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Pharmacological and DBS-induced changes in cerebral serotonin release

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Ph.D. thesis

Pharmacological and DBS-induced changes in cerebral serotonin release

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Paper 1 Cerebral 5-HT release correlates with [^{11}C]Cimbi36 PET measures of 5-HT2A receptor occupancy in the pig brain. Jørgensen LM, Weikop P, Villadsen J, Visnapuu T, Ettrup A, Hansen HD, Baandrup AO, Andersen FL, Bjarkam CR, Thomsen C, Jespersen B, Knudsen GM. *Journal of Cerebral Blood* Flow & Metabolism 2017, 37(2), 425–434.

Paper 2 Cerebral serotonin release correlates with [^{11}C]AZ10419369 PET measures of 5-HT$_{1B}$ receptor binding in the pig brain. Jørgensen LM, Weikop P, Svarer C, Feng L, Keller SH, Knudsen GM. *Journal of Cerebral Blood Flow & Metabolism* 2017 Jan (epub ahead of print).

Paper 3 Parkinson patients display a presynaptic serotonergic deficit: A dynamic DBS-STN PET study. Jørgensen LM, Henriksen T, Mardosiene S, Keller SH, Stenbæk DS, Hansen HD, Jespersen B, Thomsen C, Weikop P, Svarer C, Knudsen GM. (in submission)

Related papers:

Serotonin 2A receptor agonist binding in the human brain with [11C]Cimbi-36. Ettrup A, da Cunha-Bang S, McMahon B, Lehel S, Dyssegaard A, Skibsted AW, Jørgensen LM, Hansen M, Baabdrup AO, Bache S, Svarer C, Kristensen JL, Gillings N, Madsen J, Knudsen GM. *Journal of Cerebral Blood Flow & Metabolism* 2014, 34(7): 1188-96

Serotonin 2A receptor agonist binding in the human brain with [11C]Cimbi-36: Test–retest reproducibility and head-to-head comparison with the antagonist [18F]altanserin. Ettrup A, Svarer C, McMahon B, da Cunha-Bang S, Lehel S, Møller K, Dyssegaard A, Ganz M, Beliveau V, Jørgensen LM, Gillings N, Knudsen GM. (2016). *NeuroImage* 2016, 130 167–174.

Automatic delineation of brain regions on MRI and PET images from the pig. Villadsen J, Hansen HD, Jørgensen LM, Keller SH, Andersen FL, Petersen IN, Knudsen GM, Svarer C. Journal of Neuroscience Methods 2017 Nov (Epub ahead of print).

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PREFACE

The work presented in this thesis was carried out during my 3-year employment as a PhD student at the Neurobiology Research Unit, Rigshospitalet 2014-2017 under the supervision of Professor Gitte Moos Knudsen.

The thesis is based on 3 studies. First, I conducted two preclinical studies in pigs, where I characterize the association between PET and microdialysis measures of the 5-HT level in the brain induced by various pharmacological challenges aimed to raise the 5-HT level differently. Last, I conducted a clinical study in patients with Parkinson's Disease (PD) and treated with Deep Brain Stimulation (DBS). Here, I used one of the PET radioligand first assessed in the preclinical study to investigate the reduction in receptor availability in patients with PD as compared to age-matched controls, the change in 5-HT level when DBS is turned off and the association to mood and clinical measures of Parkinson's Disease.

In this thesis, I first present a general introduction to PD, surgical therapies, serotonergic involvement in PD, synaptic serotonergic neurotransmission, and the PET radioligands used to target the 5-HT system here in sight. The experimental part of the thesis covers various aspects of the surgical procedure, microdialysis, PET and MRI as well as evaluations of mood and clinical measures of PD.

During the years of my PhD studies, I have acquired both technical and research experience in clinical and pre-clinical PET imaging studies, microdialysis, and psychological testing. I have also implemented a surgical procedure for microdialysis and an anaesthetic setup in the MRI scanner as an add-on to the pig model used in NRU. Although not included in the work of this thesis, I have also implemented a set-up for DBS surgery in pigs and conducted simultaneous PET/fMRI and microdialysis experiments with DBS being turned on and off within scans.

I received the following contributions to raw data: In study 1-3, the production of PET radioligands and reconstructions of the PET scans were performed by employees at the PET and Cyclotron Unit, Rigshospitalet. In study 1-2, I received raw data based on blood and metabolite analyses performed by Agnete Dyssegaard (NRU), and HPLC analyses of the microdialysate samples performed by Pia Weikop (NPlab). The structural MRI in humans (study 3) were performed by employees at Rigshospitalet.

SUMMARY

Parkinson's Disease (PD) is one of the most common movement disorders in the world affecting 1% of the population above 60 years of age causing both motor and non-motor symptoms. Although focus has initially been on dopaminergic dysfunction, there are vast amount of research supporting the involvement of other brain monoamine systems in PD, such as serotonin (5-HT).

Deep Brain Stimulation (DBS) is a potential reversible surgical treatment used to alleviate motor symptoms in a selected group of patients with PD, but DBS also has the potential to treat other neuropsychiatric disorders, such as depression, of which serotonergic modulation at the synaptic level constitute an established treatment. Even though the therapeutic effect of DBS in movement disorders is well established, the mechanism underlying its effect is still unclear. Brain imaging of the 5-HT system and its functions is important to characterize neuropsychiatric disorders and to identify treatment options. Understanding the DBS-induced mechanisms may contribute to improve medical therapy of such brain disorders and support the transition of DBS-treatment to new applications.

The aim of this thesis is to characterize the association between microdialysis and Positron Emission Tomography (PET) measures of changes in cerebral 5-HT level, which will serve as a tool for translating the outcome from future PET imaging studies, as subsequently conducted here in a PET study of patients treated with DBS.

The thesis is based on three studies. In the first two studies in pigs, the association between microdialysis and PET measures of changes in 5-HT level is characterized upon various pharmacological interventions with the aim to raise the extracellular cerebral 5-HT level differently. In the last study, we used the PET radioligand first evaluated in the preclinical study to investigate the presynaptic serotonergic function in patients with PD treated with DBS and the association to mood and clinical measures of PD.

We demonstrate that both PET radioligands, the 5-HT_{2A} receptor agonist [^{11}C]Cimbi-36 and the 5-HT_{1B} partial agonist [^{11}C]AZ10419369, are sensitive to 5-HT, but only when the release is sufficiently high. While the sensitivity of [^{11}C]AZ10419369 to detect 5-HT is comparable to that of [^{11}C]raclopride to detect dopamine (DA), [^{11}C]Cimbi-36 is three times more sensitive than

[^{11}C]AZ10419369, but an advantage of [^{11}C]AZ10419369 is that it can be used in a within-scan design.

With [^{11}C]AZ10419369, we show that patients with PD treated with DBS exhibit a region specific loss of serotonergic presynaptic terminals associated with an incapacity to elicit a serotonergic response when DBS was turned off. The serotonergic dysfunction is to some extent correlated with motor symptom severity.

The thesis confirms that data from PET imaging comply with the competition model and diagrams of the association between cerebral 5-HT level and PET measures allow to translate outcome from new PET 5-HT studies as changes in interstitial 5-HT. With this novel technology we observed a presynaptic serotonergic deficit in patients with PD treated with DBS. The observed serotonergic deficits may play an important role in both motor and non-motor symptoms in patients with PD. The outcome from this thesis expand the existing knowledge of serotonergic involvement in PD and the underlying mechanism of DBS. Moreover, it provides an important tool to translate PET images to cerebral 5-HT release in future studies.

DANSK RESUME

Parkinsons sygdom er en af de mest hyppige bevægeforstyrrelser i verden idet den rammer 1% af befolkningen over 60 år og den medfører både motoriske og non-motoriske symptomer. Selvom fokus hidtil primært har været rettet mod den dopaminerg dysfunction, så er der omfattende forskning, som peger på at Parkinsons sygdom også påvirker andre monoamin transmittersystemer i hjernen, herunder serotonin (5-HT).

Deep Brain Stimulation (DBS) er en potentiel reversibel kirurgisk behandling som kan bruges til at afhjælpe motoriske symptomer hos udvalgte patienter med Parkinsons sygdom, men DBS kan potentielt også anvendes i behandlingen af andre neuropsykiatriske sygdomme, som f.eks. depression, hvor modulation af 5-HT på det synaptiske niveau udgør en etableret behandling. Selvom den terapeutiske effekt af DBS på Parkinsons sygdom er veldokumenteret, så er den underliggende mekanisme stadig ukendt. Billeddannende undersøgelser af 5-HT systemet og dets funktioner er vigtige metoder til udforskning af neuropsykiatriske sygdomme og identificere behandlingsmuligheder. Forståelse af DBS' virkningsmekanismer kan ligeledes bidrage til at forbedre medicinsk behandling af hjernesygdomme og bane vejen for nye behandlingsmuligheder med DBS.

Formålet med denne afhandling er at beskrive sammenhængen mellem ændringer i hjernens 5-HT niveau målt ved mikrodialyse og Positron Emission Tomography (PET), som et vigtigt redskab til brug ved fortolkning af resultater fra PET studier, som kan gennemføres på patienter med Parkinsons sygdom i behandling med DBS.

Afhandlingen er baseret på tre studier. De første to studier er udført på grise, hvor der beskrives sammenhængen mellem ændringer i 5-HT niveauet målt ved mikrodialyse og PET efter forskellige farmakologiske interventioner, der har til formål at øge det ekstracellulære 5-HT niveau i forskellig grad. I det sidste studie anvendte vi een af de to PET radiologander, som var valideret i det prækliniske studie, til at undersøge den præsynaptiske serotonerge funktion hos patienter med Parkinsons sygdom i behandling med DBS. Vi undersøgte også om der var sammenhæng med sindsstemning og kliniske parametre for Parkinsons sygdom.

Vi påviser, at begge PET radioligander, 5-HT_{2A} receptor agonisten [^{11}C]Cimbi-36 og 5-HT_{1B} receptor partial agonisten [^{11}C]AZ10419369, kan måle ændringer i 5-HT, når det frigives i tilpas

store mængder. Hvor sensitiviteten af [^{11}C]AZ10419369 for 5-HT er sammenligneligt med sensitiviteten af [^{11}C]raclopride for dopamin, så er [^{11}C]Cimbi-36 tre gange så sensitivt som [^{11}C]AZ10419369 til at detektere ændringer i 5-HT, men modsat [^{11}C]Cimbi-36, så kan [^{11}C]AZ10419369 anvendes i et within-scan design.

Vi viser med [^{11}C]AZ10419369 og PET, at patienter med Parkinsons sygdom i behandling med DBS frembyder en regionsspecifik tab af serotonerge presynaptiske terminaler som medfører en reduceret evne til at frigive 5-HT, når DBS slukkes. Den serotonerge dysfunktion er i nogen grad korreleret til sværhedsgraden af motoriske symptomer.

Afhandlingen bekræfter at billeddannende PET undersøgelser af 5-HT systemet med de to radioligander er i overensstemmelse med okkupansmodellen og korrelationen mellem ændringer i hjernens 5-HT niveau og PET mål muliggør fortolkning af fremtidige PET studier. Med anvendelse af denne nye teknologi observerede vi en presynaptisk serotonerg dysfunktion i patienter med Parkinsons sygdom i behandling med DBS. Den observerede serotonerge dysfunktion kan udgøre et vigtig bidrag til de motoriske og non-motoriske symptomer hos patienter med Parkinsons sygdom. Resultaterne fra denne afhandling tilføjer ny viden til det eksisterende kendskab til serotonerge forandringer ved Parkinsons sygdom og til de underliggende mekanismer ved DBS. Derudover bibringer den et vigtigt redskab hvormed PET billeddannelse kan anvendes til at bestemme relative ændringer i frigivelse af 5-HT i hjernen, hvilket kan anvendes til en lang række fremtidige studier.

ABBREVIATIONS AND TERMINOLOGY

5-HT: 5-Hydroxytryptamine (serotonin)

5-HTIAA: 5-Hydroxyindoleacetic acid (5-HT metabolite)

5-HT_{1B}: Serotonin 1B Receptor

5-HT_{2A}: Serotonin 2A Receptor

5-HTT: Serotonin Transporter (also known as SERT)

[^{11}C]Cimbi-36: A PET receptor agonist radioligand for imaging the 5-HT_{2A} Receptor

[^{11}C]AZ10419369: A PET receptor partial agonist radioligand for imaging the 5-HT_{1B} Receptor

ACC: Anterior cingulate cortex

Amy: Amygdala

ANOVA: Analysis of variance

BG: Basal ganglia

BP_{ND}: Non-displaceable binding potential

Cau: Caudate nucleus

COV: Covariance

DA: Dopamine

DBS: Deep Brain Stimulation

dlPFC: Dorsolateral prefrontal cortex

DRN: Dorsal raphe nucleus

FC: Frontal cortex

GABA: Gamma-aminobutyric acid

GPe: External globus pallidus

GPi: Internal globus pallidus

HPLC: High performance liquid chromatography

IC: Insular cortex

LC: Limbic cortex

MDI: Major Depression Inventory

MDS-UPDRS: Unified Parkinson Disease Ranking Scale (revised by the Movement Disorder Society)

Mid: Midbrain

MIFG: Medial inferior frontal gyrus

MITG: Medial inferior temporal gyrus

mPFC: Medial prefrontal cortex

MRI: Magnetic Resonance Imaging

OFC: Orbitofrontal cortex

PC: Parietal cortex

PCC: Posterior cingulate cortex

PD: Parkinson's Disease

PFC: Prefrontal cortex

PMC: Primary motor cortex (postcentral gyrus)

PET: Positron Emission Tomography

POMS: Profile of Mood State

PPN: Pedunculopontine nucleus (Superior colliculi)

Put: Putamen

ROI: Region of Interest

SD: Standard Deviation

SEM: Standard Error of Mean

SERT: Serotonin Transporter (also known as 5-HTT)

SFG: Superior frontal gyrus

SN_r: Substantia nigra pars reticularis

SN_c: Substantia nigra zona compacta

SSC: Somatosensory cortex

SSRI: Selective serotonin reuptake inhibitor

STN: Subthalamic nucleus

STG: Superior temporal cortex

TC: Temporal cortex

Tha: Thalamus

vlPFC: ventrolateral prefrontal gyrus

V_{ND}: Non-displacable Distribution Volume

VTA: Ventral tegmental area

V_T: Volumes of distribution

VOI: Volume of interest

BACKGROUND

Parkinson's Disease

Parkinson's Disease (PD) is one of the most common neurological disorders affecting 1% of people above 60 years of age (Goetz & Pal, 2014). It was first described as a syndrome in 1817 by James Parkinson in his 'Essay on the Shaking Palsy'. Later, the legendary neurologist Jean-Martin Charcot added features to the description in his famous Salpêtrière lectures in the 1870-80's (Goetz, 2011; Obeso et al., 2017). Hoehn and Yahr introduced the first staging system (I-V) of PD in 1967 (Hoehn & Yahr, 1967) still commonly used for measuring disease progression.

PD is classified as a movement disorder characterized by four cardinal features, namely, rest tremor, bradykinesia, rigor, and postural instability. The first symptoms usually present in the 5th decade of life and rest tremor is the initial symptom in 70% of the patients, but non-tremor dominant types of PD are not rare (Winn, 2011b). The idiopathic form of PD is most common, but there exist several familial subtypes (5-10%) as well as many disorders associated with Parkinsonism (Winn, 2011a).

The histopathological and biochemical classification of PD is as an α-synucleinopathy, although several other noxious factors have also been related to PD. The α-synuclein protein deposits in the pre-synaptic terminals as aggregates of misfolded oligomers, known as Lewy bodies, which cause neuronal dysfunction and eventually lead to neuronal death (Winn, 2011a). According to Braak's observations (Braak et al., 2003), Figure 1, the neuronal damage does not appear randomly, but begins in two induction spots in the medulla oblongata from where it ascends in a topographical predictable sequence eventually reaching the cortex and very last affecting the primary motor and sensory areas. While the disease may progress in a sequential distinct manner and ultimately involve the entire brain, it is not clear to which extent neurons are affected across brain regions.

The neuropathological characterization of PD is neuronal degeneration and progressive loss of primarily dopamine (DA) producing cells in the dopaminergic nigrostriatal pathways. Eventually, this lead to dopaminergic dysfunction that underlies two of the cardinal symptoms in PD, namely bradykinesia and rigidity, but there is a preclinical phase of several years before biochemical DA deficiency presents with symptoms in patients (Winn, 2011a).

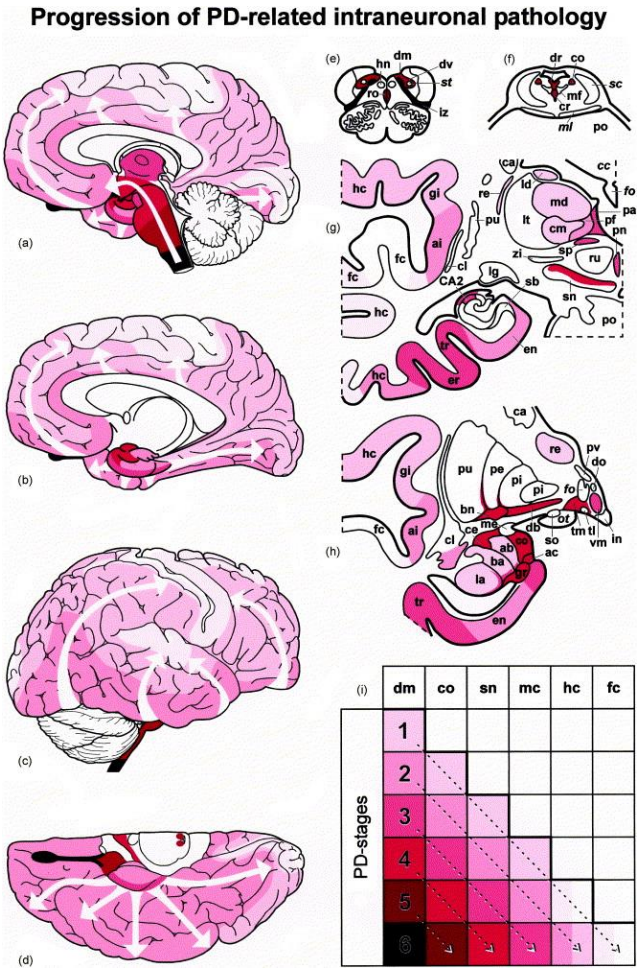

Figure 1. Progression of PD-related interneuronal pathology. Lesions begin in the IX/X motor nucleus and aolfactory nucleus and expands in the brainstem, taking an upward course (white arrows) and eventually reach the cortex. The growing severity of the pathology is represented by shading (stage 1-6) in the diagram (i) and brain structures (a-h). In stage 6, there is involvement of the entire brain, although pre- and primary motor and sensory areas may only be mildly affected.
Adapted from Braak et al, 2003.

The first modern medicine used to treat PD motor symptoms was levodopa, which was introduced in 1961 (Goetz, 2011). Levodopa has since been established as the premier agent for DA replacement therapy in PD, although other drug options are available (Goetz, 2011). Most pharmacological therapies used in PD aim to alleviate motor symptoms and executive deficits through modulation of the dopaminergic system, but increasing attention is given to neuroprotective agents and drugs targeting non-motor symptoms of PD as well as. More recently, gene-based therapies have entered clinical trials (Goetz & Pal, 2014).

Surgical therapy and Deep Brain Stimulation

Beginning as early as the 1900's, several types of surgical therapy has been attempted for treatment of PD. Although some were quickly abandoned, such as excision of cerebral cortex and arterial occlusion, other types of ablation of spinal and cerebral targets became therapeutic options. All of them became near obsolete with the advent of levodopa up through the 1960's

due to its dramatic effect on symptom relief in patients with PD (Goetz, 2011). The discovery of pharmaceutical DA replacement initiated the search of cell based DA replacement that lead to neuronal grafting starting in the 1970's. Today, experimental human embryonic stem cell transplants still have the potential to become a therapeutic option in treatment of PD (Barker, Drouin-Ouellet, & Parmar, 2015; Winn, 2011f).

Although DA replacement therapy was an enormous therapeutic breakthrough in PD, the limitations of long-term treatment with levodopa soon appeared in terms of development of side effects such as dyskinesia, motor fluctuations, psychiatric side effects and treatment resistance (Winn, 2011f). Because of the shortcomings of long-term treatment with levodopa combined with general advances in surgical procedures and safety, surgical therapy of PD regained importance during the 1990's (Duker & Espay, 2013), although the ablative procedures had largely been replaced by imitation of surgical lesions known as deep brain stimulation (DBS) (Winn, 2011c). DBS is a potentially reversible surgical treatment that deliver an adjustable high frequency stimulus to a cerebral target, e.g. the subthalamic nucleus (STN) or internal globus pallidus (GPi), by an implanted electrode connected to an impulse generating unit (IPG) placed under the skin of the chest (Figure 2). Surgical lesioning of deep brain structures, however, may still be indicated, although limited to special circumstances (Winn, 2011d).

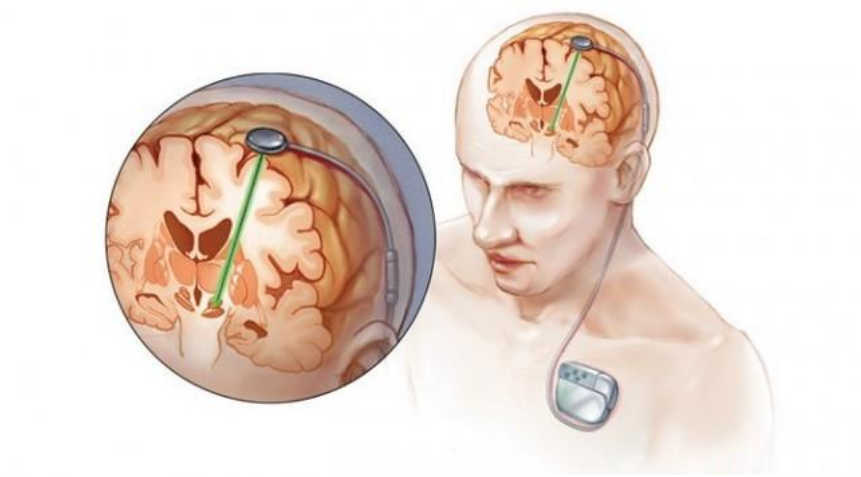

Figure 2. Illustration of the DBS system. An implanted electrode to a cerebral target is connected to an IPG unit placed under the skin of the chest.

DBS is applicable to a selected group of PD patients, but also has the potential to treat other neurological disorders, such as essential tremor, dystonia, and Tourette's syndrome as well as neuropsychiatric disorders, e.g., depression, obsessive-compulsive disorder, and addiction (Holtzheimer & Mayberg, 2011).

Even though the therapeutic effect of DBS in movement disorders is well established, the underlying mechanism is still unclear. DBS may act by inducing changes in neurotransmitter levels in target and connected regions (van Dijk, Mason, Klompmakers, Feenstra, & Denys, 2011), activation of afferents and efferents causing distant effects (Luigjes et al., 2012), long-term effects on neuroprotection and neural networks (Winn, 2011e), receptor regulations, a combination or more complex mechanisms. Understanding the DBS-induced mechanisms on relevant brain targets may contribute to improve medical therapy of such brain disorders and support the transition of DBS-treatment to patients with such disorders not controlled by medicine.

Serotonergic involvement in Parkinson's Disease

Although focus has initially been on degeneration of the nigrostriatal dopaminergic system, increasing attention has been given to other brain monoamine systems such as 5-HT, (Politis & Niccolini, 2015) beyond the nigrostriatal pathways. As reviewed (Huot, Fox, & Brotchie, 2011), there is evidence from both biochemical, imaging and post-mortem studies that 5-HT and its biomarkers are reduced in PD and various 5-HT markers present a reduction with a regional distribution distinct from that of dopamine.

5-HT is involved in regulation of several physiological functions (Barnes & Sharp, 1999). Concomitant with medical side effects, premorbid psychiatric vulnerability and psychosocial factors (Voon, Kubu, Krack, Houeto, & Tröster, 2006), deficits in the 5-HT system may be an important element underlying another clinical feature of PD, namely the so called non-motor symptoms, e.g., sleep disturbances, autonomic dysfunction, cognitive deficits, mood disorders and fatigue (Goldman & Postuma, 2014; C. P. Müller & Jacobs, 2010). Neuropsychiatric disorders, such as depression commonly seen in patients with PD, are generally associated with serotonergic dysfunction of which pharmacologically modulation of 5-HT at the synaptic level is an established treatment (Moret & Briley, 2000). The non-motor symptoms constitute a huge burden on quality of life in a vast number of PD patients with particular importance to depression and fatigue (B. Müller, Assmus, Herlofson, Larsen, & Tysnes, 2013; Politis et al., 2010). Patients often experience minor cognitive dysfunction in early stage of PD and a decline during the course of disease progression (Winn, 2011b), and dementia is common in late stage PD with a life-time incidence rate 4-6 times higher than that of age-matched controls.

While dopaminergic replacement therapy is currently effective to treat both motor and non-motor symptoms in PD, it may worsen certain non-motor symptoms and can eventually lead to complications such as motor fluctuations known as levodopa-induced dyskinesia (Chaudhuri & Schapira, 2009). Increasing attention has been given to non-dopaminergic (ND) medications targeting, e.g., the 5-HT system with the purpose to alleviate non-motor symptoms and allow for reduction in doses of levodopa treatment (Freitas & Fox, 2016).

A vast amount of research indicates that the 5-HT system is involved also in DBS treatment in PD, as DBS induce both temporary and stationary effects on mood and behavior (Castrioto, Lhommée, Moro, & Krack, 2014; Kurtis, Rajah, Delgado, & Dafsari, 2017; Voon et al., 2006),. While up to 25% of patients with PD experience affective side effects within three months of DBS surgery, other studies and multiple case series suggest that DBS may also alleviate non-motor symptoms (Castrioto et al., 2014; Kurtis et al., 2017; Voon et al., 2006). A recent review concludes that there is level I evidence on the effect of DBS on mood: improvement of anxiety has been reported by a class I trial while two randomized prospective studies reported no change in depression in PD patients treated with DBS (Kurtis et al., 2017).

Serotonergic modulation of the Basal Ganglia

The basal ganglia (BG) is generally associated with control of voluntary movements, and impaired in PD. The general concept is, that the BG exerts an overall GABAergic (inhibitory) effect on the glutaminergic (excitatory) thalamocortical projections, thereby decreasing cortical activity in motor function areas (Ding & Zhou, 2014; Miguelez, Morera-Herreras, Torrecilla, Ruiz-Ortega, & Ugedo, 2014; Politis & Loane, 2011). The STN, which is spontaneous active, exert a strong excitatory drive on the two GABAergic BG output nuclei, the GPi and SNr, thereby playing an important role in propelling the GABAergic output from the BG (Ding & Zhou, 2014).

The 5-HT system is involved in regulation of the BG, and it receives a dense innervation from DRN. The connectivity of the BG nuclei and interactions with various neurotransmitter system, such as 5-HT, is truly complex, but the combined effect of the 5-HT system on the BG nuclei is a net decrease in the GABAergic output from the BG, thereby acting to release the thalamocortical pathways to motor function areas (Ding & Zhou, 2014).

Of main importance is the STN, which receives a dense serotonergic innervation, expressed by somatodendritic excitatory (5-HT_{2C} and 5-HT_4) and inhibitory (5-HT_{1A}) receptors that modulate

the intrinsic STN activity. However, the net inhibitory effect of 5-HT on the BG is primarily driven by the presynaptic inhibitory 5-HT_{1B} receptors sited on predominantly glutaminergic (excitatory) STN afferents and efferents (Ding & Zhou, 2014), thereby reducing the intrinsic activity in the STN activity as well as decreasing presynaptic signaling at the STN axonal terminal.

Moreover, 5-HT_{1B} receptors on axonal terminals projecting from the two BG output nuclei, SNr and GPi, to the thalamocortical pathways also contribute to an overall reduction of BG output (Ding & Zhou, 2014). The 5-HT system thereby play an important role in modulating the interaction between the BG and cortical motor areas (Ding & Zhou, 2014; Miguelez et al., 2014).

The interplay with serotonergic modulation on the BG nuclei and thalamocortical projections is illustrated in Figure 3 with serotonergic action sites of main importance.

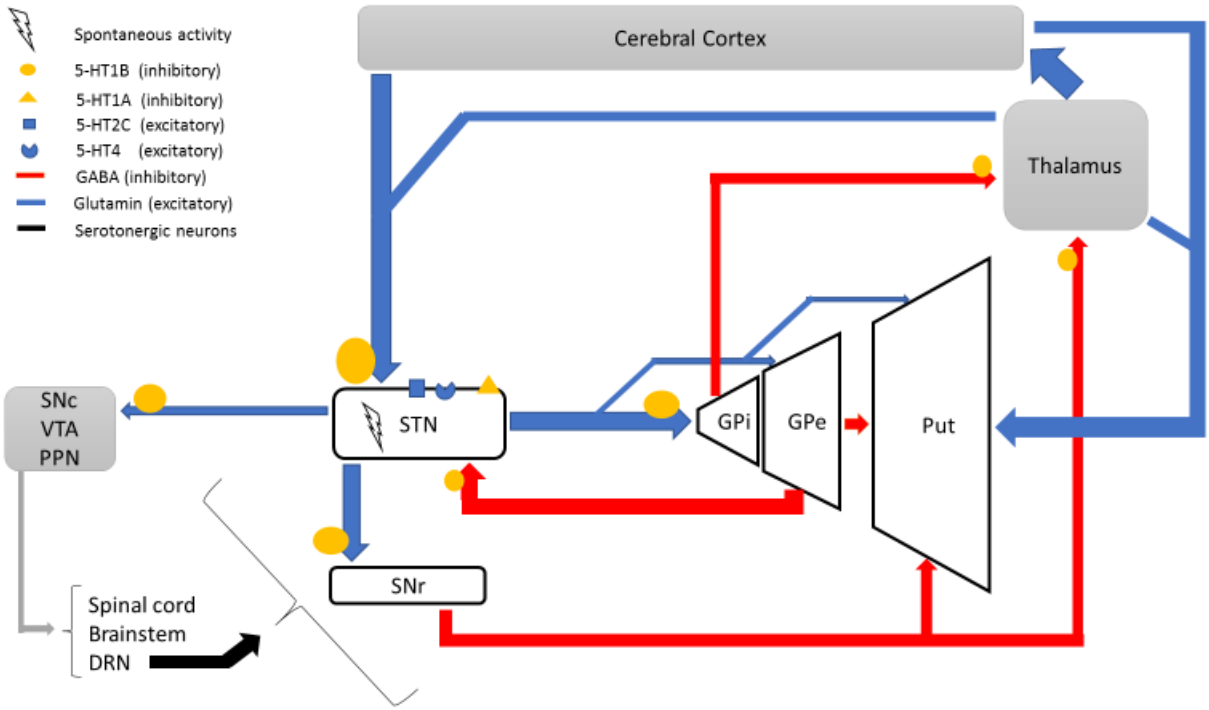

Figure 3. Serotonergic modulation of the STN and basal ganglia output. The GABAergic (red) and glutaminergic (blue) connectivity between basal ganglia nuclei (white) and extrastriatal regions (grey) are illustrated with serotonergic action points (5-HT receptors and dorsal raphe nucleus (DRN)).

In PD, the spontaneous STN firing rate is 4-6 times above normal, which result in an excessive BG GABAergic output which inhibit the thalamocortical pathways and cause motor deficits

(Ding & Zhou, 2014). Preclinical studies in non-human primates and rats show that 5-HT deficiency as well as lesions in the DRN, both known to occur in Parkinson's Disease (Huot et al., 2011), lead to an increase in STN firing rate (Ding & Zhou, 2014; Miguelez et al., 2014), which can be reverted by either serotonin reuptake inhibitors (SSRI) (Aristieta et al., 2014), 5-HT_{1B} agonists, DA replacement (Ding & Zhou, 2014), and high frequency stimulation in the STN (Meissner et al., 2005). Reduction of excessive STN firing rate is currently considered the prime candidate mechanism underlying the therapeutic effect of DBS-STN (Ding & Zhou, 2014).

As such, serotonergic degeneration could be a constituent of the pathologically high STN firing rate observed in PD, concomitant to dopaminergic deficits in the nigrostriatal pathways. The 5-HT system tempers the BG output associated with voluntary motor control, and impaired serotonergic modulation of the BG, particularly the STN, may thus worsen motor symptoms in PD beyond the theory (Politis et al., 2014) of serotonergic neurons mishandling exogenous levodopa associated with levodopa-induced dyskinesia (LID).

