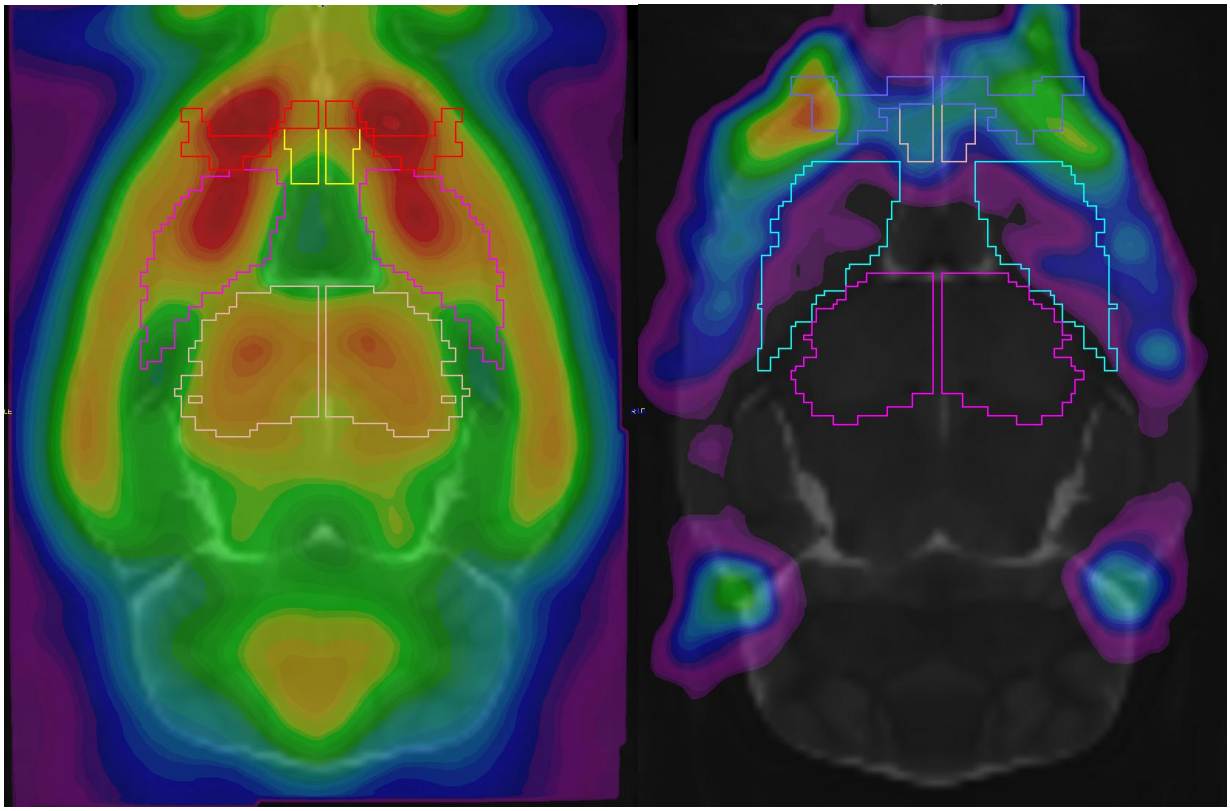
UNIVERSITY OF COPENHAGEN
FACULTY OF HEALTH AND MEDICAL SCIENCES
DEPARTMENT OF DRUG DESIGN AND PHARMACOLOGY



Master Thesis

Naja Støckel Jessen

The long-term effects of psilocybin in Long Evans rats

Supervisor: Jesper Tobias Andresen

External supervisor: Mikael Kjærby Palner

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Preface and acknowledgements

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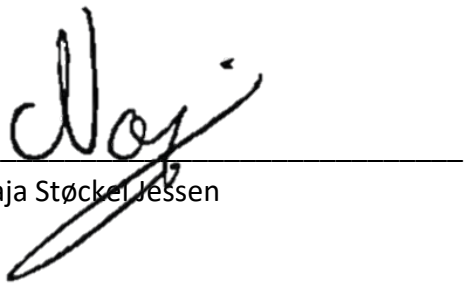

Naja Støckel Jessen

Abstract

In Denmark upwards of 33% of the population will at one point in their life, suffer from a mental illness. The most prevalent mental illness is depression, with a prevalence of 10%. Depression is characterised by increased negative affect and decreased positive affect. An old hypothesis of depression is the monoamine hypothesis. Newer hypotheses are emerging, among those the neuroplasticity hypothesis. The neuroplasticity hypothesis emphasises on decreased plasticity of neurons and abnormally functioning areas of the brain- including neuronal networks.

The first choice for treatment of depression in Denmark is the drug, Sertraline a serotonin reuptake inhibitor. Unfortunately, many patients are not satisfied with this treatment, and many patients experience only partial remission and apathic recovery. Newer antidepressant drugs are emerging, these include ketamine and psychedelics including psilocybin, which seem to work very well on depression with few side-effects.

In depression many neuronal networks seem to be faulty, many of these networks include the cortical-striatal-thalamic-cortical loops. Psilocybin seems to disrupt the cortical-striatal-thalamic-cortical loops. It is therefore relevant to investigate the mechanisms of psilocybin on these loops.

One aim of this thesis is therefore to establish if a dose of 1mg/kg in male Long Evans rats can be characterised as being a psychedelic dose. The thesis also tries to investigate if a single dose of 1mg/kg psilocybin produces changes in the metabolism in the brain areas of the cortical-striatal-thalamic-cortical loops, both acutely and a week after administration.

The method used in this thesis is positron emission thermography scans. Scans with the selective $5HT_{2a}$ receptor antagonist [^{18}F]-MHMZ was used to measure the occupancy of the $5HT_{2a}$ by psilocybin after a dose of 1mg/kg in male Long Evans rats. The radioactive glucose analogue [^{18}F]-fluorodeoxyglucose was used to measure glucose metabolism at baseline, acutely after a 1mg/kg dose of psilocybin and a week after dosing.

The experiments showed that a 1mg/kg dose of psilocybin produces an occupancy of 61% $\pm$11% at the $5HT_{2a}$ receptor in male Long Evans rats. This is comparable to the occupancy observed in humans that cause a psychedelic experience.

The metabolism experiment only shows a significantly lowered metabolism in the striatum acutely after psilocybin dosing (P-value: 0.0010). Though the results were not significant several trends in the data were observed. These trends need to be investigated further.

Table of contents

Abbreviations

GABA- γ-aminobuteric acid
AMPA- α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
NMDA- N-methyl-D-aspartate receptor
GPCR- G protein coupled receptor
5HT- 5-hydroxytryptamine (serotonin)
SERT- serotonin reuptake transporter
MAO- monoamine oxidase
cAMP- cyclic adenosine monophosphate
CREB- cAMP response element binding protein
BDNF- brain derived neurotropic factor
mPFC- medial prefrontal cortex
SSRI- Selective serotonin reuptake inhibitor
NDRI-noradrenaline and dopamine reuptake inhibitors
NRI-noradrenaline reuptake inhibitors
MAOI- monoamine oxidase inhibitors
TCA-Tricyclic antidepressants
OCD - obsessive compulsive disorder
PTSD- post-traumatic stress disorder
RADS- Råd for anvendelse af dyr sygehusmedicin
FDA- The Food and Drug Administration
EMA- The European Medicines Agency
DMT- N, N-Dimethyltryptamine
LSD- Lysergic acid diethylamide
DEA- Drug enforcement administration
K_i- Inhibitory constant
MRI-Magnetic resonance imaging
PET- Positron emission thermography
CSTC loops- Cortical-striatal-thalamic-cortical loops
DLPFC- dorsolateral prefrontal cortex
[^{18}F]-FDG- [^{18}F]-fluorodeoxyglucose
BPnd- non displaceable binding potential
V_T- equilibrium volumes of distribution
GLUT- glucose transporter
IV- intravenous
IP-interperitoneally
SC-subcutaneously
BS- blood sugar
VOI- Voxel of interest
SUV- Standardised uptake values
SUVwb- Standardised uptake value whole brain normalised
REBUS- relaxed beliefs under psychedelics mode

1.Introduction

1.1. The cost and prevalence of psychiatric diseases

It is said that a third of the Danish population, will at some point in their life be diagnosed with a mental illness. On a yearly basis 700.000 persons of the adult Danish population, show symptoms of mental illnesses [1]. It is estimated that the yearly cost of mental illnesses and mental health issues in Denmark is 110 billion dkk, equivalent to 5.4% of the Danish gross domestic product according to the Organisation for Economic Co-operation and Development[2]. This includes the cost of early retirement, long term sick-leave and reduced work capacity caused by mental illnesses. The yearly cost of the psychiatric sector in Denmark in 2017 was 10.05 billion dkk, where the biggest cost comes from psychiatric hospitals[3].

By far the most abundant mental illness in Denmark is depression having a prevalence of 10% in the Danish population. After that comes substance abuse with a prevalence of 1.3%. All other categories of mental illness have a prevalence between 1.1% in personality disorders and 0.2 % in obsessive compulsive disorders and autism spectrum disorders. These numbers are based on registry data from the Danish health authorities [4]. Hvidberg et.al. suggests that depression (10%) is the 4th most prevalent chronic disease in Denmark after hypertension (23.3%), respiratory allergy (18,5%) and disorders of lipoprotein metabolism (14.3%). The above-mentioned diseases are easily treated with e.g. ACE inhibitors for hypertension, antihistamines for respiratory allergy and statins for disorders of lipoprotein metabolism. Generally, these treatments have a high efficacy and are well tolerated by patients [5–7]. In Denmark depression is most commonly treated with Sertraline, a selective serotonin reuptake inhibitor(SSRI) [8]. Unfortunately many patients experience problems with this treatment [9,10].

1.2. Glutamate and GABA transmission

Glutamate and GABA (γ-aminobuteric acid) are the two major neurotransmitters in the central nervous system. Glutamate being the major excitatory neurotransmitter and GABA being the major inhibitory neurotransmitter. These neurotransmitters are thereby almost always involved directly or indirectly in all aspects of normal brain functioning.

Glutamate interact with ionotropic glutamate receptors, which are ligand gated ion channels (AMPA, NMDA and kainate) and metabotropic glutamate receptors which are G protein coupled receptors. GABA interacts with the two types of GABA-receptors, of which the $GABA_a$ receptors are ligand gated ion channels and the $GABA_b$ receptors are G protein coupled receptors [11].

In the brain, several types of neuronal cells exist. Some of the most important types are pyramidal cells and interneurons (basket neurons, chandelier neurons, bouquet neurons). The pyramidal cells are the drivers of the cortical circuits. They receive glutamatergic input from several other brain areas depending on their location, in the different cortical lamina. They also send out glutamatergic output to several brain areas depending on their location. The interneurons are often GABAergic, meaning that they will send an inhibitory output to e.g. pyramidal cells. To put it simply, pyramidal cells receive input and forwards a signal. GABA interneurons counteract the input so nothing/or a

diminished signal is transduced. But as a pyramidal cell often will receive input from several places, finetuning is needed, so that only the right information is transduced. This is where many of the other neurotransmitters e.g. the monoamines (serotonin(5HT), dopamine and noradrenaline) come into play. They regulate the signal transduction by acting either inhibitory or excitatory. The action depends on the neurotransmitter and the receptor they interact with. Generally, the monoamines regulate the signal transduction, by eliminating noise and enhancing the signal of interest. Therefore, the monoamines are a very important part of neurotransmission [11].(Figure 1)

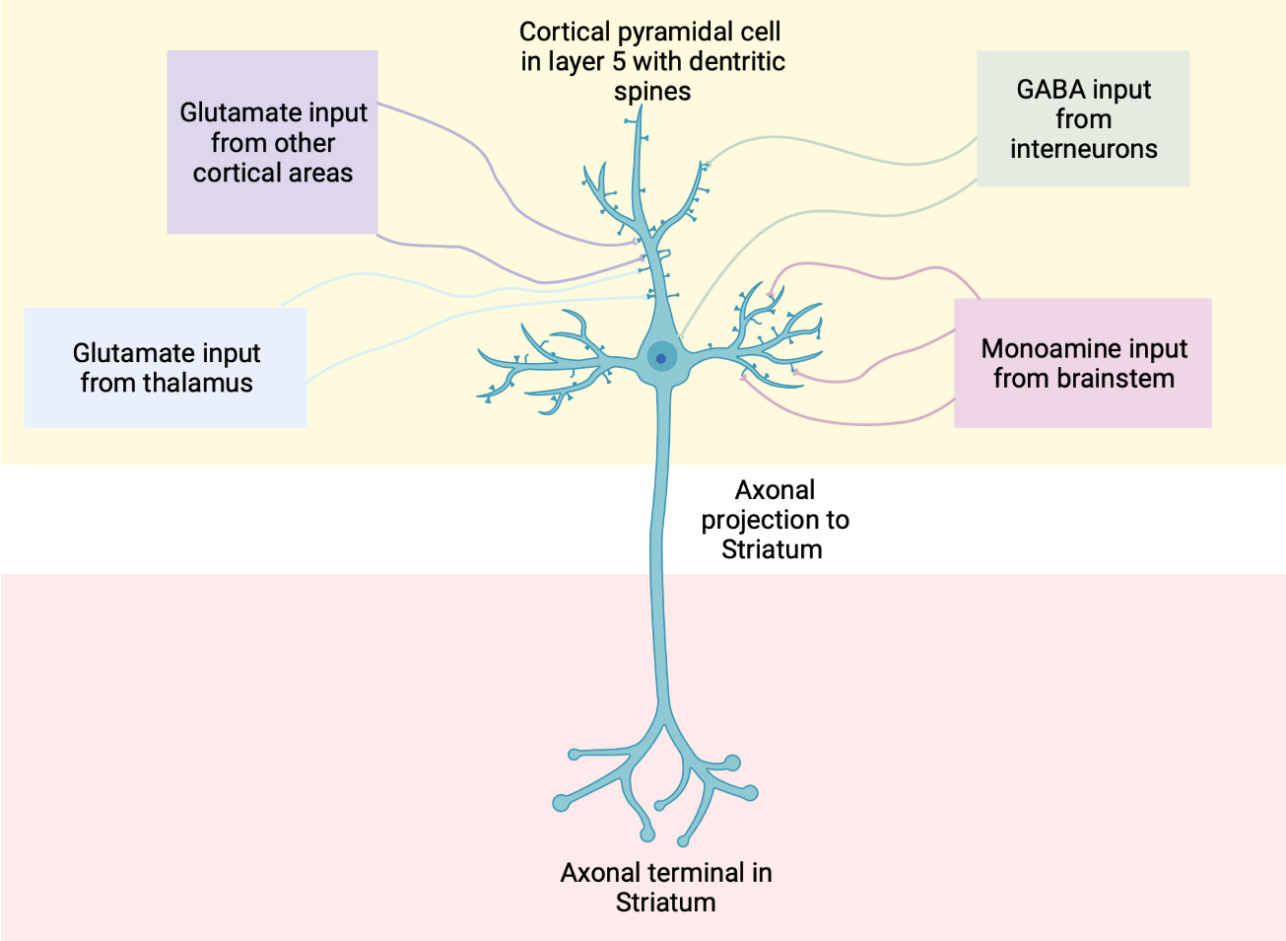

Figure 1: Schematic explanation of cortical glutamatergic pyramidal cells in cortical layer 5, with input from GABA interneurons, glutamate input from other cortical areas, and from the thalamus and monoamine input from the brainstem.

1.3. The serotonergic system

Serotonin is an endogenous monoamine neurotransmitter with the systematic name 5-hydroxytryptamine, therefore often abbreviated 5HT. Serotonin is predominantly present in the gut where it causes vasoconstriction. It is also present in the brain where it is a neurotransmitter, regulating e.g. mood, sleep, appetite and cognition.

In the central nervous system, serotonin has serotonergic projections that stem from the raphe nuclei in the brain stem. The descending projections regulate pain in the body. The major ascending projections project to the cortex (importantly the prefrontal cortex), basal forebrain, striatum, nucleus accumbens, thalamus, amygdala, hippocampus and the cerebellum. (Figure 2)

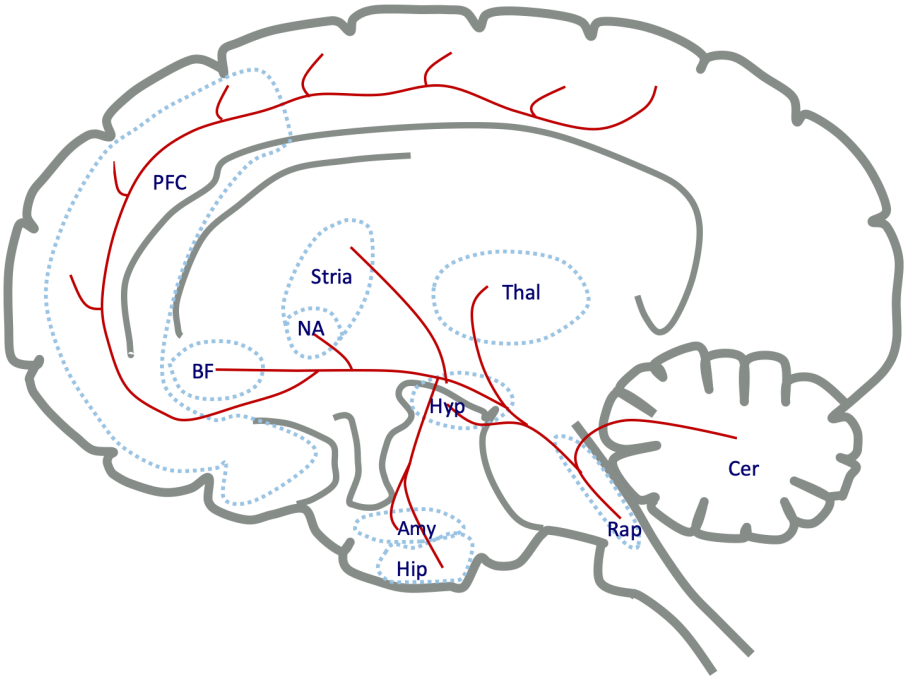

Figure 2: Serotonergic projections in the human brain. PCF: prefrontal cortex, BF: Basal forebrain, NA: Nucleus Accumbens, Amy: Amygdala, Hyp: Hypothalamus, Hip: Hippocampus, Thal: Thalamus, Cer: Cerebellum, Rap: Raphe nuclei. Inspired by Stahl [11]

1.3.1. Serotonin in neurotransmission

Serotonin is released by presynaptic vesicles and acts on the various receptors both presynaptically and postsynaptically. Serotonin is transported back into the presynaptic cell via the serotonin reuptake transporter (SERT) and repacked into presynaptic vesicles for reuse. Some of the serotonin is also degraded in glia-cells by the monoamine oxidase A enzyme (MAO_a). (Figure 3)

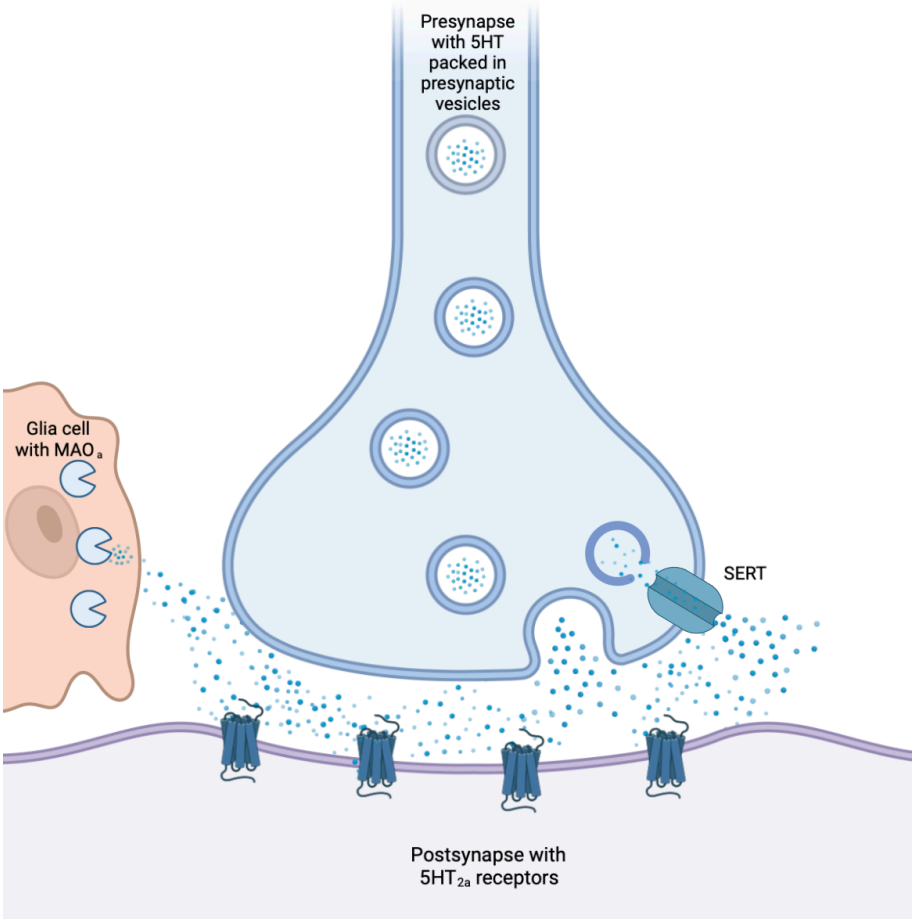

Figure 3: Schematic representation of serotonergic transmission. SERT: Serotonin reuptake transporter, 5HT: serotonin, $5HT_{2a}$: serotonin 2a receptor, MAO_a: Monoamine oxidase A. figure created in biorender.com.

The serotonergic system is regulated by many of the other neurotransmitters and the serotonergic system regulate other neurotransmitters as well. The system is complex and differs between brain areas[11].

Serotonergic receptors can be found presynaptically and postsynaptically. Depending on the type some are excitatory, and some are inhibitory.

Most serotonergic receptors are G protein coupled receptors, but the $5HT_3$ subtype is a ligand gated ion channel (figure 4).

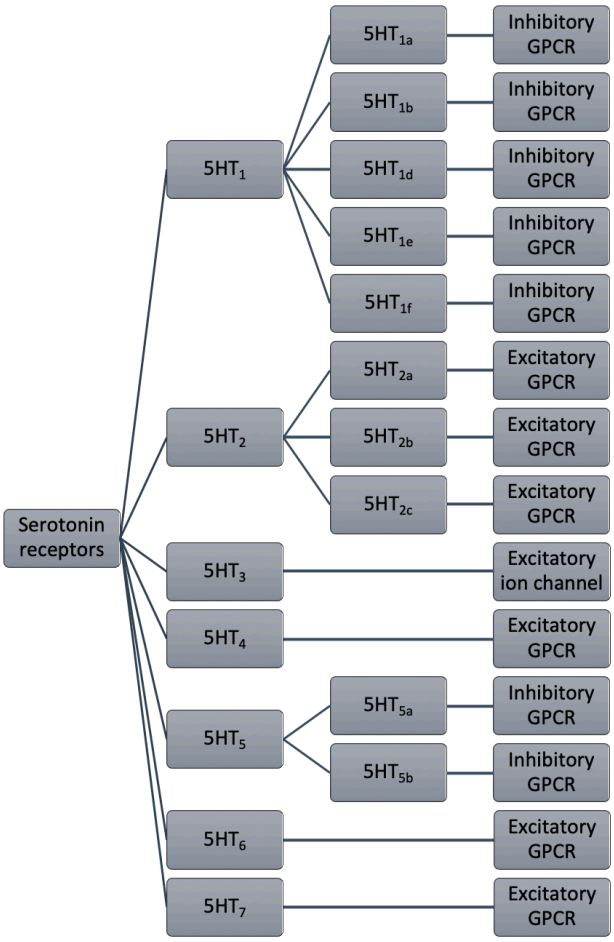

Figure 4: Simplified figure of types of serotonin receptors and their basic functions. GPCR: G-protein coupled receptor.

