{11}C]AMT-PET measures 5-HT synthesis rates [20], this may not be true under pathological conditions which are accompanied by neuroinflammation.

Another option to measure 5-HT synthesis with PET, is labeling the endogenous precursor of serotonin, [^{11}C]5-HTP [21, 22]. A great advantage of this compound is that it is metabolized exactly in the same way as endogenous 5-HTP. Several studies with [^{11}C]5-HTP have indicated that this tracer can be used to measure 5-HT synthesis rates by kinetic modeling of PET data. The rate constant for accumulation of [^{11}C]5-HTP, K_{acc}, calculated using a two-tissue compartment model with irreversible tracer trapping, reflects 5-HT synthesis rates [23, 24].

The trapping of radioactivity in brain regions represents the production of [^{11}C]5-HT and its metabolite [^{11}C]5-HIAA. When the enzyme MAO, converting 5-HT to 5-HIAA, is inhibited in monkeys, the trapping of radioactivity is unchanged [24]. This indicates that within a period of 60 min there is no significant loss of radiolabeled metabolites from monkey brain, and the trapping of ^{11}C can reflects 5-HT synthesis. Inhibition of AADC, and thus the conversion of 5-HTP to 5-HT, reduces

the value of k_3, which suggests that this rate constant is closely related to the decarboxylation of [^{11}C]5-HTP [24].
PET studies with [^{11}C]5-HTP have only been performed in monkeys or humans. To our knowledge, only one study applied [^{11}C]5-HTP in rodents [25]. Rats were sacrificed 40 min after [^{11}C]5-HTP injection and radioactive metabolites in striatum and cerebellum were determined by HPLC. The results of this analysis raise the question whether pharmacologically induced changes of 5-HT synthesis in the rodent brain can be detected also with PET. If so, small animal imaging could be used to examine the mechanism of action of antidepressants.

The enzymatic steps in 5-HT synthesis can be inhibited by administering various drugs, thus one could investigate what [^{11}C]5-HTP is exactly measuring. Levels of the precursor Trp can be reduced by feeding animals a special, Trp-free diet, lacking Trp but containing identical levels of other amino acids as the control diet. Since Trp is the direct precursor of 5-HT, we expect a decrease of 5-HT synthesis in animals fed a Trp-free diet. The first enzymatic step in 5-HT synthesis, hydroxylation of Trp by TPH, can be inhibited by para-chlorophenylalanine (PCPA), which strongly reduces 5-HT content in the rat brain [26]. As PCPA treatment reduces the conversion from Trp to 5-HTP, 5-HT synthesis should decrease as well. The second step in 5-HT synthesis, decarboxylation of 5-HTP by AADC, can be inhibited in peripheral organs by carbidopa, without effecting AADC activity in the brain [27]. Treatment of animals with carbidopa should not influence cerebral rates of 5-HT synthesis, but only reduce the peripheral metabolism of 5-HTP. When peripheral metabolism of [^{11}C]5-HTP is reduced, more parent tracer becomes available for uptake in the brain. A final compound, NSD-1015, can inhibit AADC activity both in the brain and periphery [28]. Treatment of animals with NSD-1015 should decrease 5-HT synthesis rates in rat brain, as the production of 5-HT from 5-HTP is directly inhibited. An overview of the manipulations used in this study is presented in Fig. 1.

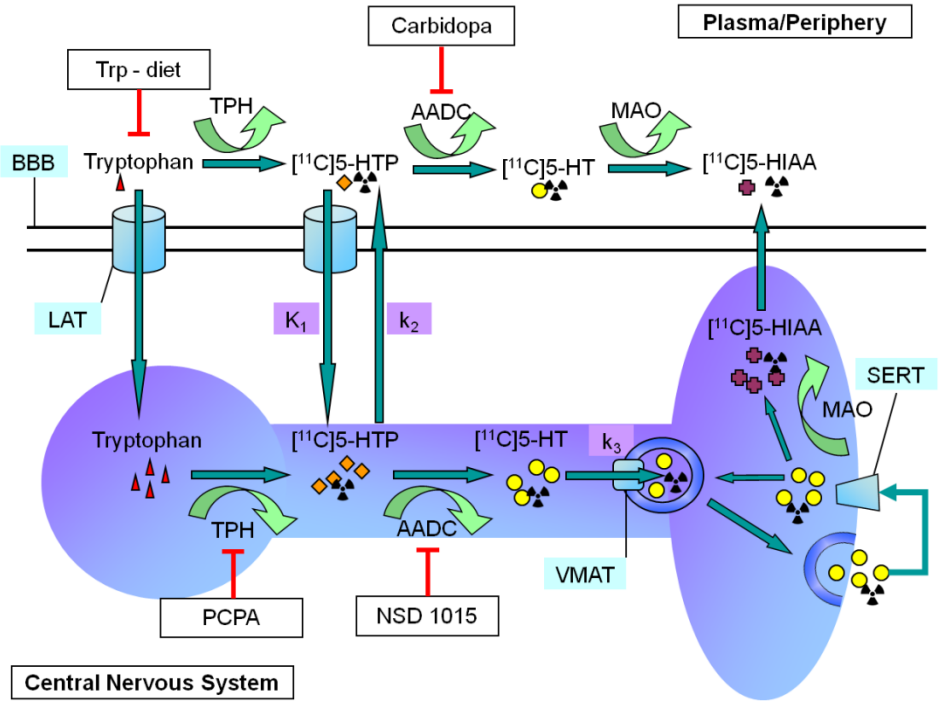

Fig 1. Manipulations in the serotonin synthesis pathway

Serotonin (5-HT) synthesis takes place within neurons in the brain and cells in the periphery. Tryptophan (Trp) is the amino acid precursor of 5-HT, which is transported by the large amino acid transporter (LAT) over the blood-brain barrier (BBB). Within the neuron, Trp is hydroxylated by tryptophan hydroxylase (TPH), the rate limiting enzyme in the synthesis pathway, to 5-HTP. In turn, 5-HTP is decarboxylated to 5-HT by aromatic amino acid decarboxylase (AADC). 5-HT is transported into vesicles by the vesicular monoamine transporter (VMAT). When these vesicles fuse with the synaptic membrane, 5-HT is released in the synaptic cleft and can be taken up by the serotonin transporter (SERT). Finally, 5-HT is metabolized to 5-hydroxyindoleacetic acid (5-HIAA) and transported out of the brain. [^{11}C]5-HTP follows the same pathway as endogenous 5-HTP. Different treatments can be used to manipulate different aspects of the 5-HT synthesis pathway. In this study we used a Trp low diet (Trp – diet) to deplete rats from the 5-HT precursor. Carbidopa was used to inhibit peripheral AADC, and NSD 1015 was used to inhibit both peripheral and central AADC. Para-chlorophenylalanine (PCPA) was used to inhibit TPH. Adjusted from Visser et al. (2010) [7].

Thus, we performed the current study which is aimed at answering the following questions: (1) Can [^{11}C]5-HTP uptake in rat brain be increased by inhibiting peripheral AADC with carbidopa? (2) Can changes of 5-HT synthesis after

pharmacologic inhibition of key enzymes be detected with [^{11}C]5-HTP and microPET?

Materials and Methods

Animals

Adult male Wistar rats (CpB:WU) weighing 317 ± 34 g were housed in pairs (Harlan, Boxmeer, The Netherlands). They were kept under a 12:12 hour light:dark cycle (light on at 7.00 a.m.) with food and water available *ad libitum*. Animals were either fed a diet with 0.025 g Trp/100 g (Trp depletion) or 0.25 g Trp/100 g (Research Diet Services, The Netherlands, for 4 days). The animal experiments were performed by licensed investigators in compliance with the Law on Animal Experiments of The Netherlands. The protocol was approved by The Institutional Animal Care and Use Committee of the University of Groningen.

Tracer and drug treatment

[^{11}C]5-HTP was produced according to a published method [22]. The average injected dose of [^{11}C]5-HTP was 12.0 ± 6.4 MBq with a specific radioactivity of 23.4 ± 11.2 GBq/µmol. All drugs were administered by i.p. injection. Carbidopa was dissolved in water (1 mg/ml or 2.5 mg/ml), and doses of 1 or 10 mg/kg were administered 1h before tracer injection. NSD 1015 was dissolved in saline at 100 mg/ml and a dose of 100 mg/kg was administered 30 min before tracer injection. Para-chlorophenylalanine (PCPA) was dissolved in saline at 150 mg/ml and a single dose of 150 mg/kg was administered on the two consecutive days before PET scanning. The second PCPA administration took place 24 hours before tracer injection.

In the initial part of this project, the dose-dependent effect of carbidopa was examined by treating groups of animals with 0, 1 or 10 mg/kg carbidopa. In the second part of the study all animals were treated with 10 mg/kg carbidopa. Separate groups of animals were used for metabolite analysis.

MicroPET acquisition and image reconstruction

Animals were anesthetized with isoflurane (5 % in medical air for induction, 2 % for maintenance). A canula was placed in the left femoral artery to enable arterial

blood sampling during PET scanning. Two canulated animals were positioned in the Siemens/Concorde MicroPET Focus220 camera, with the brain in the field of view. A 515 s-transmission scan with a ^{57}Co source was made, to allow correction of the PET images for attenuation and scatter. Tracer solution was injected through the penile vein. MicroPET data was acquired, using a list mode protocol of 96 min. The camera was started at the moment of injection of the first animal, whereas the second animal was injected after 6 min. During microPET scanning, 15 arterial blood samples (volume 0.1-0.15 ml) were drawn at different time points: 15, 30, 45, 60, 75, and 90 s; 2, 3, 5, 7.5, 10, 15, 30, 60, and 90 min. The fifteen blood samples for determination of input curves were centrifuged for 5 minutes at 13,000 rpm. Plasma was collected (25 µL) and radioactivity was measured in a calibrated γ-counter.

List mode data were reframed into 8 frames of 30 s, 3 of 60 s, 2 of 120 s, 2 of 180 s, 3 of 300 s, 3 of 600 s, 1 of 720 s and 1 of 960 s. Data of each frame were reconstructed employing an interactive algorithm (OSEM2D with Fourier rebinning, 4 iterations and 16 subsets). The final data sets consisted of 95 slices with a thickness of 0.8 mm and an in-plane image matrix of 128 x 128 pixels. Voxel size was 0.5 mm x 0.5 mm x 0.8 mm. Linear resolution of the images at the center of the field-of-view was about 1.5 mm.

Ex-vivo Biodistribution

After scanning, animals were sacrificed by extirpation of the heart under deep isoflurane anesthesia and blood was collected. The blood was centrifuged for 10 min at 6000 rpm, and plasma and a cell fraction were collected separately. Samples of different tissues were taken and weighed. Radioactivity in all samples was measured in a γ counter. Standard uptake values (SUV) were calculated using the formula:

$$\frac{Tissue\,activity\,(MBq/g)}{(Injected\ dose(MBq)/\,Bodyweight\,(g))}$$

The following brain regions were dissected: bulbi olfactorii, hippocampus, striatum, cortex, pons, medulla, cerebellum and rest of the brain.

Tryptophan concentration

Blood samples were taken after extirpation of the heart, and plasma was collected as mentioned above. About 0.3 ml of plasma was pipetted on an ultrafilter with 25 kDa cut-off (Amicon). Free and bound Trp were separated by 30 min of centrifugation at 3,000 rpm. The ultrafiltrate was frozen (-20°C) to determine free Trp at a later moment. A sample of unfiltered plasma was used to assess total Trp. Free and total Trp were measured as described previously [29].

Metabolite analysis

In separate groups blood samples (0.8-1.5 ml) were drawn for metabolite analysis at 5, 10, 20, 40, and 60 min after tracer injection. The blood was centrifuged (2 min at 13 000 rpm). Plasma was collected and 5 % perchloric acid was added. After Vortex mixing, proteins were removed by 2 min of centrifugation at 13.000 rpm. The supernatant was analyzed by high-performance liquid chromatography (HPLC), using a X-Terra C18-column (Waters, 4.6 x 250 mm). The HPLC was equipped with a UV detector (set at 254 nm). Parent [^{11}C]5-HTP was separated from radioactive metabolites using gradient elution (buffer A: 50 mM potassium dihydrogen phosphate, pH 3.3, buffer B: 30 % acetonitrile in buffer A). The gradient was programmed as follows: 0-2 min 90 % A and 10 % B; 2-10 min 60 % A and 40 % B; 10-10.1 min 0 % A and 100 % B; 10.1-15 min 0 % A and 100 % B; 15-15.1 min 90 % A and 10 % B; 15.1-17 min 90 % A and 10 % B. Flow rate was 1 ml/min and the gradient was linear.

The eluate was collected by a fraction collector, every 30 sec and every 20 sec during peak collection, and measured in a γ-counter. Fractions of parent tracer and metabolites were calculated from the radiochromatograms.

MicroPET data analysis

A volume of interest (VOI) of the whole brain was drawn on a template of the rat brain (rat scanned with [^{11}C]verapamil and treated with cyclosporine). PET images were re-aligned to this template, and the VOI was used to calculate time-activity curves (TACs). Model fits to these TACs were performed using in-house MatLab software. Using metabolite corrected plasma data as input function, a one-tissue compartment model (1TCM) or a two-tissue compartment model (2TCM) with irreversible tracer trapping were applied to fit the TACs. Blood volume was fixed to 3.6 % [30]. Only the first 62 min of the scans were analyzed. Since Patlak

analysis requires an equilibrium state, which is reached after 20 min, a 40 min data set is too short. When a longer (90 min) data set was used, there is a higher change of washout of [^{11}C]5-HIAA from the brain. We therefore used 62 min of data. The accumulation constant, K_{acc}, was calculated with the formula:

$$K_{acc} = \frac{K_1 * k_3}{k_2 + k_3}$$

A Patlak plot of the data was also made, as this graphical method can also be used to estimate the accumulation constant independent of the specific trapping model [31, 32].

Statistical analysis

For analysis of TAC's, metabolite curves and plasma curves, repeated measures ANOVA with Bonferroni posttest were used. One-way ANOVA was used for comparison of the values of K_1, k_2, k_3, K_{acc}, and Patlak constant in the different groups. Dunnett's Multiple Comparison tests were applied for further analysis of group differences. Correlations between the Patlak constant and K_{acc} were examined by Spearman rank correlation. Since we expected to observe a decrease in Trp levels in animals exposed to a low-Trp diet, measured plasma Trp-concentrations in the control and Trp depleted groups were directly compared using Student's t-test. The Akaike criterion of 1TCM and 2TCM were compared by a paired t-test, where we expect a better fit of the 2TCM. All statistical analysis was performed with GraphPad Prism 5.

Results

Tryptophan depletion

Direct comparison of the Trp depleted (N=9) and control group (N=12) indicated no significant change of total Trp, but there was a significant decrease of free Trp (-28.5 %) in plasma of animals which had been fed a low-Trp diet (t-test, t = 2.636, Df = 19, P = 0.016). One-way ANOVA showed a significant effect of treatment on total Trp levels (control, NSD, PCPA and Trp low diet) (F = 4.64, Df = 3, P = 0.009). A significant decrease in total Trp levels (-24 %) occurred in animals treated with PCPA (Dunnett's Multiple Comparison, q = 2.99, P < 0.05). The other treatments

did not affect either total or free Trp. Neither total nor free levels of Trp were correlated with K_{acc}.

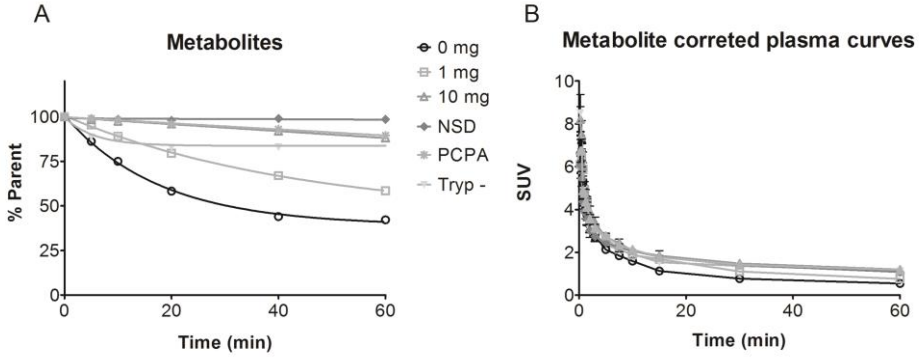

Fig 2. Metabolites and metabolite corrected plasma

A. Especially the different doses of carbidopa significantly affect metabolism of [^{11}C]5-HTP in plasma. B. Metabolite corrected plasma input curves only significantly differ in some of the first few time-points. Data are depicted as mean ± SEM.

Metabolite analysis

For all groups metabolite analysis was performed. There was a significant difference in metabolism between the different treatment groups (repeated measures ANOVA, F = 93.74, Df = 5, P < 0.0001). After Bonferroni posttests it appeared that 10 mg/kg carbidopa decreased metabolism (also in combination with the other treatments).

After metabolite correction, there was a significant difference between plasma curves (repeated measures ANOVA, F = 9.96, Df = 5, P < 0.0001). After Bonferroni posttest it appeared that this significant difference was present between almost all treatment groups, but only in the early time-points of the plasma peak. See Fig 2 for metabolite and metabolite corrected plasma curves.

Tracer uptake

Since visual inspection of microPET images and biodistribution data indicated a homogeneous uptake of [^{11}C]5-HTP in rat brain, we used TACs of whole brain ROIs for kinetic modeling.

TACs of whole brain of animals treated with different carbidopa concentrations were significantly different (repeated measures ANOVA, interaction, F = 2.4, Df =

40, P < 0.0001). After Bonferroni correction, an increase in [^{11}C]5-HTP uptake was observed between animals treated with 0 mg/kg and 10 mg/kg carbidopa (Fig 3 A). Biodistribution data also indicated a significant increase of tracer SUV in brain after carbidopa treatment (2-way ANOVA, F = 49.89, Df = 2, P < 0.0001).

Comparison of the different doses with Bonferroni posttests revealed that the SUV in the cortex was increased in animals treated with 1 mg/kg carbidopa, as compared to untreated controls, with an average SUV of 0.48 ± 0.07 for animals treated with 1 mg/kg carbidopa versus 0.15 ± 0.02 for untreated animals. Trends towards an increase were also seen in hippocampus, cerebellum, pons, medulla, and rest of the brain. At the higher dose used (10 mg/kg), carbidopa increased tracer uptake in all studied brain regions, although the change in striatum and hippocampus did not reach statistical significance (Fig 4 A). Since the effect of carbidopa appeared to be dose-dependent, we used 10 mg/kg carbidopa in all subsequent experiments in order to increase tracer delivery to the brain.

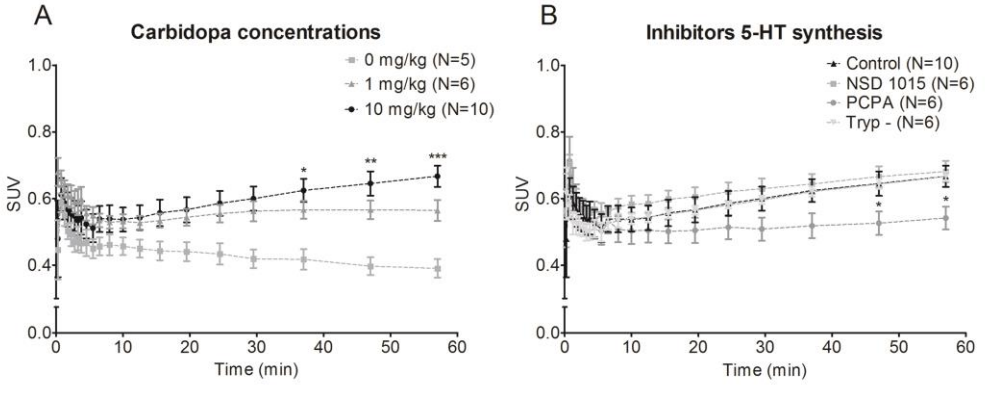

Fig 3. Time-activity curves

Time-activity curves were obtained from PET images by drawing a ROI of the whole brain. Data are depicted as mean ± SEM.

A. Both 1 mg/kg and 10 mg/kg increase the uptake of [^{11}C]5-HTP over time, although this is only significant for the 10 mg/kg dose.

B. Only PCPA had an effect on tracer uptake of [^{11}C]5-HTP over time, compared to control (10 mg/kg carbidopa).

Brain TACs indicated no significant difference in [^{11}C]5-HTP uptake in animals treated with 10 mg/kg carbidopa or the combination of 10 mg/kg carbidopa with either NSD, PCPA or a Trp low diet (repeated measures ANOVA, F = 0.45, Df = 2, P

= 0.72) (Fig 3 B). Biodistribution did indicate a significant decrease in tracer uptake after the different treatments (two-way ANOVA, F = 20.25, Df = 3, P < 0.0001). Bonferroni posttest revealed a significant difference in the PCPA treated group, in hippocampus and medulla, and a trend towards a decrease in all the other brain regions investigated. NSD 1015 and a low Trp diet did not affect brain uptake of [^{11}C]5-HTP (Fig 4 B).

Analysis of tracer uptake in peripheral organs indicated a significant increase of radioactivity due to carbidopa treatment (two-way ANOVA, F = 4.73, Df = 2, P = 0.0095). Bonferroni posttest revealed an increased radioactivity in urine after treatment of animals with 1 mg/kg carbidopa, although a large individual variation was observed. The higher dose of 10 mg/kg had the opposite effect, as the amount of radioactivity in urine was tremendously reduced. Carbidopa did not affect tracer uptake in any of the other organs, although uptake in red blood cells and pancreas tended to be increased after treatment with 10 mg/kg carbidopa, compared to untreated controls (Fig 4 C).

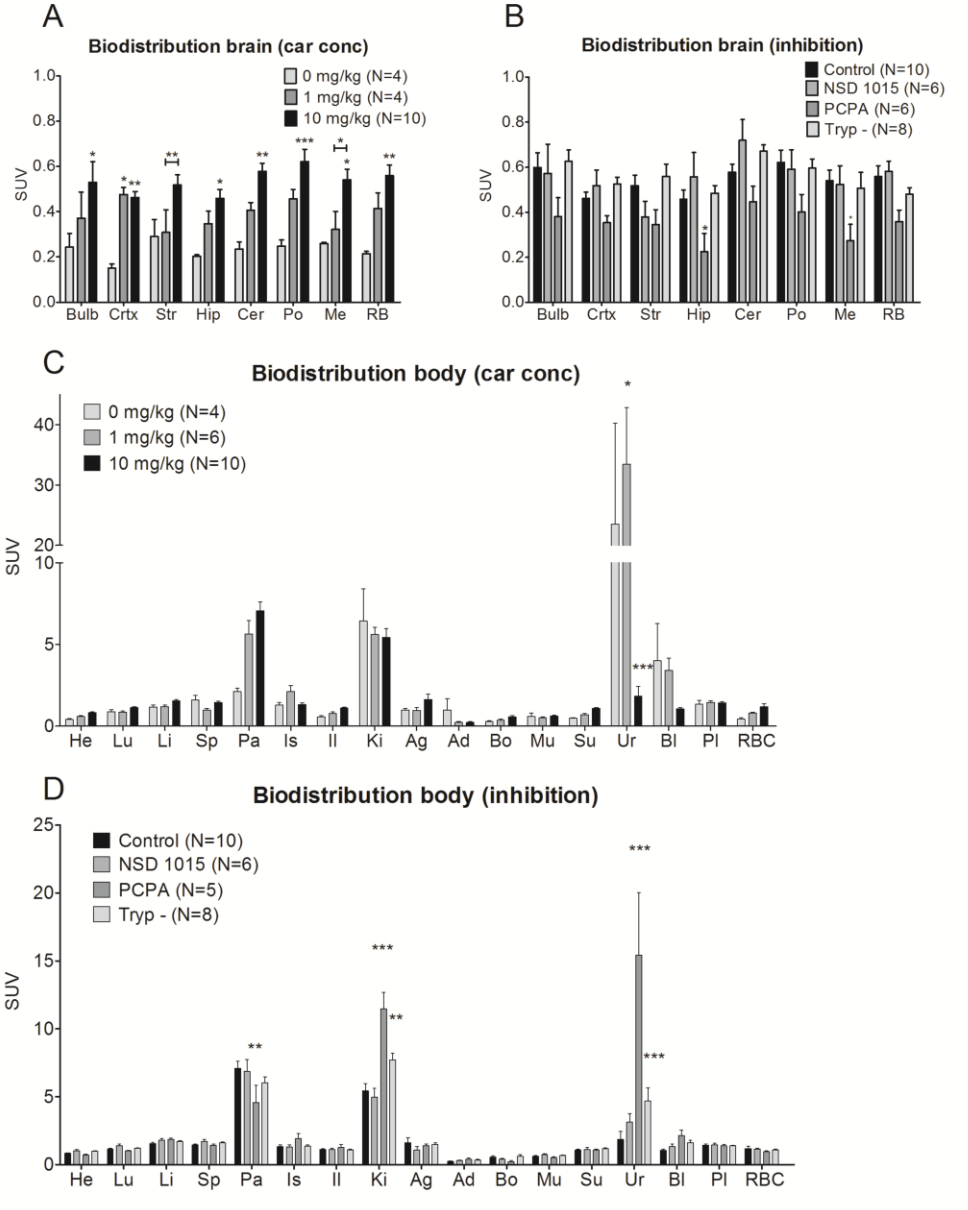

Fig 4. Biodistribution of brain regions (A, B) and peripheral organs (C, D).

