Academic year 2012-2013

Faculty of Pharmaceutical, Biomedical and Veterinary Sciences
Educational Committee for Biomedical Sciences

MOLECULAR IMAGING OF PHARMACOLOGICAL MODULATION IN THE PREFRONTAL CORTEX AS A REFERENCE FOR rTMS

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Master dissertation submitted in completion of the degree of

Master in Biomedical Sciences: Neurosciences

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ABSTRACT

Repetitive transcranial magnetic stimulation (rTMS) is a non-invasive neurostimulation technique, which makes it possible to evoke an electrical field in the brain. This field can then induce a depolarization or hyperpolarization of neurons. By stimulating at a low frequency (≤1 Hz) it is commonly accepted that an inhibition can be induced. Stimulating at higher frequencies (>1 Hz) is mostly associated with an excitatory effect. Through propagation of the signal by interconnected networks, the effect can also indirectly influence other connected regions.

Research has already shown that rTMS has promising therapeutic effects for a variety of neurological diseases. In many of these diseases, the dorsolateral prefrontal cortex (dlPFC) plays a major role, making it often the target of an rTMS treatment. In certain circumstances rTMS is already being used to treat depression. A potential therapeutic benefit has also been suggested for other diseases such as addiction and schizophrenia. This has led to a substantial increase in studies investigating the therapy in the recent years. There is however still much uncertainty about the exact working mechanism, which leads to difficulties in improving protocols and developing new therapeutic applications. Because there are strict ethical legislations regarding clinical trials, animal research will have an important role in the clarification of this working mechanism. For this reason there is need for a protocol optimized for small animals, which approaches the human therapy as good as possible.

In this study the stimulation of the prelimbic cortex in the rat, the equivalent of the dlPFC in human, was investigated by a direct intracranial pharmacological intervention. By implanting a cannula in this region, a GABA-A receptor antagonist (bicuculline) or a GABA-A receptor agonist (muscimol) could be administered. Every rat received each treatment and functioned as its own control by administration of saline. After each injection the animals were scanned with a PET/CT-scanner. By administration of 1 mCi tracer (^{18}F-FDG) changes in glucose metabolism due to the administration of the (ant)agonist could be visualized. This allowed us to assess the network connectivity of the region and the focality of the stimulation. Bicuculline induced an overall increase in glucose metabolism, most clearly seen in the sensory regions and the regions associated with the memory. Muscimol administration decreased the glucose metabolism in the regions around the prelimbic cortex, the striatum and the thalamus. These results could be used as a golden standard for further rTMS studies focusing on this region.

By this study insight on the network connectivity and the effect of a stimulation of the prelimbic cortex in small animals was gained. We have also shown that it was possible to visualize these effects by PET-FDG. This study can thus function as a golden standard to further optimize rTMS in a small animal model for a great variety of neuropsychological and neurological diseases such as depression and addiction.

SAMENVATTING

Repetitieve transcraniële magnetische stimulatie (rTMS) is een niet-invasieve, neurostimulatietechniek die het mogelijk maakt om een elektrisch veld in de hersenen op te wekken. Dit veld kan op zijn beurt een depolarisatie of hyperpolarisatie van neuronen induceren. Door te stimuleren aan een lage frequentie (≤1 Hz) kan er een inhiberend effect geïnduceerd worden. Stimulatie met een hoge frequentie (>1 Hz) gaat meestal gepaard met een exciterend effect. Door propagatie van het signaal doorheen verbonden netwerken, kan het effect ook indirect in andere verbonden regio's worden waargenomen.

Er is reeds aangetoond dat rTMS veelbelovende therapeutische effecten heeft voor verschillende neurologische aandoeningen. In deze ziekten speelt de dorsolaterale prefrontale cortex meestal een grote rol, waardoor deze dikwijls ook het doelwit is van rTMS. Zo wordt depressie in bepaalde omstandigheden reeds met rTMS behandeld. Ook is er potentieel voor het gebruik ervan in behandelingen voor andere aandoeningen zoals verslaving en schizofrenie. Deze mogelijkheden hebben geleid tot een grote hoeveelheid onderzoek in de therapie gedurende de afgelopen jaren. Er is echter nog veel onzekerheid over het exacte werkingsmechanisme, wat leidt tot moeilijkheden in het verbeteren van protocols en het ontwikkelen van nieuwe therapeutische applicaties. Door de vele ethische beperkingen in klinische studies, zullen proefdieren een belangrijke rol spelen in de verduidelijking van dit werkingsmechanisme. Hiervoor is er nood aan een protocol dat geoptimaliseerd is voor proefdieren en de humane therapie zo juist mogelijk benadert.

In deze studie werd de stimulatie van de prelimbische cortex in de rat, het equivalent van de dlPFC in de mens, onderzocht door middel van een directe intracraniële farmacologische interventie. Door middel van een implantatie van een cannula in deze regio, kon een GABA-A receptor antagonist (bicuculline) of een GABA-A receptor agonist (muscimol) toegediend worden. Elke rat kreeg elke behandeling en diende als zijn eigen controle door administratie van saline. Na elke injectie werden de dieren gescand met een PET/CT-scanner. Door toediening van 1 mCi tracer (^{18}F-FDG), konden de veranderingen in het glucosemetabolisme door de stimulatie vastgelegd worden. Dit liet ons toe om uitspraken te doen over de netwerkconnectiviteit en de focaliteit van de stimulatie. Bicuculline induceerde over het algemeen een toename in glucose metabolisme, met name in de thalamus, het striatum, de sensorische regio's en de regio's geassocieerd met het geheugen. Muscimol toediening verlaagde het glucose metabolisme in de regio's rond de prelimbische cortex, het striatum en de thalamus. Deze resultaten kunnen dienen als gouden standaard voor verdere rTMS studies die zich richten op deze regio.

Dankzij deze studie kon een inzicht geleverd worden in de netwerkconnectiviteit en het effect van een stimulatie van de prelimbische cortex in kleine proefdieren. Verder werd getoond dat deze effecten ook gevisualiseerd konden worden door PET-FDG. Deze studie kan dus fungeren als een gouden standaard voor de verdere optimalisatie van rTMS in een proefdiermodel voor een grote variëteit aan neuropsychologische en neurologische ziekten zoals depressie en verslaving.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

^{18}F-FDG	:	2-deoxy-2-^{18}F-fluoro-β-D-glucose
A	:	Ampere
ACC	:	Anterior Cingulate Cortex
AP	:	Anteriorposterior
Bicu	:	Bicuculline
CT	:	Computed Tomography
DA	:	Dopamine
DBS	:	Deep Brain Stimulation
dlPFC	:	dorsolateral Prefrontal Cortex
DV	:	Dorsoventral
ECT	:	Electroconvulsive Therapy
EEC	:	European Ethics Committee
EEG	:	Electroencephalography
EMG	:	Electromyography
fMRI	:	Functional Magnetic Resonance Imaging
FOV	:	Field Of View
GABA	:	γ-Aminobutyric Acid
GPe	:	Globus Pallidus externa
GPi	:	Globus Pallidus interna
Hz	:	Hertz
IV	:	Intraveneous
KeV	:	Kiloelectronvolt
LTP	:	Long Term Potentiation
mCi	:	Millicurie
MDD	:	Major Depressive Disorder
MEP	:	Motor Evoked Potential
MICA	:	Molecular Imaging Center Antwerp
ML	:	Mediolateral
mPFC	:	Medial Prefrontal Cortex
MR	:	Magnetic Resonance
Musc	:	Muscimol
NAc	:	Nucleus Accumbens
PET	:	Positron Emission Tomography
PFC	:	Prefrontal Cortex
PL	:	Prelimbic
rTMS	:	repetitive Transcranial Magnetic Stimulation
SNr	:	Substantia Nigra pars reticulate
SPM	:	Statistical Parametric Mapping
STN	:	Subthalamic Nucleus
TES	:	Transcranial Electrical Stimulation
VOI	:	Volume of Interest
VTA	:	Ventral Tegmental Area

1) TRANSCRANIAL MAGNETIC STIMULATION

1.1 BACKGROUND

Transcranial Magnetic Stimulation (TMS) is a non-invasive neurostimulation technique making it possible to stimulate cortical tissue by inducing an electric field in the brain (FIGURE 1). Since it became available in the late 1980s, the technique has evolved from a simple tool for neurophysiologists to a novel method for neuromodulation (Kapogiannis and Wassermann, 2008). In contrast to transcranial electrical stimulation or TES, TMS is not painful to the person receiving the stimulation (Nitsche et al., 2008). General complaints mostly include very mild discomfort due to the direct stimulation on the skull, although these diminish rapidly in incidence after the first treatment week. Another source of discomfort is caused by the clicking sound originating from the stimulating coil. This however, is easily resolved by wearing earplugs (O'Reardon et al., 2007).

FIGURE 1: Transcranial Magnetic Stimulation on a patient. In this figure TMS is being applied to the brain of a patient by placing a figure-8-coil on the skull.

1.2 DIFFERENT TYPES OF rTMS

According to the pattern of the delivered pulses, different types of TMS can be distinguished. The term single-pulse TMS is used when the stimulus is applied just once. The first TMS systems used this technique to record motor evoked potentials (MEP's) in response to a cortical stimulation (Barker et al., 1985). The amplitude of this MEP can be seen as a measure of the excitation state of output cells in the motor cortex (Rothwell et al., 1991). This technique showed that TMS preferentially depolarizes axons running in the plane of the stimulating current (parallel to the plane of the coil). With paired-pulse stimulation, the stimuli are delivered in pairs within a variable interval by using two stimulators discharging through the same coil. This can be used to explore the effects of drugs and the correlates of genes and behavioural traits in cortical physiology (Kapogiannis and Wassermann, 2008).

When lasting inhibitory or facilitatory effects are desired, trains of stimuli are applied, which is called repetitive TMS (rTMS). Applying rTMS at a low frequency (≤ 1Hz) is hypothesized to achieve a synaptic depression because the incoming pulse arrives during the later inhibitory phase, which is produced by the previous pulse. Using higher frequencies (> 1Hz) could lead to a synaptic potentiation since this time the incoming pulse coincides with the depolarizing phase of the previous pulse (Reithler et al., 2011).

1.3 Therapeutic Potential of rTMS

Being able to non-invasively modulate brain activity in a specific cortico-subcortical network opens up a new variety of treatments for different neuropsychiatric conditions such as depression, bipolar disorders, schizophrenia and drug craving (Marangell et al., 2007; Wassermann and Zimmermann, 2012). Because there is no need for a post-surgery recovery period and a rapid evaluation is possible, other neurological diseases such as Parkinson's disease and epilepsy would possibly benefit from such a non-invasive technique (Fregni, 2005).

At the moment, rTMS has already proven its validity in the treatment of depression. Several studies have shown that stimulation of the left dorsolateral prefrontal cortex (dlPFC) by rTMS caused a reduction in depressive symptoms (Berman et al., 2000; George et al., 2000; Schutter, 2011), making it a valuable alternative tool to pharmacotherapy for the treatment of depression. When TMS is delivered over several hundreds of pulses within one session, these beneficial effects are observed beyond the stimulation period as after-effects. Moreover, when these sessions are repeated over several weeks, rTMS has been reported to induce long-lasting antidepressant effects, highlighting the therapeutic potential. Additionally, it has been suggested that rTMS may be able to enhance the action of antidepressant drugs, resulting in a faster or increased clinical response (Bortolomasi et al., 2007). Although the exact mechanism of the antidepressant action of rTMS has not been fully elucidated, it is believed that activation of the left dlPFC induces activation of the dopamine (DA) reward system and neuroplastic changes in connected limbic regions such as the subgenual cingulate cortex and amygdala (Nakamura, 2012).

Not only has rTMS already attained FDA approval for the treatment of depression, the list of disorders for which rTMS could be used, is growing steadily. An important potential therapeutic role of rTMS can be found in the treatment of drug addiction. Repeated exposure to addictive drugs produces long-lasting neuronal adaptations in reward-related areas such as the prefrontal cortex (PFC). This region has a major function in the regulation of the DA reward system by releasing this neurotransmitter as a response to rewarding experiences (Arias-Carrión and Pöppel, 2007). If the addictive drug-induced adaptations could be manipulated, causing a reduced response to drugs or drug-associated cues, the drug-associated behaviour may be less pronounced (Levy et al., 2007). A study has shown that dlPFC rTMS has this ability by modulating the DA release (Cho and Strafella, 2009). Also researchers studying tobacco addiction showed that dlPFC rTMS could influence the cortical excitability by modulating neurotransmitters as DA and GABA (gamma-Aminobutyric acid) (Addolorato et al., 2011). Another study researching the effect of high frequency dlPFC rTMS on cocaine-dependence showed a reduction in drug craving in patients, emphasizing the possible use of rTMS as a therapeutic tool for the treatment of drug addiction (Camprodon et al., 2007).

This great potential of rTMS highlights the need for an in-depth knowledge of the pathophysiology for each of the above described diseases as well as the mechanisms by which rTMS can induce plastic changes in the functioning of brain networks involved in these pathologies.

1.4 Technical Aspects of TMS

The technique itself harnesses Faraday's principles of electromagnetic induction (Pascual-Leone et al., 2000). By sending a varying electric current through the coil, a changing magnetic field is generated in the direct vicinity of the coil. When placed over the head, this variable magnetic field is able to penetrate the scalp, skull and meninges, to eventually induce an electric field in the brain (FIGURE 2). This causes a flow of ions, which alters the electric charge stored on both sides of the cell membranes. This causes neurons to depolarize or hyperpolarize (Haraldsson et al., 2004; Rossi et al., 2009). The standard apparatus consists of a wire coil that is encased in plastic. An electric cable attaches

the coil, which can be placed over a subject's head, to a stimulator with one or more large capacitors. These capacitors are charged by a power source, while the discharging happens through the coil when the device is triggered. Firing of neurons by induction requires a very strong and brief current pulse in the coil, because the strength of the induced magnetic field is proportional to the rate of change of the current through the coil. Since this places difficulties on stimulator design, rTMS became commercially available only decades after the introduction of the technique (Wassermann and Zimmermann, 2012).