Serotonergic neurotransmission

5-HT is a neurotransmitter derived from the essential amino acid tryptophan. It is synthesized inside the pre-synaptic neuron and stored in vesicles until released by exocytosis into the synaptic cleft upon activation of the pre-synaptic neuron. Here, 5-HT acts by rapid diffusion and binding to its receptor, thereby initiating a response (Figure 4). The 5-HT action is terminated by either; 1) diffusion of 5-HT away from the synaptic junction; 2) reuptake of 5-HT back into the pre-synaptic terminal by the serotonin reuptake transporter (SERT), where it is restored in pre-synaptic vesicles; or 3) metabolism of excess 5-HT in the synaptic cleft or presynaptic cytosol by monoamino-oxidase (MAO) (Devlin, 1997; Guyton & Hall, 1996).

There are seven major classes of 5-HT receptors (5-HT_{1-7}) divided in fourteen mammalian 5-HT receptor subtypes. Each receptor subtype acts by specific cellular mechanisms, has a distinct regional distribution in the brain and are involved in a wide range of various behavioural and physiological responses (Barnes & Sharp, 1999; Beliveau et al., 2017). Most of the 5-HT receptors are located post-synaptically, where they act as 5-HT heteroreceptors modulating other neurotransmitter systems such as e.g. DA, acethylcholine, GABA, and glutamate. Other 5-HT receptors are located pre-synaptically such as 5-HT_{1A} or 5-$HT_{1B/1D}$ auto- and heteroreceptors

modulating the firing rate, synthesis, and release of neurotransmitter into the synaptic cleft (Barnes & Sharp, 1999; Fuller, 1994).

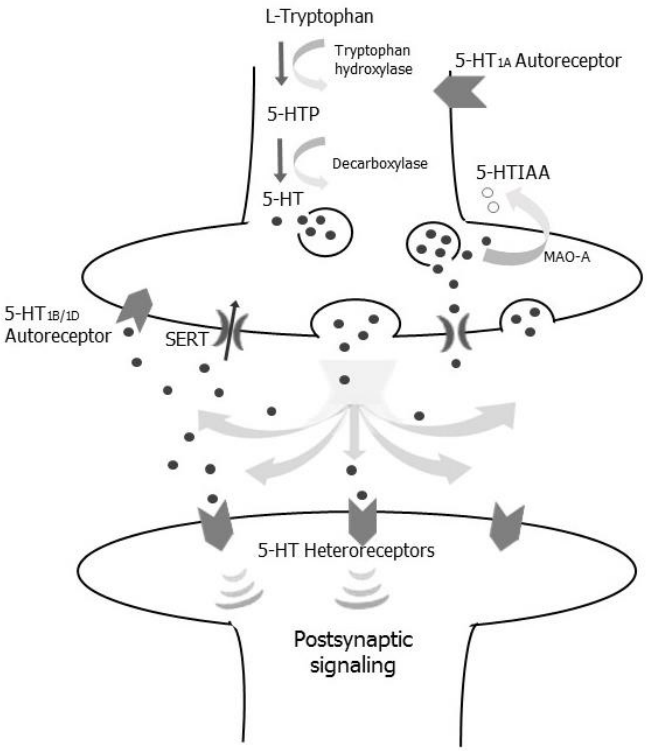

Figure 4. Illustration of the synaptic terminal and the main steps of 5-HT synthesis, metabolism, and neurotransmission.

An immense amount of research has explored 5-HT action and intervention sites of drugs aiming to modulate synaptic neurotransmission, and some are used in medical therapy of cardiological and neuropsychiatric disorders. Depression is common in patients with PD and approximately one quarter is taking antidepressants, such as SSRIs (Richard, Kurlan, & Parkinson Study Group, 1997). Whereas MAO-inhibitors mainly increases intracellular 5-HT (Perry & Fuller, 1992), the SSRIs lead to accumulation of extracellular 5-HT resulting in an increased and prolonged postsynaptic signalling (Invernizzi, Belli, & Samanin, 1992; Perry & Fuller, 1992). However, although SSRIs increase synaptic 5-HT concentration, it also leads to adaptive changes as of a decrease in the firing rate of the serotonergic neuron and dampening the effect on extracellular 5-HT level (Artigas, Romero, de Montigny, & Blier, 1996). Although these autoregulatory processes lessen with prolonged use of SSRIs, the time-delay can be surpassed by adding a 5-$HT_{1A/1B}$ autoreceptor antagonist, such as pindolol (Artigas et al., 1996; Fuller, 1994). And so it has also been speculated if these autoregulatory processes is an underlying mechanism behind the delayed onset of therapeutic effect of antidepressant treatment with SSRIs (Fuller, 1994), and if adding a 5-$HT_{1A/B}$ antagonist could reduce this delay (Artigas et al., 1996; Whale, Terao, Cowen, Freemantle, & Geddes, 2010).

PET imaging and the competition model

Positron Emission Tomography (PET) is a nuclear imaging technique that allows for detection and quantification of neuroreceptors as well as measuring dynamic changes in synaptic neurotransmission *in vivo*. The PET scanner detects the emission from a radioligand (tracer) injected into the individual placed in the scanner, and the recordings are then computed into 3-dimensional images of the radioligand concentration in e.g. the brain. The injected radioligand is made by labelling an isotope, such as ^{11}C, to a biologically active precursor with the ability to access and bind to a specific target of interest, e.g. the 5-HT_{1B} receptor in the brain.

The principle of PET imaging is based on measuring the binding of the PET radioligand relative to the synaptic neurotransmitter level known as the competition model (Figure 5). The PET radioligand and the neurotransmitter compete with binding to the same receptor, so when the synaptic neurotransmitter level increase then the PET radioligand binding decrease.

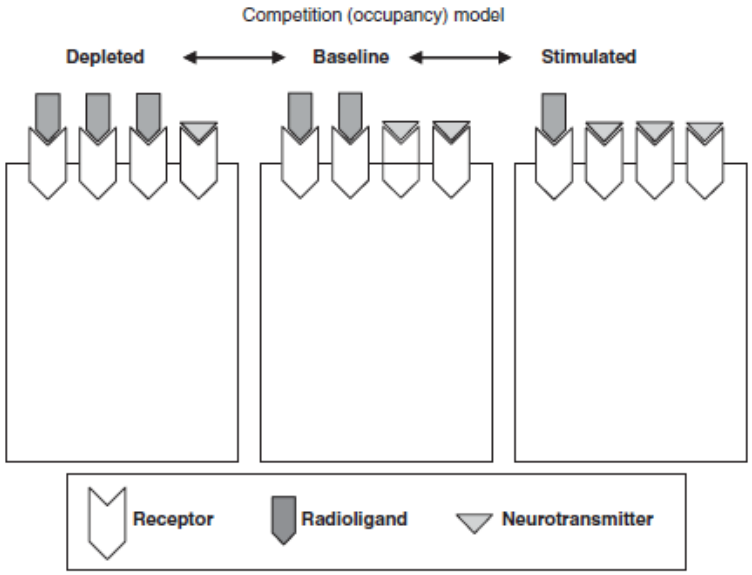

Figure 5: The figure shows binding of the neurotransmitter and the tracer to its receptor in three situations; at baseline and at low (depleted) or high (stimulated) synaptic neurotransmitter level. Adapted from Paterson et al., 2010

This relationship can be described according to the competition model (Paterson, Tyacke, Nutt, & Knudsen, 2010).

$$Occupancy = \frac{\Delta F_{NT}}{K_{NT}+F_{NT}+\Delta F_{NT}} \qquad \text{(eq.1)}$$

Three factors determine the ability of a radioligand to detect changes in synaptic neurotransmission: affinity of the neurotransmitter for its receptor, K_{NT}, the basal neurotransmitter concentration in the interstitial fluid, F_{NT}, and the change in neurotransmitter concentration, ΔF_{NT} (Paterson et al., 2010). This means, the PET radioligand detect changes in binding more readily, if the changes in neurotransmitter concentration, ΔF_{NT}, are much larger as compared $K_{NT} + F_{NT}$.

Direct measures of changes in extracellular 5-HT level relative to baseline, such as done with microdialysis, and simultaneous measures of the corresponding PET occupancy would serve to confirm that the PET radioligand comply with the competition model (eq 1) and allow for calculation of K_{NT}. Moreover, plotting this relationship would be an important tool when interpreting the outcome of future PET studies. As such, diagrams of the occupancy model allow to translate PET imaging into changes in extracellular cerebral 5-HT level.

PET radioligands

Several studies have investigated PET radioligands for their ability to be displaced by endogenously released 5-HT elicited by a serotonergic challenge. From a clinical perspective, the ability of PET radioligands to image changes in serotonergic neurotransmission would be an important tool for investigating the serotonergic mechanisms and specific 5-HT subtypes involved in neuropsychiatric disorders and to identify therapeutic options. While PET radioligands more readily detect large increases in synaptic 5-HT level elicited by potent serotonergic challenges (e.q.1), the sensitivity may be limiting with weaker serotonergic challenges. Outcomes from clinical trials have not yet convincingly shown that the radioligands were capable of detecting the expected effect of pharmacological interventions supposed to increase the extracellular 5-HT level (S. J. Finnema et al., 2015; Paterson et al., 2010).

In this thesis, two PET radioligands targeting the 5-HT_{1B} and the 5-HT_{2A} receptors are investigated to assess if the binding behavior complies with the competition model. Both receptors have been associated with neuropsychiatric disorders and considered potential targets for investigation of disease mechanisms and pharmacological therapy.

Several neuropsychiatric disorders such as depression, anxiety and obsessive compulsive disorder (Moret & Briley, 2000), aggression and psychopathy (da Cunha-Bang et al., 2016) have been associated with 5-HT_{1B} receptor function and to modulation of the BG output which is

impaired in PD (Ding & Zhou, 2014). The 5-HT_{2A} receptor is the main excitatory 5-HT receptor and has been associated with hallucinogenic effects, depression and schizophrenia and is a target for antipsychotic drugs action with 5-HT_{2A} receptor antagonists (Barnes & Sharp, 1999).

In humans, the 5-HT_{2A} receptor has a dense cortical distribution across brain regions, while the 5-HT_{1B} receptor has the most dense locations in the subcortical regions and in the occipital cortex (Beliveau et al., 2017). The occurrence of 5-HT_{1B} autoreceptors versus heteroreceptors across brain regions are unknown, and PET radioligands does not allow to discriminate between them.

5-HT_{2A} receptor PET radioligands

The 5-HT_{2A} antagonist radioligand [^{18}F]altanserin, was used in three human studies, where two of them demonstrated a decrease in radioligand binding in several brain regions following a serotonergic challenge of clomipramine (Larisch et al., 2003) and dexfenfluramine (Quednow et al., 2012), while the third study showed an increase in radioligand binding during sleep, consistent with the decreases in 5-HT level known to occur during sleep (Elmenhorst, Kroll, Matusch, & Bauer, 2012). On the other hand, these encouraging outcomes were not supported in three other human studies using [^{18}F]altanserin and [^{18}F]setoperone, which is another 5-HT_{2A} antagonist, where there were no displacement of the radioligand following a pharmacological challenge of either paroxetine (Meyer, Cho, Kennedy, & Kapur, 1999), intravenous ketamine (Matusch et al., 2007) or citalopram plus pindolol (Pinborg et al., 2004).

However, it has been suggested that agonist radioligands are more sensitive to changes in neurotransmitter level as compared to antagonists (Narendran et al., 2004; Willeit et al., 2007), which would favour the use of agonist radioligands. Not long ago, the first 5-HT_{2A} receptor PET radioligand agonist [^{11}C]Cimbi-36 were developed and assessed in humans (Ettrup et al., 2014), and a recent study in non-human primates indicates that [^{11}C]Cimbi-36 is sensitive to changes in 5-HT level induced by a high dose of the potent 5-HT releaser fenfluramine (5 mg/kg) (Yang et al., 2017). In this thesis, we investigate the sensitivity of [^{11}C]Cimbi-36 to detect changes in 5-HT level.

5-HT_{1B} receptor radioligands

The 5-HT_{1B} receptor partial agonist radioligands [^{11}C]AZ10419369 and the 5-HT_{1B} receptor antagonist [^{11}C]P943, used in challenge-studies in non-human primates, have shown a dose-dependent decrease in radioligand binding across brain regions following a serotonergic

challenge of fenfluramine or a high dose of escitalopram (a SSRI) (Cosgrove et al., 2011; S.j. Finnema et al., 2010; Nord, Finnema, Halldin, & Farde, 2013).

However, strong pharmacological serotonergic challenges are not applicable in human studies, and instead SSRI in a therapeutic dose is often used as a tool to induce 5-HT increases in the human brain. Three human studies have investigated the sensitivity of a PET radioligands to detect changes in 5-HT level following a clinically relevant dose of SSRI. A PET study with [^{11}C]AZ10419369 (Nord et al., 2013), showed an *increase* in binding following a clinical relevant dose of escitalopram, which was also observed in another PET study with [^{11}C]CUMI-101 (Selvaraj et al., 2012), a 5-HT$_{1A}$ receptor agonist following citalopram. On the other hand, this unexpected finding was not seen in another [^{11}C]CUMI-101 study (Pinborg et al., 2012). These studies question if an acute intervention with SSRI is capable of eliciting an increase in synaptic 5-HT concentration, as both studies interpreted the unexpected finding in humans as being caused by autoreceptor function, where stimulation of 5-HT$_{1B}$ or 5-HT$_{1A}$ autoreceptors inhibit 5-HT release leading to a *decrease* in synaptic 5-HT level (Nord et al., 2013; Selvaraj et al., 2012).

Such paradoxical SSRI-induced effects in serotonergic neurotransmission have not been observed in animals with microdialysis, which is the state-of-the-art measurement used to investigate changes in neurotransmitter levels (Cosford, 1996). Microdialysis studies show, that 5-HT$_{1B}$ autoreceptor function can dampen or even cancel the SSRI-induced effect on 5-HT release in a region-dependent matter, but autoregulation does not *decrease* the 5-HT level as compared to baseline level (Artigas et al., 1996; Gobert, Rivet, Cistarelli, & Millan, 1997; Hjorth, 1993). Still, microdialysis studies in rodents only investigate SSRI doses manifold above the therapeutic interval, which may explain the conflicting outcomes in the human and non-human primate PET studies. The matter would be easily settled, however, in the presence of combined study of PET imaging and a direct measure of changes in cerebral 5-HT level, such as microdialysis.

Serotonergic PET studies in PD

Neuroimaging with PET offer a unique opportunity to investigate the serotonergic system in patients with PD. Table 1 on the next page presents an overview of previous PET studies in non-depressed patients PD, as previously reviewed (Huot et al., 2011; Varrone et al., 2014).

Several studies describe a considerable and significant decrease in the 5-HT transporter (SERT) availability, while the somatodendritic receptors (5-$HT_{1A,}$ 5-HT_{2A}, 5-$HT_{3,}$ and 5-HT_4) seem less affected, or even increased (Huot et al., 2011). One study showed a reduction in the presynaptic 5-HT_{1B} receptor availability in the orbitofrontal cortex (Varrone et al., 2014). To the best of our knowledge, there are no scientific reports so far on the DBS-induced effect on serotonergic neurotransmission in humans, most likely because the technology for such an investigation has been missing.

The following will describe the association between microdialysis and PET measures of two radioligands, the 5-HT_{2A} receptor agonist [^{11}C]Cimbi-36 and the 5-HT_{1B} receptor partial agonist [^{11}C]AZ10419369. The latter was subsequently used to investigate group differences in 5-HT_{1B} receptor availability between patients with PD treated with DBS compared to controls and the change in regional cerebral 5-HT level, when DBS is turned off.

BP_{ND} RATIO (PD / HC)							FRONTAL								TEMPORAL			PARIETAL		LIMBIC					OCCIPITAL	SUBCORTICAL						MIDBRAIN	
Target	Study	PD (n)	HC (n)	Mean age (PD,HC)	HYR (med.off)	PET radioligand	FC	PMC	PFC	SFG	dlPFC	OFC	MIFG	vlPFC	TC	STG	MITG	PC	SSC	LC	IC	ACC	PCC	Amy	OC	vStr	Cau	Put	Tha	SNr	Gpi	Mid	DRN
SERT	**In vivo**																																
	Politis 2010	10	10	67, 64	3.4 (off)	DASB			**.60**												**.44**	**.70**	**.64**	**.75**			**.69**	**.66**	**.70**	**.71**				**.80**
	do	10	10	62, 64	3.1 (off)	DASB			**.60**												**.58**	**.70**	**.69**	(.83)			(.86)	**.70**	**.77**	**.76**				(.84)
	do	10	10	66, 64	1.9 (off)	DASB			(.70)												(.67)	**.68**	(.78)	(.87)			(.87)	**.72**	(.87)	**.83**				(.85)
	Guttman 2007	9	13	65, 63	2.6	DASB					(.93)	**.78**									(>.90)	(>.90)				(>.90)		**.70**	**.64**	(> .95+)			**.71**	
	Strecker 2011	9	9	59, 57	1.7	DASB					(1.04 | 1.04)			(1.01)						(1.01)	(1.05)		(1.02)		(1.01)		(1.00)	(1.03)	(1,00)			(1.07)	(1.08)
	Albin 2008	5	8	64 ,53	1.7	DASB															**.53**	**.53**	**.48**	**.64**			**0.68**	**.68**	**.69**	**.71**	**.77**		**.73**	
	Kerenyi 2003	13	13	65, 63	2.1	McN5652																						**.50**	**.65**	(.75)				
	In vitro																																
	Kish 2008	23	31	75, 76	4.1	SERT immunoreactivity																						.45	(.70)					
	D'Amato 1987	7	7	71, 76	-	[3H]citalopram	**.52**								**.53**																			
	Chen 1998	8	20	-	-	Paroxetine						**.77**					**.98**																	
	Chinaglia 1993	11	8	81, 81	-	[3H]citalopram	**.39**														**.25**					**.29**	**.57**	**.39**	**.46**		**.52**	**.43**		(.79)
5-HT_{1B}	**In vivo**																																
	Varrone 2014	12	12	65, 65	1.7 (1.5)	[11C]AZ10419369						↓*																						
	do	12	12	65, 65	1.7 (1.5)	[11C]AZ10419369					(.96)	**.86**	(.97)		**.86**							**.79**	(.85)	(.91)	(.93)		(.89)	(.96)	(1.04)	(.98)	(.90)			
5-HT_{1A}	**In vivo**																																
	Ballanger 2011	8	7	63, -	-	[18F]MPPF							↓*			(↓)	(↓)		(↓)		↓*	(↓)					(↓)							
	Doder 2003	23	8	58, 57	-	[11C]WAY 100635																											**.77**	
	In vitro																																
	Chen 1998	8	20	-	-	Paroxetine						**.69**					**.74**																
5-HT_{2A}	**In vitro**																																
	Chen 1998	8	20	-	-	[3H]Ketanserin						(.74)					(.79)																
	Cheng 1991	6	6	73, 78	-	[3H]Ketanserin									**.86**																		
	Maloreaux 1988	21	21	71, 72	-	[3H]Ketanserin	(.83)								**.60**														(.82)				
	Perry 1984	3	7	78, 81	-	[3H]hydroxytrypta mine												(1.37) (0.94)													ns			
5-HT_{2C}	**In vitro**																																
	Fox and Brotchie	5	6	81, -	[3-5]	[3H]mesulergine / SB200646A																						(.92)	(.77)		**2.08**	(.68)		
5-HT_{3}	**In vitro**																																
	Steward 1993	6	5	68, -	-	[3H]granisetron																						(.94)						
5-HT_{4}	**In vitro**																																
	Reynolds 1995	6	6	-	-	[3H]GR 113808																							(.81)		(.76)			

Signatures

Bold: Significant difference in BP_{ND} between groups

Parentheses ( ) : Non-significant

↓ : Parametric analysis, decreased BP_{ND} in PD as compared to controls

Table 1. Overvirew of PET studies in non-depressed patients with PD

AIMS AND HYPOTHESES

The overall aim of this thesis is to characterize the association between changes in extracellular cerebral 5-HT level and PET measures of 5-HT release, which will serve as a tool for translating the outcome from PET imaging of the entire brain into regional changes in extracellular 5-HT level. Diagramming the association between microdialysis and PET measures of cerebral 5-HT level would be a useful tool for translating the outcome from future PET imaging studies, such as conducted here in a PET study of patients treated with DBS.

We will do so by measuring 5-HT with simultaneous PET and microdialysis in the pig brain following various pharmacological interventions aiming to raise the cerebral 5-HT level differently. Then we will diagram and fit the data according to the competition model (eq.1). Then, we will use this diagram to interpret the outcome from a clinical PET study when DBS is turned off in patients with PD and finally, we will correlate the outcomes from PET with clinical measures of Parkinson's patients treated with DBS.

Study 1

The aim of study 1 was to assess the effect of different serotonergic challenges on the cerebral 5-HT level as measured by microdialysis and to correlate this outcome to the occupancy of the 5-HT_{2A} receptor agonist PET radioligand [^{11}C]Cimbi-36. We obtained simultaneous measures of the pharmacologically induced changes in the 5-HT level in the medial prefrontal cortex (mPFC) and changes in [^{11}C]Cimbi-36 binding *in vivo* in the pig brain. We hypothesized that the different challenges would induce varying increases in the regional cerebral 5-HT level as measured with microdialysis, and a corresponding decline in binding of [^{11}C]Cimbi-36 to the 5-HT_{2A} receptor consistent with the competition model.

Study 2

The aim of study 2 was to measure the effect of serotonergic challenges - also in clinically relevant doses - on the cerebral 5-HT level as measured by microdialysis and to correlate this outcome to the non-displaceable binding potential (BP_{ND}) of the 5-HT_{1B} receptor partial agonist PET radioligand [^{11}C]AZ10419369 *in vivo* in the pig brain. We hypothesized that the challenge-induced acute increases in extracellular brain 5-HT level were associated with a decline in binding of [^{11}C]AZ10419369 to the 5-HT_{1B} receptor binding consistent with the competition model.

Study 3

The aim of study 3 was to investigate changes in cerebral 5-HT levels, as indexed by regional binding of the 5-HT_{1B} receptor partial agonist PET radioligand [^{11}C]AZ10419369, when DBS was turned off in patients with PD treated with DBS-STN. A group of age-matched healthy controls was included to identify group differences in baseline 5-HT_{1B} receptor availability and to investigate if such a difference in 5-HT_{1B} receptor availability was associated with clinical characteristics of PD.

We hypothesized that the extent to which patients with PD had regional lower 5-HT_{1B} receptor availability would be proportional to symptom severity, as measured by motor scores. Secondly, we hypothesized that turning off DBS-STN in patients with PD would be associated with a change in cerebral 5-HT, as indexed by an inverse change in 5-HT_{1B} receptor binding of [^{11}C]AZ104193695. Thirdly, we anticipated a concomitant worsening of mood symptom scores with DBS turned off, proportional to the change in cerebral 5-HT level or the 5-HT_{1B} receptor binding at baseline level.

MATERIAL AND DESIGN

Study population

Animals (study 1-2)

Danish Landrace female pigs age 9-10 weeks were used in study 1 (N =13) and study 2 (N = 10) with a mean ± SD weight of 24 ± 1.5 kg and 22 ± 1.3 kg respectively. The pigs were delivered from a local farmer, housed and acclimatized for 1 week prior to the investigation.

Humans (study 3)

We included 13 patients with PD treated with DBS-STN, which were recruited from the Movement Disorder Clinic at the Department of Neurology in Bispebjerg Hospital by their consultant neurologist. Eleven age-matched healthy volunteers served as controls; six of whom had entered in parallel studies (da Cunha-Bang et al., 2017; Deen et al., 2017). One control had to discontinue for reasons of discomfort while being placed in the PET scanner, so the final group of age-matched controls included 10 participants. Demographics of all participants are shown in Table 2 and detailed in Paper 3.

The participants were interviewed and selected according to the in- and exclusion criteria. General exclusion criteria were severe or symptomatic medical, psychiatric or neurological illness not related to Parkinson's Disease, use of medicine which may influence the research results, severe cognitive deficits or Mini Mental State Examination (MMSE) (Folstein, Folstein, & McHugh, 1975) scores < 27, severe hearing or visual impairment, non-fluent Danish speakers and current substance or alcohol abuse. All participants complied to these criteria. Specific inclusion criteria for the patients were treatment with DBS-STN and exclusion criteria were DBS surgery within 3 months from PET scan or dysregulated PD.

None of the participants presented with abnormalities on their cerebral Magnetic Resonance Imaging (MRI), at least not when age was considered. Some participants appeared with slightly elevated blood cholesterol, but blood chemistry was otherwise unremarkable. All participants were screened drug negative on urine (Rapid Response^{TM} Multi-Drug Test Panel; BTNX Inc., Markham, Ontario, Canada) except for one patient who reported having self-medicated an acute onset of low back pain with a single tablet of 5 mg morphine 5 days prior to the scanning.

Patient	Sex	Age	Years since surgery	L-DOPA equivalents	UPDRS DBS-ON	UPDRS-motor DBS-ON	UPDRS-motor DBS-OFF	HYR DBS-OFF
1	M	55	7	531	n.a	n.a	n.a	2
2	M	66	1.7	469	48	20	47	2
3	M	53	3.4	967	15	7	34	2
4	M	59	2.7	391	34	25	66	2
5	M	72	5.5	815	44	11	37	3
6	M	67	0.5	430	9	0	26	2
7	F	56	0.8	532	21	4	15	1
8	F	63	2.5	479	22	7	41	2
9	F	50	1.2	500	36	7	55	2
10	F	65	1.8	305	36	7	29	2
11	M	50	2.6	385	36	7	28	2
12	M	62	2.6	1896	73	27	41	2
13	M	56	0.7	835	22	0	23	2
Mean ± SD		60±7	2.5 ± 1.8	656 ± 405	33 ± 16	10 ± 9	37 ± 14	2.0 ± 0.4

Table 2 (Paper 3). Patient characteristics.
Male (M), Female (F), levodopa (L-DOPA), Deep Brain Stimulation (DBS), Unified Parkinson Disease Rating Scale (UPDRS) and motor scores part III (UPDRS-motor), Hoehn and Yahr Rating scale (HYR).

Experimental day and study design

Animals (study 1-2)

An overview of the experimental day is given in Figure 6. On the morning of investigation, we performed tranquilization, anaesthesia, intubation, installation of arterial and venous intravenous lines followed by continuous monitoring until sacrifice in the late afternoon as previously detailed (Andersen et al., 2015). After the pre-operative anaesthetic procedures, the pig underwent surgery with implantation of microdialysis probes. The surgical procedure is detailed in Paper 1. Next, the pig was transported to the PET scanner where it was placed in the prone position and allowed a two-hour wash-out period in order to let neurotransmitters reach baseline level.

The pigs were PET scanned twice with an interval of 30 minutes. Microdialysis sampling was conducted throughout in 15 minutes samples. The serotonergic challenge was given prior to the second scan (study 1) or within scans (study 2). After the second PET scan the pig was euthanized (study 2) or taken for a MRI scan before euthanizing (study 1). Finally, brain tissue

from the site of the implanted microdialysis probes were excised before disposing the pig. The brain tissue was quickly frozen on dry ice and stored in a -80C freezer until further analyses.

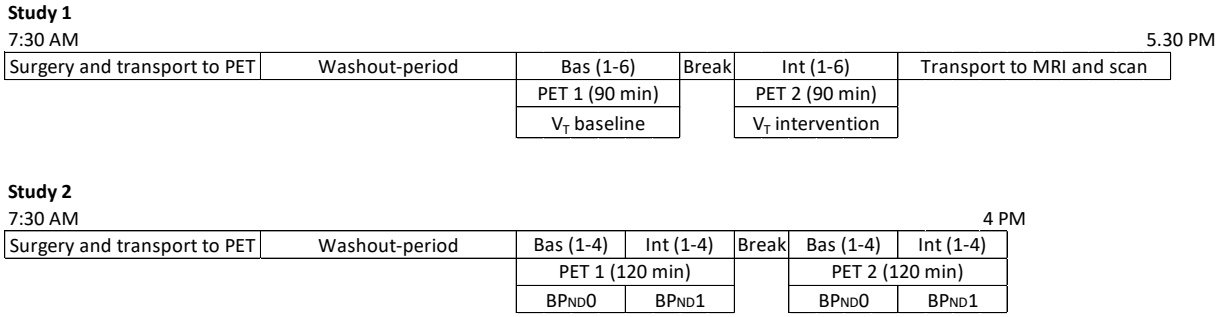

Figure 6. Schematic overview of the experimental day in study 1-2. Microdialysis sampling was conducted throughout the PET scans at baseline (Bas) and following the intervention (Int) in 15 minutes samples. In study 1, the pharmacological interventions were given in between PET scans and the distribution volumes of the radioligand (V_T) were calculated from each scan. In study 2, the interventions were given as a within scan challenge of saline in the first PET scan and a serotonergic challenge in the second PET scan with $BP_{ND}0$ (baseline) and $BP_{ND}1$ (intervention) obtained from both scans.

Humans (study 3)

The day before the PET scan, patients were admitted to the Department of Neurology and offered admission until the following day of the PET scan for reassurance after having their DBS turned off. On the day of PET scan, the patient was attended by their designated DBS nurse and a neurosurgeon. After the PET scan, upon return to the ward, all patients reported themselves in habitual condition and were discharged by own choice with the exception of 3 patients living alone or at long distance from the hospital, who decided to stay overnight as planned.

The study design is illustrated in Figure 7. In all patients, the IPG unit was turned off 45 minutes from start of PET scan in a within scan design. The PET scan was analysed in all participants according to the two conditions: $BP_{ND}0$ (DBS-ON equal to 0-44 min) and $BP_{ND}1$ (DBS-OFF equal to 45 min - end of scan) with the occupancy being calculated as the relative change in BP_{ND}. All patients were assessed at baseline with POMS and MDS-UPDRS (part I-IV) in the DBS-ON condition with repeated measures of POMS and MDS-UPDRS (part III) post scan in the DBS-OFF condition to allow for measures of change in mood and motor scores. The age-matched controls were also evaluated twice with POMS with the exception of the healthy controls, who had entered parallel studies and not undertaken a second POMS.

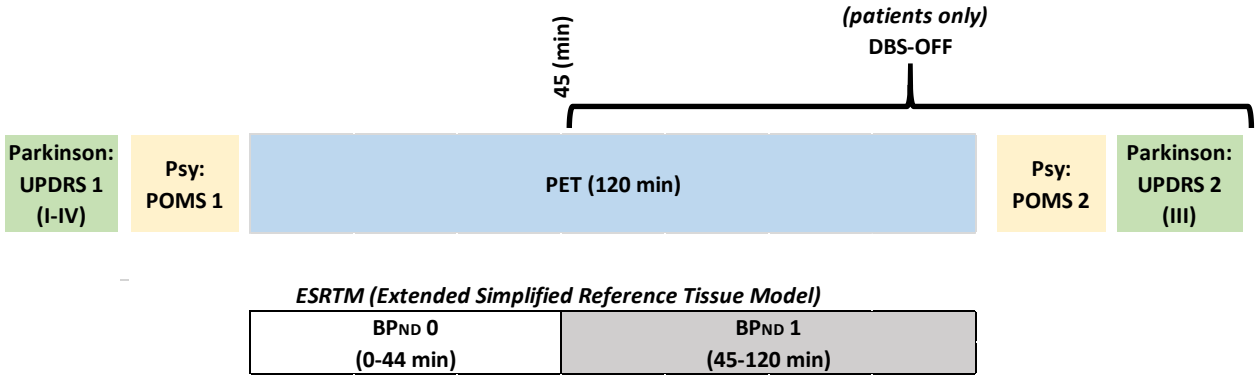

Figure 7 (Paper 3). Experimental design in the study 3. Unified Parkinson's Disease Rating Scale (UPDRS) revised by the Movement Disorder Society UPDRS part I-IV, neuropsychological Profile of Mood Scale (POMS) and non-displacement binding potential of the PET radioligand (BP_{ND}).

Interventions and blinding

Pharmacological interventions (animals)

The serotonergic challenges used in study 1 and 2 aimed to increase the extracellular 5-HT concentration in the pig brain to various degrees as measured by simultaneously microdialysis and PET. An overview of the pharmacological interventions is given in Table 3, and their different actions points in the serotonergic neurotransmission are illustrated in Figure 8.

Escitalopram (SSRI) act by blocking the SERT hereby increasing synaptic 5-HT concentration. Although studies generally investigate SSRIs in doses manifold above the therapeutic interval, such as done in study 1, escitalopram is given in a therapeutic relevant dose in study 2.

Pindolol (5-HT_{1A} and 5-HT_{1B} receptor antagonist) was given to a subgroup of pigs in addition to escitalopram with the purpose to block autoreceptor functions.