The G protein coupled receptors couple to many different G-proteins. Generally, the $5HT_1$ family and $5HT_5$ family couple to the $G\alpha_{I/O}$ protein. The $5HT_4$, $5HT_6$, $5HT_7$ couple to the $G\alpha_s$ protein whilst the $5HT_2$ family couple to the $G\alpha_{q/II}$ [12]. The $G\alpha_{I/O}$ protein inhibit the adenylate cyclase protein and the $G\alpha_s$ protein stimulates the adenylate cyclase protein. Adenylate cyclase produce cyclic adenosine monophosphate(cAMP) which activate protein kinase A, which in turn will start a signalling cascade, ending in formation of various transcription factors.
The signal cascade of the $G\alpha_{q/II}$ protein at a $5HT_{2a}$ receptor can be described by the following figure. (figure 5)

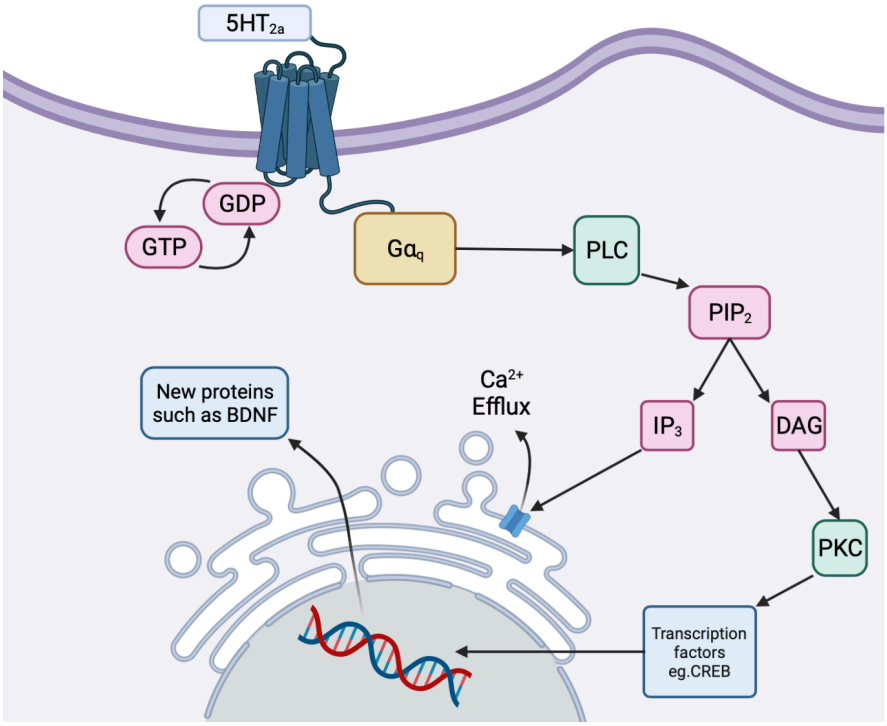

Figure 5: GTP: Guanosine Triphosphate, GDP: Guanosine diphosphate, cAMP: cyclic adenosine , PLC: phospholipase C, PIP2: Phosphatidylinositol 4,5-biphosphate, IP3: inositol triphosphate, DAG: diacylglycerol, PKC: protein kinase C, CREB: cAMP (cyclic adenosine monophosphate) response element binding protein, BDNF: Brain derived neurotropic factor. Figure created in biorender.com.

As shown, the $5HT_{2a}$ will be activated by a ligand, which will activate the $G\alpha_{q/II}$ protein. $G\alpha_{q/II}$ activates phosphor lipase C, that will cleave phosphatidylinositol 4,5-biphosphate into inositol triphosphate and diacylglycerol. Inositol triphosphate will go to the endoplasmic reticulum, where it will activate calcium transporters. These will raise the intracellular concentration of calcium, thereby hyperpolarising the cell, and allowing for calcium dependant signalling. Diacylglycerol will activate protein kinase C, which will start a cascade leading to formation of transcription factors, such as the cAMP (cyclic adenosine monophosphate) response element binding protein (CREB). CREB will go to the nucleus where it will regulate the transcription of genes. This will end in transcription of genes, such as the brain derived neurotropic factor (BDNF) gene among others. BDNF is a protein that is involved in neurogenesis and thereby neuronal plasticity [13].

1.4. Hypothesis of depression

In depression several hypotheses have been developed to explain the symptomatology. An older and once widely accepted hypothesis is the monoamine hypothesis, where monoamine transmission (serotonin, noradrenaline and dopamine) is deficient. Many of the specific symptoms have been related to the monoamines. Generally, a deficient serotonin transmission and noradrenaline transmission in specific brain areas are attributed to increased negative affect.

Deficient dopamine and noradrenaline transmission in other brain areas are attributed it the decreased positive affect [11]. Therefore, most of the current treatments of depression try to normalise the deficient monoamine system, by raising the working time/concentration of the monoamines in the synaptic clefts. Several other hypotheses have also been developed, as not only antidepressants that raise the working time/concentration of monoamines in the synaptic cleft have shown efficacy.

1.4.1. Neuroplasticity hypothesis

A newer and now much accepted hypothesis is the neuroplasticity hypothesis. This is based on the fact that NMDA receptor antagonists have antidepressant effects, and that the downstream molecular events of traditional antidepressants, result in altered gene expression. This hypothesis is a lot more complex than the monoamine hypothesis, and stress is recognised as one of the main culprits of the development of depression.

In a review by Pittenger and Duman (2008) the authors summarise the findings that have led to the formulation of the neuroplasticity hypothesis of depression [14]. The authors emphasise that one of the defining symptoms of depression is the impairment of learning and memory, and that many brain areas related to these functions appear to function abnormally in depressed individuals.

For instance, altered function of the prefrontal cortex and hippocampus, seems to be some of the leading causes of memory and learning impairment. Sustained/chronic stress with high levels of glucocorticoids seems to have an influence of the hippocampal neuroplasticity, by causing atrophy and retraction of dendrites and suppression of neurogenesis. This in turn, causes problems with memory and learning.

It also seems that stress and high levels of glucocorticoids have an influence on the medial prefrontal cortex(mPFC), causing regression of apical dendrites and a reduction of glia cells. This regression of the apical dendrites of the mPFC, is proposed to cause problems with attention shifting in depression. The reduced number of glia cells is hypothesised to cause altered functioning of the pyramidal cells [14].

Pittenger and Duman also emphasise that an increased volume and activity of the amygdala caused by stress, play a part in the symptomology of depression. Pittenger and Duman emphasise that this increased volume and activity, have a massive impact on amygdala-based fear-learning. This contributes to overactivation of neuronal circuits that control fear, anxiety and emotion. Pittenger and Duman finally emphasise on the attenuated response of the ventral striatum, including the nucleus accumbens, seen in depressed individuals. The attenuated response is thought to relate to the anhedonia seen in depression [14].

1.4.1.1. Molecular and cellular mechanisms of the neuroplasticity hypothesis

In the review by Pittenger and Duman, the authors also describe the cellular and molecular mechanisms thought to be involved in depression. The main emphasis is on what can cause changes in neuroplasticity. Emphasis is laid on calcium influx and on raised concentration of the second

messenger cAMP, as a result of activation of receptors, such as the NMDA receptors and $5HT_4$, $5HT_6$, $5HT_7$ receptors.
Both of these types of events lead to signalling cascades ending in regulation of genes, and thereby regulation of synaptic change and neuroplasticity. One of the main proteins that seem to be disrupted in depression, is the transcription factor CREB. CREB is responsible for the regulation and transcription of several genes, some of which are responsible for long term synaptic change. These genes include the BNDF gene. In depression CREB seems to be inhibited and therefore synaptic plasticity is inhibited. Pittenger and Duman emphasise on the fact that an increasing amount of studies show that antidepressants introduce neuroplasticity and neurogenesis. Evidence is also emerging that some antidepressants upregulate CREB. This increases expression of BDNF etc. causing the neurogenesis and antidepressant effect. What can be learned by the neuroplasticity hypothesis, is that stress can cause decreased neuroplasticity, which can lead to depression. Treatment with antidepressants cause neurogenesis and strengthening of neuroplasticity. The neuroplasticity hypothesis of depression also gives an explanation as to why atypical antidepressants and newer rapid acting antidepressant work [14]. Several other theories have also been developed, but this researcher finds, that the neuroplasticity best captures the evidence emerging.

1.5. Clinical symptoms of depression

Unipolar depression is characterised by depressed mood and or apathy as its core symptoms. But several other symptoms must also be present to clinically diagnose a patient with depression. These symptoms can amongst others be; change in appetite and weight, sleep disturbances, psychomotor disturbances such as agitation and/or retardation, general fatigue, a sense of guilt and worthlessness, executive dysfunction and suicidal ideation[15]. The symptoms can be characterised into general reduced positive affect and increased negative affect[11].

Table 1: The symptoms of depression categorised into reduced positive affect and increased negative affect.

Reduced positive affect.	Increased negative affect.
Anhedonia	Ruminative thoughts
Apathy	Guilt
Decreased alertness	Disgust
Decreased energy	Fear/anxiety
Decreased enthusiasm	Hostility/irritability
Decreased self-confidence	Loneliness/worthlessness
Impaired concentration	Suicidal ideation

For patients diagnosed with depression, several cognitive tests have been used to see how depression affects executive function. In the Wisconsin card sorting test, depressed individuals

perform worse than non-depressed individuals. This indicates difficulties with problem solving, abstract thinking, sustained attention, working memory, cognitive flexibility and disengagement from negativity [16]. Individuals with depression also show difficulties performing The Stroop test compared to non-depressed individuals. The emotional Stroop test also show that depressed individuals have a higher bias towards negative stimuli than controls [17]. These problems with executive function can also be explained by the neuroplasticity hypothesis described above [14].

1.6. Types of antidepressants

Currently most of the used treatments for unipolar depression work on the monoamine neurotransmitter systems and most work on the serotonin system. The mechanisms of these typical antidepressants, working on the monoamine system, was once described by the monoamine hypothesis, but as the neuroplasticity hypothesis, have emerged the mechanisms have been revised [14].

Antidepressants are categorised in up to 7 categories. Selective serotonin reuptake inhibitors (SSRI's), serotonin and noradrenaline reuptake inhibitors (SNRI's), noradrenaline and dopamine reuptake inhibitors (NDRI's), noradrenaline reuptake inhibitors (NRI's), monoamine oxidase inhibitors (MAOI's), Tricyclic antidepressants (TCA's) and atypical antidepressants (which do not target transporters but receptors). There are also newer prospects that do not fall into the above-mentioned classes.

1.6.1. Mode of action of antidepressants

SSRI's work by inhibiting the reuptake of serotonin thereby raising the concentration in the synaptic cleft. The same mechanism is seen for the SNRI's, except SNRI's also work by inhibiting the noradrenaline reuptake transporters. NDRI's work by inhibiting the noradrenaline and dopamine reuptake transporters. NRI's only inhibit the noradrenaline reuptake transporters. The MAOI's work by inhibiting the monoamine oxidase enzyme, which catabolise the monoamines. This means that the concentration of monoamines is raised in the synaptic cleft. The TCA's have the similar mechanism as SNRI's or SSRI's. Some TCA's have high affinity towards receptors and have their main mechanism via this interaction. TCA's are an older class of drugs that have a lot of side-effects, which current prescribers do not tolerate. The atypical antidepressants work directly on receptors, they do not work by raising the concentration of monoamines in the synaptic cleft, as typical antidepressant such as SSRI's do. Examples could be drugs such as mianserine and mirtazapine, which antagonise the noradrenergic α_1 receptor and block the $5HT_3$, $5HT_{2a}$ and $5HT_{2c}$ receptors along with the histamine 1(H_1) receptors. Several other atypical antidepressants exist and all of them have vastly different mechanistic profiles and most of their mechanisms can be described by the neuroplasticity hypothesis[11]. (figure 4)

1.6.2. Tolerability and efficacy of antidepressants

In Denmark the first choice for treatment of unipolar depression is Sertraline(SSRI) [8]. Sertraline is also prescribed for obsessive compulsive disorder (OCD), social anxiety, panic disorder and post-traumatic stress disorder (PTSD). In a meta-analysis from 2018, 21 antidepressants were examined for efficacy and tolerability. In the study Sertraline was shown to have great efficacy compared to placebo[9]. Unfortunately, the tolerability of Sertraline was not better than placebo. The same picture was seen for 18 of the other frequently prescribed antidepressants. The meta-analysis even showed that for Clomipramine (TCA) placebo was favoured in terms of tolerability. Only the drugs agomelatine (atypical) and fluoxetine (SSRI) showed favourability for the drug over placebo in terms of tolerability. In terms of efficacy the study showed that all the examined antidepressants were efficacious compared to placebo [9].

1.6.2.1. Side-effects

Antidepressants generally have a high occurrence of adverse events and side-effects. For many antidepressants the frequency of reported side-effects is high, compared to other commonly prescribed drugs. For more than 10% of the patients treated with Sertraline, the following side-effects have been reported; nausea, dizziness, headaches, delayed or missing ejaculation (male population), tiredness, insomnia etc. [18]. Looking at the recommendation for general practitioners from "Råd for anvendelse af dyr sygehusmedicin" (RADS), the most well tolerated antidepressants, agomelatine and fluoxetine, are seen in the 3rd choice category. This means that practitioners are not advised to prescribe these drugs to patients, before trying other types [8,9].
The recommendation from RADS is based on side-effect profiles, interactions with other drugs and the need for regular monitoring from a healthcare professional.

1.6.2.2. Partial remission

A problem with the current treatments of unipolar depression is partial remission and residual symptoms [19]. Depending on the study, up to 50% of treated patients will have residual symptoms after a pharmacological intervention with antidepressants. The most prevalent residual symptoms reported from patients in full remission, are sleep disturbances (44%), fatigue (38%) and diminished interest or pleasure (27%) [20]. A review form Tranter et al. showed that patients who have residual symptoms and partial remission, have a bigger chance of relapse than patients without [20]. It is hypothesised that when unipolar depression is recurrent, it can develop into chronic recurrent depression. Here the severity becomes higher with each episode, and the remission period becomes shorter until there is none [21]. Recurrent depression is also hypothesised to be neurotoxic and causing problems with impaired memory [22], poor social recovery [23] and higher risk of dementia etc. [24].

1.6.2.3. Apathetic recovery

Another problem that many patients with unipolar depression experience, is SSRI induced apathy syndrome. This means that the patient experience that the increased negative affect, such as worthlessness and anxiety are diminish, but that the reduced positive affect persists.
This means that the patient still experiences symptoms, such as apathy and decreased enthusiasm. Some patients explain it as having a lack of motivation or just being "fine". Patients also describe it as flatlining, feeling no form of joy but also no form of sadness, and feeling that their emotional range is much smaller than before [25,26]. This can be argued to be a problem for patients, as only part of their problems are solved by giving them SSRI's as a pharmacological treatment.
Taking all of the above into account, it is evident that the current treatments are not sufficient and that the way we treat patients is not satisfactory to them. It is of the utmost importance that the scientific community finds a way to treat these patients. First of all, to end their suffering, but also to potentially save money and make the mental healthcare system more effective.

1.6.3. New prospects

There is a newer type of rapidly acting antidepressants with completely different modes of action. Their mechanisms of action can be explained by the neuroplasticity hypothesis.
In the US, FDA (The Food and Drug Administration) and in the EU, EMA (The European Medicines Agency) has approved the nasal spray Spravato® as a rapid acting antidepressant. It contains esketamine which works by antagonising the NMDA receptor. Esketamine is the s (+) enantiomeric form of the dissociative anaesthetic ketamine, which has been used for a long time in the clinic. In sub-anaesthetic doses, it has been shown to decrease depressive symptoms within a few hours. It has also been shown to work well as an add-on for oral antidepressants such as SSRI's [27].
Several other drug candidates are also currently under investigation. Many of these drugs fall under the category psychedelics. Psychedelics have previously been investigated, but were abandoned in the 60's and 70's.

1.6.3.1. Psychedelics

Psychedelics are a class of drugs that have been used traditionally for millennia all over the world. They are classified into 3 classes; tryptamines, phenethylamines and lysergamides. Many of the drugs come from natural products e.g. Psilocybin (psilocybe mushrooms), mescaline (peyote cacti) and N, N-Dimethyltryptamine (DMT) (*Psychotria viridis*). Several other synthetic psychedelics have been developed e.g. Lysergic acid diethylamide (LSD) and the NBOMe's (e.g. 25C-NBOMe). All of these drugs have in common that they produce changes in cognitive processes, mood and cause hallucinations. All of these drugs are classified as class 1 drugs by the DEA (drug enforcement administration) in the US, meaning that they are "*…defined as drugs with no currently accepted medical use and a high potential for abuse* "[28]
In Denmark the psychedelics are classified differently. LSD is classified as a class A drug, meaning that it is fully illegal and must not be possessed in Denmark. All other psychedelics are classified as class B drugs, meaning that they can only be used for medicinal or research purposes [29]. This

means that research in LSD is difficult and why current research is focused on the other types of psychedelics in Denmark.

1.6.3.1.1. What is psilocybin?

Psilocybin is an indolamine, naturally occurring in psilocybe mushrooms. Psilocybin is considered one of the classic serotonergic psychedelics along with LSD, mescaline and DMT.
The compound psilocybin is closely related to serotonin and is essentially a prodrug, as it gets rapidly dephosphorylated, to the active compound, psilocin in the gastrointestinal tract and in the blood. Afterwards it is transported to the site of action via the bloodstream[30]. (Figure 6)

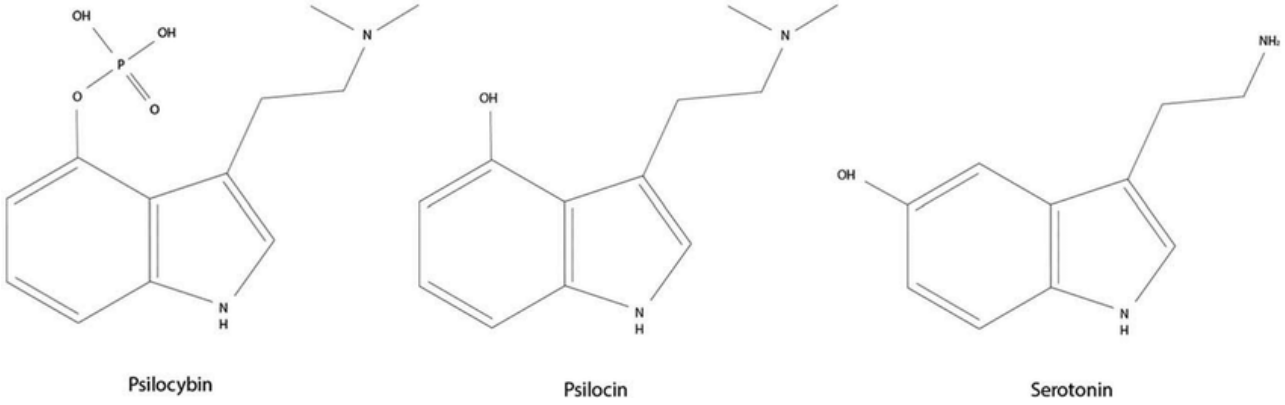

Figure 6: Chemical structure of psilocybin, psilocin and serotonin.

Psilocybin and the active metabolite psilocin have affinity for most of the serotonin receptors. But as psilocybin is rapidly dephosphorylated, psilocin is believed to be the main contributor to the mode of action. The affinity of psilocin has been investigated and estimated by various researchers. The affinity (K_i (inhibition constant)) of psilocin to some of the serotonin receptors, can be found in the following table, with information of the used competitor, species of origin and source. (table 2)

Table 2: overview of K_i values of psilocin at various 5HT receptors. Information found at The Psychoactive Drug Screening Program.

Receptor	K_i	Competitor	Species	Source
$5HT_{1a}$	49 nM	^{3}H-8-OH-DPAT	Human	Cloned
$5\text{-}HT_{2A}$	12 nM	^{125}I-DOI	Rat	Cerebral cortex
$5\text{-}HT_{2A}$	25 nM	^{125}I-DOI	Rat	Cloned
$5\text{-}HT_{2A}$	370 nM	^{3}H-Ketanserin	Bovine	Cerebral cortex
$5\text{-}HT_{2B}$	450 nM	^{3}H-Ketanserin	Bovine	Cerebral cortex
$5\text{-}HT_{2C}$	10 nM	^{125}I-DOI	Rat	Cloned

As evident psilocin has the highest affinity towards the $5HT_{2c}$ receptor (10 nM in rat) and after that comes the $5HT_{2a}$ receptor (12-25 nM in rat). It is evident that the affinities of psilocin at the $5HT_{2c}$ and $5HT_{2a}$ in rats is within relevance.

As mentioned, psilocybin is classified a class 1 drug by the DEA. This classification came with the Controlled Substances act form 1971, where the American president Nixon declared "the war on drugs". Before the 1970's several clinical studies/trials were performed with psilocybin showing great promise for several different mental illnesses[30].
After the classification as a class 1 drug in the US, the number of studies with psilocybin decreased drastically. But since the 1990's the number of studies with psilocybin has been steadily increasing again. The trials from the 1960's and 1970's has many times been shown to be flawed in their design and causing more harm to the patients than good [31]. Luckily the newer trials are much better constructed and the welfare of patients and trail subjects has been improved dramatically [32,33].

Psilocybin in both preclinical and clinical trials, show great promise for treatment of major depression [32–36]. Clinical trials are also currently done for e.g. use disorders/substance abuse, bipolar II disorder, PTSD, OCD, anorexia nervosa and many other illnesses according to the clinical trials database form the National Institutes of Health.
The clinical trials that have been published with psilocybin show, that a single treatment with a psychedelic dose of psilocybin and the right psychological support, alleviate many of the depressive symptoms that patients experience. The improvement of symptoms persist for a long time – upwards of 6 months after treatment [32–34]. They also show that the general tolerability in patients with treatment resistant depression is great. Not many side-effects/adverse events have been reported in these studies. The most prevalent adverse event in an Carhart-Harris study was headaches that occurred within 24 hours after dosing [32,33]. Taking all this information into consideration, psilocybin seems like a good candidate for treatment of unipolar depression. Unfortunately, still very little is known as to what happens on a molecular level when we treat patients with psilocybin. Further research is needed to fully assess the safety profile. Unearthing the molecular mechanisms of psilocybin will hopefully, also help in the understanding of the molecular mechanisms behind depression and add to the neuroplasticity hypothesis.

1.7. Depression as a symptom of faulty neuronal networks

More and more evidence is pointing towards depression being a symptom of faulty neuronal networks, such as the default mode network, task positive network and salience network [37]. Mounting evidence from connectivity studies (MRI (magnetic resonance imaging), PET (positron emission thermography) etc.) show that in depressed individuals, many neuronal networks have altered connectivity compared to healthy individuals. Many neuronal networks with functional connectivity, have in common that they involve cortical, striatal and thalamic areas of the brain [37].

Cortical-striatal-thalamic-cortical (CSTC) loops are therefore very essential in the study of depression.

1.7.1. Cortical- striatal- thalamic-cortical loops

Cortico-striatal-thalamic-cortical loops are very essential neuro circuits in the brain. These loops allow information to be sent downstream from cortical regions (often prefrontal cortex), to lower regions and to get feedback about how the information has been processed downstream. Generally, the striatum and thalamus are organised, so that they only interact with certain areas of the cortex. In the striatum it is common that the loop has another synapse through another part of the complex, before the loop goes to the thalamus. When reaching the thalamus, the loop will relay back to the cortex and even right back to the same pyramidal cell that started the loop. The loops go through 3 distinct areas allowing for regulation by the neurotransmitters at each site.
Looking at the serotonergic projections from the dorsal raphe nuclei, it is evident that the projections protrude to all the areas of the CSTC loops, thereby giving serotonin the ability to regulate the CSTC loops closely (figure 2).
The CSTC loops have the capacity to transform simple inputs from the cortex into complex outputs, because of the complexity the loops entail. These complex outputs ultimately mediate brain function and behaviour.
Several CSTC loops have been described and linked to functions within the brain, depending on which parts of the cortex, striatum and thalamus are involved.
A loop stemming from the dorsolateral prefrontal cortex (DLPFC) to the rostal part of the caudate, down to the thalamus and back to the DLPFC, is hypothesised to regulate executive function.
Another loop stemming from the dorsal anterior cingulate cortex to the bottom of the striatum and then to the thalamus, is thought to regulate selective attention.
Others are hypothesised to regulate emotions, compulsivity, motor activity such as psychomotor agitation/retardation[11,38].

Many of these functions are known to be faulty in unipolar depression (table 1) and mounting evidence show that faulty CSTC loops, of for instance the salience network, is related to the pathology and symptomology of depression [37,39,40].
Therefore, it is very relevant to look at how the brain areas of the CSTC loop behave when drugs that should improve depressive symptoms, are administered. In OCD faulty CSTC loops are found to be restored to normal function, after treatment with antidepressant SSRI's [41]. This further underline the need for investigation of the CSTC loops in depression treatment. Psilocin the active metabolite of psilocybin, is known to interact with serotonin receptors acting as an agonist on these receptors. Therefore, it could be hypothesised that psilocin could interfere with the regulation of CSTC loops involved in the symptomology of depression. Thereby treatment with psilocybin, could help change the way theses loops are regulated.