Although the underlying principles are relatively simple and the passage of the magnetic field through the different tissues does not significantly affect it, the complex shape and variable conductivity of the cervical tissue greatly hinder an anatomically precise understanding of where TMS acts. Additionally, because induced current is divided among the infinite number of possible pathways according to their conductances, it will preferentially flow through areas containing cerebrospinal fluid, due to their high conductance. This means that the current may concentrate at points that are not positioned directly under the coil. Knowledge of the ultrastructure of the brain can increase our understanding of where and why TMS acts, since it has been shown that axons are very susceptible to stimulation at bends in their courses (Amassian et al., 1992).

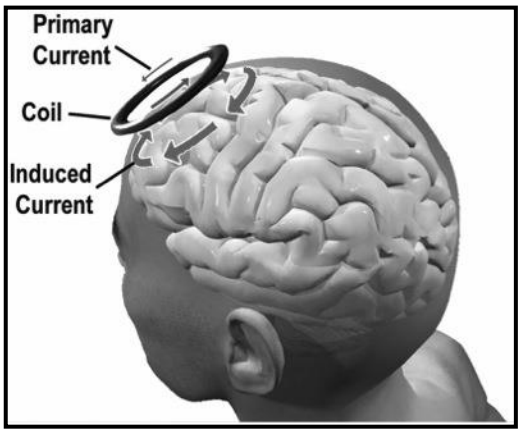


FIGURE 2: Generation of an electrical field in the brain
A varying current driven through a coil generates a fluctuating magnetic field around the coil. By directing this magnetic field to the brain an electrical field can be induced in the cortical tissue.

Source: The Oxford handbook of transcranial stimulation. Oxford University Press; Oxford; New York: 2007.

1.5 Parameters for rTMS

Optimal stimulation parameters such as the size of the coil, the frequency of pulsing of the magnetic field, the strength of the magnetic field generated or the duration of the electrical current are yet to be established. It is likely that these optimal parameters will vary depending on the stimulation target and the psychiatric or neurological application (Mozeg and Flak, 1999).

Depending on the parameters of stimulation, the resulting net effect i.e. cortical excitability or inhibition, can be altered by changing the rTMS stimulation frequency (Haraldsson et al., 2004; Fitzgerald et al., 2006). In general, it is believed that frequencies lower or equal to 1 Hz inhibit neuronal firing in a localized area, which is used to induce virtual brain lesions (Haraldsson et al., 2006). The term 'high-frequency rTMS' is used when frequencies larger than 1 Hz are applied (Rossi et al., 2009). High-frequency rTMS is believed to be excitatory in nature and might result in neuronal depolarization under the stimulating coil. Important is that the net effect of rTMS on the cortical excitability will be influenced by the basic excitability level, which might explain different neurobiological effects in similar stimulation conditions (Wing et al., 2012). Furthermore, several remote indirect effects, due to propagation of the delivered signal throughout the connected network, have been witnessed (Laird et al., 2008).

With regard to the design of the coil, different types of coils can be used to induce TMS stimuli. Depending on the stimulation intensity, they are able to modulate neurons at a depth of 1.5 to 3.0 cm

beneath the scalp. The most common types of coils are the figure-8-coil, producing a more focal and shallower stimulation, and the circular coil, which is used for the stimulation of deeper brain regions due to its larger diameter (Rossi et al., 2009).

1.6 Repetitive Transcranial Magnetic Stimulation in Small Animals

The promising therapeutic effects in various disorders and the non-invasiveness of rTMS, have led to a vast amount of research and clinical interest in the therapy over the recent years. However, despite this substantial amount of research investigating the efficacy of rTMS, much work still needs to be done in unravelling its mechanism of action, optimization of protocols and the development of new therapeutic applications (Haraldsson et al., 2006; Martin et al., 2003). Because of the ethical limitations in human testing and the necessity for large and homogeneous patient populations, animal models play and will continue to play a major role in the clarification of the working mechanism behind rTMS and the development and standardisation of therapeutic treatments.

To this day, rTMS is administered to animals by the use of several different types of magnetic coils, of which the most common are the circular and figure-8-coil. All commercially available coils are designed for human use, and thus outlimit the dimensions of the animal's head (Tischler et al., 2011). Furthermore, human-oriented coils have a slower decay over space, affecting deeper structures in the smaller animal's brains due to the difference in the ratio of rTMS coil size to head size (FIGURE 3). This severely hampers the focality of the stimulation in animals and thus results in different response mechanisms than those in humans (Tischler et al., 2011). Although the use of a smaller coil limits the current going through the coil (and thus the field strength) due to overheating of the wires (Vahabzadeh-Hagh et al., 2012), the development of miniaturized coils and stimulators specifically designed for small animals, would allow a more focal stimulation. An experimental setup has already been proposed (Wyckhuys et al., 2012) to investigate different coil sizes, shapes and orientations in order to achieve a more focal stimulation.

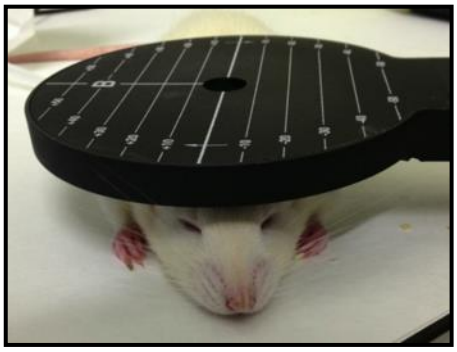


FIGURE 3: A human coil positioned on a rat head. Human coils are specifically designed for humans. Applying these types of coils to the rat head results in a significant loss of focality, making it impossible to target a specific region.

Because the dlPFC plays a major role in conditions such as depression and addiction (Qi et al., 2012; Wing et al., 2012) and is the most common target for rTMS in patients, it would be interesting to stimulate this brain region in animals. Although the dlPFC is unique in primates, the functional and anatomical properties attributed to the dlPFC can also be found in rats in the prelimbic (PL) region (Uylings et al., 2003). Therefore, the PL cortex does provide a useful analogue for aspects of the human dlPFC, making the rat a good model to investigate the working mechanism and beneficial effects of rTMS.

To direct the rTMS-treatment to the PL cortex of a rat, it is crucial that the animal's head is immobilized during the procedure. Since experimenters are not able to control volitional motor activity in rats, they have to use a fixed stereotactic frame combined with an anaesthetic to limit the discomfort. The use of sedatives however, may mask the neurophysiological response induced, since it appears that rTMS effects are strongly dependent on the brain state at the time of stimulation (Vahabzadeh-Hagh et al., 2012). To achieve an accurate and reproducible positioning of the coil without the use of anaesthesia, a restrainer can be used. This procedure does however require behavioural training to minimize stress.

1.7 Other Neurostimulation Techniques

Except for rTMS, other techniques are readily available to induce electric currents into the brain. One other example, currently being used to activate or inactivate certain brain areas, is Deep Brain Stimulation (DBS). In this technique, microelectrodes are implanted into the target brain area by stereotactic techniques to deliver electric pulses into the surrounding tissue. This makes it possible to stimulate these areas much more focally compared to rTMS, which targets a more superficial, larger area. Another difference between rTMS and DBS is the higher frequency rate of DBS (low frequency: 20-60 Hz; high frequency: >100 Hz) at which the pulses are delivered (Goddard et al., 1969).

DBS is currently being used in Parkinson's disease, essential tremor and dystonia (Blomstedt and Hariz, 2010) although the exact neurobiological mechanisms of the modulatory effects of DBS are not yet fully understood. A few hypotheses have been brought forward, including the depolarisation blockade of current dependent ion channels (Beurrier et al., 2001), exhaustion of the neurotransmitter pool (Zucker et al., 2002), synaptic inhibition (Dostrovsky et al., 2000) and the neural activation of certain stimulated areas (Jech et al., 2001). In most psychiatric illnesses, there are several brain structures that presumably play different roles in the development and the maintenance of symptoms instead of a single pathological structure. This results in problems for target selection of a focal technique, like DBS, since the net-effect is highly dependent on the target and the different stimulation parameters. Moreover, because of the invasiveness of the technique, there are also substantial risks like bleeding and infection that can occur (Schläpfer and Bewernick, 2009). These features cause a controversy in the use of DBS for neuropsychiatric and neuropsychological diseases when there are non-invasive alternatives, like rTMS, available.

Another method often done in small animals to stimulate or inhibit a brain region is the direct intracranial pharmacological injection of drugs, by means of a cannula. This focal administration is regarded as a golden standard in animal research to induce focal neuronal inhibition or excitation. Numerous studies have been conducted using this technique to investigate several neuropsychiatric and neurological diseases. Slattery et al. (2011) revealed the positive effect on depressive behavior when the infralimbic region of the mPFC was inactivated by injection of a GABA-A agonist, muscimol. Gilmartin et al. (2012) managed to inactivate the PL cortex with muscimol to investigate its role in trace and contextual fear conditioning, revealing an impaired functioning after a bilateral microinjection. Similarly, neuronal activation can be achieved by using a GABA-A antagonist, such as bicuculline. This approach has shown that the PL region is also thought to modulate neuroendocrine responses to psychogenic and systemic stressors (Jones et al., 2011b).

Finally, electroconvulsive therapy (ECT) is a method used in humans to induce seizures electrically to achieve a therapeutic effect. To this date, ECT is the most successful treatment in severe depression and schizophrenia (Perrin et al., 2012; Fink, 2001). However, despite the clinical efficacy, sever problems remain, such as the high relapse rates and considerable side-effects (Hoy and Fitzgerald, 2010). The exact mechanism of action is not yet fully revealed (Bolwig, 2011).

1.8 Neuro-Imaging

A growing amount of evidence from neuropsychological, neurophysiological and neuroimaging studies indicate that the interactions between brain regions underlie cognitive processing and determine behaviour. Defining these network interactions is thus crucial to understand cognition, brain disorders and brain reorganization. The effects of rTMS are not restricted to the area of stimulation, but can also be detected at a distance through connected sites within the same functional circuit (Siebner, 2003). Neuro-imaging makes it possible to identify these integrated circuits (Thut and Pascual-Leone, 2010).

To directly record the electric neural activity, TMS can be combined with Electroencephalography (EEG) (Reithler et al., 2011). Because of the high temporal resolution of this technique the direct consequences of TMS can be separated from adaptive responses in the distributed networks (Thut and Pascual-Leone, 2010).

Repetitive TMS can also be combined with functional Magnetic Resonance Imaging (fMRI), providing unique insights into causal interactions between brain regions. This allows visualisation of the spatial topography of local and remote rTMS effects and how these effects vary with several psychological factors (Bestmann et al., 2008).

By combining TMS with Positron Emission Tomography (PET) imaging, activation maps can be constructed (Reithler et al., 2011). PET is a nuclear medicine imaging technique that produces a three-dimensional image representing functional processes in the body. It is particularly useful as an objective tool to understand the state of functional connectivity without the requirement of the subject to engage in any specific behaviour (Paus et al., 1997). The technique requires biomolecules to be labelled with a positron-emitting isotope. The emitted positron will annihilate with an electron, resulting in the production of two photons with 511 KeV 180 degrees apart. The PET camera then detects these photons (FIGURE 4). The most commonly used biologically active molecule for PET is 2-deoxy-2-^{18}F-fluoro-β-D-glucose (^{18}F-FDG), which is an analogue for glucose. Following introduction of ^{18}F-FDG in the body, glucose-using cells in the brain easily take it up by the activity of glucose transporters. However, in contrast to glucose, the metabolite misses its 2'-hydroxyl group, which is needed for further glycolysis. The phosphorylation prevents that the molecule is being released resulting in an entrapment of the molecule inside the cell. By analysing the ^{18}F-FDG PET images, it is possible to determine the concentration of tracer that accumulated in the body, reflecting the metabolic activity of the tissue (Alauddin, 2012). Furthermore it has previously been shown that the majority of cortical energy production supports functional glutamatergic neuronal activity, making it thus an indirect measurement for neuronal activity (Sibson et al., 1998).

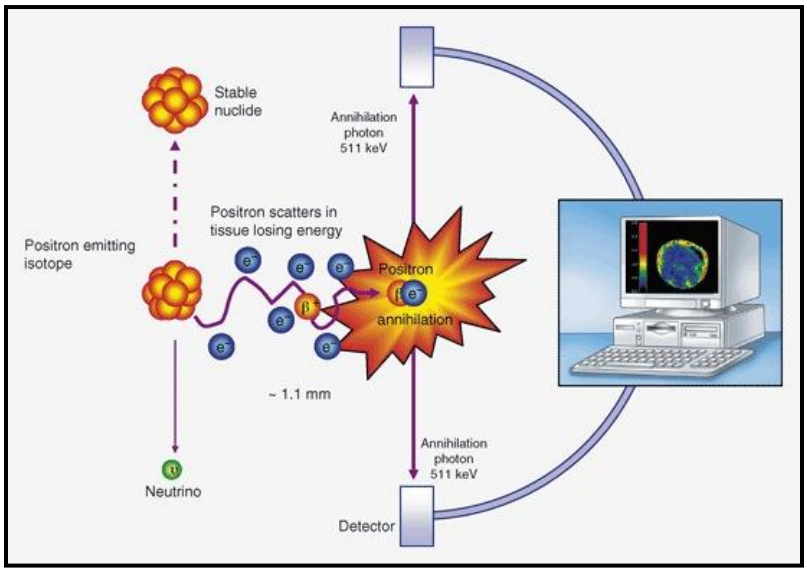


FIGURE 4: Annihilation reaction of a positron emitted by an isotope and an electron in a PET scan.
A tracer containing a radioactive isotope is intravenously injected. The isotope emits a positron that will collide with an electron in an annihilation reaction. This then results in the production of two photons with 511 KeV 180 degrees apart. The PET camera is then able to detect these photons and send the information to a computer to reconstruct the images displaying the uptake of the tracer in the different regions.

Source: quantumtunnel.wordpress.com

2) TARGET REGION

2.1 PREFRONTAL CORTEX

In mammals, the PFC functions as the brain's executive center, synthesizing information from a wide range of brain systems (FIGURE 5), taking part in motor planning, organization, decision making, complex cognitive behavior, the processing of stressful information and episodic memory retrieval (Miller et al., 2002; Jones et al., 2011a). Functional imaging has already demonstrated that a great variety of these tasks can activate the dlPFC (Duncan and Owen, 2000), further highlighting its role in these activities. A dysfunction in this region is believed to be involved in a variety of pathologies such as depression, antisocial behavior, post-traumatic stress disorder, schizophrenia and bipolar disorder (Qi et al., 2012); (Rektorova et al., 2007; Wing et al., 2012; Yang and Raine, 2009). Previous research has already shown that the PL cortex of rats, the analogue of the human dlPFC, is connected to a great variety of brain regions, including the hypothalamus, the thalamus and the regulatory regions for the limbic system (including the amygdala and the hippocampus) (Vertes, 2004). Targeting this region with several neurostimulation techniques provided further evidence for this theory.