Fenfluramine acts as a potent 5-HT releaser.

Para-chlorophenylalanine (pCPA) acts as an irreversible inhibitor of the enzyme tryptophan hydroxylase, which catalyses the rate-limiting step in the 5-HT biosynthesis (Javed, Van De Kar, & Gray, 1997). A subgroup of pigs in study 1 underwent a four-day pre-treatment regime with pCPA or saline prior to the experimental day according to a validated paradigm (Ettrup,

Kornum, Weikop, & Knudsen, 2011) with the purpose to substantially reduce the 5-HT level in the brain. This would serve to confirm that the fenfluramine-induced displacement of the radioligand was in fact driven by 5-HT release and not caused by direct interaction between fenfluramine and the 5-HT receptor as speculated by others (S. J. Finnema, Varrone, Hwang, Halldin, & Farde, 2012).

Saline interventions were used as a control.

Table 3. Pharmacological interventions used in study 1-2.

	Study 1			Study 2		
Interventions	**Dose mg/kg**	**Pigs N**	**Time to start PET 2**	**Dose mg/kg**	**Pigs N**	**Time from start PET 1 and 2**
Saline	-	2	-15 min	-	(10)	56.5 min
Escitalopram	2.0	2	-15 min	0.28	5	56.5 min
Pindolol +Escitalopram	1.0 / 2.0	3	-30 /-15 min			
Fenfluramine	0.5	4	-15 min	0.5	5	56.5 min
Fenfluramine (pCPA pre-treatment)	0.5	2	-15 min			

The dose (mg/kg), number of pigs (N) and time of intervention are listed for all interventions given as intravenous injections.

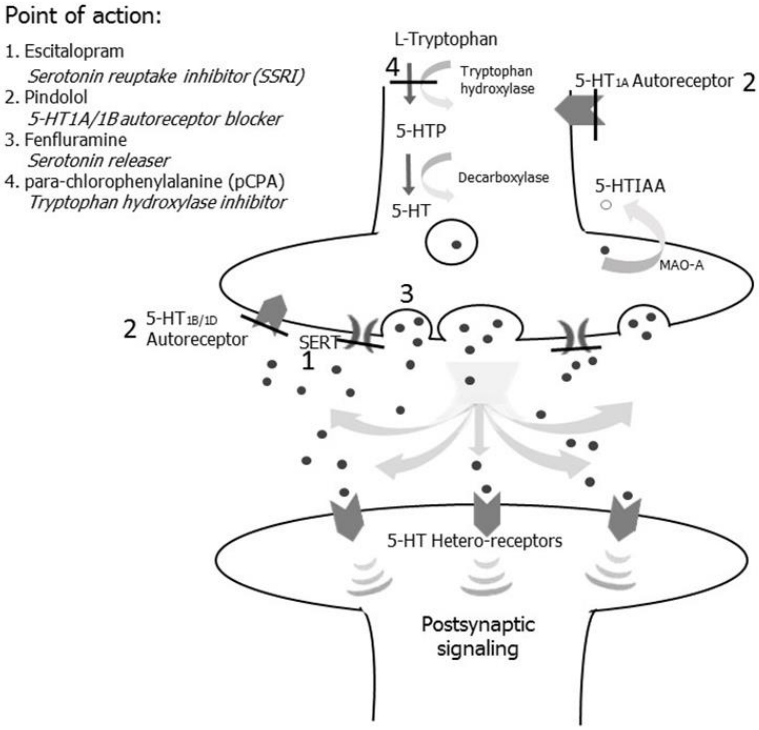

Figure 8. Illustration of the synaptic serotonergic neurotransmission with action points of the pharmacological interventions used in study 1-2.

Unexpectedly, two of the four pigs assigned for fenfluramine in study 1, did not elicit a serotonergic response as evaluated by microdialysis, PET, and clinical observation. These two studies were conducted 5 months after the previous fenfluramine experiments, which led to speculations of whether fenfluramine had either decomposed in between experiments or the content had been replaced by some unknown inactive material. A new batch of fenfluramine was acquired and compared to the remaining powder in the bottle by visual inspection, HPLC analysis, and new trials in mice and pigs, which all supported the assumption that the original content in the bottle had been replaced. These two pigs were then excluded from the group analyses of fenfluramine, but they were not excluded from the correlation analysis of microdialysis and PET measures.

Deep Brain Stimulation (humans)

The DBS was turned off 45 minutes into the PET scan starting from the time of radioligand injection. The DBS was turned back on after the patient had completed the PET scan and the subsequent assessments of POMS and MDS-UPDRS (part III). The patients were allowed to take their regular medication during the experimental day.

The investigators were not blinded from the interventions.

Measures

Microdialysis

Animals (study 1-2)

Procedural details of the microdialysis properties regarding probes, perfusion and recovery, biochemical assays of high-performance liquid chromatography (HPLC) and computation are described in Paper 1-2. A sample of the excised pig brain block was homogenised and analysed in order to measure the 5-HT concentration (ug/g brain tissue) in the subgroup of pigs who underwent pre-treatment with saline or pCPA as detailed in Paper 1.

The baseline 5-HT level was delineated as means of three samples collected prior to the intervention and set to 100%. The 5-HT level in the following samples were calculated as relative to baseline level. Microdialysis experiments do not generate absolute 5-HT

concentrations unless performed as a zero net flux microdialysis study (Olson & Justice, 1993), which has been done in mice (Calcagno, Canetta, Guzzetti, Cervo, & Invernizzi, 2007; Deltheil et al., 2008; Gardier et al., 2003; Guiard et al., 2008; Tao, Ma, & Auerbach, 2000). This is a very time-consuming method and incompatible with the use of short-lived radioisotypes and the need to conduct two PET studies. Instead, the stable baseline level (100%) of pig cerebral interstitial fluid 5-HT was assumed to be about 1.7 nM, equal to what had been measured in mice. ΔF_{NT} was then computed as 1.7 nM times the relative peak increase in 5-HT level minus 1.7 nM.

Magnetic Resonance Imaging

Animals (study 1)

All pigs had a postoperative MRI from a 3.0 T MRI Siemens VERIO MR scanner, which served to verify the correct position of the microdialysis probe in the gray matter of the pig brain. This was done by manual co-registration of the MRI to a histology slice by use of the Brainlab Stereotactic Planning Software.

Humans (study 3)

For purpose of co-registration and alignment to the PET scan, an MRI brain scan was conducted in-house on a 3 T MRI Prisma scanner (Siemens, Erlangen, Germany) using a 64-channeled head coil to obtain structural T1 and T2 weighted whole brain images in all age matched controls, with the exception of one participant, who only had a T1 image.

For reasons of relative contraindications and artefacts to MRI, we used the preoperative MRI scans in patients with DBS implants. The pre-operative scans had all been conducted in-house on a 1.5 T MRI system and whole brain images were available only as T2 weighted images with the exception of one patient, who had a T1 whole brain image only. Time interval between the pre-operative MRI and the PET scan was 2.6 ± 1.8 years.

Because only T2 weighted images was available in PD patients, we evaluated the impact on PET outcomes using T1 versus T2 weighted images, available in nine controls. The relative difference between the use of T1 and T2 was not significant in either $BP_{ND}0$ (0.7%) or $BP_{ND}1$ (1.3%), and therefore we used the T2 weighted images for analyses in all participants except for the two participants with only a T1 available.

Positron Emission Tomography

PET Scanning protocol

All PET scans were obtained in list mode with a high-resolution research tomography (HRRT) PET scanner (CTI/Siemens, Knoxville, TN, USA). Both radioligands, [^{11}C]AZ10419369 or [^{11}C]Cimbi-36 were given as an intravenous bolus injection and data acquisition began at the time of injection. The radioligands were given in very low doses with high specific radioactivity not to induce a pharmacological effect or significant cold saturation radioligand binding. The PET protocols are detailed in Paper 1-3. The synthesis and radiochemical labelling of [^{11}C]Cimbi-36 and [^{11}C]AZ10419369 is previously described (da Cunha-Bang et al., 2016; Ettrup et al., 2014).

In study 1-2 conducted in animals, additional measurements of arterial whole blood radioactivity were obtained during the PET scan. In the beginning continuously for 20 minutes using an ABSS autosampler (Allogg Technology, Mariefred, Sweden). Later, 8 blood samples were drawn during the course of the scan and the radioactivity in whole blood and plasma were measured in a well counter (Cobra 5003, Packard Instruments, PerkinElmer, Skovlunde, Denmark) that was cross-calibrated to the HRRT scanner and autosampler. Finally, radiolabeled parent compound and metabolites were measured in plasma using HPLC with online radioactivity detection (Gillings, 2009). In study 1, The parent compound fraction-time curve as well as the plasma and full blood radioactivity concentration curves were used as input functions in the subsequent kinetic modelling of the PET data. In study 2, which was designed as a within-scan challenge, the blood sampling served to confirm that the pharmacological challenges did not induce changes in blood [^{11}C]AZ10419369 time activity curves (TAC).

Quantification of PET data

The [^{11}C]Cimbi-36 and the [^{11}C]AZ10419369 PET scans were reconstructed into 38-45 dynamic frames of increasing length, depending on the duration of the PET scan, using attenuation correction as detailed in Paper 1-3.

In the pigs, PET images were motion corrected and co-registered to a standardized MRI based atlas in study 1 or an in-house pig atlas (Villadsen et al., 2018) in study 2 based on an PET template made of PET and MR images obtained from study 1. The details are given in Paper 1-2.

Each co-registration was verified by visual inspection before extraction of time-radioactivity curves (TACs) from the volumes of interest (VOIs) in the pig brain.

In humans, the PET images were motion corrected and co-registered to the T2 weighted image. Each co-registration, motion curve and all TAC curves of the participants were visually inspected. If sudden and substantial movement were identified, the PET raw data were recomputed and reconstructed using attenuation correction with μ-maps aligned to each frame producing the final motion corrected images as detailed in Paper 3.

[^{11}C]Cimbi-36 binding to the 5-HT$_{2A}$ receptor was quantified with Logan invasive modelling and the volumes of distribution (V_T) for the VOIs were calculated with PMOD software (version 3.0; PMOD Technologies, Zürich, Switzerland) as detailed in Paper 1. The occupancy was determined from V_T of the baseline and intervention scan using the occupancy plot (Cunningham, Rabiner, Slifstein, Laruelle, & Gunn, 2010). Data with standard coefficient of variance (COV) above 10% for all regional V_Ts were excluded from further analyses.

[^{11}C]AZ10419369 binding to the 5-HT$_{1B}$ receptor was quantified using the Extended Simplified Reference Tissue Modelling (ESRTM) (Zhou et al., 2006) with the intervention time at 57 minutes (study 2, pharmacological intervention) or 45 minutes (study 3, DBS) to estimate $BP_{ND}0$ and $BP_{ND}1$ for each region of interest (ROI) as detailed in Paper 2-3. The cerebellum was used as a reference region, as the cerebellar level of 5-HT$_{1B}$ receptors is insignificant (Varnäs, Halldin, & Hall, 2004). The occupancy was determined by the relative change in BP_{ND}. Data with COV above 15% for all regional BP_{ND}'s were excluded from further analyses.

Paraclinical and Clinical Evaluation (Study 3)

On the day of PET scan, all participants underwent a physical examination, urine drug screening, and psychological trait and state measures before undergoing one PET scan. The participants included as age-matched controls also had blood samples for biochemical evaluation, which had been sampled from the patients with PD upon admission to the Department of Neurology.

Blood chemistry

All participants were screened for basic metabolic disorders: (P-thyrotropin, P-Glucose, HBA_{1C}); kidney (P-Creatinine); electrolytes (P-Sodium, P-Potassium); infection (B-Leucocytes, B-

Leucocytes DIFF) and blood counts (B-Haemoglobin, B-Trombocytes); Coagulation (Coagulation Factors II, VII, and X) and Albumin.

UPDRS

The Unified Parkinson Disease Rating Scale (UPDRS) is a widely used clinimetric rating scale for assessing disability in patients with PD and the longitudinal course of the disease. It was revised in 2007 (Goetz et al., 2008) by The Movement Disorder Society (MDS-UPDRS) and cover four parts, namely, I: Non-motor Experiences of Daily Living (13 items such as daytime sleepiness or urinary problems); II: Motor Experiences of Daily Living (13 items such as salivation, eating and dressing); III: Motor Examination (33 scores based on 18 items such as facial expression, movements or tremor from both left and right side); IV: Motor Complications (6 items such as dyskinesia or motor fluctuations). Each subscale is rated on a 5-point Likert scale from 0 (normal) to 4 (severe). Part III is clinician-scored while the other parts are self-reported by the patient and/or the caretaker based on recollection from the past week.

In this thesis, the total MDS-UPDRS score (part I-IV) and the Motor Examination score (part III) obtained in the pre-scan DBS-ON condition and the corresponding Motor Examination scores (part III) in the post-scan DBS-OFF condition are used. The Motor Examination was documented on video and all MDS-UPDRS scores were evaluated on the day of scanning and in the patients with PD only.

Hoehn and Yahr

The Hoehn and Yahr Rating (HYR) is another widely used clinimetric rating scale describing the degree of disability in patients with PD (Hoehn & Yahr, 1967), and has also been incorporated in the MDS-UPDRS Motor Examination (part III). It consists of the following 5 scores (1-5); 1: Unilateral involvement only; 2: Bilateral involvement without impairment of balance; 3: Bilateral disease with mild to moderate disability and impaired postural reflexes; 4: Severely disabling disease although still able to walk or stand unassisted; 5: Confinement to bed or wheelchair unless aided. Here, the HYR score were assessed in all PD patients on the day of scanning in the two conditions DBS-ON and DBS-OFF. Patients were scored 0 in case they did not present any motor involvement in the DBS-ON condition.

The Mini Mental State Examination

The Mini Mental State Examination (MMSE) (Folstein et al., 1975) is the most frequent clinimetric scale used for screening of cognitive function. It consists of 30 scores based on 8 items, namely, Orientation (10), Episodic Memory (6), Concentration (5), Language (3), Executive Function (3), Reading (1) and Writing (1) skills and Visuospatial ability (1). Low scores is associated with dementia, and although various cut-off points are in use, a score < 27 may suggest dementia (Schultz-Larsen, Kreiner, & Lomholt, 2007). We used MMSE scores < 27 as exclusion criteria of all subjects.

Mood rating scales

The Profile of Mood States

The Profile of Mood States (POMS) is a self-reported rating scale used to assess transient, distinct mood states (McNair & Heuchert, 2007). It consists of six factors and a total score of mood disturbance (TMD) rated by 65 adjectives (e.g. “Furious”, “Hopeless” and “Carefree”) on a 5-point Likert scale from 1 (not at all) to 5 (extremely) based on the present mood state. The six factors include: Tension or Anxiety (T), Anger or Hostility (A), Vigor or Activity (V), Fatigue or Inertia (F), Depression or Dejection (D), Confusion or Bewilderment (C) (Stenbæk et al., 2015). The test takes approximately 5 minutes to complete. The POMS was completed twice the same day immediately before and after the PET scan in the DBS-ON and DBS-OFF conditions to allow for measurement of change in mood.

The Major Depression Inventory

The Major Depression Inventory (MDI) is a self-reported rating scale for measuring DSM-IV and ICD-10 diagnoses of major depression (Bech, Rasmussen, Olsen, Noerholm, & Abildgaard, 2001) according to presence of depressive symptomatology. It measures the total sum of 10 items rated on a 6-point Likert scale from 0 (at no time) to 5 (all the time) based on recollection of the last 2 weeks. The MDI was completed once in all participants prior to the PET scan. MDI total scores < 20 were not considered suggestive of depression.

Statistical Analyses

Pharmacological interventions (study 1 and 2)

The cerebral 5-HT level was compared between groups of saline or pCPA pre-treatment with un-paired two-tailed t-test (study 1). The mean change in BP_{ND} (PET) and 5-HT (microdialysis) were analyzed post hoc for significant group difference between saline and either two interventions (fenfluramine or escitalopram) using the Wilcoxon signed rank test for each region of interest (study 2).

The competition model (study 1 and 2)

The microdialysis sample (left or right) with the highest peak increase in extracellular 5-HT level relative to baseline was correlated with the PET occupancy. The microdialysis data, F_{NT} , $\Delta F_{NT,}$ and the corresponding PET occupancy of radioligand, [^{11}C]Cimbi-36 and [^{11}C]AZ10419369, were fitted to the model given in eq.1 with a non-linear regression analysis. The Wald Runs-Test for randomness were used to test if the curve fitted by non-linear regression to the competition model deviated from the data.

Test-retest analysis (study 2)

The reproducibility of [^{11}C]AZ10419369 binding were assessed within the individual scan and between the first part of the two scans before the interventions by test-retest analysis of $BP_{ND}0/1$ (PET 1) and $BP_{ND}0$ (PET 1 and 2) in neocortex. The data was assessed for significant order effect by the Wilcoxon signed-rank test before estimation of the Intraclass Correlation Coefficients (ICC) modelled as a two-way mixed ANOVA with absolute agreement and average measurement in a within-subject design. Finally, the required sample sizes to reach significance level of the neocortex region were calculated for each pharmacological intervention.

Clinical DBS study (study 3)

Group differences in demographics and radioligand injection dose (mass per kilogram bodyweight) were evaluated with unpaired 2-tailed t-test. The POMS subscale scores (A, V, and F) at baseline were evaluated for Gaussian distribution and tested for group differences at

baseline with the Mann-Whitney test. The difference in patient state POMS scores (DBS OFF – ON) was evaluated with the paired Wilcoxon test.

The $BP_{ND}0$ were tested for group differences by multiple linear regression analyses with predictors of group (controls, patients) and age, since previous PET studies have demonstrated a negative correlation between [^{11}C]AZ10419369 BP_{ND} and age (Matuskey et al., 2012; Nord et al., 2014; Varrone et al., 2014). Although the specific radioactivity of [^{11}C]AZ10419369 was high in all cases, we initially included the injected mass per kilogram of bodyweight as a covariate, because of theoretical effects of [^{11}C]AZ10419369 on BP_{ND}. However, given that injected mass was not identified as a significant covariate, it was not included in the final analyses. To test for differences between $BP_{ND}0$ and $BP_{ND}1$, we tested each regional $BP_{ND}0$ with paired sample t-test, independently for both groups.

The association between regional BP_{ND} and the DBS induced change in POMS subscale measures was evaluated by multiple linear regression analysis with either $BP_{ND}0$ or relative change in BP_{ND} as the dependent and the following predictors: age, L-DOPA equivalents, and difference (off-on) in the POMS subscale measures with a demonstrated significant difference between the DBS ON and OFF condition.

Significance level was set at p-value of .05, and the primary analysis outcome was corrected for multiple comparisons by the Bonferroni-Holm method. The post hoc analyses were not corrected for multiple comparisons.

Ethical Approvals

The clinical study was approved by the Capital Regions ethics committee (H-1-2014-002, H-3-2013-100, H-6-2014-057 and H-KF-2006-20) and the Danish Data Protection Agency (30-1450). All participants provided written informed consent following full description of the procedures. The age-matched controls received monetary compensation for their participation.

All animal experiments were performed in accordance with the European Communities Council Resolves of 22nd of September 2010 (2010/63/EU), approved by the Danish Veterinary and Food Administration's Council for Animal Experimentation (Journal No. 2012-15-2934-00156), and is in compliance with the ARRIVE guidelines (www.nc3rs.org.uk/arrive-guidelines).

RESULTS

Surgical procedure for implantation of microdialysis probes (study 1-2)

The surgical procedure used for implantation of the microdialysis probes in the mPFC was based on coordinates relative to bregma and validated in study 1 for correct position of the probes (Figure 9).

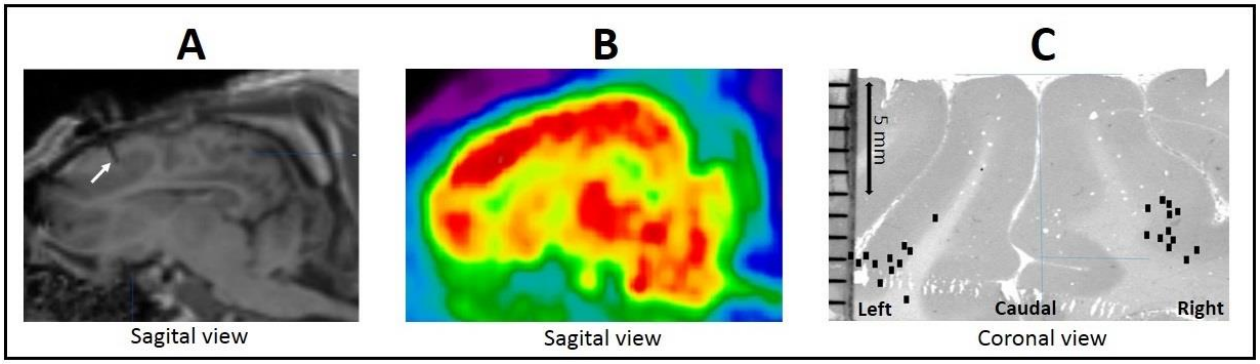

Figure 9 (Paper 1). (A) MRI of the pig brain showing the implanted microdialysis probe (white arrow) and (B) [^{11}C]Cimbi-36 PET image of the pig brain. The placement of the tip of the microdialysis probes in the mPFC, as identified from the MRI scan, is indicated on a histology slice (c) and marked with a black dot. The microdialysis probe extends from the tip (black dot) and 4 mm cortically, so the active part of the probe is embedded in the grey matter of the mPFC.

Pharmacological interventions (study 1-2)

In each individual pig, the peak 5-HT increase was identified on the microdialysis time course (Figure 10-A), and the associated 5-HT$_{2A}$ occupancy of [^{11}C]Cimbi-36 was quantified using the Lassen plot (Figure 10-B). The distribution volumes (V_T) of the pig brain regions and the non-displaceable distribution volume (V_{ND}) of [^{11}C]Cimbi-36 are specified in Paper 1. Accordingly, the time-dependent effects of all the serotonergic challenges used in study 1 is illustrated in Figure 11 relative to baseline level.

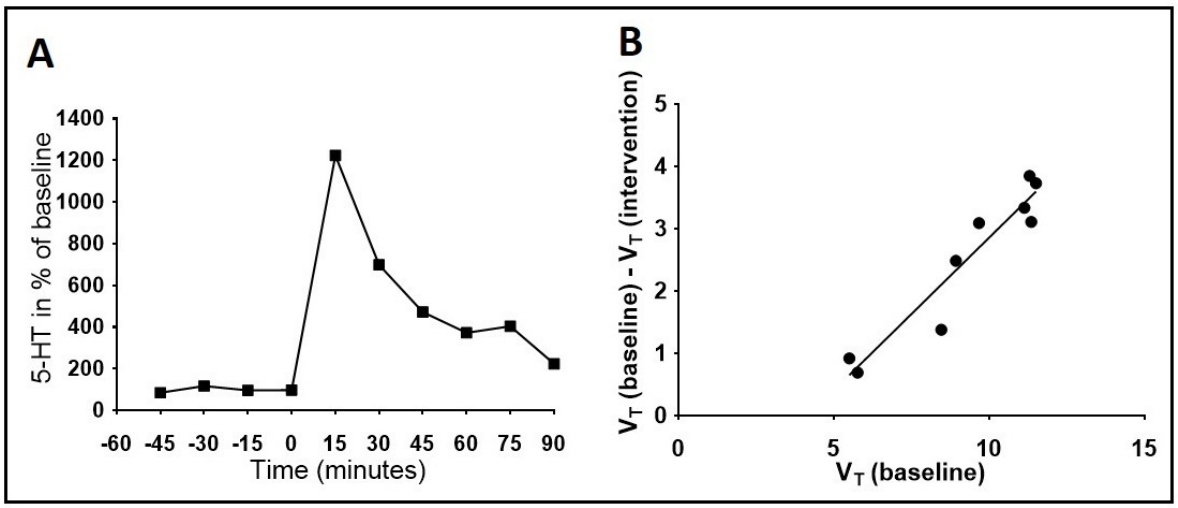

Figure 10 (Paper 1). Example of the microdialysis time course (A) and the [^{11}C]Cimbi-36 PET occupancy plot (B) from a single pig upon a serotonergic challenge of fenfluramine given at time 0. Panel A shows the change in 5-HT level relative to baseline (100%) over time. Panel B shows the corresponding occupancy plot based on volumes of distribution (V_T) in the VOIs (mL/cm3). The 5-HT_{2A} occupancy is read as the slope on the regression line (B).

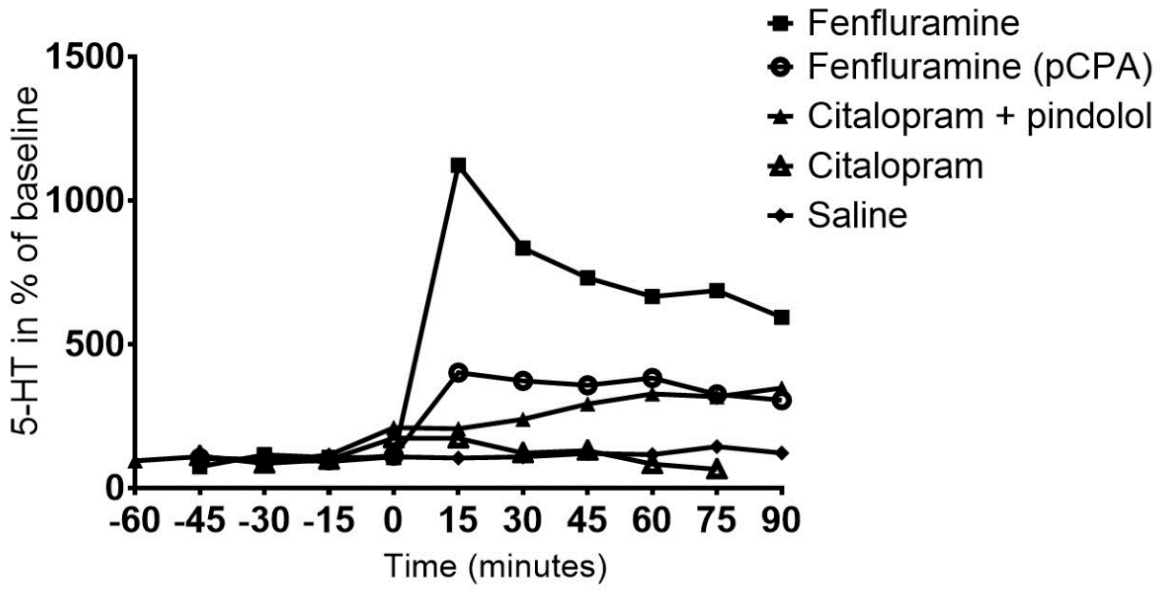

Figure 11 (Paper 1). Time-dependent effect of the intervention injected at 0 min on 5-HT release relative to baseline level (100%), as measured by microdialysis in the mPFC. Values are given as means of 2-3 measurements.

The change in 5-HT level and the associated region dependent relative decrease in 5-HT_{1B} binding of [^{11}C]AZ10419369 was calculated. The average change in BP_{ND} and 5-HT level are shown in Figure 12.

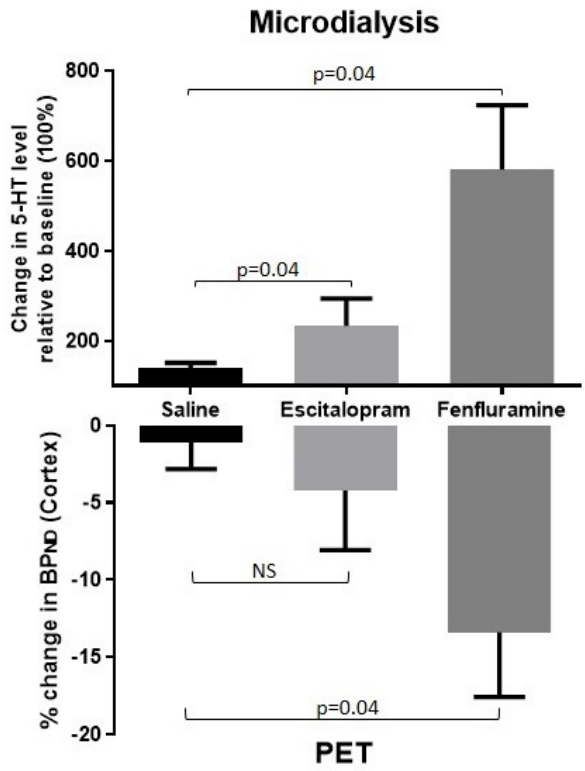

Figure 12 (Paper 2). Changes in the peak extracellular 5-HT level (mean ± SEM) relative to baseline (100%) (upper panel) and changes in BP_{ND} (mean ± SEM) of [^{11}C]AZ10419369 (lower panel) following a within-scan challenge of saline, escitalopram, or fenfluramine. Group difference in 5-HT release were tested for escitalopram and fenfluramine relative to saline with the Wilcoxon signed-rank test.

An overview of the effect of the various serotonergic challenges on 5-HT release as measured by PET and microdialysis are given in Table 4. Fenfluramine produced the largest increase in extracellular 5-HT level, and the effect was blunted with pCPA pre-treatment. Escitalopram produced the lowest response, but adding pindolol enhanced and prolonged the serotonergic response to escitalopram. The 5-HT level did not change upon intervention with saline. Unexpectedly, the SSRI-induced effect on 5-HT release was not lower following a therapeutic dose of escitalopram (study 2) as compared to a 7-fold higher dose (study 1).

Table 4. Microdialysis and PET measures of 5-HT levels

	Study 1				Study 2			
Pharmacological interventions	Dose mg/kg	Pigs N	Peak 5-HT %	Cimbi-36 Occupancy (%)	Dose mg/kg	Pigs N	Peak 5-HT %	AZ10419369 ΔBP_{ND} (%)
Saline	-	2	151 ± 63	17 ± 5	-		140 ± 31	-1 ± 5
Escitalopram	2.0	2	217 ± 98	19 ± 2	0.28	5	233 ± 120	-4 ± 8
Pindolol +Escitalopram	1.0 / 2.0	3	441 ± 78	28 ± 4				
Fenfluramine	0.5	2	1123 ± 144	38 ± 3	0.5	5	580 ± 286	-13 ± 8
Fenfluramine (pCPA pre-treatment)	0.5	2	516 ± 159	44 ± 3				

Combined results from study 1-2 showing the dose-dependent peak (mean ± SD) increase in 5-HT level relative to baseline (100%) as measured by microdialysis in the mPFC and the change in radioligand binding (mean ± SD) of [^{11}C]Cimbi-36 (study 1) and [^{11}C]AZ10419369 (study 2) in neocortex. The results from other ROIs in study 2 are detailed in Paper 2. The doses and number of pigs are listed for each intervention.

The re-test variability of [^{11}C]AZ10419369 (study 2) was very small and the corresponding ICC values were excellent (ICC 0.94-0.98) when measured both within scan ($BP_{ND}0/1$ of PET scan 1) and between scans ($BP_{ND}0$ of PET scan 1 and 2). The required sample size to statistically identify a fenfluramine associated 13% decline in BP_{ND} in neocortex or an escitalopram associated 4% decline in BP_{ND} was N = 5 and N = 11 respectively in a within scan design. The results from the test-retest analysis are detailed in Paper 2.

Diagrams of the competition model (study 1-2)

The corresponding measures of the ΔF_{NT} and the occupancy of the two PET radioligands, the 5-HT_{2A} receptor agonist [^{11}C]Cimbi-36 and the 5-HT_{1B} partial agonist [^{11}C]AZ10419369, fitted to the competition model (eq.1.) are displayed in Figure 13 and 14 respectively. For both PET radioligands, the correlated PET and microdialysis measures complied with the competition model, and the data did not deviate significantly from the model when evaluated by the Wald Runs-test for randomness.

K_{NT} of the 5-HT_{2A} receptor agonist [^{11}C]Cimbi-36 was calculated from eq.1 to 14.3 nM, about 8 times higher than the assumed baseline interstitial 5-HT concentration of 1.7 nM. Correspondingly, the K_{NT} of the 5-HT_{1B} receptor partial agonist [^{11}C]AZ10419369 was 74 nM, about 43 times higher than the baseline brain interstitial 5-HT concentration.