1.7.1.1. Psilocybin and CSTC loops

Psilocybin/psilocin is known to cause hyperactivation of the $5HT_{2a}$ receptors. This alters the functioning of some CSTC loops. The hyperactivation of the $5HT_{2a}$ by psychedelics causes disruption of the thalamic gating/filtering of sensory and cognitive information. This disruption overloads the cortical areas with sensory information as the information is relayed from the thalamus to the cortical areas. This overload and disruption is hypothesised to be the what causes the perceptional distortions (hallucinations) from psilocybin [41]. (Figure 7)

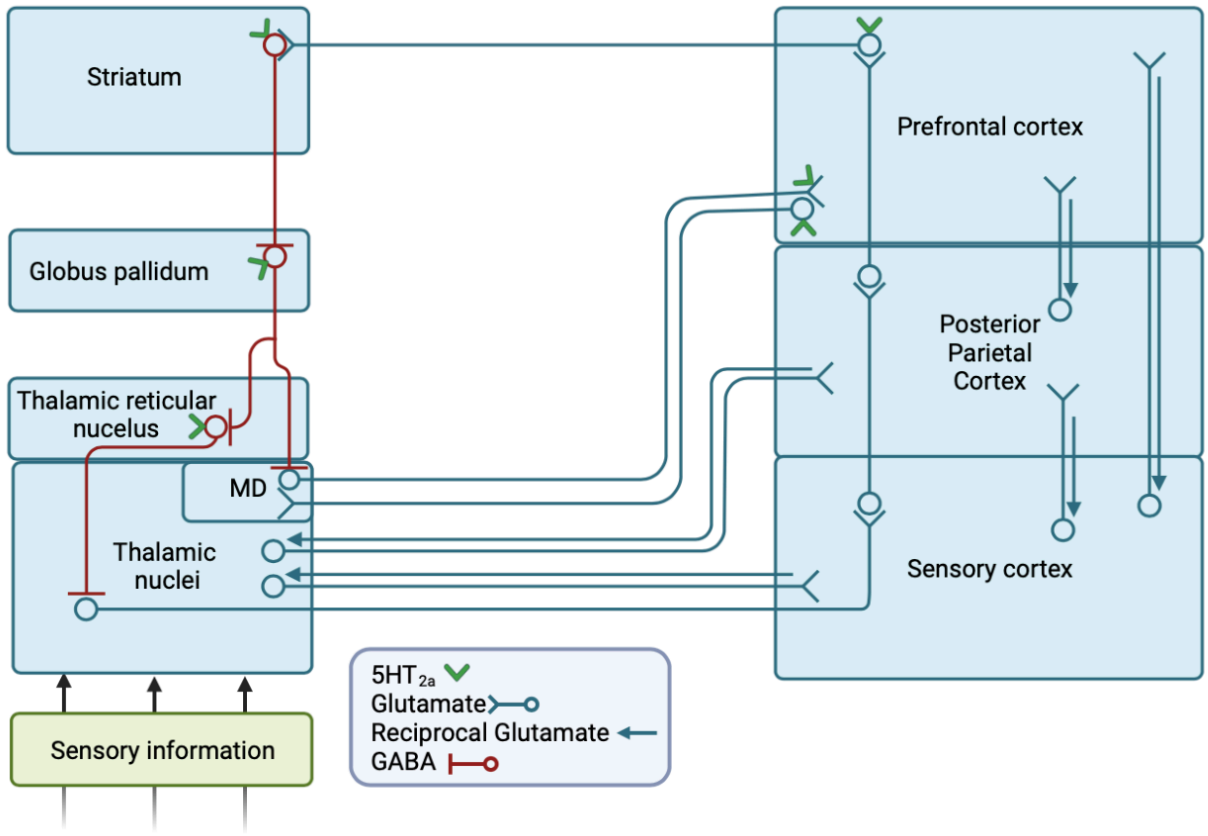

Figure 7: Simplified figure of the brain areas, projections and neurotransmitters of the hypothesised CSTC loop where $5HT_{2a}$ receptor is thought to mediate the psychedelic drug action of psilocybin. MD: mediodorsal nucleus. GABA: γ-aminobuteric acid. Figure is inspired by Doss et. al. [42] *Figure created in Biorender.com.*

It is hypothesised that the disruption of the CSTC loop causes a "reset" to faulty CSTC loops, meaning that normal function is restored after treatment, at least in diseases like OCD, where some CSTC loops are known to function abnormally [41].
From this it is possible to extent the hypothesis to depression where some CSTC loops are faulty and involved in the symptomology [37,39,40].

2.Preliminary findings

A study by researchers at The Neurobiological Research Unit at Rigshospitalet was done before this thesis. The author of this thesis was the main researcher of the study.

Here the molecular changes in the brain of male Long Evans rats, was investigated up to a week after an acute dose of psilocybin. A dose of 1 mg psilocybin/kg rat was given subcutaneously (SC). The animals were euthanised on day 1, day 2, day 3, day 5 and day 7 after dosing. A control group which had not received psilocybin was also included. After euthanasia the brains were extracted and cryo-sectioned to perform autoradiographic studies. The data has not yet been published publicly but none the less, the outcomes laid grounds to some of the decisions made in this project. The autoradiographic studies showed that there were significant changes in the density of $5HT_{2a}$ receptors (P-value 0.034) in the prefrontal cortex of the animals 1 day after dosing. The data also suggests that changes happened in the density of $5HT_{1a}$, $5HT_{2c}$ receptors in the cortex and the $5HT_7$ receptor in the thalamus. These findings were not significant, but trends were still seen. Several other areas of the brain were also investigated. In all brain areas, trends of molecular changes were observed. From this study it is suggested that molecular changes in the brain of psilocybin-treated rats, persisted for a rather long time after dosing. They persisted even after psilocybin is thought to be cleared from the body of the animal. (Figure 8).

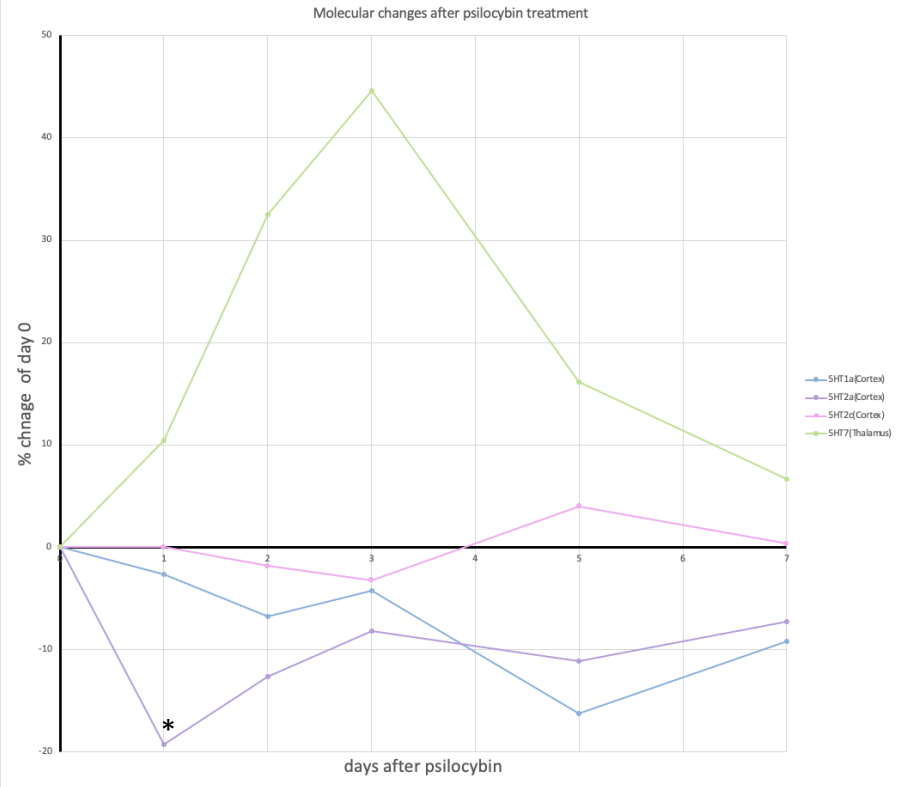

Figure 8: Data showing molecular changes in the brain of Long Evans rats in 1-7 days after an acute dose (1 mg/kg) of psilocybin. Changes are shown for density of $5HT_{1a}$ $5HT_{2a}$, $5HT_{2c}$ in the cortex and $5HT_7$ in the thalamus. The statistical tests have been done on the actual measurements and not percentages. Change in percentages are based on means and are shown for easy understanding. See annex 1 for raw data.

3.The aim of the study

One of the aims was to investigate the occupancy of the $5HT_{2a}$ receptor by psilocybin at a dose of 1 mg/kg psilocybin. An occupancy PET study using a $5HT_{2a}$ antagonist has been used to measure the occupancy of the $5HT_{2a}$ receptor and give an estimate of if the dose is of psychedelic relevance.
A study by Madsen et. al. from 2019 found that the occupancy of the $5HT_{2a}$ receptor is correlated with the intensity of the subjective experience of the psychedelic effect [35]. It has been shown that a psychedelic dose is needed to produce good psychological results in patients [32,43]. Another study by Donovan et. al. showed that pigs show characteristic behavioural changes, corresponding to a psychedelic experience when the occupancy of psilocybin is 67% of the $5HT_{2a}$ receptors with the tracer [^{11}C]-Cimbi-36 [44]. Therefore, this study aimed to investigate if the chosen dose of 1 mg psilocybin/kg rat, was sufficient enough to produce a level of occupancy at the $5HT_{2a}$ receptor that could be characterised to be psychedelic, compared to the occupancy in humans and pigs.

Another aim of this study was to look at how and if psilocybin influences the brain areas of CSTC loops in male Long Evans rats after a single 1 mg/kg acute dose.

The metabolism was investigated using PET and the radioactive glucose analogue [^{18}F]-fluorodeoxyglucose ([^{18}F]-FDG) as the metabolism can be used as a proxy of neuronal activity [45]. Previous studies have shown that the positive effects of psilocybin on mood etc. persist for a long time after the administration of psilocybin [32,33]. Molecular changes can be seen up to a week after administration, as described above. Therefore, it was chosen to look at the metabolism of the brain areas of the CSTC loops while psilocybin was present in the body and a week after administration. This was done to make sure that possible changes in metabolism could not be attributed to the molecular changes. It was also done to investigate if the persistent changes on mood, could be attributed to the possible change in the metabolism of the areas of the CSTC loops.

Therefore, the main questions this thesis aimed to answer were:

- Does a dose of 1 mg/kg psilocybin in male Long Evans rats give an occupancy of the $5HT_{2a}$ receptors that can be said to be psychedelic?
- Does a single treatment with 1 mg/kg psilocybin in male Long Evans rats change the metabolism of the brain areas involved in CSTC loops acutely? If so, how?
- Can changes in the metabolism of the brain areas involved in the CSTC loops, be seen 7 days after dosing with 1 mg/kg psilocybin in male Long Evans rats? If so, how?

4. Methodology

4.1. Radioactive decay

In this project radioactive isotopes are used and especially radioactive isotopes that decay via β^+. There are 6 types of radioactive decay: α, β^+, β^-, electron capture, γ and spontaneous fission. The β^+ decay can be described by the following equation

$$ {}^{A}_{Z}x \rightarrow {}^{A}_{z-1}x' + e^+ + v_e \quad \text{(I)} $$

β^+decay emits a positron and an electric neutrino, while a proton is converted into a neutron in the nucleus of the isotope.

β^+isotopes are used in positron emission tomography (PET) and a list of the most commonly used isotopes with corresponding half-life can be seen in table 3

Table 3: β^+isotopes used in PET and their corresponding half-life

Isotope	^{82}Rb	^{15}O	^{14}N	^{11}C	^{68}Ga	^{18}F	^{64}Cu	^{55}Co	^{89}Zr	^{52}Mn
Half-life	76 sec.	122 sec.	9.97 min.	20.364 min.	67.81 min.	109.771 min.	12.7 hours	17.5 hours	78.4 hours	5.6 days

4.2. PET- positron emission tomography

Positron emission tomography imaging is based on the detection of the energy charge emitted by the decay of a β^+ isotope.

The emitted positron collides with a nearby electron and annihilates, creating 2 gamma rays (photons) at 511 keV at an angle of 180° between each other (Figure 9)

This energy charge is what is measured in a PET scan. A PET scanner is a circular scanner with PET detectors all around. When a photon is detected at one site, then another photon should be detected around the same time at a detector 180° away from the first. This is called coincidence detection. There will be a small time difference between the events, and this time difference is what is used to calculate where the annihilation occurred, if the PET scanner uses time of flight.

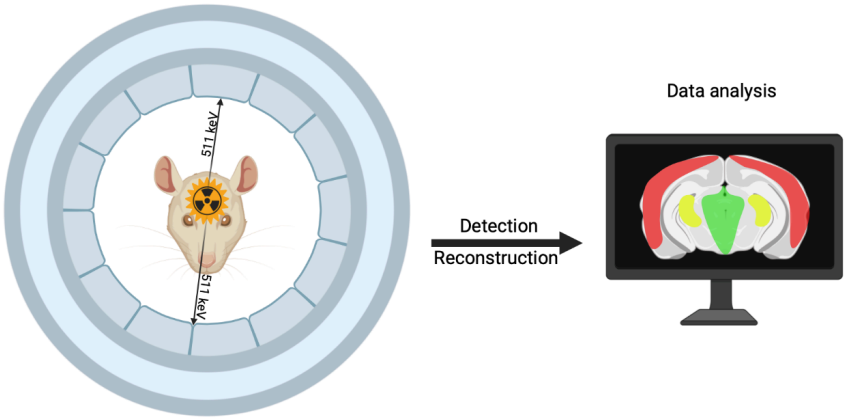

Figure 9: Simplified schematic view of the PET scanner principle. Created in biorender.com

Depending on the type of physiological process one tries to image, a radioisotope with a suitable half-life is needed. Generally, if it is not to study biodistribution of long-lived pharmaceuticals (e.g. Antibodies) a PET isotope with a short half-life is used. The most commonly used PET radioisotopes are ^{11}C, ^{13}N, ^{15}O, ^{18}F, ^{64}Cu and ^{68}Ga.

^{11}C, ^{13}N, ^{15}O are used as they can be substituted with their non-radioactive atom in many molecules. Regarding ^{18}F, hydroxyl groups are often substituted with ^{18}F as the electron cloud volume and polarity closely resembles that of a hydroxyl group. See figure 10 where a hydroxyl group is substituted for ^{18}F atom.

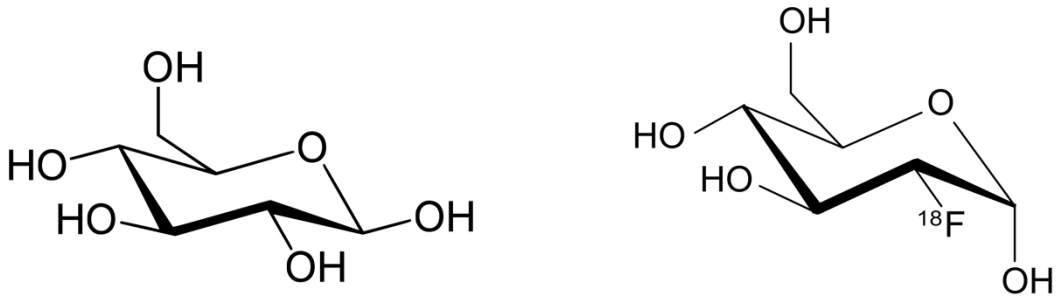

Figure 10: Molecular structures of glucose and [^{18}F]- fluorodeoxyglucose

Because of the very short half-lives of ^{11}C, ^{13}N, ^{15}O one would need to have a cyclotron onsite to produce isotopes for PET scanning. Chemical synthesis with ^{11}C, ^{13}N, ^{15}O needs to be very short to ensure sufficient radiochemical yield for imaging. With ^{18}F the longer half-life of almost 2 hours makes it possible to transport the finished radiochemical to other facilities relatively close by, without losing too much activity on the way. The longer half-life also makes it possible to have chemical synthesis lasting longer. The longer half-life of ^{18}F also makes it possible to study the compound of interest after state of equilibrium has been reached.

4.2.1. The use of PET in the clinic and clinical studies

PET scans can have many applications clinically. PET scans are frequently used in cardiology, oncology and neurology to assess the state of disease. In neurology PET is used clinically to assess e.g. Alzheimer's disease ([^{18}F]-FDG) and severity of ischemic stroke ([^{18}F]-FDG). Many radiotracers used for neuroimaging have been developed for imaging of specific receptors and transporters in the brain. These tracers are rarely used in the clinic. [^{18}F]-FDG is used in both humans and in animals and have also been used to assess cerebral function in patients with major depression [46]. In this project it is used to assess metabolism after psilocybin treatment. [^{11}C]-Cimbi36 a $5HT_{2a}$ receptor agonist and [^{18}F]-MHMZ, a $5HT_{2a}$ receptor antagonist, have been developed for imaging of the $5HT_{2a}$ receptor. The tracers have many applications, but they can help assess the distribution and density of the receptor.

[^{18}F]-MHMZ is used in this thesis to measure the non-displaceable binding potential (BPnd) and estimate the occupancy of psilocybin/psilocin in rat brain tissue using PET. [^{18}F]-MHMZ is shown to have an affinity towards the $5HT_{2a}$ receptor with a K_i value of 9.0 ± 0.10 nM. This affinity is lower than the affinity of the commonly used [^{11}C]-MDL 100,907 which has a K_i 2.10 ± 0.13 nM. The K_i of [^{18}F]-MHMZ is still within the nanomolar range which is acceptable for PET scans [47]. In this project [^{18}F]-MHMZ is used because of the longer half-life compared to ^{11}C. (Figure 11)

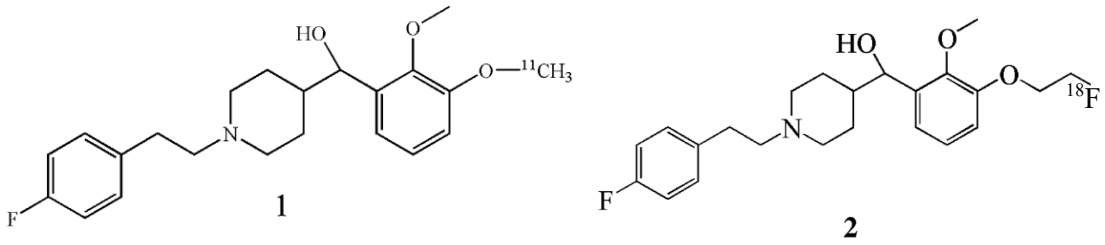

Figure 11: Molecular structure of ^{1}C-MDL 100,907 (1) compared to the molecular structure of [^{18}F]-MHMZ (2)

PET studies are also used in this project to investigate the metabolism of the brain after treatment with psilocybin. Here the [^{18}F] substituted glucose analogue [^{18}F]-Fluorodeoxyglucose ([^{18}F]-FDG) is used (figure 10).

4.3. Equilibriums of PET tracers

When injecting a tracer or drug into the body several equilibriums will be established. First of all, there will be an equilibrium between the blood and the brain, because of the transport of the tracer/drug between the plasma and the brain over the blood brain barrier.

There will also be an equilibrium between the free tracer in the brain and the specific binding of the tracer to the receptor of interest.

When trying to estimate the binding potential one would normally take blood samples to get an estimate of the amount of free ligand. But if it is known that a reference tissue that is devoid of the receptor of interest, the concentration of tracer in this area can be used as an estimate of the amount of free tracer and nonspecific binding [48]. If a reference tissue is found, it is assumed that the equilibrium between the tissue and the plasma, is the same as the equilibrium between the tissue of interest and the plasma. (Figure 12)

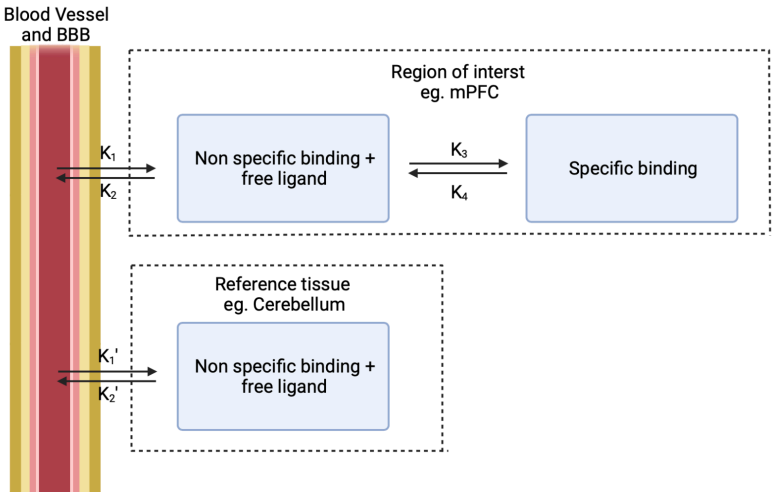

Figure 12: Model of reference tissue model with equilibriums between the plasma and the region of interest denoted $\frac{K_1}{K_2}$*, between the free ligand and the bound ligand in the region of interest denoted* $\frac{K_3}{K_4}$ *and between the plasma and the reference tissue which is assumed to be equal to the equilibrium between plasma and the region of interest and therefore denoted* $\frac{K_1'}{K_2'}$ *it is also assumed that the reference tissue does not have any specific binding. Figure created in biorender.com.*

The model above is called the full reference tissue compartment model and is the gold standard for PET experiments with reference tissues.
In this model the non-displaceable binding potential will essentially be the ratio between K_3 and K_4.

$$BPnd = \frac{K_3}{K_4} \tag{II}$$

4.4. Non-displaceable binding potential

Doing an occupancy study, it is important to understand what the non-displaceable binding potential is an estimate of.
The *in vitro* binding potential can be expressed as a function of B_{max} and K_d, meaning that the binding potential is the ratio between the maximum concentration of available receptors denoted B_{max} and the affinity of the drug to the receptor. The affinity of a drug can be expressed as the inverse dissociation constant denoted K_d

$$BPnd = \frac{B_{max}}{K_d} \tag{III}$$

Traditionally the relationship at equilibrium, between the concentration of the free ligand, bound ligand, Bmax and K_d can be expressed with the following formula

$$[bound\ ligand] = \frac{B_{max} * [free\ ligand]}{K_d + [free\ ligand]} \tag{IV}$$

To get an estimate of the K_d and the Bmax, several experiments with varying doses would need to be conducted, to make a saturation curve or a Scatchard plot. In a Scatchard plot the concentration of the free ligand, bound ligand, Bmax and K_d have the following relationship

$$\frac{[Bound\ ligand]}{[Free\ ligand]} = -\frac{1}{K_d} * [Bound\ ligand] + \frac{B_{max}}{K_d} \quad \text{(V)}$$

In PET studies the tracer principle is being used. This means that the concentration of the tracer is very low and therefore the concentration of bound ligand approximates 0. Therefore, in PET studies the relationship can be reduced to the following.

$$\frac{[Bound\ ligand]}{[Free\ ligand]} = \frac{B_{max}}{K_d} = BPnd \quad \text{(VI)}$$

Meaning that the binding potential can be expressed as the ratio between free and bound ligand at equilibrium. This means that only one PET study is needed to find the BPnd of the ligand [48].

4.4.1. The Logan reference tissue model.

In the experiments of this thesis, instead of the full reference tissue compartment model, a Logan reference tissue model was used. Here the equilibrium volumes of distribution (V_T) are used. The distribution volume ratio between the tissue of interest and the reference is calculated as a measure of non-displaceable binding potential.

$$BPnd = \frac{{V_T}^{tissue}}{{V_T}^{ref}} - 1 \quad \text{(VII)}$$

In the Logan reference tissue model the equilibriums have to be established before modelling begins. In the experiments of this thesis, the equilibrium was assumed to have been reached as the scans were started after 40-45 minutes after injection (see annex 2). The mathematical function used to describe this model is linear and using the PMOD programme, Logan plots with reference tissues can be made easily. In these plots the slope of the linear fit will be the BPnd[49].

As described, the occupancy of psilocybin on the $5HT_{2a}$ receptor at a dose of 1 mg/kg was investigated. This was done with the radioactive ligand [^{18}F]-MHMZ which as described is a selective antagonist of the $5HT_{2a}$ receptor. For the 5-HT_{2a} receptor it is known that the cerebellum is completely devoid of the 5-HT_{2a} receptor [50]. [^{18}F]-MHMZ is known to show a level of binding in the cerebellum, at the level of non-specific binding [47]. Therefore, the equilibrium volume of distribution in the cerebellum was used as the ${V_T}^{ref}$

4.5. Occupancy

Receptor occupancy is a measure of how big a percentage of the available receptors are occupied by the drug in question

$$Occupancy(\%) = \frac{BPnd_{baseline} - BPnd_{drug}}{BPnd_{baseline}} * 100 \quad \text{(VIII)}$$

The BPnd is the binding potential between the receptor in question and a radioactive ligand. When adding the drug that binds to the same receptor as the radioactive ligand, the drug will displace the ligand and therefore the BPnd will be lowered. This principle is used to find the occupancy of the drug and is similar to the principles used in a traditional K_i study.

From the equation above it is evident that two PET scans are needed, to obtain the BPnd data needed to calculate the occupancy of 1mg/kg psilocybin at the $5HT_{2a}$ receptor.