After scanning, animals were sacrificed and different organs were collected for analysis in the γ-counter. Data are depicted as mean ± SEM.

A. 10 mg/kg carbidopa increased the uptake of [^{11}C]5-HTP in almost all brain regions, whereas 1 mg/kg only increased the uptake in the cortex.

B. Inhibition of TPH by PCPA decreased the uptake of [^{11}C]5-HTP in hippocampus and medulla.

C. 1 mg/kg carbidopa increased excretion of radiolabeled compounds through the urine, while a 10 mg/kg dose largely decreased excretion of these compounds. There is also high uptake of [^{11}C]5-HTP in pancreas, which tends to increase by treatment with carbidopa.
D. Inhibition of TPH by PCPA decreases uptake of [^{11}C]5-HTP in pancreas and increased uptake in kidney and urine. Trp also increased the uptake in kidney and urine.
Abbreviations A/B: Bulb: bulbus olfactorii; Crtx: cortex; Str: striatum, Hip: hippocampus; Cer: cerebellum; Po: pons, Me: medulla, RB: rest brain.
Abbreviations C/D: He: heart; Lu: lung; Li: liver; Sp: spleen; Pa: pancreas; Is: intestine small; Il: Intestine large; Ki: kidney; Ag: adrenal gland; Ad: adipose tissue; Bo: bone; Mu: muscle: Su: submandibular gland; Ur: urine; Bl: bladder; Pl: plasma; RBC: red blood cells.

PCPA reduced [^{11}C]5-HTP uptake in pancreas and increased radioactivity in kidney and urine (two-way ANOVA, F = 13.49, Df = 3, P < 0.0001). Feeding rats a low-Trp diet also increased tracer elimination via the renal route (Fig 4 D).

Kinetic modeling

A 2TCM fitted whole brain TACs better than a 1TCM, as shown by lower values of the Akaike criterion (AICc corrected for small sample size; paired t-test, t = 9.07, Df = 46, P < 0.0001), although both fits were good with an average Akaike value of 239 ± 1.8 for the 1TCM and 225 ± 2.6 for the 2TCM. Cerebral blood volume was fixed at 3.6 % in kinetic modeling, in order to avoid model fits with physiologically irrelevant high values (>> 10%) for blood volume. In some cases, fixing the blood volume also resulted in a visually better fit.

Calculated values for the Patlak rate constant correlated well with K_{acc} (r = 0.733, P = 0.0001), therefore only K_{acc} is presented in Fig 5. Data analysis with one-way ANOVA revealed that carbidopa treatment did not result in significant changes in K_{acc} (F = 0.13, Df = 2, P = 0.88) (Fig 5 A). NSD 1015, PCPA, and Trp depletion did not result in significant changes of K_{acc} either, although a trend towards a decrease was seen after 2 days of treatment with PCPA (one-way ANOVA, F = 2.76, Df = 3, P = 0.0655).

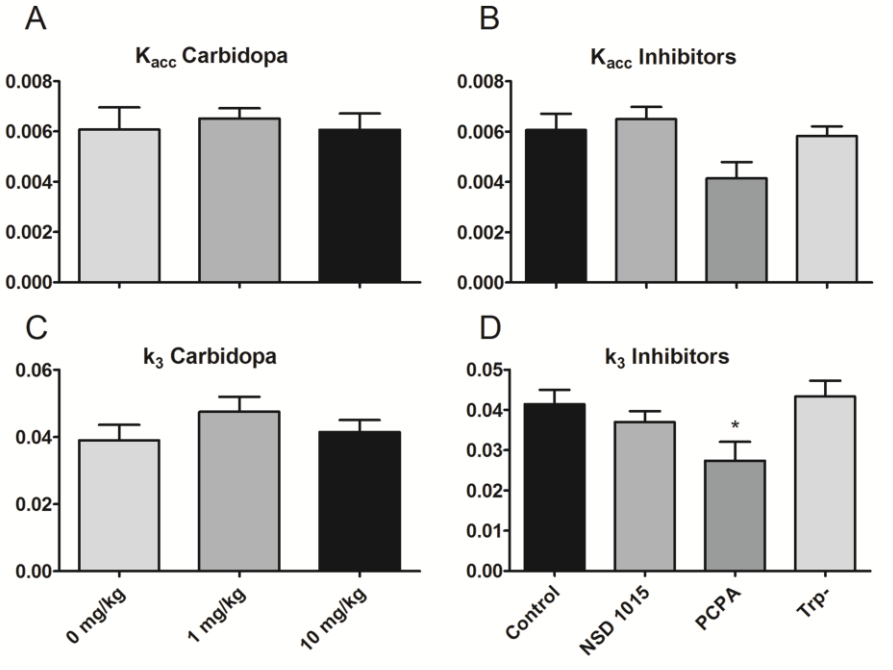

Fig 5. Rate constants

Time-activity curves and plasma-input curves were modeled in a 2TCM, rendering K_{acc} and k_3. Data are depicted as mean ± SEM.

A. Carbidopa does not influence K_{acc}

B. There is no significant effect of inhibition of TPH by PCPA, AADC by NSD 1015 or Trp depletion on K_{acc}, although a trend towards a decrease is seen after PCPA treatment.

C. Carbidopa does not influence k_3.

D. Only PCPA decreases k3 significantly.

Inter-individual variation of K_1 and k_2 was high in all groups, but values for k_3 were more stable. As this rate constant possibly reflects the enzymatic activity of AADC, it is of equally great interest as K_{acc}. There was no significant effect of carbidopa treatment on k_3 (one-way ANOVA, F = 0.9767, Df = 2, P = 0.40) (Fig 5 C). There was a significant effect of treatment on k_3 (one-way ANOVA, F = 3.26, Df = 3, P = 0.04), attributable to a reduction in k_3 after PCPA treatment (Dunnet's Multiple Comparison, q = 2.70, P < 0.05) (Fig 4 D). None of the other constants (K_1 or k_2) showed significant difference between groups, which is probably caused by the high variability between individual animals. The kinetic modeling data are summarized in Table 1.

TABLE 1. Summary of parameters determined with kinetic modeling

	K_1	k_2	k_3	K_{acc}	Patlak	AICc	C_{Trp} tot (µmol/L)	C_{Trp} free (µmol/L)
0 mg/kg car	0.37 ± 0.51	2.61 ± 3.95	0.04 ± 0.01	0.006 ± 0.002	0.003 ± 0.001	220 ± 5.59	56.3 ± 13.4	17.7 ± 3.51
1 mg/kg car	0.70 ± 0.41	4.58 ± 4.07	0.05 ± 0.01	0.007 ± 0.001	0.005 ± 0.001	228 ± 5.54	61.9 ± 4.89	21.3 ± 6.80
10 mg/kg car (control)	0.27 ± 0.36	1.75 ± 2.22	0.04 ± 0.01	0.006 ± 0.002	0.004 ± 0.001	229 ± 24.4	56.6 ± 8.67	17.7 ± 4.10
NSD 1015	0.16 ± 0.07	0.88 ± 0.42	0.04 ± 0.01	0.006 ± 0.001	0.004 ± 0.002	238 ± 5.87	55.4 ± 10.6	16.5 ± 3.05
PCPA	0.42 ± 0.40	2.69 ± 2.65	0.03 ± 0.01 [‡]	0.004 ± 0.002	0.003 ± 0.001	222 ± 11.5	41.3 ± 7.38[‡]	15.7 ± 4.02
Trp-	0.12 ± 0.07	0.88 ± 0.65	0.04 ± 0.01	0.006 ± 0.001	0.004 ± 0.001	213 ± 14.6	57.1 ± 7.54	13.3 ± 2.69[§]

K_1, k_2, k_3 and K_{acc} are determined by a two-tissue compartment model with irreversible tracer trapping. In addition, the Patlak value, Akaike criterion (AICc), and total (C_{Trp} tot) and unbound (free, C_{Trp} free) tryptophan concentrations are shown. Data are depicted as mean ± SD. [‡] Comparison of NSD 1015, PCPA and Trp- to controls, $P < 0.05$. [§] Direct comparison of Trp- to controls, $P < 0.05$.

Discussion

This study focused on two questions: (1) whether carbidopa can dose-dependently increase [^{11}C]5-HTP uptake in rat brain, and (2) whether enzyme inhibitors or Trp depletion affect [^{11}C]5-HTP trapping in the rat brain. Since [^{11}C]5-HTP is metabolized in exactly the same way as endogenous 5-HTP and [^{11}C]5-HIAA is the main radiolabeled metabolite contributing to the cerebral signal after 40-60 min, trapping of [^{11}C]5-HTP should reflect the rate of 5-HT synthesis [25].

Both the microPET and biodistribution data indicate that carbidopa increases brain uptake (SUV) in a dose-dependent way, with the greatest effect occurring at a dose of 10 mg/kg. Increased tracer uptake in the brain could result from a decrease in the peripheral breakdown of [^{11}C]5-HTP. The reduction of urinary radioactivity after carbidopa treatment, indicating decreased tracer excretion, confirmed this hypothesis. Carbidopa treatment does not influence K_{acc} or k_3, indicating that AADC activity in the brain is the same as under control conditions. Therefore, we used 10 mg /kg carbidopa in all other treatment groups.

We expected to observe a large reduction of [^{11}C]5-HTP trapping after inhibition of AADC in the brain with NSD, as this enzyme converts [^{11}C]5-HTP to [^{11}C]5-HT. Surprisingly, 100 mg/kg of NSD had no significant effect on tracer trapping. After inhibition of AADC with NSD, the total uptake of radioactivity may not be altered since PET cannot distinguish between [^{11}C]5-HT and [^{11}C]5-HTP. In order to see differences in k_3 or K_{acc}, [^{11}C]5-HTP should exit the brain and not be trapped. Such trapping was in fact measured in rat brain by Lindner and colleagues [25]. In a study with [^{11}C]5-HTP in monkeys, the brain uptake of radioactivity was even increased after administration of NSD1015, although a decrease in K_{acc} was observed [33]. The observed increase of radioactivity in monkey brain could be due to inhibition of peripheral AADC by NSD1015, an effect which we also observed in rats after carbidopa treatment. There seems to be a species difference between monkeys and rats, where the k_3 in monkeys is influenced by NSD 1015 and in rats it is not.
Besides NSD 1015, we examined the effect of PCPA, an inhibitor of the enzyme TPH, which is located upstream in the 5-HT synthesis pathway. Surprisingly, PCPA

had a significant effect on TACs calculated from the microPET data and SUV values from the biodistribution study. TPH inhibition additionally decreased k_3, and tended to decrease K_{acc}. Since neither [^{11}C]5-HTP nor [^{11}C]5-HT are a substrate for TPH, these were surprising findings. Decreases in SUV and k_3 in the brain, coincided with a decrease of the levels of total Trp in plasma, although Trp levels were not correlated with any of these values. The most likely explanation is that PCPA competes with [^{11}C]5-HTP for transport over the BBB. Competition at the level of LAT is highly probable, since PCPA has been shown to compete with Trp for BBB transport [34]. However, this competition should influence the K_1 (and thus K_{acc}), but not the decarboxylase activity rate of AADC (k_3). Another explanation is that reduced 5-HT synthesis (by inhibition of the rate-limiting enzyme TPH) also influences enzymatic activity more downstream, e.g. AADC activity.

Trp depletion did not affect the brain uptake of [^{11}C]5-HTP, although it increased tracer excretion via the renal route. We expected to see a decrease in levels of total and free (unbound) Trp in plasma, but only the free fraction was found to show a decline. The relatively minor effect of the low-Trp diet on plasma levels of free Trp explains why we could not measure any effect on serotonin synthesis rates in the brain.
An interesting observation in biodistribution studies was the high specific trapping of radioactivity in the pancreas. This may be physiologically meaningful, because 5-HT regulates insulin secretion by β-cells in the islets of Langerhans [35]. Indeed, both in vitro and in vivo studies of our laboratory have shown that serotonin synthesis and trapping of [^{11}C]5-HTP and its metabolites in rat pancreas are specific to the β-cells compared to exocrine cells [36]. So this may be an interesting finding for future studies on imaging of pancreatic β-cells.

In summary, almost all results indicate that assessment of the validity to use [^{11}C]5-HTP PET as a measure for 5-HT synthesis rates in rat failed to convincingly show alterations in response to various interventions. Drug effects on enzyme and transport activities in the metabolic pathway of [^{11}C]5-HTP cannot always be distinguished, probably causing the aforementioned problem. Kinetic modeling is based on a mathematical approximation, and it may be hard to relate individual k-values to a physiological process. A simplified model, like the 2TCM, may pool

multiple metabolic steps into a single mathematical compartment and the data presented here does not allow us to accurately decompose this process. This is probably also the reason why Lindner and colleagues could find a difference in k_3 after NSD 1015 treatment, as they assessed all the different metabolites of [^{11}C]5-HTP post mortem, in brain homogenates. Thus, they knew exactly how much radioactivity belonged to a certain compartment of the kinetic model. A PET camera cannot distinguish different radioactive species, and although k_4 seems negligible, other processes like transport and retention of unmetabolized [^{11}C]5-HTP could influence the model parameters. Eventually, this may lead to inaccurate interpretation of the individual k-values. A major source of variation may be the low uptake of [^{11}C]5-HTP in rat brain.
Although our data indicate that [^{11}C]5-HTP is not useful for measuring serotonin synthesis in rat brain, the tracer may be useful in primates since inhibitors of central AADC reduce the measured values of k_3 and K_{acc} in monkeys [33].

Conclusion

Further validation of [^{11}C]5-HTP for measurement of 5-HT synthesis rates in human brain is definitely of great interest, especially for evaluation of 5-HT synthesis rates in patients with major depression. But based on our current data, [^{11}C]5-HTP and microPET are not suitable for pharmacodynamic studies in the rodent brain.

Conflict of interest

There are no conflicts of interest.

Acknowledgements

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CHAPTER 4

Analysis of 5-HT_{2A} Receptor Binding with [^{11}C]MDL 100907 in Rats: Optimization of Kinetic Modeling

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Abstract

Purpose Preclinical PET studies are important to follow disease progression and develop new pharmacological agents. We investigated whether kinetic modeling of 5-HT_{2A} tracer [^{11}C]MDL 100907 is possible in rats.

Procedures and results Kinetic modeling with either a metabolite-corrected plasma curve or with cerebellum as a reference tissue, resulted in a good correlation of non-displaceable binding potential (BP_{ND}) calculated from a 2-tissue compartment model (2TCM) or different reference tissue models. Injecting the tracer by a slower bolus decreases the variation in 2TCM outcome parameters and results in a good correlation between k_3/k_4 and the other models. Application of 0.2 mg/kg cold MDL 100907 resulted in almost complete occupancy of 5-HT_{2A} receptors.

Conclusions A reference tissue model can be used for [^{11}C]MDL kinetic modeling in rats, which is preferable in pharmacological or longitudinal studies.

Keywords: [^{11}C]MDL 100907, PET, serotonin, 5-HT2a receptor, kinetic modeling, reference tissue

Introduction

The serotonergic system has many receptor subtypes (1-7) which are differentially distributed throughout the brain [1, 2]. The 5-HT_{2A} receptor is particularly of interest as it is present in high densities on pyramidal neurons of the prefrontal cortex, a brain area associated with many psychiatric diseases. This receptor is implicated in the pathology of anorexia nervosa [3], addiction [4], depression [5], aggression [6, 7], and anxiety [8].

Especially the therapeutic efficacy of atypical antipsychotics is attributed to their action on 5-HT_{2A} receptors [9], but also the therapeutic efficacy of antidepressants is associated with this receptor subtype [10]. Although research advances in the understanding of the involvement of the serotonergic system in psychiatric disorders and its interaction with other neurotransmitter systems, there is still no optimal treatment for these disorders and there is an urgent need for new pharmaceutical developments.

In order to understand the mechanisms underlying the pathologies and the actions of pharmaceutical therapy, physiological processes like serotonergic neurotransmission need to be measured in living human and animals. For example, it has already been shown that 5-HT_{2A} binding correlates positively with neuroticism, a personality trait often related to psychiatric pathology [11]. An advanced tool for tracing these processes in the living brain is Positron Emission Tomography (PET).

A promising ligand is the highly selective carbon-11 labeled antagonist [^{11}C]MDL 100907 ([^{11}C]MDL) with specificity for the 5-HT_{2A} receptor in the nanomolar range and at least 100 times lower affinity for other receptors [12]. This tracer has been validated in humans and monkeys. [^{11}C]MDL appears to be highly selective, does not produce lipophilic radioactive metabolites that cross the blood brain barrier (BBB), and the distribution in the brain is in accordance with the distribution of 5-HT_{2A} receptors known from post mortem studies [13, 14]. These characteristics make [^{11}C]MDL advantageous over [^{18}F]setoperone or [^{18}F]altanserin.

In both monkeys and humans, a two-tissue compartment model (2TCM) with metabolite-corrected arterial input function seemed to give the best fit to the time-activity data for calculating BP_{ND} of [^{11}C]MDL [15, 16]. If a reference tissue model can be used with cerebellum as a reference region, arterial blood sampling is no longer required to model the data. Indeed, the cerebellum appeared to be a good reference region, as no significant blocking of [^{11}C]MDL binding in this brain region was observed after treatment with mirtazapine, an antidepressant with high affinity for 5-HT_{2A} receptors [15]. However, the question whether a reference model can be used for kinetic modeling of [^{11}C]MDL is still debated, as a change of tracer binding in cerebellum after administration of unlabeled MDL in monkey was found [16]. Recently, Meyer and colleagues compared several reference tissue models with the established 2TCM as a golden standard [17]. They concluded that the non-invasive Logan's graphical analysis (NIGA) correlated best with 2TCM, and the ratio method, where BP_{ND} is estimated from late scan time tissue activity concentration ratios, showed the largest bias compared to 2TCM. Also the simplified reference tissue model (SRTM) correlated well with the 2TCM.

All these studies were performed in humans or monkeys. Only a single study focused on rodents [18]. In that study, Hirani and colleagues used volume of interest (VOI) to cerebellum ratios, obtaining total to non-specific binding ratios over a 40-min time frame, as resolution of the scans was too low to perform proper modeling. Although this approach to quantification is not ideal, their results revealed that [^{11}C]MDL cannot measure serotonin release, and that results from isoflurane anesthetized and non-anesthetized animals were closely correlated. Validation of the use of [^{11}C]MDL in rodents is important as much research focusing on psychiatric disorders is performed in these animals. Longitudinal studies can more easily be performed when arterial blood sampling is not necessary. In the future, we would like to investigate the longitudinal effects of stress on 5-HT_{2A} binding and possibly the effects of antidepressant therapy on 5-HT_{2A} binding.

Therefore, we investigated whether kinetic modeling is possible in rats, and most importantly, whether a simplified reference tissue model can be used. We used the unlabeled compound MDL 100907 to specifically block 5-HT_{2A} receptors and compared the results of various kinetic models.

Materials and methods

Animals

Adult male Wistar rats (Harlan CpB:WU) weighing 317 ± 49 g were housed in pairs and used throughout the experiment. They were kept under a 12:12 hour light:dark cycle with lights on at 7.00 a.m. with food and water available *ad libitum*. The animal experiments were performed by licensed investigators in compliance with the Law on Animal Experiments of The Netherlands. The protocol was approved by The Institutional Animal Care and Use Committee of the University of Groningen.

Tracer and drugs

[^{11}C]MDL 100907 was produced according to a published method [13]. The average injected dose was 25 ± 11 MBq with a specific activity of 63.8 ± 48.6 GBq/µmol (> 33.6 GBq/µmol). A stock solution of 1 mg/ml unlabelled MDL 100907 (ABX, Germany) was prepared in saline with 10% ethanol. This stock solution was added to the tracer, and the final concentration injected was 0.2 mg/kg in every animal from the block group. The tracer/MDL 100907 solution or tracer/vehicle solution was intravenously injected in a volume of 0.5 ml.

MicroPET scanning and reconstruction

Before PET acquisition, a femoral artery canula was placed under isoflurane anaesthesia (5% in medical air for induction, 2 % for maintenance) to enable arterial blood sampling during acquisition. After canulation, two animals were simultaneously placed, with brain in the field-of-view, in the Siemens/Concorde microPET camera (Focus220, 1.5 mm linear resolution at the centre of the field-of-view) and maintained under isoflurane anaesthesia. The scanner bed was always placed in the same position. First, a transmission scan was made with a ^{57}Co point source for 515 sec, enabling attenuation and scatter correction of the PET images. Tracer solution was administered by a bolus injection (taking approximately 10-15 sec) through the penile vein, and microPET data was acquired using a list mode protocol of 96 minutes, with brain in field-of-view. The first animal was injected at time point 0 and the second animal after 6 min to enable blood sampling. Each animal's scan was reconstructed separately, so data of each animal was

comprised of 90 min of scanning data after injection. During scanning, 15 arterial blood samples of approximately 0.1 ml were drawn at 15, 30, 45, 60, 75, and 90 s; 2, 3, 5, 7.5, 10, 15, 30, 60, and 90 min. Blood samples were centrifuged for 5 min at 6000 rpm, and radioactivity in 25 µl plasma was measured in a calibrated γ counter for input function calculation.

During reconstruction, list mode data was reframed into 8 frames of 30 s, 3 of 60 s, 2 of 120 s, 2 of 180s, 3 of 300 s, 3 of 600 s, 720 s and 960 s. Data was reconstructed per timeframe, employing an iterative algorithm (OSEM2D with Fourier rebinning, 4 iterations and 16 subsets). The final data sets consisted of 95 slices with a slice thickness of 0.8 mm and an in-plane image matrix of 128 x 128 pixels. Voxel size was 0.5 mm x 0.5 mm x 0.8 mm.

Biodistribution

After PET acquisition (96 min), anesthetized animals were sacrificed and blood was collected. The blood was centrifuged at 6000 rpm for 10 min to collect plasma and a cell fraction. Tissue samples and several brain regions were dissected, measured for radioactivity in the γ counter, and weighed. Standard uptake values (SUV) were calculated as follows:

$$SUV = \frac{Tissue\,activity\,(MBq/g)}{Injected\ dose(MBq)/\,Body\,weight(g)}$$

Metabolite analysis

For each animal, 0.3 ml of extra blood was taken for metabolite analysis at time intervals: 1, 5, 10, 30, 60, and 90 min. These samples were centrifuged for 5 min at 13.000 rpm. Plasma was collected and diluted with 2 volumes of 0.01 M $(NH_4)HPO_4$. A SEPPAK cartridge (Oasis HLB 1 cc (30g)) was used for separation of [^{11}C]MDL from its metabolites. First, diluted plasma was passed through the cartridge rendering polar metabolites in the eluate. Then, the cartridge was washed with 2 ml 0.01 M $(NH_4)HPO_4$, which also elutes polar metabolites. Unchanged parent was eluted with 1.5 ml of 30% acetonitrile in 0.1 M aqueous NaH_2PO_4. Samples were measured in the γ counter together with residual activity on the cartridge. The metabolite fraction was calculated with the formula:

$$\%Metabolites = \frac{A_{eluate}}{A_{wash} + A_{eluate}} * 100\%$$

This analytical technique was validated by HPLC analysis of the elution fractions, demonstrating that metabolites and intact tracer were well separated. Recovery of radioactivity from the SEPPAK was > 97 %.

PET data analysis

Volumes of interest (VOIs) were drawn on an MRI template [19] in Inveon Research Workplace (Siemens), and PET images were co-registered to the MRI template by manually placing markers on the PET image and MRI image. Time-activity curves were calculated for the VOIs and were fitted to the reversible 2-tissue compartment model (2TCM), which was used as the golden standard, using metabolite-corrected plasma data as input function. The ratio of radioactivity in plasma and blood cells (biodistribution assay) was used to calculate the apparent activity of whole blood. The K_1/k_2 ratio in the 2TCM was fixed by taking the average ratio of the different brain regions. In addition, the blood volume was fixed to 3.5 %.