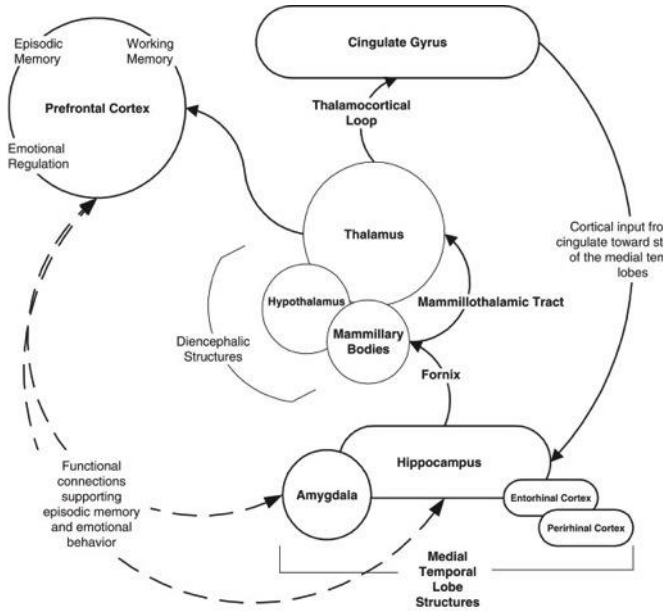


FIGURE 5: The prefrontal cortex functions as the brain executive.
The PFC synthesizes information from a wide variety of different brain systems and brain areas such as the limbic system the hippocampal associated regions. It plays a major role in an equal wide variety of functions such as motor planning, organization, decision-making and episodic memory.

Source: *Handbook of Behavioral Neuroscience. Elsevier Science; Amsterdam 2013.*

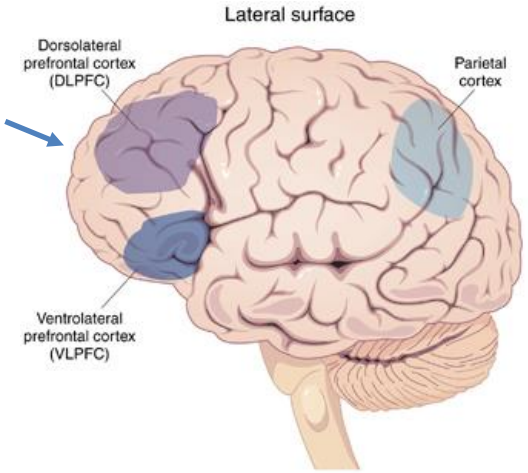


FIGURE 6: Location of the prefrontal cortex.
The PFC, containing the dlPFC, is marked by a blue arrow.

Source: *nature.com (Neuropsychopharmacology, 2010)*

2.2 PFC Related Diseases

2.2.1 Depression

Major depressive disorder (MDD) is a common, recurrent and disabling disease. According to a study conducted by the World Health Organization in 2008, it is the third leading cause of global disease burden and a leading cause of disability worldwide (World Health Organization, 2008). It is estimated that 16.6% of individuals in the United States will meet the criteria for MDD at least once in their life (Kesser et al., 2005). Furthermore it is estimated that the work-related costs in the United States linked to depression exceed 50 billion dollars each year (Greenberg et al., 2003). When pharmacological treatments are no longer effective or if the depression becomes life threatening, the most effective treatment is ECT. However, despite widespread use, there are concerns about significant cognitive side effects (UK ECT Review Group, 2003).

The disease itself probably originates from a dysfunction in the PFC, resulting in a disturbance in the regulation of stress hormone secretion due to endogenous or exogenous stressors, rather than a deficit in a single gene, brain region or neurotransmitter system (Mayberg et al., 2005).

A variety of studies have shown that patients with unipolar depression show prefrontal abnormalities, predominantly in the left hemisphere (Drevets et al., 2008). Also reports on decreased neuronal activities in the prefrontal regions and in the rostral anterior cingulate cortex (ACC), closely connected to the dlPFC are common in depression. It are these frontal hypoactivities (FIGURE 6) which result in the apathy, psychomotor slowness and impaired executive function, associated with depression (Baeken and de Raedt, 2011).

It is estimated that 20% to 40% of patients with MDD do not benefit sufficiently from treatments with antidepressants or psychotherapy (O'Reardon et al., 2007). This substantial proportion of patients manifests a chronic, treatment-resistant course of illness. Furthermore is the likelihood for a complete recovery even lower. Researchers have shown that only one in three patients achieve remission (Trivedi et al., 2006). Furthermore it is estimated that 40% of the patients achieving remission will relapse within 2 years (Boland and Keller, 2002).

2.2.2 Addiction

Addiction is characterized by a relapsing cycle of intoxication, binging, withdrawal and craving eventually resulting in excessive drug use, despite severe adverse consequences (Koob and Volkow, 2009). It is estimated that between 149 million and 271 million people worldwide used an illicit drug at least once (data for 2009), which is likely to be an underestimation still (Degenhardt et al., 2012). Another national survey estimated that there were 22.5 million Americans that were illegal drug users in 2011. Additionally, alcohol, not being an illegal drug, is not included in these numbers, although it is estimated that more than 50% of Americans older than 12 are alcohol users. It is also noted that illegal drug use is often associated with significant alcohol usage (Department of health and human services, 2012). Accordingly, the amount of people affected by addiction is thus enormous. Furthermore, the societal costs associated with addiction are substantial, reaching over 500 billion dollars (Surgeon's General Report, 2004; ONDCP, 2004; Harwood et al., 2000).

Many of the drugs abused by humans, increase DA in the reward circuit. This is believed to underlie the rewarding effects they induce. Some drugs of abuse can induce a release of 2 to 10 times as much DA as a natural reward. In some cases, the rewarding daze can also last much longer. The powerful DA surge resulting from such drugs strongly motivates people to take the drug again. Another effect of these strong increases in DA is that the brain adjusts itself by producing less DA or reducing the number of DA receptors (Volkow et al., 2001) (FIGURE 7). This results in an under-expressing reward

circuit causing the user to become insensitive for natural rewards and in need of higher dosages of drugs.

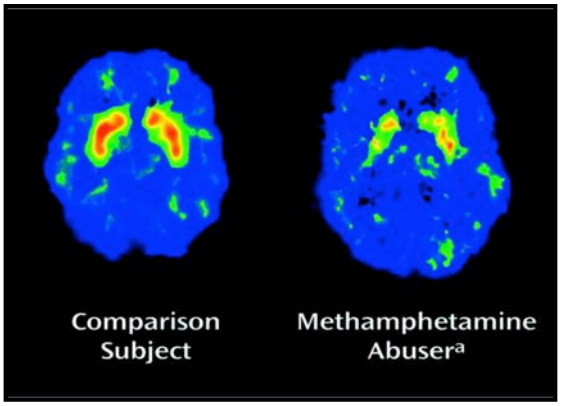


FIGURE 7: Striatal distribution volume of the DA transporter ligand [11C] d-threo-Methylphenidate of a healthy patient and a methamphetamine abuser.
It can clearly be seen that there is a significant decrease in signal when comparing the brain of a healthy patient (left) to the brain of a methamphetamine abuser (right).

Source: *Volkow et al. (2001)*

Nearly all of the dopaminergic cells reside in the ventral part of the mesencephalon. Several pathways make up the dopaminergic system: the mesolimbic pathway, the mesocortical pathway, the nigrostriatal pathway and the tuberoinfindubular pathway (FIGURE 8). The mesolimbic pathway includes the ventral tegmental area (VTA) that projects mainly to the nucleus accumbens (NAc) as well as the olfactory tubercle innervating the septum, hippocampus and amygdala. The mesocortical pathway on the other hand is built out of the fibers running from the VTA to the prefrontal, cingulate and perirhinal cortex. This total circuit is referred to as the mesocorticolimbic system (Arias-Carrión and Pöppel, 2007). The nigrostriatal pathway transmits DA from the substantia nigra to the striatum and is strongly associated with movement. The tuberoinfundibular pathway delivers DA coming from the hypothalamus to the pituitary gland and thereby influences the secretion of hormones.

Much of the studies that have been done investigating this increase in DA focused on the midbrain DA producing areas like the area VTA and the substantia nigra. Other studies investigated the closely associated basal ganglia structures, such as the ventral and dorsal striatum (Everitt et al., 2001; Wise, 1996). However, it has been brought to light that the PFC plays an important role in addiction and DA release as well (Taber and Fibiger, 1995; Volkow and Fowler, 2000). A hypothesis that has been brought forward is that disrupted functioning of the PFC leads to an impaired response inhibition and craving, causing an excessive desire to the drug and drug-related cues and a decreased sensitivity to non-drug reinforcers. Furthermore there is a decreased ability to inhibit maladaptive behaviors. These aspects cause drug seeking and drug taking to become a main motivational drive, even occurring at the expense of other activities while going through extreme measures in order to obtain drugs (Goldstein and Volkow, 2011).

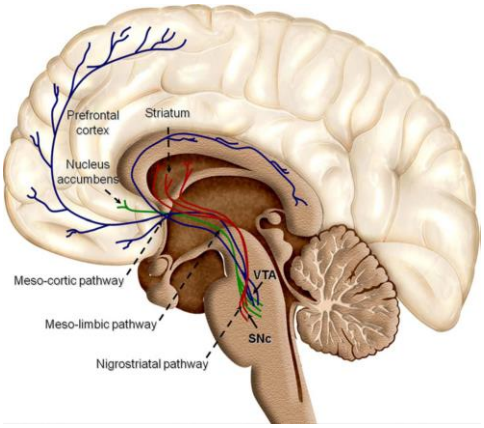


FIGURE 8: Dopaminergic pathways in the brain.
The mesolimbic pathway consists of projections of the VTA to the nucleus accumbens and olfactory tubercle. The mesocortical pathway is built out of fibers from the VTA that are running towards the prefrontal, cingulate and perirhinal cortex. The nigrostriatal pathway connects the substantia nigra with the striatum while the tuberoinfundibular pathway transmits DA from the hypothalamus to the pituitary gland.

Source: Arias-Carrion et al., 2010

2.2.3 Schizophrenia

Another disease with a significant role of the PFC is schizophrenia. In this neurodevelopmental disease both genetic and environmental factors play a role although the exact etiology currently remains unknown. Several of the risk factors include hypoxia, prenatal infection, season of birth, drug abuse and migration. A current hypothesis is that this gene-environmental interaction might intervene with epigenetic alterations such as DNA methylation and histone acetylation. Additionally GABA and glutamate alterations possibly play a role as well (Schmitt et al., 2011).

Studies using brain-imaging technologies to examine functional differences in brain activity have already shown differences to occur in the frontal region, hippocampus and temporal lobes (Laureys, 2005). For example, animal models with developmental hippocampal lesions, known to be associated with schizophrenia, are causing disconnectivity of the PFC (Schmitt et al., 2011). Moreover, fMRI studies in patients have revealed a decrease in effective connectivity between the posterior hippocampus and the PFC during working memory tasks (Henseler et al., 2010).

3) GOALS

This study will focus on the visualization of the effects on the glucose metabolism of an intracranial pharmacological intervention in the PL region of a rat by using PET-FDG. By administrating bicuculline (GABA-A antagonist) and muscimol (GABA-A agonist) directly into this region, we hope to respectively activate or inhibit. By combining this direct injection with PET imaging using ^{18}F-FDG, we will be able to visualize changes in glucose metabolism, giving us an insight in the network connectivity and the effects of modulating the activity of the mPFC. The goal is to acquire results that can function as a golden standard for a novel, non-invasive technique, rTMS, thereby facilitating future research exploiting this technique as a therapeutic tool for these conditions.

4) Material and Methods

4.1 Determination of Stereotactic Coordinates

To be able to stimulate the PL cortex, the coordinates for stereotactic placement of the cannula needed to be determined.

4.1.1 Animals

Male Sprague-Dawley rats (n=3, 250-300g) were housed individually, in a temperature (20-23 °C) and humidity-controlled room (50-55%), with food and water available *ad libitum*. They received a total of 12 hours light and 12 hours dark. All animals were treated according to guidelines approved by the European Ethics Committee (86/609/EEC). The study protocol was approved by the Ethical Committee for Animal Experiments (2012-50).

4.1.2 Experimental procedure

One week prior to the placement of the guide cannula, the animals were handled every day for a few minutes to acclimate to the laboratory environment. After this period they were implanted with a cannula into the PL region by means of stereotactic placement. With an internal cannula, fountain pen ink was administered to determine the correctness of the stereotactic coordinates. When the ink administration was done, slices of the brain were made to determine the location of the cannula.

4.1.3 Cannula placement

First, the animals received an induction dose of isoflurane 5% by placing them into an induction box. After achieving unconsciousness the animals were positioned onto a stereotactic frame where they were placed on a heat pad to maintain their body temperature. Two earbars were inserted in the ears to make sure the animal was firmly fixed into the frame (FIGURE 9). After this, an analgesic was given subcutaneous in the neck region (0.05 mg/kg Temgesic). The dose of isoflurane was lowered to 2%, while the breathing rate of the animal was closely monitored. If necessary, the dose was adjusted before continuing the procedure.