After calculation of the BPnd for both types of scans on a VOI (Voxel of interest) level, an occupancy plot can be produced, by plotting the difference in BPnd for each area on the y axis, and the baseline BPnd for each area on the x axis. The slope of the linear fit line will be the occupancy which is similar to a Lassen plot [51,52].

4.6. [^{18}F]-FDG PET scans

In this project PET scans with the radioactive tracer [^{18}F]-fluorodeoxyglucose (FDG) were done. [^{18}F]-FDG is a glucose analogue meaning it will be taken up by cells via glucose transporters (GLUT's) like normal glucose. Especially when fasted [^{18}F]-FDG is rapidly taken up as blood glucose levels are low. In the cells [^{18}F]-FDG is rapidly phosphorylated by hexokinase enzymes into [^{18}F]-FDG-6-phosphate, which cannot escape the cells without being dephosphorylated. [^{18}F]-FDG-6-phosphate will not travel further down the glycolytic or oxidative pathway and will therefore stay within the cell until it is dephosphorylated by glucose-6-phosphotase and exported out of the cell via GLUTs. The dephosphorylation and transport is not favoured when the body is fasted, and therefor [^{18}F]-FDG-6-phosphate will be trapped metabolically within the cell and decay here. (figure 13)

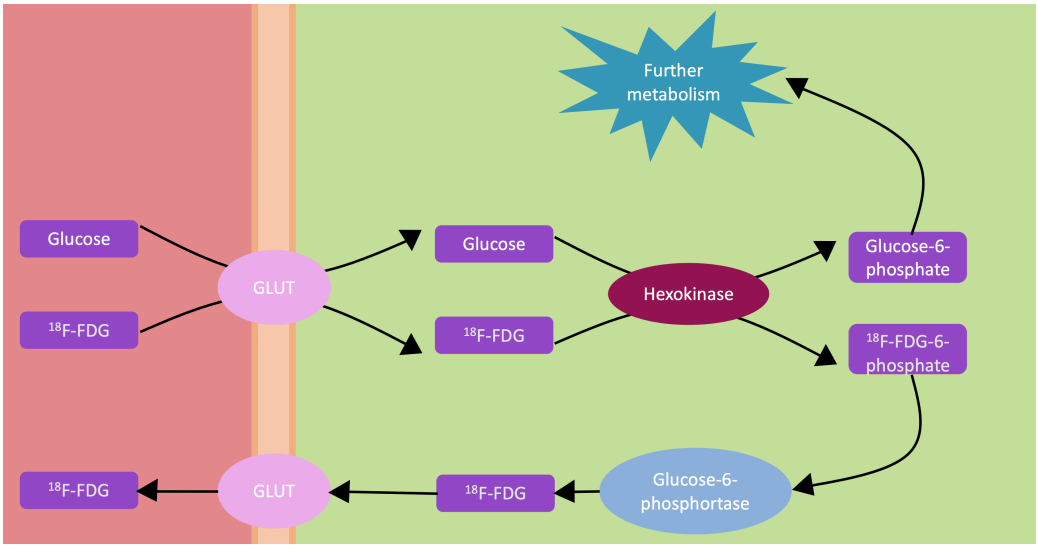

Figure 13: A simplified schematic overview of [^{18}F]-FDG metabolism compared to normal glucose metabolism. The figure shows cellular uptake by GLUT (glucose transporters), phosphorylation by hexokinase and dephosphorylation by glucose-6-phosohortase and cellular excretion by GLUT.

[^{18}F]-FDG will decay within the cell and by using a PET scanner, one will be able to see how glucose uptake is distributed throughout the organism. In the brain, glucose metabolism is correlated to neuronal activity and therefore if there is higher uptake in certain areas, it is believed that these areas have higher neuronal activity [45].

5. Method

In the following section the methods used in this thesis will be described.

5.1. Animals

Long Evans rats (n=14), weighing 259.7 $\pm$ 15.4g were purchased from Janvier. Animals were housed with ad libitum access to food and water and kept on a 12-hour light/dark cycle. Scans were performed during the light phase of the cycle.

Animals were housed 3-4 per cage with environmental enrichment. They were moved to the PET facility in conventional cages 3 days before scanning to ensure acclimatisation. In the PET facility the animals were housed in conventional cages and kept on dust free bedding (alpha dri®) in a scantainer set to 27^{o}C. Animals were earmarked the same day as they were moved to PET facility. The animals were quickly anaesthetised with isoflurane and pure oxygen before ear clipping. All procedures with animals were done in accordance with FELASA guidelines and approved by the Danish Animal Experiments Inspectorate (2021-15-0201-00909).

5.2. [^{18}F]-MHMZ scans

The animals were not fasted for the scans. For the baseline scan the animals were injected with 0.2 mL saline SC 15 minutes before injection with [^{18}F]-MHMZ. For psilocybin scans the animals were injected SC with 1 mg/kg of a 1mg/mL saline solution of psilocybin 15 minutes before [^{18}F]-MHMZ injection.

[^{18}F]-MHMZ was produced at Rigshospitalet and shipped immediately to Odense University Hospital after production.

Approximately 10 minutes after subcutaneous injection, the animals were anesthetised with 4-5% isoflurane in pure oxygen. After it was ensured the animals were without reflexes, they were placed with their noses in a nosecone to secure continued anaesthesia. An intravenous (IV) cannula with injection port was placed in one of the tail veins of the animal and flushed with saline to make sure it was placed correctly. The rats were then injected with a dose of [^{18}F]-MHMZ(6.36$\pm$1.42mBq) (table 4) through the cannula. The [^{18}F]-MHMZ was mixed with saline in the syringe to make sure the injection did not contain more than 10% ethanol. This meant that the injections were up to about 0.8 mL. After injection, the cannula was flushed with 0,15 mL saline. The animals were allowed to recover from the anaesthesia in a solitary box lined with medical cloth and darkened with a

blanket. After recovery, the animals were allowed to return to their home cages, which were covered with dark cloth and roam around for approx. 45 min before being anesthetised with 3-4% isoflurane in pure oxygen. The animals were then moved to the scanner, where they were kept under anaesthesia during the scan (1-2 % isoflurane in pure oxygen). The animals were secured with meditape during the scan and Viscotears® 2mg/mL carbomer gel was applied to the eyes of the animals. Respiration rate was measured during the scan and the quality of the respiration was visually assessed frequently. Isoflurane and oxygen flow were adjusted accordingly.
The animals were CT scanned for 5 minutes and PET scanned for 30 minutes (dynamic scan with 5-minute frames) on a Simens ® inveon animal scanner. When the scan was finalised, the animals were retrieved and allowed to recover from the anaesthesia in a solitary box, lined with medical cloth and darkened with a blanket. After the experiment, the animals were returned to their home cages, given access to food and provided with a sugary treat (Piña colada ball) to raise blood sugar and ease recovery after anaesthesia.

Table 4: overview of rats included, the weight of rats, injected dose in mBq and absolute dose of MHMZ

	Baseline			Acute		
Rat ID	**Weight(g)**	**Injected dose(mBq)**	**Injected dose of MHMZ nmol**	**Weight(g)**	**Injected dose(mBq)**	**Injected dose of MHMZ nmol**
MKP-0062	275	5.25	0.3511	300	8.836	0.1102
MKP-0063	280	5.722	0.5149	320	7.223	0.1244
MKP-0064	276	5.182	0.6369	308	5.944	0.1335

When doing the psilocybin scan it was visually checked that the animals had head twitch responses, as it is known to be a rodent response to psychedelics/hallucinogens [53]. The radiochemical purity of [^{18}F]-MHMZ was >97% both days of scanning. The molar activity was estimated to be 69 Gbq/μmol at production the day of the baseline scan. At the acute scan the molar activity was estimated to be 52 Gbq/μmol at production.

5.3. [^{18}F]-FDG scans

[^{18}F]-FDG scans were preformed similarly to [^{18}F]-MHMZ scans with the modifications described.
The animals were fasted the night before scanning but still allowed free access to water.
On the day of the scan the animals were weighed, and blood sugar was measured (see table 5). For the baseline and one-week scans, animals were injected with 0.2 mL of saline (0,9%) SC 5 minutes before interperitoneally (IP) injection of [^{18}F]-FDG, which was produced at the Department of Nuclear Medicine and Preclinical Research at University Hospital of Odense (OUH).

For the acute scan, animals were injected subcutaneously with 1 mg/kg of a 1mg/mL saline solution of psilocybin. The animals were injected with 7.55± 1.11MBq [^{18}F]-FGD in 0,2 mL saline interperitoneally (IP) (see table 5)

Table 5: overview of rats included, the weight of rats, injected dose in of [^{18}F]-FDG in mBq and blood sugar (BS)(mmol/L).

	Baseline			Acute			One-week post		
Rat ID	**Weight(g)**	**Injected dose(mBq)**	**BS**	**Weight(g)**	**Injected dose(mBq)**	**BS**	**Weight(g)**	**Injected dose(mBq)**	**BS**
MKP-0001	253	8	5.1	260	7.73	6.5	284	7.13	4.7
MKP-0002	255	9.9	6.1	260	5.09	5.4	285	6.82	4.7
MKP-0003	252	7.2	5.3	262	7.89	5.4	300	7.44	4.7
MKP-0004	228	7.1	4.8	240	8.13	4.7	266	9.28	4.9
MKP-0005	266	6.0	5.3	270	9.06	5.2	300	8.04	4.7
MKP-0006	260	7.1	5.2	266	7.2	5.6	292	7.19	5.1
MKP-0007	252	5.5	5.8	260	7.62	6.0	296	7.00	6.8
MKP-0008	262	8.3	5.3	266	8.76	5.3	295	7.64	6.4

Similar to above, the animals were returned to their home cage for approx. 45 min. before being anesthetised and placed in the scanner. After CT and PET scan the animals were returned to their home cage as described above.

The psilocybin scans were preformed 3 days after the baseline scans and the one-week post scans were performed 7 days after the psilocybin scans. When doing the psilocybin scans, it was visually checked that the animals had head twitch responses.

5.4. Image matching and data extraction

Image and data processing were done in PMOD version 4.301, PMOD Technologies LLC.

5.4.1. [^{18}F]-MHMZ scans

The final 4D images was checked for missing data. An average over the timeframe was produced, and the head was cropped out from both the averaged and dynamic scan pictures. The averaged picture was then matched to a standard [^{18}F]-MHMZ picture and the transformation matrix was applied to the dynamic picture. A set of standard atlases of voxels of interest (VOIs) was applied to the averaged image. The VOIs were fitted onto individual images manually by dragging and

stretching all the VOI's simultaneously. When VOI's fitted the images, the VOI's were put on to the dynamic pictures.
The binding potential in each region were calculated using the Logan reference tissue model and using the cerebellum grey matter as reference tissue [49]. The time was set to 0 minutes after scan start, as the scans was only started, when a pseudo equilibrium was assumed to have been reached (see annex 2). The occupancy was calculated with formula VII and occupancy plots for regions with high density of the $5HT_{2a}$ were made. A paired two-sided T-test was done to asses if binding potential was significantly lowered after psilocybin administration.

5.4.1.1. Extraction of [^{18}F]-MHMZ BPnd pictures

Pictures were extracted from PMOD with pixelwise calculation of the BPnd. To calculate the BPnd the Logan reference tissue model was used in the kinetic module of PMOD. The cerebellum grey matter was used as the reference tissue and the mPFC used as the target tissue. The K_2 was estimated based on the cerebellum. Pictures were made for each scan. After pictures were made, an average picture from the 3 rats was produced. A 3D Gaussian blur filter (0.6 mm XYZ) was added to smooth out pictures. Then the averaged pictures were overlaid a standardised MRI scan for structural identification.

5.4.2. [^{18}F]-FDG scans

Done similar to above, but with slight modifications as described.
Averaged cropped scans were opened in the rodent brain plugin and matched to an [^{18}F]-FDG brain PET atlas from Wistar rats. The sampling was set to 0.6 mm. After matching, a standard brain atlas with VOI's was overlaid the PET image. Almost no adjustments were made by hand to minimise bias. After matching, standardised uptake values (SUV's) were drawn out. When all SUV's were collected, a whole brain SUV for each scan was calculated by finding the mean of all SUV's.
SUV's were wholebrain normalised by finding the mean of all the whole brain SUV's, and then calculating the ratio between the mean whole brain SUV and the whole brain SUV for each scan. The ratio for each scan was multiplied with the SUV for each VOI.
Statistical analysis was done for the areas of interest. Areas were chosen based on easy recognition and size. In the cortex, the medial prefrontal cortex was chosen, as metabolism was high in this area (see section 6.2.1) and therefore easy to recognise. The thalamus and striatum were also chosen based on easy recognition and size. See annex 3 for structural MRI reference. Furthermore, the standardised PET [^{18}F]-FDG brain atlas, did not include smaller compartments of e.g. the striatum and thalamus. Problems with matching could also give bigger uncertainties for small brain areas than bigger ones. (see discussion)
One-way anova analyses with Tukeys multiple comparisons and repeated measures were done, and it was taken into account that the experiment was done with repeated measures for each rat.

5.4.2.1. Picture extraction of [^{18}F]-FDG scans

The whole brain normalisation constant for each scan was multiplied with the pixel values of each scan, to be able to show comparable pictures. An averaged picture of all the rats was made for the baseline scan, acute scan and the one-week scan. A 3D Gaussian blur filter (at 0,6 mm XYZ) was added to smooth out pictures. The averaged pictures were overlaid a standardised MRI scan for Long Evans rats, to easily identify brain structures. The slices with areas of interest drawn in, were selected and exported along with MRI slices. Other cortical areas than the mPFC were drawn in for reference.

6. Results

The results, statistical analysis and power calculations of the above-described experiments will be presented below. Selected pictures from the scans will also be shown.

6.1. Results of [^{18}F]-MHMZ occupancy study

In the following section the results of the ^{18}F-MHMZ occupancy study will be presented.

6.1.1. Scan pictures from [^{18}F]-MHMZ scans

From the visual check of animal behaviour, it was confirmed that all rats had a dose of psilocybin capable of inducing head twitches. [^{18}F]-MHMZ binding distribution is shown in figure 14, where high binding is visual throughout the cortex. (Figure 14)

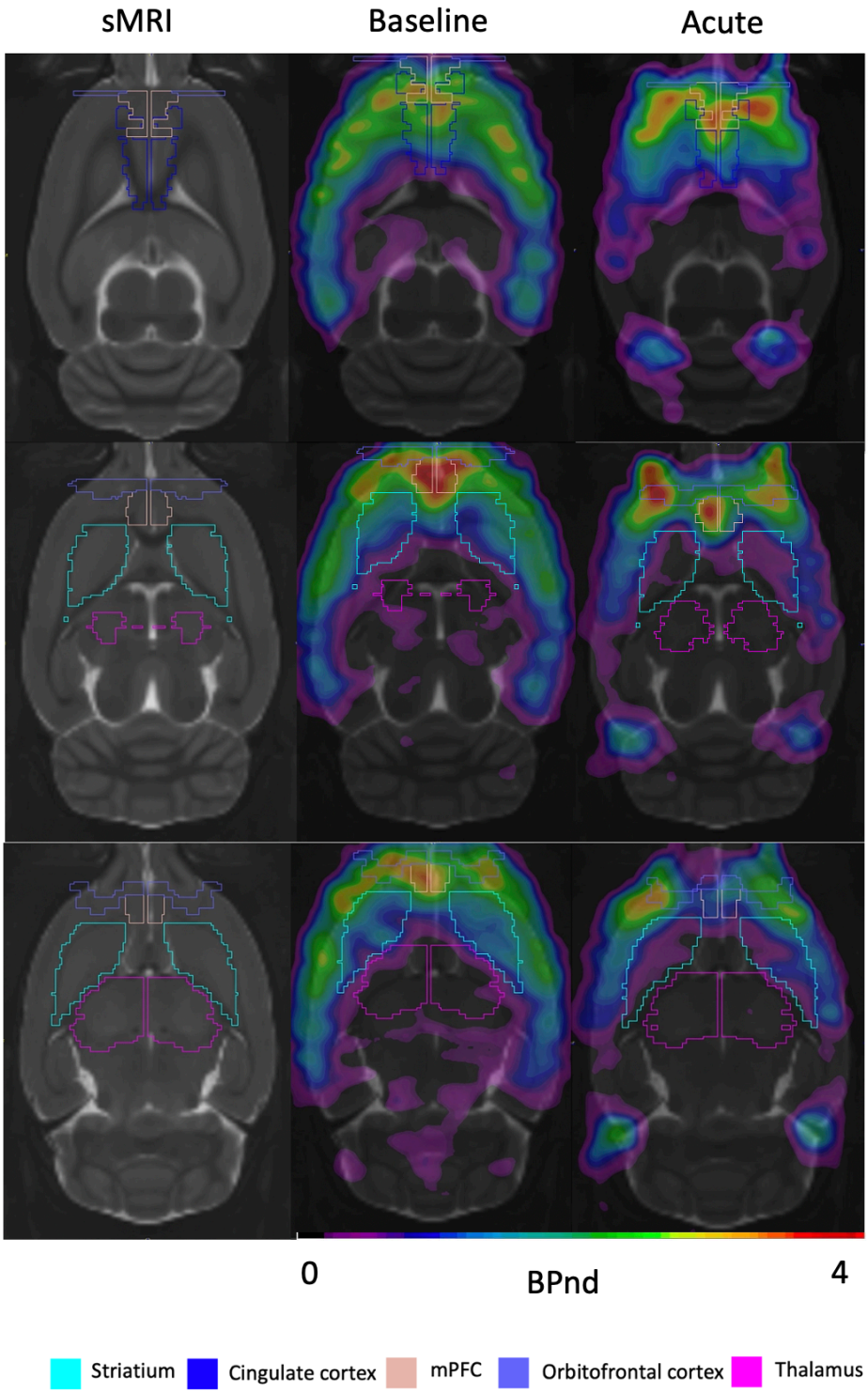

Figure 14: Averaged binding potential of rat 62, 63 and 64 from [^{18}F]-MHMZ baseline and acute psilocybin scan. Column 1: Reference structural MRI regions of interest. Column 2: Averaged BPnd of [^{18}F]- MHMZ at baseline scan. Column 3: Averaged BPnd [^{18}F]- MHMZ acutely after psilocybin dosing. Areas of interest are drawn in with turquoise: striatum, navy blue: cingulate cortex, beige: medial prefrontal cortex, cornflower: orbitofrontal cortex, pink: thalamus. Examples of non-manipulated scan pictures from each individual scan can be found in annex 4

6.1.2. Analysis of MHMZ dose

Regions rich of $5HT_{2a}$ receptors were included in the analysis.

To make sure that the injected dose of MHMZ did not have an influence on BPnd, a plot showing dose and binding potential for areas rich in $5HT_{2a}$ receptors was made. (figure 15)

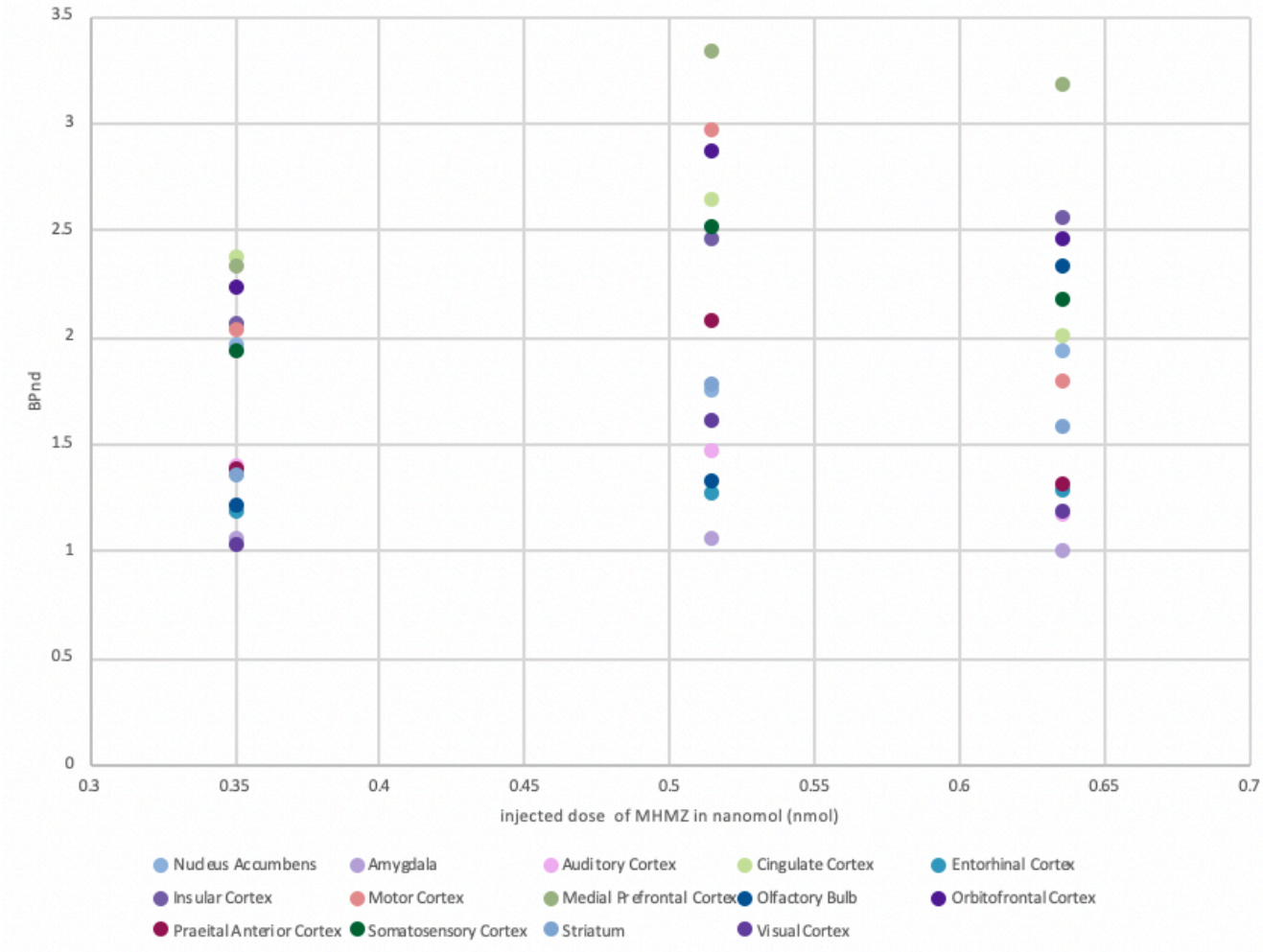

Figure 15: Plot showing BPnd for areas rich in $5HT_{2a}$ receptor and injected dose of MHMZ at baseline.

Figure 15 shows that the injected dose did most likely not have an effect on the BPnd. Therefore, it must be concluded that the tracer principle in fact was used.