Volumes of distribution (V_T) and binding potential values (BP_{ND}) were calculated from the 2TCM fit.

$$V_T = \frac{K_1}{k_2} * \left(1 + \frac{k_3}{k_4}\right)$$

$$BP_{ND} = \frac{V_T^{target} - V_T^{cerebellum}}{V_T^{cerebellum}}$$

To compare reference tissue models to the 2TCM, BP_{ND} was additionally calculated by SRTM [20] and NIGA [21] analysis, with cerebellum as a reference tissue. For NIGA analysis a population k_2' is needed for proper analysis. This constant was estimated by taking the average k_2' from all brain regions as calculated by the SRTM. Also an approximation of BP_{ND} was calculated by taking

the ratio of target and cerebellum SUV of the last 3 frames, consisting of totally 38 min: (SUV_T / SUV_R)-1, to see if the results of [21] are valid.
Total occupancy and non-displaceable volume of distribution (V_{ND}) were estimated using an occupancy plot as previously described [22]. In this plot, the slope of the regression line resembles total occupancy and the intercept with the x-axis resembles V_{ND}.
Delineation of the VOIs in rat brain is shown in Fig1.

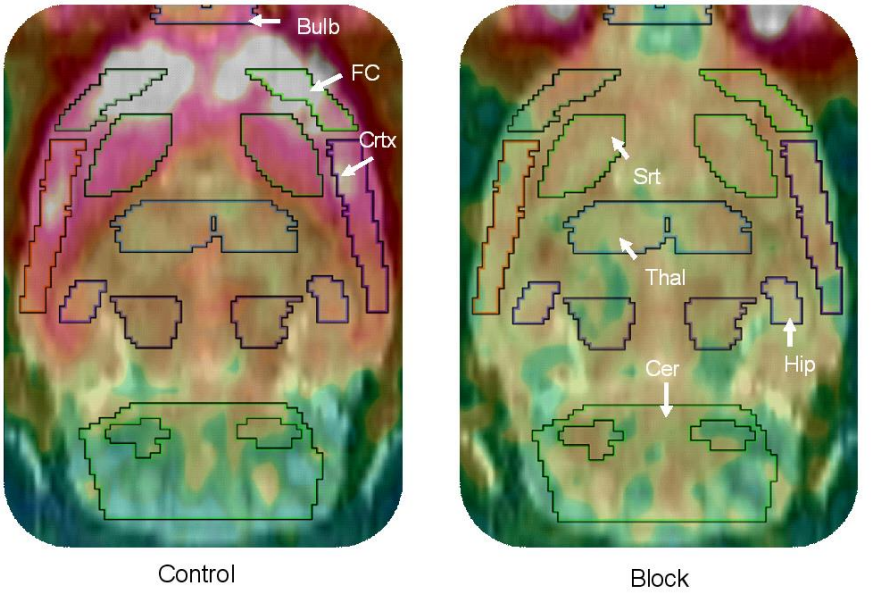

Fig 1 Delineation of ROIs
Tracer uptake and ROI delineation in a summed picture (5-74 min, time at frame midpoint) of a control and blocked rat. Abbreviations: Bulb: bulbus olfactorii; Cer: cerebellum; Crtx: cortex; FC: Frontal cortex; Hip: hippocampus; Str: striatum; Thal: thalamus.

Prolonged injection of [^{11}C]MDL 100907

Additionally, BP_{ND} was calculated by k_3/k_4 from the 2TCM, which is a less robust measure than the calculation of BP_{ND} from V_T. This measure appears to show a great variance in the calculated BP_{ND} between control animals, possibly because tracer concentration in plasma peaked before the first plasma sample was taken. Therefore, we tested if we could reduce inter-individual variability by infusing 0.5 ml [^{11}C]MDL over a 1 min period through a canula in the femoral vein, using a pump, and by taking four extra blood samples at 5-s intervals after the start of

infusion. Processing of plasma samples and kinetic modeling were performed as described before, except that the blood volume was set to unconstrained fitting, as physiologically possible values were estimated by the model, which was not the case after a fast bolus injection. To compare the amount of variation in the outcome values of the different kinetic models and injection methods, the coefficient of variation was calculated

Statistical analysis

Comparison of SUV, V_T and BP_{ND} values of the blocked and control group was conducted by two-way ANOVA with Bonferroni post-hoc test, to correct for the number of brain regions analyzed. To compare the BP_{ND} calculated by 2TCM to the BP_{ND} calculated by SRTM, NIGA or ratio method, a linear regression was used. Linear regression was also used for drawing the occupancy plot and correlating SUV of biodistribution to V_T. Metabolite curves were fitted to a two-phase decay model, with a plateau going to zero. Differences in plasma curves between blocked and control group, were investigated with repeated measures ANOVA. Differences between fast bolus and slow bolus protocol were compared by an unpaired student's t-test.

Results

TAC's and biodistribution

There was a clear difference in time-activity curves (TAC) between different brain regions, with uptake at 90 min being in the following order: FC> Str > Crtx > Bulb > amygdala > Hip > hypothalamus > Po and Med > Cer. Average SUV in frontal cortex was around 2.5 (Fig2 A/B). This was in accordance with the distribution of 5-HT_{2A} receptors known from immunohistochemical and autoradiographic studies [23, 24]. In this study, we did not observe any difference between left and right hemispheres, therefore the brain regions in each hemisphere were pooled in all further analysis.

When unlabeled MDL-100907 was co-injected with the tracer, SUV in cerebellum was not significantly different compared to controls, as shown by *ex-vivo* biodistribution. SUV in all other regions was significantly different from controls after blocking, except in Hip and Med (where a trend towards a decrease was

seen) (ANOVA, P < 0.0001; Fig2 B). Note that this is also apparent from the TAC's, as the control and blocked condition differ in regions like FC, Str, and Hip, but not in Cer (Fig2 A). The number of animals included in the study is described in the figure legends.

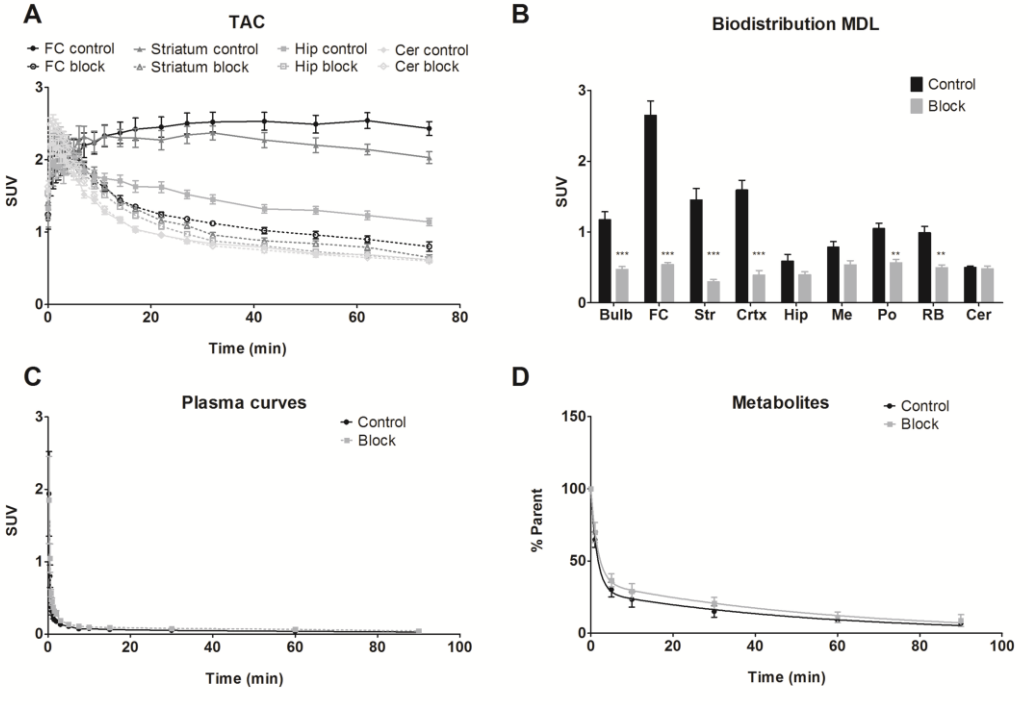

Fig 2 Activity measurements of [^{11}C]MDL

A. Time-activity curves (TAC) of different brain regions (Mean ± SEM, N = 6 for both groups).

B. Biodistribution data for different brain regions (Mean ± SEM, controls N = 6; blocked N = 7).

C. Metabolite corrected plasma curves (controls N = 6; blocked N = 6).

D. Percentage of parent tracer in plasma (controls N = 5; blocked N = 6).

Abbreviations: Bulb: bulbus olfactorii; Cer: cerebellum; Crtx: cortex; FC: Frontal cortex; Hip: hippocampus; Me: medulla; Po: pons; RB: rest brain; Str: striatum. (* P < 0.05, ** P < 0.01, *** P < 0.001). Depicted are mean ± SEM.

Plasma input curve

Metabolism of [^{11}C]MDL in plasma was fast, as after 10 minutes only 23.4 ± 5.3 % parent tracer was left in controls and 29.0 ± 5.3 % in blocked animals. There was no significant difference between the groups. The plasma input curves of all animals could be individually corrected for metabolites, as analysis was performed

with a SEPPAK cartridge, which required only small amounts of plasma. There was very little variation in metabolism between animals (Fig2 C/D).

Kinetic modeling

The data fitted well to a 2TCM with reversible binding, using the metabolite corrected plasma curve as input function, a representative fit is shown in Fig3 A. The fit for the 2TCM was better than the fit for 1TCM, as shown by a lower Akaike value for all animals of the control group, in each brain region and for almost all animals of the blocked group (ANOVA, $P < 0.001$). After Bonferroni correction, the averages in the control group are only significantly different in FC and the rest of the cortex, none of the average Akaike values are significantly different in the blocked group.

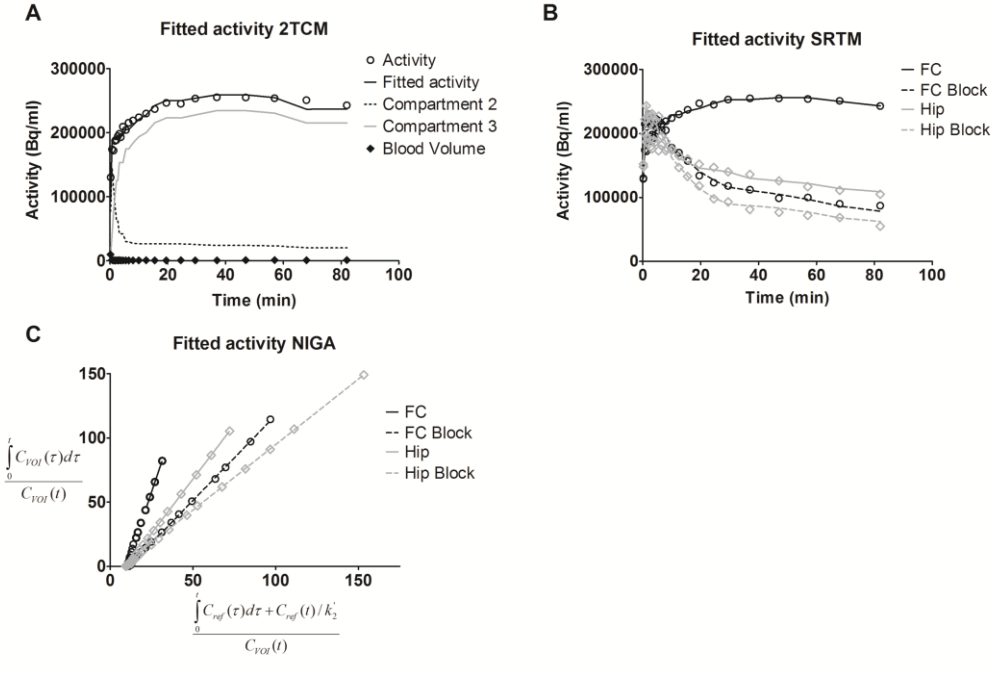

Fig 3 Kinetic model fits

A. Example of a 2TCM fit in frontal cortex of a control rat.

B. Example of a SRTM fit in frontal cortex and hippocampus of a control and blocked rat.

C. Example of a NIGA fit in frontal cortex and hippocampus of a control and blocked rat.

There was a good correlation between biodistribution and V_T after 90 min of scanning (r^2 = 0.90). Blocking the 5-HT_{2A} receptor with unlabeled MDL-100907 caused a significant reduction in V_T in all brain regions, except in cerebellum (ANOVA, P < 0.001). Bonferroni post-hoc tests also showed that tracer uptake in none of the brain regions in the blocked group was significantly different from cerebellum (Fig4 A) and BP_{ND} calculated by k_3/k_4 in cerebellum did not give a significant difference between controls (1.82 ± 1.56) and blocked animals (2.43 ± 1.83) either.

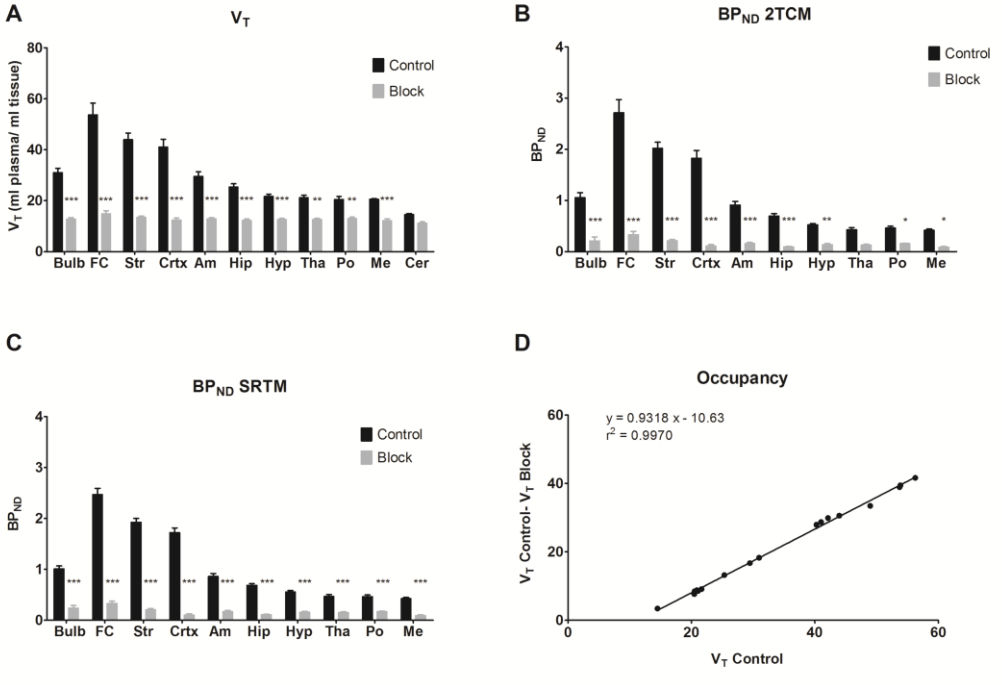

Fig 4 Kinetic modelling of [^{11}C]MDL

A. V_T calculated with 2TCM (controls N = 5; block N = 7).

B. BP_{ND} calculated with 2TCM (controls N = 5; block N = 7).

C. BP_{ND} calculated with SRTM (controls N = 7; block N = 7).

D. Occupancy plot. Linear regression indicated 93 % occupancy and a V_{ND} of 11.4.

Abbreviations: Am: amygdala; Bulb: bulbus olfactorii; Cer: cerebellum; Crtx: cortex; FC: Frontal cortex; Hip: hippocampus; Hyp: hypothalamus; Me: medulla; Po: pons; Str: striatum; Thal: thalamus. (* P < 0.05, ** P < 0.01, *** P < 0.001). Depicted are mean ± SEM.

All brain regions showed a significant reduction in BP_{ND} after blocking as calculated by 2TCM (ANOVA, P<0.001), except in the thalamus where the

difference was not significant after Bonferroni correction (Fig4 B). There was little variation in BP_{ND} calculated by 2TCM, as the COV was on average 19 % of the mean in the control group.

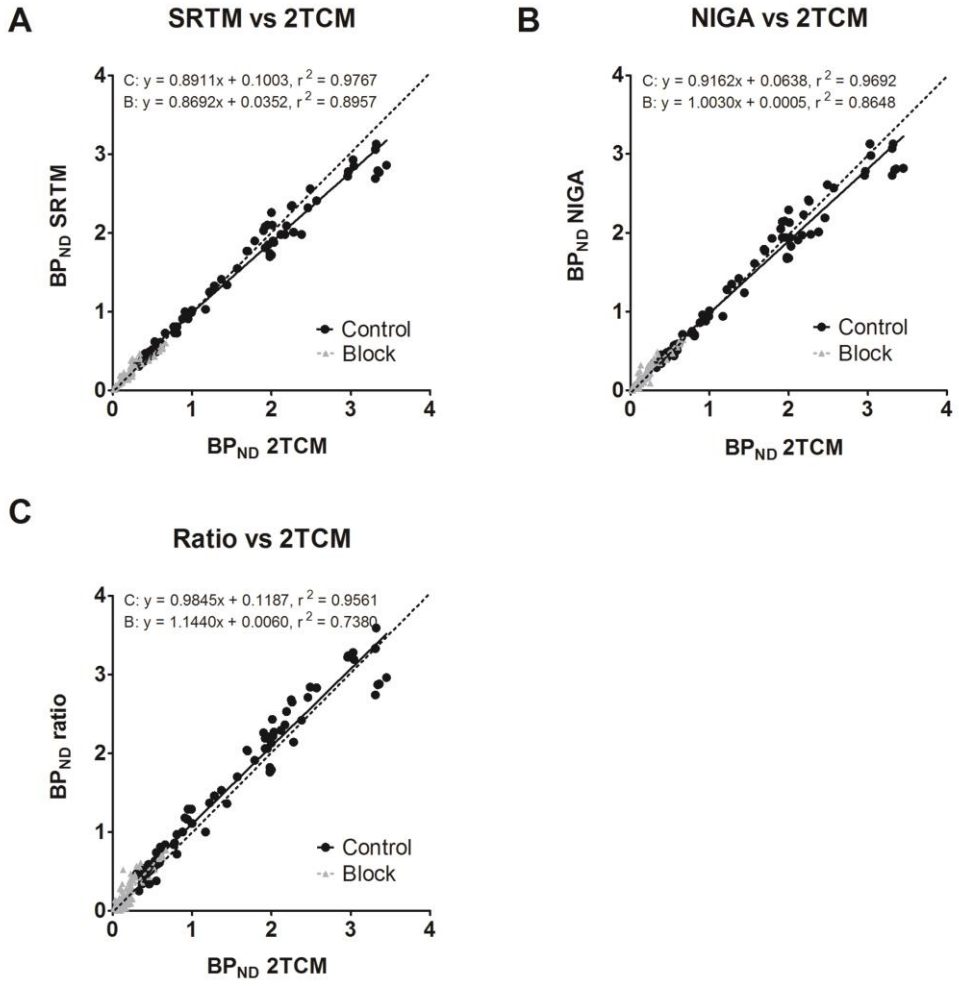

Fig 5 Comparison of 2TCM to SRTM, NIGA and ratio method

Each point represents a different brain region of an individual animal. The dotted line indicates the line of identity.

A. Linear regression 2TCM and SRTM.

B. Linear regression 2TCM and NIGA.

C. Linear regression 2TCM and ratio method.

Also the SRTM fitted well to the data (Fig3 B). Blocking by cold MDL 100907 caused a significant reduction of BP_{ND} in all brain regions investigated (ANOVA, $P < 0.001$), even after Bonferroni correction (Fig4 C). Variation in BP_{ND} was low with an average COV of 15.5 % in the control group. The correlation with 2TCM was good for both control and blocked group, however, it was better for the control group then for the blocked group (control $r^2 = 0.98$, blocked $r^2 = 0.90$) (Fig5 A).

A NIGA plot is shown in Fig 3 C, and shows a nice fit to the data. Linearization of the NIGA fit occurred approximately 20 min after the start of the scan, as was visible from the modeled graph. Therefore, data points after 22 min of scanning (frame 17) were used for the linear regression analysis of the NIGA. By blocking with cold MDL 100907, there was a significant reduction in BP_{ND} in all brain regions in a similar way as the SRTM (therefore not shown) (ANOVA, $P < 0.001$). The COV was comparable to the SRTM and on average 15 % of the mean. The correlation with 2TCM was good for both groups, but better for the control group (control $r^2 = 0.97$, blocked $r^2 = 0.86$) (Fig5 B).

When the SUV ratios, between the target region and cerebellum, of the last 38 min of the scan are used, a comparable BP_{ND} to the other models is obtained. However, there is a bit more variation, as shown by a higher COV of 22 % of the mean in the control group. The goodness of fit of the linear regression is better for the control group than for the blocked group (control $r^2 = 0.96$, blocked $r^2 = 0.74$) (Fig5 C).
For an overview of all outcome measures see table 1.

Occupancy

The occupancy of the tracer can be estimated by plotting the average V_T of all brain areas measured with the unblocked condition on the x-axis and the control minus the blocked condition on the y-axis (Fig4 D). The occupancy is defined by the slope of the linear regression ($r^2 = 0.997$), and in this study the occupancy of MDL-100907 was 93 %. The intercept of the x-axis can be used to estimate V_{ND}, which should be close to the V_T of a brain area with no specific binding. Indeed, the V_{ND} (11.4) estimated from the occupancy plot is close to the V_T of cerebellum (14.5 ± 0.92), however under blocked condition the V_T (11.1 ± 1.5) is even more close to the V_{ND}. Although there is no significant difference between the control

and blocked conditions, this indicates some amount of specific binding in cerebellum.

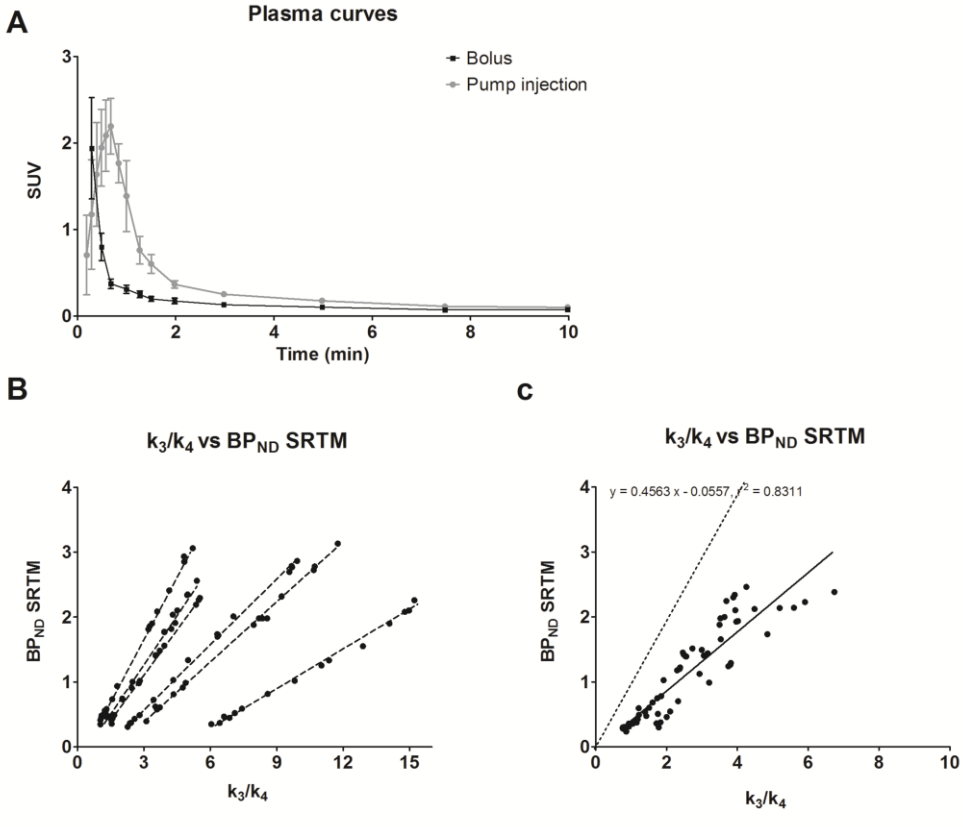

Fig 6 Bolus injection versus pump injection

A. First 10 min of the metabolite corrected plasma curves after bolus injection and infusion (Mean ± SEM, bolus N = 6; pump injection N = 4).

B. Relation k_3/k_4 and BP_{ND} SRTM bolus injection. Each point represents a different brain region of an individual animal. Each dotted line resembles one animal (6 controls).

C. Linear regression k_3/k_4 2TCM and BP_{ND} SRTM pump injection. Each point represents a different brain region of an individual animal (4 animals in total). The dotted line indicates the line of identity.