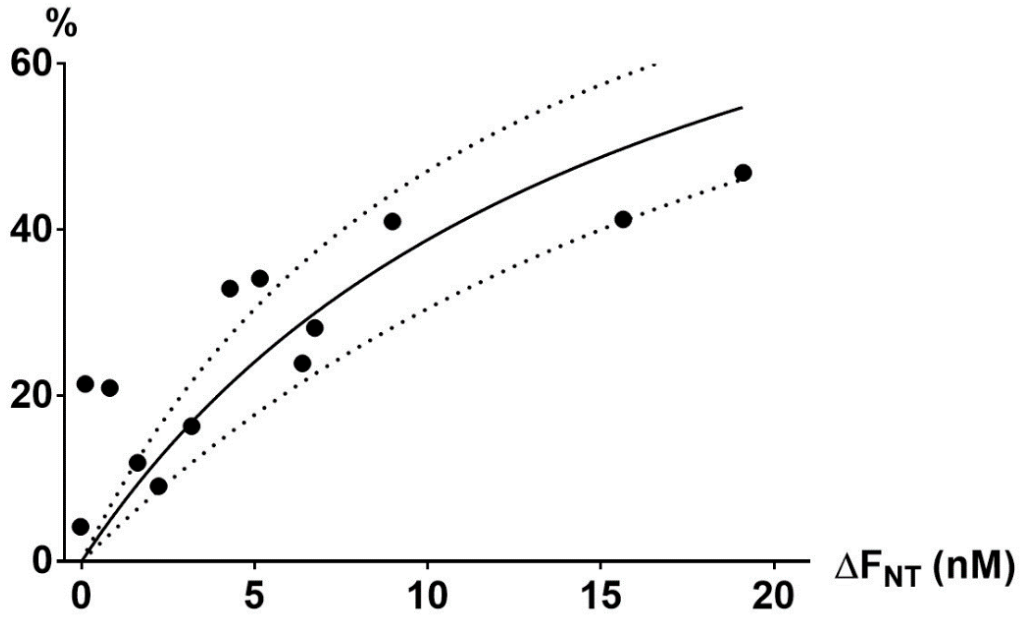

Figure 13. [^{11}C]Cimbi-36 PET radiologand (Paper 1). The ΔF_{NT} (nM) and the corresponding 5-HT_{2A} receptor occupancy (%) with 95% confidence interval of all 13 pigs (study 1) were fitted with nonlinear regression analysis according to the competition model.

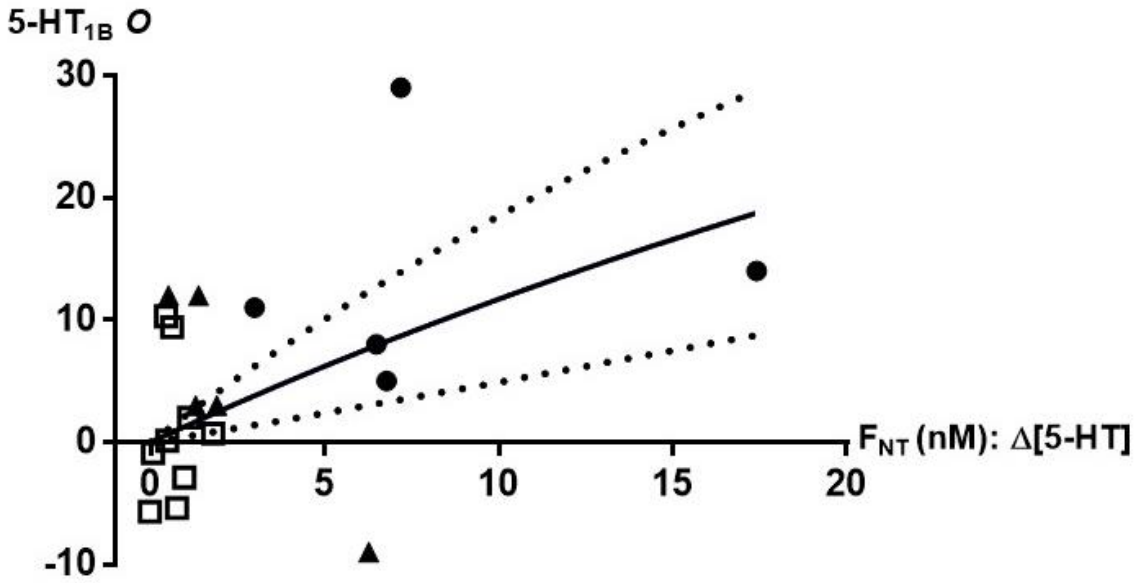

Figure 14 [^{11}C]AZ10419369 PET radiologand (Paper 2). The ΔF_{NT} (nM) and the corresponding 5-HT_{1B} receptor occupancy (%) with 95% confidence interval of all 10 pigs (study 2), each with two PET scans, were fitted with nonlinear regression analysis according to the competition model.

Cross-study comparisons

To put the findings in perspective, the sensitivity of the two PET radioligands, [^{11}C]Cimbi-36 and [^{11}C]AZ10419369, to detect 5-HT was compared to the sensitivity of [^{11}C]-raclopride to DA, a much used approach to investigate changes in cerebral DA (Laruelle, 2000).

Figure 15 illustrates the fitted curves of the two radioligands (*blue and red line*) as well as the results with [^{11}C]raclopride (*black circle*) from human challenge-studies reported in literature. A single dose of amphethamine 0.3 mg/kg is known to induce an increase in DA level of approximately 8-fold (*dotted line*) (Laruelle, 2000) and the corresponding decrease in [^{11}C]raclopride binding is 13-16% (*black circle*) (Breier et al., 1997; Martinez et al., 2005, 2007; Schneier et al., 2009) consistent with findings in non-human primates of 15-24% decrease in binding of [^{11}C]raclopride (Drevets et al., 1999; Narendran et al., 2004; Tsukada et al., 1999).

With [^{11}C]Cimbi-36 and [^{11}C]AZ10419369, the PET occupancy at an 8-fold increase in 5-HT baseline level (1.7 nM) is 46% (CI 38-55%) and 15% (CI 7, 24%) respectively. This demonstrate that when the 5-HT level is increased by 8-fold, the sensitivity of [^{11}C]Cimbi-36 to detect changes in 5-HT level is 3 times that of [11C]raclopride to DA, while the sensitivity of [^{11}C]AZ10419369 to detect 5-HT is comparable to that of raclopride to DA.

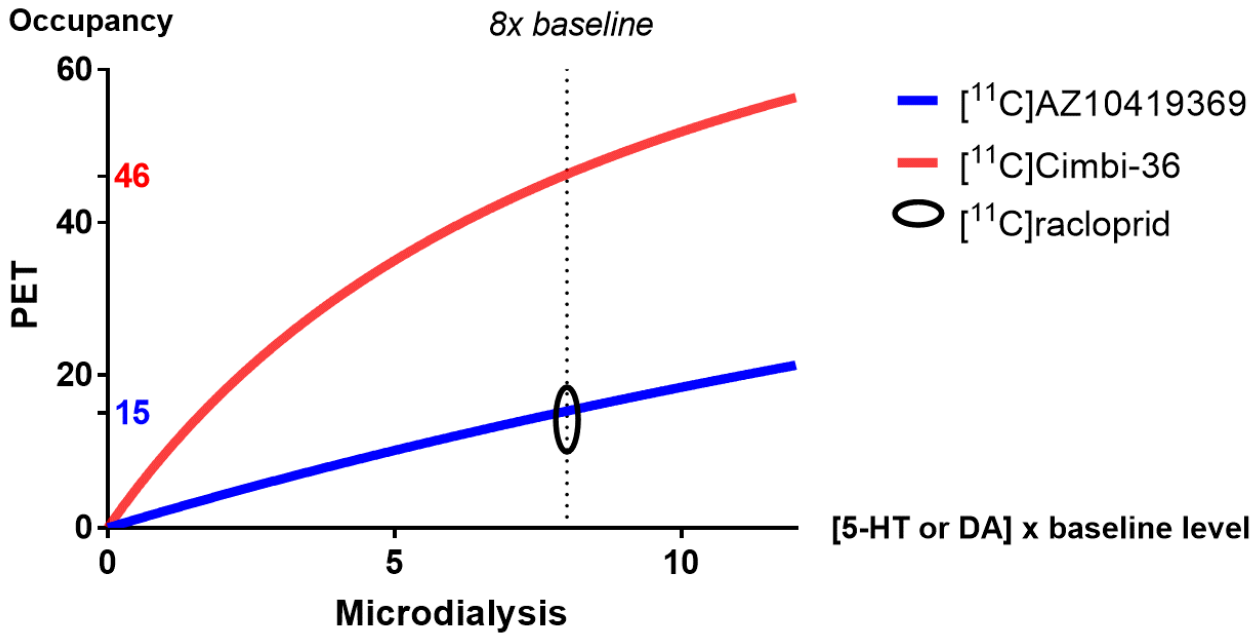

Figure 15. The sensitivity of the two 5-HT receptor PET radioligands *(blue and red)* at an 8-fold increase in neurotransmitter level (dotted line) compared to the sensitivity of [^{11}C]raclopride to detect DA, as reported in literature (black circle).

In microdialysis studies, the peak increase across several samples is the generally accepted reported outcome measure. Instead, when the data is computed to the model with AUC instead of peak, K_{NT} of [^{11}C]Cimbi-36 is 8.2 nM (instead of 14.3 nM) and associated with a 57% change in occupancy at an 8-fold increase in 5-HT rather than 46%. Correspondingly, K_{NT} of [^{11}C]AZ10419369 is 27 nM (instead of 74 nM) and associated with a 32% decline in BP_{ND} rather than 15%. The data would still conform to the competition model in both cases.

DBS-STN in patients with PD (study 3)

Demographichs and clinical measures

Demographic and clinical measures were given in table 2 and detailed in Paper 3. Before PET scan, the POMS A subscale was significantly different between groups, with patients being less angry and hostile (2.0 ± 2.1) as compared to controls (5.1 ± 2.0) ($p = 0.007$, Mann-Whitney and corrected for 7 comparisons). None of the other POMS subscales differed between groups.

PET reconstruction and quantification

The patients with PD only displayed slightly more head movements than controls during the PET scan, judged insufficient to create any consistent bias in the observed BP_{ND} (Paper 3, Supplementary Material). An example of a fitted TAC curve by the ESRTM modelling with cerebellum as the reference region is shown in Paper 3 (Supplementary Material).

DBS-STN treated patients with PD versus controls

Table 5 shows $BP_{ND}0$ (based on PET data acquired 0-44 min after injection) in patients (with DBS on) and age matched controls. We found that the regional $BP_{ND}0$ values were numerically consistently lower in patients with PD and the patients displayed a significantly lower $BP_{ND}0$ in the frontal and parietal cortices.

The UPDRS score was inversely correlated with frontal cortex $BP_{ND}0$ (Figure 16), meaning that patients with PD who suffered most impairment when evaluated clinically also had the lowest frontal cortex $BP_{ND}0$.

Table 5. The 5-H T_{1B} receptor binding potential

	$BP_{ND}0$			ΔBP_{ND}(%)			
ROI	PD	Controls	p-value (group)	PD	P-value	Controls	P-value
Frontal Cortex	0.85 ± 0.17	1.12 ± 0.27	.01 *	-5 ± 11	.08	6 ± 12	.12
Temporal Cortex	0.79 ± 0.15	0.95 ± 0.25	.09	-11 ± 9	.002 **	3 ± 11	.21
Parietal Cortex	0.79 ± 0.13	1.00 ± 0.21	.01 *	-2 ± 11	.33	12 ± 12	.02
Limbic Cortex	1.02 ± 0.21	1.28 ± 0.34	.05	-9 ± 12	.01 *	2 ± 7	.17
Occipital cortex	1.06 ± 0.16	1.16 ± 0.22	.25	-8 ± 9	.02 *	0 ± 8	.74
Post hoc analysis							
Neocortex	0.84 ± 0.14	1.05 ± 0.24	.02	-7 ± 10	0.03	6 ± 10	ns
Superior frontal gyrus	0.80 ± 0.20	1.07 ± 0.25	.01	-6 ± 12	ns	5 ± 13	ns
Primary motor cortex	0.86 ± 0.19	1.11 ± 0.27	.02	-6 ± 16	ns	7 ± 14	ns
Dorsolateral prefrontal cortex	0.75 ± 0.22	1.12 ± 0.31	.005	-2 ± 19	ns	8 ± 1	ns
Ventrolateral prefrontal cortex	1.02 ± 0.19	1.26 ± 0.32	.05	-2 ± 11	ns	9 ± 12	.04
Medial inferior frontal gyrus	0.90 ± 0.18	1.19 ± 0.29	.01	-2 ± 12	ns	8 ± 12	ns
Orbitofrontal gyrus	0.86 ± 0.19	1.00 ± 0.27	ns	-11 ± 14	.03	4 ± 19	ns
Superior temporal gyrus	0.79 ± 0.18	0.97 ± 0.31	ns	-15 ± 10	.002	4 ± 12	ns
Medial inferior temporal gyrus	0.80 ± 0.14	0.94 ± 0.22	.10	-6 ± 8	.02	2 ± 10	ns
Somatosensory cortex	0.73 ± 0.19	1.00 ± 0.24	.009	-5 ± 15	ns	8 ± 12	ns
Anterior cingulate cortex	1.09 ± 0.23	1.38 ± 0.33	.03	-7 ± 10	.05	2 ± 11	ns
Posterior cingulate cortex	0.88 ± 0.19	0.89 ± 0.21	ns	-17 ± 15	.05	-5 ± 11	ns
Insular cortex	0.99 ± 0.21	1.23 ± 0.36	ns	-11 ± 13	.02	1 ± 6	ns
Caudate	0.66 ± 0.28	1.07 ± 0.37	.006	2 ± 29	ns	8 ± 21	ns
Putamen	1.25 ± 0.25	1.42 ± 0.46	ns	4 ± 12	ns	20 ± 13	.004
Thalamus	0.49 ± 0.12	0.55 ± 0.14	ns	-16 ± 21	ns	-3 ± 14	ns

$BP_{ND}0$ in PD patients and age-matched controls and the relative change in BP_{ND} (%) after switching DBS-STN off, as measured by ($BP_{ND}1$-$BP_{ND}0$)/ $BP_{ND}0$) *100. Values of all ROIs are given as mean ± SD of 13 PD patients and 10 controls. For reason of COV > 15%, occipital cortex, dlPFC, and superior frontal gyrus BP_{ND}'s were excluded from one patient, and in the caudate and thalamus from two other patients. In two controls, thalamus BP_{ND}'s were excluded. The asterix marks the significance level at p =.05 (*), 0.01 (**) of the primary ROIs (n=5) that survived multiple comparisons after correction with the Bonferroni-Holm method.

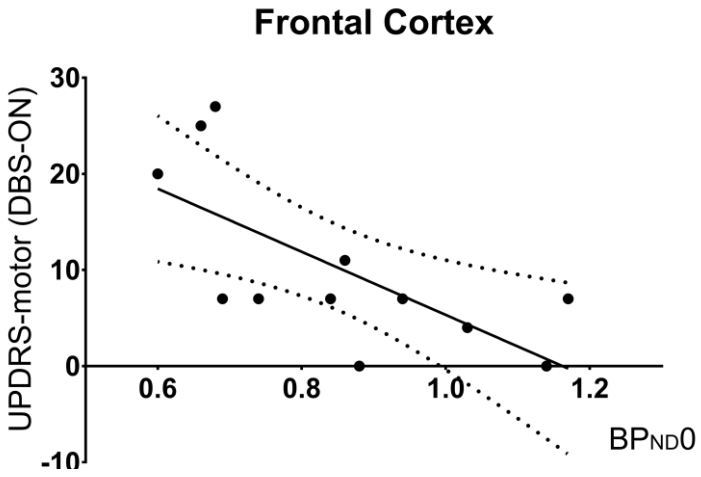

Figure 16 (Paper 3). The $BP_{ND}0$ and the UPDRS motor scores at baseline are inversely correlated (p = .01) as evaluated by multiple linear regression with $BP_{ND}0$ as the dependent and the following predictors: UPDRS-motor score (DBS-ON), age and levodopa-equivalents.

Turning the STN-DBS off

When the DBS stimulator in the patients was turned off, we observed a decrease in binding potential in the temporal, limbic and occipital cortex, namely the primary regions with $BP_{ND}0$ not significantly different from controls (Table 5, Figure 17). This pattern also applied to the regions in the post-hoc analyses (Table 5).

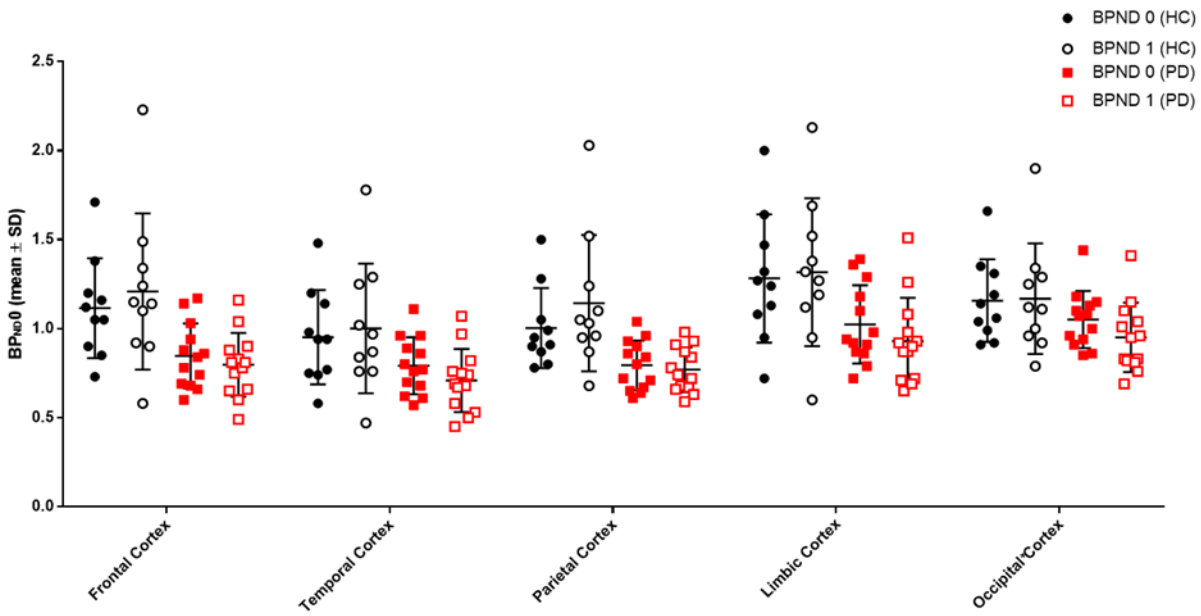

Figure 17 (Paper 3). Binding potentials of all participants. The binding potential $BP_{ND}0$ (0-44 min) and $BP_{ND}1$ (45 min – end of scan) of patients with Parkinson's Disease (red) and age-matched controls (black) equivalent to the two conditions DBS-ON and DBS-OFF for each region of interest.

In a post hoc analysis across all brain regions, the extent to which patients with PD had regionally preserved $BP_{ND}0$ as compared to their age-matched controls was related to the regional brain response to turning DBS-STN off. Interestingly, we found a significant correlation between the relative change in BP_{ND} when DBS is turned off and preservation of $5\text{-}HT_{1B}$ receptor binding in PD patient (Figure 18). That is, in the least affected brain regions in patients with PD, 5-HT was released when DBS was turned off, whereas no 5-HT release was detected in the most affected brain regions.

When correcting for multiple comparisons, the POMS V subscale changed significantly (p=.005, paired t-test) in response to turning DBS off (13.8 ± 8.3) compared to DBS-ON (24.0 ± 2.6), meaning that the DBS-OFF condition was associated with a decrease in self-reported activity and vigor. An inverse relationship was observed between change in POMS V scores (OFF - ON),

when DBS was turned off, and $BP_{ND}0$ in temporal and limbic cortex (Figure 19); meaning that a high BP_{ND} at DBS ON was associated with the large decrease in self-reported activity and vigor. We did not find any association between change in POMS score and regional change in BP_{ND}.

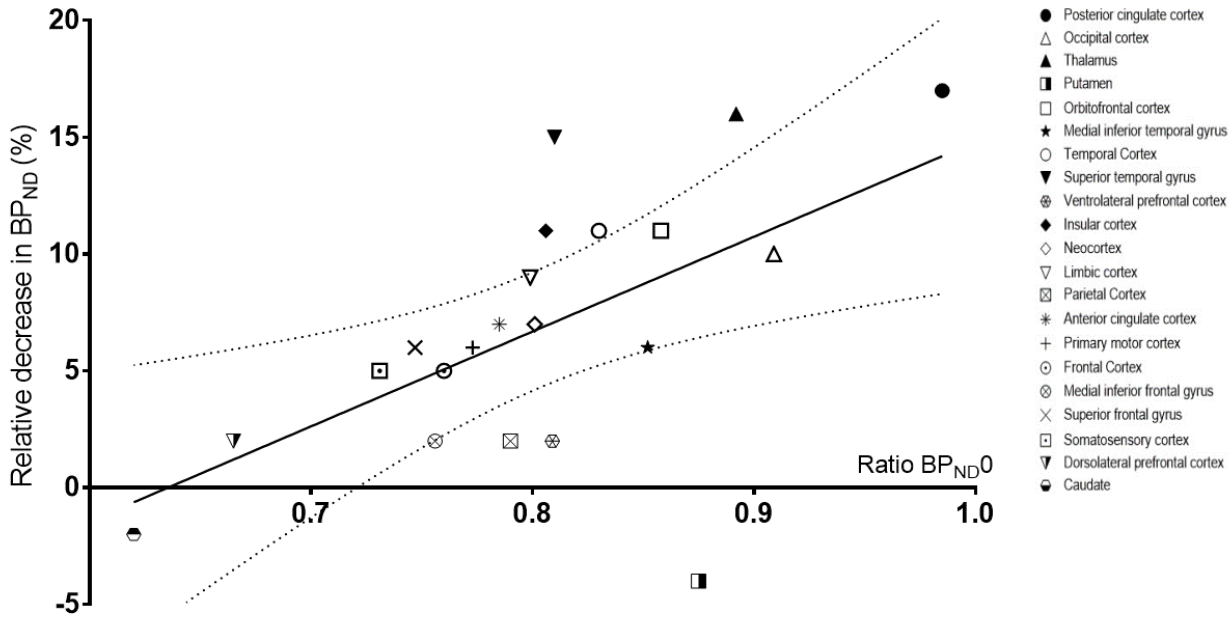

Figure 18 (Paper 3). Percent decrease in BP_{ND} when turning DBS off versus the ratio between BP_{ND} of patient's and control's across regions. There was a significant correlation between the two measures ($p = .005$, $r=0.62$). Only regions with more than 50% of the subjects' data fitted the kinetic model with less than 15% covariance were included. Regions comprising sub-regions (neocortex, FC, PC, LC and TC) were excluded from the statistical regression analysis. The putamen was excluded, since the healthy controls showed an increase in $BP_{ND}1$ as detailed in paper 3.

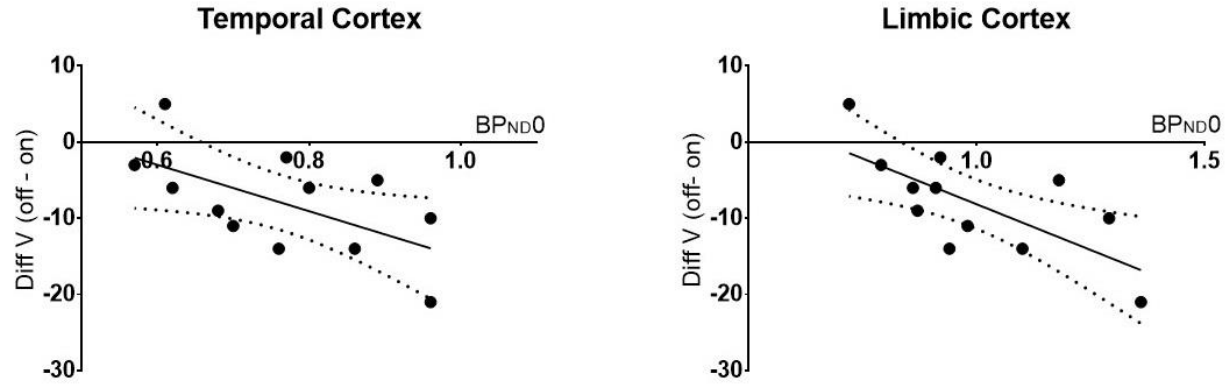

Figure 19 (Paper 3). The association between $BP_{ND}0$ and the change (off - on) in POMS V when DBS is turned off as evaluated by multiple linear regression with regional $BP_{ND}0$ as the dependent and the following predicors: age and difference in POMS subscale measure (V_{off} - V_{on}), evaluated for each region independently, the temporal cortex ($p = .04$) and the limbic cortex ($p = .03$).

DISCUSSION

The 5-HT system plays a critical part in regulating various physiological responses and has been associated with many neuropsychiatric disorders. The work presented here in this thesis contribute to advance knowledge on serotonergic involvement in Parkinson's Disease and DBS therapy. Moreover, the diagrams of the competition model in specific PET radioligands produced in this work also make an important contribution to understand how outcomes from PET imaging in future studies translate to relative changes in cerebral 5-HT level by plain curve reading. One of these radioligands were used in the clinical study of this thesis to characterize the serotonergic dysfunction in patients with PD and the serotonergic changes related to DBS. The following sections give an overall interpretation of the outcomes from the three studies included in this thesis, while Paper 1-3 provide a more comprehensive discussion of the outcomes and comparison with existing literature.

Main findings

Consistent with our a priori hypothesis in the preclinical studies (Study 1-2), we first showed how various pharmacological interventions directly increased the extracellular 5-HT level in the pig brain as measured by state-of-the-art microdialysis. Secondly, we demonstrated an orderly decrease in binding of the two PET radioligands, the 5-HT_{2A} receptor agonist [^{11}C]Cimbi-36 and the [^{11}C]AZ10419369 partial agonist, in response to these interventions consistent with the competition model (eq.1.). The observed correlation between changes in the extracellular 5-HT level in the pig brain and both the $5HT_{2A}$ and the 5-HT_{1B} receptor occupancies indicates that [^{11}C]Cimbi-36 and [^{11}C]AZ10419369 binding is sensitive to changes in endogenous 5-HT levels, but only when the 5-HT release is sufficiently high.

Consistent with the a priori hypothesis in the clinical study of DBS-STN in patients with PD (study 3), we first showed that patients with PD treated with DBS have lower 5-HT_{1B} receptor availability in the frontal and parietal cortex as compared to controls, and that the frontal 5-HT_{1B} receptor availability is negatively correlated with motor symptom severity in PD. Secondly, when DBS is turned off, we observed a decrease in 5-HT_{1B} receptor binding in temporal, limbic, and occipital cortex, interpreted as representing an increase in 5-HT levels. Importantly, the extent to which 5-HT_{1B} receptor binding is reduced when turning DBS off is proportional to the regional preservation of 5-HT_{1B} receptors in PD. Finally, we demonstrate a negative correlation

between temporal and limbic cortex 5-HT_{1B} receptor availability at baseline when DBS is on, and a reduction in vigor and activity when DBS is turned off.

Pharmacological interventions effect on 5-HT levels

The serotonergic challenges induced a dose-dependent increase in extracellular 5-HT level of 2-11 fold of baseline level in the mPFC in the pig brain with fenfluramine (0.5 mg/kg) being the most potent releaser. Our findings are consistent with microdialysis studies in rats and non-human primates, where fenfluramine (1-10 mg/kg) induced a 5-35 fold increase in the extracellular 5-HT level compared to baseline (Laferrere & Wurtman, 1989; Schwartz, Hernandez, & Hoebel, 1989; Tao et al., 2002; Udo de Haes et al., 2005; Udo de Haes, Harada, Elsinga, Maguire, & Tsukada, 2006).

In line with (Ettrup et al., 2011), pretreatment with pCPA (100 mg/kg) effectively reduced the 5-HT level to ~20% as measured in the brain homogenate. Despite the vast reduction in total 5-HT level in pigs pretreated with pCPA, the fenfluramine induced 5-HT release was still half of that released in pigs pretreated with saline. This could be caused by compensatory mechanism, e.g., serotonergic neurons more efficiently storing and releasing 5-HT from the synaptic terminal. Alternatively, given that our microdialysis measurements only generate relative increases in extracellular 5-HT level, if pCPA-treated pigs had reduced baseline 5-HT levels to 20% of the saline-pretreated pigs, then the absolute amounts of released 5-HT could have been substantially smaller. However, based on the PET occupancy plot (Figure 13), the latter did not seem to be the case.

Attenuation of the serotonergic response by presynaptic 5-HT autoreceptor regulation has been demonstrated in preclinical studies with rats (Gobert et al., 1997; Hjorth, 1993), and blocking the 5-HT autoreceptors potentiate the serotonergic response to SSRIs (Artigas et al., 1996; Invernizzi et al., 1992). We also observed a 4-fold higher serotonergic response to escitalopram in combination with pindolol (1 mg/kg) as compared to the observed 2-fold increase with mono-intervention with escitalopram (study 1).

In general, preclinical microdialysis studies investigate drugs in doses manifold above what is clinically relevant, which we also did in study 1. However, we deliberately chose to investigate escitalopram equivalent to a therapeutic and lower dose in study 2, in order to compare the

outcome with previously reported clinical studies (Nord et al., 2013; Pinborg et al., 2012; Selvaraj et al., 2012). Somewhat unexpectedly, we did not observe that using a low dose of escitalopram in study 2 (escitalopram 0.28 mg/kg) was associated with substantially less 5-HT release, when compared to study 1 (escitalopram 2 mg/kg). This is in line with studies in rats, where a high dose of citalopram (5-10 mg/kg) in rats (Fuller, 1994; Invernizzi et al., 1992) induced a 2-4 fold increase in extracellular 5-HT level, while a lower dose of citalopram (1 mg/kg) still produced a 2-fold change in serotonergic response (Invernizzi et al., 1992).

Although all the serotonergic challenges were given in smaller doses in our pig studies as compared to the studies in rats and non-human primates, we observed an increase in extracellular 5-HT level comparable to other challenge studies in rats and non-human primates. This efficacy of lower doses in our studies of the pig brain could be ascribed to species difference, or due to awake experiments in some of the rodent studies or differences in the administration route of the drugs (Schwartz et al., 1989; Tao et al., 2002; Udo de Haes et al., 2005, 2006). In any instance, based on pilot studies, prior to the ones described her, we found that a dose of 1.5 mg/kg was the maximal dose of fenfluramine that the pigs tolerated.

A limitation of the microdialysis studies are that they only allow for measurements in a few selected brain regions, such as done here bilaterally in the mPFC. PET, on the other hand, can generate information about 5-HT release in the entire brain as indexed by an inverse relationship to binding of the PET radioligand.

Pharmacological interventions effect on BP_{ND}

Depending on the pharmacological intervention, the average change in 5-HT_{1B} receptor binding ranged between 4-16% as measured by the PET radioligand [^{11}C]AZ10419369, and the average 5-HT_{2A} receptor occupancy ranged between 19-44% as measured by the PET radioligand [^{11}C]Cimbi-36. As expected, we observed a higher change in receptor binding with more powerful serotonergic challenges.

Somewhat puzzling, the two pigs receiving saline intervention measured with [^{11}C]Cimbi-36 had a 5-HT_{2A} receptor occupancy of 12% and 21% (average 17%), which is unexpected and also differing from the absence of 5-HT release demonstrated by microdialysis. However, in the two animals which had received inactive fenfluramine, the 5-HT_{2A} receptor occupancy was only 4%

and 9% and in accordance with the microdialysis measures. We have no explanation for this variability in occupancy, but speculate that use of local anesthetics instituted after the initial scans could be a factor (Kupers et al., 2009).

Finnema et al (S. J. Finnema et al., 2012) speculates on how regional differences in SERT, 5-HT_{1B} auto –and heteroreceptor may impact BP_{ND} by separate mechanisms. For instance, high-density SERT regions may express a higher SSRI-induced increase in 5-HT level as compared to low-density SERT regions. Due to autoreceptor regulation, differences in density of the 5-HT_{1B} receptor across brain regions may also influence the BP_{ND}, but given that the regional relationship between 5-HT_{1B} auto- and heteroreceptor densities is largely unknown, it is difficult predict regions which would be more sensitive to serotonergic challenges.

The decrease in BP_{ND} of [^{11}C]AZ10419369 was only statistical significant in high binding regions or when a large brain volume was encountered, suggesting that good count statistics is needed to detect smaller changes in BP_{ND}.