6.1.3. Statistical analysis of BPnd and occupancy

BPnd data from baseline and acute scans have been plotted and a paired T-test was made (P-value <0.0001****). Baseline and acute scan BPnd's are therefore significantly different form each other. (Figure 16)

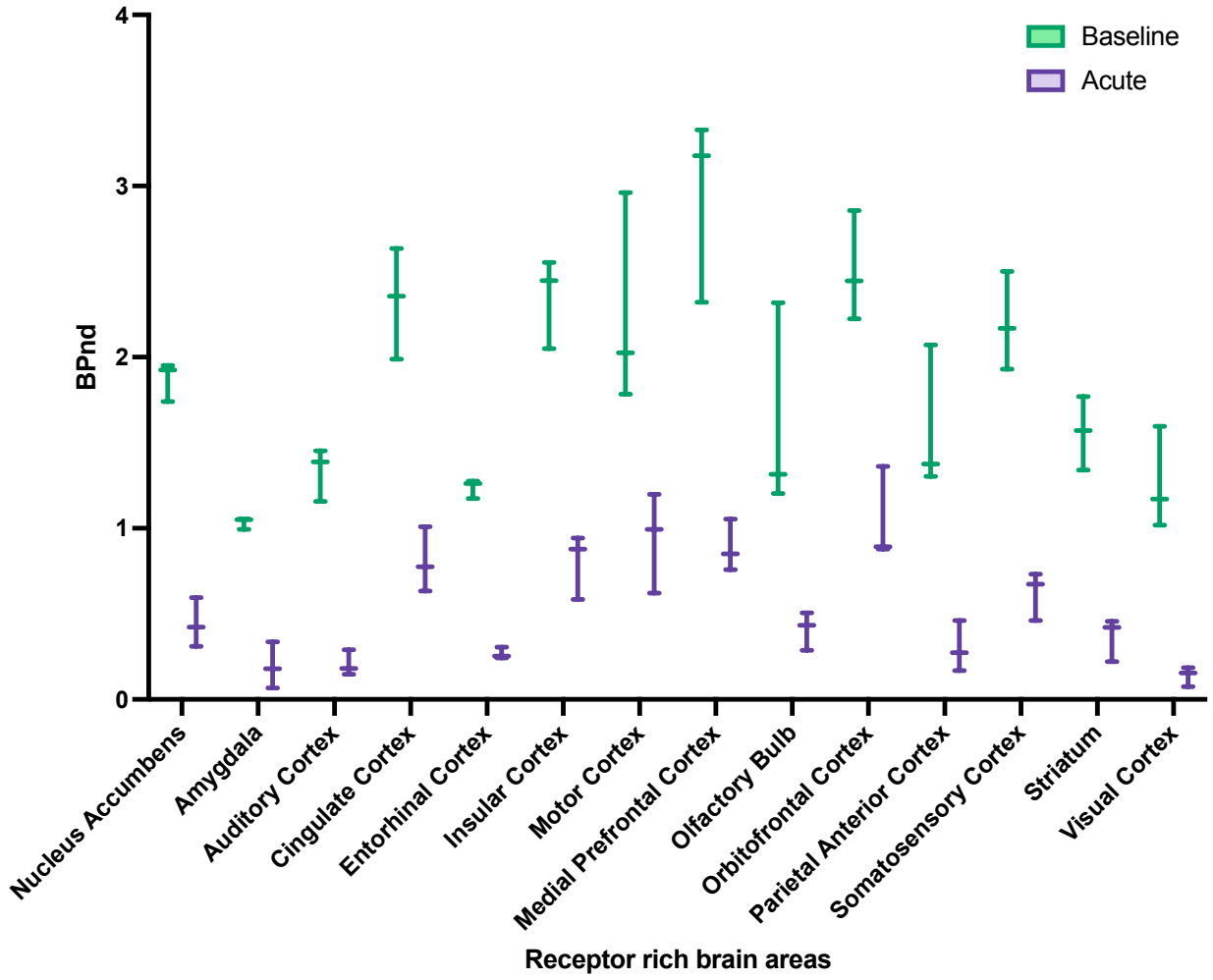

Figure 16: Plot over BPnd data from [^{18}F]-MHMZ experiments n=3. Areas that are receptor-rich are plotted. Green bars show BPnd data from baseline scans with [^{18}F]-MHMZ. Min., max, and median values. Purple bars show BPnd data form acute scans with psilocybin and[^{18}F]-MHMZ. Min., max. and median. (see annex 5 for raw data).

The occupancy for psilocybin at the $5HT_{2a}$ receptors in receptor-rich areas was calculated using formula VIII. A two-way anova with multiple comparisons was performed. The test showed that animals were not significantly different form each other (P-value 0.0708(F (2,26) =2.937)). The test also showed that the areas were not significantly different from each other (P-value 0.1586(F (13,26) =3.546)). Tukeys multiple comparisons test showed that the cingulate cortex was significantly different from pareital anterior cortex (adjusted P-value: 0.0065). It also showed that the insular cortex was significantly different form the visual cortex (adjusted P-value: 0.0418) and that the somatosensory cortex was significantly different form the visual cortex (adjusted P-value: 0.0483).(Figure 17)

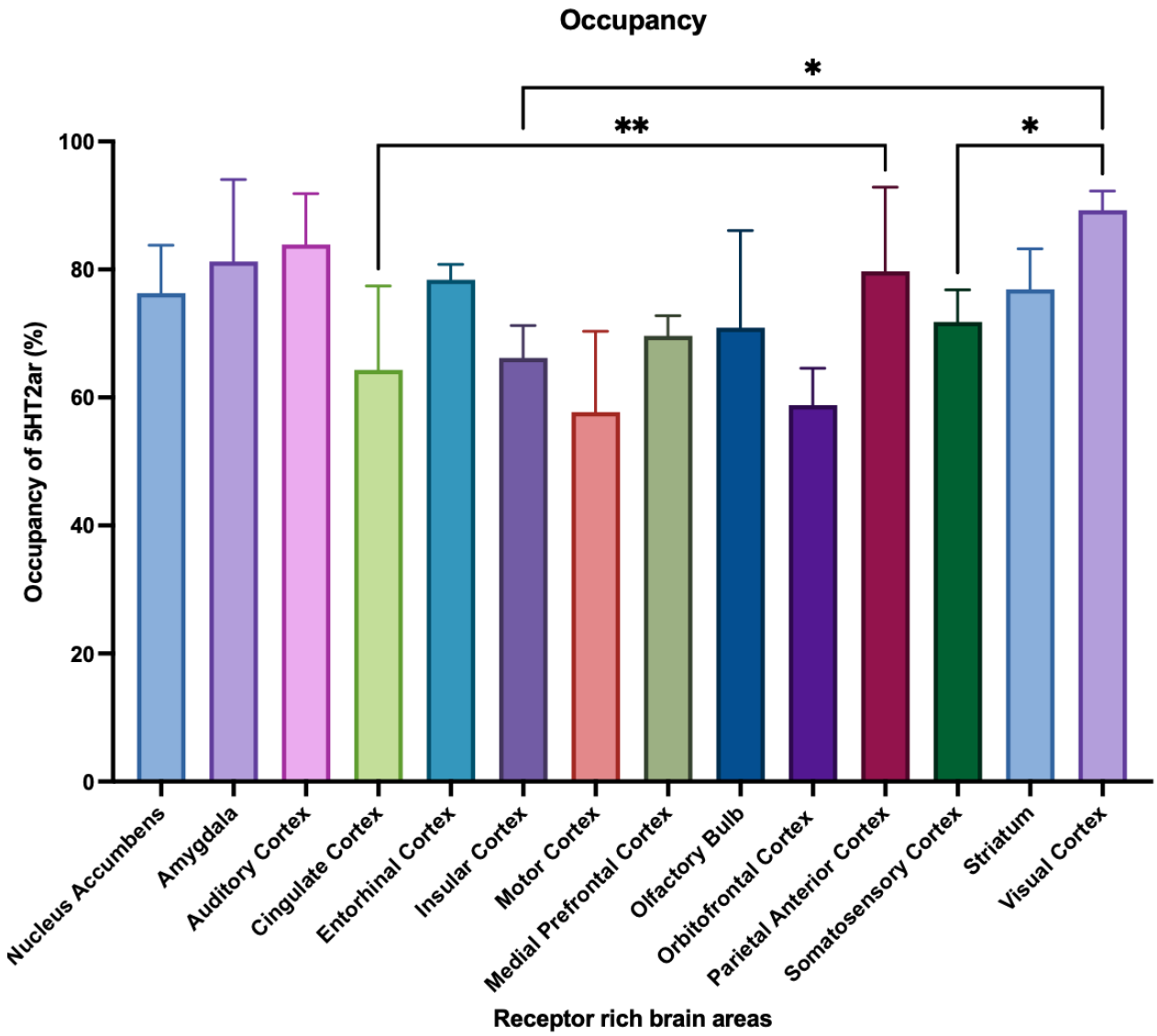

Figure 17: Occupancy % of psilocybin in areas rich in $5HT_{2a}$ receptors. Bars are shown with standard deviations and areas that are significantly different from each other are indicated.

The occupancy of psilocybin globally at $5HT_{2a}$ receptors was calculated by making an occupancy plot. Again, here only receptor rich areas were included in the analysis. A plot was made for each rat and the mean occupancy in % was calculated. (Figure 18)

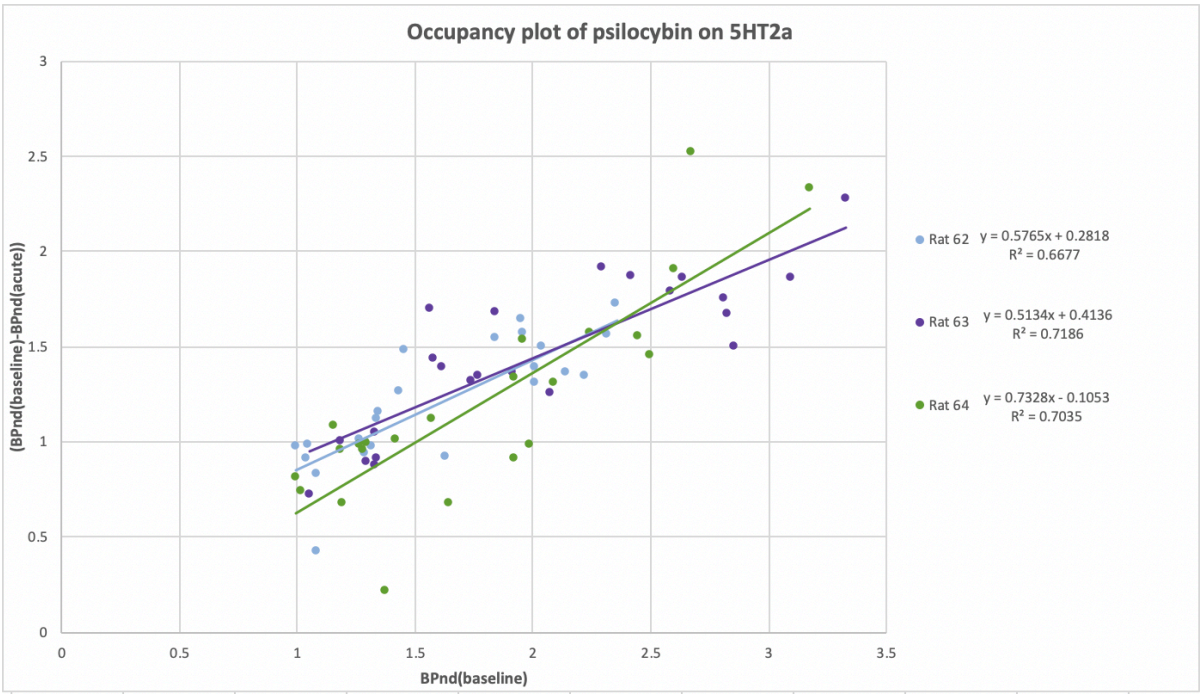

Figure 18: Occupancy plot for psilocybin at $5HT_{2a}$ receptors globally. Plots are made for each rat. 62(blue), 63(purple) and 64(green). Single values are plotted for each region for interest. A linear regression is made for each rat shown with solid lines coloured corresponding to the rat. The formula for each regression line and R^2 values are shown on the right.

From figure 18 it is evident that the slope of the linear regression fit for rat 62 is 0.5765 corresponding an occupancy of 57.56%. For rat 63 the slope is 0.5134 yielding an occupancy of 51.34%. For rat 64 the slope the 0.7328 equivalent to an occupancy of 73.28%.
The mean occupancy must therefore be 61% $\pm$11%
This is somewhat lower than the occupancies that are calculated above. Calculating a mean of the calculated occupancies for each brain area it is 72% $\pm$12%.
The above differences in results are to be expected as the 61% occupancy is based on regression fits where the R^2 is 0.70$\pm$ 0.03. The mean occupancy at 72% is based on exact measures and not a fit. The correct way of calculating the occupancy is based on the regression fit.

6.2. Results of Psilocybin scans

In the following section the results of the psilocybin [^{18}F]-FDG scans will be presented.

6.2.1. Scan pictures from [^{18}F]-FDG scans

From the scan pictures it was evident that the IP injection of [^{18}F]-FDG in rat 1 at the chronic scan was done wrong. The whole brain uptake was calculated to be 2.1 SUV compared to approximately 13 for the other rats. Rat 1 was therefore excluded from the analysis.
[^{18}F]-FDG uptake distribution is shown in figure 19.

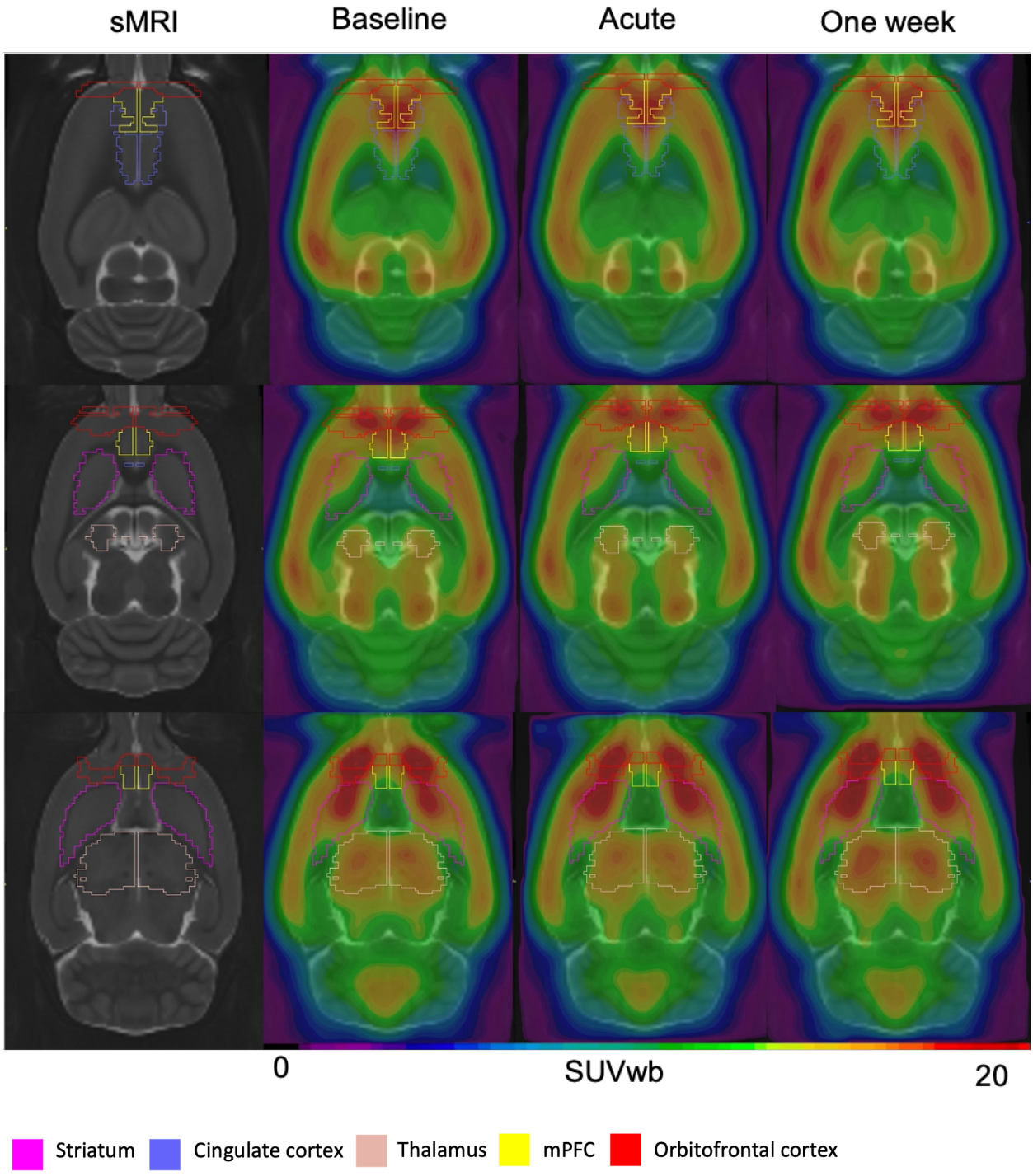

Figure 19: [^{18}F]-FDG PET images from rats. Column 1: Reference structural MRI regions of interest. Colum 2: Averaged SUVwb from [^{18}F]-FDG PET scan at baseline. Column 3: Averaged SUVwb from [^{18}F]-FDG PET after acute psilocybin. Column 4: Averaged SUVwb from [^{18}F]-FDG PET one-week post psilocybin. Areas of interest are drawn in with the following colours; pink: striatum, cornflower: cingulate cortex, beige: thalamus, yellow: orbitofrontal cortex, yellow: medial prefrontal cortex, red: orbitofrontal cortex. Examples of non-manipulated scan pictures from each individual scan, can be found in annex 6.

6.2.2. Statistical analysis of [^{18}F]-FDG experiments

The wholebrain normalised SUV (SUVwb) from the medial prefrontal cortex, striatum and thalamus was plotted against the days of scanning. (figure 20).

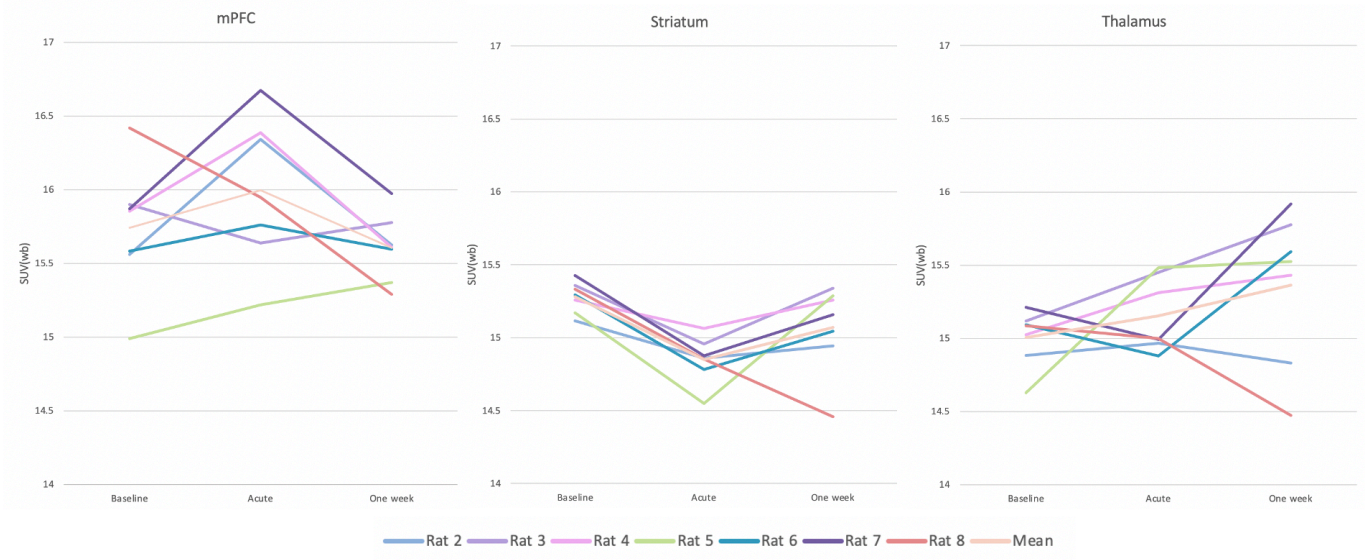

Figure 20: Plots of the whole brain normalised SUVwb data from the mPFC(A), Thalamus(B) and Striatum(C) for baseline, acutely after psilocybin dosing and one week after psilocybin dosing.

Table 6: Overview of mean SUVwb values and changes from medial prefrontal cortex(mPFC), striatum (Stria) and thalamus (Thal).

Area	Baseline (SUVwb)	Acute (SUVwb)	One week (SUVwb)	Δ Baseline vs. Acute (SUVwb)	Δ Baseline vs. Acute (%)	Δ Baseline vs. One week (SUVwb)	Δ Baseline vs. One week (%)	Δ Acute vs. One week (SUVwb)	Δ acute vs. One week (%)
mPFC	15.74 ±0.43	15.99 ±0.50	15.61 ±0.23	0.26± 0.49	1.6± 3.1%	-0.13± 0.48	-0.8± 3.0%	-0.39± 0.41	-2.4± 2.6%
Stria	15.28 ±0.10	14.84 ±0.16	15.07 ±0.30	-0.43± 0.16	-2.8 ±1.0%	-0.21± 0.33	-1.4± 2.1%	0.22± 0.34	1.5± 2.3%
Thal	15.00 ±0.19	15.16 ±0.25	15.36 ±0.52	0.15± 0.38	1.0± 2.6%	0.36± 0.52	2.4± 3.5%	0.21± 0.49	1.4± 3.3%

From figure 20 and table 6 it is evident that in the mPFC, the mean [^{18}F]-FDG uptake is raised acutely after dosing (1.6±3.1%) and then falls back to a little below baseline a week after dosing (-0.8±3.0%). In the striatum the mean uptake falls acutely after dosing (-2.8±1.0%) and then comes back to almost baseline a week after dosing (1.5±2.3%). In the thalamus the mean [^{18}F]-FDG uptake is raised (1.0±2.6%) acutely after dosing of psilocybin and is then further raised one week after dosing (1.4±3.3%).

From figure 20 it is evident that most of the rats follow the same pattern, but some show a completely different pattern compared to the rest. For instance, rat 8 generally shows a fall in metabolism from baseline to acute (-0.47 SUVwb(mPFC), -0.48 SUVwb (striatum), -0.09 SUVwb (thalamus)) and from acute to one week (-1.13 SUVwb (mPFC), -0.87SUVwb (striatum), -0.61SUVwb (thalamus)) which is a pattern that no other rat shows. Rat 3 shows a fall in metabolism in the mPFC acutely after dosing (-0.26 SUVwb) opposite to almost every other rat. Rat 5 show a pattern in the thalamus that is also a little different compared to the other rats. It shows a high increase in metabolism acutely after dosing (0.85 SUVwb) and then almost no change in the metabolism a week after dosing compared to acutely after dosing (0.04SUVwb). (See annex 7 for SUVwb data)

A one-way anova test with repeated measures and Tukey's multiple comparisons was done for each area of interest. Post hoc power calculations were done using formula 1, see annex 8.1

Table 7: Overview of statistical comparisons with P-values and corresponding power.

mPFC	**P-values**	**Significant?**	**Power**
Baseline vs acute	0.4117	No	17.2%
Baseline vs. one week	0.7505	No	10.8%
Acute vs. one week	0.1070	No	46.1%
Striatum	**P-values**	**Significant?**	**Power**
Baseline vs acute	0.0010**	Yes	100%
Baseline vs. one week	0.2571	No	40.6%
Acute vs. one week	0.2571	No	39.8%
Thalamus	**P-values**	**Significant?**	**Power**
Baseline vs acute	0.5879	No	23.4%
Baseline vs. one week	0.2435	No	39.6%
Acute vs. one week	0.5386	No	15.7%

From table 7, it is evident that there is only a significant difference in means in the striatum between the baseline scan and the acute scan. All other comparisons show a P-value above 0.05 indicating that no significant changes are found.

From the power calculations it is evident that the power of the study is low for many of the comparisons. Generally, in research, the aim is to have a power >80%.

Based on the comparison with the lowest power, a sample size that would have yielded a power of 80% was calculated using formula 2 see annex 8.2

Based on the calculation, the sample size should have been 54 rats to have a power of 80% and be able to detect significance in the experiment. (see annex 8.3.)

7 Discussion

7.1. Discussion of [^{18}F]-MHMZ occupancy study

The $5HT_{2a}$ occupancy study of this thesis, shows a significant reduction in [^{18}F]-MHMZ BPnd following 1 mg/kg psilocybin. The $5HT_{2a}$ receptor occupancy by psilocybin is 61% ±11% at a dose of 1 mg/kg psilocybin given subcutaneously.

7.1.1. The occupancy $5HT_{2a}$ needed to produce a psychedelic trip

In the study by Madsen et al. the authors correlate the psychedelic effect, to the occupancy of the $5HT_{2a}$ receptors by psilocybin, in cortical areas of the human brain [35]. Here the lowest found occupancy was 42,9% at a dose of 0.05mg/kg, and the highest measured occupancy was 72.4% at a dose of 0.2 mg/kg. At the highest doses of 0.3 mg/kg an occupancy of 65.6%±0.4% was measured. In the Madsen et. al. study, the participants who received the dose of 0.3mg/kg rated the psychedelic experience (intensity rating) the highest. Participants who had a lower dose (0.14 mg/kg) rated the psychedelic experience similarly. This was probably because the occupancy level was almost the same (66.4%) as for the participants that received 0.3mg/kg. This just goes to show, that the occupancy most definitely has a part in the intensity of the psychedelic experience. A thing to note here was that the sample size was very small.

In the study by Madsen et al. the PET tracer [^{11}C]-Cimbi-36 was used. Cimbi-36 is a $5HT_{2a}$ receptor agonist but it also has some affinity towards the $5HT_{2c}$. Therefore, it could be postulated that some of the occupancy measured comes from the $5HT_{2c}$ receptor occupancy.

From table 2 it is evident that the active metabolite psilocin, has a higher affinity towards $5HT_{2c}$ than $5HT_{2a.}$ Therefore, it could be postulated that the actual occupancy of only the $5HT_{2a}$ might be lower than what Madsen et. al. measured.