Pump injection of [^{11}C]MDL 100907

Although the 2TCM fitted well for each animal, there was a large variation in k_3/k_4 ratio per brain region between animals (Fig6 B). This could be explained by a large variation in K_1 and k_2 values. Injection of [^{11}C]MDL over a 1 min period through a

canula in the femoral vein rendered plasma peak concentrations 41 sec after the start of infusion with an average SUV of 2.20 ± 0.32, while after a bolus injection, lasting 10-15 sec, the highest average SUV of 1.94 ± 0.58 was measured at the first sample, at an average time point of 19 sec (see Fig6 A).

Indeed, most striking are the effects of slow tracer administration on K_1 and k_2, where the average values are much lower and more in a physiologically feasible range, this is significant for K_1 in Po and Med (ANOVA, $P < 0.001$). The average COV over the different brain areas is significantly lower for both K_1 and k_2 when [^{11}C]MDL is injected over 1 min (t-test, $P < 0.0001$). The COV of K_1 after bolus injection is on average 59.4 ± 4.00 % and after pump injection 16.5 ± 6.43 %, the COV of k_2 values over the different brain areas after bolus injection is on average 105 ± 14.7 % and after pump injection 35.6 ± 8.8 %. Consequently, there is a significant reduction in variation in the k_3/k_4 ratio calculated by the 2TCM, where the COV reduces from 66.6 ± 12.6 % to 32.7 ± 10.1 % (t-test, $P< 0.0001$). There is an increase in COV of k_3 and k_4 though, increasing from 66.5 ± 12.6 % to 83.4 ± 19.6 % for k_3 (t-test, $P = 0.025$) and from 36.5 ± 11.9 % to 53.2 ± 13.9 % for k_4 (t-test, $P = 0.007$). Because pump injection reduced the inter-individual variation of k_3/k_4, a good correlation between k_3/k_4 and SRTM was observed ($r^2 = 0.83$), however there is a bias between the two methods of 54 ± 3 %. The correlation between k_3/k_4 and BP_{ND} SRTM as calculated by a fast bolus or by pump injection are shown in Fig6 B/C.

TABLE 1. SUV, VT and BPND values of control and blocked groups

	SUV		V_T		BP_{ND} 2TCM		BP_{ND} SRTM		BP_{ND} NIGA		BP_{ND} ratio	
Region	**Control**	**Blocked**	**Control**	**Blocked**	**Control**	**Blocked**	**Control**	**Blocked**	**Control**	**Blocked**	**Control**	**Blocked**
Bulbus	1.17 ± 0.28 †	0.47 ± 0.11 *	31.0 ± 3.95 †	12.7 ± 1.60 *	1.06 ± 0.22	0.21 ± 0.21 *	1.01 ± 0.17	0.23 ± 0.13 *	0.97 ± 0.14	0.19 ± 0.10 *	1.22 ± 0.09	0.29 ± 0.16 *
Frontal cortex	2.65 ± 0.50 †	0.54 ± 0.08 *	53.7 ± 10.2 †	14.8 ± 3.15 *	2.71 ± 0.58	0.33 ± 0.18 *	2.47 ± 0.32	0.33 ± 0.13 *	2.54 ± 0.31	0.35 ±0.14 *	2.75 ± 0.45	0.42 ± 0.18 *
Striatum	1.45 ± 0.40 †	0.30 ± 0.08 *	44.0 ± 5.81 †	13.4 ± 1.65 *	2.02 ± 0.27	0.21 ± 0.08 *	1.93 ± 0.20	0.20 ± 0.07 *	1.98 ± 0.23	0.19 ± 0.08 *	2.19 ± 0.29	0.20 ± 0.12 *
Cortex	1.59 ± 0.34 †	0.39 ± 0.17 *	41.0 ± 6.90 †	12.3 ± 2.04 *	1.82 ± 0.35	0.11 ± 0.08 *	1.72 ± 0.24	0.10 ± 0.06 *	1.75 ± 0.27	0.10 ± 0.07 *	1.93 ± 0.34	0.12 ± 0.08 *
Amygdala			29.48 ± 4.16 †	12.8 ± 1.35 *	0.91 ± 0.16	0.16 ± 0.07 *	0.86 ± 0.16	0.17 ± 0.07 *	0.81 ± 0.14	0.15 ± 0.08 *	1.02 ± 0.17	0.19 ± 0.10 *
Hippocampus	0.60 ± 0.23 †	0.40 ± 0.12	25.3 ± 2.84 †	12.2 ± 1.67 *	0.69 ± 0.11	0.09 ± 0.03 *	0.69 ± 0.09	0.11 ± 0.04 *	0.66 ± 0.06	0.07 ± 0.04 *	0.83 ± 0.13	0.06 ± 0.03 *
Hypothalamus			21.6 ± 2.06 †	12.5 ± 1.44 *	0.52 ± 0.06	0.13 ± 0.06 *	0.55 ± 0.10	0.16 ± 0.05 *	0.51 ± 0.08	0.16 ± 0.08 *	0.55 ± 0.12	0.18 ± 0.08 *
Thalamus			21.1 ± 2.46 †	12.5 ± 1.41 *	0.43 ± 0.09	0.13 ± 0.05	0.47 ± 0.10	0.15 ± 0.04 *	0.45 ± 0.08	0.08 ± 0.06 *	0.51 ± 0.10	0.09 ± 0.08 *
Pons	1.05 ± 0.19 †	0.57 ± 0.14 *	20.5 ± 2.54 †	12.8 ± 1.88 *	0.46 ± 0.10	0.15 ± 0.03 *	0.46 ± 0.09	0.17 ± 0.03 *	0.44 ± 0.11	0.14 ± 0.05 *	0.48 ± 0.19	0.12 ± 0.06 *
Medulla	0.79 ± 0.19 †	0.54 ± 0.16	20.5 ± 0.77 †	12.1 ± 1.81 *	0.42 ± 0.06	0.09 ± 0.04 *	0.42 ± 0.07	0.09 ± 0.05 *	0.41 ± 0.06	0.06 ± 0.04 *	0.46 ± 0.11	0.04 ± 0.05 *
Cerebellum	0.50 ± 0.05	0.48 ± 0.11	14.5 ± 0.92	11.1 ± 1.51								

Mean ± SD, * blocked significantly different from control; † significantly different from cerebellum within group

Discussion

In this study, we show that an SRTM or NIGA with cerebellum as a reference tissue can be applied in rats. Our results show that there is no significant reduction in SUV or V_T in the cerebellum after 5-HT_{2A} receptor blocking, although a trend towards decrease was seen in V_T. As the difference was not significant, the amount of 5-HT_{2A} receptors in the rodent cerebellum is probably negligible. In addition, neither SUV nor V_T of the cerebellum in the blocked group was significantly different from any of the other brain regions. Finally, the V_{ND}, estimated from the occupancy plot, was similar to V_T of cerebellum, strengthening the point of cerebellum being a good reference tissue.

Indeed, when the 2TCM is compared to either the SRTM or NIGA there is a high correlation between the methods and there is hardly any bias between the BP_{ND} estimations, although there is a small tendency to an underestimation of BP_{ND} by the SRTM and NIGA. This is smaller than described in [20]. There is no big difference between SRTM and NIGA in the quality of the correlations with 2TCM. Therefore, both methods seem suitable for measuring 5-HT_{2A} binding by using the cerebellum as a reference region. It can be discussed that the SRTM actually assumes a 1TCM for both the reference region and the target region, while for [^{11}C]MDL the 2TCM gives the best fit [20]. However, there are only small differences in the quality of 2TCM and 1TCM fits. Additionally, the BP_{ND} calculated from the different models correlates well and as this assumption is not an issue in the NIGA, it can be concluded that the SRTM is applicable even when 2TCM fits a bit better. Especially the calculation of BP_{ND} does not seem to be as restricted to the assumption that the target region fits a 1TCM [25]. Also the ratio method correlates well with the 2TCM and only shows a bit more variation in the outcome measures as compared to SRTM and NIGA. Probably the ratio method is suitable for analysis as well, if full kinetic modeling is not possible.

Administration of 0.2 mg/kg unlabeled MDL-100907 resulted in an almost complete blocking of the 5-HT_{2A} receptor, as the occupancy of the receptor by the unlabeled compound was 93 %. It appears that receptor blocking in the frontal

cortex was not complete, as BP_{ND} of this brain area was significantly different from other brain areas in the blocked group. The density of 5-HT_{2A} receptors in rat brain is highest in the frontal cortex. This may explain the residual difference between frontal cortex and cerebellum in the blocked group [23]. A higher dose of unlabeled MDL-100907 is required to saturate the 5-HT_{2A} receptors in the frontal cortex. Another option is that the application of a co-injection rather than a consecutive tracer injection may generate a small offset in the apparent occupancy values.

When examining the individual constants obtained by the 2TCM, the K_1 and k_2 varied greatly, suggesting that the peaks of the plasma input curves were missed during blood sampling. The first plasma samples were taken after (not during) bolus injection, which increases the chance of missing the tracer peak during blood sampling.

Indeed, when the tracer was injected over a 1 min period through a venous canula and blood samples were drawn every 5 sec during tracer injection, the peak of radioactivity in plasma could be measured. This resulted in less variation in K_1 and k_2 and there was less variation in k_3/k_4 as calculated from the 2TCM, consequently, k_3/k_4 and SRTM correlated well (r^2 = 0.83). However, there was a large bias between k_3/k_4 and the SRTM BP_{ND}, where k_3/k_4 is more than twice as high as the BP_{ND} obtained with SRTM (or 2TCM and NIGA). The cause of this discrepancy between the models is not easily identified, but we cannot rule out that the bias is caused by a small amount of specific and a considerable amount of nonspecific ($V_{ND} \approx 11.4$) tracer binding in cerebellum. In the 2TCM more parameters are calculated (including k_3 and k_4), making the individual parameters more sensitive to variation, while the SRTM and NIGA are more robust in their outcome parameters, but also with a higher risk that the assumptions of the model are not met. Despite the bias observed between k_3/k_4 and the BP_{ND} calculated from SRTM or NIGA, they still correlate significantly and therefore we can conclude that reference models can be used to compare different groups, even if there would be a small amount of specific binding in cerebellum.

Conclusion

In future research, the SRTM or NIGA can be used for modeling [^{11}C]MDL data in rats, which makes blood sampling superfluous. Thus, longitudinal studies can be more easily performed in pharmacological research. Injection of the tracer as a slow bolus (e.g. 1 min) can greatly reduce variation in kinetic modeling outcome parameters.

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Conflict of interest

The authors declare that they have no conflict of interest.

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CHAPTER 5

Acute social defeat does not alter cerebral 5-HT_{2A} receptor binding in male Wistar rats

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Submitted

Abstract

It has been hypothesized that effects of uncontrollable stress on serotonin receptor expression contribute to the etiology of stress-related disorders like depression. While the serotonin-2A receptors (5-HT_{2A}R) are thought to be important in this context, only few studies examined effects of stress on this receptor subtype. In the present study we therefore assessed acute and long-term changes in 5HT_{2A}R binding after social defeat stress in rats.

Male Wistar rats were subjected to social defeat by placing them in the home cage of an aggressive, dominant Long Evans rat. Acute social defeat suppressed growth, but did not affect anxiety-like behavior in an open field test. A PET scan with the 5-HT_{2A}R tracer [^{11}C]MDL 100907 one day and three weeks after defeat did not show significant changes in receptor binding. To verify these results, [^{3}H]MDL 100907 binding assays were performed in prefrontal cortex and hippocampus, not showing changes in B_{max} or K_d.

These findings do not support the hypothesis that changes in 5-HT_{2A}R function are a vital mechanism through which uncontrollable stress contributes to stress-related pathologies such as depression. It remains to be determined whether effects of stress on 5HT_{2A}R binding depend on the nature of the stressor or on the characteristics of the rat strain.

Keywords: positron emission tomography, serotonin 2A receptors, social defeat, stress, anxiety, depression

Introduction

Depression is a multi-symptom pathology associated with dysfunctions in several neurotransmitter systems and molecular signaling pathways. Yet, the serotonergic system is likely to play an important role modulating the activity of many other neurotransmitter pathways in the brain [1-4].

Early studies in patients with major depression have implicated altered serotonergic signaling as a possible cause of the mood disturbance. Depressed patients often have lower levels and decreased turn-over rates of serotonin (5-hydroxytryptamine, 5-HT). Drugs and therapies that increase 5-HT levels improve mood in patients [5, 6] and the administration of the 5-HT precursor, 5-hydroxytryptophan (5-HTP) reduces relapse rates [7-9]. In contrast to the conventional view, other studies report increased 5-HT turnover rates in unmedicated patients with major depression [10]. Other aspects of the serotonergic system have been related to the pathology of depression as well, including 5-HT reuptake transporter, 5-HT synthesis and several 5-HT receptor subtypes, among others the 5-HT_{2A} receptor (5-HT_{2A}R).

Post-mortem studies indicate increased levels of 5-HT_{2A}R in frontal cortex of subjects with major depressive disorder and suicide victims [11-14]. These results are supported by imaging studies showing an increase in 5-HT_{2A}R binding in cortical regions of euthymic and medication free depressive patients [15]. Additionally, the personality trait neuroticism, known to be associated with increased risk of developing depression, is associated with higher frontolimbic 5-HT_{2A}R binding [16].

Preclinical studies support the hypothesis that 5-HT_{2A}R are involved in stress-related diseases like depression. It was shown that 5-HT_{2A}R expression is changed following an inescapable tail shock in rats. Both mRNA and protein levels are decreased in hippocampus after acute stress, while after subchronic stress it is only reduced in those animals that display learned helplessness behavior. Conversely, 5-HT_{2A}R expression in frontal cortex (FC) is increased, but only after subchronic tail shocks in the learned helpless animals [17]. Similar results were found through autoradiography, where one day of inescapable tail shock resulted in decreases of 5-HT_{2A} binding. Depending on whether animals were learned

helpless or nor, different brain regions were effected [18]. Inescapable tale shock (3 days) also causes weight loss and an elevated acoustic startle response, which both could be prevented by administration of a 5-HT_{2A} antagonist before the stress paradigm. The elevated acoustic startle response could even be prevented by administration after the stress paradigm [19].

The above mentioned clinical and preclinical studies indicate that the 5-HT_{2A}R is an interesting target for stress and depression research; yet, the available data are limited.

Social defeat stress is a commonly used and well-validated model of uncontrollable stress in rodents, in which an experimental animal is subjected to a social conflict with a dominant aggressor. Social defeat induces an acute neuroendocrine stress response that is stronger than with almost any other form of stress [20]. While these classical stress responses are often diminished within hours after exposure to the stressor, several studies have shown more persistent changes in behavior and physiology that resemble human depression, even after one or two days of defeat [20]. For example, social defeat stress was associated with reduced explorative activity, social activity, and food intake [21]; disturbances in daily rhythmicity [22, 23]; changes in the regulation of the hypothalamus-pituitary-adrenal axis [24]; and changes in serotonin receptor sensitivity [25]. Some of these changes persist for days up to weeks after the actual conflict. However, previous studies did not examine effects of social defeat stress on 5-HT_{2A}R.

A powerful in-vivo approach to investigate both the acute and long-term effects of stress on 5-HT_{2A}R function is positron emission tomography (PET). Non-displaceable binding potential (BP_{ND}) to 5-HT_{2A}R can be measured in rats with [^{11}C]MDL 100907 . We previously showed that cerebellum can be used as a reference tissue, so 5-HT_{2A}R density and affinity can be repeatedly measured in the same animal [26]. This tracer is a specific 5-HT_{2A}R antagonist [27, 28].

In the present study we investigated the acute and long-term effects of two days of subsequent social defeat on [^{11}C]MDL 100907 binding. We verified the results using receptor binding assays on hippocampal and cortical tissue, using the tritiated form of MDL 100907.

Materials and Methods

Ethics statement

The experiments were performed by licensed investigators in compliance with the Law on Animal Experiments of The Netherlands. The protocol was approved by The Institutional Animal Care and Use Committee of the University of Groningen (DEC 5529G). Scans were performed under isoflurane anesthesia. The animals were monitored for body weight and health appearance in order to minimize suffering.

Animals

The study was performed with adult, male Wistar rats weighing 353 ± 22 g at the start of the experiment (Crl:WI, Charles River, France). These Wistar rats were individually housed during the whole experiment and equally divided between two groups: home cage control (N = 8) and social defeat (N = 8). Animals were kept under a 12:12 hour light:dark cycle with lights on at 7.00 a.m., had *ad libitum* access to food and water, and were weighed every day. Ambient temperature was 21 ± 1 ºC.

Social defeat

Half of the experimental animals were subjected to social defeat stress by introducing them into the home cage of a dominant male Long Evans rat (outbred, LE/CpbHsd, Harlan, The Netherlands). These resident Long Evans rats were kept in a separate experimental room in large cages (80 x 50 x 40 cm) with a female (tubal ligated) to stimulate territorial aggression. The long Evans residents were trained and screened for aggressive behavior at least three times prior to the experiment. Only residents that attacked an intruder test animal within 1 min were used for the actual social defeat experiment.

The social defeat experiment took place at the beginning of the dark phase (red light). Females were removed before the experimental Wistar rat was introduced to the resident Long Evans. Total duration of the interaction between resident and intruder was 60 min, but physical interaction was limited to a maximum period of 10 min or ended when the Wistar rat showed clear submissive behavior (i.e. supine posture). Hereafter the animal was placed in a wire mesh cage and returned to the cage of the resident for the remainder of 60 min. In the wire mesh

cage, the experimental animals were protected from further attacks but were still in the threatening presence of the aggressor. The subsequent day, the Wistar rat underwent the same procedure, but with another resident. During the experiment home cage controls remained in their room, but were handled in a similar way to the experimental animals.

MicroPET scan, reconstruction and analysis

One day and three weeks after the second defeat experience, all animals underwent a 90-min PET scan with the 5-HT_{2A}R tracer [^{11}C]MDL 100907. Tracer was produced as previously described [28]. The average injected dose was 10.4 ± 5.0 MBq for scan 1 and 13.4 ± 5.4 MBq for scan 2. Two animals were simultaneously placed in a Siemens/Concorde microPET camera (Focus220, 1.5 mm linear resolution at the centre of the field-of-view), with the brain in the field-of-view. During the scan, animals were anesthetized with isoflurane mixed with medical air (induction 5%, maintenance 2-1.5%). First, a transmission scan was made with a ^{57}Co point source for 515 sec, enabling attenuation and scatter correction of the PET images. Tracer was injected through the penile vein and microPET data was acquired using a list mode protocol of 92 min. The first animal was injected at time point 0 and the second animal after 2 min. Each animal's scan was reconstructed separately, so data of each animal was comprised of 90 min of scanning data after injection. List mode data was reframed into 23 dynamic frames of increasing length (8 x 30 s, 3 x 60 s, 2 x 120 s, 2 x 180s, 3 x 300 s, 3 x 600 s, 1 x 720 s, 1 x 960 s). Data was reconstructed per time frame, employing an interactive algorithm (OSEM2D with Fourier rebinning, 4 iterations and 16 subsets). The final data sets consisted of 95 slices with a slice thickness of 0.8 mm and an in-plane image matrix of 128 x 128 pixels. Voxel size was 0.5 mm x 0.5 mm x 0.8 mm.

Each scan was modeled to a simplified reference tissue model (SRTM), with cerebellum as a reference tissue [29]. BP_{ND} was calculated and compared between the different groups, using INVEON.

Open field

Two days after each PET scan, the animals were subjected to a 5-min open field (OF) test to assess the effects of social defeat on explorative and anxiety-related behavior. The OF tests took place at the beginning of the dark phase under dim

light conditions in a separate test room. The animals were placed in the middle of an oval arena (126 x 85 cm) and their locomotion was recorded and analyzed with a camera and specialized tracking software (Ethovision, Noldus, Wageningen, The Netherlands). As anxious animals tend to avoid open spaces, a zone in the center of the arena was defined and the distance moved within that zone was determined. The following parameters were determined: first latency to leave the inner zone, distance moved in the inner zone, total distance moved in the whole arena, and average velocity.

Tissue homogenization

One day after the last OF test, the animals were sacrificed by decapitation under anesthesia (5 % isoflurane for 3 min). Frontal cortex and hippocampus were dissected and quickly snap frozen in liquid nitrogen. Tissues were homogenized in homogenization buffer (50 mM Tris-HCL, 150 mM NaCl and 20 mM EDTA, pH 7.4), whereafter the homogenate was centrifuged at 33 000 g for 10 min at 0-2 ºC. The supernatant was discarded and the pellet was homogenized in lysis buffer (50 mM Tris-HCL and 5 mM EDTA, pH 7.4), whereafter the homogenate was centrifuged at 1 000 g for 1 min at 0-2 ºC. This procedure was repeated and the supernatant of both runs was centrifuged at 33 000 g for 10 min at 0-2 ºC. The pellet was resuspended in 50 mM Tris-HCL containing a protease inhibitor cocktail (complete tablet, Roche). Protein concentrations were measured with a DC Protein Assay Kit (Biorad, Hercules, CA).

Binding assay

[^{3}H]MDL 100907 was kindly provided by Prof. Christer Halldin, Karolinska Institute. Brain homogenates (25 µL) of FC and hippocampus were added to different concentrations of [^{3}H]MDL 100907 (0.04-1.25 nM). The different concentrations of [^{3}H]MDL 100907 were diluted in assay buffer (50 mM Tris-HCl, pH 7.4), and the homogenate was added to 975 µL of this solution. For the determination of non-specific binding 10 µM of ketanserin was added to the assay buffer, further applying the same procedure as for total binding. All measurements were performed in duplicate. Once homogenate was added to the radioligand solutions, they were incubated on a shaker, in a stove at 37 ºC, for 90 min. After incubation, the samples were passed over a Whatman GF/B glass-fiber filter, which was pre-soaked in a 1% polyethylenimine solution. Subsequently, the filters

were washed with ice-cold assay buffer for three times. The filters were placed in scintillation tubes and 2 mL scintillation fluid (Ultima Gold) was added. Samples were incubated overnight at 4 ºC and the next day counted in the scintillation counter.

Average counts of the duplicate samples were used to configure nonlinear regression curves to model total and non-specific binding. By subtracting non-specific from total binding, specific binding was calculated. From these non-linear regression lines K_d and B_{max} were calculated using Graphpad Prism 5.0. BP was then calculated by dividing B_{max} by K_d, assuming 10 % protein/mg and a density of 1 g/mL. The free fraction of tracer in tissue can now be calculated by a formula including the BP_{ND} from the PET scan and B_{max} and K_d from the binding assay:

$$BP_{ND} = f_{ND} * \frac{B_{\max}}{K_d}$$

Statistics

All statistics were performed with GraphPad Prism 5.0. OF behavior and PET scan measures were analyzed by a two-way ANOVA with Bonferroni post-hoc test to correct for multiple comparisons. The difference in body weight gain was analyzed by repeated measures ANOVA. Outcome parameters of the binding assay were analyzed by a Student's two-sample *t*-test. The Student's two-sample *t*-test was also used to see if there were differences in OF behavior between stressed and non-stressed animals without PET scan or anesthesia. The level of significance was set at $P < 0.05$.

Results

Social defeat

All experimental Wistar rats were readily attacked by the resident cage owner within 30 sec. The average attack latency of the residents was 9.5 ± 9.8 sec on the first day, and 9.4 ± 5.2 sec on the second day. Most Wistar rats showed submission on both days. Only two animals did not show clear signs of submission on one of the days, but did show fleeing and freezing behavior. Average time to submission was 3.7 ± 2.9 min on the first day of social defeat and 4.4 ± 3.5 min on

the second day. Most experimental animals had minor bite wounds, but they healed quickly.

Body weight gain and open field

Social defeat stress significantly suppressed growth (repeated measures ANOVA, F = 9.5, Df = 1, P = 0.002). On the day of the PET scan (day 2) control animals gained 9.6 ± 1.9 g compared to day 0, while social defeat animals lost 6.6 ± 2.8 g. The effect on body weight lasted until after the PET scan, but was restored three weeks after defeat, when the second PET scan was made (Fig 1 A).