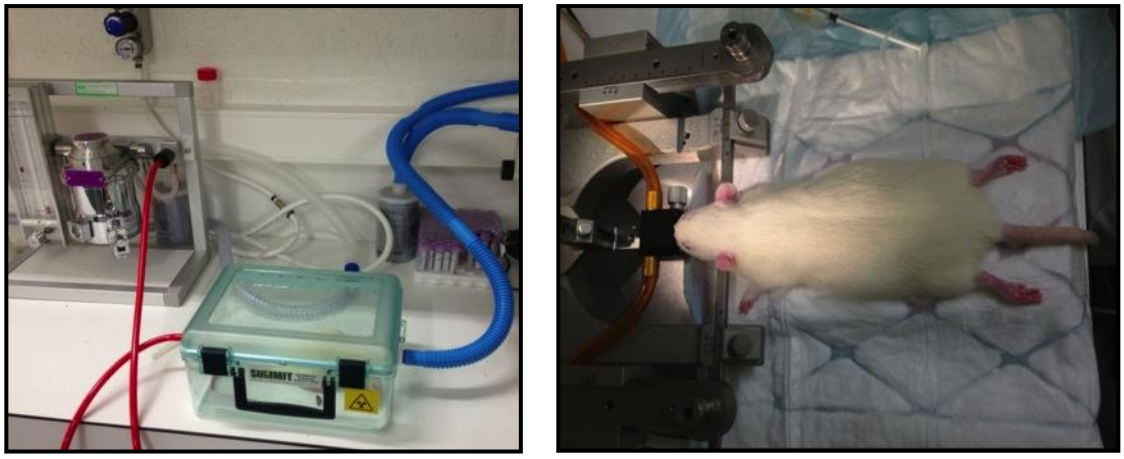


FIGURE 9: Anaesthetizing the animal.
The animals were placed into an induction box where they received a dose of 5 % isoflurane until they achieved complete unconsciousness. After this they were transported to the stereotactic frame where the dose of isoflurane was lowered to 2%.

After these initial steps a sagittal incision following the superior sagittal suture was made along the skull. The intersection of the coronal suture with the perpendicular sagittal suture is called bregma

(FIGURE 10c) and functioned as a reference point for stereotactic implantation. With the help of a stereotactical device, the double-barrel guide cannulae was implanted into the PL cortex (FIGURE 10a) at an angle of 22°, to avoid the rupture of the sagittal sinus (FIGURE 10b). The coordinates that were used were calculated on the anterioposterior axis (AP), the mediolateral axis (ML) and the dorsoventral axis (DV), relative to the position of bregma. These 3 axes allowed a precise three-dimensional placement of the cannula. To insert the cannula in the PL cortex the following coordinates were chosen: AP +0.37 mm; ML +0.20 mm; DV -0.50 mm (Paxinos et al., 2007).

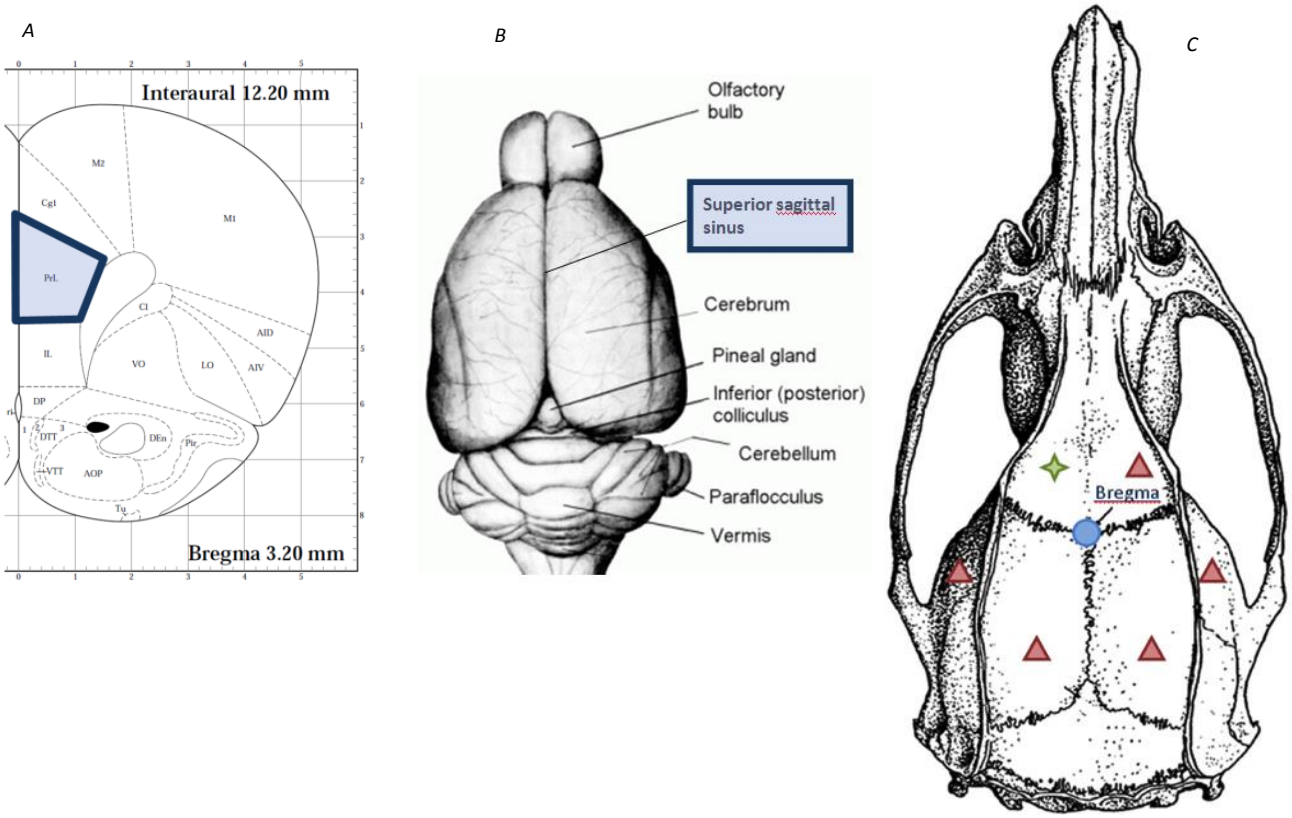


FIGURE 10A: A coronal slice of the left hemisphere of the brain from a rat

The blue box marks the location of the PL cortex where the cannula will be placed Paxinos et al., 2007).

FIGURE 10B: A dorsal view of the rat brain

The line near the blue box marks the location of the Superior Sagittal sinus. The rupture of this sinus was avoided to prevent excessive blood loss (adapted from Anatomical Foundations of Neuroscience).

FIGURE 10C: A dorsal view of the skull of the rat

The blue dot marks the location of bregma. The red triangles mark the position of the 5 screws. Finally, the green star marks the place where the cannula was inserted during the operation (adapted from Paxinos et al., 2007).

By drilling 5 additional holes with a diameter of 1 mm into the skull, 5 small screws were inserted as shown in FIGURE 10c. With the help of dental cement the guide cannula was secured into the skull (FIGURE 11).

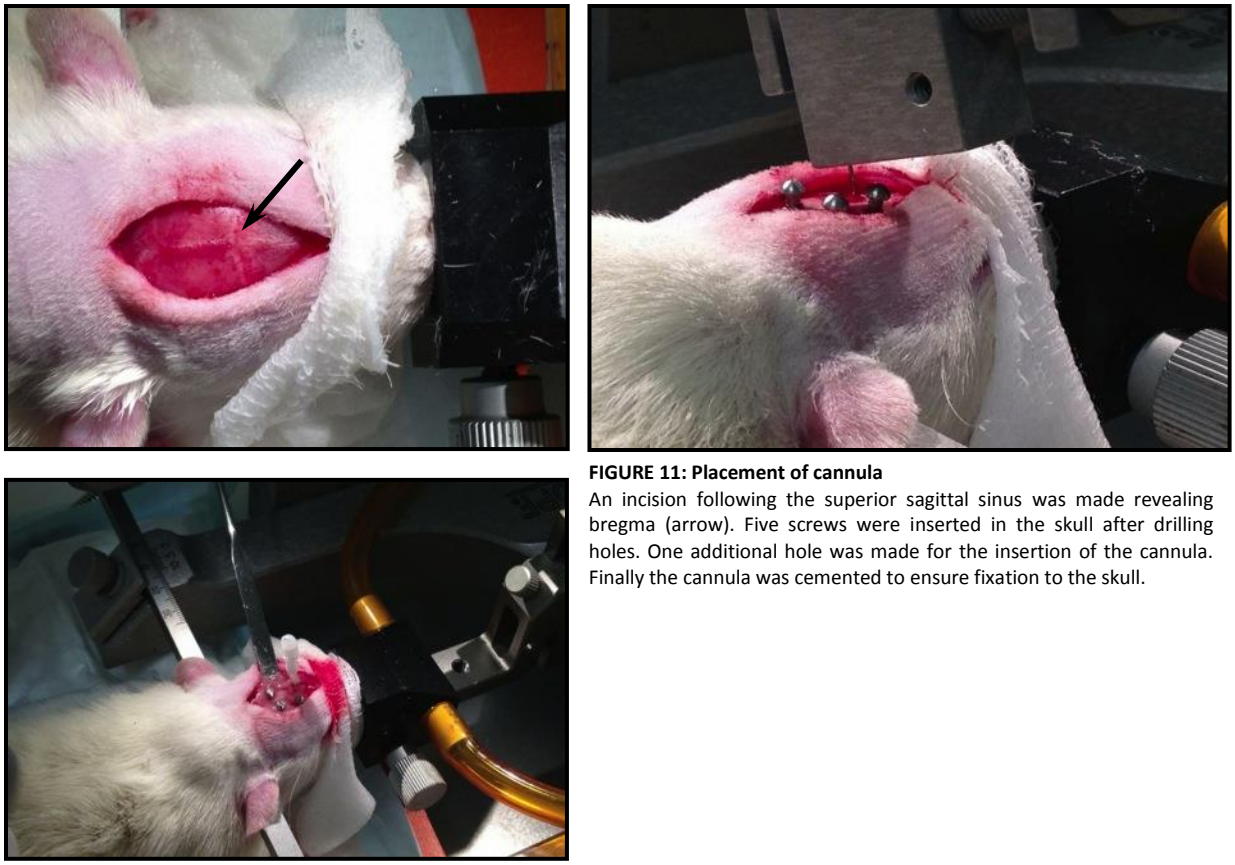


FIGURE 11: Placement of cannula
An incision following the superior sagittal sinus was made revealing bregma (arrow). Five screws were inserted in the skull after drilling holes. One additional hole was made for the insertion of the cannula. Finally the cannula was cemented to ensure fixation to the skull.

4.1.4 Ink injection

Fountain pen ink (0.5 µL) was injected via an internal cannula at a rate of 0.5 µL/min with a microsyringe pump and a Hamilton syringe, to check whether the coordinates of the stereotactical placed cannulae were correct (Jones et al., 2011b). The internal cannula, which extends 1 mm beyond the tip of the guide cannula, had to be left in place for an additional minute to allow diffusion of the solution before removal of the guide cannula (Doherty and Gratton, 1999). After this, the animal was sacrificed by an intraperitoneally administered overdose of Nembutal (150 mg/kg). Next, the brains were removed, snapfrozen by liquid nitrogen using isopentane (2-methyl-butane, Sigma-Aldrich Co. LLC ReagentPlus) and stored at -20 °C.

4.1.5 Microtome slices

The next day, the brains were placed into a microtome (Leica CM 1950) where 30 µm slices were made to determine the injection place. This location was visible due to the blue colour of the fountain pen ink. The coordinates were assessed and since the blue stain was too deep, the coordinates were adjusted to AP +0.37 mm; ML +0.20 mm; DV -0.40 mm. The protocol was repeated as described above and it was concluded that these new coordinates would be used for the further progress in the experiment.

4.2 Pharmacological Administration

4.2.1 Animals

Male Sprague-Dawley rats (n=12, 250-300g) were housed individually, in a temperature (20-23 °C) and humidity-controlled room (50-55%), with food and water available *ad libitum*. They received a total of 12 hours light and 12 hours dark. All animals were treated according to guidelines approved by the European Ethics Committee (86/609/EEC). The study protocol was approved by the Ethical Committee for Animal Experiments (2012-50).

4.2.2 Experimental procedure

One week prior to the placement of the cannula, the animals were handled every day for a few minutes to acclimate to the laboratory environment and manipulation. Following implantation, the animals were allowed to recover for one week prior to a habituation period (10 days).

On the test days, 0.5 µL of saline, bicuculline or muscimol, was administered to each rat. To allow uptake of the solution by the brain, 10 minutes was waited before 1mCi of the ^{18}F-FDG was intravenously injected while the rats were awake. To be positioned on the µPET/CT scan, the animal was anaesthetized by isoflurane.

To ensure the washout of the activity and the drug, meanwhile allowing for a fasting duration of at least twelve hours, a minimum of 48 hours was maintained between different scans. The order at which the rats were subjected to saline, bicuculline or muscimol, was randomized. Each rat thus acted as its own control.

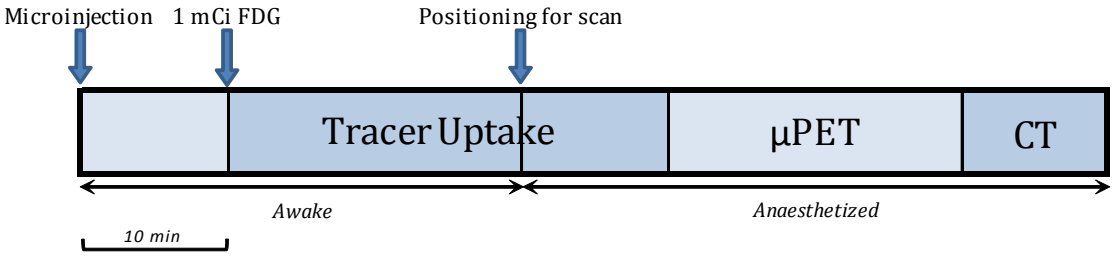


FIGURE 12: Scan Session Protocol Pharmacological Experiment

10 minutes after the injection of the drug or saline solution, the ^{18}F-FDG-tracer will be injected intravenously. 10 minutes before the start of the µPET-CT-scan the animal is anaesthetized and placed on the thermostatically heated bed of the scanner. This results in a total tracer uptake time of 30 minutes. The total duration of the scan is 30 minutes.

4.2.3 Cannula placement

After optimisation of the coordinates, the implantation was performed using the optimized coordinates according to the protocol described previously (2.1.1.3).

4.2.4 Habituation period

Following the recovery period (1 week) there was a habituation period (10 days). The following table (TABLE 1) describes the several steps that were taken in the 10-day habituation period to adjust the animals to the handling that needed to be done during the pharmacological administrations.

During this period the animals were getting used to the operator's handling of the cannula and manual immobilization of their head. This was necessary because the rats would not be allowed to move for 2 minutes when the compounds were being administered via the cannula. The handling gradually evolved from simply holding the rats, to eventually keeping their head still for 2 minutes under light

manual restraint, so the guide cannula could be opened and the internal cannula could be inserted. Also the pump used to drive the solution into the brain was turned on to accustom the animals to the sound and prevent stress when the administrations started.