In the study from Donovan et.al. the occupancy of $5HT_{2a}$ in pigs that received a dose of 0.08mg/kg was measured. These animals showed characteristic behavioural changes corresponding to a psychedelic experience. Here the pigs had an occupancy of 67% at the $5HT_{2a}$. In this study the PET tracer [^{11}C]-Cimbi-36 was used, which again raises the question of whether the measured occupancy can only be attributed to the $5HT_{2a.}$

In a study from 2022 Barrett et al. measured the occupancy of psilocybin at the $5HT_{2a}$ with the PET tracer [^{11}C]-MDL-100,907[54] which as described above, is much more like the tracer used in this project. This tracer is a very selective antagonist towards the $5HT_{2a.}$Therefore, it is estimated that the results of the Barrett et al study, are more comparable with the results found in this project. Barrett et. al. finds an overall occupancy of 39.5% ± 10.9% in their participants with a dose of 10mg/70 kg (equivalent to ≈ 0.142mg/kg). In a parent study of the Barrett et. al. study they also found that the dose 10mg/70 kg psilocybin in humans, resulted in a psychedelic experience for the participants [55]. Compared to the Madsen et al. study, this dose gave an occupancy of 66.4 %. In this thesis the overall occupancy of the $5HT_{2a}$ was found to be 60.75% ±11.29% which is similar to the occupancy found by Madsen et al., which caused a psychedelic experience in humans. Barrett et. al. calculates the occupancy based on specific VOI's as this thesis has done.

One problem is that in this thesis, an antagonist is used to measure the occupancy. When using an antagonist, the compound will bind to the total amount of receptors. An agonist will only bind to receptors which are in their high affinity state [56].Therefore, the occupancy using an antagonist will probably be estimated lower, than the actual occupancy of functional receptors. To investigate the actual occupancy, a selective agonist could have been used. Unfortunately, no such tracer has been developed except [^{11}C]-CIMBI-36. An example of a possible selective agonist on the $5HT_{2a}$ receptor, that could be used as a PET tracer, could be 25CN-NBOH[57]. But no PET tracer of this compound has been developed.
In this thesis it was also noted if the rats had head twitch responses, which as described is known to be a rodent response to psychedelics/hallucinogens [53]. It must therefore be concluded that the dose of 1mg/kg can in fact be considered to be a psychedelic dose in male Long Evans rats. But as discussed, it would be relevant to do the same type of experiment, with an agonist of the $5HT_{2a}$ receptor to determine the actual occupancy.

7.1.2. Limitations in the [^{18}F]-MHMZ study

There are some limitation and biases in the occupancy study, in particularly spatial variances in the measured occupancy, the dose of MHMZ and blinding of the researcher.

7.1.2.1. Spatial variances in estimation of occupancy in [^{18}F]-MHMZ/psilocybin scans

With the calculation of BPnd and occupancy, some assumptions are made. For instance, it is assumed that the non-specific binding is the same throughout the brain. It is also assumed that the occupancy of psilocybin is uniform. From the calculations made on a VOI basis, it is evident that this is not entirely the case. The occupancy varies spatially in the brain. An example could be the cingulate cortex and the pareital anterior cortex (adjusted P-value: 0.0065). This spatial variation in occupancy has also been observed by other studies [58,59].
In this project the Logan reference tissue model is used. Here the cerebellum grey matter is used as the reference, as it is assumed to be devoid of the $5HT_{2a}$ receptors. However, many experiments find that the non-specific binding can vary spatially throughout the brain[60]. It can therefore be argued that the spatial variance needs to be considered, when calculating the occupancy to avoid biases.
In an article from 2020, Bart et. al. suggests that it is possible to add a local neighbourhood filter to Lassen plots to create more accurate estimates of BPnd and occupancy [51]. An estimation and voxel-based correction of the variance in occupancy and non-specific binding, would therefore have been preferable in this project.
Bart et. al. proposes that a regional difference in occupancy is still controversial, but some studies have observed and explored this phenomenon e.g. an article by Martinez et. al. from 2001 [58]. Martinez et.al. saw that there is a regional difference, in the occupancy of Pindolol at the $5HT_{1a}$ receptors. Martinez et.al. suggests that there can be several potential mechanisms to this regional difference they observed. Martinez et.al suggest that some regions might contain a higher fraction of high activity state receptors. These regions might exhibit larger displacements of pure antagonist

tracers by agonists. This will lead to higher than average occupancy. Another hypothesis by Martinez et.al. propose that the tracer and drug of interest, might have different *in vivo* affinities for internal versus external receptors. Therefore, regions with different ratios of internal vs. external receptors will display variances in occupancy, if the tracer and drug have different capabilities of crossing the cell membrane and thereby binding to the internalised receptor.
Another hypothesis could be that the affinity of the tracer and drug of interest are different to each other. For instance, if the tracer had higher affinity than the drug for the receptor, it would result in lower apparent occupancy in regions with an abundance of the receptor in question. This could for instance be the reason to why a lower occupancy is seen in the cingulate cortex, compared to the pareital anterior cortex.
Irrelevant of which mechanism might be the underlying cause, it is evident that these regional variances should be investigated when trying to determine occupancy with occupancy plots. Therefore, it could be relevant, in future work, to investigate if the observed spatial variances have an influence on the calculated occupancy. Secondly it would also be relevant to investigate how to correct for the variances with filters as Bart et.al have tried to do with the Lassen plot.

7.1.2.2. 5HT2a regulation after MHMZ administration

It is known that chronic administration of both agonists and antagonists of the $5HT_{2a}$ receptor will cause downregulation of the receptor [61]. It is therefore relevant to wonder if the administration of MHMZ could cause a downregulation of the $5HT_{2a.}$ But the thing to keep in mind here, is that a tracer principle is utilised. This means that only a very small amount of the compound is administered. Such a low amount that it should not elicit a pharmacological effect.
Looking at the injected dose and the corresponding BPnd, it is evident that the dose did in fact not produce a pharmacological response. Therefore, it must be assumed that the administration of MHMZ did not influence the regulation of the $5HT_{2a.}$ Furthermore, MHMZ was only administered once at the baseline scan, and a very long washout period of 12 days was allowed before the administration of psilocybin.

7.1.2.3. Blinding of the researcher

In these experiments there were no control group as the rats served as their own baseline. This gave problems with blinding of the researcher, as the researcher knew on which days the rats received psilocybin, which could cause unconscious difference in the handling of the animals. The researcher was also not blinded for the data processing in PMOD, which is also problematic, because unconscious bias can occur. In this experiment, it was difficult to blind the researcher on the scan days because of the time aspect. The animals further behaved differently because of the psilocybin treatment. It would have been possible to blind the researcher during the data processing to avoid bias. The biggest bias in the data processing came from fitting the VOI atlas. For the [^{18}F]-MHMZ scans most of the fitting was done by hand, resulting in bias of the data.

7.2. Discussion of [^{18}F]-FDG metabolism study

The metabolism studies of this thesis only show a significantly lowered metabolism in the striatum acutely after dosing of psilocybin (P-value 0.0010). No other significant results are seen for the metabolism studies.

Power calculations show underpowering of the study. The underpowering can be attributed to a high degree of variance and a small sample size.

7.2.1. The changes in glucose metabolism.

The only significant finding in this study, is a significantly lowered [^{18}F]-FDG uptake in the striatum acutely after dosing of 1mg/kg psilocybin.

Stimulation of $5HT_{2a}$ receptors by psilocybin have a big influence in the striatum, as evident from the proposed mechanism of action of psilocybin on the CSTC loops (figure 7). The prefrontal cortex has glutamate projections with $5HT_{2a}$ receptors, that project to GABAergic neurons in the striatum. In the striatum CSTC loops often have another synapse through the complex, before going to the thalamus. This could in part explain the lowered metabolism acutely. Furthermore, in the striatum the $5HT_{2c}$ receptor, for which psilocin has a high affinity (see table 2), is expressed on many of the GABAergic neurons. This could also explain some of the lowered metabolism[62].

Looking at the mPFC, the mean uptake of [^{18}F]-FDG is raised acutely after dosing (1.6±3.1%). This makes sense as the $5HT_{2a}$ receptor is abundant in this area [50]. Psilocybin is known to hyperactivate the $5HT_{2a}$ receptor and being excitatory on glutamate projections. It makes sense that an area abundant with the receptor will have higher metabolism acutely after administration. These findings are also consistent with PET [^{18}F]-FDG studies in humans, where hyperfrontality is seen acutely after administration of a psychedelic dose of psilocybin. This is further underlined by the proposed mechanism of action of psilocybin on CSTC loops (figure 7) [63,64].

From the preliminary study with psilocybin in Long Evans rats (see section 2), it was evident that $5HT_{2a}$ receptors in the cortex became down-regulated one day after administration of 1mg/kg psilocybin SC. However, the density was back to baseline seven days after administration. This could explain why the measured metabolism of the mPFC, is approximately the same as before the administration (-0.8±3.0%). Based on these findings, a hypothesis could be that the metabolism will be down-regulated in the mPFC, one day after administration of psilocybin. This could therefore be interesting to investigate in further work.

The [^{18}F]-FDG study showed that the mean uptake in the striatum is lowered acutely after dosing (-2.8±1.0%) compared to baseline. One week after dosing the mean uptake is lowered compared to baseline (-1,4±2.1%). One week after dosing the mean uptake is raised (1.5±2.3%) compared to acutely after dosing. The lowered uptake can be explained by the influence of $5HT_{2a}$ hyperactivation by psilocybin, in the CSTC loops as described. The slightly lowered metabolism one week after psilocybin dosing is as mentioned not significant, but a slight trend is observed which could be interesting to investigate further.

In the thalamus the mean metabolism is raised acutely after dosing (1.0±2.6%) and further raised one week after dosing (2.4±3.5%). However, looking at the individual rats it is evident that the

variance is very high in the thalamus. For 3 rats (3(0.33 SUVwb),4(0.28 SUVwb), and 5(0.85 SUVwb)) the metabolism is raised, and for 3 rats the metabolism falls acutely (6(-0.21 SUVwb),7 (-0.22 SUVwb) and 8(-0.09 SUVwb)). One week after psilocybin treatment 3 of the rats(3(0.32 SUVwb), 6 (0.71 SUVwb) and 7(0.92 SUVwb)) have a raised metabolism, 3 rats have a metabolism that is almost the same as at the acute scan (rat 2(-0.14 SUVwb),4(0.12SUVwb) and 5(0.04SUVwb). Then there is one rat (8) where the metabolism falls throughout the experiments(-0.61SUVwb). Some of the results seen could be explained by the disruption of the thalamic gating when the $5HT_{2a}$ receptors are hyperactivated. Some papers suggest that this hyperactivation causes increased activity in the thalamus, but several studies report both increases and decreases in activity of the thalamus acutely after administration of psychedelics [42]. This is consistent with the findings of this thesis. Looking at the models of the psychedelic mechanism of action, it is clear that it is a very complex system that is still heavily debated [42,64].

Several studies have researched how connectivity changes after administration of psychedelics, and many of them look at connectivity at the nuclei level of the thalamus. From these studies it has been shown that the connectivity changes in very complex ways, that are beyond the scope of this thesis. It could be relevant to look further into how, and if the activity/metabolism of these thalamic nuclei changes with administration of psilocybin, both acutely and after a week administration, as it is evident from this thesis that the changes on a regional level are inconsistent.

7.2.2. Limitations of the [^{18}F]-FDG study

In the [^{18}F]-FDG study there are some limitations, the most prevalent one being blinding of the researcher.

7.2.2.1. Blinding of the researcher

For the [^{18}F]-FDG study, the researcher was not blinded on the scan days or for the data processing. Blinding should have been done for the data processing. But as most of the data processing was automated and as the researcher chose not to alter the matching after the program had matched the pictures and VOI's, it is estimated that the chance of bias has been minimalised.

7.3. General limitations of PET in preclinical studies

There are some general limitations in preclinical PET studies. In the following section these will be discussed.

7.3.1. Matching in PMOD

First of all, the atlas used for the matching in PMOD is based on scans of Wistar rats (an inbred strain). The rats used in this study are Long Evans rats (an outbred stock). Generally the Wistar rats have wide heads [65]. Therefore, there might be some problems with the matching, if the head and thereby brain have a different form in the atlas compared to the actual form of the scanned rats. It was also noticed when matching, that there were some differences in the shapes of the brains. Because of these differences in the head shape of the rats, the shapes and sizes of the atlas do not

fit completely. To account for this, one could have dragged and expanded VOI's to better fit the scan. But this would result in experimenter biases and therefor it was chosen not to do this, at least in the [^{18}F]-FDG study as it was evaluated to have resulted in bigger problems with the validity and reproducibility. A way to solve this issue, could be to do several baseline [^{18}F]-FDG scans in Long Evans rats, and produce an atlas for matching based on these scans, and a VOI atlas based on the scans as well. This would result in better matching and better placement of VOI's in scans, where Long Evans rats are used. This could result in much lower variance, as the VOI's would fit to the actual anatomy of the rats. Doing this would be beyond the scope of this project and would be costly and time consuming. Therefore, it was not chosen to do this.
Another possibility could have been to draw in the VOI's by hand by the researcher. Doing this would result in researcher biases and problems with reproducibility, and therefore the validity of the study. It should always be favoured to have a high degree of reproducibility, as it gives an experiment much higher validity. Therefor every measure that can be taken to minimise experimenter biases should be taken.

7.3.2. Inbred strain vs outbred stock

Another issue with this study is that Long Evans rats are an outbred stock type of rat. This means that there will generally be much more variability within a population of these rats, mainly caused by genetic variability, compared to an inbred strain, such as the Wistar rats. The problem with using an inbred strain is that you will lose some of the construct validity, which is very important to have when doing translational research. Construct validity is a measure of if the experiment/model is representative of what it aims to measure. As this experiment aims to be representative of a human population, where there will be a high degree of genetic variability, an outbred stock will give better representation. Using an inbred strain could possibly have resulted in less variance within the population, but some of the construct validity would have been lost. Construct validity is estimated to be an important part of this project. Based on the considerations above, the Long Evans rat was chosen. The Long Evans rats are much more resilient and can handle being under anaesthesia for a long time, which is preferable when doing longer scans [65]. Long Evans rats are also good for behavioural studies which could be an area of interest in future work.

7.3.3. Stress level of rats

In animal studies it is known that the stress level can impact the outcome of an experiment [66]. The cortisol/corticosterone levels become elevated if the animals are stressed. Elevated cortisol/corticosterone raises metabolism in the animals. This could be an important factor to take into account especially in the [^{18}F]-FDG metabolism experiments. Also, it must be taken into account that some brain areas will be more active depending on the stress and fear level of the animal. For instance, if the animal is fearful the amygdala will be more active[67].
Another thing to keep in mind is that the animals will become more and more accustomed to the researcher and handling. This will decrease the stress levels with time. The animals had not grown

accustom to the researcher before the experiments started. Therefore, there is a chance that the animals were less stressed on the last day of scans compared to the first.
To circumvent this source of error, sessions of gradual increases in handling and acclimatisation towards the researcher, could have been done before the actual experiments. This would reduce the stress levels of the animals.
In these experiments, several measures were taken to minimise stress in the animals. The animals were acclimatised for at least 2 weeks after arriving in animal facility. The animals were also moved to the PET facility 3 days before scanning to allow for acclimatisation. On the day of the scan the cages of the animals were covered with dark cloth to minimise light exposure. Handling was done as quickly as possible and when solutions were administered via syringe in awake animals, the animals were wrapped in cloth and eyes were covered to calm them. The animals were also allowed to roam around their home cages, while equilibriums of the radioactive compounds were allowed to adjust. All of these measures must have reduced stress levels.
Unfortunately, the experiments were done during the light phase of the animals' day cycle. Because of this, the stress levels might have increased a bit compared to doing the experiments during the dark phase. This could have been adjusted if the researcher had been willing to do the experiment during the dark phase, but this would have cost unnecessary strain on the researcher and it was therefore not chosen in this study.

7.3.4. Effects of anaesthesia

In these experiments the animals were anaesthetised with isoflurane mixed with pure oxygen. A study has found, that using isoflurane can alter brain connectivity and gene expression for up to a month after exposure in rats [68]. The study finds that isoflurane strengthens thalamo-cortical and hippocampal-cortical functional connectivity, and that the expression of several cortical genes are altered after isoflurane exposure. Many of these genes are involved in neuronal signal transmission [68]. It could therefore be discussed whether or not isoflurane is a good form of anaesthesia for brain studies in rats, where neuronal activity is investigated.
However, it is preferable that the animals are in a state of stable anaesthesia, and that it is easy to regulate the level of anaesthesia based on vital signs. This leaves out anaesthesia with bolus injections. As the animals are contained within a PET scanner, it makes it difficult to access them during the scan. Another option is to have an IV infusion of anaesthesia. With an IV infusion it is possible to easily regulate the infusion rate based on vital signs. It is also possible to regulate the infusion rate outside the scanner if the IV line is long enough. But then comes the problems with placing an IV cannular in an awake animal. This is impossible without restraining the animal and causing stress.
As described above it is preferable to cause minimal amounts of stress in the animals. Doing this type of procedure would need extensive training of the animals to grow familiar with the restraining. Using a type of inhalation anaesthesia is therefor often the best option. Halothanes (e.g. desflurane, sevoflurane) are the most commonly used types of inhalation anaesthesia, along with isoflurane. Other types of inhalation anaesthetics that could have been used, are nitrous oxide (N_2O) and

Xenon. The problem is that Xenon is very expensive and that nitrous oxide can be neurotoxic [69]. The best option is therefore halothanes and as isoflurane is widely available, it was chosen in these experiments.

There are problems with using 100% oxygen as the flow gas. Firstly, it has been shown that rats that receive 100% oxygen with isoflurane, go into acute respiratory acidosis. The mean arterial blood pressure will also be elevated [70]. Hyperoxic ventilation (>21% O_2) is also known to cause responses in the human brain. E.g. areas of autonomic and hormonal control such as the hippocampus, insula, thalamus, hypothalamus and cerebellum, show pronounced responses after only 2 minutes of hyperoxic treatment[71].

When hyperoxic treatments is used in humans, the insulin and glucagon levels are raised causing changed metabolism compared to non hyperoxic treatment [71]. This could cause a problem at least in the [^{18}F]-FDG experiments, as a sugar analogue is used as the tracer.

Non hyperoxic (< 21% O_2) flow gas mixtures should have been used in the experiments.

As described, there was no control group in this study. Based on knowledge that isoflurane can influence functional connectivity and gene expression, it would have been advisable to have a control group, to make sure that the observed changes, could not be attributed to the administration of isoflurane.

7.4. Translatability

When doing preclinical studies, it is important to keep the translatability in mind. In the experiments done in this thesis, several points in terms of translatability need to be discussed. For instance, representation, external validity and the use of an animal model.

7.4.1. Representation in the studies

It is problematic that only male rats were included in this study. Preclinical studies have historically had problems with including female animals [72], and it is known that sex, hormones and genotypes have a massive impact on e.g. symptomology in psychiatric diseases and efficacy of medicines [73]. Therefore, to have a truly representative study, one needs to include both female and male subjects and take sex into account when doing statistical analysis.

When speaking of psychiatric diseases more females (15,5%) report to have bad mental health compared to men (10,7%) [1]. Therefor it is of the utmost importance to balance the studies because there is a real chance, that different outcomes can be seen across sexes.

This being said, there are some experimental considerations to take into account. Female rats have cyclic hormonal changes because of their ovarian cycles. It is known that whilst in proestrus and oestrus female rats have changes in behaviour. E.g. in motivation, where female rats would rather seek to access males than palatable food whilst in oestrus [74]. Therefore, it would be advisable to measure serum oestrogen and progesterone and correlated it to the observed changes. It is also known that aggression in both male and female rats can be driven by hormones. Aggression in females is much higher in the presences of male rats [75]. Therefore, it would be necessary to control the exposure of hormones of the opposite sex, to keep control of the aggression and

hormonal cycles of female rats. This would be labour intensive and add an extra layer of complexity to the experiments. It would therefore be something that would be advisable to do later on, in the process to understand the neuronal processes of psilocybin across sexes.

7.4.2. The concepts of external validity

In relation to model validity/external validity, some things also need to be noted about the experiments at hand. The external validity is a measure translatability. The 3 types of validity criteria one can look at in terms of external validity are:

1. the construct validity, meaning to which extend both the animal model and the human phenomenon can be explained by the same theory.
2. the face validity, meaning if there is a similar appearance in the animal compared to the human phenomenon.
3. the predictive validity, meaning if the performance of the test predicts performance in the human phenomena that is being modelled.

Starting with the occupancy study is estimated to have good construct validity, as the same processes of psilocybin binding can be translated between animals and human. The face validity is also estimated to be relatively high, as the appearance is similar and as the head twitch response is used as a proxy of a psychedelic experience in rodents [53]. The predictive validity is also high as similar results are seen in this thesis and the Madsen et al. study in humans [54]. The study was done to show that a dose of 1 mg/kg in rats could be characterised as a psychedelic dose.

For the [^{18}F]-FDG experiments many of the same things apply and the construct validity and face validity, are also estimated to be relatively high. The predictive validity is a bit more uncertain. This comes from the fact that the human studies are not conclusive and the fact that the variance is high in this experiment.

There are problems with applying the concepts of external validity to the experiments. The experiments do not model a specific disease but are supposed to model the general population. Therefore, the concepts of external validity do not fully apply, as these are made to assess the validity of disease models in animals.

7.4.3. Use of an animal model

As described above the principles of external validity are hard to translate to the experiments of this thesis, as a model of diseases is not used. Examples of depression models, could be the learned helplessness protocol, chronic mild stress protocol, social stress protocol and the maternal deprivation protocol [76]. These protocols generally take a long time to perform and are very labour intensive. Also, reproducibility is often problematic. With these types of models, the predictive validity is often lost [76].

Then lesions (e.g. olfactory bulbectomy) or pharmacological interventions (reserpine, tryptophan, psychostimulant withdrawal) could be used instead [76]. These models are not super labour intensive, but a lot of the construct validity is lost in these models [76]. A genetic model could be

used (genetic engineering or selective breeding) but the construct validity would also be lost here [76]. Another problem with animal models of psychiatric diseases, is that the researcher cannot ask the animal about how it is feeling, as one can with human participants. A researcher can only perform tests to see if the animal behaves, in a way that is somewhat different to normal animal behaviour. Tests with elevated plus or O mazes, forced swim tests, tail suspension tests, sucrose preference tests, intercranial self-stimulation etc. are only proxies for how the animals "feel". Many of the tests only give a measure of one aspect or symptom of the modelled disease. A lot of the face validity is lost in animal models of psychiatric diseases, because we are not able to fully understand how the animals are feeling. In humans, questionnaires such as the Quick Inventory of Depressive Symptomatology–Self-Report, or the Warwick- Edinburgh Mental Wellbeing score are used to assess the mental state of patients [33]. Essentially, we are not able to assess the psychiatric diseases the same way in animals and humans.
Several tests looking at executive functioning in humans have now been developed for animals. For instance, The Continuous Performance test and The Reversal Learning test. These tests have a high face validity, as the same types of problems with executive functioning can be assessed.

The knowledge and promise that psilocybin work well and without side-effects in patients with depression, anxiety, OCD and many other psychiatric diseases is amazing. But if psilocybin is to obtain regulatory approval and market authorisation by the FDA, EMA etc., more knowledge about mechanisms of actions are needed. From a clinical trial point of view, we have to establish these mechanisms in healthy individuals before moving on to patients. The same goes for animals. Therefore, experiments such as the ones done in this thesis with healthy animals, can help lay the foundation for psilocybin used as a treatment. In the near future it will be relevant to use a disease animal model, in the same type of experiment to establish the mechanisms and efficacy of psilocybin on the disease.

8. Future prospects and considerations

Regarding the occupancy study, it would be interesting to investigate if the same occupancy at a dose of 1mg/kg could be achieved in an actual disease model. It would also be interesting to see if the same occupancy could be achieved in rats with an agonist. Here the PET tracer [^{11}C]-CIMBI-36 could be used, to be able to fully compare to the study by Madsen et al.
In relation to this, it would also be interesting to investigate the plasma level of psilocin after administration, to investigate if the psilocin level in rats have a similar clearance as in humans. A PET tracer of the compound 25CN-NBOH could also be interesting to investigate, as it has higher selectivity towards the 5HT$_{2a}$ receptor, than the 5HT$_{2c}$ compared to [^{11}C]-CIMBI-36. Unfortunately, no PET tracer of this compound has been made yet.

It is clear that there is an under-powering of the [^{18}F]-FDG/psilocybin experiments, it would be relevant to add more animals to the experiment, to be able to detect significance. Further, it would also be relevant to examine whether or not the anaesthesia has an effect on the results.

Therefore, addition of control animals which only receive saline injections and no psilocybin would be relevant. Including female animals in the study is also relevant and easy to implement. To begin with it would be relevant to not control for ovarian/hormonal cycles in the female animals, to demonstrate proof of concept. Further on it would be relevant to control for hormonal cycles. After establishment of mechanisms in healthy animals, it would be relevant to look at how the metabolism could change in an actual disease model. Different models would be relevant, but further investigation into which model is the best, for this type of experiment is needed.

8.1. Other networks of interest

Many hypotheses of how psychedelics elicit their mode of action have been proposed. In this thesis, the main focus has been on the disruption of the CSTC loops. In reviews by Vollenweider and Preller, and Doss et. al. the REBUS (relaxed beliefs under psychedelics mode) model is also proposed as a mechanism of action of psychedelics [42,64]. In this model it is believed that psychedelics disrupt the nature of the hierarchy that is present in the brain.