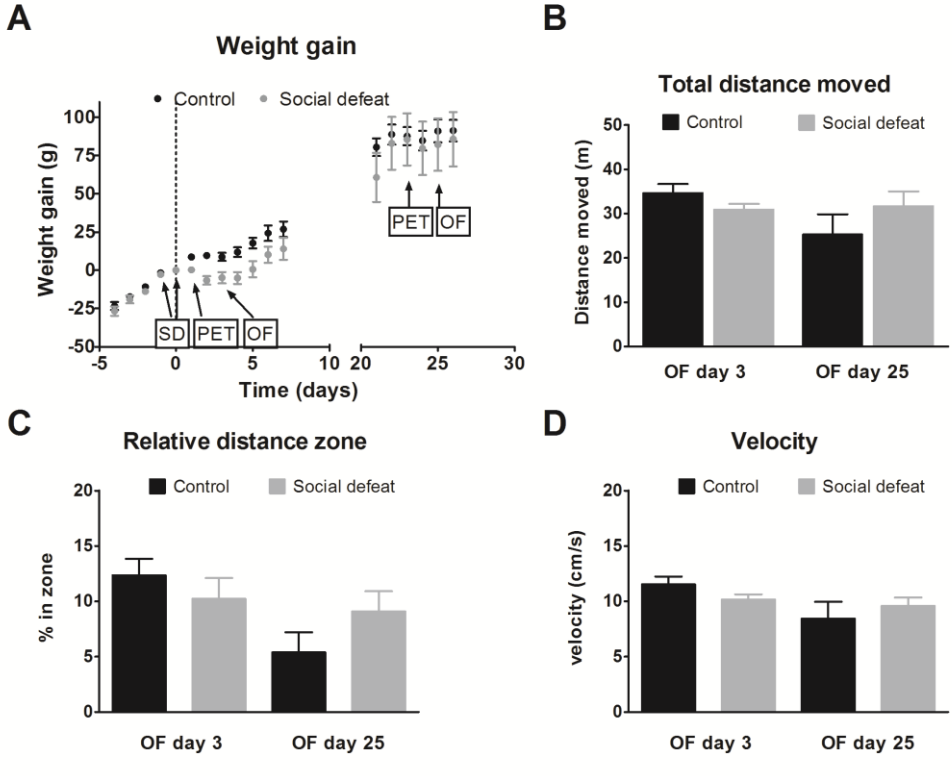

Fig 1 Body weight gain and open field

Social defeat caused a small but significant decrease in body weight gain (A). The animals recovered after three weeks. There are no significant differences in total distance moved due to defeat (B), relative distance in zone (C) or velocity (D). SD = social defeat, OF = open field. Depicted are mean ± SEM.

There were no significant differences between the groups or scan dates in the total distance moved in the OF (ANOVA, F = 2.95, Df = 3, P = 0.22, Fig 2B). The total distance moved by control animals was on average 34.6 ± 5.6 m in the first test and 25.3 ± 10.2 m in the second test. For socially defeated animals, the average total distance moved was 30.9 ± 3.7 m in the first test and 31.7 ± 9.4 m in the second test (Fig 1 B). Also distance within the inner zone (Fig 1 C), velocity (Fig 1 D), and time to leave the inner zone (not shown) did not differ between the groups. To exclude that the PET scan and the concomitant anesthesia two days before the OF influenced the behavior in the OF, we tested OF behavior in a separate group of animals, undergoing two days of defeat, but no PET scan (N = 8 for each group). Also in these groups, no effect of defeat was seen in the OF, as for example the total distance moved (t-test, t = 0.80, Df = 14, P = 0.44; data not shown).

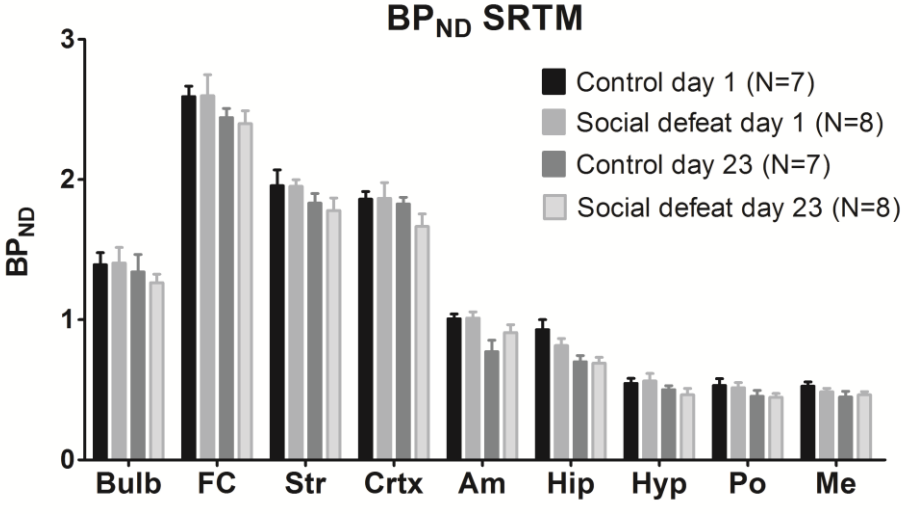

Fig 2 Binding potential PET scan [^{11}C]MDL 100907

The binding potential was calculated by the simplified reference tissue model (SRTM). There were no differences between the treatment groups. Depicted are mean ± SEM. Bulb = bulbus olfactorius, FC = frontal cortex, Str = striatum, Crtx = rest cortex, Am = amygdala, Hip = hippocampus, Po = pons, Me = medulla.

PET scan

Animals were scanned 1 day and 3 weeks after social defeat. There was no significant difference in BP_{ND} between the control and the defeat group in the first

scan (two-way ANOVA, F = 0.15, Df = 1, P = 0.70), neither was there any difference in the second scan (two-way ANOVA, F = 0.72, Df = 1, P = 0.40). The BP_{ND} between the first and second scan differed significantly and is similar for both groups (two-way ANOVA, F = 13, Df = 1, P = 0.0004), but after Bonferroni correction this effect was not significant for any individual brain region. We had to exclude one animal from the control group, because, for unknown reasons, its BP_{ND} deviated more than two times the standard deviation from the mean. The BP_{ND} is depicted in Fig 2.

Binding assay

To verify whether the absence of a social defeat effect on BP_{ND} was not caused by opposite effects on B_{max} or K_d, we conducted binding assays with [^{3}H]MDL 100907 on tissue homogenates from hippocampus and FC, collected 2 days after the last scan (Fig 3).

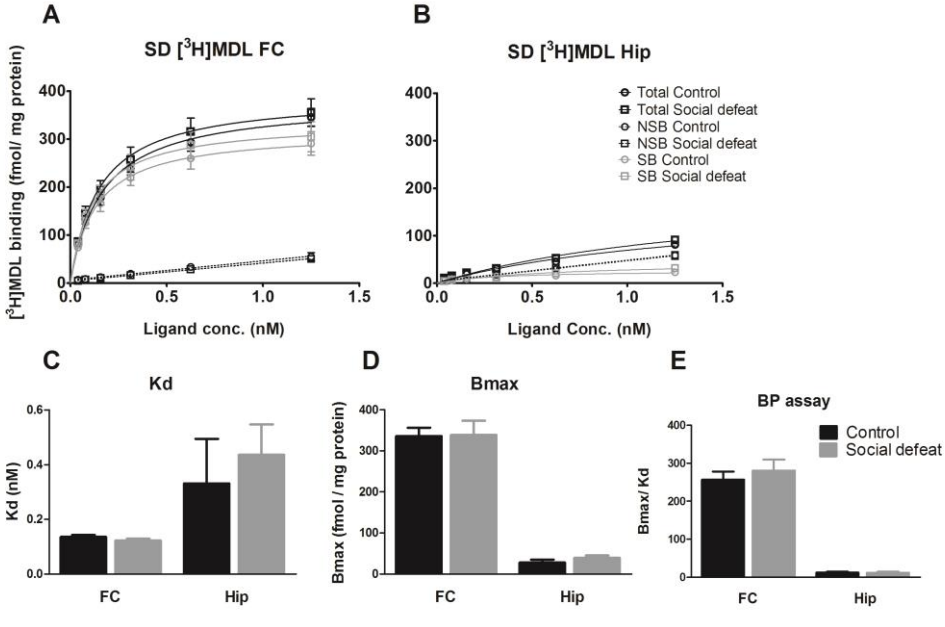

Fig 3 Binding assay [^{3}H]MDL 100907

No differences in the 5-HT_{2A} receptor affinity or concentration between social defeat and control animals were found. There were also no differences in specific binding (SB), non-specific binding (NSB) in frontal cortex (FC, A) or hippocampus (Hip, B). K_d (C), B_{max} (D) binding potential (BP), as calculated by B_{max}/K_d (E). Depicted are mean ± SEM.

No difference in binding of [^{3}H]MDL 100907 between control animals and animals subjected to defeat was found. None of the binding assay parameters (B_{max}, K_d and BP) showed significant differences between control and defeated animals (*t*-test, $P > 0.05$).

As there are far less receptors present in hippocampus, it was not surprising we found a lower affinity (higher K_d) and lower density (lower B_{max}) in this brain region. The variation in hippocampus is much greater, though, and we even had to exclude 2 animals in each group (these were outliers, as they deviated more than two times the standard deviation from the mean).
Indeed, there was a greater tissue free fraction in hippocampus, compared to FC. On average the f_{ND} in hippocampus for controls was 0.07 ± 0.04 and in defeated animals 0.07 ± 0.03. In FC the f_{ND} was on average 0.01 ± 0.003 for the control group and 0.008 ± 0.002 for the defeated group. There were no significant differences in f_{ND} between controls and defeated rats.

Discussion

In this study we investigated the acute and long-term effects of social defeat stress on cerebral 5-HT_{2A}R binding. This was done by two different methods. First, the acute and long-term effects were investigated within the same animals by means of small-animal PET. There were no effects on BP_{ND} as measured by PET 1 day or 3 weeks after social defeat. Second, these results were verified by looking at receptor numbers (B_{max}) and affinity (K_d) by using receptor binding assays in hippocampus and FC brain homogenates. Results from the binding assay confirmed the results from the PET scan, indicating that there were no differences in receptor number or affinity between control animals and animals subjected to social defeat. In addition, there were no effects found in the OF, a behavioral measure for levels of anxiety. The only effect seen after social defeat was on body weight gain, where defeated animals gained less weight than control animals.

If social defeat has opposite effects on K_d and B_{max}, BP_{ND} may be unchanged, since this parameter is calculated as B_{max}/ K_d. Because the binding assay was solely performed after three weeks we were only able to verify the long-term effect of

defeat on 5-HT_{2A}R density and affinity. However, since the results of the binding assay are in line with the PET scan, we find it well substantiated that there was no effect of defeat on 5-HT_{2A}R and combining two different analysis methods limits the chances of a Type II error. As we both calculated BP_{ND} and B_{max}/K_d, we could calculate the free fraction of radioligand in tissue. The f_{ND} was about 1 % in FC and about 7 % in hippocampus, these values correspond well to the f_{ND} of other tracers [30], and therefore show good correspondence between the PET-imaging and binding assay data in our study. High variation of B_{max} and K_d was found in hippocampus, probably caused by the low receptor concentrations in this brain area. It was indeed shown that receptor concentrations and the presence of competitive inhibitor concentrations during receptor preparation can influence B_{max} and K_d [31, 32]. However, the bias seems to be higher with increasing concentrations of the receptor, rather than lower concentrations, and is therefore unlikely to explain our variation.

There is no difference in BP_{ND}, as measured by PET, between control and stressed animals, however there seems to be a small decrease in 5-HT_{2A}R binding over time, rather than an effect of treatment. However, this effect is the same for both control and defeated animals. Human 5-HT_{2A}R binding decreases during aging, especially in early adulthood [33, 34]. This may explain why we found a difference in the BP_{ND} of [^{11}C]MDL 100907 between the first scan and the scan three weeks later. Another explanation could be the effect of anesthesia on 5-HT_{2A}R. It was shown that the standard uptake value (SUV) of [^{11}C]MDL 100907 was decreased during anesthesia in rats, although there was still a good correlation with the SUV under non-anaesthetized conditions [35]. The anesthesia effects could be long-lasting, but are equal in both the control and social defeat group.

The anesthesia or the PET scan procedure did not influence OF behavior, as was shown in a separate study where no PET scan was included in the social defeat paradigm. Therefore, we can conclude that acute social defeat does not affect OF behavior or 5-HT_{2A}R binding in the Wistar strain from Charles River. Strain differences could explain the discrepancies with other studies. Some studies did find an effect of social defeat on OF behavior, using Tyron Maze Dull S3 rats or a Wistar strain from Taconic [21, 36]. Most convincingly, direct comparison between two different rat strains revealed that social defeat stress could elicit

different effects in both anxiety-like behavior and on 5-HT_{2A}R binding in different strains [37]. However, in this study they used a chronic stress paradigm, instead of an acute stressor.

The effects of repeated defeat stress on body weight gain and food intake were more severe in Lewis rats than in spontaneous hypertensive rats (SHR) and only Lewis rats showed an increase in 5-HT_{2A}R binding of [^{3}H]ketanserin (5-HT_{2A}R antagonist) in the cortex, one day after the last defeat [37]. The explanation for this difference may be related to differences in anxiety-like behavior, as shown by the elevated plus maze. Lewis rats show increased anxiety following defeat, which was not seen in the SHR strain. If changes in anxiety and 5-HT_{2A}R binding are related, it might explain why the rat strain we used did not show differences in the OF or in 5-HT_{2A}R binding. Indeed, the 5-HT_{2A}R is known to be involved in anxiety-related behaviors [38, 39]. As the inhibited function of 5-HT_{2A}R in htr2a$^{-/-}$ mice resulted in decreased anxiety-like behavior in the OF, elevated plus maze and dark/light box, it is likely that an increase in 5-HT_{2A}R functioning can cause an increase in anxiety-like behavior, as observed in the Lewis rats.

Also, in another study, animals that showed learned helpless behavior showed much stronger effects of tail shock stress on 5-HT_{2A}R mRNA and protein levels than animals that were not learned helpless [17]. This suggests that certain strains or animals with certain characteristics are more vulnerable for stress effects on 5-HT_{2A}R. A human study has indeed shown that neuroticism and especially vulnerability relates to 5-HT_{2A}R binding [16].

Besides strain differences, the type of stressor could also contribute to the absence of an effect of social defeat. In this study we wanted to start with an acute social stressor and investigate the short- and long term effects. It is important to take temporal effects into account as previous studies have shown that social defeat can cause effects that are stronger after 2 weeks compared to one day after defeat, for example on 5-HT_{1A} receptor sensitivity. Longitudinal effects of a single day of social defeat on OF behavior were also found in the study of Meerlo and colleagues (1996), hence the choice of this behavioral test in this study. Rats showed a reduction in OF activity which lasted until 7 days after the defeat. The latency to move from the inner zone to the outer zone of the arena

was even increased up to 4 weeks after the defeat. Also other effects (behavioural, neurochemical, neuroendocrine, and physiological) of social defeat are temporally dynamic in nature, where some effects are acute (hours to a few days), others intermediate (up to one week), and others longlasting (until weeks after the defeat). These effects are nicely described in previous studies [20, 25].

A logical next step would be to investigate the chronic effects of social defeat, as effects might be more robust. However, that was not the question that we wanted to answer within this study. It might be that physical stress (e.g. tail shock, which is a widely used stress paradigm) would have a greater impact on 5-HT_{2A} receptors as compared to social stress.

For future research, it is warranted to further elucidate the effects of stress in animals with different behavioral traits or characteristics in anxiety- or depressive-like behavior. This may reveal more clearly what defines the conditions under which the density and affinity of 5-HT_{2A}R are influenced, and whether 5-HT_{2A}R binding is a state or trait characteristic. Because, so far, conflicting results exist concerning whether 5-HT_{2A}R binding can be influenced by a changing environment.

Conclusion

Acute social defeat, for two consecutive days, did not trigger changes in 5-HT_{2A}R binding in our Wistar rat strain. This strain did not show signs of increased anxiety in OF behavior after defeat, which can be related to the lack of change in 5-HT_{2A}R binding. It may be that the effects of stress on 5-HT_{2A}R are related to personality traits or characteristics of the animal strain. Future research should be aimed at elucidating this relationship.

Conflict of interest

There are no conflicts of interest to declare.

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CHAPTER 6

Identical serotonin-2A receptor binding in rats with different coping styles or levels of aggression

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Submitted

Abstract

Individuals in a population may consistently differ in the way they cope behaviorally and physiologically with stressors. Individual differentiation in coping styles emerges as a function of underlying variability in the activation of a mesocorticolimbic brain circuitry that includes the prefrontal cortex and hippocampus, and its key neurochemical signaling pathways. Among these neurochemical mechanisms, variability in the functional dynamics of particularly serotonin (5-HT)activity seems to play an important role. Serotonin-2A receptors (5-HT_{2A}R) within the prefrontal cortex represent a potential molecular mechanism through which 5-HT can modulate this mesocorticolimbic circuitry. Therefore, In this study we assessed serotonin-2A receptor binding in individual animals differing in coping style (proactive vs reactive) that originated from two different rodent strains: the Wild-type Groningen (WTG, high- and low aggressive) and Roman high (RHA)- and low avoidance rats (RLA). WTG rats were characterized by their level of aggressiveness, as tested in the resident-intruder test, and Roman's were bred for their different behavior in the active avoidance task. 5-HT_{2A}R binding in (pre)frontal cortex (FC) and hippocampus was investigated in binding assays, using a radiolabeled antagonist ([^{3}H]MDL 100907, [^{3}H]MDL) and agonist ([^{3}H]Cimbi-36).

[^{3}H]MDL displayed a higher specific-to-nonspecific binding ratio than [^{3}H]Cimbi-36. Binding parameters determined in FC were accurate but more difficult to assess in hippocampus, probably because of a lower 5-HT_{2A}R density in this region. No differences in 5-HT_{2A}R binding (B_{max}, K_d or BP) were observed in animals with different coping styles (in WTG or Roman strain) or levels of aggression (in WTG).

Our findings suggest that 5-HT_{2A}R binding is not an important molecular marker for coping style. Since neither an antagonist nor an agonist tracer showed any binding differences, it is unlikely that the affinity state of the 5-HT_{2A}R is co-varying with levels of aggression or active avoidance.

Keywords: 5-HT2A receptor, coping style, serotonin, aggression, stress

Introduction

A large number of studies have shown that individuals of the same species consistently differ in the way they cope behaviorally and physiologically with stress along an axis polarized at the two extremes by proactive or active and reactive or passive responses [1]. A proactive coping style is generally characterized by a high level of active avoidance, aggression, impulsivity and other bold/extraverted actions indicating active attempts to counteract a stressful stimulus. Reactive coping, on the other hand, involves immobility, low aggressiveness and impulsivity, and a general tendency to passively accept or introvertedly shy away from similar stressful situations [2]. In addition, these different behavioral coping styles are also associated with distinct patterns of both autonomic nervous and neuroendocrine (re)activity patterns [1]. It is generally accepted that this individual differentiation in behavioral and physiological coping styles emerges as a function of an underlying variability in the activation pattern of a basic mesocorticolimbic brain circuitry that includes the prefrontal cortex, amygdala, hypothalamus, hippocampus and their common output projection nodes. The functioning of this brain network is tightly controlled by the brainstem ascending monoaminergic inputs .

Especially the serotonergic system has been intensively investigated in relation to coping style. Animals with different coping styles show differences in functional sensitivity of the 5-HT1A receptor, expressed as autoreceptor on cell bodies in the raphe nucleus and postsynaptically on neuronal terminals [1-4]. These results indicate that the responsiveness of the serotonergic system may be related to coping style, and therefore be a characteristic of an individual.

Another candidate, which is less thoroughly investigated, is the 5-HT_{2A} receptor (5-HT_{2A}R). Various clinical studies have shown differences of 5-HT_{2A}R binding in subpopulations but a relationship with coping style has not been clearly established. Instead, the 5-HT_{2A}R has been investigated most frequently in relation to aggressiveness, which in turn may be related to general coping style. However, literature findings are not consistent. Violent aggression in humans was related to *decreased* binding potential (BP_{ND}) of the PET tracer [^{18}F]setoperone in prefrontal cortex, especially at young age [5]. Similarly, using the PET tracer

[^{11}C]MDL 100907, *lower* 5-HT$_{2A}$R availability was found across cortical regions in males with extreme levels of impulsive aggression without callous unemotional traits as compared to males with low impulsive traits [6]. On the other hand, two other PET-studies have reported that prefrontal cortex 5-HT2A receptor binding is *higher* in physically aggressive patients with impulsive-aggressive personality disorder [7], and in patients with borderline personality disorder [8] as compared to healthy controls. Another interesting finding is that 5-HT$_{2A}$R binding in the human brain is related to neuroticism, especially the subscale vulnerability, perhaps indicating that this receptor is a trait characteristic [9]. In addition, a post-mortem study has shown that 5-HT2A receptor expression in the prefrontal cortex is positively correlated with life-time aggression in subjects who committed suicide, but not in subjects who died from non-neurological causes [10]. However, another recent study using a large sample of healthy individuals was unable to find a consistent relationship between frontal cortex 5-HT$_{2A}$R binding and trait aggression and trait impulsivity [11]. As these human studies involved different patient groups and employed different radiotracers for 5-HT$_2$ receptor imaging, results cannot be directly compared.

Concerning studies in animals, the relationship between 5-HT2AR properties and aggression are studied as well [12, 13]. There is no change in functional sensitivity of 5-HT$_{2A}$R in Norway rats bred for high aggression levels, compared to rats without high aggression levels. Also 5-HT$_{2A}$R levels did not differ [12]. In hamsters, 5-HT$_{2A}$R expression did not change after a social defeat challenge, either in subordinate or dominant animals, as tested by immunohistochemistry [13]. It must be noted, however, that the specificity of antibodies for 5-HT$_{2A}$R staining is questionable [14]. Other studies did show differences in 5-HT$_{2A}$R binding, as studied by SPECT in impulsive, aggressive dogs. These dogs showed increased 5-HT$_{2A}$R binding in cortical areas, which could be ameliorated by administration of the antidepressant citalopram [15, 16].

Supporting evidence for the existence of general individual differences in coping style comes from research focusing on the amount of aggression displayed by rodents, when they encounter an opponent. One animal strain showing such differences is the Wild-type Groningen (WTG) rat, which is bred from animals originating in the wild. WTG rats not only differ in their level of aggression, but

show also either proactive (active) or reactive (passive) interaction with a changing environment [17-19].

Differences in coping style have also been noted when the active avoidance of a shock was examined in rodents. This research has led to the Roman high- and low avoidance rat strains (RHA vs RLA). The difference in active avoidance of these two genetically selected strains could be interpreted as RHA being proactive and RLA being reactive copers [20-22]. However, when tested in behavioral paradigms for anxiety, the distribution range was different from that when animals were tested for coping style, so RHA and RLA differ in both coping style and anxiety, whereas these are independent vectors [22]. This is the basis for the two-tier model, wherein emotional reactivity is a separate dimension from coping style. Coping style in the WTG rat is also not correlated with anxiety, as measured in the elevated plus-maze [17]. While WTG rats are selected on the basis of their aggression level, RHA and RLA are selected based on their genetics. They do not differ in offensive aggression, but rather in anxiety [23, 24]. One animal study found an increased binding of [^{3}H]ketanserin (a 5-HT$_{2A}$R antagonist) in RHA compared to RLA rats [25], indicating that 5-HT$_{2A}$R might be related to coping style. In conclusion, it remains unclear what the relation is between aggression, coping style and 5-HT$_{2A}$R.

The current study aimed to investigate if the binding of the ligands [^{3}H]MDL 100907 ([^{3}H]MDL, 5-HT$_{2A}$antagonist) and [^{3}H]Cimbi-36 ([^{3}H]Cimbi, 5-HT$_{2A/2C}$ agonist) differs between rats displaying different coping styles [26-29]. Comparing an antagonist with an agonist may be interesting as it is hypothesized that an agonist mainly binds to high affinity states of the receptor, while an antagonist binds to both the low- and high affinity states [30].

Material and Methods

Animals

We used two different rat models to assess the relationship between coping style and 5HT$_{2A}$R binding. The first model consisted of the adult, male WTG rats (16) and the second model of RHA and RLA rats (16, outbred). All animals were 3 months old and bred in our own facilities. Proactive and reactive WTG rats can be selected through their level of aggression in the resident-intruder test (high- and

low agression levels, respectively), while the RHA and RLA animals are selectively bred for their proactive and reactive coping styles, or high- and low avoidance.
All animals were kept under a 12:12 hour light:dark cycle with lights on at 19:00h and food and water available *ad libitum*. The animal experiments were performed by licensed investigators in compliance with the Law on Animal Experiments of The Netherlands. The protocol was approved by The Institutional Animal Care and Use Committee of the University of Groningen.