Table 1: Scheme habituation period

Day 1	Taken out of cage; Placed on table 5 times	/
Day 2	See previous; Head was held gently	Briefly
Day 3	See previous	10 seconds
Day 4	See previous; Cannula screwed loose	10 seconds
Day 5	See previous; Exposed to sound of pump	10 seconds
Day 6	See previous	20 seconds
Day 7	See previous	40 seconds
Day 8	See previous	1 minute
Day 9	See previous	1.5 minutes
Day 10	See previous; Cannula inserted	2 minutes

On the first day of this period the animals were taken out of their cage for 5 minutes during which they were placed 5 times on the table for a few seconds. On day 2 the same procedure was followed but this time the head was held gently as well. On day 3 the duration was increased to 10 seconds. On day 4 the cannula was opened for the first time, which was followed with the sound of the pump on day 5. After this the procedure was repeated as on day 5, but the duration was increased each day from 20 seconds, to 40, to 60 to 90 and eventually 120 seconds. On the last day the internal cannula was inserted for the first time after the removal of the dummy cannula.

4.2.5 Microinjections

On the test days, the dummy cannula was removed and replaced by an internal injector cannula under light manual restraint. Because of the habituation period preceding the pharmacological administrations, the animals were used to these handlings and did not show any signs of stress or struggling. The wound on the head healed nicely, and no signs of pain or discomfort were noticed (FIGURE 13).

FIGURE 13: Rat after recovery period After the recovery period, the rats were fully restored and the wound on their heads closed.

The drug solutions were prepared on the day before the injection would take place. They consisted out of muscimol (1 mg/mL) (Gilmartin et al., 2012), and bicuculline (0.1 mg/mL) (Doherty and Gratton, 1999; Enomoto et al., 2011). The saline solution that was used to dissolve the drugs, constitutes the control. Both muscimol and bicuculline are known to interact with the GABA neurotransmitter system.

Next, 0.5 µL (0.5 µL/min) of the drug solution or control solution was injected via the cannula, with a 2.5 µL Hamilton syringe. To allow diffusion of the solution, the injection cannula had to remain in place for 1 min before it was removed from the guide cannula and replaced with the dummy (Doherty and Gratton, 1999).

4.2.6 PET-FDG scan

One day prior to the scan, the animals were fasted to lower blood glucose levels. Ten minutes after the administration of the compound, 1 mCi ^{18}F-FDG was injected intravenously in the tail. After this procedure the animal stayed in a warm environment for approximately 20 minutes to allow optimal tracer uptake (Sunwoo et al., 2012). Next, the rats were anaesthetized by a mixture of isoflurane and oxygen (5% induction, 1.5% maintenance) after which the animal was placed onto the thermostatically

heated bed of the µPET scanner (Stolc et al., 2011). The microPET images were acquired using two Siemens Inveon µPET small-animal scanner (Siemens Preclinical Solution, Knoxville, TN).

4.2.7 Ink injection

At the end of the experiment, the cannula placements were verified according to the protocol described previously (2.1.1.4 Ink injection).

4.2.8 Microtome slices

The next day, the brains were placed into a microtome (Leica CM 1950) where 30 µm slices were made to determine the injection place. This location was visible due to the blue colour of the fountain pen ink. This location was photographed (FIGURE 14) and marked on a template while the important slices were stained with haematoxylin to store them for later use.

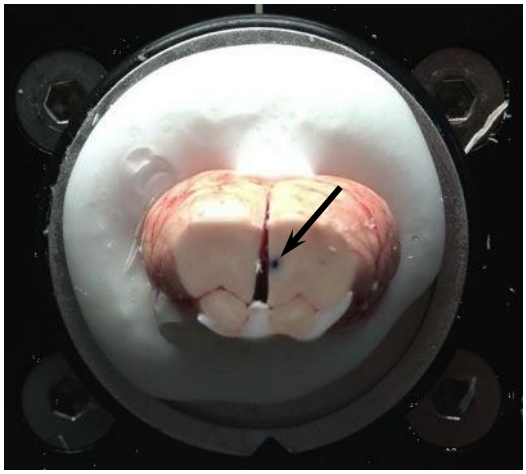


FIGURE 14: Slice at the location of injection
This picture shows the result of an ink injection via the internal cannula. The arrow marks the blue ink visible in the prelimbic region at a slice with coordinates 12.7 mm from the interaural line and 3.7 mm from bregma.

4.2.9 Normalisation and analysis of data

The µPET images were cropped, transformed into the space of an ^{18}F-FDG template, masked to remove extracerebral activity and count normalized by dividing the values with the injected dose of tracer in PMOD v3.3 (PMOD Technologies, Switzerland).

Using SPM8 (SPM8, Welcome Department of Cognitive Neurology, London, UK), hyper- and hypometabolism T-maps of treatment versus saline were achieved for muscimol and bicuculline (for each paradigm 9 pairs of data). These T-maps were overlaid on a 9.4 T MR rat brain image (PMOD v3.3) and overlaid with a predefined rat brain volume of interest (VOI) template delineating brain regions, which is available in the software. For all T-maps and for each brain region, we determined the percentage of significant metabolically changed volume per total VOI volume, the maximal T-value and the total number of significant voxels ($p < 0.05$, i.e. T-value > 1.860 or T-value < -1.860, degrees of freedom = 8).

A Gaussian filter of 0.5 mm was applied to make the images visually smoother. Hyper-regions were given a “yellow-orange-red hot” color, while the hypo-regions are visible by values of “blue and green cold” colours according to their intensity.

When averages were calculated, the standard error is determined.

5) RESULTS

5.1 RESULTS HISTOLOGY

The cannula placement of each rat is depicted in FIGURE 15. Since one rat did not survive the IV-injection of FDG, due to stress, only 11 dots are visible. The placement was successful in 9 out of 11 rats. Since correct positioning of the cannula was disputable in two rats (marked in red), these were not included in further analysis to prevent a misinterpretation of the results, giving a total of 9 rats (marked in green) for further analysis.

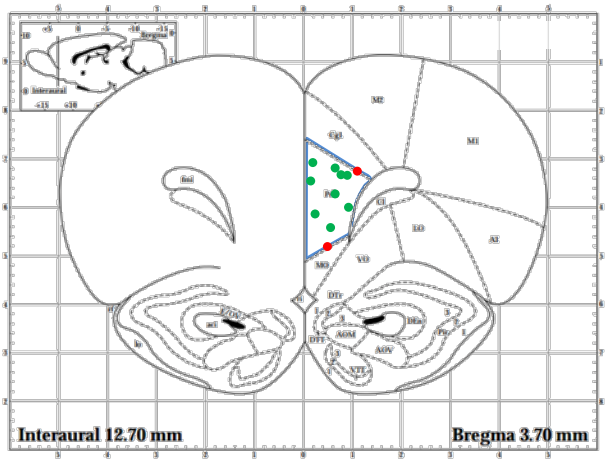
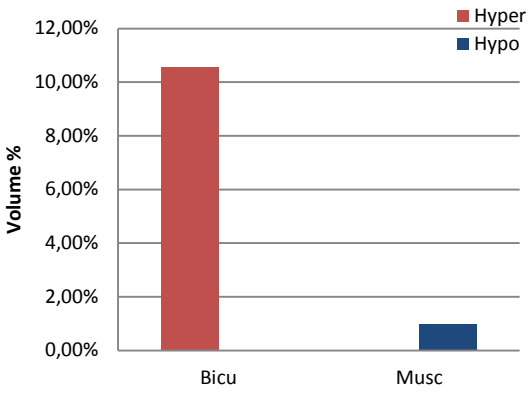


FIGURE 15: Locations of cannulae
The prelimbic area is marked with a blue cadre. Nine cannulae were placed with certainty in the prelimbic region (marked in green). To avoid a misinterpretation of the results, the 2 animals with a cannula that might or might not be placed in the prelimbic area (marked in red) were left out of the analysis.

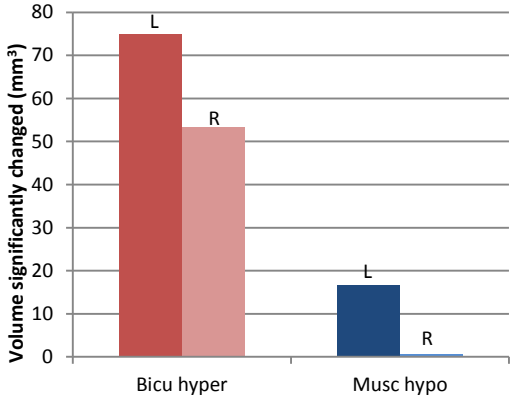
5.2 OVERALL CHANGES IN METABOLISM

Compared to saline, administration of bicuculline induced significant increases in glucose metabolism in 10.57 % of the total brain volume, while no significant volume with a decreased glucose metabolism was visible. Muscimol induced significant decreases in glucose metabolism in 0.99 % of the brain, while no significant amount of volume was increased in metabolism (GRAPH 1).



GRAPH 1: The percentage of whole brain that shows significant metabolic changes after drug administration.
The percentage of volume increased in glucose metabolism is displayed in red, while the percentage of volume showing a decreased glucose metabolism is characterized by a blue color.

Since the administration was done in the left mPFC the significantly changed volume of the left and the right hemisphere was compared for each drug and plotted in a graph. The volume significantly increased in glucose metabolism was more pronounced in the left hemisphere when compared to the right with 58% of the volume with an increased metabolism present in the left hemisphere, while 42% of the total increased metabolism was found in the right hemisphere. Also for the muscimol administration, more volume that was decreased in glucose metabolism was found in the left hemisphere as well (96% left vs 4% right) (GRAPH 2).

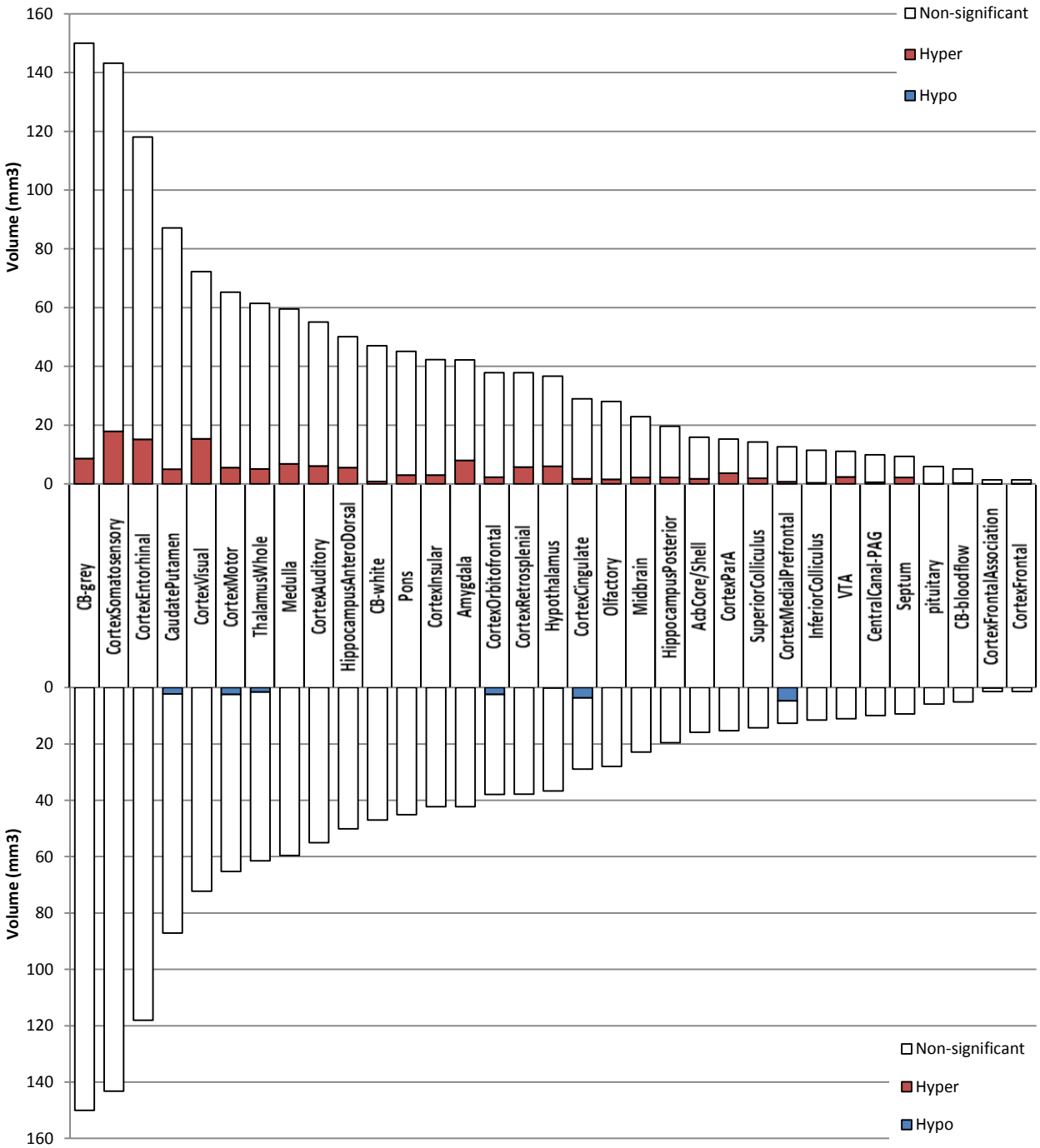


GRAPH 2: The significantly changed volume of each hemisphere after drug administration
For bicuculline only the amount of volume increased in metabolism (red colors) in each hemisphere (L and R) is displayed, since no hypo volume was present. Because muscimol did not induce any hyper metabolism, only the volume significantly decreased in glucose metabolism in each hemisphere is displayed.

5.3 REGIONAL CHANGES IN METABOLISM

Administration of bicuculline induced a clear increase in metabolism in many of the different regions (GRAPH 3). This increase was most notable in the sensory regions with 21.29% of the total regional volume (rV) of the visual cortex and 11% of the rV of the auditory cortex affected. Also the somatosensory and olfactory cortex showed an increase in metabolism (12.50% and 5.40% of their rV's respectively). Other regions that were characterized by a hypermetabolism were the motor cortex (8.44% rV), the thalamus (8.26% rV), the anterodorsal hippocampus (11.11% rV), the retrosplenial cortex (15.10% rV) and the entorhinal cortex (12.86% rV).