It is hypothesised that the constraints of top down control are reduced, which results in increased influence of low-level areas on the cortical areas (bottom up control). In this model, higher levels of the brain are defined as prefrontal and parietal association cortexes, such as the default mode, frontoparietal and salience networks. The lower levels are said to include areas such as the hippocampus, parahippocamal gyrus, amygdala and thalamus. In the REBUS model, the default mode network is of special interest. Unfortunately studies have not been consistent in whether or not activity is raised or lowered with the administration of psychedelics [42]. It could therefore be interesting to look at these areas that the REBUS model propose are implicated, in the mode of action of psychedelics. The review by Doss et.al. also introduce the cortical-claustro-cortical model. In this model the claustrum is implicated. This area of the brain is located lateral to the putamen, medial to the insula and in between the external and extreme capsules. This area of the brain densely expresses the $5HT_{2a}$ receptor and has bidirectional glutamatergic connections with a major part of the cortex. The claustrum is thought to disrupt higher level cortical networks through the bidirectional connections, when psychedelics are administered. The disruption is thought to lead to aberrant cognitive control, by decoupling of the prefrontal cortex and claustrum by the $5HT_{2a}$ receptor. This may lead to attenuation of cortical networks, such as the frontoparietal network and the default mode network. In humans the administration of psilocybin has been shown to decrease the resting state activity of the claustrum[42]. It could therefore be interesting to also look at the activity of the claustrum. The neuroplasticity hypothesis further underlines the fact, that many of the implicated brain areas of depression, are involved in the potential mechanisms of psychedelics and are therefore relevant to investigate. Furthermore, it also seems relevant to include other areas of interest, based on the hypothesis that depression is a product of faulty neuronal networks. It would also be relevant to look at the connectivity of these areas, to be able to investigate if connectivity changes with the administration of psilocybin.

8.2. The promise of psychedelic therapy

The revival of psychedelic research is currently blooming. An enormous amount of clinical trials and preclinical research in these drugs, are currently being done all over the world. This brings hope to the many patients suffering from psychiatric diseases [1]. But is it even feasible to think that psychedelics will find their way into the clinic? There will be many bumps on the road.

First of all, the psychedelics, such as psilocybin, DMT, mescaline and LSD are old compounds and pharmaceutical companies are not able to directly patent them. Then it is not financially feasible to go through the process of pharmaceutical development and regulatory approval, which is very costly, only to have another company, create a generic product right after marketing authorisation. Then an option could be to make analogues or other compounds, with the same properties as the currently used psychedelics and patent them. This would help in protection of income after pharmaceutical development. But then comes the problem of establishing efficacy. With the way drugs are currently approved, a company have to establish that the drug they wish to have approved have better efficacy than standard placebo. The double blinded placebo-controlled trial is the gold standard. But it is impossible to establish blinding of patients with psychedelic drugs. Then a company would have to compare the drug, with a currently approved pharmaceutical, that should produce the same net effect, as the drug in question. But as no psychedelic is currently approved, the only option is to compare to drugs (e.g. SSRI's) that do not produce a psychedelic experience. These types of trials have been done and they show great efficacy, but blinding is still problematic [33]. New ways of designing trials are needed or the regulatory bodies will have to reform the approval system, to pave the way for psychedelic drugs.

Then there's the problem of the right psychological support and the right setting in psychedelic therapy. Minimum requirements need to be investigated, and effects of setting and psychological support, would also need to be evaluated, to obtain regulatory approval. Then comes the problem of reimbursement. If psychedelic therapy is ever approved by medicines agencies, then national health technology assessment bodies, will need to allow for the usage of a drug with a probable high immediate cost and uncertainty of efficacy in some patient groups.

There might also be a problem in the general public and with legislators. Currently, most of the public and legislators see psychedelics as dangerous substances that need to be prohibited. A lot of this fear of psychedelics, come from the war on drugs in the 70's and anecdotal tales from hippies who were carefree in the usage and cost harm to themselves. The fear also comes from the badly constructed experiments of the 60's and 70's[31]. Horror stories form these trials emerge frequently in media and scare the public each time[77]. A shift in paradigm is needed to be able to implement psychedelics into the health care system. Luckily this seems to be happening. Younger generations are becoming more and more positive towards psychedelic therapy. More and more of the public and legislators are also becoming aware of the fact, that current treatments of psychiatric diseases are insufficient. Hopefully psychedelics, including psilocybin, will be part of the solution for a part of the 33% of Danes who at one point in their life, will suffer from mental illness.

9. Conclusion

In this thesis the following questions were wished to be answered.

- Does a dose of 1 mg/kg psilocybin in male Long Evans rats, give an occupancy of the $5HT_{2a}$ receptors that can be said to be psychedelic?
- Does a single treatment with 1 mg/kg psilocybin in male Long Evans rats, change the metabolism of the brain areas involved in CSTC loops acutely? If so, how?
- Can changes in the metabolism of the brain areas involved in the CSTC loops be seen 7 days after dosing with 1 mg/kg psilocybin in male Long Evans rats? If so, how?

It can be concluded that the dose of 1 mg/kg psilocybin in male Long Evans rats give an occupancy of 61% $\pm$11% at the $5HT_{2a}$ receptors. Therefore, the dose can be characterised as being a psychedelic dose, based on comparisons of occupancy of the $5HT_{2a}$ receptors (65.6%$\pm$0.4%) in humans that cause a psychedelic trip. This is further underlined by the characteristic head twitch response that was observed.
This finding creates the bases for further preclinical research in how a psychedelic dose of psilocybin works on a molecular level.

A single treatment with 1mg/kg psilocybin in male Long Evans rats lowers the metabolism significantly in the striatum acutely after dosing, compared to baseline (P-value 0.0010). A significant change in metabolism acutely after dosing of 1mg/kg psilocybin is not seen in the mPFC or the thalamus.
No significant changes are seen in any of the brain areas of the CSTC loops one week after dosing with psilocybin. Both compared to baseline and acutely after dosing.
The negative results can mainly be attributed to underpowered experiments with a big variation and a small sample size.
Even though not significant, trends can be seen in the data. The mean for the mPFC shows a raised metabolism (1.6±3.1%) acutely after dosing, and lowered metabolism one week after dosing (-0.8-0.8±3.0%). The mean metabolism in the striatum is significantly lowered (-2.8%±1.0%) acutely after dosing. A week after dosing, the mean metabolism is still a little lowered (-1.4±2.1%) but increased (1.5±2.3%) compared to acutely after dosing. The mean metabolism of the thalamus is increased (1.0±2.6%) acutely after dosing and further increased (2.4±3.5%) one week after dosing compared to baseline.
These findings underline the further need for investigation of the CSTC loops in relation to psilocybin treatment.

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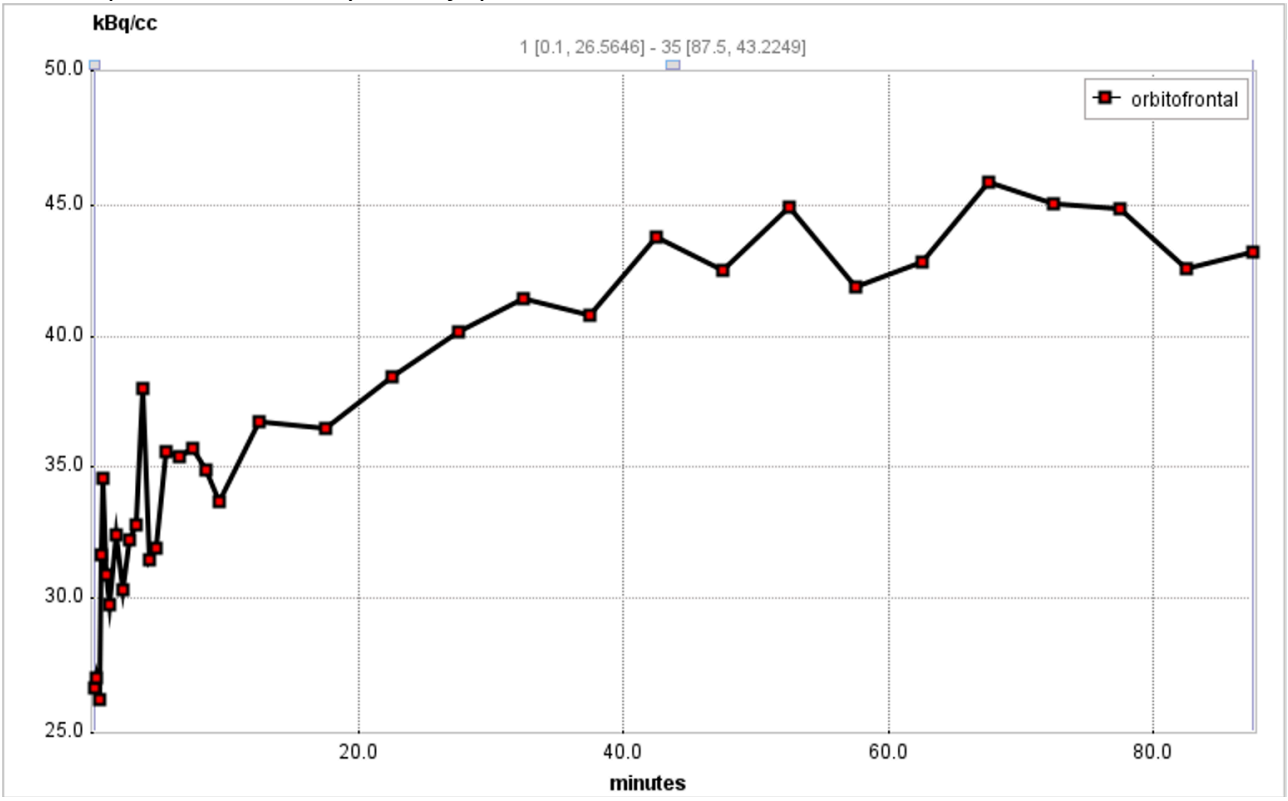
Annexes

Annex 1 – Raw data of autoradiography studies

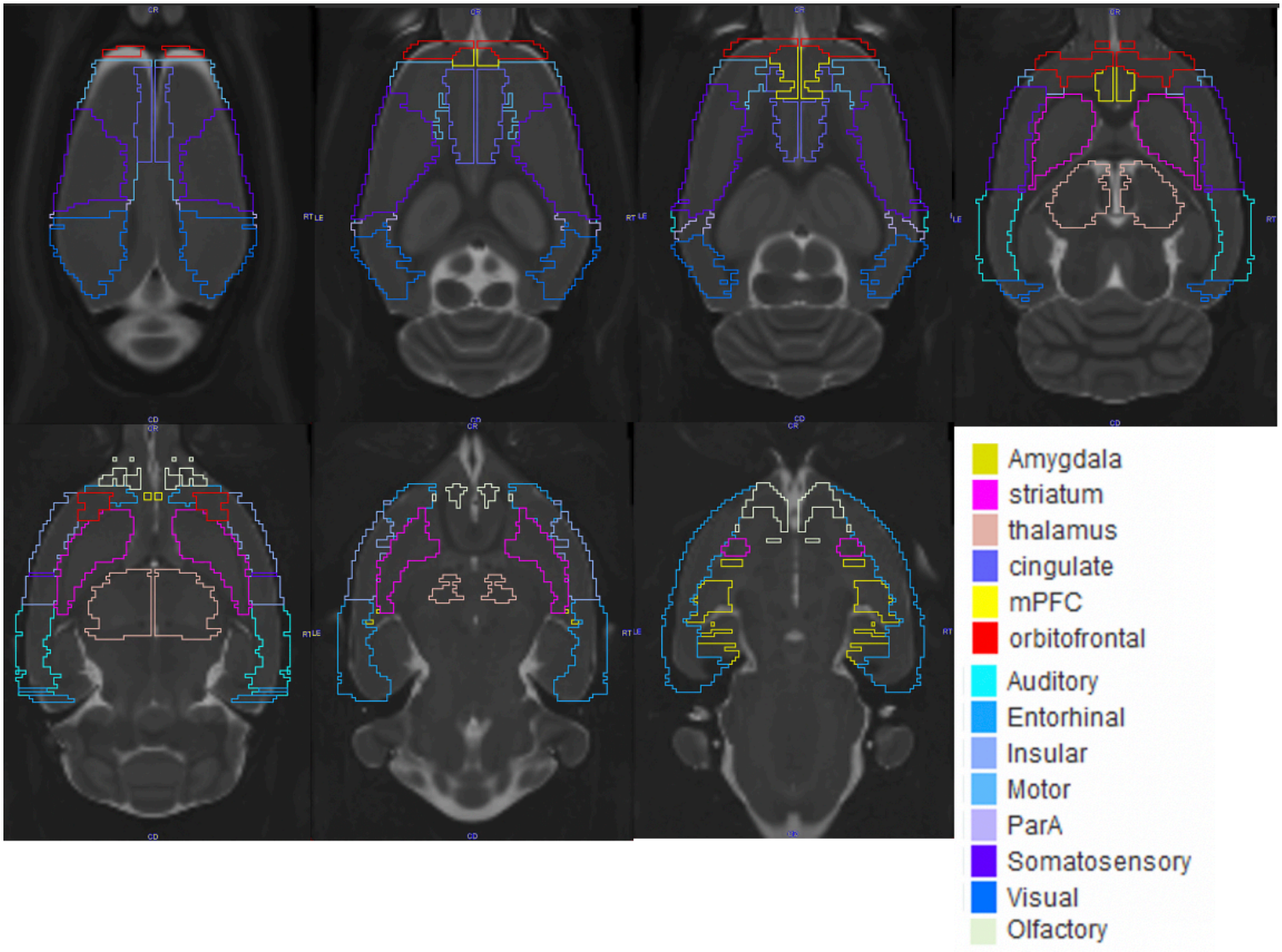
Animal number	Days after psilocybin dosing	5HT2a (fmol/mgTE)	5HT5c (fmol/mgTE)	5HT7 (fmol/mgTE)	5HT1a (fmol/mgTE)
05	0	48.8869048	32.0080659	32.0080659	111.363317
11		40.4107143	31.5164744	31.5164744	89.3993205
19		41.2858699	28.1299555	28.1299555	81.2036859
30		43.3586463	27.8131521	27.8131521	79.3106312
33		33.9979586	23.0720257	23.0720257	70.5219914
36		40.2531699	25.9341804	25.9341804	70.4839783
02	1	37.7202381	26.6333326	26.6333326	75.2964428
07		31.0238095	29.2770022	29.2770023	83.5605011
09		32.7916667	28.5450772	28.5450772	106.771329
16		28.1964286	24.7980579	24.7980579	73.0612697
26		36.0928643	28.2501223	28.2501223	77.7520921
27		34.4036621	27.8350006	27.8350007	86.1758056
03	2	42.1130952	28.6543197	28.6543197	82.5493513
12		42.3630952	31.3416864	31.3416864	86.6471687
15		35.6785714	30.6097613	30.6097613	72.4150462
23		34.9864	23.9131933	23.9131933	78.2234551
29		32.1538515	25.3879676	25.3879676	71.2366386
31		29.4171965	23.1157227	23.1157227	77.835721
01	3	41.9166667	30.6643826	30.6643826	73.2513354
10		36.7440476	29.6047299	29.6047299	82.6709934
17		36.172619	28.2719708	28.2719708	73.1677065
18		43.6666667	32.6088999	32.6088999	89.3537048
20		37.5902792	29.1459112	29.1459112	76.1859504
32		31.7186422	24.8963762	24.8963762	81.3785464
04	5	42.6785714	33.2534309	33.2534309	98.5604891
06		35.4285714	27.977016	27.977016	58.1905264
14		29.5357143	27.0266058	27.0266058	69.1915313
21		41.9054899	30.0417001	30.0417001	69.0090682
28		39.876972	28.2938193	28.2938193	82.3896961
34		31.0768929	22.4821159	22.4821159	73.2893486
08	7	41.1845238	31.8442021	31.8442021	96.6522291
13		34.0416667	27.2450909	27.2450909	76.5508767
22		38.3279221	29.178684	29.178684	74.4449483
24		38.8959071	26.5131658	26.5131658	78.0257868
25		37.6124085	25.3661191	25.3661191	84.3511745
35		40.0908885	26.4913173	26.4913173	73.2513354

Annex 2- [^{18}F]-MHMZ TAC of orbitofrontal cortex in Long Evans rats

From experiments made by Nikolaj Speth 2019



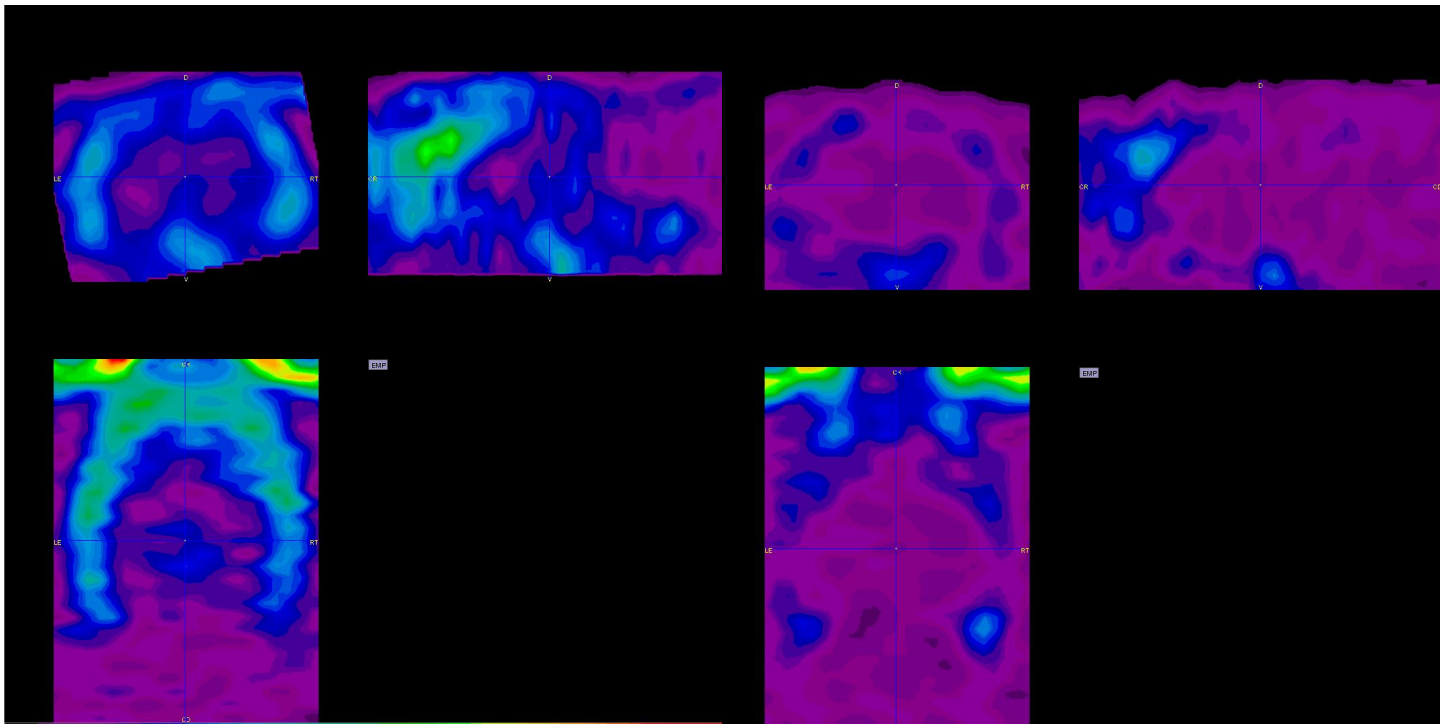
Annex 3-Stuctual MRI references.