Resident-intruder test
To select aggressive (proactive) rats and non-aggressive (reactive) rats form the local breeding colony, individuals were screened for aggression upon reaching adulthood. 24 Male WTG rats were housed together with a tubaligated female WTG rat to stimulate territorial aggression. The cage had sliding, plexiglass doors, so the interaction of animals could be filmed at the time of the resident-intruder test. The female was taken out of the homecage one hour before a male Wistar intruder was placed in the cage with the male WTG. First, the attack latency of all WTG rats was tested for 3 days, where the Wistar rat was taken out of the cage after the WTG rat attacked (full clinch). Second, all WTG rats were characterized for their aggressive behaviour on a fourth day by placing the Wistar in its cage for 10 min. The animals always encountered an unfamiliar opponent. The different behaviours displayed, during these 10 min, were analyzed by scoring the percentage of time spent on that behaviour. The behaviours scored, included in aggressive behaviour, were: lateral threat, keep down, clinch/attack bite, chase, fight upright. Other behaviours scored were: social exploration, ambulant behaviour, rearing, grooming and inactivity. From the 24 animals tested, the 8 most aggressive and the 8 least aggressive were included in the high- and low aggressive groups, respectively. The average time spent on aggressive behaviour in the high aggressive group was 68 ± 7 %, and in the low aggressive group was 19 ± 9 %. Brains were collected at least one week after the last test, together with the brains of the Roman rats.

Brain disection and homogenization
Blood was removed from the brain by perfusing the animals for 1 min with a solution of saline with 0.5 % heparin. Frontal cortex (FC) and hippocampus (Hip)

were isolated and snap frozen in liquid nitrogen, then stored at -80 ºC until further processing.
Brains were homogenized with ten volumes of homogenization buffer (50 mM Tris-HCl, 150 mM NaCl and 20 mM EDTA, pH 7.4), then the homogenate was centrifuged at 33.000 g for 10 min at 0-2 ºC. The supernatant was discarded and the pellet was homogenized in lysis buffer (50 mM Tris-HCl and 5 mM EDTA, pH 7.4), whereafter the homogenate was centrifuged at 1.000 g for 1 min at 0-2 ºC. This procedure was repeated and the supernatant of both runs was centrifuged at 33.000 g for 10 min at 0-2 ºC. The pellet was resuspended in 50 mM Tris-HCl with protease inhibitor (complete tablet, Roche). Protein concentration was determined by a DC Protein Assay Kit (Biorad, Hercules, CA).

Binding assay
[^{3}H]MDL and [^{3}H]Cimbi were kindly provided by Prof. Christer Halldin, Karolinska Institute. Saturation binding to 5-HT_{2A}R was assessed by adding 25 µL of tissue homogenate to six different concentrations (0.06-2 nM) of [^{3}H]MDL or [^{3}H]Cimbi in 975 µL assay buffer (50 mM Tris-HCl for [^{3}H]MDL and 50 mM of Tris-HCl with 0.1% BSA and 4 mM $CaCl_2$ for [^{3}H]Cimbi). Non-specific binding was determined by addition of 10 µM of ketanserin in repeated and adjacent samples. All dilutions were made in duplicate. Subsequently, the samples were incubated on a shaker, in a stove at 37 ºC for 90 min.
After incubation, the reaction mixture was flushed over a microfiber filter, which was soaked in 1% polyethylenimine solution, with ice cold assay buffer by a harvester. Scintillation fluid (2 mL, Ultima Gold) was added to the filters and incubated overnight at 4 ºC. The next day, samples were counted in a scintillation counter.

Affinity (K_d) and the maximum number of availible binding sites (B_{max}) were calculated from a nonlinear regression analysis of the total- and non-specific binding in Graphpad Prism 5.0. The binding potential (BP) was calculated as B_{max}/K_d. As [^{3}H]Cimbi has high nonspecific binding, we did not measure 5-HT_{2A}R binding in the Hip, where receptor levels are low.

Statistics

Outcome measures of the binding assay were analyzed by a two-tailed student's *t*-test for statistical significance. The level of statistical significance was set at $P < 0.05$.

Results

Binding assay in RHA and RLA

Both [^{3}H]MDL and [^{3}H]Cimbi show specific binding to 5-HT$_{2A}$R in FC, as shown by specific blocking with saturating concentrations of ketanserin. However, the non-specific binding of [^{3}H]Cimbi is larger than of [^{3}H]MDL (Fig 1). The average ratio between total and nonspecific binding at the highest concentration of 2 nM was on average 15 for [^{3}H]MDL and 5.5 for [^{3}H]Cimbi. In Hip, greater variability in K_d and B_{max} of [^{3}H]MDL binding was found.

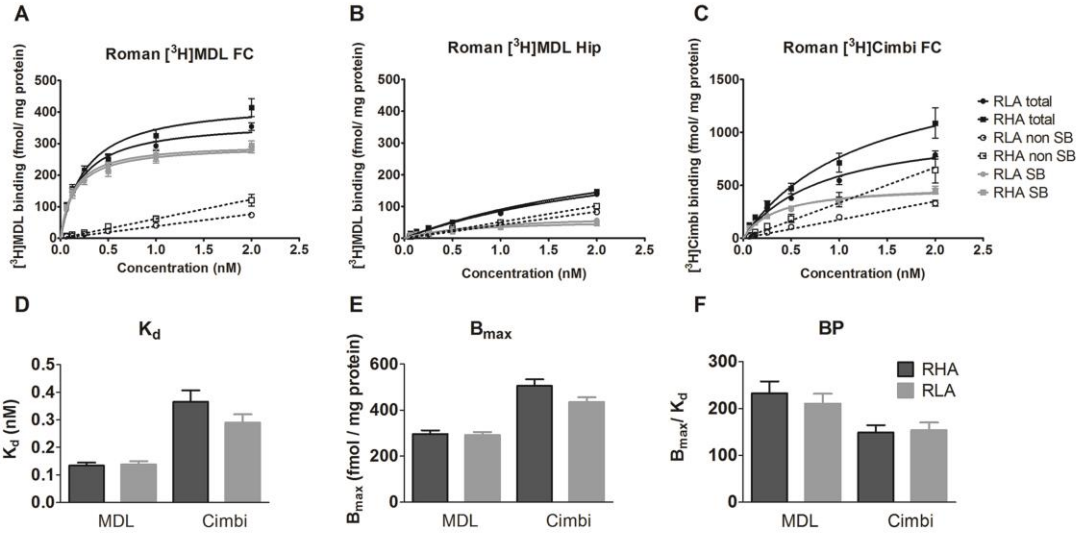

Fig 1 5-HT$_{2A}$ receptor binding in Roman High-Avoidance (RHA) and Roman Low-Avoidance (RLA) rats

Upper panels: (non)linear regression of total binding, nonspecific binding (non SB) and specific binding (SB). A. [^{3}H]MDL in frontal cortex (FC), B. [^{3}H]MDL in hippocampus (Hip), and C. [^{3}H]Cimbi-36 in FC. Lower panels: bar graphs of affinity (K_d, D), maximum amount of binding sites available (B_{max}, E) and binding potential (B_{max}/K_d = BP, F) in FC. Depicted are mean ± SEM.

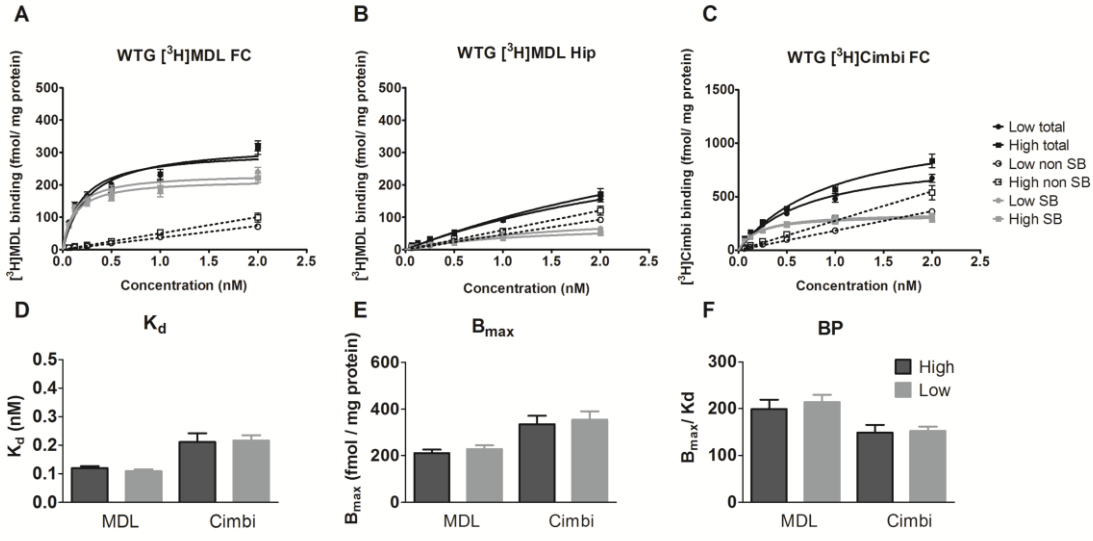

Fig 2 5-HT$_{2A}$ receptor binding in Wild-type Groningen (WTG) rat showing high and low aggression
Upper panels: (non)linear regression of total binding, nonspecific binding (non SB) and specific binding (SB). A. [^{3}H]MDL in frontal cortex (FC), B. [^{3}H]MDL in hippocampus (Hip), and C. [^{3}H]Cimbi-36 in FC. Lower panels: bar graphs of affinity (K_d, D), maximum amount of binding sites available (B_{max}, E) and binding potential (B_{max}/K_d = BP, F) in FC. Depicted are mean ± SEM.

Neither K_d (t = 0.21, Df = 13, P = 0.84) nor B_{max} (t = 0.23, Df = 14, P = 0.82) of [^{3}H]MDL binding was different in FC of RHA and RLA rats (Fig 1). Similar findings were made in Hip. B_{max} of [^{3}H]Cimbi tended to be decreased in FC of RLA rats compared to RHA. However, in a two-tailed *t*-test this difference was not statistically significant (t = 2.02, Df = 12, P = 0.07). An overview of the outcome measures is given in Table 1.

Binding assay in high- and low aggressive WTG rats

As expected, both [^{3}H]MDL and [^{3}H]Cimbi showed specific binding to 5-HT$_{2A}$R in FC of WTG rats, and also in this strain, [^{3}H]Cimbi displayed a higher nonspecific binding (Fig 2). The average ratio between total and nonspecific binding at the highest concentration of 2 nM was 13 for [^{3}H]MDL, but only 5.5 for [^{3}H]Cimbi. Low levels of 5-HT$_{2A}$R expression in Hip resulted in higher variation of the K_d and B_{max} values for [^{3}H]MDL in this region.

There is also no difference between either K_d (t = 1.19, Df = 12, P = 0.26) or B_{max} (t = 0.77, Df = 13, P = 0.46) of [^{3}H]MDL binding in the FC of high- and low aggressive WTG rats (Fig 2). The same observation (lack of differences) was made for [^{3}H]MDL binding in Hip or [^{3}H]Cimbi binding in FC. The outcome measures for the WTG rat are listed in Table 2.

Table 1 5-HT$_{2A}$ binding assay parameters Roman rats

	Roman high avoidance			Roman low avoidance		
	[^{3}H]MDL		[^{3}H]Cimbi	[^{3}H]MDL		[^{3}H]Cimbi
	FC	Hip	FC	FC	Hip	FC
K_d	0.13 ± 0.03	0.84 ± 0.47	0.37 ± 0.11	0.14 ± 0.03	1.04 ± 0.55	0.29 ± 0.08
B_{max}	296 ± 46	65.0 ± 20	506 ± 75	291 ± 38	79 ± 28	435 ± 54
BP	232 ± 72	9.41 ± 3.91	150 ± 48	210 ± 59	8.39 ± 2.52	160 ± 47

B_{max} = maximum number of binding sites available (fmol/mg protein), BP = binding potential, FC = frontal cortex, Hip = hippocampus, K_d = affinity (nM). Depicted are mean ± SD.

Table 2 5-HT$_{2A}$ binding assay parameters Wild-type Groningen rats

	High aggressive			Low aggressive		
	[^{3}H]MDL		[^{3}H]Cimbi	[^{3}H]MDL		[^{3}H]Cimbi
	FC	Hip	FC	FC	Hip	FC
K_d	0.12 ± 0.01	1.53 ± 0.85	0.21 ± 0.08	0.11 ± 0.02	1.78 ± 0.96	0.22 ± 0.05
B_{max}	211 ± 40	93 ± 43	335 ± 95	228 ± 47	122 ± 49	354 ± 103
BP	199 ± 53	6.70 ± 1.74	149 ± 40	214 ± 45	7.52 ± 2.80	152 ± 24

B_{max} = maximum number of binding sites available (fmol/mg protein), BP = binding potential, FC = frontal cortex, Hip = hippocampus, K_d = affinity (nM). Depicted are mean ± SD.

Discussion

This study investigated whether there are differences between 5-HT_{2A}R binding in animals with different coping styles. Two different animal models, the RHA and RLA rats and high- and low aggressive WTG rats, were used to compare differences in coping style within these animal models.
We found no difference in 5-HT_{2A}R binding in the FC or Hip of either strain, neither with a radiolabeled antagonist ([^{3}H]MDL), nor with an agonist ([^{3}H]Cimbi). Therefore, our results do not support the hypothesis that there are differences of 5-HT_{2A}R expression in animals with different coping styles, although [^{3}H]Cimbi did show a trend towards increased binding in RHA compared to RLA. This could either mean that there is a small difference between RHA and RLA in the high affinity state of 5-HT_{2A}R, which is only detected by the agonist tracer, or that there is a small difference in 5-HT_{2C}R expression between RHA and RLA. [^{3}H]MDL is selective for 5-HT_{2A}R whereas [^{3}H]Cimbi binds both to 5-HT_{2A} and 5-HT_{2C} receptors.

A lower selectivity of [^{3}H]Cimbi could also explain why the B_{max} values for this ligand are higher, since ketanserin blocks both 5-HT_{2A} and 5-HT_{2C} receptors (the latter with lower affinity) [31].
Our observation that binding of [^{3}H]Cimbi in the FC of WTG rats with different levels of aggression is not significantly different suggests that aggression is not related to the affinity state of 5-HT_{2A}R, since 5-HT_{2A}R agonists are known to bind mainly to the high affinity state [32]. Because of the high nonspecific binding of [^{3}H]Cimbi and low expression of 5-HT_{2A}R in Hip it was not possible to perform accurate measurements with this tracer in this brain area.

Even with the highly specific tracer [^{3}H]MDL such measurements were difficult. Therefore, data on 5-HT_{2A}R binding shows greater variability in Hip than in FC, where the density of 5-HT_{2A}R is much higher [33]. Yet, the same result was acquired in two different strains and with two different radioligands.
In contrast to our current findings, a previous publication reported significant differences in binding of the 5-HT_{2A}R ligand [^{3}H]ketanserin between RHA and RLA rats [25]. However, these data concerned a Roman inbred strain, while in the

current study we used an outbred strain. Since RHA and RLA from inbred and outbred strains show similar behavioral differences, interpretation of the role of 5-HT_{2A}R is complex. We can at least conclude that 5-HT_{2A}R do not play a crucial role in coping style.

As the WTG rats differed in their level of aggression, it is also unlikely that 5-HT_{2A}R binding is different between low- and high aggressive animals. With respect to levels of aggression, the literature is inconsistent. Some studies in humans have reported differences in 5-HT_{2A}R binding [7, 34], while a study in rodents did not find any [35]. In the human studies all aggressive subjects were “pathologically” aggressive, whereas rodents displayed natural aggressive behavior. The aggressive dogs used in the study that did find an increased 5-HT_{2A}R binding, were besides aggressive also impulsive [16]. Thus, it could be hypothesized that 5-HT_{2A}R are changed only in pathologic aggression.

We did not compare 5-HT_{2A}R binding before and after a stressful challenge in order to examine the involvement of 5-HT_{2A}R in stress sensitivity. As there is evidence in the literature that 5-HT_{2A}R binding is positively correlated to higher scores for the personality trait neuroticism (a risk factor for affective disorders), and especially to vulnerability for stress, it may be interesting to investigate whether there are any differences between proactive and reactive copers in their response to stress and the effect of acute stress on 5-HT_{2A}R binding [9].
In conclusion, we did not observe any difference of baseline 5-HT_{2A}R binding in rats with different coping styles and in rats displaying different levels of aggression. This result was acquired both with a radiolabeled antagonist [^{3}H]MDL, and agonist, [^{3}H]Cimbi, suggesting that the fraction of 5-HT_{2A}R in the high affinity state is also not different. Future studies could focus on the effects of stress on 5-HT_{2A}R in animals with different coping styles.

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CHAPTER 7

Serotonin-2C antagonism augments the effect of citalopram on serotonin and dopamine levels in the ventral tegmental area and nucleus accumbens

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Submitted

Abstract

Not all patients with major depression respond properly to selective serotonin reuptake inhibitors (SSRIs). Augmentation strategies by combining a 5-HT_{2C} antagonist with an SSRI may improve efficacy by increasing serotonin (5-HT) and counteracting the inhibitory effect of an SSRI on dopamine (DA). Therefore, male Wistar rats were acutely treated (s.c.) with citalopram (Cit, 5 mg/kg), SB 242084 (SB, 2 mg/kg), or Cit + SB, to explore the effects on 5-HT and DA levels by microdialysis in the ventral tegmental area (VTA) and nucleus accumbens (NAcc). In a separate experiment animals were subchronically treated with vehicle, Cit (20 mg/kg/d), SB (2 mg/kg/d) or Cit + SB for 2 days (s.c.). On the day of microdialysis, 5-HT_{2C} receptor sensitivity was assessed through a challenge with SB. Acutely, Cit + SB increased 5-HT levels in the NAcc more than an SSRI alone. SB alone increased DA levels in the NAcc (not in VTA), but when administered together with Cit, this effect was abolished. Subchronic treatment with Cit or Cit + SB increased 5-HT levels in both VTA and NAcc, at baseline. Cit + SB augmented the effect of Cit alone in the VTA. Only Cit + SB significantly increased DA levels in VTA and NAcc at baseline. In conclusion, the combination of Cit + SB has beneficial effects on 5-HT and DA levels after subchronic treatment, probably related to decreased tonic inhibition via 5-HT_{2C} receptors. There are regional differences and future studies should also elucidate if there are benefits on a behavioural level.

Keywords: 5-HT2C receptor; SSRI; augmentation; microdialysis; dopamine; serotonin

Introduction

Life time prevalence of major depressive disorder (MDD) is approximately 15 %, thereby causing a large socioeconomic burden [1] . MDD was the fourth leading cause of disability worldwide around 1990, but it has been estimated to climb to the second rank in 2020 [2] . SSRIs are the first treatment option for MDD. Yet, a large percentage of patients (40-50 %) does not respond satisfactorily to SSRIs. Moreover, their delayed onset of action might negatively influence patient's compliance [3]. Clearly, innovative treatment strategies are warranted to improve the efficacy of SSRIs.
The main route of action of SSRIs is an increase of extracellular serotonin (5-HT) levels. However, other monoamines, such as dopamine (DA) and norepinephrine (NE), have also been linked to the pathology of depression. For instance, two core symptoms of MDD are anhedonia and reduced motivation, which are both believed to involve diminished activity of the mesolimbic DA system [4].

So ideally, antidepressants should increase both 5-HT and DA, without causing severe side-effects. Several studies, however, indicate that SSRIs actually reduce DA neuronal activity and reduce DA release [5-7]. This could be a factor in the delayed onset of action and lack of antidepressant response [8, 9]. The reduction of DA neuronal activity is probably related to the interaction between 5-HT and DA in the mesolimbic system, through 5-HT_{2C} receptors (5-HT_{2C}R) [10]. The mesolimbic DA system consists of neurons projecting from the ventral tegmental area (VTA) to the nucleus accumbens (NAcc). It was demonstrated that dopaminergic neurons in the VTA are under tonic inhibition by 5-HT through stimulation of 5-HT_{2C}R positioned on GABA-ergic or dopaminergic neurons [11-13].

Previous studies have already shown an augmentation of the effects of an SSRI on extracellular 5-HT by a 5-HT_{2C}R antagonist [14]. Notably, this effect has been related to a reduction of GABA release following 5-HT_{2C}R antagonism [15, 16]. See Fig 1 for a schematic few of the hypothesized mechanism.

In the present study, we have investigated the effects of 5-HT_{2C}R antagonism on SSRI induced changes of extracellular 5-HT and DA levels in both VTA and NAcc, using intracerebral microdialysis. As acute effects may differ from longitudinal effects, we additionally looked at subchronic effects of combining an SSRI with a 5-HT_{2C}R antagonist. The same treatment regimen was used as in the study of Dremencov et al (2009), as this seemed to reduce VTA neuronal activity.

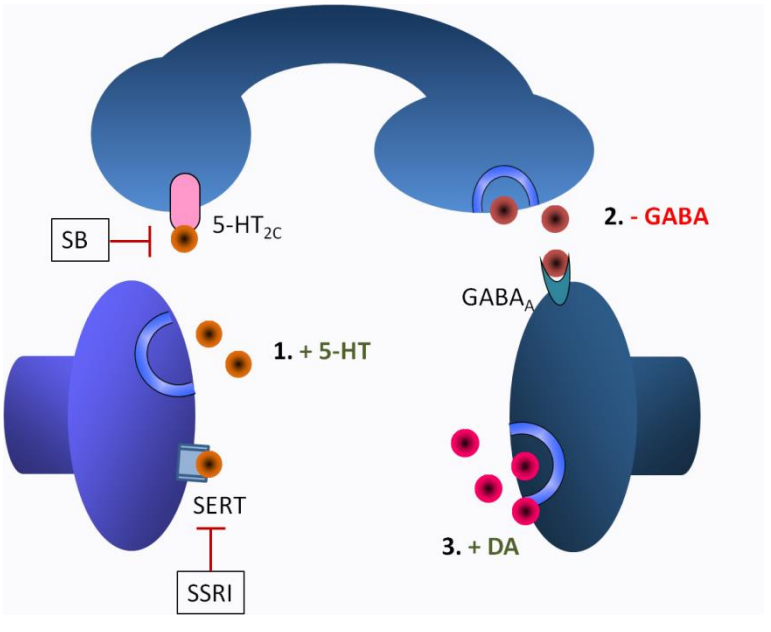

Fig 1 The interaction between serotonin and dopamine through 5-HT_{2C} receptors.
Under normal circumstances, 5-HT is released by 5-HT neurons and stimulates 5-HT_{2C} receptors on GABA-ergic neurons. The inhibitory neurotransmitter GABA in turn tonicly inhibits the release of dopamine (DA). An SSRI increases extracellular levels of 5-HT, and therefore could increase GABA release and finally decrease extracellular DA levels. When combining the treatment of an SSRI and a 5-HT_{2C} antagonist, SB 242084 (SB), 5-HT is increased by the SSRI (1). SB decreases GABA release, and blocks the increased release of GABA due to increased 5-HT after SSRI treatment (2), preventing the decrease in DA, or even increasing DA (3).

Experimental procedures

Animals

Adult male Wistar rats (Harlan, Zeist, The Netherlands), weighing 332 ± 14 g, were individually housed with *ad libitum* food and water and on a 12:12 h light: dark cycle, with lights on at 7.00 a.m. After surgery, rats were housed in Plexiglas cages (35 x 35 x 40), specifically suitable for microdialysis experiments. The animal experiments were performed by licensed investigators in compliance with the Law on Animal Experiments of The Netherlands. The protocol was approved by The Institutional Animal Care and Use Committee of the University of Groningen.

Treatment

In the acute microdialysis experiment, three different treatments were applied (at timepoint 0): citalopram (Cit) (N = 5), SB 242084 (SB) (N = 5), and Cit + SB (N = 4). In the subchronic experiment animals were treated for 2 days with: vehicle + saline (N = 4), Cit + saline (N = 4), vehicle + SB (N = 5), and Cit + SB (N = 5). During the subchronic microdialysis experiment a challenge with SB was applied at timepoint 0 in all groups.

Citalopram (Trademax Pharmaceuticals & Chemicals Co., China) was dissolved in UP water. During the acute experiment 5 mg/kg was applied subcutaneously and in the subchronic experiment 20 mg/kg/day was applied subcutaneously through osmotic minipomps (Alzet). The 5-HT_{2C}R antagonist SB 242084 (Tocris Bioscience, USA) was dissolved in 0.9 % saline with 10 % β-cyclodextrin and in both the acute and subchronic experiment 2 mg/kg was applied through subcutaneous injections. During the acute experiment the combination of Cit and SB was dissolved in Ultra Pure water and applied by a subcutaneous injection, in the same concentrations as used for in the single administration.

Microdialysis procedure

Microdialysis probes (9 mm, 1.5 mm exposed membrane; polyacrylonitril, MWcut-off 40–50 kDa; Brainlink, the Netherlands) were simultaneously implanted in the VTA (coordinates from Bregma and Dura; Paxinos and Watson, 2007: A/P −5.0, L/M 0.9, V/D −8.2) and NAcc shell (coordinates: A/P 2.0, L/M 1.2, V/D −7.9) under

isoflurane anesthesia (induction 5 %, maintanance 2-2.5 %). Directly after insertion of the microdialysis probes the minipumps for the subchronic experiment were subcutaneously implanted. Bupivacaine and fynadine (2 mg/kg, s.c.) were applied as local- and postoperative analgetics, respectively.