When muscimol was administered, a decrease in metabolism in certain areas was visible as shown in GRAPH 3. Regions that were directly noted for their hypometabolism were the regions close to the mPFC such as the cingulate and orbitofrontal cortex (12.79% rV; 6.34% rV). The dorsal striatum, which contains the caudate nucleus and the putamen (2.53% rV), and the thalamus (2.50% rV) were also characterized by a decreased glucose metabolism. Also a portion of the motor area (3.73%) was characterized by a decrease in metabolism as well.

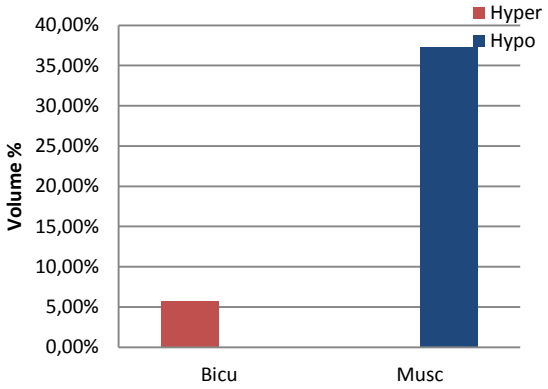


GRAPH 3: Significant changes in metabolism for the different regions after administration of bicuculline and muscimol in the mPFC
In this graph all the volumes of interests were arranged according to size (large to small) after administration of bicuculline (top) and muscimol (bottom) in the prelimbic region, which is part of the medial prefrontal cortex. Volumes are given in mm^3. The red part marks the amount of the total volume that is increased in glucose metabolism in the region, while the blue part is the amount that is decreased in glucose metabolism. The remainder non-significantly changed volume is marked in white.
CB: Cerebellum
Acb: Nucleus Accumbens
CortexParA: Paracortex
VTA: Ventral Tegmental Area
PAG: Periaqueductal Gray

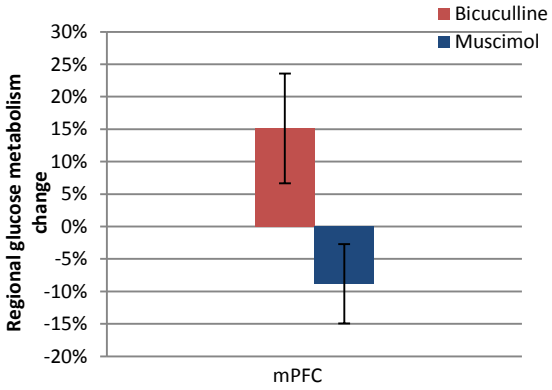
5.3.1 Medial Prefrontal Cortex

After injection of bicuculline in the left mPFC, 5.71% of the volume of the mPFC showed a significant increase in metabolic activity resulting in an increase in this region of 15.12% (±8.5%) when compared to the control condition (GRAPH 4 & 5; FIGURE 16a).

Muscimol on the other hand decreased the overall metabolism of this region with 8.84% (±6.1%), affecting 37.31% of its volume (GRAPH 4 &5; FIGURE 16b).



GRAPH 4: The percentage of the total medial prefrontal cortex that shows significant metabolic changes after drug administration.
The percentage of volume increased in glucose metabolism is displayed in red, while the percentage of volume showing a decreased glucose metabolism is characterized by a blue color.



GRAPH 5: The percentage of average change in glucose metabolism in the total medial prefrontal cortex.
The percentage of change in glucose metabolism is displayed in red for bicuculline, while the percentage of change for muscimol is characterized by a blue color. The error bars display the standard error.

Both the left and the right mPFC were affected by the drugs although the effects were more pronounced in the left hemisphere. For bicuculline 9.90% of the left mPFC was increased in glucose metabolism, versus 1.52% in the right mPFC. The effect was even more pronounced after administration of muscimol with 70.05% of the left mPFC decreased in glucose metabolism and 4.57% of the right mPFC affected.

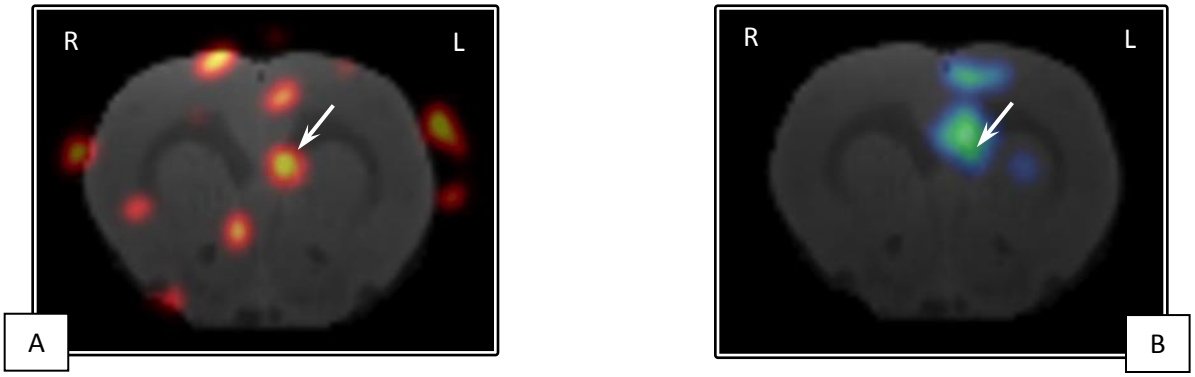
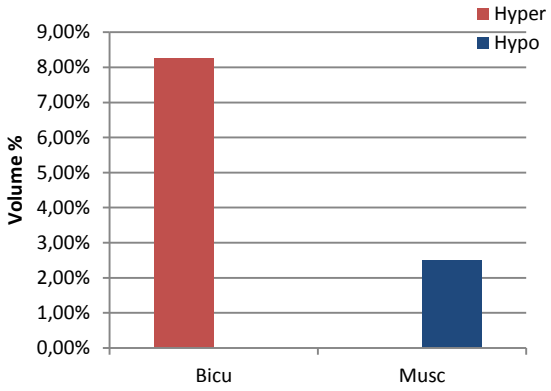


FIGURE 16: PET-FDG image of prelimbic region.
Volumes significantly increased in glucose metabolism are displayed with a "yellow-orange-red hot" color. The "blue and green cold" color characterizes a decreased glucose metabolism in that region. The arrow marks the location of the cannula.
(A) The PET-FDG image after bicuculline administration.
(B) The PET-FDG image after muscimol administration.

5.3.2 Thalamus

After administration of bicuculline there was a portion (8.20%) of the total thalamus that showed an increase in glucose metabolism (GRAPH 6; FIGURE 17a). This increased the total regional metabolism with 12.33% (±7.3%).

Muscimol however, decreased the glucose metabolism in a small portion (2.50%) of the thalamus (GRAPH 6; FIGURE 17b). This decreased the total regional metabolism with 2.39% (±6.0%)



GRAPH 6: The percentage of the total volume of the thalamus that shows significant metabolic changes after drug administration.
The percentage of volume increased in glucose metabolism is displayed in red, while the percentage of volume showing a decreased glucose metabolism is characterized by a blue color.

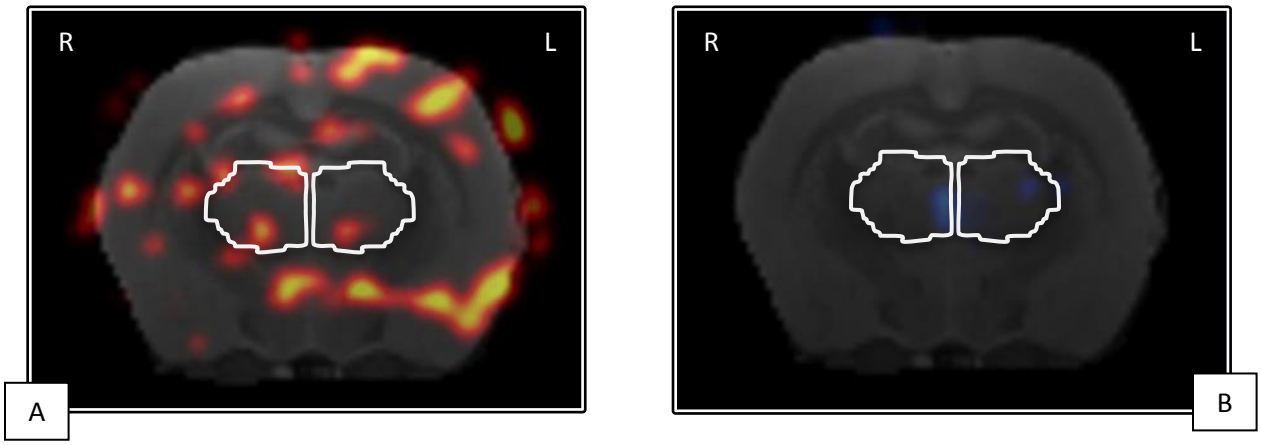
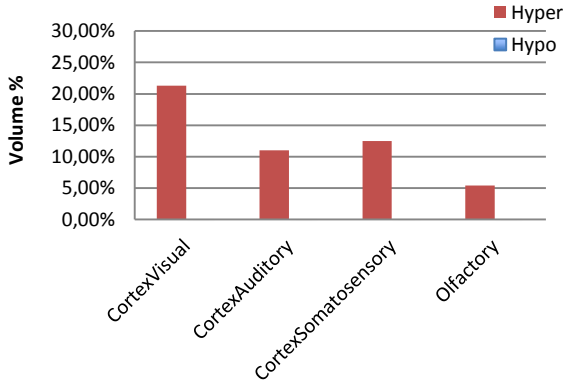


FIGURE 17: PET-FDG image of the thalamus.
Volumes significantly increased in glucose metabolism are displayed with a "yellow-orange-red hot" color. The "blue and green cold" color characterizes a decreased glucose metabolism in that region. The white box marks the location of the thalamus.
(A) The PET-FDG image after bicuculline administration.
(B) The PET-FDG image after muscimol administration.

5.3.3 Sensory regions

After administration of bicuculline in the prelimbic region there was a significant increase in the sensory regions, including the visual cortex (21.29% of the regional volume), auditory cortex (11%), somatosensory cortex (12.50%) and the olfactory cortex (5.40%) (GRAPH 7; FIGURE 18a).

Administration of muscimol in the prelimbic region did not result in any significant glucose metabolism changes in the sensory regions (FIGURE 18b).



GRAPH 7: The percentage of the total volume of the sensory regions that shows significant metabolic changes after administration of bicuculline.
The percentage of volume increased in glucose metabolism is displayed in red, while the percentage of volume showing a decreased glucose metabolism is characterized by a blue color.

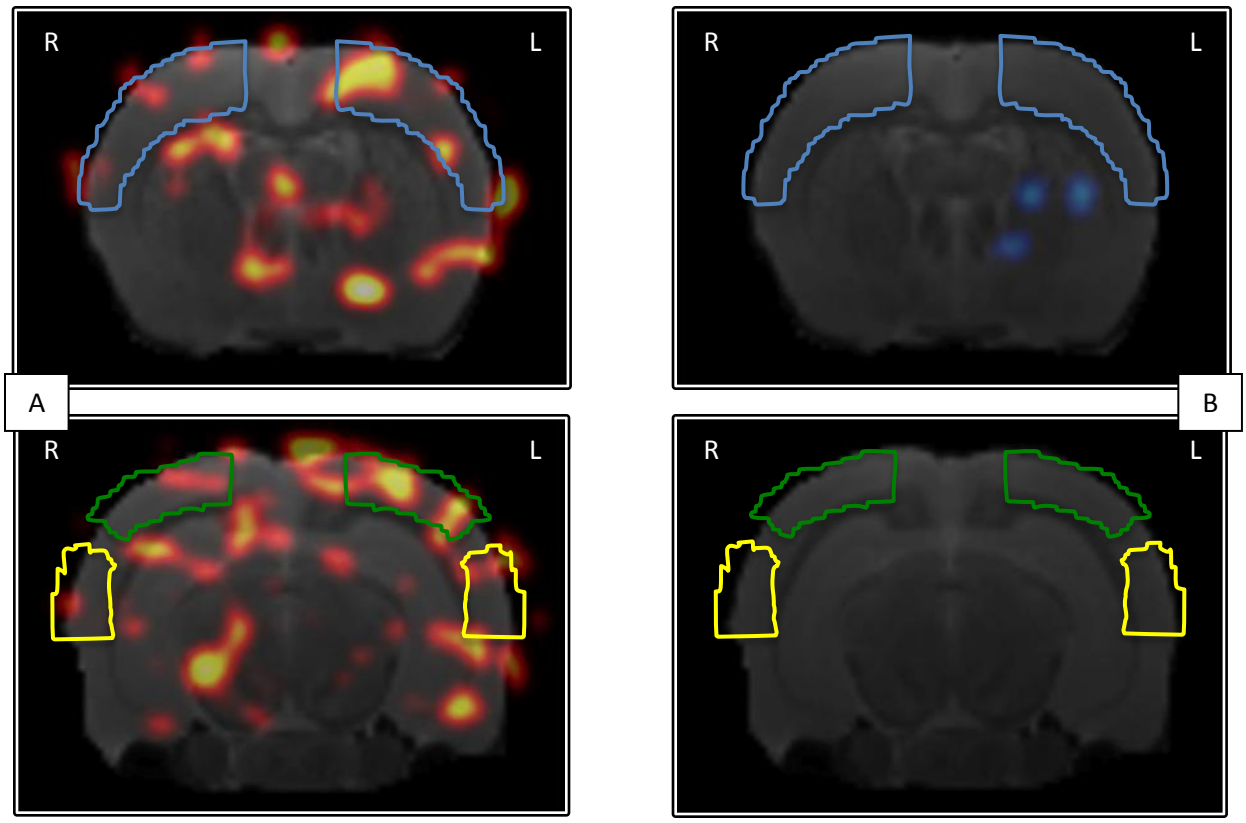
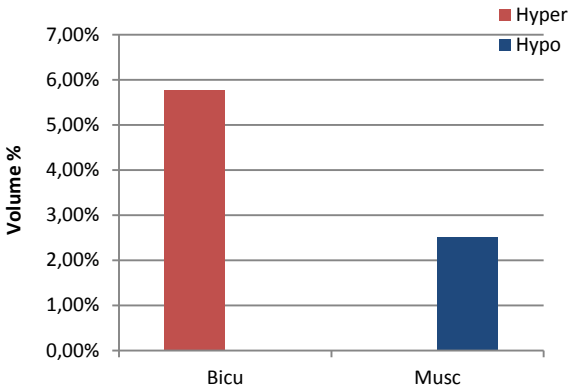


FIGURE 18: PET-FDG image of the sensory regions.
Volumes significantly increased in glucose metabolism are displayed with a "yellow-orange-red hot" color. The "blue and green cold" color characterizes a decreased glucose metabolism in that region. The blue box marks the location of the somatosensory cortex, the green box of the visual cortex and the yellow of the auditory cortex.
(A) The PET-FDG image after bicuculline administration.
(B) The PET-FDG image after muscimol administration.