Annex 4- non manipulated [^{18}F]-MHMZ scan pictures

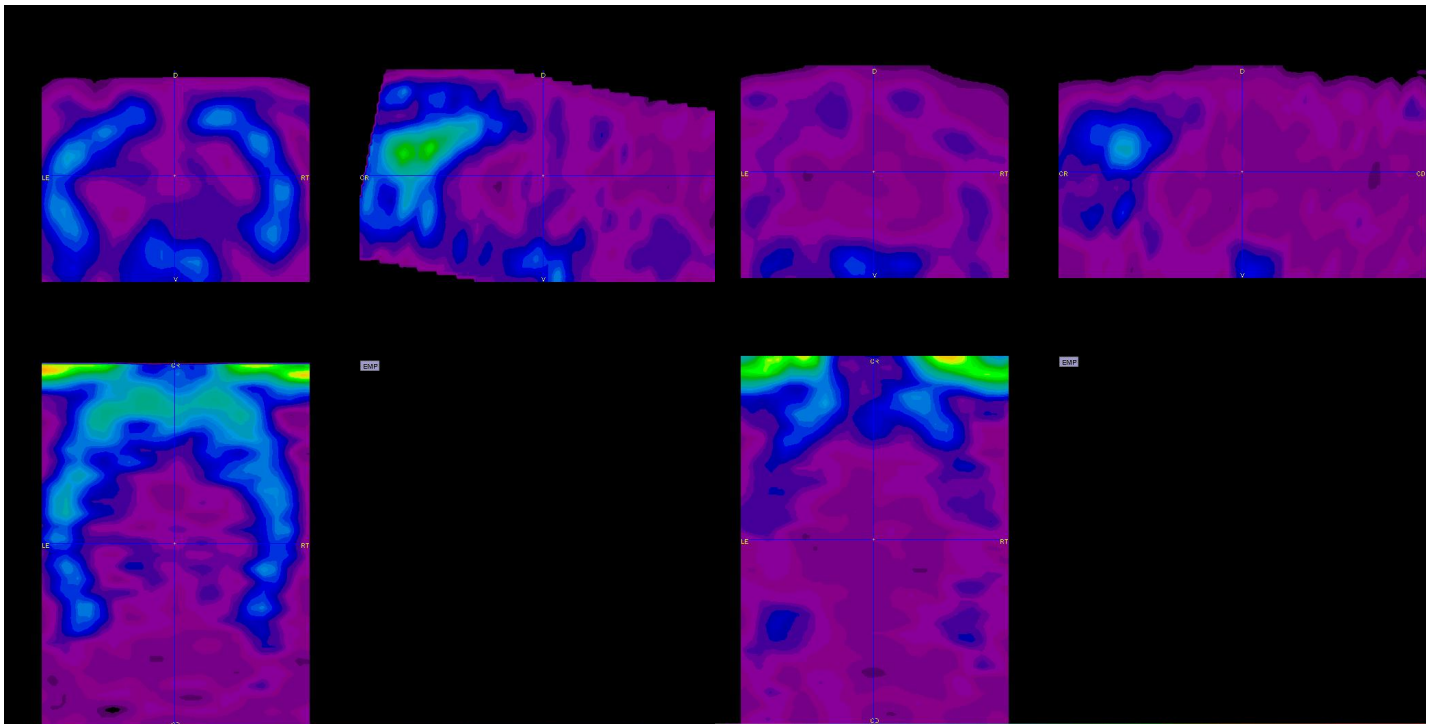
4.1 rat 62 Baseline and Acute

Baseline Acute



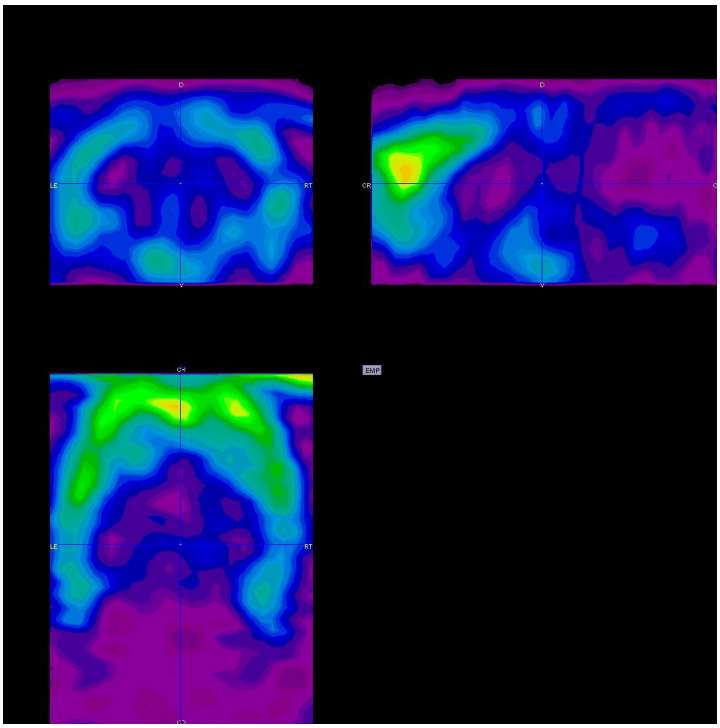
4.2 rat 63 Baseline and Acute

Baseline Acute

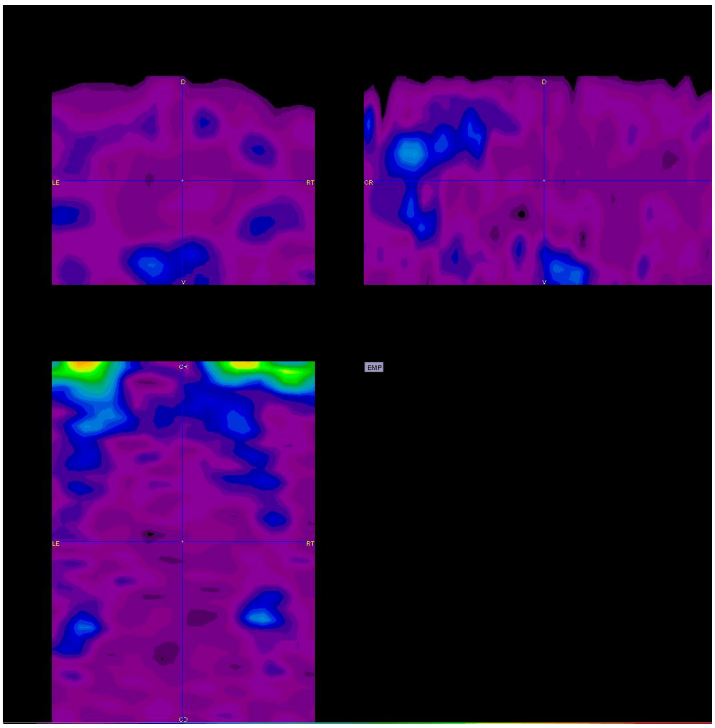


4.3 rat 64 baseline and acute

Baseline



Acute



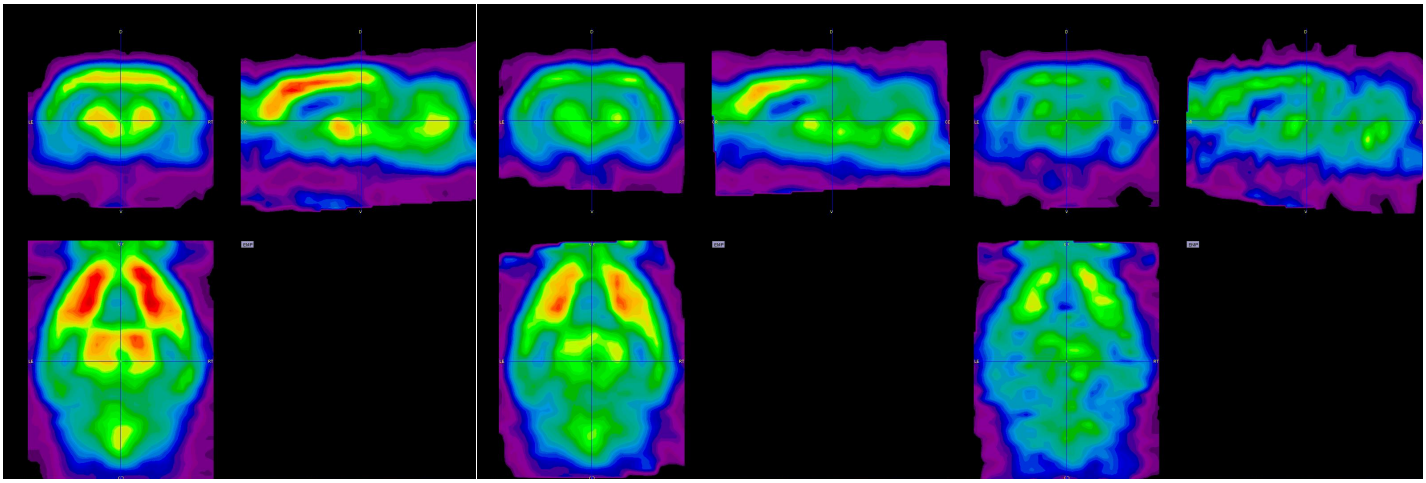
Annex 5- Raw data from MHMZ BPnd

	Baseline (BPnd)			Acute (BPnd)		
	rat 62	rat63	rat64	rat 62	rat63	rat64
Nucleus Accumbens	1.951177	1.740111	1.92596	0.310108	0.423396	0.594372
Amygdala	1.050531	1.053165	0.994555	0.066291	0.336195	0.179672
Auditory Cortex	1.38864	1.452043	1.1569	0.180915	0.147008	0.290814
Cingulate Cortex	2.357156	2.635456	1.988842	0.633067	0.775792	1.00967
Entorhinal Cortex	1.173665	1.261815	1.27484	0.253134	0.242438	0.306163
Insular Cortex	2.049827	2.446793	2.552958	0.584119	0.943382	0.878159
Motor Cortex	2.025994	2.961037	1.783768	0.620999	1.198744	0.993832
Medial Prefrontal Cortex	2.320557	3.328038	3.176297	0.758537	1.054109	0.851002
Olfactory Bulb	1.203542	1.315875	2.31723	0.505463	0.433076	0.286885
Orbitofrontal Cortex	2.22476	2.856388	2.445903	0.877694	1.361469	0.892729
Parietal Anterior Cortex	1.375916	2.07178	1.303883	0.168106	0.273141	0.461879
Somatosensory Cortex	1.930049	2.501507	2.169162	0.461659	0.67364	0.731519
Striatum	1.340087	1.769324	1.571434	0.220375	0.421494	0.456716
Visual Cortex	1.018216	1.596126	1.171414	0.075317	0.185278	0.154494

Annex 6- non manipulated [^{18}F]-FDG scan pictures

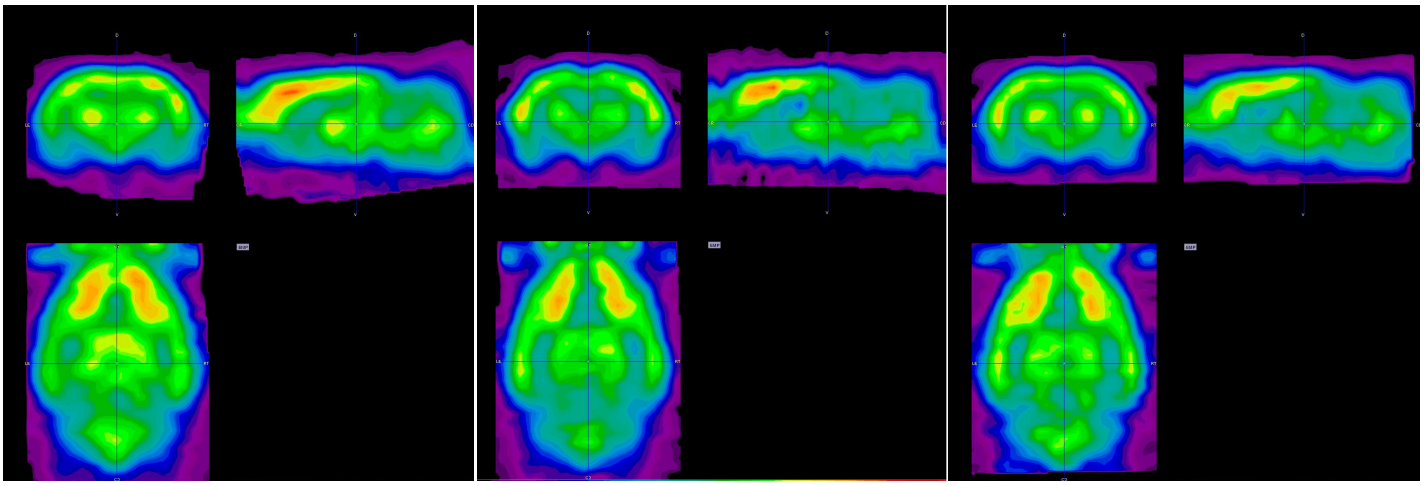
6.1 rat 1 baseline, acute and one week

Baseline acute one week



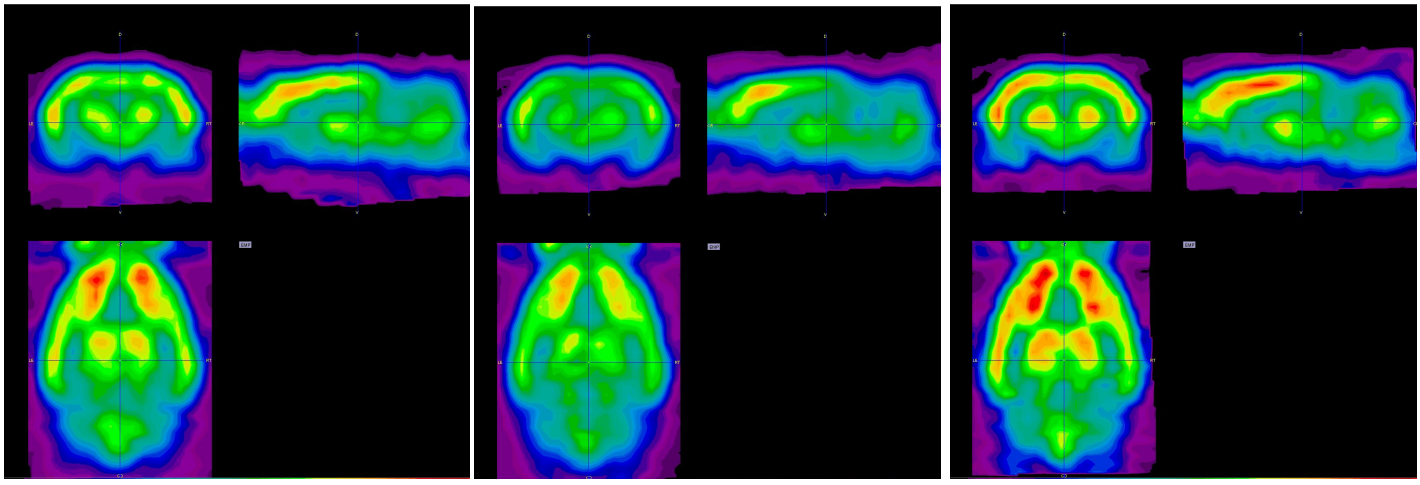
6.2 rat 2 baseline, acute and one week

Baseline acute one week



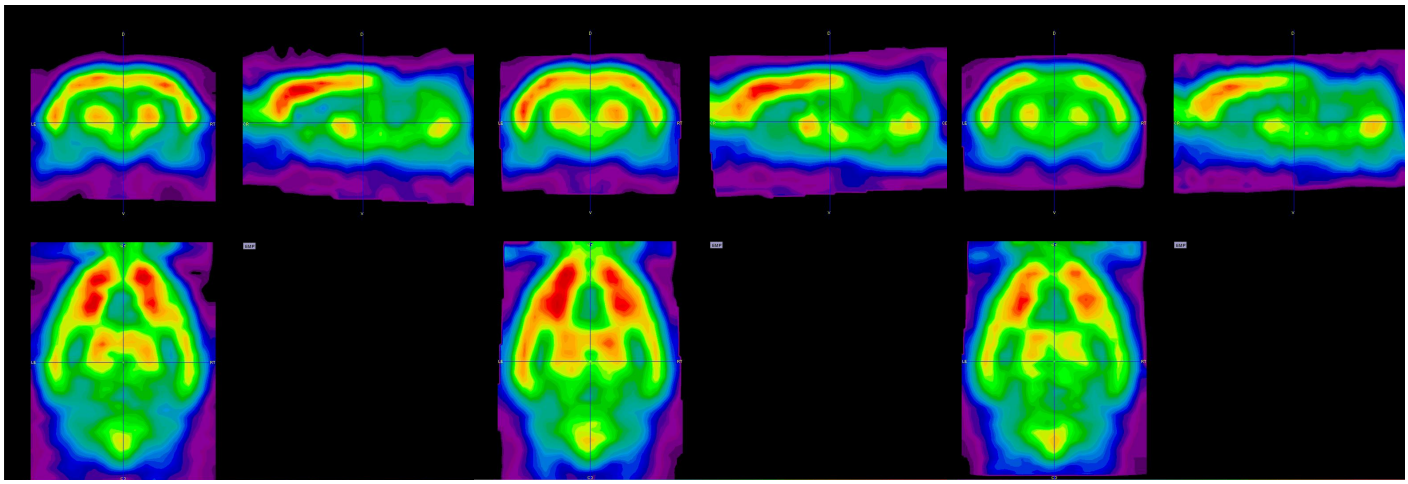
6.3.rat 3 Baseline, acute and one week

Baseline acute one week



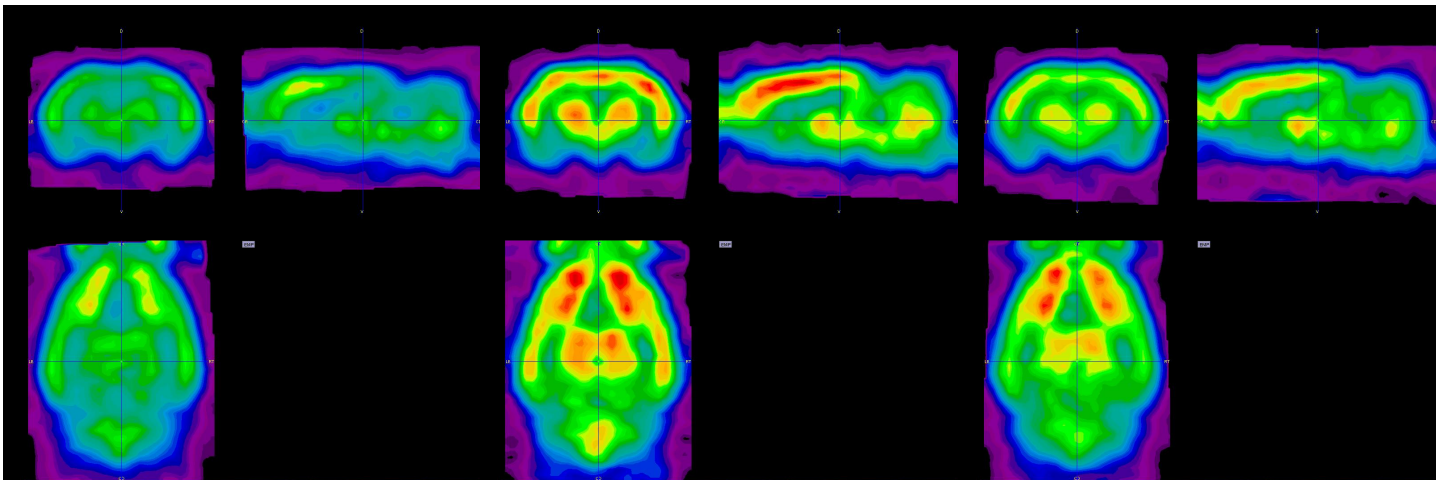
6.4 rat 4 baseline, acute and one week

Baseline acute one week



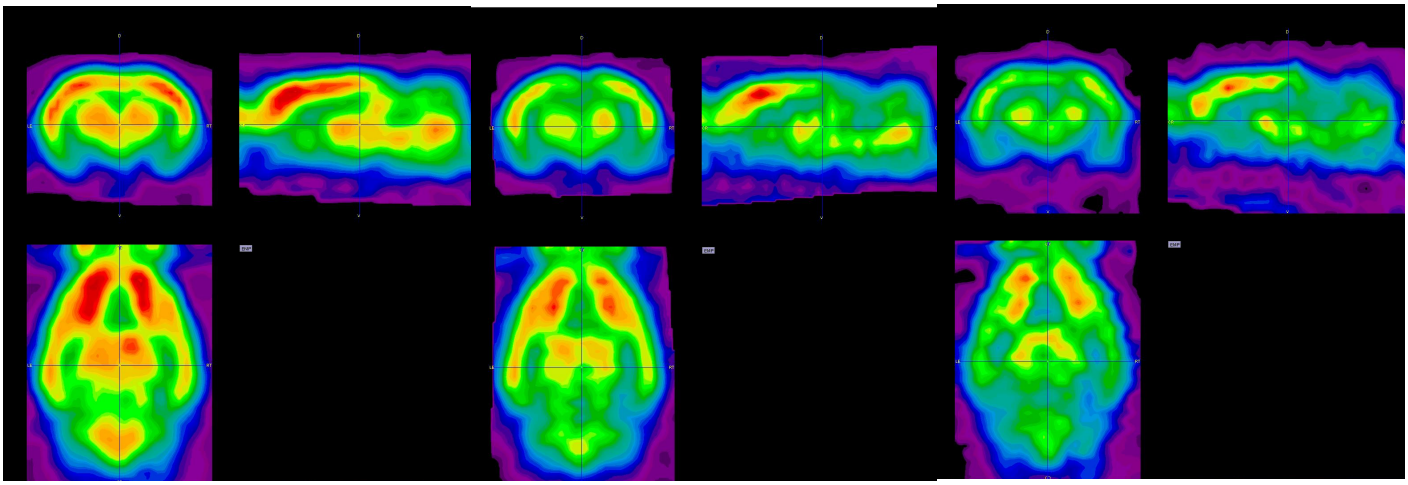
6.5 rat 5 baseline, acute and one week

Baseline acute one week



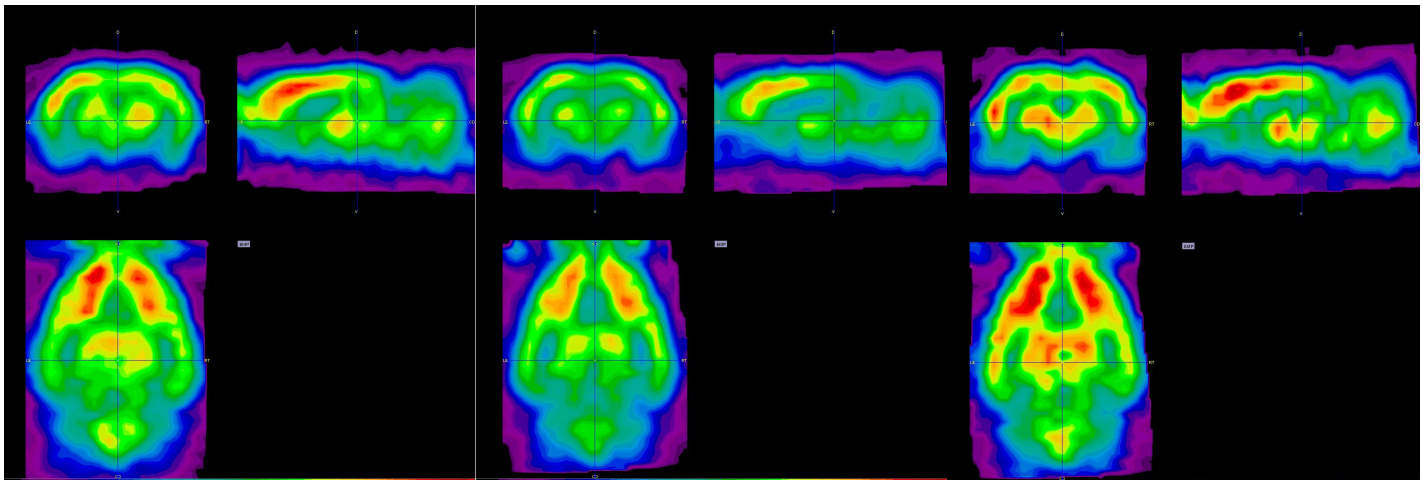
6.6 rat 6 baseline, acute and one week

Baseline acute one week



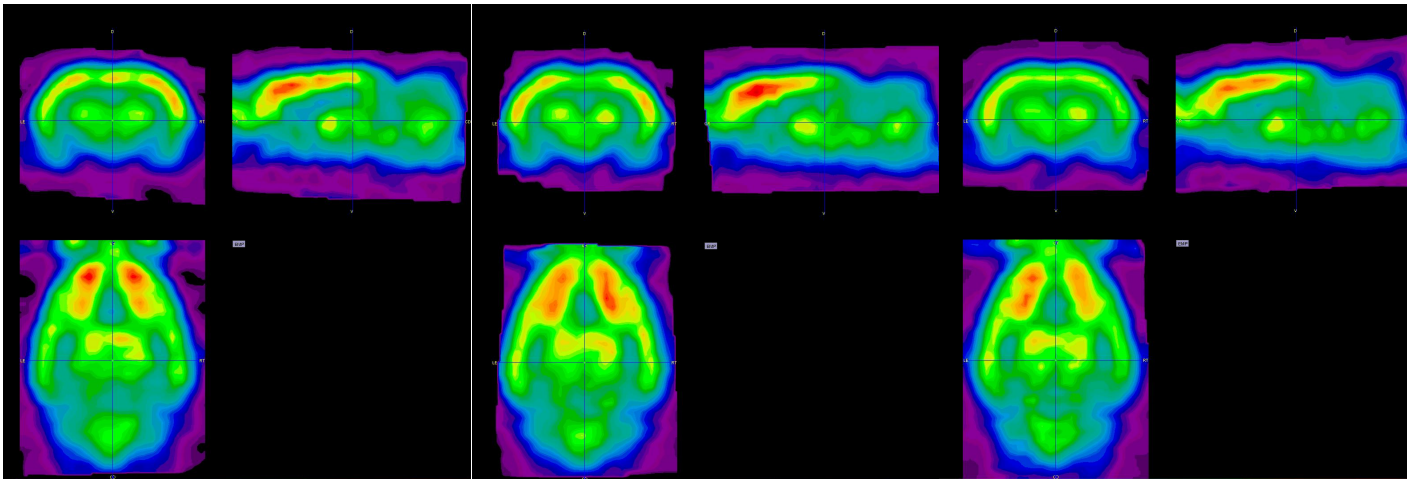
6.7 rat 7 baseline, acute and one week

Baseline acute one week



6.8 rat 8 baseline, acute and one week

Baseline acute one week



Annex 7- SUVwb data

mPFC	Baseline	Acute	One week
Rat 2	15.56188	16.34119	15.62487
Rat 3	15.8987	15.63726	15.77744
Rat 4	15.85287	16.38689	15.60789
Rat 5	14.99091	15.2189	15.37138
Rat 6	15.58482	15.76241	15.59571
Rat 7	15.87139	16.67425	15.97318
Rat 8	16.41984	15.94689	15.29006
Mean	15.7400592	15.9954007	15.6057876
SD	0.43480359	0.50357376	0.23077751
Striatum	**Baseline**	**Acute**	**One week**
Rat 2	15.11645	14.85893	14.9427
Rat 3	15.35951	14.95688	15.33884
Rat 4	15.25835	15.06453	15.25911
Rat 5	15.1721	14.54776	15.28829
Rat 6	15.29499	14.78086	15.04504
Rat 7	15.42617	14.87611	15.15793
Rat 8	15.33142	14.8545	14.45693
Mean	15.2798553	14.84851	15.0698345
SD	0.10756175	0.1603848	0.30444051
Thalamus	**Baseline**	**Acute**	**One week**
Rat 2	14.88395	14.96831	14.83049
Rat 3	15.11996	15.452	15.77548
Rat 4	15.02372	15.31134	15.43224
Rat 5	14.62924	15.48171	15.52525
Rat 6	15.09246	14.88012	15.59095
Rat 7	15.21275	14.99388	15.91646
Rat 8	15.08547	14.99797	14.4753
Mean	15.0067906	15.1550481	15.3637356
SD	0.19436746	0.25180311	0.52139488

Annex 8- power calculations

8.1. Formula 1 Post hoc power test

$$Power = \phi\left\{-z_{1-\frac{\alpha}{2}} + \frac{\Delta}{\sqrt{\frac{\sigma_1^2}{n_1} + \frac{\sigma_2^2}{n_2}}}\right\}$$

Here the critical value$\left(-z_{1-\frac{\alpha}{2}}\right)$ for the α(0.05) is 1.96

The σ are the variances in the means in the 2 groups

The Δ is the absolute difference in means.

The n is the number of test subjects (the sample size).

7.2. Formula 2 sample size calculation.

The following formula was used.

$$n_1 = \frac{\left(\sigma_1^2 + \frac{\sigma_2^2}{k}\right) * \left(z_{1-\frac{\alpha}{2}} + z_{1-\beta}\right)^2}{\Delta^2}$$

Here the sample ratio (k) is equal to 1 as repeated measure are done.

The significance level (α) is 0.05.

The power level (β) is set to 0.2.(equivalent 80% power)

The σ is the variance.

The Δ is the absolute difference in means.

Z is the critical value for the given significance and power level. At a significance level of 0.05 the $z_{1-\frac{\alpha}{2}}$ is 1.96 and with a power level of 0.2 the $z_{1-\beta}$ is 0.84

8.3. Actual calculation of sample size.

$$n_1 = \frac{\left(0.434803576^2 + \frac{0.50357375\text{^}2}{1}\right) * (1.96 + 0.84)^2}{(15.74005915 - 15.99540068)^2} = 53.22607799$$