After 24 hours of recovery in the acute experiment, and 12 hours after the last treatment-injection in the subchronic experiment, the probes were flushed with Ringer solution (140 mM NaCl, 4 mM KCl, 1.2 mM $CaCl_2$, and 1 mM $MgCl_2$), at a flow rate of 1.5 µl/min. Microdialysis samples from VTA and NAcc were collected every 15 min by a fraction collector and diluted with 7.5 µl formic acid, to prevent breakdown of monoamines, before storage at – 80 ºC until further analysis. The first 5 samples were used as baseline, whereafter the different challenges were applied by s.c. injection in the neck region. After the experiment, animals were sacrificed by extirpation of the heart and brains were fixed in 4 % paraformaldehyde to verify probe possition.

Initially, samples from the VTA were analyzed by HPLC with electrochemical detection, but it appeared necessary to switch to LC-MS/MS to enable a more sensitive and reliable analysis. Accordingly, DA and 5-HT were analyzed by isotope dilution mass spectrometry using serotonin-d4 and dopamine-d4 as internal standard. However, the results from the acute experiment in the VTA were not reliable and therefore not shown.

Data analysis

Data are presented as a percentage of baseline 5-HT and DA concentrations (average value of first 5 samples). Statistical significance of the change in neurotransmitter levels was examined by repeated measures ANOVA (two-way), with Student-Newman-Keuls as post-hoc test. Changes in baseline levels after subchronic treatment was examined by one-way ANOVA, with Student-Newman-Keuls as post-hoc test (Sigmaplot). The value at t = 0 was used as the reference value to look at treatment effects over time. Thresholds of significance were set at $P < 0.05$.

Results

Acute experiment

In the acute experiment, there was a significant interaction between time and treatment on 5-HT levels in the NAcc ($F_{(28, 110)}$ =4.64, P<0.001). Post-hoc analysis revealed that there was a significant increase in 5-HT after Cit and after Cit + SB, compared to SB alone (from t = 30). Cit + SB showed increased 5-HT release compared to Cit alone from t = 75-135. After a Cit or a Cit + SB challenge, almost all time points differed significantly from t = 0, except t = 15 (see Fig 2A).

In the NAcc, there was also a significant interaction effect between time and treatment ($F_{(28, 145)}$ =1.57, P=0.046). Post-hoc analyses revealed that SB increased DA release compared to Cit (t=15, 90, 105, 135) and Cit + SB (t=15, 45). Thus, the significant increase in DA levels was abolished by co-administration of Cit. At t = 90, a Cit challenge decreased DA levels compared to the other groups. After an SB challenge, DA levels on t = 15 were significantly increased compared to baseline (see Fig 2B).

Subchronic experiment

When comparing 5-HT levels at baseline in the subchronic experiment, there was a significant difference between the groups in the NAcc ($F_{(3, 85)}$ =52.9, P<0.0001). Post-hoc analysis showed an increase in 5-HT levels after Cit (437 %) and Cit + SB (524 %), compared to vehicle + saline (Fig 3A). Also in the VTA there was a significant difference between the treatment groups ($F_{(3, 81)}$ =136.5, P<0.0001). Post-hoc analysis showed an increase of 5-HT levels after Cit (274 %) and after Cit + SB treatment (849 %). The combination of Cit with SB significantly augmented the effect of SB or Cit alone (Fig 3B).

At baseline, treatment increased DA in both NAcc ($F_{(3, 86)}$ =2.8, P=0.04) and VTA ($F_{(3,77)}$=16.9, P<0.0001). Post-hoc analyses showed that Cit + SB (162 %) increased DA levels in NAcc compared to vehicle + saline. Other treatments did not lead to significant changes in DA levels in NAcc, although a trend was observed for both SB and Cit treatment alone (Fig 3C). Cit + SB also significantly increased DA levels (221 %) in VTA, compared to the vehicle + saline group. Moreover, Cit + SB increased DA in the VTA compared to treatment with SB or Cit alone as well (Fig 3D).

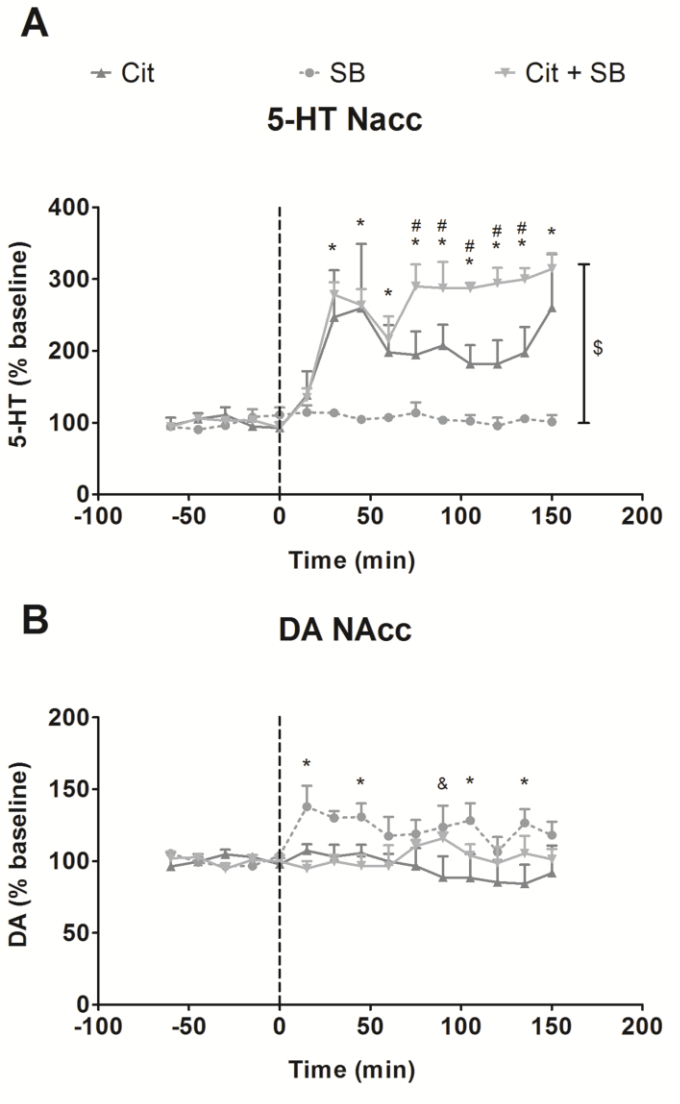

Fig 2 Effect of acute treatment effects on 5-HT and DA in NAcc.
Challenges of either citalopram (Cit), SB 242084 (SB), or the combination were applied s.c. at t = 0. Effects on extracellular serotonin (5-HT, A) or dopamine (DA, B) in NAcc. * SB differs significantly ($P < 0.05$) from the other groups. # Cit + SB differs significantly ($P < 0.05$) from Cit alone. & Cit differs significantly ($P < 0.05$) from the other groups. $ Significant effect of time ($P < 0.05$). Depicted are mean ± SEM.

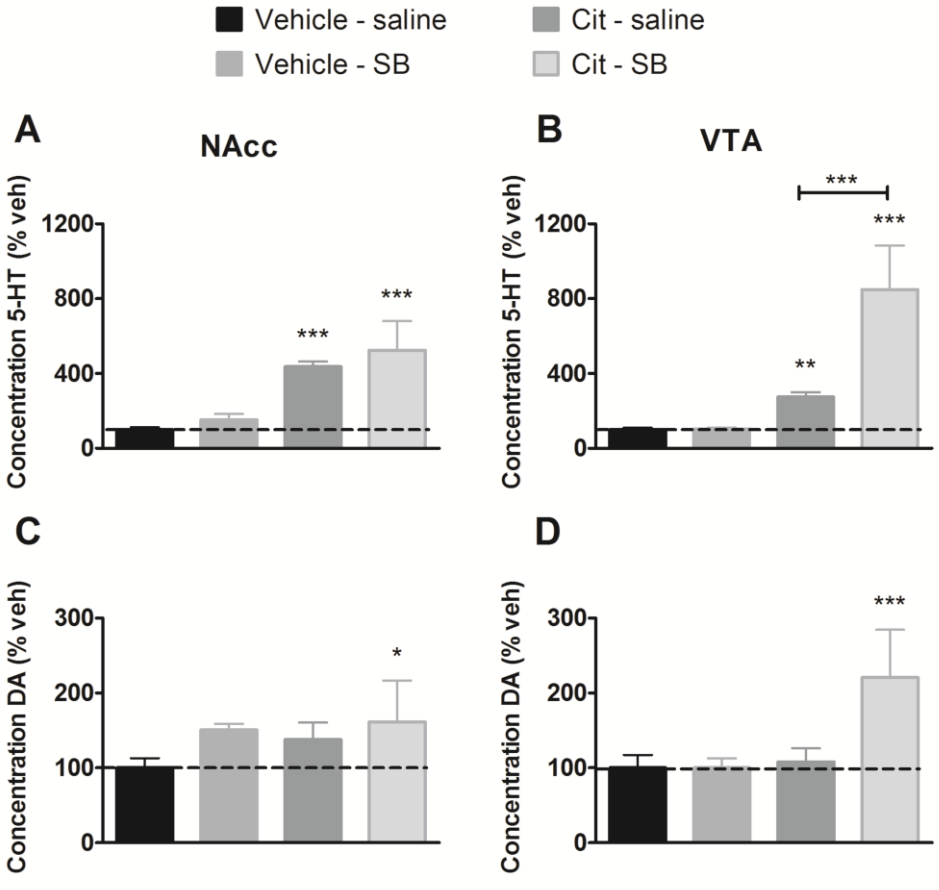

Fig 3 Baseline levels of 5-HT and DA after subchronic treatment

Concentrations of serotonin (5-HT) and dopamine (DA) are expressed as the percentage of the vehicle-saline group after subchronic treatment (s.c). Both citalopram (Cit) and Cit + SB 242084 (SB) increase 5-HT in the NAcc (A) and VTA (B). Only Cit + SB increases DA in the NAcc (C) and VTA (D). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, depicted are mean ± SEM.

Subchronic treatment did not change effects of an SB challenge on 5-HT levels in the NAcc ($F_{(3,14)} = 1.70$, $P=0.21$). There was an interaction between time and treatment though ($F_{(42, 188)} = 1.47$, $P=0.044$). Post-hoc analysis revealed a significant increase in 5-HT levels in the Cit group at t= 45 and t = 75 (Fig 4 A).

Subchronic treatment did also not change the effect of an SB challenge on 5-HT levels in the VTA ($F_{(3, 14)}$ =1.99, P=0.16). There was an effect of time ($F_{(14, 186)}$ =7.17, P<0.001), and a trend towards an interaction effect between time and treatment ($F_{(42, 186)}$ =1.42, P=0.061), but this was not significant. Therefore, SB caused a similar increase in 5-HT levels in all groups (Fig 4 B).

The changes in DA levels in the NAcc after an SB challenge did not significantly differ between groups ($F_{(3, 14)}$ =0.94, P=0.45). There was only an effect of time ($F_{(14, 42)}$ =5.04, P<0.001). Neither did SB elicit different changes in DA levels in the VTA ($F_{(3, 14)}$ =2.38, P=0.11). Also here there was an effect of time ($F_{(14, 42)}$ =7.89, P<0.001). This suggests that SB increases DA in the NAcc and VTA in a similar way in all groups, despite the respective pre-treatment (Fig C and D).

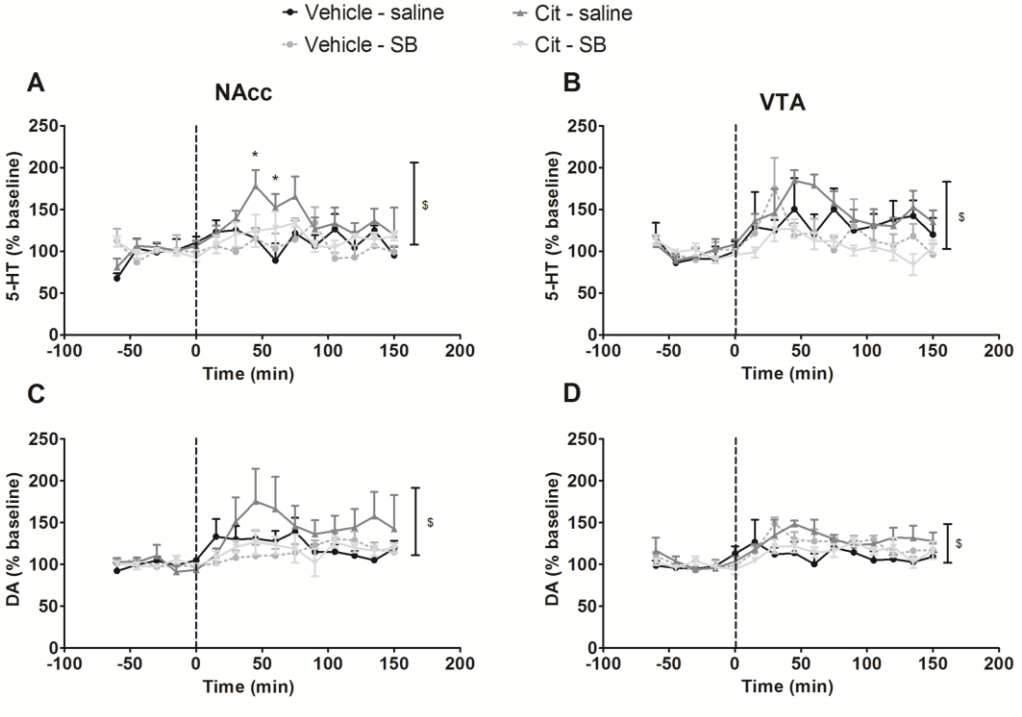

Fig 4 Changes in 5-HT and DA release after subchronic treatment and a challenge of SB
A challenge of SB 242084 (SB, s.c.) was applied at t = 0, after subchronic treatment. Effects on extracellular serotonin (5-HT) were measured in NAcc (A) and VTA (B). Also effects on dopamine (DA) release were measured in NAcc (C) and VTA (D). * Citalopram (Cit) differs significantly (P < 0.05) from vehicle. $ There is a significant (P < 0.05) time effect. Depicted are mean ± SEM.

Discussion

According to the monoamine hypothesis both 5-HT and DA are deficient in MDD, so ideally both neurotransmitters should be increased by antidepressant treatment. In this study we investigated if this aim could be achieved by combining an SSRI with a 5-HT_{2C}R antagonist.

Indeed, we show that acute administration of the combination of an SSRI and a 5-HT_{2C}R antagonist has greater effects on extracellular 5-HT levels in the NAcc than administration of each compound alone. This is in agreement with a previous study reporting a similar augmentation of extracellular 5-HT levels in rat hippocampus and frontal cortex [14].

In addition we show that an acute challenge with SB increases DA in the NAcc, and that this effect is abolished by Cit, suggesting an inhibitory effect of Cit on DA release. However, the administration of Cit alone did not significantly decrease DA release in the NAcc. This is at variance with a previous study showing a significant decrease of DA levels, albeit with a longer exposure to a higher dose of the SSRI [6].

Dremencov and colleagues have shown that 2 days of treatment with SSRIs induces reduced DA neuronal activity in the VTA [5]. Accordingly, we also explored the effects on extracellular neurotransmitter levels after this extended treatment period.
Indeed, the largest augmenting effects were observed after subchronic treatment. At baseline, the combination of Cit and SB increased 5-HT and DA in both VTA and NAcc, compared to the control group. In the VTA, Cit + SB (849 %) increased 5-HT levels more than Cit alone (275 %). Moreover, increases of DA in VTA and NAcc were only significant in the group receiving both Cit and SB, suggesting that this combination increases both 5-HT and DA levels in VTA and NAcc.

To investigate whether subchronic treatment affected 5-HT_{2C}R sensitivity, in each group an SB challenge was administered. Although there were no significant differences between the groups, levels of 5-HT in the NAcc were significantly

different from t = 0 in the Cit group, while this was not the case for the other treatments. This might suggest a gradual sensitization of 5-HT_{2C}R regulating 5-HT release after Cit treatment in the NAcc, although the effect is rather small.

Additionally we found regional differences in the effect of 5-HT_{2C}R antagonism. For example, there is no tonic inhibition of DA release in the VTA, while there is in NAcc. Another study showed that there is tonic inhibition of DA and NE release in the prefrontal cortex, but not of 5-HT release [17]. 5-HT_{2C} receptors are not only present on GABA-ergic neurons, but also on dopaminergic neurons [11] and probably on other neurons as well. As the effects of 5-HT_{2C}R stimulation are excitatory [18] , stimulation would have opposite effects compared to the 5-HT_{2C}R on GABA-ergic neurons. Therefore, the release of DA could also depend on the balance between excitatory and inhibitory effects of 5-HT_{2C}R stimulation.

By administering an SSRI, this balance may be disturbed in favour of decreased DA release in the mesolimbic system. Co-administration with a 5-HT_{2C}R antagonist may alleviate the negative effects on DA and even increase DA levels. Moreover, increased efficacy of an SSRI after co-administration of a 5-HT_{2C}R antagonist was demonstrated in the forced swim test, a behavioural test that measures depressive-like behaviour. Mice, not responding to Cit in this test, did respond after co-administration with a 5-HT_{2C} antagonist [9]. So there is both neurochemical and behavioural evidence that combining an SSRI with a 5-HT_{2C}R antagonist may increase antidepressant efficacy.

Indeed, there are antidepressants available with 5-HT_{2C}R antagonistic properties. Agomelatin, for example, is an antagonist for 5-HT_{2C}R and an agonist for melatonin receptors. However, the increases in DA and NE in frontal cortex upon treatment can be attributed to its action on 5-HT_{2C}R, rather than melatonin [19]. Mirtazapine and fluoxetine are other examples of antidepressants with 5-HT_{2C}R antagonistic properties [20, 21].

Future research is mandatory to confirm our hypothesis, investigating the behavioural effects (e.g. anhedonia) and effects of long-term administration of the combination SSRI/5-HT_{2C}R antagonist. Yet, this study has its limitations, in particular the rather small number of animals and the incomplete data set.

Nonetheless we conclude that the combined action of an SSRI/5-HT_{2C}R antagonist could have substantial impact on treatment outcomes and therefore needs further investigation. We believe that this combination holds promise in both targeting dopamine- and serotonin related symptoms of depression, including anhedonia and a depressed mood. The combination might also positively influence treatment response and onset of action of SSRIs.

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CHAPTER 8

General discussion and future perspectives

Dysfunctions of monoamine systems (serotonin, norepinephrine and dopamine) may have a causal relationship with major depression [1, 2]. According to the monoamine theory, major depression is caused by an impaired monoamine neurotransmission, resulting in decreased extracellular norepinephrine (NE) and/or serotonin (5-HT) levels [3, 4]. Diminished concentrations of 5-HT and its metabolites have been demonstrated in cerebrospinal fluid [5] and in post mortem brain tissue of depressed patients [6]. There are also reports of altered platelet 5-HT transporter function [7] and of 5-HT_2 receptor function in the brain [8] and platelets [9]. Studies investigating NE and 5-HT metabolites in cerebrospinal fluid, blood or urine of patients and post-mortem studies seem to support the monoamine hypothesis of major depression [10]. Serotonin is the neurotransmitter most frequently associated with stress related disorders such as major depression. Yet, antidepressants capable of increasing extracellular 5-HT levels are only moderately effective in a small majority of patients. This could indicate that other monoamines such as norepinephrine and dopamine are also involved in the aetiology of major depression, making them viable targets for pharmacological treatment.

One of the greatest challenges in psychiatry is to enable treatment of patients on a more individual basis by taking into account the divergent subtypes and symptom profiles of major depression. To achieve this goal new treatment strategies have to be developed. Clearly, such an approach demands a thorough knowledge of the relation between the clinical manifestations of major depression and the underlying biochemical processes, especially for patients with treatment resistant depression. An additional argument for improving pharmacological treatment is that treatment resistant depression is a large socio-economic burden.

A large body of evidence regarding the biological processes underlying major depression is derived from *in vitro* and *in vivo* research performed with laboratory animals and it must be realized that several confounding factors exist when translating animal data to clinical manifestations. First, animal research concerning major depression is based on models trying to mimic the human condition. Clearly such models have to fulfill stringent criteria such as construct (etiology), face (depression-like behavior) and predictive validity (antidepressant

effect) [11, 12]. Second, marked differences exist between species both in neuronal organization and plasticity. Such differences may even be present among different rat strains (see e.g. chapter 6). Finally, overt differences in pharmacokinetics and pharmacodynamics have been reported especially between rodents and humans [13-15]. Post mortem studies certainly have scientific merit [16], but the progressive character of many neuropsychiatric diseases and the fact that many patients have been treated with drugs for a substantial part of their lives limit the value of many post mortem data. In addition, it is difficult to judge the consequences of dying for brain physiology, such as the inactivation of enzymes and an instantaneous and massive release of neurotransmitters. On the other hand neuroimaging has matured although the number of molecular targets that can be studied is still limited. Because PET studies can be performed in patients as well as in animal models they are in the unique position to bridge the gap between clinical and preclinical research. Moreover, PET-imaging enables longitudinal studies in small groups of humans and animals.

An important issue that might be related to the moderate efficacy of antidepressants is whether 5-HT synthesis is capable of replenishing 5-HT stores when reuptake of this monoamine is blocked. Some studies even suggest that antidepressants actually reduce 5-HT synthesis, but this is still a matter of debate (see [17] and references therein). Because most techniques are either invasive (e.g. lumbar puncture) or give indirect information about brain physiology (e.g. blood platelets, white blood cells and neuroendocrine strategies) it is difficult to ascertain whether 5-HT synthesis is indeed a limiting factor with antidepressant activity. The 5-HT synthesis tracer [^{11}C]5-HTP, which was shown capable of measuring 5-HT synthesis in non-human primates and humans (chapter 3 of this thesis), seems an appropriate tool to answer this question. Accordingly, we have performed a micro-PET study with [^{11}C]5-HTP to validate the tracer for use in rodents. However, it appeared very difficult to unravel the different kinetic steps in the 5-HT synthesis chain with micro-PET in rats. A PET tracer must fulfil several criteria to enable estimation of synthesis rates, which in rodents [^{11}C]5-HTP apparently does not. An explanation for this species discrepancy may be that unmetabolized [^{11}C]5-HTP does not readily leave the rodent brain within the time frame of the PET scan, therefore preventing the ability to distinguish between [^{11}C]5-HTP and [^{11}C]5-HT. The reason why this process is different in non-human

primates and humans remains the question, somehow the excretion of [^{11}C]5-HTP must be faster in humans. Our study indicates that for translational purposes it is absolutely necessary to validate PET tracers in several species.

Neurotransmission is not only characterized by presynaptic activity, including synthesis, but also by the number and affinity of postsynaptic receptors. Arguably, kinetics are somewhat more straight forward for a receptor tracer than for a synthesis tracer such as [^{11}C]-5-HTP. Given previous reports on 5-HT$_{2A}$ receptor function in major depression (e.g.[8, 9]) we have validated the 5-HT$_{2A}$ receptor tracer [^{11}C]MDL 100907 (Chapter 4). Our study indicates that this radio-ligand is applicable for preclinical research, with the major advantage that a reference region can be used to quantify tracer kinetics. It would be even more advantageous if a compound with similar kinetic characteristics was labelled with fluor-18, which has a longer half-life thus enabling transportation to other facilities. Attempts have been made to synthesize such a compound, but tracer kinetics seem less favourable than for [^{11}C] MDL 100907. Future studies should focus on the optimization of ^{18}F-labelled compounds with high affinity for 5-HT$_{2A}$ receptors and also on developing compounds with full agonist properties that preferentially bind to the high affinity state of the receptor which may be more relevant in disease state conditions. [^{11}C]Cimbi-36 is possibly the first 5-HT$_{2A}$ receptor ligand that can discriminate between the low and high affinity state of the receptor. However, more studies are required to prove that it really preferably binds to the high affinity state of the 5-HT$_{2A}$ receptor.

As [^{11}C]MDL 100907 seemed suitable for measurement of 5-HT$_{2A}$ receptor binding in rodents we investigated the impact of a severe social stressor, social defeat on apparent 5-HT$_{2A}$ receptor densities in the rat brain (Chapter 5). Although previous studies (not involving imaging) suggested that stress would cause an increase of tracer binding in the frontal cortex and a decrease in hippocampus, we were unable to show such effects in male Wistar rats. An explanation for this unexpected finding is that most previous studies used a chronic, physical stressor, which may have a larger effect on 5-HT$_{2A}$ receptors than the acute stress which we applied. Future studies could examine the effects of chronic social defeat on 5-HT$_{2A}$ binding. Besides unaltered 5-HT$_{2A}$ receptor binding, we also did not find any effect on behaviour in the open field, which is a behavioural test for anxiety.