5.3.4 Striatum

The dorsal striatum, which contains the caudate nucleus and the putamen, was another region affected by the administration of bicuculline in the prelimbic area. This resulted in an increased glucose metabolism in 5.77% of the total region (GRAPH 8; FIGURE 19a) giving a total increase in glucose metabolism of 12.27% (±7.5%) of the entire region.

Muscimol however showed a decreased glucose metabolism in 2.53% of this region (GRAPH 8; FIGURE 19b). This gave a total decrease in glucose metabolism of 2.37% (±5.8%)



GRAPH 8: The percentage of the total volume of the striatum that shows significant metabolic changes after drug administration.
The percentage of volume increased in glucose metabolism is displayed in red, while the percentage of volume showing a decreased glucose metabolism is characterized by a blue color.

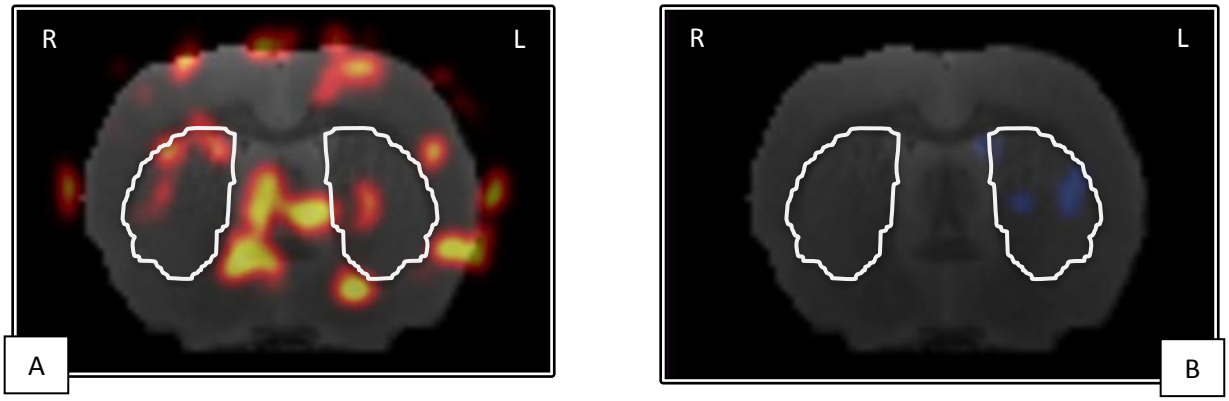
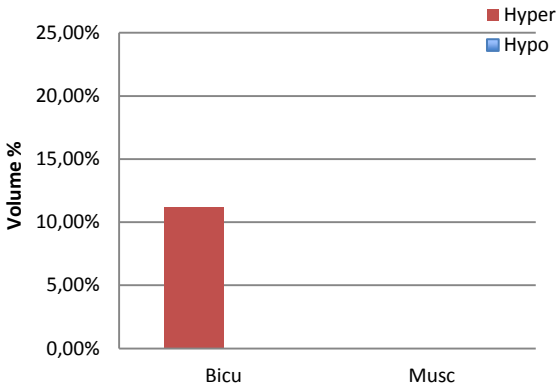


FIGURE 19: PET-FDG image of the striatum.
Volumes significantly increased in glucose metabolism are displayed with a "yellow-orange-red hot" color. The "blue and green cold" color characterizes a decreased glucose metabolism in that region. The white box marks the location of the striatum.
(A) The PET-FDG image after bicuculline administration.
(B) The PET-FDG image after muscimol administration.

5.3.5 Hippocampus

Another structure strongly affected by the prelimbic administration of bicuculline was the anterodorsal hippocampus. A total of 11.18% of the entire region was increased in glucose metabolism (GRAPH 9; FIGURE 20a). This gave a total increase in glucose metabolism in this region of 13.63% (±7.3%).

Muscimol on the other hand did not affect the glucose metabolism in the hippocampus (GRAPH 9; FIGURE 20b).



GRAPH 9: The percentage of the total volume of the hippocampus that shows significant metabolic changes after drug administration.
The percentage of volume increased in glucose metabolism is displayed in red, while the percentage of volume showing a decreased glucose metabolism is characterized by a blue color.

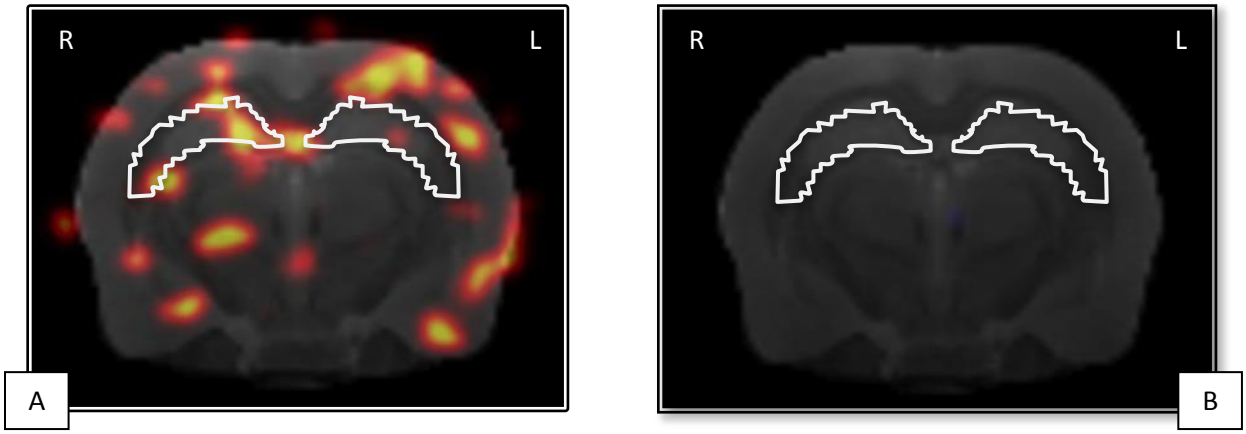
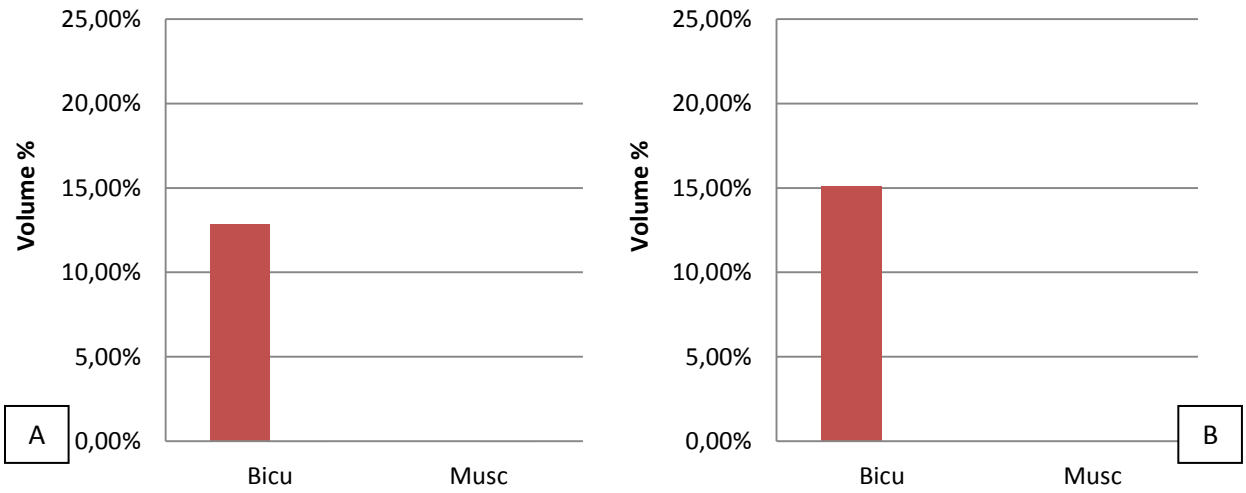


FIGURE 20: PET-FDG image of the hippocampus.
Volumes significantly increased in glucose metabolism are displayed with a "yellow-orange-red hot" color. The "blue and green cold" color characterizes a decreased glucose metabolism in that region. The white box marks the location of the hippocampus.
(A) The PET-FDG image after bicuculline administration.
(B) The PET-FDG image after muscimol administration.

5.3.6 Memory associated areas

Except for the hippocampus, other memory-associated regions are also affected by the administration of bicuculline in the prelimbic area. There is a clear increase visible in 15.10% of the retrosplenial cortex and in 12.86% of the entorhinal cortex. (GRAPH 10; FIGURE 21a).

Muscimol on the other hand did not affect the glucose metabolism in either of these regions (GRAPH 10; FIGURE 21b).



GRAPH 10: The percentage of the total volume of the retrosplenial (A) and entorhinal (B) cortex that shows significant metabolic changes after drug administration.
The percentage of volume increased in glucose metabolism is displayed in red, while the percentage of volume showing a decreased glucose metabolism is characterized by a blue color.

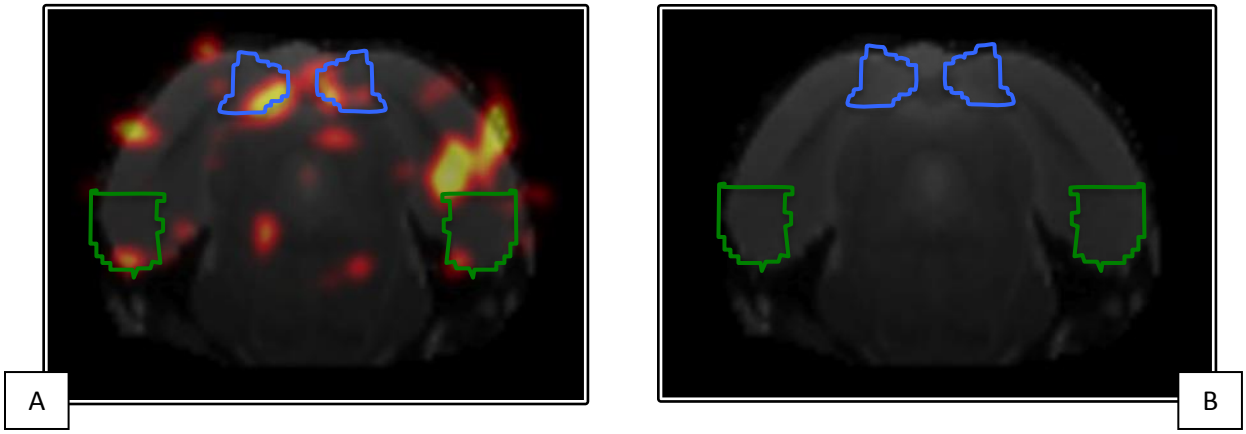


FIGURE 21: PET-FDG image of the entorhinal and retrosplenial cortex.
Volumes significantly increased in glucose metabolism are displayed with a "yellow-orange-red hot" color. The "blue and green cold" color characterizes a decreased glucose metabolism in that region. The green box marks the location of the entorhinal cortex and the blue box marks the location of the retrosplenial cortex.
(A) The PET-FDG image after bicuculline administration.
(B) The PET-FDG image after muscimol administration.

6) DISCUSSION

6.1.1 HISTOLOGY

Since the ink injection showed a clear and focal blue mark in the prelimbic region, it could be concluded that the effects visible in this region were a direct effect of the administration of the drug. The increase or decrease in metabolism visible in the mPFC is thus believed to be a direct consequence of the administration of bicuculline or muscimol.

Changes in metabolic activity in other brain regions are therefore believed to reflect an indirect consequence of the changed metabolic activity in the prelimbic region, through direct or indirect functional connectivity with the target region.

6.1.2 OVERALL EFFECTS

In this study we have shown that an administration of bicuculline, a GABA-A receptor antagonist, in the prelimbic cortex of a rat caused a widespread increase in metabolism in the entire brain. GABA is the main inhibitory neurotransmitter for the central nervous system of mammals. Except for regulating neuronal excitability, it is also responsible for the regulation of muscle tone. Currently there are 2 different classes of receptors known on which GABA can bind. The first class comprises the GABA-A receptor (FIGURE 22), which is part of the ligand-gated ion channel complex mediating the passing of chloride ions across the membrane thereby hyperpolarizing and thus inhibiting neurons (Devlin, 2001). The other class consists of the GABA-B receptor, which is a G-protein-coupled receptor mediated by intermediaries. Bicuculline, being a GABA-A receptor antagonist, hinders the passage of chloride ions and thus prevents hyperpolarisation, explaining the visible increase in metabolism. Muscimol on the other hand, being a GABA-A agonist, caused a more focal decrease in metabolism in the brain, which was most pronounced in the left hemisphere and more specifically in the target region mPFC. This drug enhances the passage of the chloride ions across the membrane, resulting in a hyperpolarization (Devlin, 2001). This limits the excitation of the neurons, explaining the hypometabolism after administration. The results also suggest that excitation is more likely to be transferred through present networks, while inhibition remains more locally based.