Several intervention-studies have investigated the sensitivity of PET radioligands to detect serotonergic changes induced by citalopram or escitalopram, such as used here in study 1 and 2. Two human studies with [^{11}C]AZ10419369 (Nord et al., 2013) and the 5-HT_{1A} receptor radioligand [^{11}C]CUMI-101 (Selvaraj et al., 2012) reported an *increase* in binding in several cortical regions, rather than the expected decrease, while no change in binding was reported in another human [^{11}C]CUMI-101 study (Pinborg et al., 2012). In both cases, this was interpreted as a consequence of autoregulation causing a temporary decrease in synaptic 5-HT level. Although in a different species, our data in pigs and other microdialysis studies in rats (Gobert et al., 1997; Hjorth, 1993) suggest that autoregulation may dampen or even cancel out the anticipated SSRI-induced serotonergic response, but the data do not lend support for an acute SSRI-induced decrease in extracellular 5-HT level. The apparently conflicting outcome in the human studies with the non-human primate studies (S. J. Finnema et al., 2012; S.j. Finnema et al., 2010; Nord et al., 2013) and microdialysis studies in rats and to some extent our pig studies may be explained by species differences, differences in study design or much higher doses of escitalopram used in animals inducing a slightly higher increase in 5-HT level more readily detected by PET.

In a human PET study, using a combined intervention with citalopram and pindolol, no change in binding of the 5-HT_{2A} receptor antagonist [^{18}F]altanserin (Pinborg et al., 2004) was observed, om apparent contrast to what we observed with [11C]Cimbi-36 in pigs. Yet, another human [^{18}F]altanserin study (Quednow et al., 2012) did report a decrease in [^{18}F]altanserin binding following an intervention with the more potent 5-HT releaser of dexfenfluramine. This discrepancy could, be ascribed to a higher sensitivity of the 5-HT_{2A} receptor agonist [^{11}C]Cimbi-36 to detect 5-HT as compared to the antagonist [^{18}F]altanserin. Moreover, our data suggest that the reason [^{18}F]altanserin binding was not reduced following an intervention with citalopram + pindolol (Pinborg et al., 2004) but only with dexfenfluramine (Sjoerd Finnema et al., 2011; Quednow et al., 2012) is because the latter is a much stronger elicitor of 5-HT release.

Test-retest analysis

In order to evaluate the stability of our design, we investigated the test-retest variability of BP_{ND} in the preclinical study 2, and we found an excellent reproducibility of [^{11}C]AZ10419369 binding to cortical 5-HT_{1B} receptors. The test-retest variability and ICC was comparable to the outcome reported in clinical studies of [11C]Cimbi-36 binding to 5-HT_{2A} receptors (Ettrup et al., 2016) and a little better than that of [11C]-SB207145 binding to 5-HT_4 receptors (Marner et al., 2009) and [11C]P943 binding to 5-HT_{1B} receptors (Saricicek et al., 2015)

The competition model

The paired data on microdialysis to both [11C]Cimbi-36 (Figure 13) and [^{11}C]AZ10419369 (Figure 14) comply with the competition model (eq.1.) supporting that the two PET radioligands are both sensitive to changes in endogenous 5-HT levels but that is only detectable with PET when the 5-HT release is sufficiently high.

F_{NT} varies across brain regions and species, but in order to relate to eq. 1, we assumed a fixed value for interstitial fluid 5-HT. K_{NT} would change accordingly, but an increase in 5-HT level, whether still result in the same percental decrease in BP_{ND}, regardless of input measures of ΔF_{NT} (nM) relative to level F_{NT} (1.7 nM) or relative 5-HT increase (%) to baseline level (100%).

Based on the data shown in Figure 13 and 14, we estimated the affinity of 5-HT to its receptor, K_{NT}, of [11C]Cimbi-36 (5-HT_{2A} receptor) and [^{11}C]AZ10419369 (5-HT_{1B} receptor), which was

14.3 nM of and 74 nM respectively. For comparison, the concentration of ^{3}H-5-HT that labels 50% of cloned human is 21nM of the 5-HT_{2A} receptors and 0.6-15.8 nM of the 5-HT_{1B} receptors (PDSP K_i database).

A limitation of the studies is that the pharmacological challenges may increase the 5-HT level differently in a region dependent pattern. Microdialysis studies show that SSRI's induce an increase in extracellular 5-HT level much larger in the DRN as compared to striatal and cortical projection areas, where the increase is lower or even absent (Adell & Artigas, 1991; Bel & Artigas, 1992; Gartside, Umbers, Hajós, & Sharp, 1995; Invernizzi et al., 1992; Malagié, Trillat, Jacquot, & Gardier, 1995). This could be caused by regulation of DRN activity and inhibition of DRN firing rate (Artigas et al., 1996; Gartside et al., 1995; Romero, Celada, & Artigas, 1994), or high density of SERT and 5-HT autoreceptors in the DRN (Cortés, Soriano, Pazos, Probst, & Palacios, 1988; Hrdina, Foy, Hepner, & Summers, 1990). As such, the change in 5-HT level measured by microdialysis in the mPFC may not reflect the SSRI induced changes in 5-HT level in other cerebral regions. However, by using the occupancy plot there is an implicit assumption that the pharmacological challenges induce a homogenous 5-HT release in the entire brain, which may not be so. Nevertheless, we found that the data did not deviate significantly from the model and with good r^2 values.

The sensitivity of the radioligands: cross-study comparisons

We find it relevant to compare the sensitivity of the two radioligands used in this thesis, [^{11}C]AZ10419369 and [^{11}C]Cimbi-36, with the sensitivity of: I) [^{18}F]altanserin (5-HT_{2A} receptor antagonist) to detect 5-HT and II) [^{11}C]-raclopride to detect DA, a well-recognized approach to detect in-vivo change in striatal DA.

In a human study (Quednow et al., 2012), [^{18}F]altanserin binding decreased to ~8% following a dose of fenfluramine (40 mg/kg) comparable to the dose (0.5 mg/kg) used in the Study 1 and 2. Thus, the sensitivity of [11C]AZ10419369 to detect changes in 5-HT is twice as good as [^{18}F]altanserin, and the sensitivity of [^{11}C]Cimbi-36 is 6 times better of [^{18}F]altanserin.

At an 8-fold increase in neurotransmitter level, it can be read from Figure 15 that the sensitivity of [^{11}C]AZ10419369 to 5-HT is comparable to the sensitivity of [^{11}C]-raclopride to detect DA as reported in literature, but 3 times less than the sensitivity of [^{11}C]Cimbi-36 to detect changes in

5-HT level. Moreover, whereas [^{11}C]-raclopride only provide reliable assessment of striatal changes in DA level, [^{11}C]Cimbi-36 and [^{11}C]AZ10419369 can – due to the widespread and relatively uniform distribution of the 5-HT$_{2A}$ and 5-HT$_{1B}$ receptor – theoretically be used to determine regional 5-HT changes in the brain.

It is an important feature of a PET radioligand to be able to identify changes also in smaller brain areas, since changes in the cerebral neurotransmitter level could occur in confined brain regions, which potentially could allow for functional imaging of 5-HT release. We observed that the decrease in BP_{ND} of [^{11}C]AZ10419369 was only statistical significant in high binding regions or when a large brain volume was encountered, suggesting that good count statistics is needed to demonstrate smaller changes in BP_{ND}.

Although the sensitivity of [11C]Cimbi-36 is 3 times better than [^{11}C]AZ10419369, there are other factors such as cerebral distribution and kinetics, which may favor the use of the less sensitive [^{11}C]AZ10419369 depending on the aim of the investigation. First, the 5-HT$_{2A}$ receptor has a solid cortical density, while the 5-HT$_{1B}$ receptor are most dense in subcortical areas (Beliveau et al., 2017). Secondly, the 5-HT$_{1B}$ receptor play an important part in the serotonergic modulation of the BG output. Finally, the kinetics of [^{11}C]AZ10419369 is fast, which allow for experimental design conducted as a within scan challenge studies, which reduce scans, lower costs and injected radioactivity and minimize total scan length, with the latter being particular important in clinical studies including disabled and vulnerable patients. All of the above favor the use of [^{11}C]AZ10419369 in PD patients treated with DBS, and when balanced against the more sensitive [^{11}C]Cimbi-36 we chose to use [^{11}C]AZ10419369 in the clinical study (study 3).

5-HT$_{1B}$ receptor availability in patients with PD

We observed that patients with PD had numerically lower 5-HT$_{1B}$ receptor binding in all brain regions (Table 5), which was only significant in the frontal and parietal cortex when correcting for multiple comparisons in the statistical analysis. This is consistent with a prior study in patients with PD PET scanned with [^{11}C]AZ10419369, where patients had a lower 5-HT$_{1B}$ receptor binding in many cerebral regions, although a significant lower binding was observed in the orbitofrontal cortex only, when PET data were analyzed voxel-based (Varrone et al., 2014). The lower 5-HT$_{1B}$ binding could, theoretically, be caused by 1) lower radioligand affinity (higher K_d), for example caused by higher 5-HT levels, 2) lower tissue free fraction (f_{ND}) or 3) a

decrease in receptor density (B_{max}). Hower, based on the outcome from literature the latter seem to be the most likely mechanism due to the following reasons. First, there is amble evidence from biochemical, imaging, and postmortem studies (Huot et al., 2011; Kish et al., 2008; Tohgi, Abe, Takahashi, Takahashi, & Hamato, 1993) that patients with PD have lower levels of 5-HT markers, 5-HT and its metabolites, which would lead to an *increase* in binding instead of the observed decrease. As such, a low level of 5-HT in PD may in fact mask an otherwise low 5-HT_{1B} availability, when measured with PET. However, we cannot exclude that there is a region variability in synaptic 5-HT level in patients with PD as compared to controls, which could potentially explain part of the regional variation in binding potential observed between groups. Second, although it cannot be ruled out, we cannot find any good explanation why patients with PD should have lower free fraction of radioligand in their brain tissue. That is, we consider a low 5-HT_{1B} receptor density, possibly due to loss of neurons that express 5-HT_{1B} receptors, the most likely cause of the low 5-HT_{1B} binding potential observed in patients with PD.

Since the 5-HT_{1B} receptor acts both as an autoreceptor modulating serotonergic release and as an heteroreceptor modulating other neurotransmitter systems (Barnes & Sharp, 1999), the lower 5-HT_{1B} receptor density could be caused by loss a reduction in either auto- or heteroreceptor density, or both. However, as reviewed (Huot et al., 2011), several in vivo and in vitro PET studies of patients with PD report widespread reductions in the SERT (entirely presynaptic) in a regional dependent pattern fairly consistent with our findings (Table 1), whereas the 5-HT_{1A}, 5-HT_{2A}, 5-HT_{3}, and 5-HT_{4} receptors (postsynaptic) are less affected or even upregulated. Loss of serotonergic neurons is known to occur in PD (D'Amato et al., 1987; Halliday et al., 1990), possibly due to aggregation of Lewy bodies in the axonal terminals, leading to cellular dysfunction and eventually neuronal death (Winn, 2011a). The observation supports that in PD, cellular structures proximate to the Lewy body deposits in the axonal terminal are more affected as compared to somatodendritic cellular components.

The role of 5-HT_{1B} receptors for motor disability in patients with PD

We observed an inverse relationship between motor symptom severity and frontal 5-HT_{1B} receptor availability in patients with PD, which suggest that preservation of the 5-HT_{1B} receptor is important for motor function or the decline in 5-HT_{1B} receptor availability is a proxy of the disease progression, or a combination. This association was not driven by any specific frontal subregion. A previous PET study in patients with PD, did not observe a statistically significant

correlation between 5-HT_{1B} receptor binding and symptom severity or staging of the disease, which the authors ascribe to lack of power or due to medication effects (Varrone et al., 2014).

In PD, the spontaneous STN firing rate is 4-6 times above normal, which result in an excessive BG output which inhibit the thalamocortical pathways and cause motor deficits (Ding & Zhou, 2014). Preclinical studies in non-human primates and rats show that 5-HT deficiency as well as lesions in the DRN, both known to occur in Parkinson's Disease (Huot et al., 2011), lead to an increase in STN firing rate (Ding & Zhou, 2014; Miguelez et al., 2014), which can be reverted by either SSRI (Aristieta et al., 2014), 5-HT_{1B} agonists and DA replacement (Ding & Zhou, 2014) and high frequency stimulation in the STN (Meissner et al., 2005). Reduction of excessive STN firing rate is currently considered the prime candidate mechanisms underlying the therapeutic effect of DBS-STN (Ding & Zhou, 2014).

As reviewed, one mechanism DBS-STN acts by is tempering the excessive STN firing rate (Ding & Zhou, 2014), known to be 4-6 fold higher in patients with PD, which lead to a reduction of the excessive GABAergic output from the basal ganglia causing motor symptoms. Like DBS, 5-HT also exert an inhibitory effect on the basal ganglia output, primarily driven by the 5-HT_{1B} receptors on STN afferents and efferents (Ding & Zhou, 2014). As such, preservation of 5-HT_{1B} receptors and the capacity to release 5-HT may be an important mechanism to temper the basal ganglia output in patients with PD, when DBS-STN is turned off.

Turning off DBS-STN in patients with PD

When we turned DBS-STN off, the 5-HT_{1B} receptor binding decreased between zero and 15% across a number of brain regions (Figure 18). Based on data from the pig brain (Jørgensen et al., 2017), (Figure 14) it is possible to estimate that a 15% decline in [^{11}C]AZ10419369 BP_{ND} corresponds to an 8-fold increase in extracellular 5-HT level, which is approximately the same 5-HT release as induced by a single intravenous dose of a potent 5-HT releaser, fenfluramine (0.5 mg/kg).

But how does turning off the DBS stimulator lead to increased cerebral 5-HT release? Preclinical studies show (Navailles, Benazzouz, Bioulac, Gross, & Deurwaerdère, 2010; Tan et al., 2012; Temel et al., 2007) that high frequency stimulation of the STN decrease both STN firing rate and the 5-HT level in frontal cortex. Here, we observe an increase when DBS is turned off. The fact

that turning off DBS-STN induce a serotonergic response beyond the BG and thalamocortical pathways suggest involvement of the raphe nuclei serotonergic components.

Interestingly, we find that the degree to which regional cerebral 5-HT_{1B} receptors are preserved in patients with PD predicts the capacity to produce a serotonergic response when DBS is turned off. However, when the 5-HT_{1B} receptor binding is reduced to 2/3 of age-matched controls, no 5-HT is released in response to turning off the stimulator. The observed association between decline in presynaptic 5-HT_{1B} receptor availability and deficiency to release 5-HT supports the assumption that the abnormalities in cerebral 5-HT_{1B} receptors found in PD patients are primarily caused by presynaptic degeneration.

A limitation of this study is, that for logistical reasons only turning off DBS-STN was investigated, and it is not given that turning DBS-STN on would lead to reverse actions. Also, we cannot assess the temporal evolvement of the response beyond 75 min since our experimental setup with PET does now allow for extended measurements of cerebral 5-HT_{1B} receptor binding in the off condition.

POMS data

If DBS-STN also act on raphe firing, increased raphe firing rate in the DBS-off condition would produce a 5-HT release in cortical projections, which could potentially regulate mood. We observed a significant worsening in POMS score of vigor and activity when DBS was turned off, proportional to the availability of the 5-HT_{1B} receptors. The fact that the negative correlation between 5-HT_{1B} receptor binding and POMS score of vigor and activity was dissociated from the MDS-UPDRS motor scores, is in line with others suggesting separate mechanisms of cognitive and physiological fatigability in PD (Lou, 2015). Patients with least motor symptom severity and best preservation of presynaptic serotonergic functions reported most worsening in vigor and activity when DBS was turned off, as compared to patients with severe serotonergic deficits and substantially worsening in motor symptoms, who only reported little or no change in vigor and activity. This seem less intuitive, and we suspect that it may be due to a report bias in the least affected patients. However, studies of mood scores usually involve sample sizes much larger than the present study, and we suggest these data should be replicated in an independent behavioral study.

CONCLUSIONS AND PERSPECTIVES

This thesis presents three studies aimed to characterize the association between PET measures and changes in 5-HT level, used to investigate the presynaptic serotonergic function in patients with PD treated with DBS.

Brain imaging of the 5-HT system and its functions is important to characterize neuropsychiatric disorders and to identify treatment options. While microdialysis is the gold standard measurement of changes in cerebral 5-HT level, it only allows for measurements in a few selected regions and being an intrathecal invasive procedure, microdialysis is not suitable for readily implementation in the armamentarium of work-ups of these disorders. PET imaging, on the other hand, is non-invasive and provide measures of the 5-HT system in the entire brain. Here, we diagrammed two different PET radioligands in the pig brain by simultaneous PET and microdialysis measures, to allow for interpretation of PET measures to cerebral 5-HT release in future studies such as in our study of patients with PD treated with DBS.

In the pig brain, we demonstrate that the change in [^{11}C]AZ10419369 and [^{11}C]Cimbi-36 PET signal correlates to pharmacologically induced changes in interstitial 5-HT brain level compatible with the competition model. The observed correlations indicate that [^{11}C]Cimbi-36 and [^{11}C]AZ10419369 are both sensitive to changes in endogenous 5-HT levels, but that is only detectable with PET when the 5-HT release is sufficiently high. Differences in earlier studies may thus be ascribed to the efficacy of the pharmacological interventions to change interstitial brain 5-HT levels. The sensitivity of [^{11}C]Cimbi-36 to detect changes in 5-HT level is 3 times higher when compared to [^{11}C]AZ10419369, but the sensitivity of [^{11}C]AZ10419369 to detect changes in extracellular 5-HT level is comparable to that of [^{11}C]raclopride to detect changes in DA level. The reproducibility of [^{11}C]AZ10419369 is excellent. Verifying the direct correlation between pharmacologically induced changes in 5-HT and [^{11}C]Cimbi-36 and [^{11}C]AZ10419369 PET occupancy is an important step prior to conduction of clinical trials and the calibration allows for estimating the regional relative change in interstitial 5-HT.

We used this novel PET methodology to investigate the dynamic integrity of the 5-HT system in patients with PD treated with DBS-STN. We find that the patients exhibit a region specific serotonergic presynaptic dysfunction expressed by a loss of serotonergic presynaptic terminals and, when DBS is turned off, an associated decrease in 5-HT releasing capacity. The serotonergic dysfunction is to some extent correlated to clinical symptom severity. These

serotonergic deficits may contribute to both motor and non-motor symptomatology in patients with PD. This thesis demonstrates that DBS-STN dynamically regulates the 5-HT system and that further studies on long-term effects on 5-HT function is warranted.

Research perspectives

Based on our findings in study 1 and 2, we suggest that both PET radioligands, [^{11}C]Cimbi-36 and [^{11}C]AZ10419369, are suitable for detecting changes in future clinical studies of patients with neuropsychiatric disorders. While [^{11}C]Cimbi-36 is 3 times as sensitive to detect 5-HT release as compared to [^{11}C]AZ10419369, other factors may support the use of [^{11}C]AZ10419369, depending on aim of investigation regarding synaptic localization of the receptor, cerebral region in sight of the investigation and study design.

Diagramming the association between cerebral neurotransmitter release and PET measures according to the competition model in a simultaneous PET and microdialysis study in a pig model, such as presented in this thesis, is a novel method applicable to characterize a broad spectrum of PET radioligands targeting different neurotransmitter systems in the brain. These diagrams are very useful for translation of PET measures to changes in actual neurotransmitter levels in future intervention studies.

While turning DBS off lead to an increase in cerebral 5-Ht release, it cannot readily be assumed that turning DBS on would result in a decrease in cerebral 5-HT level. An important next step would be to investigate the effect of turning DBS on de novo in patients with PD. Moreover, it would also be important to study the effect of DBS on serotonergic function during the longitudinal course of the disease progression.

The present thesis examines acute effects of pharmacological and DBS interventions, while the long-term effects of these interventions or having DBS turned off for an extended period remain unknown.

Based on the outcome from study 3, we find that POMS is a candidate for investigating state mood and interactions with the serotonergic system. However, larger behavioural studies are needed to better investigate the DBS induced changes in mood and affective symptoms.

Based on data from study 3, we find that 5-HT_{1B} receptor binding correlate with clinical scores of motor symptoms in PD, and the 5-HT_{1B} receptor may be a predictor of disease progression. Moreover, since patients with better preserved presynaptic function and less motor symptoms, but report the worst symptoms in mood score when DBS is turned off, the 5-HT_{1B} receptor may be a predictor of self-reported outcome of DBS surgery. More studies are needed to investigate any beneficial effects of brain imaging of the 5-HT_{1B} receptor in both the diagnostic and pre-operative work up in patients with PD.

Increasing attention is given to new application of DBS therapy within the neuropsychiatric field. However, the outcomes from case reports and clinical trials show conflicting results, as well as ethical concerns related to psychosurgery. Future studies with brain imaging of the physiological changes underlying these disorders and the effects of DBS may support the transition of DBS treatment to new applications.

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APPENDICES (PAPER 1, 2 and 3)

Paper 1: Cerebral 5-HT release correlates with [^{11}C]Cimbi-36 PET measures of 5-HT2A receptor occupancy in the pig brain.

Paper 2: Cerebral serotonin release correlates with [^{11}C]AZ10419369 PET measures of 5-HT$_{1B}$ receptor binding in the pig brain.

Paper 3: Parkinson patients display a presynaptic serotonergic deficit: A dynamic DBS-STN PET study.

Paper 1

Original Article

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Cerebral 5-HT release correlates with [^{11}C]Cimbi36 PET measures of 5-HT2A receptor occupancy in the pig brain

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Abstract

Positron emission tomography (PET) can, when used with appropriate radioligands, non-invasively generate temporal and spatial information about acute changes in brain neurotransmitter systems. We for the first time evaluate the novel 5-HT_{2A} receptor agonist PET radioligand, [^{11}C]Cimbi-36, for its sensitivity to detect changes in endogenous cerebral 5-HT levels, as induced by different pharmacological challenges. To enable a direct translation of PET imaging data to changes in brain 5-HT levels, we calibrated the [^{11}C]Cimbi-36 PET signal in the pig brain by simultaneous measurements of extracellular 5-HT levels with microdialysis and [^{11}C]Cimbi-36 PET after various acute interventions (saline, citalopram, citalopram + pindolol, fenfluramine). In a subset of pigs, para-chlorophenylalanine pretreatment was given to deplete cerebral 5-HT. The interventions increased the cerebral extracellular 5-HT levels to 2–11 times baseline, with fenfluramine being the most potent pharmacological enhancer of 5-HT release, and induced a varying degree of decline in [^{11}C]Cimbi-36 binding in the brain, consistent with the occupancy competition model. The observed correlation between changes in the extracellular 5-HT level in the pig brain and the 5-HT_{2A} receptor occupancy indicates that [^{11}C]Cimbi-36 binding is sensitive to changes in endogenous 5-HT levels, although only detectable with PET when the 5-HT release is sufficiently high.

Keywords

Positron emission tomography, 5-HT, brain imaging, kinetic modelling, neurosurgery

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Introduction

Positron emission tomography (PET), when used with appropriate radioligands can non-invasively generate temporal and spatial information about acute changes in neurotransmitter systems in the brain; in particular, imaging of changes in the cerebral dopamine levels. For example, the PET radioligands [^{11}C]-raclopride and [^{123}I]IBZM have expanded the knowledge of the dopaminergic mechanisms involved in disorders such as schizophrenia, Parkinson's, and stimulant abuse.[1] Only to a lesser extent has this approach been successfully applied to imaging of other neurotransmitter systems, such as the opioid,[2] serotonin,[3] and other neurotransmitter systems.[4]

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Neuropsychiatric disorders such as depression and migraine are ameliorated by pharmacological modulation of the serotonin (5-HT) system and this constitutes an established treatment for these disorders.[5] PET imaging of the cerebral dynamic changes of the 5-HT level would make an important contribution to the understanding of the serotonergic mechanisms involved in these disorders, the underlying mechanisms behind the effect of, for example, pharmacological treatment or other interventions and could potentially lead to new treatments. Several studies have investigated PET radioligands for their ability to be displaced by endogenously released 5-HT. Five studies with the 5-HT_{2A} antagonist radiotracer [^{18}F]altanserin and [^{18}F]setoperone were performed in humans, with various pharmacological challenges. A single dose of paroxetine,[6] intravenous ketamine[7] and pindolol plus citalopram[8] did not induce any significant changes in [^{18}F]altanserin binding or [^{18}F]setoperone binding. Clomipramine[9] and dexfenfluramine[10] (40 or 60 mg single oral dose) dose-dependently decreased [^{18}F]altanserin binding in several brain regions. In addition, during sleep in sleep-deprived individuals, [^{18}F]altanserin binding increases, consistent with lower 5-HT levels, known to occur during sleep.[11]

According to the competition model, there are three factors, which determine the ability of a tracer to detect changes in synaptic neurotransmission: affinity of the neurotransmitter for its receptor, K_{NT}, the basal neurotransmitter concentration in the interstitial fluid, F_{NT}, and the challenge-induced change in neurotransmitter concentration, ΔF_{NT}. The relationship between the resulting receptor occupancy and these variables is described by the equation[3]

$$Occupancy = \frac{\Delta F_{NT}}{K_{NT} + F_{NT} + \Delta F_{NT}} \quad (1)$$

That is, if the challenge-induced change in 5-HT concentration is much larger than $K_{NT} + F_{NT}$, then changes in radioligand binding with PET may be much more readily detected. Accordingly, we speculate that the different outcomes of the studies mentioned above[7–10] may be due to the extent to which the different challenges affect synaptic 5-HT levels.

We recently developed and assessed the first 5-HT_{2A} receptor agonist PET radioligand [^{11}C]Cimbi36 in humans.[12] Agonists have generally been suggested to be more sensitive to neurotransmitter release as compared to antagonist radioligands[13,14] and preliminary data in non-human primates suggest that [^{11}C]Cimbi36 binding is reduced following intravenous fenfluramine (5 mg/kg).[15] For clinical studies, however, fenfluramine cannot be given at such high doses, and instead the 5-HT transporter selective inhibitor (es)citalopram has often been used as a tool to induce 5-HT increases in the human brain. Two recent PET-studies in humans question, however, if intravenous citalopram is capable of acutely increasing 5-HT levels and they both suggest that due to the 5-HT autoreceptors, an acute SSRI intervention results in a *decrease* in brain interstitial fluid 5-HT levels.[16,17] In the absence of a direct measure of brain interstitial 5-HT concentration, such as done by state-of-the-art *in vivo* cerebral microdialysis,[18] the matter is, however, difficult to settle.

The aim of the present study was to measure the effect of different pharmacological interventions on 5-HT by cerebral microdialysis and to correlate this outcome to the occupancy of the 5-HT_{2A} receptor agonist radioligand [^{11}C]Cimbi36. We measured pharmacologically induced changes in 5-HT levels in the medial prefrontal cortex (mPFC) *in vivo* in pigs and assessed simultaneously changes in [^{11}C]Cimbi36 binding. We hypothesized that the different challenges would induce varying increases in the regional 5-HT brain level as measured with microdialysis, and a corresponding decline in [^{11}C]Cimbi36 receptor binding in the brain.

Material and methods

Animals

We used 13 female 9- to 10-week-old Danish Landrace pigs weighing 24 ± 1.5 kg (mean $\pm$ SD) in this study. All animal experiments were performed in accordance with the European Communities Council Resolves of 22 September 2010 (2010/63/EU) and approved by the Danish Veterinary and Food Administration's Council for Animal Experimentation (Journal No. 2012-15-2934-00156), and is in compliance with the ARRIVE guidelines (www.nc3rs.org.uk/arrive-guidelines). We housed the pigs and performed tranquilization, anesthesia, intubation, installation of arterial and venous intravenous lines in the morning, monitoring and sacrifice of animals late afternoon as previously described.[19] On the day of investigation, we implanted microdialysis guide cannulas bilaterally into the medial prefrontal cortex (mPFC). After midline sagittal incision and exposure of the skull, we placed two burr holes 25 mm anterior and 8 mm lateral to each side of bregma, incised dura and secured hemostasis. Then we placed an anchor screw anterolateral to the right burr hole and decorticated the skull to allow fixation of the 10 mm CMA 12 guide cannulas (CMA, Kista, Sweden), which we introduced through each burr hole in a direction perpendicular to the skull and fixed with Dentalon Plus Cement (Heraeus Kulzer GmbH, Hanau, Germany). After surgery, we inserted a CMA

12 metal-free microdialysis probe (CMA Microdialysis) with a membrane length of 4 mm and a molecular weight cut-off value of 20 kDa through each of the cannulas. The coordinates allowed full embedment of the membrane in grey matter of mPFC. A postoperative MRI from a 3.0 T Siemens VERIO MR scanner, which we analyzed with the Brainlab Stereotactic Planning software and manual co-registration to a histology slice, served to confirm the correct position of the probe in the grey matter of mPFC of the pig brain.

Study design

All baseline and challenge PET scans were conducted on the same day, in fixed order. Microdialysis probes were implanted bilaterally in the mPFC and we confirmed the correct position with a postoperative brain MRI, as described above. A 2-h wash-out period was allowed for the microdialysis to reach a stable baseline level. The two PET scans, each lasting 90 min, were conducted with at least 30 min apart. After completion of the first PET scan, we administered the pharmacological interventions intravenously, with the aim to acutely increase the extracellular 5-HT levels.

After the last PET scan, we excised tissue from the mPFC where the microdialysis probe had been implanted. The tissue was quickly frozen on dry ice and stored in a −80°C freezer until further analyzed for either content of 5-HT or sliced in a microtome and stained to obtain a histology slice that could be compared to the MRI, for establishment of probe placement in the grey matter of mPFC.

Pharmacological interventions

To confirm that a change in radioligand binding was not caused by direct interaction between fenfluramine and the 5-HT_{2A} receptor[20,21] we gave two pigs that later received fenfluramine intramuscular para-chlorophenylalanine (pPCA)[22] prior to the day of the PET-scan. pCPA is an irreversible inhibitor of the enzyme tryptophan hydroxylase, which catalyzes the rate-limiting step in 5-HT biosynthesis, and we have previously shown that pCPA substantially reduces 5-HT level in the pig brain without altering 5-HT_{2A} receptor density.[23] Thus, nine of the 13 pigs received a four-day pretreatment of intramuscular saline 10 mL (N = 7) or pCPA 100 mg/kg (N = 2) in a solution of 2 ml/kg. The intramuscular injections required sedation of the pigs with a solution of dexmedetomidin 50 μg/kg, butorphanol 0.2 mg/kg and midazolam 0.15 mg/kg on the first day. On the following days, propofol 4 mg/kg and midazolam 0.1 mg/kg were given intravenously in a catheter placed in the ear. After the procedures, the pigs were given atipamezole 200 μg/kg for reversal of anesthesia.

In pilot experiments prior to the ones described here, we tested the effect of three different doses of fenfluramine; 0.05 mg/kg, 0.5 mg/kg and 2.0 mg/kg, but when 2 mg/kg was given, the pig started to shiver and showed a large increase in vital signs, so the injection was interrupted after ¾ had been given. Since the dose of 0.05 mg/kg did not induce any significant alterations in microdialysate 5-HT levels, we chose to give a final dose of 0.5 mg/kg.

Interventions between the two PET-scans were given 15 min prior to the second scan and consisted of either

- Saline (Controls, $N = 2$)
- Citalopram 2 mg/kg (Selective 5-HT reuptake inhibitor (SSRI), $N = 2$)
- Citalopram 2 mg/kg, preceded by pindolol 1 mg/kg, given 30 min prior to the second scan (5-HT_{1A} autoreceptor antagonist, $N = 3$)
- Fenfluramine 0.5 mg/kg (5-HT releaser, $N = 4$)
- Fenfluramine 0.5 mg/kg with pCPA pre-treatment (5-HT depletion, $N = 2$)

Unexpectedly, two pigs assigned for fenfluramine 0.5 mg/kg intervention, did not respond to the challenge as evaluated by both microdialysate 5-HT levels and PET measures. These studies were conducted five months after the initial experiments, which raised the concern that in between the experiments, the fenfluramine had either decomposed or the content had been replaced by some unknown 5-HT inactive material. We acquired a new batch of fenfluramine and compared it to the remaining powder in the vial. Visual inspection, HPLC analysis and new trials in mice and pigs supported that the vial content had been replaced. Accordingly, we excluded these two pigs from the group analyses of pharmacological interventions with fenfluramine, but we did not exclude them from the correlation analysis of microdialysis and PET measures.

Microdialysis and 5-HT measurements

We placed the pig in the PET scanner and allowed a 2-h washout period to obtain steady baseline microdialysis level. The probes were perfused with a standard Ringer solution (147 mM NaCl, 4 mM KCl and 2.3 mM CaCl2, adjusted to pH 6.5) at a flow rate of 1.0 μL/min. From both probes, we collected 15 min time-series of samples of extracellular fluid to be analyzed off-line for monoamines by HPLC. After a 2-h washout period, we collected 16 samples with 15 min interval. The microdialysate samples were quickly frozen on dry ice and stored in a −80°C freezer until further analyzed.