However, effects of social defeat in the open field test were repeatedly observed by Meerlo et al. (1996)[18]. These effects were not only acute, but lasted up to a week, which raises the question why they were not observed in our study. The most probable answer is that we used a different rat strain. Individual differences in stress response or coping style may play a crucial role in the impact of stressors on behaviour and physiology. For that reason, we wanted to investigate if there are differences of 5-HT_{2A} binding in rats with different coping styles.

A previous study found that there is a difference in 5-HT_{2A} receptor binding between high- and low avoidance rats of the Roman strain. These animals either actively avoid a foot shock or passively cope with this shock and were bred for these behaviours. As a lot of research in Groningen is focused on individual differences, like coping with stress, we wanted to repeat these results and additionally show that differences in coping style are also present in the Wild-type Groningen rat strain (Chapter 6). Although animals from this strain are selected for aggression, they show similar differences as Roman rats in coping with a stressful situation. However, neither in the Roman nor in the Wild-type Groningen rat strain we could show differences in 5-HT_{2A} receptor binding related to coping style. An explanation for this negative finding may be that the Roman strain we used was an outbred strain, while in previous studies an inbred strain was employed.

From our studies we can conclude that 5-HT_{2A} receptors do not seem to have a prominent role in stress-related behaviour and coping style. Maybe this receptor is more related to vulnerability to stress, and is only altered in a subgroup of animals (or animal strains) and possibly also only in some humans. The hypothesis of subgroups with various 5-HT_{2A} receptor expression and stress vulnerability is still worth investigating since clinical research aims at more personalized treatment of diseases like major depression. This patient group shows a large variety of symptoms which are most likely related to different neurobiological characteristics.

In addition, it might be more worthwhile to study networks instead of a single neurotransmitter system. The brain is a network of interconnecting neurotransmitter systems and signalling pathways. Measuring interactions between neurotransmitter systems might give much more information about the

underlying mechanisms of complex diseases like major depression. In the case of depression, the interaction between 5-HT and dopamine is of great importance, as both are involved in the most common symptoms of depression: reduced mood and motivation, respectively. However, it was found that selective serotonin reuptake inhibitors (SSRIs) actually reduce dopamine release. Ideally, both 5-HT and dopamine should be increased by antidepressant treatment. This can be achieved by augmenting the increase in 5-HT seen after antidepressant treatment by additionally giving a specific antagonist for the 5-HT_{2C} receptor. Indeed, we showed for the first time that systemically applying an SSRI (citalopram) together with a 5-HT_{2C} antagonist could increase both extracellular 5-HT and dopamine, as measured by microdialysis (Chapter 7).

Future studies should elucidate what the effect of this combined treatment is on a behavioural level. There are also other options to augment antidepressant treatment, like a combination of SSRI's with an anti-inflammatory drug or with a kappa opioid receptor antagonist. These treatments are focused on reducing neuroinflammation and anhedonia, respectively. Depending on the clinical characteristics of the patient, either 5HT2c antagonists or anti-inflammatory medicine or kappa-opioid antagonists could be the most beneficial adjuvants. PET could play a major role in identifying which patient will be sensitive to which treatment and in monitoring the treatment effects.

In summary major depression probably is a disorder where networks rather than a single neurotransmitter system are disturbed. Within these networks neurotransmitters act through receptors and signalling pathways that have a certain sensitivity set-point for signal transduction. If these set-points are changed by disease, treatment could lead to a normalization of the sensitivity of signalling pathways resetting the patient's "state of mind".

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CHAPTER 9

Summary

Introduction (Chapter 1)

The serotonergic system is a complex neurotransmitter network, whereof the cell bodies lie in the raphe nuclei and project to all brain regions and spinal cord. When deregulated, the serotonergic system is a key player in pathologies like major depression and is involved in the therapeutic effect of antidepressants.

Serotonin is produced in serotonergic neurons in two steps: hydroxylation of the amino acid tryptophan (Trp) to 5-hydroxytryptophan (5-HTP), followed by its decarboxylation to 5-HT. The rate limiting step in this process is the availability of the precursor Trp. Trp is not only used in serotonin synthesis, but is also incorporated in proteins and metabolized by the enzyme 2,3-indolamine deoxygenase (IDO). As IDO is activated during inflammation, it is possible that under inflammatory conditions less serotonin is produced.
Another important component of the serotonergic system is the 5-HT_2 receptor. There are three different subtypes (a,b,c) whereof 5-HT_{2A} receptors are most abundant. This receptor is related to the pathology of depression, the efficacy of antidepressants and the anti-hallucinogenic effects of antipsychotics. 5-HT_{2C} receptors are also widely distributed and modulate the release of dopamine (DA).

The components of the serotonergic system in the living brain can be measured by different techniques. One is positron emission tomography (PET), through which 5-HT synthesis can be measured with [^{11}C]5-HTP and 5-HT_{2A} receptor binding with [^{11}C]MDL 100907.
Another technique is microdialysis, where a small dialysis membrane is inserted in the brain, allowing the exchange of small molecules between a perfusion fluid and the surrounding extracellular fluid. Concentrations of neurotransmitters such as monoamines can be measured in microdialysates using liquid chromatography followed by electrochemical detection or mass spectrometry.

The aim of the thesis is to validate different serotonergic PET tracers in rodents so that they can be used to study the physiology of stress and to investigate strategies to improve the efficacy of antidepressants.

Measuring 5-HT synthesis with [^{11}C]5-HTP PET (Chapter 2 & 3)

Many aspects of the functioning of the serotonergic system are still unclear, partially because of the difficulty to measure physiological processes in the living brain. An example is the measurement of 5-HT synthesis. The conventional methods, like measuring metabolites in cerebrospinal fluid or measuring 5-HT concentrations in blood platelets, are indirect and may not always resemble the real synthesis rates. New possibilities for directly measuring synthesis rates arose when PET techniques were advancing.

There are two options if one wants to measure 5-HT synthesis by PET: labeling an analogue of Trp with a radioactive isotope or labeling an analogue of 5-HTP. Under some conditions the Trp analogue α-[^{11}C]methyl-tryptophan ([^{11}C]AMT) may match the real 5-HT synthesis rates (it is not incorporated into proteins), although under inflammatory conditions this is still questionable. Another available tracer is [^{11}C]5-hydroxytryptophan ([^{11}C]5-HTP), which has exactly the same properties as endogenous 5-HTP. However, this tracer is difficult to produce as enzymatic reactions are needed to synthesize [^{11}C]5-HTP.

Both synthesis tracers have been validated for use in humans. However, [^{11}C]AMT and [^{11}C]5-HTP kinetics are differently affected by Trp depletion or changes of mood, indicating that these tracers are associated with different enzymatic processes.
As [^{11}C]5-HTP probably resembles 5-HT synthesis rates best, we wanted to validate the use of this tracer in rodents with the ultimate aim to investigate the effect of antidepressant treatment on cerebral 5-HT synthesis. We did this by pharmacologic inhibition of the different steps in the pathway of 5-HT synthesis. Unfortunately quantification of synthesis rates appeared to be difficult.

The uptake of [^{11}C]5-HTP in the rat brain is low, because the tracer is quickly metabolized by peripheral aromatic amino acid decarboxylase (AADC). Uptake can be increased by the administration of carbidopa, which inhibits peripheral AADC activity without affecting the rate constant of [^{11}C]5-HTP trapping in the brain (k_3).

However, inhibiting the enzymatic activity of AADC in the brain by NSD 1015 does not change the rate constant of [^{11}C]5-HTP trapping either, indicating that the measured amount of radioactivity in the brain does not reflect the breakdown of [^{11}C]5-HTP to [^{11}C]5-HT and [^{11}C]5-HIAA. A possible explanation is that parent [^{11}C]5-HTP is also trapped (together with its radioactive metabolites) and the PET scanner cannot distinguish between parent [^{11}C]5-HTP and these metabolites. Therefore, we conclude that [^{11}C]5-HTP is not suitable for measuring 5-HT synthesis in rodents, but it may be applicable in monkey and human.

Kinetic modeling of [^{11}C]MDL 100907 in rats (Chapter 4)

The most suitable tracer for measuring binding to 5-HT$_{2A}$ receptors is the antagonist [^{11}C]MDL 100907. This tracer was validated in humans, but not in rodents. As we wanted to measure longitudinal changes of 5-HT$_{2A}$ receptor binding in rats, we needed to validate various simplified modeling approaches in order to exclude invasive arterial blood sampling from the protocol.

In animals pre-treated with a vehicle solution (controls) or cold MDL 100907 (blocked group) different kinetic models were applied. The two-tissue compartment model (2TCM) was used as a gold standard and compared with the simplified reference tissue model (SRTM), non-linear graphical analysis (NIGA), and the target region- cerebellum ratio, which do not require arterial blood sampling.

Although all the above mentioned models fitted the data properly, there was a huge variation in k_3/k_4 values (2TCM) between animals. A possible explanation is that by using a rapid (5-10 sec) bolus injection and manual blood sampling, the radioactive peak in plasma is missed. Indeed, injecting the tracer as a slow (1 min) bolus decreases the variation in 2TCM outcome parameters, resulting in a good correlation between the 2TCM and the reference tissue models. Application of 0.2 mg/kg cold MDL 100907 resulted in almost complete occupancy of 5-HT$_{2A}$ receptors.

Thus, the tracer [^{11}C]MDL 100907 can be used to measure 5-HT$_{2A}$ binding in longitudinal studies in rodents.

The effect of social stress on 5-HT$_{2A}$ receptor binding (Chapter 5)

A frequently used rodent model for depression is the social defeat model in rats. In previous studies it was shown that there are both acute and more long-term effects on the serotonergic system in animals that underwent defeat stress. Considering the role of 5-HT in depression and the efficacy of antidepressants, we investigated the effect of acute social defeat on 5-HT$_{2A}$ binding of [^{11}C]MDL 100907 directly after the defeat and three weeks later. The open field (OF) test was used as a measure of anxiety.

Although rats lost weight after social defeat, there were no effects on behavior in the OF or on 5-HT$_{2A}$ receptor binding. To verify these findings, an *ex vivo* binding assay with [^{3}H]MDL 100907 was performed on hippocampus and prefrontal cortex tissue. These assays showed the same results as the PET study. As literature indicates different findings, the effect of stress on 5-HT$_{2A}$ receptors may depend on the characteristics of the rat strain or on the type of stressor used.

Serotonin-2A binding in rats differing in coping style (Chapter 6)

If individual differences determine the response of 5-HT$_{2A}$ receptors to stress, there may be a difference in expression at baseline conditions depending on how animals cope with a stressful environment. At the University of Groningen we have two rat lines available that differ in coping style. The Roman High- and Low-avoidance (RHA and RLA) are selected for actively or passively avoiding an electrical shock and the Wild-type Groningen (WTG) rat shows natural variation in coping behavior. WTG rats are selected for their levels of aggression and not bred for behavioral characteristics like the Roman strain.

Binding assays with an agonistic and an antagonistic radioligand ([^{3}H]Cimbi-36 and [^{3}H]MDL 100907 respectively) were performed to investigate receptor affinity and expression in hippocampus and prefrontal cortex. There were no differences in 5-HT_{2A} receptor binding between the rats that were characterized as active copers or rats characterized as passive copers, neither with the agonist nor the antagonist radioligand.

Thus, our data do not provide evidence for a major role of $5HT_{2A}$ receptors in stress response or the characterization of coping style.

Serotonin- dopamine interaction and the efficacy of SSRIs (Chapter 7)

Serotonin modulates the release of other neurotransmitters, like DA. In relation to major depression this is an important issue as both 5-HT and DA are involved in the pathophysiology of depression and the efficacy of antidepressants. Serotonin tonically inhibits DA release through 5-HT_{2C} receptor stimulation on GABAergic neurons. If antidepressant treatment with SSRIs increases the level of 5-HT in the synaptic cleft, it can even further decrease DA release.
Ideally both 5-HT and DA should be increased by antidepressant treatment to improve mood and motivation, respectively. This can be accomplished by co-administration of a specific 5-HT_{2C} antagonist and an SSRI like citalopram.

Indeed, in chapter 7 we show that by adding a 5-HT_{2C} antagonist to citalopram treatment, both 5-HT and DA can be increased in the ventral tegmental area and the nucleus accumbens, and the combination treatment shows increased effects compared to treatment with the 5-HT_{2C} antagonist or citalopram alone (as measured by microdialysis). This was particularly evident for the baseline concentrations of 5-HT and DA after subchronic treatment.

General discussion and future perspectives (Chapter 8)

There is a need for new techniques to non-invasively measure serotonin neurotransmission, especially in serotonin-related pathophysiology like stress and

major depression. PET is such a technological platform, where radioactive ligands can be used to measure biological processes in the brain, if the right prerequisites are met.

An important aspect in the use of PET is the validation of these radioactive ligands to ensure that they actually measure the process of interest.
Here we show that a complex process, like 5-HT synthesis, including different transporters and enzymes that define the amount of tracer trapped in the brain, is difficult to quantify. More straight-forward is the measurement of 5-HT_{2A} receptor binding by PET, which is quantifiable with a reference tissue model.
Although we could not show an effect of social defeat or coping style on 5-HT_{2A} receptor binding, it would still be interesting to investigate individual differences and the effect of chronic stress.

Especially interesting for future studies are the augmentation strategies of antidepressant treatment to improve their efficacy. As shown in the microdialysis study, both 5-HT and DA can be increased by combining an SSRI with administration of a 5-HT_{2C} antagonist. Future studies should focus on the effects of this combination therapy on the behavioral level.

CHAPTER 10

Nederlandse samenvatting

Serotonine (5-HT) is een neurotransmitter in het brein die betrokken is bij de totstandkoming van veel natuurlijk gedrag, zoals slapen en eten, maar ook bij de reactie van het lichaam op stress en bij het ontstaan van depressie. Daarom is het van belang om het signaalproces van 5-HT in de hersenen te kunnen meten. In levende organismen is dit heel lastig, omdat het invasieve procedures zoals het nemen van een monster van de cerebrospinale vloeistof vereist. Het gebruik van beeldvormende technieken zoals positron emissie tomografie (PET) en microdialyse biedt nieuwe mogelijkheden om het signaalproces van 5-HT te begrijpen, zowel tijdens gezond, natuurlijk gedrag als in het geval van aandoeningen zoals depressie.

De huidige medicatie voor depressie grijpt voornamelijk aan op het signaalproces van 5-HT, maar blijkt niet goed te werken in een groot aantal van de patiënten. Selectieve serotonine heropname remmers (SSRIs) zijn er op gericht om 5-HT concentraties in de hersenen te verhogen, door de heropname van 5-HT dat door neuronen is afgegeven te remmen. Als het signaal van 5-HT wordt versterkt door een SSRI, kunnen er compensatie-mechanismen optreden die de signaaloverdracht vervolgens weer verzwakken. Doordat het lastig is om de signaaloverdracht van 5-HT in levende mensen en dieren te meten weten we niet precies hoe een SSRI de nieuwe aanmaak van 5-HT beinvloedt.

Het onderzoek beschreven in dit proefschrift is erop gericht om verschillende serotonine PET tracers te valideren in ratten, zodat deze gebruikt kunnen worden om de fysiologie van stress te onderzoeken. Daarnaast hebben we een mogelijkheid onderzocht om de werking van antidepressiva te verbeteren.

In **hoofdstuk 2 en 3** van dit proefschrift is beschreven hoe we de 5-HT synthese proberen te meten met PET. De chemische voorloper van 5-HT is het aminozuur tryptofaan (Trp), dat na het passeren van de bloed-hersen barriere in de neuronen wordt omgezet in 5-hydroxytryptofaan (5-HTP) en uiteindelijk in 5-HT. De huidige methoden om dit proces in mensen te meten zijn invasief, zoals een punctie van de cerebrospinale vloeistof. PET maakt het in principe mogelijk om het proces op een niet invasieve manier te volgen. De twee beste opties zijn radioactief gelabelde varianten van Trp en 5-HTP. De eerste variant heeft als

nadeel dat bij ontstekingen t Trp ook wordt omgezet in andere stoffen dan 5-HTP. Door deze directe precursor, 5-HTP, radioactief te labelen zou dit probleem voorkomen kunnen worden, maar [^{11}C]5-HTP is lastig te produceren.

Om te kijken of de tracer [^{11}C]5-HTP inderdaad de synthese van 5-HT meet, hebben we de verschillende stappen in het 5-HT productieproces geblokkeerd in ratten en gekeken wat er gebeurde met het radioactief signaal in de hersenen en de tracerconcentratie in het bloed. Door deze data in een wiskundig farmacokinetisch model in te voeren kan de productiesnelheid van 5-HT worden berekend. Waarschijnlijk omdat de synthese van 5-HT uit meerdere stappen bestaat bleek het lastig om de snelheid van de synthese daadwerkelijk te meten. Het grootste probleem is waarschijnlijk dat [^{11}C]5-HTP niet volledig wordt omgezet in [^{11}C]5-HT en zelf ook in de hersenen blijft, waardoor het gebruikte farmacokinetische model niet helemaal klopt.

Een ander belangrijk element in de signaaloverdracht van 5-HT is de 5-HT$_{2A}$ receptor. Deze receptor is ook in verband gebracht met aandoeningen als psychose en depressie. Bijna alle antipsychotica grijpen aan op deze receptor. De PET tracer [^{11}C]MDL 100907 lijkt geschikt om 5-HT$_{2A}$ binding in de menselijke hersenen te meten, maar is nog nooit uitgetest in ratten. Om farmacologisch onderzoek en onderzoek naar biologische processen met deze tracer in ratten te kunnen doen moet de imaging procedure eerst gevalideerd worden (**hoofdstuk 4**). Er is eerst gekeken welk farmacokinetisch model het meest geschikt is om de data mee te fitten. We hebben kunnen aantonen dat [^{11}C]MDL 100907 geschikt is om 5-HT$_{2A}$ receptor binding in de hersenen van knaagdieren te meten. Een groot voordeel is dat er een vereenvoudigd farmacokinetisch model kan worden gebruikt, waarbij het niet nodig is om ook bloedmonsters af te nemen. Daardoor kunnen er meerdere PET scans worden gemaakt in één rat.

In **hoofdstuk 5** hebben we deze techniek toegepast in onderzoek naar het effect van stress op 5-HT$_{2A}$ receptoren, waarbij zowel op de dag direct na de stress als drie weken later een PET scan gemaakt is. Omdat de rol van de 5-HT$_{2A}$ receptor bij de symptomen van stress nog niet helemaal duidelijk is, hebben we in een diermodel van stress gekeken hoe de binding van [^{11}C]MDL 100907 over de tijd veranderde. In dit model worden ratten verslagen door een sterkere

tegenstander. Dit verlies veroorzaakt veel stress en gaat gepaard met een duidelijk aantoonbaar gewichtsverlies. De dieren vertoonden echter geen symptomen van angst in de open field test, in tegenstelling tot eerdere bevindingen in een andere rattenstam. Ook waren er geen veranderingen te zien in de 5-HT_{2A} binding van [^{11}C]MDL 100907 na één dag of na drie weken. De resultaten van de PET scan worden ondersteund door een additionele *ex vivo* 5-HT_{2A} receptor binding studie in weefsel van de hippocampus en frontale hersenschors.

Omdat in andere rattenstammen een duidelijker effect van stress was gevonden, zouden er verschillen tussen rattenstammen en tussen individuele ratten kunnen bestaan in de manier waarop de dieren omgaan met stress, wat ook wel 'coping style' wordt genoemd. Roman High- en Low- Avoidance ratten zijn speciaal gefokt om hun verschil in coping style. Groningen Wildtype ratten vertonen een natuurlijke variatie in gedrag, onder andere op het gebied van agressie, dat mede is gerelateerd aan verschillen in coping style. In deze twee rattenstammen hebben we in **hoofdstuk 6** gekeken naar de binding van radioactieve stoffen ([^{3}H]MDL 100907 en [^{3}H]Cimbi-36) aan de 5-HT_{2A} receptor in de hippocampus en frontale cortex. We konden geen verschil in binding vinden bij dieren met een verschillende coping style.

Uit bovenstaande resultaten kunnen we concluderen dat 5-HT_{2A} receptoren waarschijnlijk geen cruciale rol spelen bij de totstandkoming van de symptomen van stress of bij verschillen in coping style. Het is wellicht interessant om na te gaan wat het effect is van langdurige stress, in plaats van twee acute stressvolle ervaringen, op de 5-HT_{2A} receptor in het brein.

Naast het begrijpen van de biologische processen die gerelateerd zijn aan stress en depressie is het belangrijk om de werking van antidepressiva, zoals SSRIs, te verbeteren. Hoewel SSRIs erop zijn gericht om de concentratie van 5-HT in de hersenen te verhogen, zijn ook andere neurotransmitters van belang voor de verbetering van de gemoedstoestand. Eén daarvan is dopamine (DA). Dopamine is vooral betrokken bij motivatie. Een optimaal antidepressivum zou zowel de 5-HT als de DA concentratie in de hersenen moeten verhogen. In de praktijk blijken SSRIs eerder het signaalproces van DA te remmen. Waarschijnlijk komt dit omdat

de verhoogde 5-HT concentratie de signaaloverdracht via 5-HT_{2C} receptoren verhoogt waardoor de activiteit van dopaminerge neuronen wordt geremd. **Hoofdstuk 7** beschrijft een microdialyse studie in proefdieren die aantoont dat een combinatie van een SSRI en een stof die de 5-HT_{2C} receptor blokkeert zowel 5-HT als DA verhoogt in hersengebieden die gerelateerd zijn aan de symptomen van depressie. Zulke "augmentatie strategieën" kunnen een belangrijke rol spelen in toekomstig onderzoek naar de verbetering van antidepressiva. In vervolgonderzoek moet allereerst worden bepaald of combinatietherapie van een SSRI met een 5-HT_{2C} antagonist ook een positief effect heeft op niveau van het gedrag.

We concluderen in **hoofdstuk 8** dat er behoefte is aan de ontwikkeling van nieuwe technieken die non-invasief 5-HT neurotransmissie kunnen meten. PET is een dergelijke techniek waarbij radioactieve liganden biologische processen in het brein kunnen meten, mits aan de voorwaarden voor een goede PET tracer wordt voldaan. Daarom is het van groot belang dat nieuw ontwikkelde PET tracers goed worden gevalideerd, zodat we precies weten welk proces er wordt gemeten.
In dit proefschrift laten we zien dat een complex proces, zoals de vorming van 5-HT waarbij verschillende enzymen en transporters zijn betrokken , niet eenvoudig te meten is met PET. Het meten van de binding van een antagonist zoals [^{11}C]MDL 100907 aan een receptor lijkt minder complex, vooral wanneer er een vereenvoudigd analysemodel gebruikt kan worden voor de kwantificering.
Voor toekomstige studies zijn augmentatie strategieën om de werking van antidepressiva te verbeteren zeer interessant. Microdialyse is een goede techniek om zulke strategieen op neurochemisch niveau te ondersteunen. Vervolgstudies in proefdieren en patienten zullen moeten uitwijzen of de theoretische voordelen van augmentatiestrategieen ook werkelijk leiden tot gedragsveranderingen en een verbetering van de gemoedstoestand.

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Curriculum Vitae

Anniek Visser was born on March 28, 1985 in Amersfoort, The Netherlands. She graduated Atheneum in 2003 at the RSG Tromp Meesters in Steenwijk. After graduation she started her study Biology at the University of Groningen. During her Bachelors she decided to focus on Behavior and Neurosciences. Her first master project focused on the role of the phosphatase calcineurin in learning and memory, under the supervision of dr. Robert Havekes and prof. Eddy van der Zee at the department of Molecular Neurobiology. The obtained experience in behavioral testing and molecular analysis was very helpful during her second master project about the effect of chronic stress on the blood-brain barrier transporter P-glycoprotein, supervised by dr. Peter Meerlo and dr. Onno de Klerk (department of Behavioral Physiology and Nuclear Medicine and Molecular Imaging (NMMI), respectively). Anniek obtained her Master of Science in Biology *cum laude* in 2009.

Right thereafter (2009) she started her PhD at NMMI under the supervision of prof. Rudi Dierckx, prof. Johan den Boer, dr. Aren van Waarde and dr. Fokko Bosker. During her masters she learned how to use the innovative scanning modality positron emission tomography (PET), which she could apply during her PhD in studies on the role of serotonin in stress-related behaviors and antidepressant efficacy. These successful projects led to the present dissertation.

Currently, Anniek is working as a research project manager for the UMCG Research BV, focusing on imaging projects of the NMMI department and the Center for Medical Imaging (CMI). She is mainly involved in the application of research grants, linking of different parties, and contract research.