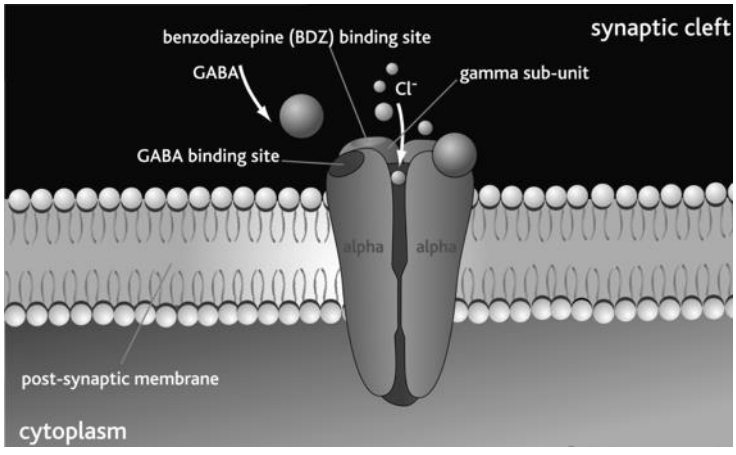


FIGURE 22: GABA-A receptor
The GABA-A receptor mediates the passing of chloride ions across the membrane after binding of GABA to the appropriate binding site.

Source: Adapted from Lundbeck institute (cnsforum.com)

6.1.3 Regional Effects

There was a significant increase in glucose metabolism in 5.71% of the total volume of the mPFC, with no hypometabolism present after administration of bicuculline. After administration of muscimol, 37.31% of the region was decreased in glucose metabolism while no hypermetabolism is present. Although there is more volume significantly changed in glucose metabolism in the mPFC after administration of muscimol, the actual change in glucose metabolism is higher after administration of bicuculline (15.12% ±8.5% vs 8.84% ±6.1%).

A region affected by each drug administration was the thalamus. The thalamus plays an important role in several key functions including sleep and wakefulness (Steriade and Llinás, 1988), motor control (Sommer, 2003) stress habituation and sensitization (Jones et al., 2011a). Furthermore it acts as a processing and relay center receiving sensory signals and sending them to the associated primary cortical areas (McCormick and Bal, 1994). The neurons of the olfactory system are the only ones that bypass the thalamus and connect directly to the forebrain (Alitto and Usrey, 2003). The other sensory systems have a thalamic nucleus that receives the sensory information before sending it to the associated cortex.

After administration of bicuculline the thalamus showed an increase in glucose metabolism. A possible explanation for this hypermetabolism, can be found in its connection with the basal ganglia. Two major pathways, a direct and an indirect one, have an influence on the activity of the thalamus (Lenglet et al., 2012). Which pathway is followed depends on the level of DA present in the striatum (Rice et al., 2011). In the presence of DA, the direct pathway is favoured, due to the stimulation of GABAergic neurons (FIGURE 23). Previously it has been shown that excitating the mPFC causes a stronger DA release in the striatum (Karreman and Moghaddam, 1996). This assumption is strengthened by the visible increase in metabolic activity of the VTA in 21.27% of the regional volume, which is one of the regions where dopaminergic cell bodies find their origin. The DA increase might have stimulated the direct pathway starting with inhibitory GABAergic projections from the striatum to the GPi and the substantia nigra pars reticulate (SNr) (Purves, 2001; Smith et al., 1998), resulting in more inhibition of these areas. This means that the GABAergic projections of these regions to the thalamus (Purves, 2001) were now inhibited, resulting in a disinhibition of the thalamus, which could explain the increased metabolism in 8.26% of this region.

There was a small volume (2.5%) that showed a significant decreased metabolism in this region when muscimol was applied. By inhibiting the mPFC, thereby decreasing DA levels, the direct pathway is not able to function optimally which causes the indirect pathway to take over (FIGURE 23) (Gerfen and Surmeier, 2011; Humphries, 2012). Since a study has already shown that a lesion of the mPFC decreases the DA level (Karreman and Moghaddam, 1996; Shahidani et al., 2012), the administration of muscimol, is likely to reveal a similar effect. This decrease in DA level would cause the balance between the pathways to overhaul to the indirect pathway. The glutamatergic connections from the cortex connect to the striatum and thereby inhibit the external part of the globus pallidus (GPe) via a GABAergic pathway. This inhibits the GABAergic projections going to the subthalamic nucleus (STN) resulting in a disinhibition of the glutamatergic pathway to the internal part of the globus pallidus (GPi). This region will then inhibit the thalamus, explaining the visible decrease in thalamic activity (Purves, 2001; Smith et al., 1998).

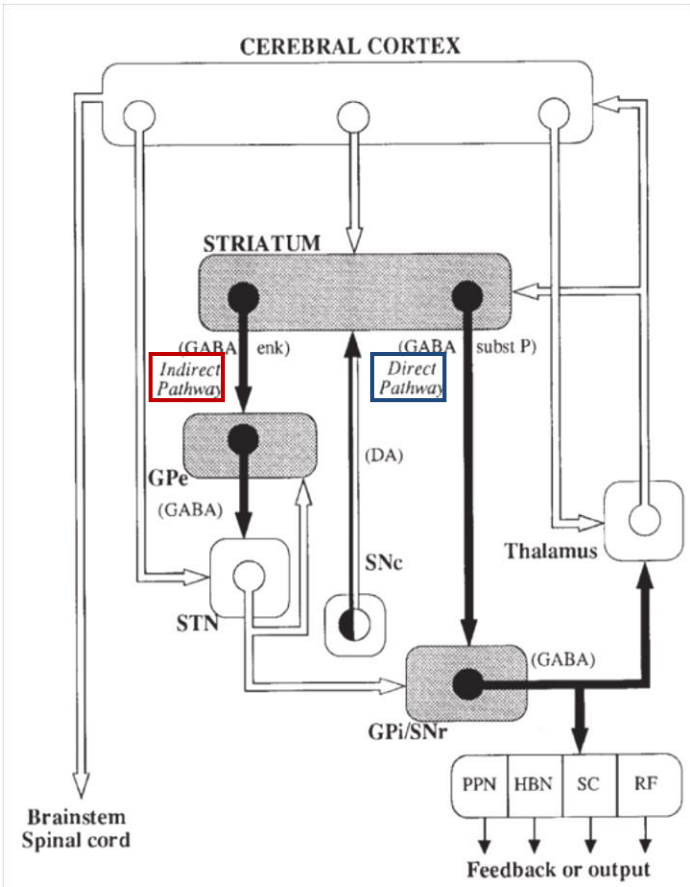


FIGURE 23: Microcircuitry of the basal ganglia
Inhibitory projections are shown as filled arrows, excitatory projections are shown as open arrows.
The cortical information that reaches the striatum is conveyed to the basal ganglia output structures (GPi/SNr) via two pathways, a direct inhibitory pathway (blue box) from the striatum to the GPi/SNr and an indirect pathway (red box), which involves an inhibitory projection from the striatum to the GPe, an inhibitory projection from the GPe to the STN and an excitatory projection from the STN to the GPi/SNr. The information is then transmitted back to the cerebral cortex via a relay in the thalamus or conveyed to various brain stem structures.

Source: Adapted from Smith et al., 1998

Since the thalamus also receives the sensory information before sending it to the associated areas, the increase in thalamic activity might also explain the hypermetabolism in the somatosensory, the visual and the auditory cortex.

Muscimol administration however did not induce any significant changes in the sensory regions, possibly due to the marginal decrease in thalamic metabolism.

Another structure characterized by an increase in glucose metabolism was the striatum (5.77% rV). As previously noted, this region has a major function in the planning and modulation of movement pathways through the thalamus, but has potentially a role in a number of other cognitive processes as well (Balleine et al., 2007). Studies have suggested that, although the PFC is linked strongly to cognitive control of executive functioning (Fuster, 2000), in reality this is a more complex form of interaction, whereby prefrontal, premotor and sensorimotor cortices are linked by the striatum through reward-related circuitry (Chang et al., 2002; Tanaka et al., 2006).

Previous studies have shown that there are anatomical connections between the striatum and the mPFC as well (Drevets et al., 2008; Ongür and Price, 2000), thereby explaining the increased striatal metabolism.

Application of muscimol in the prelimbic area induced a decreased metabolism in a small but noticeable volume of the striatum (2.53% rV) that might be explained by the interaction with the mPFC as well.

Administration of bicuculline also induced significant changes in portions of the memory associated areas. In the hippocampus, which has an important role in the consolidation of short-term to

long-term memory as well as in spatial navigation, hypermetabolism was visible in 11.18% of the rV. This might be due to the hyperexcitability of the hippocampus and the epileptic characteristics of bicuculline (Uva et al., 2005). Moreover since this region is innervated by midbrain DA neurons (Gasbarri et al., 1997) which are activated by surprising, novel, aversive and rewarding events (Bromberg-Martin et al., 2010) as well as reward locating (Puryear et al., 2010), the bicuculline induced increase in DA (Karreman and Moghaddam, 1996) may affect the hippocampal activity as well. The increase in DA would then trigger the hippocampus to learn the circumstances in which this neurotransmitter was given which could explain the increase in metabolism in the hippocampus and associated structures such as the entorhinal cortex and the retrosplenial cortex as well as the increase in the sensory areas.

6.1.4 EVALUATION

6.1.4.1 Normalisation

Different techniques can be used for the normalisation of the cropped PET images. Normalizing using the whole brain as a reference is a common technique where every voxel of the image is compared to the total sum of values from the entire brain. However, when there is a general increase or decrease over the entire brain, this effect might be over- or underestimated when only this value is taken into account (Borghammer et al., 2009). Another possibility is normalizing for the injected dose of radioactivity. Here, the voxels reflecting the metabolic activity are divided by the tracer dose at the time of the scan. This however does not take into account the weight of the animal, which has a role in tracer distribution as well since the metabolism (and thus the tracer distribution) increases with weight resulting in less uptake in the brain. When weight is taken into account, the images are normalized by the standardized uptake value (SUV). This value expresses the amount of metabolic activity in a certain region, normalized to the bodyweight and the amount of tracer given (Boellaard, 2009). If there is a region that is not affected by the challenge, it is possible to use this area as a reference structure for the normalisation. All voxel values are then divided by the average value of this brain structure.

Since (i) we expected to see overall changes, (ii) the weight varied strongly during the experiment, and (iii) the cerebellum was affected by mPFC stimulation of the mPFC, the data was normalized to the injected dose of radioactive tracer only being the most conservative approach.

6.1.4.2 Dosage Drugs

The effects of bicuculline and muscimol were regarded as separate and could not be compared to each other in terms of efficacy, since each of the two drugs has their own characteristics regarding diffusion rate, receptor binding rate, efficacy, etc. The effects of the drug on certain brain regions might be more or less pronounced depending on these factors. Moreover, since bicuculline is often used to evoke epileptic seizures (Uva et al., 2005), the dosage of this drug was kept at a minimum. An epileptic seizure would cause a major increase in brain activity, which affects the regional glucose metabolism and thus the eventual microPET results.

Also it is possible that the dosage of muscimol was too low, causing an underestimation of the inhibition effect.

7) Conclusion and Future Perspectives

Administration of bicuculline in the prelimbic cortex in the rat brain induces a significant increase in metabolism over the entire brain, especially in the striatal and thalamic regions, the sensory cortices and the memory-associated regions. Administration of muscimol in the prelimbic cortex induces a decrease in metabolism in regions in close proximity with the mPFC and in the striatum and the thalamus. These indirect effects of the drugs are possibly caused by modulation of the DA levels of the connected regions and the vast amount of connections originating from the mPFC.

The intracranial pharmacological administration of agents in the prelimbic cortex functions as a good golden standard for the evaluation of the effects and focality of other upcoming neurostimulation techniques, such as rTMS, targeting the same area, allowing a better interpretation of the results and more accurate conclusions. Furthermore the pharmacological administration of the two drugs illustrates the vast connectivity of the mPFC and the ability of PET-FDG to visualize this. The findings of this study thus allow further research that is testing and fine-tuning rTMS-therapies targeting the PL cortex in small animals. A first step would be to investigate the effect of targeting this region with different stimulation frequencies and comparing the data with the pharmacological study that was described above. Also future research investigating the role of DA in diseases such as addiction and depression, and the ability of rTMS to modulate these DA levels should provide additional insights and add to the clinical value of current treatments. By combining this research with imaging techniques such as PET, rTMS treatments can be further optimized and become an even more valuable tool in a clinical setting.

DANKWOORD

Eerst en vooral wil ik graag mijn promotor, prof. **Steven Staelens**, en co-promotor, prof. **Sigrid Stroobants**, bedanken voor mij de kans te geven om mee te mogen werken aan een boeiend en uitdagend onderzoek. Ik was zeer onder de indruk van jullie expertise en wil jullie oprecht bedanken voor de begeleiding die ik heb mogen ontvangen tijdens mijn onderzoek. Verder stel ik het ook ten zeerste op prijs hoezeer ik vanaf dag één bij het lab betrokken werd, alsook de verantwoordelijkheid waarmee ik werd toevertrouwd.

Verder was ook de begeleiding die ik ontving van Dr. **Tine Wyckhuys** en Drs. **Joke Parthoens** uitmuntend.
Tine, bij jou kon ik altijd terecht met elke vraag die ik had. Bedankt om hier altijd tijd voor vrij te maken ondanks je drukke agenda!
Joke, de positieve instelling die je telkens weer toonde tijdens de vele (soms late, soms vroege) uurtjes in het lab en achter de computer, maakten alles zoveel sneller vooruit gaan. Soms verloor ik zelfs de tijd uit het oog! Je hebt me enorm veel bijgeleerd! Nogmaals sorry voor de neus trouwens…

Philippe en **Lauren**, bedankt voor het zorgen van de dieren en de vele technische hulp!

Jeroen en **Steven**, heel erg bedankt voor de begeleiding (en het geduld) tijdens de beeldverwerking. En uiteraard ook voor de hulp als het internet of de computers eens tegenwerkten!

Ook de rest van het lab wil ik bedanken voor al hun hulp. Het was een zeer fijn gevoel om zelf deel te mogen uitmaken van een ambitieus team waarbij iedereen zijn eigen sterk punt heeft.

Sven en **Viki**, mijn twee thesis-collega's, ik vond het zeer gezellig in ons hoekje, ook al werd het af en toe wat krap. Dat maakte ons echter des te hechter! Bedankt voor jullie hulp tijdens de stressmomentjes!

Verder wil ik mijn **ouders** ook bedanken om tijdens de weekends Antwerpen toch nog maar eens een keertje op te zoeken en in mij te blijven geloven. Ook mijn zus, **Anne**, die (ondanks haar examens) nog altijd tijd maakte om eens goed te kunnen lachen. Tenslotte bedank ik nog graag mijn vriendin, **Carol**, wiens steun mij gemotiveerd hield om er telkens weer voor te blijven gaan.

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