We determined the relative changes in 5-HT concentrations in the dialysates and the absolute 5-HT

concentration in brain tissue samples by HPLC, with electrochemical detection. The column was a Prodigy 3 µ ODS (3) C18 (DA 2 mm × 100 mm, particle size 3 µm, Phenomenex, Torrance, CA, USA). The mobile phase consisted of 55 mM sodium acetate, 1 mM octanesulfonic acid, 0.1 mM Na2EDTA and 8% acetonitrile, adjusted to pH 3.2 with 0.1 M acetic acid, and was degassed with an on-line degasser. Ten microliters of the dialysate samples was injected, and the flow rate was 0.15 mL/min. The electrochemical detection was done with an amperometric detector (Antec Decade, Antec, Leiden, the Netherlands) with a glassy carbon electrode set at 0.8 V, with an Ag/AgCl reference electrode.

The excised pig brain blocks were homogenised in perchloric acid 0.1 N. After centrifugation at 14,000 r/min for 30 min, 200 µL of the supernatant was filtered through a glass 0.22 µm filter Avantec 13CP020AS. The 5-HT tissue concentrations in the pig brain homogenate was calculated as 5-HT ug/g brain tissue and represent as mean ± SEM for the saline and the pCPA-treated group.

The output of the HPLC was recorded by the program "LC solution" (Simadzu, Columbia, MD, USA), which also was used to calculate the peak areas. We determined the baseline level on the basis of the mean peak area obtained by the HPLC from the three samples preceding the pharmacological intervention. We used the baseline level to calculate the relative change in the 5-HT levels of the following samples. In 10 of the 13 pigs, stable baseline 5-HT levels were achieved on both sides; in three pigs, only one probe returned stable baseline levels.

PET scanning protocol

PET scans were obtained in list mode with a high-resolution research tomography (HRRT) scanner, with the pig in the prone position. [^{11}C]Cimbi36 was given as an intravenous bolus injection and data acquisition began at the time of injection. The synthesis and radiochemical labeling of [^{11}C]Cimbi36 has previously been described.[12]

The mean injected [^{11}C]Cimbi36 was 471 MBq (range, 197–510 MBq; $N = 26$), and the average injected mass was 1.20 µg (range 0.10–3.55 µg; $N = 26$). During the first 20 min of the PET scan, whole blood radioactivity was continuously measured using an ABSS autosampler (Allogg Technology, Mariefred, Sweden) counting coincidences in a lead-shielded detector. In addition, blood samples were manually drawn at 2.5, 5, 10, 20, 30, 50, 70 and 90 min and the radioactivity in whole blood and plasma was measured in a well counter (Cobra 5003, Packard Instruments, PerkinElmer, Skovlunde, Denmark) that was cross-calibrated to the HRRT scanner and autosampler. Radiolabeled parent compound and metabolites were measured in plasma using HPLC with online radioactivity detection as previously described.[24] An average parent compound fraction-time curve was generated, based on all baseline scans in this study and the data were fitted to a bi-exponential function. This function was used with the individual plasma radioactivity concentration curves from the combined baseline and intervention scans to generate the individual plasma parent compound input functions.

Quantification of PET data

The [^{11}C]Cimbi36 HRRT PET data were reconstructed into 38 frames of increasing length (6×10, 6×20, 4×30, 9×60, 2×180, 8×300, 3×600 s). Images consisted of 207 planes of 256×256 voxels of $1.22 \times 1.22 \times 1.22$ mm. Summed images of all counts in the 90 min scan were reconstructed for each pig and used for co-registration to a standardized MRI-based atlas of the Danish Landrace pig brain, similar to that previously published for the Göttingen minipig,[25] using the software Register, as previously described.[26]

Each coregistration was verified by visual inspection before extraction of time-radioactivity curves (TACs) from the volumes of interest (VOIs), and adjusted if needed. The TACs were determined for the following VOIs: Neocortex and cortical white matter, cerebellum, hippocampus, striatum (caudate and putamen) and thalamus (medial and lateral thalamus).

We quantified the 5-HT$_{2A}$ receptor binding with [^{11}C]Cimbi36 both with the 2TC and Logan invasive modeling; the latter model generated a higher number of converging regional fitting and was thus chosen for further analysis. We calculated the volumes of distribution (V_T) for the VOIs with PMOD software (version 3.0; PMOD Technologies, Zürich, Switzerland). The V_T of the baseline and intervention scan was used to determine occupancy by use of the occupancy plot.[27,28] The standard coefficient of variance (COV) was below 10% for all regional V_Ts. Data that did not fulfill this criterion were not included in the analysis.

Statistical analysis

We correlated the occupancy as measured by PET with the highest peak increase (of the left or right microdialysis probe) in extracellular 5-HT levels. Unless performed with very time-consuming methods[29] incompatible with the use of short-lived radioisotypes and the need to conduct two PET studies, microdialysis experiments do not generate absolute 5-HT concentrations. Instead, we assumed a fairly stable baseline concentration of the pig cerebral interstitial fluid 5-HT of

about 1.7 nM, equal to what has been found in mice,[30–34] and ΔF_{NT} was then computed as 1.7 nM times the relative peak increase in 5-HT level minus 1.7 nM. The data were fitted to the model given in equation (1) with a non-linear regression analysis. The groups of pre-treatment with saline or pCPA were compared with unpaired two-tailed t-test.

Results

Probe placement

Figure 1 illustrates the placement of the tip of the probe, from which the membrane extends 4 mm upwards in the grey matter. Except for one pig with one misplaced probe, both probes were correctly positioned in all pigs.

Tissue concentrations of 5-HT

The mean tissue concentrations of 5-HT in pigs pre-treated with saline was 0.25 ± 0.05 µg/g tissue (mean ± SD, $N = 7$) cortical tissue. Pre-treatment with pCPA resulted in lower 5-HT tissue concentrations, 0.05 µg/g tissue ($N = 2$), in line with previous observations.[23]

In vivo *microdialysis*

Figure 2 illustrates time-dependent effects of the interventions on changes in cerebral 5-HT as measured by microdialysis in the mPFC. Fenfluramine produced an immediate and powerful increase in extracellular 5-HT level to a peak $1123 \pm 144\%$ ($N = 2$) relative to baseline level (100%). The 5-HT releasing effect of fenfluramine was blunted by pCPA pre-treatment, with a peak 5-HT increase to $516 \pm 159\%$ ($N = 2$). Citalopram produced an immediate increase in 5-HT, to 217% ($N = 2$), that was normalized or perhaps even reversed after 30 min. The combination of pindolol and citalopram intervention prolonged and enhanced the serotonergic response as compared to citalopram alone, leading to a sustained mean increase of up to $441 \pm 78\%$ ($N = 3$) of 5-HT baseline level. Saline intervention did not change the 5-HT level in the pig brain relative to baseline level.

In vivo *PET imaging*

The V_T in the baseline scans was 7.3 ± 1.7 mL/cm^3 ($N = 13$) in the neocortex grey matter and 3.6 ± 1.0 mL/cm^3 ($N = 13$) in the cerebellum. The 5-HT occupancy was quantified using the Lassen plot in each individual pig. The 5-HT_{2A} receptor occupancy was $17 \pm 5\%$ ($N = 2$) with saline, whereas the occupancy after pharmacological intervention with citalopram was $19 \pm 2\%$ ($N = 2$), with combined citalopram

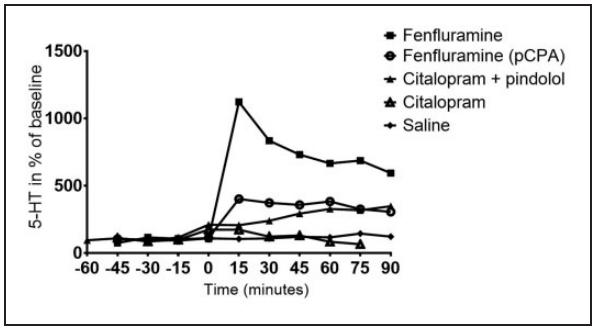

Figure 2. Time-dependent intervention effects on the changes in the relative cerebral extracellular 5-HT levels as measured by microdialysis in the mPFC. Values are given as means of 2–3 measurements. All V_T values are listed in the Supplementary Material, Table 1.

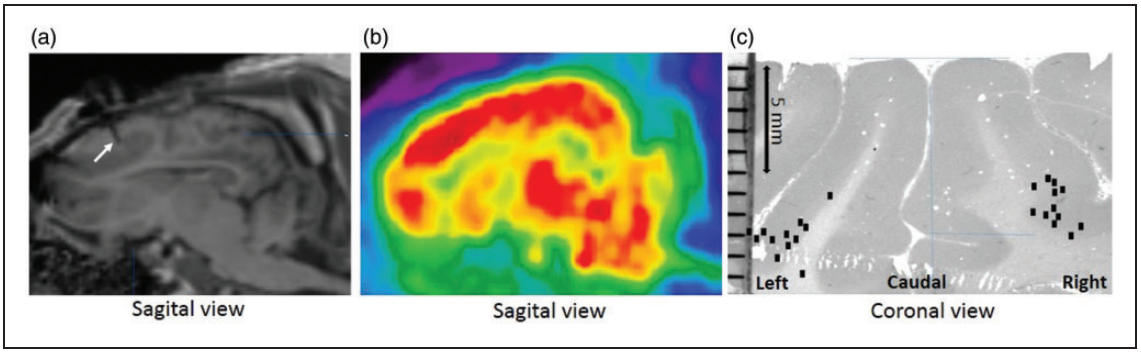

Figure 1. (a) MRI of the pig brain showing the implanted microdialysis probe (white arrow) and (b) [^{11}C]Cimbi36 PET image of the pig brain. The placement of the tip of the microdialysis probes in the mPFC, as identified from the MRI scan, is indicated on a histology slice (c) and marked with a black dot. The microdialysis probe extends from the tip (black dot) and 4 mm cortically, meaning that the relevant part of the probe is embedded within the grey matter of the mPFC.

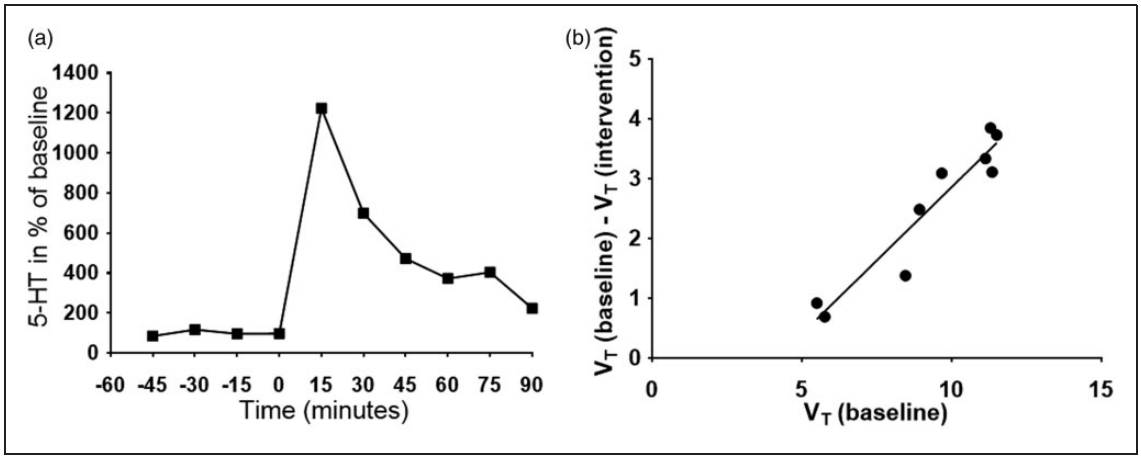

Figure 3. Example of microdialysis time course and an occupancy plot in a single pig. Panel A shows the relative change in 5-HT (given in % of baseline levels) over time, after fenfluramine 0.5 mg/kg i.v. was given at time=0 min. Panel B shows the corresponding occupancy plot based on volumes of distribution (V_T) in the left and right VOIs (mL/cm^3).

and pindolol 28 ± 4% ($N = 3$), fenfluramine with pCPA pre-treatment 38 ± 3% ($N = 2$) and fenfluramine with no pre-treatment 44 ± 3% ($N = 2$). Based on the baseline and the intervention scans, the non-displaceable distribution volume (V_{ND}) of [^{11}C]Cimbi36 was determined to 2.7 ± 0.5 mL/cm^3 ($N = 9$).

Correlation of microdialysis and PET measures

Figure 3 shows an example of microdialysis data and an occupancy plot obtained in one pig. The corresponding measures of the PET occupancy and the ΔF_{NT} were fitted to equation (1) (Figure 4). K_{NT} can be calculated from equation (1) and was determined to be 14.3 nM, i.e. about eight times higher than the baseline brain interstitial 5-HT concentration. The observed correlation measures complied with the competition model (equation (1)).

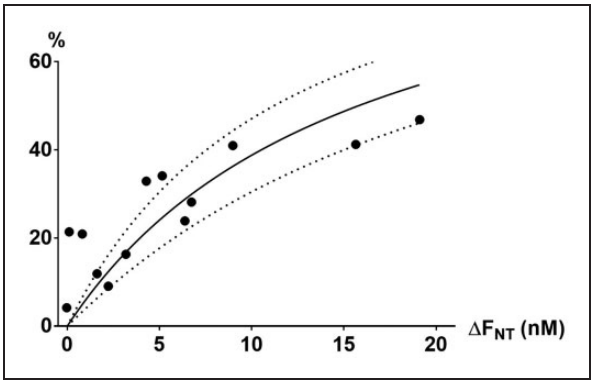

Figure 4. The ΔF_{NT} (nM) and the corresponding 5-HT$_{2A}$ receptor occupancy (%) with 95% confidence interval of all 13 pigs were fitted with nonlinear regression analysis according to the occupation model (equation (1)). The points did not deviate significantly from the model.

Discussion

We here show, first, how different pharmacological interventions directly change the cerebral interstitial fluid concentration of 5-HT in the pig brain. Second, we demonstrate how the cerebral binding of 5-HT$_{2A}$ receptor agonist radioligand [^{11}C]Cimbi36 changes in response to these interventions.

The pharmacological interventions induced a dose-dependent increase in cerebral 5-HT release of 2–11-fold of baseline level in the mPFC in the pig brain. This is in line with microdialysis studies in rats and non-human primates where the increase in the cerebral 5-HT level was 4–35 fold with fenfluramine (1–10 mg/kg)[35–39] and we observed an 11-fold increase with fenfluramine (0.5 mg/kg). Further, in rats citalopram (5–10 mg/kg)[40] induced a 2–4-fold increase in 5-HT level compared to the 2-fold increase with citalopram (2 mg/kg) in our study. A lower dose of citalopram (1 mg/kg) did not change the extracellular 5-HT level in the rat, but when the 5-HT$_{1/2}$ receptor blocker methiothepin was added, 5-HT levels increased four times as compared to the baseline level.[41] This increase was equivalent to the four-fold increase with citalopram (2 mg/kg) plus pindolol (1 mg/kg) used in our study. Although we used smaller doses of all the pharmacological interventions in the pig as compared to the studies in the rat, we observed an increase in extracellular 5-HT level comparable to those in the rats. This efficacy of lower doses could be due to species difference, or could be ascribed to the absence of anaesthesia in some of the rodent studies or differences in the administration route of the drugs.[35–37,39] In any

instance, we found that a dose of 1.5 mg/kg was the maximal dose of fenfluramine that the pigs tolerated.

pCPA-pretreatment effectively reduced the brain 5-HT levels to ~20%, which is in line with Ettrup et al.[23] Although a higher percentage of depletion (5–10%) was achieved in rats with pCPA-pretreatment of 200–400 mg/kg,[42] Ettrup et al. did not in pigs find an added effect of increasing pCPA dose from 50 to 100 mg/kg.[23] This lack of a clear dose–response relationship has previously been ascribed to a threshold effect or to a limited bioavailability from intramuscular administration in pigs compared to intraperitoneal administration in rats. Somewhat unexpectedly, this vast reduction in total 5-HT level only reduced the fenfluramine induced 5-HT release to about half as much as compared to saline-pretreated pigs. This could be caused by the serotonergic neurons more efficiently storing and releasing 5-HT in the vesicles of the synaptic terminal. Alternatively, given that our microdialysis measurements only generate relative increases in interstitial fluid 5-HT, if pCPA-treated pigs had reduced interstitial fluid baseline 5-HT levels to 20% of the saline-pretreated pigs, then the absolute amounts of released 5-HT could have been substantially smaller. Based on the occupancy plot (Figure 4), the latter did, however, not seem to be the case.

Depending on the pharmacological intervention, the 5-HT_{2A} receptor occupancy as measured by the PET radioligand [^{11}C]Cimbi36 ranged between 19 and 44%. The paired data on 5-HT microdialysis and [^{11}C]Cimbi36 occupancy measures comply with the competition model (equation (1)) and indicate that [^{11}C]Cimbi36 was sensitive to changes in endogenous 5-HT levels, but that was only detectable with PET when the 5-HT release was sufficiently high. As anticipated, we observed a higher occupancy with more powerful serotonergic challenges and also a higher occupancy when citalopram was combined with pindolol.

Based on the data shown in Figure 4, we estimated K_{NT} (the affinity of 5-HT to the 5-HT_{2A} receptor) to be about 14.3 nM. For comparison, the concentration of ^{3}H-5-HT that labels 50% of cloned human 5-HT_{2A} receptors is 21 nM (PDSP K_i database).

To put our findings in perspective, one can compare the sensitivity of [^{11}C]-Cimbi36 to 5-HT to that of [^{11}C]-raclopride to dopamine, a much used approach to detect *in vivo* change in cerebral dopamine. From microdialysis studies in non-human primates, it is known that when amphetamine 0.3 mg/kg is given intravenously, the interstitial dopamine concentration increases approximately 8-fold.[43–45] In non-human primates, an 8-fold increase in dopamine results in a decrease in [^{11}C]-raclopride BPnd of 13%.[46] This is consistent with findings in humans, where intravenous amphetamine 0.2–0.3 mg/kg results in a decrease in striatal BPnd of 15.5%[46] or 13%.[47–49] From Figure 4 it can be seen that when interstitial 5-HT is pharmacologically increased by 8-fold, and [^{11}C]-Cimbi36 BPnd decreases by 46% (CI 38-55%), i.e. [^{11}C]-Cimbi36 is over three times more sensitive to changes in 5-HT than [^{11}C]-raclopride is to dopamine. Moreover, while [^{11}C]-raclopride can only be used to reliably assess dopamine changes in striatum, [^{11}C]-Cimbi36 can – due to the widespread and relatively uniform distribution of the 5-HT_{2A}R – theoretically be used to determine regional 5-HT changes in the brain.

As expected, saline intervention did not change microdialysis 5-HT brain levels, and still we somewhat puzzlingly observed 5-HT_{2A}R occupancy in the two saline-treated animals, 12 and 21% (average 17%). In two animals that received inactive fenfluramine, however, the 5-HT_{2A}R occupancy was only 4% and 9%. We have no explanation for this variability in occupancy, but speculate that anesthesia or a better peripheral pain alleviation instituted after the initial scans could play a role.[50]

In a previous PET study in humans using the 5-HT_{2A} receptor antagonist [^{18}F]altanserin, intervention with citalopram + pindolol did not change the occupancy.[8] This difference could, however, also be ascribed to a higher sensitivity to 5-HT competition with the 5-HT_{2A} receptor agonist [^{11}C]Cimbi36 compared to the antagonist [^{18}F]altanserin, which binds to both low and high affinity receptors. Our data suggest that the reason why a reduction in [^{18}F]altanserin PET was seen after dexfenfluramine[10,15] but not after citalopram + pindolol[8] is because dexfenfluramine is a much stronger elicitor of 5-HT.

Three studies in humans tested the sensitivity of the 5-HT_{1A} receptor radioligand [^{11}C]CUMI-101[16,51] and the partial 5-HT_{1B} agonist radioligand [^{11}C]AZ10419369[17] to pharmacological changes with escitalopram or citalopram. While Pinborg et al.[51] did not observe any significant change in [^{11}C]CUMI-101 binding following an intravenous infusion of citalopram (~0.15 mg/kg), Selvaraj et al.,[16] using a similar dose of citalopram (~0.15 mg/kg) found a mean increase of 7% in BP_{ND} in several cortical regions, but no change in the dorsal raphe nucleus (DRN). Likewise, in humans, Nord et al.[17] reported increased binding of [^{11}C]AZ10419369 in serotonergic projection areas following a clinically relevant peroral dose of 20 mg escitalopram (~0.28 mg/kg), whereas there was a statistical trend for a decreased raphe nuclei binding. Thus, two studies in humans showed an *increase* in [^{11}C]CUMI-101[16] and [^{11}C] AZ10419369[17] binding, rather than the expected decrease. This was in both cases interpreted as a consequence of the pharmacological challenge inducing a temporary decrease in 5-HT, due to 5-HT_{1A} autoreceptor regulation. Although in a different species, our data do not lend support for

an acute decrease in cerebral interstitial 5-HT levels, at least not when giving what corresponds to a 3–4 times higher dose of citalopram. Currently, no studies have investigated the effect of lower doses of citalopram on brain interstitial 5-HT levels.

A limitation of this study is that microdialysis allows only for measurements in a few selected brain regions, while PET, on the other hand, can generate information about changes in the 5-HT of the entire brain. Other microdialysis studies[41,52–55] show that the SSRI-induced elevation in the extracellular 5-HT level is higher in the DRN compared to striatal and cortical projection areas, where the 5-HT increase is lower or even absent. This region-dependent pattern may be caused by a higher density of the 5-HT transporter and 5-HT_{1A} receptors in the DRN[56,57] and inhibition of DRN firing rate to projection areas.[55,58,59] Therefore, the Δ[5-HT], as measured by microdialysis in the mPFC, may not necessarily reflect the different regional changes in the interstitial 5-HT level. By using the occupancy plot, we implicitly assume a uniform release of 5-HT throughout the brain. While this assumption may not be justified, we nevertheless found that the occupancy plots generally had statistically significant correlations with good r-square values. The low number of pigs (2–3) in each group of the serotonergic challenges did not allow for a statistical comparison of the different pharmacological intervention effects, but that was not the main goal of the study. Rather, we wanted to asses the sensitivity of [^{11}C]Cimbi36 to detect changes in 5-HT brain levels.

In conclusion, we demonstrate that the change in the [^{11}C]Cimbi36 PET signal correlates to pharmacologically induced changes in interstitial 5-HT brain level. The observed correlation between changes in the extracellular 5-HT level in the pig brain and the 5-HT_{2A} receptor occupancy indicates that [^{11}C]Cimbi36 is sensitive to changes in endogenous 5-HT levels, but that is only detectable with PET when the 5-HT release is sufficiently high. Differences in earlier studies may thus be ascribed to the efficacy of the pharmacological interventions to change interstitial brain 5-HT levels. Verifying the direct correlation between pharmacologically induced changes in 5-HT and [^{11}C]Cimbi36 PET occupancy is an important step prior to conduction of clinical trials and the calibration allows for estimating the regional relative change in interstitial 5-HT in patients in future studies.

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Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Authors' contributions

All authors revised and gave final approval of the manuscript. LMJ, PW, AE and GMK contributed to the conception of the article, and LMJ, PW, HDH and GMK contributed to the drafting of the article). All authors contributed to the design (with exception of HDH and JV), acquisition of data (with exception of HDH and GMK) and analysis and interpretation of data (with the exception of JV, AOB and FLA)

Supplementary material

Supplementary material for this paper can be found at http://jcbfm.sagepub.com/content/by/supplemental-data

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Cerebral serotonin release correlates with [^{11}C]AZ10419369 PET measures of 5-HT$_{1B}$ receptor binding in the pig brain

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Abstract

Positron emission tomography (PET) can, when used with appropriate radioligands, non-invasively capture temporal and spatial information about acute changes in brain neurotransmitter systems. We here evaluate the 5-HT$_{1B}$ receptor partial agonist PET radioligand, [^{11}C]AZ10419369, for its sensitivity to detect changes in endogenous cerebral serotonin levels, as induced by different pharmacological challenges. To enable a direct translation of PET imaging data to changes in brain serotonin levels, we compared the [^{11}C]AZ10419369 PET signal in the pig brain to simultaneous measurements of extracellular serotonin levels with microdialysis after various acute interventions (saline, escitalopram, fenfluramine). The interventions increased the cerebral extracellular serotonin levels to two to six times baseline, with fenfluramine being the most potent pharmacological enhancer of serotonin release. The interventions induced a varying degree of decline in [^{11}C]AZ10419369 binding in the brain, consistent with the occupancy competition model. The observed correlation between changes in the extracellular serotonin level in the pig brain and the 5-HT$_{1B}$ receptor occupancy indicates that [^{11}C]AZ10419369 binding is sensitive to changes in endogenous serotonin levels to a degree equivalent to that reported of [^{11}C]raclopride to dopamine, a much used approach to detect in vivo change in cerebral dopamine.

Keywords

Positron emission tomography, serotonin, brain imaging, kinetic modelling, neurosurgery

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Introduction

Several studies have investigated PET radioligands for their ability to be displaced by endogenously released serotonin. Such PET radioligands would be an important tool to investigate disorders associated to specific serotonin subtypes and serotonergic dysfunction as well as pharmacological treatments targeting these receptors. Unfortunately, the outcomes in humans have not convincingly shown the expected effect of pharmacological interventions supposed to increase brain interstitial serotonin.[1,2]

The 5-HT$_{1B}$ receptor is one of 14 mammalian serotonin receptor subtypes; each receptor is associated with specific physiological impact and has a distinct regional distribution in the brain.[3,4] The 5-HT$_{1B}$ receptor is located on the terminals both presynaptically, where it acts as a 5-HT autoreceptor regulating serotonin release, and postsynaptically as a heteroreceptor, where it interacts with other neurotransmitter systems such as acetylcholine, glutamate, GABA and dopamine.[3] The receptor is involved in regulation of several physiological functions such as increased secretion of corticosterone and prolactin, sexual function, appetite, thermoregulation and locomotion.[3] Further, the 5-HT$_{1B}$ receptor has been associated to many

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neuropsychiatric disorders such as depression, anxiety and OCD.[5] From a clinical perspective, modulation of serotonin at the 5-HT_{1B} receptor has a high therapeutic potential. Hence, the ability to image changes in serotonin would constitute an important contribution to unveil disease mechanisms and to identify therapeutic options.

PET studies with the 5-HT_{1B} receptor radioligands [^{11}C]AZ10419369[6,7] or [^{11}C]P943[8] in humans and non-human primates have investigated the extent of radioligand displacement from the receptor by an increase in endogenous serotonin elicited by a serotonergic challenge. In non-human primates, studies[6,7] demonstrate a dose-dependent decrease in radioligand binding across brain regions following intervention with fenfluramine (a strong serotonin releaser) or a high dose of the serotonin reuptake inhibitor (SSRI) escitalopram. In humans, however, an increased radioligand binding was observed following a clinical relevant dose of escitalopram.[7] Similarly, in another study in humans, the cerebral binding of the 5-HT_{1A} receptor PET radioligand [^{11}C]CUMI-101 was also found to increase following a citalopram[9] challenge although no change was found in another [^{11}C]CUMI-101 study.[10] This unexpected finding in humans was interpreted in both studies as being caused by autoreceptor function, with 5-HT_{1B} or 5-HT_{1A} autoreceptor stimulation inhibiting serotonin release.

Such paradoxical SSRI-induced effects on interstitial brain serotonin level have not been observed in microdialysis studies, which is the gold standard procedure to investigate changes in neurotransmitter levels. Microdialysis studies demonstrate that 5-HT_{1B} autoreceptor function can dampen or even cancel out the anticipated SSRI-induced increase in cerebral serotonin level in a region-dependent way, but it does not decrease the serotonin level compared to baseline.[11–13] However, microdialysis studies to our knowledge have so far only investigated SSRI doses manifold above clinically relevant doses. Therefore, the conflicting outcomes of the human and the non-human primate PET studies may be explained by species differences or differences in the doses applied since a high SSRI dose would be expected to lead to a larger increase in brain interstitial serotonin levels.

The aim of the present study was to measure the effect of pharmacological challenges – also in clinically relevant doses – on the serotonin level by cerebral microdialysis and to correlate this to the change in the non-displaceable binding potential (BP_{ND}) of the 5-HT_{1B} receptor partial agonist radioligand [^{11}C]AZ10419369 in the pig brain.

We hypothesized that pharmacologically induced acute increases in extracellular brain serotonin level were associated with a decline in [^{11}C]AZ10419369 5-HT_{1B} receptor binding, in consistency with the competition model.[1]

Material and methods

Animals

We used 10 9-10-week old Danish Landrace female pigs weighing 22 ± 1.3 kg (mean $\pm$ SD) in this study. All animal experiments were performed in accordance with the European Communities Council Resolves on 22 September 2010 (2010/63/EU) and approved by the Danish Veterinary and Food Administration's Council for Animal Experimentation (Journal No. 2012-15-2934-00156), and is in compliance with the ARRIVE guidelines (www.nc3rs.org.uk/arrive-guidelines). We performed tranquilization, anesthesia, intubation, installation of arterial and venous intravenous lines in the morning, monitoring and sacrifice of animals late afternoon was done as previously described.[14] On the day of investigation, we performed surgery with implantation of microdialysis guide cannulas and CMA 12 metal-free microdialysis probe (CMA Microdialysis) bilaterally into the medial prefrontal cortex (mPFC) using a well-validated procedure to allow full embedment of the active part of the microdialysis probe in only the grey matter of cortex. The procedural details are given in our previous study[15] and a schematic diagram of the events on the investigation day in Figure 1.

Study design

Both PET scans were conducted on the same day as within scan challenge with saline (scan 1) and escitalopram or fenfluramine (scan 2). Upon insertion of

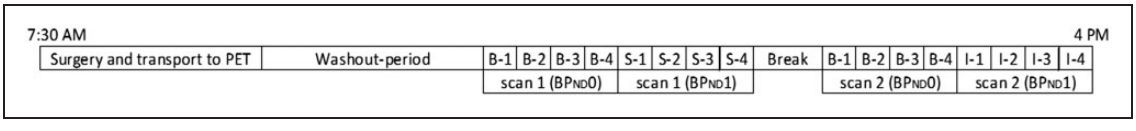

Figure 1. Microdialysis measurements were obtained with a time resolution of 15 min at baseline (B1–B4) prior to intervention with saline (S1–S4) or escitalopram/fenfluramine (I1–I4).

microdialysis probes bilaterally in the mPFC, we placed the pig in the PET scanner and allowed a 2 h-washout period to obtain steady serotonin baseline level. Two PET scans, each lasting 120 min, were conducted 30 min apart. We administered the pharmacological interventions intravenously 56.5 min after injection of the radioligand with the aim to acutely increase the extracellular serotonin levels. The interventions consisted of either saline (controls, $n = 10$), escitalopram 0.28 mg/kg (selective serotonin reuptake inhibitor (SSRI), $n = 5$) or fenfluramine 0.5 mg/kg (serotonin releaser, $n = 5$). The investigators were not blinded to the interventions but the pigs were randomized.

In pilot experiments prior to a previous study,[15] we tested the effect of three different doses of fenfluramine; 0.05 mg/kg, 0.5 mg/kg and 2.0 mg/kg, but when 2 mg/kg was given, the pig started to shiver and showed a large increase in vital signs, so the injection was interrupted after ¾ had been given. Since the dose of 0.05 mg/kg did not induce any significant alterations in microdialysate 5-HT levels, we chose to give a dose of 0.5 mg/kg. This dose has shown to elicit a 9–11-fold increase in serotonin level consistent with the spectrum that allows comparison of changes in BP_{ND} at an 8-fold increase in neurotransmitter level such as reported for [11C]raclopride to dopamine.[16]

Microdialysis and serotonin measurements

The probes had a membrane length of 4 mm and a molecular weight cut-off value of 20 kDa. They were perfused with a standard Ringer solution (147 mM NaCl, 4 mM KCl and 2.3 mM CaCl2, adjusted to pH 6.5) at a flow rate of 1.0 uL/min. From both probes, we collected 15-min time-series of samples of extracellular fluid to be analyzed off-line for monoamines by HPLC. After a 2 h-washout period, we collected 20 samples with 15 min interval. The microdialysate samples were quickly frozen on dry ice and stored in a $-80\,^{\circ}C$ freezer until further analyzed.

We determined the relative changes in serotonin concentrations in the dialysate samples by HPLC with electrochemical detection. The column was a Prodigy 3 µ ODS (3) C18 (DA 2 mm × 100 mm, particle size 3 µm, Phenomenex, Torrance, California, USA). The mobile phase consisted of 55 mM sodium acetate, 1 mM octanesulfonic acid, 0.1 mM Na2EDTA and 8% acetonitrile, adjusted to pH 3.2 with 0.1 M acetic acid, and was degassed with an on-line degasser. Ten microliters of the dialysate sample were injected, and the flow rate was 0.15 mL/min. The electrochemical detection was done with an amperometric detector (Antec Decade, Antec, Leiden, the Netherlands) with a glassy carbon electrode set at 0.8 V, with an Ag/AgCl reference electrode.

The output of the HPLC was recorded by the program "LC solution" (Shimadzu, Columbia, Maryland, USA), which also was used to calculate the peak areas. We determined the baseline level on the basis of the mean peak area obtained by the HPLC from the four samples preceding the pharmacological intervention of each PET scan. We used the baseline level to calculate the relative change in the serotonin levels of the four samples following the pharmacological intervention during the remaining period of each PET scan. In 19 of the performed 20 pig scans, stable baseline serotonin levels were achieved on one or both sides. In one pig, stable baseline level was not reached at the saline intervention and therefore this microdialysis data were excluded from the saline intervention analyses.

$$Occupancy\ (O) = \frac{\Delta F_{NT}}{K_{NT} + F_{NT} + \Delta F_{NT}} \quad (1)$$

According to the competition model,[1] there are three factors, which determine the ability of a tracer to detect changes in synaptic neurotransmission: affinity of the neurotransmitter for its receptor, K_{NT}, the basal neurotransmitter concentration in the interstitial fluid, F_{NT}, and the challenge-induced change in neurotransmitter concentration, ΔF_{NT}.

PET scanning protocol

PET scans were obtained in list mode with a high resolution research tomograph (HRRT) scanner, with the pig in the prone position. [11C]AZ10419369 was given as an intravenous bolus injection and data acquisition began at the time of injection. The synthesis and radiochemical labeling of [11C]AZ10419369 has previously been described.[17]

The average injected radioactivity of [11C]AZ10419369 was 466 MBq (range, 399–510 MBq; $n = 20$) and the average injected mass was 1.75 µg (range 0.17–9.58 µg; $n = 20$) with no significant difference between scans (paired t-test). To ensure that the pharmacological challenges did not induce changes in blood [11C]AZ10419369 time activity curves (TAC), we measured arterial whole blood radioactivity, in the beginning continuously for 20 min using an ABSS autosampler (Allogg Technology, Mariefred, Sweden) counting coincidences in a lead-shielded detector, and later blood samples were manually drawn at 2.5, 5, 10, 20, 30, 50, 70 and 90 min after injection and the radioactivity in whole blood and plasma samples was measured in a well counter (Cobra 5003, Packard Instruments, PerkinElmer, Skovlunde, Denmark) that was cross-calibrated to the HRRT scanner and autosampler. Radiolabeled parent

compound and metabolites were measured in plasma using HPLC with online radioactivity detection as previously described.[18]

Quantification of PET data

The [^{11}C]AZ10419369 HRRT PET data were acquired in 3D list mode and reconstructed using a 3D–OSEM–PSF algorithm[19] with MAP-TR attenuation correction[20] into a dynamic image dataset consisting of 44 frames of increasing length (6×10, 6×20, 4×30, 9×60, 6×120, 4×180, 2×240, 1×300, 1×360, 1×420, 1×540, 1×780, 1×1380, 1×660 s). Images consisted of 207 planes of 256×256 voxels of $1.22 \times 1.22 \times 1.22\,mm^3$. For each pig, a time-weighted average image was calculated including all frames. This image was used for automatic atlas labeling of the individual brain PET images.

Our in-house pig atlas[21] was made from transferring atlas labels from a recently published MRI-based pig atlas with 178 segmented regions[22] to an average PET template made of PET and MR images in pigs obtained from a recently published study with the PET radioligand 5-HT$_{2A}$ receptor agonist [^{11}C]Cimbi-36.[15]

Further processing of the [^{11}C]AZ10419369 PET scans in this study was done by generating an average PET scan, which was cropped and co-registered to our in-house pig atlas using the Flirt algorithm from the FSL software package (FSL 5.0.8, FMRIB Software Library, Release 5.0 (c) 2012, The University of Oxford, UK). The estimated 12 parameter affine transformation matrix was then used for transforming the atlas labels into individual pig spaces.

Each coregistration was verified by visual inspection before extraction of TACs from the following volumes of interest (VOIs) combined from the atlas labels: neocortex (28.0 cc) (frontal cortex (4.9 cc), somatosensorimotor cortex (5.9 cc), occipital cortex (5.6 cc), insula (2.8 cc) and temporal cortex (3.8 cc)), thalamus (1.4 cc), striatum (2.0 cc) and hippocampus (0.8 cc). We used cerebellum (4.4 cc) as the reference region. TACs for these VOIs were extracted from the dynamic PET dataset.

We estimated the 5-HT$_{1B}$ receptor binding of [^{11}C]AZ10419369 using the extended simplified reference tissue model (ESRTM)[23] before and after the serotonergic challenge given at 56.5 min. This would allow a 30-s administration interval until splitting of the dataset at 57 min, which we in pilot studies found gave stable $BP_{ND}0/1$ estimates before and after challenge. Frames acquired at time interval 0–57 min formed the basis for calculation of $BP_{ND}0$ and frames acquired at time interval 57–120 min for $BP_{ND}1$.

The relative decrease in BP_{ND} in the VOI was calculated as

$$\%\Delta BP_{ND} = -O = 100 * \frac{BP_{ND}1 - BP_{ND}0}{BP_{ND}0} \quad (2)$$

To reject outliers, the standard coefficient of variance (COV) was calculated for all regional BP_{ND}s quantified by the ESRTM model. Data with COV larger than 15% were excluded, and regions with excluded data ($n < 5$) were not included in further analysis.

Statistical analysis

Similar to our previous study in pigs with [^{11}C]Cimbi-36,[15] we correlated the occupancy as measured with PET with the highest peak increase (of the left or right microdialysis probe) in extracellular 5-HT levels. Unless performed with very time-consuming methods[24] incompatible with the use of short-lived radioisotopes and the need to conduct two PET studies, microdialysis experiments do not generate absolute serotonin concentrations. Instead, we assumed a fairly stable baseline concentration of the pig cerebral interstitial fluid serotonin of about 1.7 nM, equal to what has been found in mice.[25–29] ΔF_{NT} was then computed as 1.7 nM times the relative peak increase in serotonin level minus 1.7 nM. The data were fitted to the competition model given in equation (1) with a non-linear regression analysis as previously described.[15] We used the Wald Runs-Test for randomness to test if the curve fitted by non-linear regression to the occupancy model deviated from the data.

The mean increase in interstitial serotonin level (5-HT), as measured by microdialysis and the mean percentual (%) change in BP_{ND} of the PET scans, calculated by equation (2), was estimated in the three conditions: saline ($n = 10$), escitalopram ($n = 5$) and fenfluramine ($n = 5$). The change in BP_{ND} (PET) and 5-HT (microdialysis) was analyzed post hoc for significance between saline and either two interventions (Wilcoxon signed rank test) for differences in each region of interest.

Our study design with a within-scan saline challenge in the first scan, followed by a second scan where the intervention was given 56.5 min after the radioligand injection allowed for an assessment of the reproducibility of [^{11}C]AZ10419369 binding, both within the individual scan and between the first part of the two scans. We performed test-retest analysis of $BP_{ND}0$ (PET 1 and 2) and $BP_{ND}0/1$ (PET 1) in neocortex. First, we tested for significant order effect (Wilcoxon signed-rank test). Then, we determined the intraclass correlation coefficients (ICC) modelled as a two-way mixed ANOVA with absolute agreement and average measurement in a within-subject design. ICC above 0.80 is considered good and ICC above 0.90 is considered excellent.

Lastly, we calculated the required sample size for each intervention of escitalopram and fenfluramine to reach significance level of 0.05 with a power of 0.8 given the effect size determined by group parameters of mean ± SD ($BP_{ND}0$ and $BP_{ND}1$) and ICC for the neocortex region.

Results

In vivo microdialysis

Fenfluramine produced a powerful increase in extracellular serotonin level to a peak 580 ± 286% ($n = 5$) relative to baseline level (100%) following the within-scan pharmacological challenge. Escitalopram produced a peak increase in serotonin to 233 ± 120% ($n = 5$) and saline intervention did not change the serotonin level in the pig brain ($n = 9$) relative to baseline level (Figure 2).

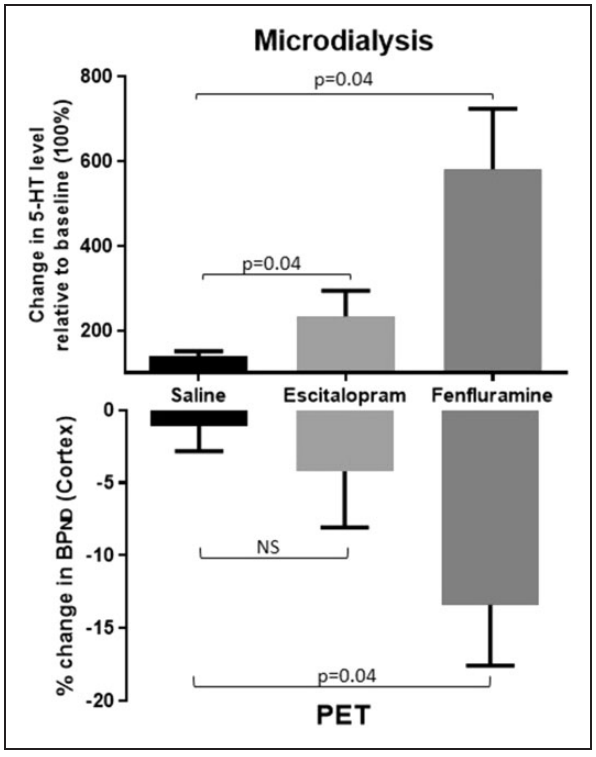

Figure 2. Changes in brain interstitial 5-HT levels (upper panel) and BP_{ND} (lower panel) following a within-scan challenge of saline ($n = 10$), escitalopram ($n = 5$), or fenfluramine ($n = 5$). The upper panel shows the changes in the peak interstitial cerebral 5-HT level relative to baseline (100 %) ± SEM as measured by microdialysis in the mPFC. The lower panel shows the relative decrease in BP_{ND} as measured by PET in the pig brain neocortex. Changes were statistically evaluated by comparing the escitalopram and fenfluramine interventions to saline with Wilcoxon signed-rank test.

The time course of the challenge-induced increase in extracellular serotonin level is given in Figure 3.

In vivo PET imaging

With PET, we measured a significant decrease in BP_{ND} of [^{11}C]AZ10419369, corresponding to an increase in interstitial serotonin level induced by escitalopram and fenfluramine (Figure 2). Saline interventions did not induce a change in BP_{ND} measures. The $BP_{ND}0$, $BP_{ND}1$ and the relative change (%) in BP_{ND} of [^{11}C]AZ10419369 in the neocortex after saline, escitalopram or fenfluramine interventions are given for all pigs individually in the supplementary material (Table 1). The average binding potential of all VOIs is listed in Table 1 in the three conditions. A significant change in binding between groups was observed in the occipital cortex ($p = 0.01$) and neocortex ($p = 0.02$) (Kruskal–Wallis test).

5-HT versus 5-HT$_{1B}$ receptor occupancy

The fenfluramine-induced changes in 5-HT level were significantly increased compared to saline, as measured by both PET and microdialysis, while the escitalopram-induced changes in 5-HT level was only significantly higher when measured by microdialysis (Figure 2).

The corresponding measures of the PET occupancy and the F_{NT} were fitted to equation (1) and the observed correlation did not deviate significantly from the occupancy model (Figure 4). The affinity of the neurotransmitter for its receptor, K_{NT}, was computed to 74 ± 27 nM, about 43 times higher than the baseline brain interstitial serotonin concentration. Figure 4 shows that when brain interstitial serotonin

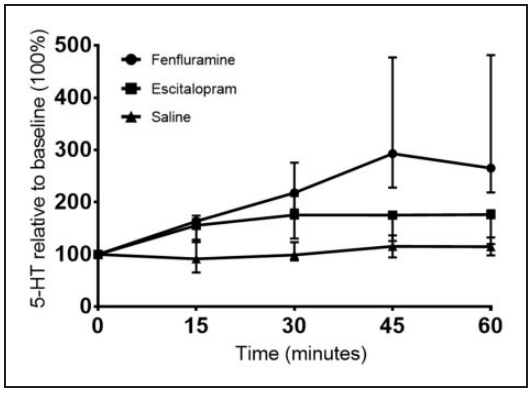

Figure 3. The median time course of the increase in extracellular cerebral 5-HT level after a pharmacological challenge of saline, escitalopram or fenfluramine administered at time 0 min. The increase in 5-HT level is presented relative to baseline (100 %) with median ± quartiles.

Table 1. Non-displaceable BP_{ND}s from the two PET scans, and % change in $BP_{ND}0$ and $BP_{ND}1$, as calculated by equation (2).

Volumes of interest	BPND0 (mean ± SD)	BPND1 (mean ± SD)	ΔBPND (%) (mean ± SD)	BPND 0/1	BPND 0/0
Saline ($n = 10$)				ICC	
Cortex (total)	0.66 ± 0.10	0.66 ± 0.13	−1 ± 5	0.98	0.94
Frontal	0.57 ± 0.11	0.54 ± 0.13	−5 ± 7	0.97	0.92
Somatosensory	0.60 ± 0.10	0.58 ± 0.14	−4 ± 8	0.96	0.95
Occipital	0.78 ± 0.11	0.74 ± 0.12	−5 ± 3	0.97	0.84
Insula	0.73 ± 0.10	0.76 ± 0.14	5 ± 8	0.91	0.87
Temporal	0.71 ± 0.12	0.72 ± 0.14	1 ± 6	0.97	0.95
Thalamus	0.90 ± 0.14	0.95 ± 0.22	4 ± 10	0.90	0.92
Dorsal striatrum	1.49 ± 0.20	1.59 ± 0.20	7 ± 5	0.85	0.80
Escitalopram ($n = 5$)				Required sample size	
Cortex (total)	0.71 ± 0.10	0.68 ± 0.12	−4 ± 8	11	19
Frontal	0.64 ± 0.12	0.60 ± 0.13	−6 ± 12	8	16
Somatosensory	0.65 ± 0.11	0.60 ± 0.13	−8 ± 9	8	9
Occipital	0.79 ± 0.11	0.73 ± 0.10	−7 ± 6	5	11
Insula	0.81 ± 0.09	0.83 ± 0.15	2 ± 9	126	149
Temporal	0.76 ± 0.10	0.72 ± 0.12	−5 ± 7	8	11
Thalamus	0.98 ± 0.11	1.03 ± 0.23	4 ± 13	67	63
Dorsal striatrum	1.64 ± 0.23	1.76 ± 0.30	8 ± 12	17	21
Fenfluramine ($n = 5$)					
Cortex (total)	0.64 ± 0.09	0.56 ± 0.12	−13 ± 8	5	6
Frontal	0.56 ± 0.11	0.47 ± 0.14	−18 ± 10	5	6
Somatosensory	0.58 ± 0.11	0.50 ± 0.14	−15 ± 10	6	6
Occipital	0.73 ± 0.07	0.61 ± 0.11	−16 ± 8	4	5
Insula	0.70 ± 0.11	0.64 ± 0.12	−8 ± 9	8	11
Temporal	0.66 ± 0.11	0.57 ± 0.15	−13 ± 8	5	6
Thalamus	0.95 ± 0.20	0.89 ± 0.22	−7 ± 8	24	20
Dorsal striatrum	1.51 ± 0.12	1.48 ± 0.19	−2 ± 6	110	131

Note: The intercorrelation coefficient (ICC) is given for both within-PET scan (BP_{ND} 0/1) and between-PET scans (BP_{ND} 0/0) along with the required sample size given a power of 0.8, significance level of 0.05 and the effect size determined by the mean $BP_{ND} \pm SD$ and the ICC.

level increases 8-fold, [11C]AZ10419369 decreases by 15% (CI7, 24%).

When we compute the data to the model with AUC instead of peak, we find that K_{NT} is 27 nM (instead of 74 nM), and the data still conform to the model. For example, a 8-fold increase in serotonin level would be associated with a 32% (rather than 15%) decline in BP_{ND}.

Test-retest analyses of [11C]AZ10419369 PET measures

In neocortex, the [^{11}C]AZ10419369 had a very small test-retest variability of $-1 \pm 5\%$ (within scan) in $BP_{ND}0/1$ (PET scan 1) and $2 \pm 9\%$ (between scans) in $BP_{ND}0$ (PET scan 1/2) and the corresponding ICC values were 0.98 and 0.94. We did not observe a consistent order effect (Wilcoxon signed-rank test, matched pairs). The required sample size to statistically identify a fenfluramine associated 13% decline in BP_{ND} in neocortex is $n = 5$ and to find an escitalopram associated 4% decline in BP_{ND} is $n = 11$ (Table 1).

Discussion

We here show first, that intravenous injections of escitalopram and fenfluramine both induce an acute increase in the cerebral interstitial fluid concentration of serotonin in the brain. Secondly, we show that

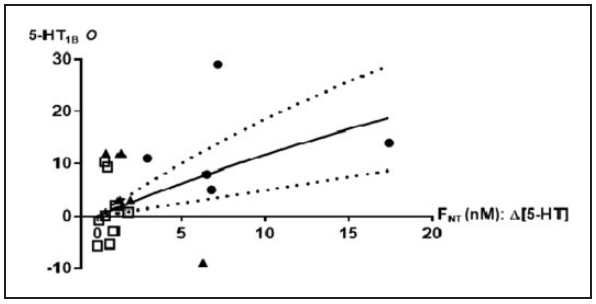

Figure 4. Relationship between relative increase in brain interstitial 5-HT levels (ΔF_{NT}) and neocortical 5-HT_{1B} receptor occupancy O (%) with 95% confidence intervals. Interventions include saline (square), escitalopram (filled triangle), and fenfluramine (filled circle). Data are based on 10 pigs with two PET scans and are fitted with nonlinear regression analysis according to equation (1), resulting in a K_{NT} of 74 nM.

the interventions induce a varying degree of decline in [^{11}C]AZ10419369 binding in the brain, consistent with the occupancy competition model. The observed correlation between changes in the extracellular serotonin level in the pig brain and the 5-HT_{1B} receptor occupancy indicates that [^{11}C]AZ10419369 binding is sensitive to changes in endogenous serotonin levels.

The pharmacological interventions induced an average 2-fold (escitalopram 0.28 mg/kg) and 6-fold (fenfluramine 0.5 mg/kg) increase in cerebral serotonin release compared to baseline level in the mPFC in the pig brain. In order to compare the outcome of our study with previously reported clinical studies,[7,9,10] we deliberately chose to use a clinically relevant dose of escitalopram. However, somewhat unexpectedly, compared to the high dose of escitalopram (2 mg/kg) given in our previous pig study, it does not seem that using a clinically relevant dose of 0.28 mg/kg is associated with substantially less serotonin release.[15] The fenfluramine-induced changes in cerebral interstitial serotonin were in line with our previous combined microdialysis and PET study in pigs with the 5-HT_{2A} receptor agonist [^{11}C]-Cimbi36[15] as well as microdialysis studies in rats[30] and non-human primates.[31]

Depending on the pharmacological intervention, the change in 5-HT_{1B} receptor binding as measured by the PET radioligand [^{11}C]AZ10419369 ranged between an average 4% (escitalopram) and 16% (fenfluramine). A statistically significant decrease in BP_{ND} was only found in brain areas with high binding (the occipital cortex) or when a large brain volume was encountered (entire neocortex), suggesting that good count statistics is important to reveal smaller changes in BP_{ND}. The paired data on microdialysis and [^{11}C]AZ10419369 (Figure 4) comply with the competition model supporting that [^{11}C]AZ10419369 PET is sensitive to changes in endogenous serotonin levels when the serotonin release is sufficiently high.

As speculated by Finnema et al.,[32] regional differences in SERT, 5-HT_{1B} auto- and heteroreceptor densities may impact the BP_{ND} by separate mechanisms. High-density SERT regions may express a higher SSRI-induced increase in synaptic serotonin level as compared to low-density SERT regions, which may produce a less or no decrease in BP_{ND}. Given that the regional relationship between 5-HT_{1B} auto- and heteroreceptor densities is largely unknown, it is difficult to predict in which regions one can expect to see the highest sensitivity to changes in synaptic serotonin.

K_{NT} varies across brain regions and species, but in order to relate to equation (1), we assumed a fixed value for interstitial fluid serotonin. KNT would change accordingly, but an 8-fold increase in serotonin level would still result in a 15% decrease in BP_{ND}. Based on the data shown in Figure 4, we estimated K_{NT} (the affinity of serotonin to the 5-HT_{1B} receptor) to be 74 nM. For comparison, K_{NT} of [11C]-Cimbi36 is 14.3 nM[15] and the concentration of ^{3}H-5-HT that labels 50% of cloned human 5-HT_{1B} receptors is 0.6–15.8 nM (PDSP K_i database).We find it relevant to compare the sensitivity of [^{11}C]AZ10419369 to serotonin with that of [^{11}C]-raclopride to dopamine, since the latter is an often used approach to detect in-vivo change in cerebral dopamine, but also to the sensitivity of [^{11}C]-Cimbi36 and [^{18}F]altanserin to detect changes in serotonin. From microdialysis studies in non-human primates, it is known that when amphetamine 0.3 mg/kg is given intravenously, the interstitial dopamine concentration increases approximately 8-fold.[33–36] In non-human primates, an 8-fold increase in dopamine results in a decrease in [^{11}C]-raclopride BP_{ND} of 13%.[16] This is consistent with findings in humans, where intravenous amphetamine 0.2–0.3 mg/kg results in a decrease in striatal BP_{ND} of 15.5%[16] or 13%.[37–39] With the 5-HT_{2A} agonist receptor [^{11}C]-Cimbi36 in pigs,[15] we have recently demonstrated that a pharmacological challenge of fenfluramine and escitalopram induces a serotonergic response compatible with the competition mode l,[1] and an 8-fold increase in the cerebral interstitial serotonin level corresponds to a 46% change in occupancy. Here we demonstrate (Figure 4) that an 8-fold increase in interstitial serotonin results in a decrease in [^{11}C]AZ10419369 BP_{ND} of 15% (CI 7, 25%), equivalent to the sensitivity of [^{11}C]-raclopride to dopamine, but three times less sensitive than [^{11}C]-Cimbi36 is to changes in the serotonin level. We here show that [11C]AZ10419369 is twice as sensitive to competition from serotonin as [11F]altanserin was in a human study,[40] following a single dose of dexfenfluramine (40 mg). Moreover, whereas [^{11}C]-raclopride can only be used to reliably assess dopamine changes in

striatum, both [^{11}C]AZ1041936 and [^{11}C]-Cimbi36 can – due to the widespread and relatively uniform distribution of the 5-HT_{1B}R and 5-HT_{2A}R – theoretically be used to determine regional serotonin changes in the entire brain. It is an important feature of a PET radioligand to be able to identify changes also in smaller brain areas, since changes in the cerebral neurotransmitter level could occur in confined brain regions, which potentially could allow for functional imaging of serotonin release. Further, it has been demonstrated in microdialysis studies in rats[11,12] that the serotonergic response is attenuated by autoregulation from negative feedback of the somatodendritic 5-HT_{1A} autoreceptors. Blocking the autoregulation by pindolol, a 5-HT_{1A} autoreceptor antagonist, thus potentiates the serotonergic response to SSRIs.[13] In pigs, we have also previously observed a higher serotonergic response to escitalopram in combination with pindolol than in mono-intervention with escitalopram.[15]

Although in a different species, our data do not lend support for an acute 5-HT_{1B} autoreceptor-induced decrease in cerebral interstitial serotonin levels upon a single and clinical relevant dose of escitalopram or citalopram as speculated in two human PET studies with [^{11}C]AZ10419369[7] and [^{11}C]CUMI-101.[9] The apparently conflicting outcome in the human studies with the non-human primate studies[6,7,32] and to some extent our pig study may be explained by species differences or differences in study design. Also, it cannot be excluded that the much higher doses of escitalopram used in the non-human primates could induce a larger serotonin release that was more easily detected by PET.

In order to assess the stability of our design, we also investigated the test-retest variability of BP_{ND} in the pig. We demonstrated an excellent reproducibility of [^{11}C]AZ10419369 binding to cortical 5-HT_{1B} receptors with a test-retest variability and ICC comparable to the outcome reported in human studies of [11C]Cimbi-36 binding to 5-HT_{2A} receptors[41] and a little better than that of [11C]-SB207145 binding to 5-HT_4 receptors[42] and [11C]P943 to 5-HT_{1B} receptors.[43]

A limitation of our study is that microdialysis allows only for measurements in a few selected brain regions, while PET, on the other hand, can generate information about changes in the serotonin level of the entire brain. The low number of pigs in the citalopram and fenfluramine groups also limits the conclusions that can be made for smaller brain regions.

In conclusion, we here show that the [^{11}C]AZ10419369 PET signal correlates to the pharmacologically induced changes in interstitial serotonin brain level compatible with the occupancy model. The observed correlation indicates that [^{11}C]AZ10419369 is sensitive to changes in endogenous serotonin levels, but that is only detectable with PET when the serotonin release is sufficiently high. The reproducibility of [^{11}C]AZ10419369 is excellent. The sensitivity of [^{11}C]AZ10419369 to detect changes in extracellular serotonin level is comparable to that of [^{11}C]raclopride to detect changes in dopamine level.

Differences in earlier studies may thus be ascribed to the efficacy of the pharmacological interventions to change interstitial brain serotonin levels. Verifying the direct correlation between pharmacologically induced changes in serotonin and [^{11}C]AZ10419369 PET occupancy is an important step prior to conduction of clinical trials and the calibration allows for estimating the regional relative change in interstitial serotonin in patients in future studies.

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Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Authors' contributions

All authors revised and gave final approval of the manuscript. Following authors contributed to the conception (LMJ and GMK), drafting of the article (LMJ, PW, CS and GMK), design (LMJ, PW, SK, LF and GMK), acquisition of data (LMJ, SK, PW) and analysis and interpretation of data (LMJ, PW, GMK).

Supplementary material

Supplementary material for this paper can be found at the journal website: http://journals.sagepub.com/home/jcb

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Paper 3

Parkinson patients display a presynaptic serotonergic deficit: A dynamic DBS-STN PET study

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Running title: DBS associated 5-HT release in Parkinson patients.

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Author Contributions Statement

All authors revised and gave final approval of the manuscript. Following authors contributed to the conception (LMJ and GMK), drafting of the article (LMJ, SHK and GMK), design (LMJ, TH, SM and GMK), acquisition of data (LMJ, TH, SM, DSS and SHK), and analysis and interpretation of data (LMJ, DSS, CS, PW, BJ, CT, SHK, HDH, and GMK).

Disclosures

TH have previously received honorary for given lectures to Abbvie, Nordic Infucare, Grünenthal, UCB, and Zambon. SM has previously received honorary for given lectures for Abbvie.

Abstract

It is increasingly acknowledged that Parkinson's Disease (PD) is not merely a motor disorder defined by its dopaminergic deficits but often non-motor symptoms constitute a problem that even more profoundly affects quality of life. Dysfunction in the serotonin (5-HT) system, known to be present in PD, may contribute to both motor and non-motor symptoms. Whereas Deep Brain Stimulation (DBS) in the subthalamic nucleus (STN) alleviates the motor symptoms in PD, the implications of DBS-STN on the 5-HT dysfunction is so far unknown. We here exploit a novel functional Positron Emission Tomography (PET) neuroimaging methodology to investigate the static and dynamic integrity of the 5-HT system in DBS-STN treated patients with PD, while turning off the DBS-STN in the scanner. Baseline cerebral 5-HT_{1B} receptor binding was investigated in the patients with [^{11}C]AZ10419369 PET and compared to age-matched controls. Next, we investigated how turning off DBS-STN induced changes in cerebral 5-HT level as indexed by [^{11}C]AZ10419369 binding and we describe the association of these PET measures with motor and non-motor functions in PD. We find that DBS-STN treated patients with PD exhibit a region-specific presynaptic serotonergic dysfunction that to some extent is correlated to clinical severity. Turning off the DBS-STN reveals that the brain regions with the best preserved presynaptic 5-HT function respond by a substantial 5-HT release, reflecting that the presynaptic terminals are still relatively preserved in those brain areas whereas the more affected brain regions have lost their 5-HT releasing capacity. These deficits in the regulation of the 5-HT may contribute to PD patients' non-motor challenges. Our study demonstrates that DBS-STN dynamically regulates the 5-HT system and further studies of the long-term effects on 5-HT function is warranted.

Background

Parkinson's Disease (PD) is one of the most common movement disorder characterized by neuronal degeneration and a well-described progressive loss of the dopamine producing cells, primarily in the nigrostriatal pathways.[1] Eventually, this dopaminergic dysfunction results in the characteristic features of bradykinesia and rigidity in PD[2], and medical therapy of PD has primarily focused on pharmacological modulation of the dopamine system, aiming to alleviate the motor symptoms and executive deficits in these patients. However, non-motor symptoms, particularly depression and fatigue,[3,4] constitute a huge burden on quality of life in many patients with PD, and dopaminergic therapy may offer both amelioration or exacerbation depending on the character of the non-motor symptoms.[5]

Although only applicable to a highly selected population of PD patients, deep brain stimulation (DBS) with electrodes placed in the subthalamic nucleus (STN) is a supplementary surgical treatment for alleviation of motor symptoms in PD. Affective side effects are commonly seen within the first three months after DBS surgery, but there are also multiple case series suggesting that DBS improves the burden of non-motor symptoms and a recent review concludes that there is level I evidence on the effect of DBS on mood: Two randomized prospective studies reported no change in depression while improvement of anxiety has been reported by a class I trial.[6] Although the therapeutic effect of DBS is well established, the physiological mechanisms underlying its effect is still unclear.[7] It has been speculated that DBS acts by inducing changes in, e.g., neurotransmitter levels in targets and connected regions,[8] changing firing rates in afferents and efferents, causing distant effects,[9] and furthermore inducing long-term reorganization of neural networks, and affect neuroprotection.[10]

Lately, increasing attention has been given to neuronal degeneration outside the nigrostriatal pathways and to other neurotransmitter systems involved in PD. As reviewed by Huot et al.,[11] a growing amount of research lends support to the presence of serotonergic deficits in PD. Post-mortem, biochemical and neuroimaging studies point to a reduction in various 5-HT associated markers with a regional distribution distinct from that of dopamine. Such deficits in the 5-HT system may account for some of the non-motor symptoms commonly found in PD patients.

Neuroimaging presents a unique opportunity to investigate the 5-HT system in PD patients. As previously reviewed,[11] several studies of non-depressed PD patients report substantial decrease in presynaptic 5-HT transporter (SERT) availability, while the somatodendritic 5-HT receptors generally seem less affected, or even increased.

To the best of our knowledge, no studies have so far investigated the effects of DBS on serotonergic neurotransmission in humans, most likely because the technology for such an investigation has been missing. However, we recently showed in a combined PET- and microdialysis pig study that the 5-HT_{1B} receptor antagonist radioligand [^{11}C]AZ10419369 is sensitive to pharmacologically induced changes in cerebral synaptic 5-HT level[12] and others have demonstrated a similar displacement in the non-human primate brain.[13]

The aim of the present study was to investigate changes in cerebral 5-HT levels, as indexed by regional [^{11}C]AZ10419369 binding, when DBS was turned off in PD patients treated with STN-

DBS. We also included age-matched healthy volunteers to identify group differences in baseline 5-HT_{1B} receptor availability and to investigate if such a difference was associated with the clinical characteristics of PD.

We hypothesized that the extent to which PD patients had regional lower 5-HT_{1B} receptor availability than controls would be proportional to PD symptom severity, as measured by motor scores. Secondly, we hypothesized that turning off the DBS-STN stimulator in PD patients would be associated with a change in cerebral 5-HT, as indexed by an inverse change in 5-HT_{1B} receptor binding. Thirdly, we anticipated a concomitant worsening of mood symptom scores, proportional to the change in cerebral 5-HT level or the 5-HT_{1B} receptor binding at baseline level.

Design and Methods

Participants

We included 13 PD patients treated with DBS-STN. The PD patients were recruited from a specialized Movement Disorder Clinic by their attending consultant neurologist. Eleven age-matched healthy volunteers served as controls; five of whom also entered in concomitant studies.[14,15] One control had to discontinue the study for reasons of discomfort while being placed in the PET scanner, so the final group of age-matched controls included 10 individuals.

All participants were assessed by a structured interview for a variety of medical disorders, major depression, severe cognitive deficits and selected according to the in- and exclusion criteria. Specific inclusion criteria for the PD patients were current DBS-STN treatment and exclusion criteria were DBS surgery within 3 months from PET scan or dysregulated PD. General exclusion criteria for all participants were severe or symptomatic medical, psychiatric or neurological illness not related to PD, use of medicine which may influence the research results, severe cognitive deficits or Mini Mental State Examination (MMSE)[16] scores < 27, severe hearing or visual impairment, being non-fluent in Danish, or current substance or alcohol abuse.

None of the participants had any significant medical, psychiatric, or neurological history or abnormalities on clinical examination other than those related to PD. None of the participants' cerebral Magnetic Resonance Imaging (MRI) scans were abnormal. Apart from slightly elevated blood cholesterol in a few participants, blood chemistry was unremarkable. All participants were screened drug negative on a urine test (Rapid Response™ Multi-Drug Test Panel; BTNX Inc., Markham, Ontario, Canada), except for one patient who reported having taken one tablet of 5 mg morphine for acute back pain 5 days prior to the scanning.

The day before the PET scan, patients were admitted to the Department of Neurology. Throughout the PET scan, the patient was attended by their designated nurse and a neurosurgeon. After the PET scan, upon return to the ward, all patients reported themselves in habitual condition and were discharged either immediately or the day after, according to their own wish.

The study was approved by the Capital Region's ethics committee (H-1-2014-002, H-3-2013-100, H-6-2014-057, and H-KF-2006-20) and the Danish Data Protection Agency (30-1450). All

participants provided written informed consent following full description of the procedures. The age-matched controls received monetary compensation for their participation.

Rating Scales

The PD patients were evaluated for staging as well as presence and symptom severity of non-motor and motor symptoms by two rating scales for PD: the Hoehn and Yahr Rating Scale (HYR)[17] and the revised Unified Parkinson's Disease Rating Scale (MDS-UPDRS)[18] carried out by a MDS-UPDRS certified neurosurgeon.

All participants were scored with the self-reported rating scales of The Major Depression Inventory (MDI)[19] and a Danish back-translated version of The Profile of Mood Score (POMS).[20] The MDI assesses depressive symptomatology and the POMS assesses transient (state-dependent) mood states described by six factors: Tension or Anxiety (T), Depression or Dejection (D), Anger or Hostility (A), Vigor or Activity (V), Fatigue or Inertia (F), and Confusion or Bewilderment (C).

Study design

The study design is illustrated in Figure 1.

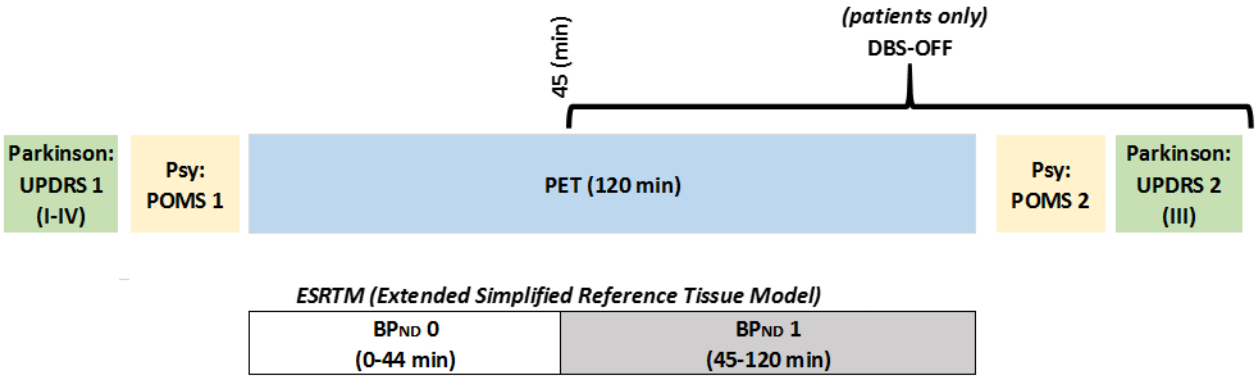

Figure 1. The experimental design of the study. In all PD patients, the DBS-STN was turned off in a within-scan design, 45 minutes after radiotracer injection. The PET scan was in all participants analyzed to generate a $BP_{ND}0$ (0-44 min, with DBS-ON) and $BP_{ND}1$ (45- end scan, with DBS-OFF). All patients were assessed at baseline in the DBS-ON condition by means of POMS and MDS-UPDRS (part I-IV) with repeated measures of POMS and MDS-UPDR (part III of motor scores) post-scan in the DBS-OFF condition.

Neuroimaging

An MRI brain scan was conducted in all age-matched controls with the purpose of co-registration and alignment to the PET scan. For reasons of relative MRI contraindications and artefacts in PD

patients with DBS implants, we used the pre-operative MRI scan of the PD patients. Time interval between the structural MRI and the PET scan was 2·6 ± 1·8 years.

The radiochemical production of [^{11}C]AZ10419369 was done as previously reported.[21] PET scanning was conducted with a high-resolution research tomography (HRRT) PET scanner (CTI/Siemens, Knoxville, TN, USA). The MRI and PET scanning protocols and quantification of [^{11}C]AZ10419369 binding are detailed in Supplementary Material.

The ROIs that entered our primary analysis included frontal, parietal, temporal, and occipital cortices and limbic cortex. We subsequently conducted a post-hoc analyses of additional ROI's. The non-displaceable binding potential (BP_{ND}) estimates with a coefficient of variation (COV) larger than 15% were excluded from the analyses. Values of all ROIs are given as mean ± SD of 13 PD patients and 10 controls. BP_{ND}'s of occipital cortex, dlPFC, and superior frontal gyrus were excluded from one patient, and in the caudate and thalamus from two other patients, as the kinetic modelling of the data could not comply to quality control. In two controls, BP_{ND}'s from thalamus were excluded.

Statistical analyses

Group differences in demographics and injected mass per kilogram bodyweight were evaluated with unpaired 2-tailed t-test. The POMS A, V, and F subscale scores at baseline were evaluated for Gaussian distribution and tested for group differences with the Mann-Whitney test. The difference in PD patients' state scores (DBS OFF-ON) was evaluated with a paired Wilcoxon test.

Group differences in regional $BP_{ND}0$ were tested by multiple linear regression analyses with predictors of group (controls, PD patients) and age. Age was included in the regression model as previous PET studies have demonstrated a negative correlation between [^{11}C]AZ10419369 BP_{ND} and age.[22–24] Although the specific radioactivity of [^{11}C]AZ10419369 was high in all cases, we initially included injected mass per kilogram of bodyweight as a covariate, because of theoretical effects of [^{11}C]AZ10419369 on BP_{ND}. However, given that we did not identify injected mass as a significant covariate, it was excluded from the final analysis. To test for differences between $BP_{ND}0$ and $BP_{ND}1$, we tested each ROI with paired sample t-test, independently for both groups.

The association between regional BP_{ND} and the change in POMS subscale measures was in the PD patients evaluated by multiple linear regression with either $BP_{ND}0$ or relative change in BP_{ND} as the dependent and the following predictors: age, L-DOPA equivalents, and difference in POMS (OFF-ON) of the subscale measures with a demonstrated significance between the DBS-ON and DBS-OFF condition.

Significance level was set at p-value of 0·05, and the primary analysis outcome was corrected for multiple comparisons by the Bonferroni-Holm method

Results

Clinical Measures

Demographic and clinical variables of the patients are displayed in Table 1. There was no significant difference between PD patients and healthy controls regarding age or sex. All patients had MMSE scores > 27 and the healthy volunteers were cognitively well-functioning, as assessed by neuropsychological testing. None of the participants met the criteria for major depression. There was no significant difference in injected mass per kg bodyweight between patients and controls (22±13 vs. 24±19 ng/kg). The patients with PD only displayed slightly more head movements than controls during the PET scan, judged insufficient to create any consistent bias in the observed BP_{ND} (see Supplementary Material).

Immediately before the PET scanning, the POMS subscale Anger and Hostility (A) was significantly different between groups, with patients being less angry and hostile (2·0 ± 2·1) as compared to controls (5·1 ± 2·0) (p = 0·007, Mann-Whitney and corrected for 7 comparisons). None of the other subscales differed between groups.

Patient	Sex	Age	Years since surgery	L-DOPA equivalents	UPDRS DBS-ON	UPDRS-motor DBS-ON	UPDRS-motor DBS-OFF	HYR DBS-OFF
1	M	55	7	531	n.a	n.a	n.a	2
2	M	66	1.7	469	48	20	47	2
3	M	53	3.4	967	15	7	34	2
4	M	59	2.7	391	34	25	66	2
5	M	72	5.5	815	44	11	37	3
6	M	67	0.5	430	9	0	26	2
7	F	56	0.8	532	21	4	15	1
8	F	63	2.5	479	22	7	41	2
9	F	50	1.2	500	36	7	55	2
10	F	65	1.8	305	36	7	29	2
11	M	50	2.6	385	36	7	28	2
12	M	62	2.6	1896	73	27	41	2
13	M	56	0.7	835	22	0	23	2
Mean ± SD		60±7	2.5 ± 1.8	656 ± 405	33 ± 16	10 ± 9	37 ± 14	2.0 ± 0.4

Table 1. Patient characteristics.
Male (M), Female (F), levodopa (L-DOPA), Deep Brain Stimulation (DBS), Unified Parkinson Disease Rating Scale (UPDRS) and motor scores part III (UPDRS-motor), Hoehn and Yahr Rating scale (HYR).

STN-DBS treated PD patients versus controls

Table 2 shows $BP_{ND}0$ in patients (with DBS-ON) and in healthy controls. We found that the regional $BP_{ND}0$ values were lower in patients with PD and the patients had significantly lower $BP_{ND}0$ in the frontal and parietal cortices. The UPDRS score was inversely correlated with frontal cortex $BP_{ND}0$ (Figure 2), meaning that PD patients who suffered the most pronounced clinical symptoms also had the lowest frontal cortex $BP_{ND}0$.

	$BP_{ND}0$			ΔBP_{ND}(%)			
ROI	PD	Controls	p-value (group)	PD	P-value	Controls	P-value
Frontal Cortex	0.85 ± 0.17	1.12 ± 0.27	.01 *	-5 ± 11	.08	6 ± 12	.12
Temporal Cortex	0.79 ± 0.15	0.95 ± 0.25	.09	-11 ± 9	.002 **	3 ± 11	.21
Parietal Cortex	0.79 ± 0.13	1.00 ± 0.21	.01 *	-2 ± 11	.33	12 ± 12	.02
Limbic Cortex	1.02 ± 0.21	1.28 ± 0.34	.05	-9 ± 12	.01 *	2 ± 7	.17
Occipital cortex	1.06 ± 0.16	1.16 ± 0.22	.25	-8 ± 9	.02 *	0 ± 8	.74
Post hoc analysis							
Neocortex	0.84 ± 0.14	1.05 ± 0.24	.02	-7 ± 10	0.03	6 ± 10	ns
Superior frontal gyrus	0.80 ± 0.20	1.07 ± 0.25	.01	-6 ± 12	ns	5 ± 13	ns
Primary motor cortex	0.86 ± 0.19	1.11 ± 0.27	.02	-6 ± 16	ns	7 ± 14	ns
Dorsolateral prefrontal cortex	0.75 ± 0.22	1.12 ± 0.31	.005	-2 ± 19	ns	8 ± 1	ns
Ventrolateral prefrontal cortex	1.02 ± 0.19	1.26 ± 0.32	.05	-2 ± 11	ns	9 ± 12	.04
Medial inferior frontal gyrus	0.90 ± 0.18	1.19 ± 0.29	.01	-2 ± 12	ns	8 ± 12	ns
Orbitofrontal gyrus	0.86 ± 0.19	1.00 ± 0.27	ns	-11 ± 14	.03	4 ± 19	ns
Superior temporal gyrus	0.79 ± 0.18	0.97 ± 0.31	ns	-15 ± 10	.002	4 ± 12	ns
Medial inferior temporal gyrus	0.80 ± 0.14	0.94 ± 0.22	.10	-6 ± 8	.02	2 ± 10	ns
Somatosensory cortex	0.73 ± 0.19	1.00 ± 0.24	.009	-5 ± 15	ns	8 ± 12	ns
Anterior cingulate cortex	1.09 ± 0.23	1.38 ± 0.33	.03	-7 ± 10	.05	2 ± 11	ns
Posterior cingulate cortex	0.88 ± 0.19	0.89 ± 0.21	ns	-17 ± 15	.05	-5 ± 11	ns
Insular cortex	0.99 ± 0.21	1.23 ± 0.36	ns	-11 ± 13	.02	1 ± 6	ns
Caudate	0.66 ± 0.28	1.07 ± 0.37	.006	2 ± 29	ns	8 ± 21	ns
Putamen	1.25 ± 0.25	1.42 ± 0.46	ns	4 ± 12	ns	20 ± 13	.004
Thalamus	0.49 ± 0.12	0.55 ± 0.14	ns	-16 ± 21	ns	-3 ± 14	ns

Table 2. $BP_{ND}0$ in PD patients and age-matched controls and the relative change in BP_{ND} (%) after switching DBS-STN off, as measured by ($BP_{ND}1$-$BP_{ND}0$)/ $BP_{ND}0$) *100. Primary ROIs (n=5) that survived multiple comparisons with correction of p-values with the Bonferroni-Holm method are labelled for significance level $p < 0{\cdot}05$ (*) and $p < 0{\cdot}01$ (**). In the post hoc analyses, p-values are not corrected for multiple comparisons.

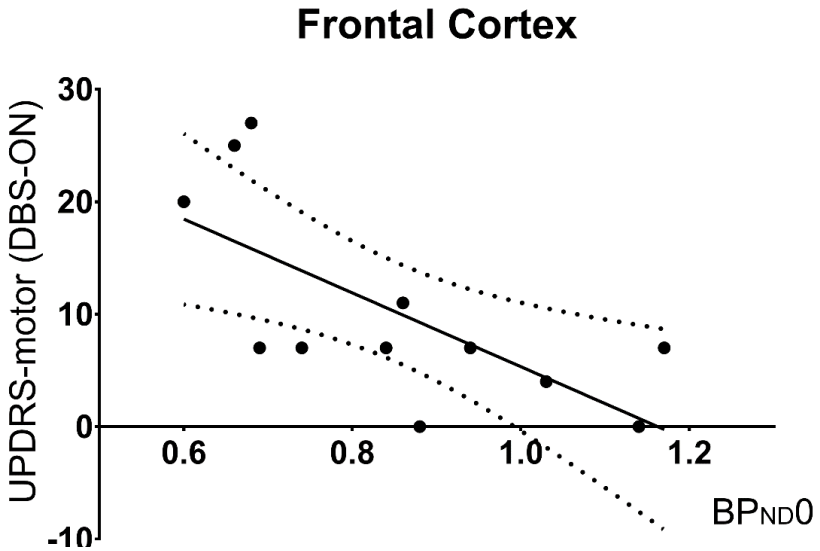

Figure 2. Association between frontal cortex $BP_{ND}0$ and the UPDRS motor scores at baseline ($p = 0{\cdot}01$) as evaluated by multiple linear regression with $BP_{ND}0$ as the dependent and the following predictors: UPDRS-motor score (DBS-ON), age, and L-DOPA equivalents.

Turning the STN-DBS off

When the DBS stimulator was turned off in the patients, we observed a significant decrease in BP_{ND} in the temporal, limbic, and occipital cortex (Table 2 and Supplementary Figure C). The controls did not show any significant changes in $BP_{ND}0$ vs. $BP_{ND}1$, except in putamen, where $BP_{ND}1$ was found to be larger than $BP_{ND}0$.

Next, in a post hoc analysis across all brain regions, we investigated if the extent to which PD patients had regionally preserved $BP_{ND}0$ as compared to their age-matched controls was related to the regional brain response to switching the DBS-STN off. We excluded putamen from the analysis, since the healthy controls showed an increase in $BP_{ND}1$, in spite of no intervention taking place (Table 2), consistent with what we have seen in previous analyses in healthy volunteers.[25] Interestingly, we found a correlation between the relative change in BP_{ND} and preservation of 5-HT_{1B} receptor binding in PD patient (Figure 3). That is, in the least affected brain regions, 5-HT was released when DBS was turned off, whereas in brain regions with the most abnormal reduction in 5-HT_{1B} receptor binding, no 5-HT release was detected.

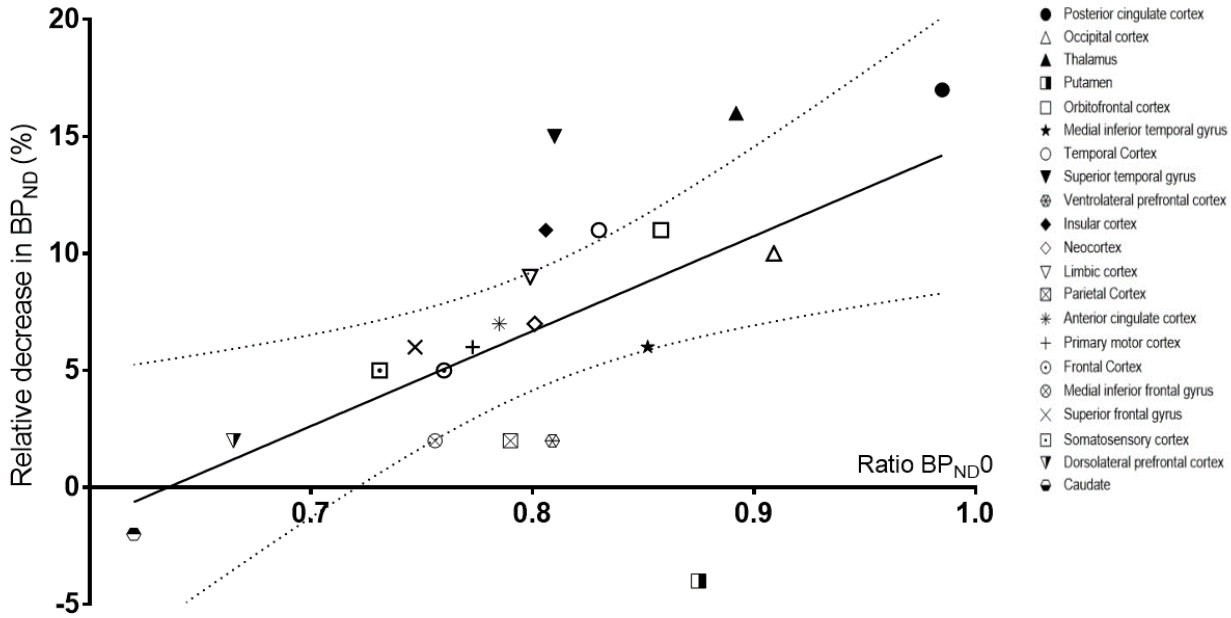

Figure 3. Percent decrease in BP_{ND} in PD patients when turning the DBS off versus the ratio between patients and healthy controls BP_{ND} across region. A significant correlation between the two measures was found, (p= 0·0005, r=0·62). Only regions where more than 50% of the subjects' data fitted the kinetic model with less than 15% covariance are included. Regions comprising sub-regions (neocortex, frontal, parietal, limbic, and temporal cortex) were not included in the statistical regression analysis. For reasons given above, putamen was not included in the regression analysis.

When correcting for multiple comparisons, the POMS subscale Vigor or Activity (V) changed significantly in response to turning DBS off compared to DBS-ON (13·8 ± 8·3 vs 24·0 ± 2·6, p=0·005, paired t-test), meaning that the DBS-OFF condition was associated with a decrease in self-reported activity and vigor. An inverse relationship was observed between POMS V (OFF - ON)

and $BP_{ND}0$ in temporal and limbic cortex (Figure 4); meaning that a high BP_{ND} in the OFF situation was associated with the largest decrease in POMS V when DBS was turned off. We did not find any association between change in POMS score and regional change in BP_{ND}.

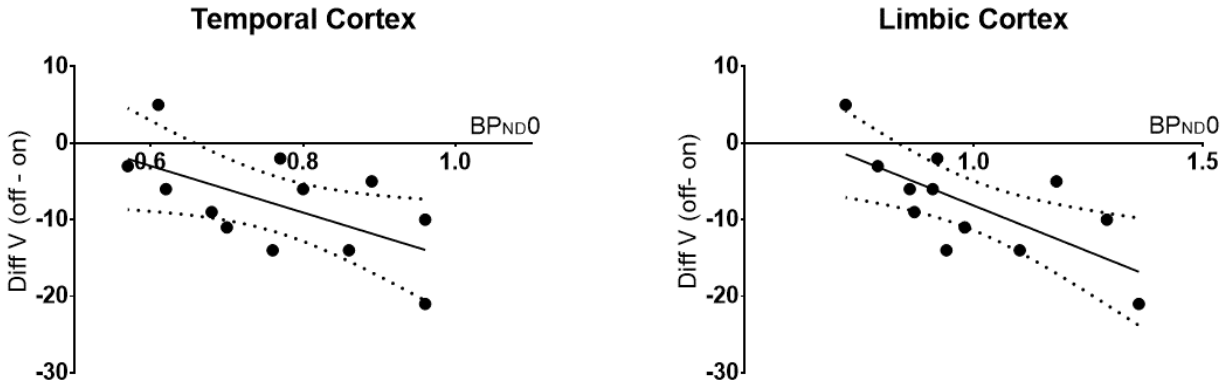

Figure 4. Association between $BP_{ND}0$ and the change (off - on) in Vigor or Activity (V) when the DBS is turned off as evaluated by multiple linear regression of the regions independently, the temporal cortex ($p = 0{\cdot}04$) and the limbic cortex ($p = 0{\cdot}03$), with $BP_{ND}0$ as the dependent and age and the difference in POMS subscale measure (V_{OFF} - V_{ON}) as predictors.

DISCUSSION

Firstly, we find that compared to controls, PD patients treated with DBS-STN have lower 5-HT_{1B} receptor availability in frontal and parietal cortex, and that the frontal cortex 5-HT_{1B} receptor availability is negatively correlated with motor symptom severity in PD. Secondly, when DBS-STN is turned off, we see a decrease in 5-HT_{1B} receptor binding, interpreted as an increase in 5-HT levels, in temporal, limbic, and occipital cortex. Importantly, the degree to which 5-HT_{1B} receptor binding is reduced after turning the DBS-STN off is proportional to the regional preservation of 5-HT_{1B} receptors in PD. Thirdly, we demonstrate a negative correlation between temporal and limbic cortex 5-HT_{1B} receptor binding measured in the ON situation and a reduction in vigor when DBS is turned off.

5-HT_{1B} receptor availability in PD patients

When conducting a rigorous statistical analysis with conservative corrections for multiple comparisons, we observed statistically significant lower 5-HT_{1B} receptor binding in frontal and parietal cortex only. As can be seen from Table 2, however, patients with PD had numerically lower 5-HT_{1B} receptor binding in all brain regions. This observation is consistent with a prior study of PD patients PET-scanned with [^{11}C]AZ10419369, where a voxel-based analysis showed lower 5-HT_{1B} receptors binding in the orbitofrontal cortex, but PD patients overall had lower cerebral 5-HT_{1B} receptor binding.[24]

Theoretically, the lower baseline 5-HT_{1B} receptor binding in PD patients could be caused by a decrease in receptor density (B_{max}), lower tissue free fraction (f_{ND}), or lower radioligand affinity (higher K_D), for example caused by higher 5-HT levels.

There is ample evidence from cerebrospinal fluid and brain autopsies studies[11,26,27] that PD patients have lower levels of 5-HT and its metabolite, 5-HIAA, but this would expectedly lead to *higher* 5-HT_{1B} receptor binding. Yet, we cannot exclude that synaptic 5-HT levels may vary regionally and thereby potentially explain (part of) the regional variation in brain binding in PD patients compared to controls. Further, although it cannot be ruled out, we fail to find any good explanation for why patients with PD should have lower free fraction of radioligand in their brain tissue. That is, our observation is most likely due to patients with PD having lower 5-HT_{1B} receptor density, possibly due to loss of brain cells that normally express 5-HT_{1B} receptors. Since the 5-HT_{1B} receptor functions both as an autoreceptor modulating serotonergic release and as an heteroceptor modulating other neurotransmitter systems,[28] the lower 5-HT_{1B} receptor density could be caused by a reduction in either autoreceptor or heteroreceptor density, or both. We find it most likely that the observed lower 5-HT_{1B} receptor density is due to presynaptic dysfunction or axonal loss. As recently reviewed,[11] several in vivo and in vitro studies have found widespread reductions in cerebral SERT in PD and in a regional pattern fairly consistent with what we observe here (Figure 3) whereas postsynaptic 5-HT receptors are better preserved or even upregulated.[11] It is also well-known that more advanced stages of PD is associated with loss of serotonergic fibres,[29,30] possibly due to Lewy bodies deposits preferentially forming at the axonal terminals, leading to cellular dysfunction and eventually neuronal death.[2] The observation supports that in PD, proximate cellular structures to the Lewy body deposits in the axonal terminal are more affected as compared to somatodendritic cellular components. Previous studies in patients with PD have shown that SERT[27,31] is relatively more reduced in caudate than in putamen and in the posthoc analysis, we saw that whereas the caudate $BP_{ND}0$ was 38% lower in PD patients, the $BP_{ND}0$ in putamen was only 11% lower than control values.

The role of 5-HT_{1B} receptors for motor disability

In the PD patients, we observed an inverse correlation between motor symptom severity and frontal cortex 5-HT_{1B} receptor availability. This suggests that either the 5-HT_{1B} receptor is important for motor function, the decline in 5-HT_{1B} receptor availability is a proxy of the disease progression, or a combination. To investigate this further, we examined if any frontal subregions seemed to drive the association, but that was not obvious. In a previous PET study of 5-HT_{1B} receptor binding they failed to identify a statistically significant correlation between 5-HT_{1B} receptor binding and stage or severity of the disease;[24] the authors ascribe this failure to lack of power or due to medication effects.

One mechanism DBS-STN acts by is tempering the excessive STN firing rate, known to occur in PD patients, thereby reducing the excessive GABAergic output from the basal ganglia causing motor symptoms. However, 5-HT also has a net inhibitory effect on the basal ganglia output primarily driven by the 5-HT_{1B} receptors on STN afferents and efferents.[32] In PD patients. preservation of 5-HT_{1B} receptors and the capacity to elicit a serotonergic response may be an important mechanism to temper the basal ganglia output, when DBS-STN is turned off.

Turning off STN-DBS in patients with PD

When we turned off the DBS-STN in patients with PD, BP_{ND} decreased between zero and 15% across a number of brain regions (Figure 3). Based on data from the pig brain,[12] one can estimate that a 7% decline in [^{11}C]AZ10419369 BP_{ND} corresponds to a 3-fold increase in cerebral 5-HT level, achievable with less than a single intravenous dose of a potent 5-HT releaser, fenfluramine (0.5 mg/kg). But how does turning off the DBS stimulator lead to increased cerebral 5-HT release? In rat studies,[33–35] high frequency stimulation of the STN caused a decrease in DRN firing and a decrease in 5-HT in prefrontal cortex. Here, we observe an increase when DBS is turned off. The fact that the serotonergic response to cessation of DBS-STN stimulation extends beyond the basal ganglia and thalamocortical circuits suggests a stimulation of the raphe nuclei serotonergic components.

Intriguingly, we find that the degree to which regional cerebral 5-HT_{1B} receptors are preserved in PD predicts the capacity to elicit 5-HT when the DBS stimulator is turned off. From Figure 3 it can be seen that when the regional 5-HT_{1B} receptor binding is normal, switching DBS off leads to a 15% decrease in BP_{ND}, corresponding to an 8-fold increase in cerebral 5-HT levels. Or conversely, when 5-HT_{1B} receptor binding is reduced in PD patients to 2/3 of control levels, no 5-HT is released in response to turning off the stimulator. This observation further supports that the abnormalities in cerebral 5-HT_{1B} receptors found in PD patients are primarily caused by presynaptic degeneration. Since our study for logistical reasons only involved turning off DBS-STN, one cannot simply assume that the reverse actions occur when turning DBS-STN on. Also, the temporal evolvement of the response cannot be assessed beyond 60 min since our experimental setup does now allow for extended measurements of cerebral 5-HT_{1B} receptor binding in the off condition.

POMS data

If DBS-STN also act on raphe firing, disinhibition of the raphe in the DBS-OFF condition would lead to an increased 5-HT release in 5-HT cortical projections, which could potentially regulate mood. We did see a significant worsening of the POMS subscale of vigor, when the DBS stimulator was turned off, proportional to the availability of the 5-HT_{1B} receptors. The negative correlation between 5-HT_{1B} binding and POMS V score was dissociated from the MDS-UPDRS motor scores, which is in line with others suggesting separate mechanisms of cognitive and physiological fatigability in PD.[36] It seems less intuitive why DBS-STN treated patients with better preservation of their presynaptic serotonergic function should report less vigor after switching the DBS off, but we suspect that it may be due to a report bias in the least affected patients. We recognize that the limited sample size imposes a challenge for interpretation of the mood scores and suggest that these data should be replicated in an independent behavioral study.

Conclusions

We here used a novel functional PET methodology to investigate the static and dynamic integrity of the 5-HT system in patients with PD. We find that DBS-STN treated patients with PD exhibit a region-specific presynaptic serotonergic dysfunction that to some extent is correlated to clinical

severity. Turning off the DBS-STN reveals that the brain regions where the presynaptic 5-HT function was best preserved responded by a substantial 5-HT release, reflecting that the presynaptic terminals are still relatively preserved in those brain areas whereas the more affected brain regions have lost their 5-HT releasing capacity. These deficits in the regulation of the 5-HT may contribute to PD patients' non-motor challenges. Our study also demonstrates that DBS-STN dynamically regulates the 5-HT system and that further studies of the long-term effects on 5-HT function is warranted.

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SUPPLEMENTARY MATERIAL

Magnetic Resonance Imaging

MRI scans of all controls were conducted in-house on a 3T MRI Prisma scanner (Siemens, Erlangen, Germany) using a 64-channeled head coil to obtain structural T1 and T2 weighted whole brain images. However, one control only had a T1 image available.

For reasons of relative MRI contraindications and artefacts in PD patients with DBS implants, we used the pre-operative MRI scan of the PD patients, which had all been conducted in-house on a 1.5 T MRI system and with only T2 weighted whole brain MR images available. Time interval between the structural MRI and the PET scan was 2.6 ± 1.8 years.

To evaluate the impact on PET outcomes by using T1 versus T2 weighted MR images, we processed the PET data with both T1 and T2, available in nine controls. Since we found no significant difference whether T1 or T2 was applied, we used the T2 weighted images for the analyses in all participants except for the one control and the one PD patient who only had a T1 weighted MRI available.

Positron Emission Tomography (PET)

PET scanning was conducted with a high-resolution research tomography (HRRT) PET scanner (CTI/Siemens, Knoxville, TN, USA). The scanning protocol for [^{11}C]AZ10419369 consisted of 2 h dynamic data acquisition, starting with a [^{11}C]AZ10419369 bolus injection administered over 20 seconds. The scan-time in the healthy volunteers was 120 min (n=5), 110 min (n=1), 90 min (n=3) and 60 min (n=1). The scan time was 120 min in all PD patients (n=13). Depending on the duration of the PET scan, [^{11}C]AZ10419369 images were reconstructed into 45 (or 39) dynamic frames (6 x 10 s, 6 x 20 s, 6 x 60 s, 8 x 120 s, 19 (or 13) x 300 s) using ordinary Poisson 3-dimensional ordered-subset expectation maximization with point spread function modeling (16 subsets, 10 iterations)[1,2] with accurate attenuation correction as previously detailed[3].

All PET images were post-reconstruction motion corrected using the AIR (Automated Image Registration, v.5.2.5, LONI, UCLA) software[4] with alignment to frame 27 (first frame of 300 seconds). All time-activity and motion curves were visually inspected to identify sudden and substantial movement. Significant patient motion was identified in 6 of 13 PD patients and so, the PET scan was re-reconstructed using HRRT Users Software with AIR following[5]. Firstly, the μ-map for attenuation correction was aligned with the PET reference frame 27 using Vinci 2.55 (http://vinci.sf.mpg.de/). Secondly, the motion was recomputed and a new reconstruction from PET raw data was performed with the μ-map aligned to each frame for optimal attenuation correction.

Figure A illustrates the average ± SD (mm) of the median head motion of the participants during the longitudinal course of the PET scan, with the PD patients generally exhibiting larger head motions as compared to controls. The difference in head movement between groups is up to 1 mm towards the end of the PET scan. However, $BP_{ND}1$ does not change, when data is truncated as to no include the last 30

minutes (6 frames, 1800 seconds). As described in the Methods, correcting for movements in 6 of 13 PD patients, did not change the outcome. Figure B illustrates an example of a ESRTM model fitted TAC.

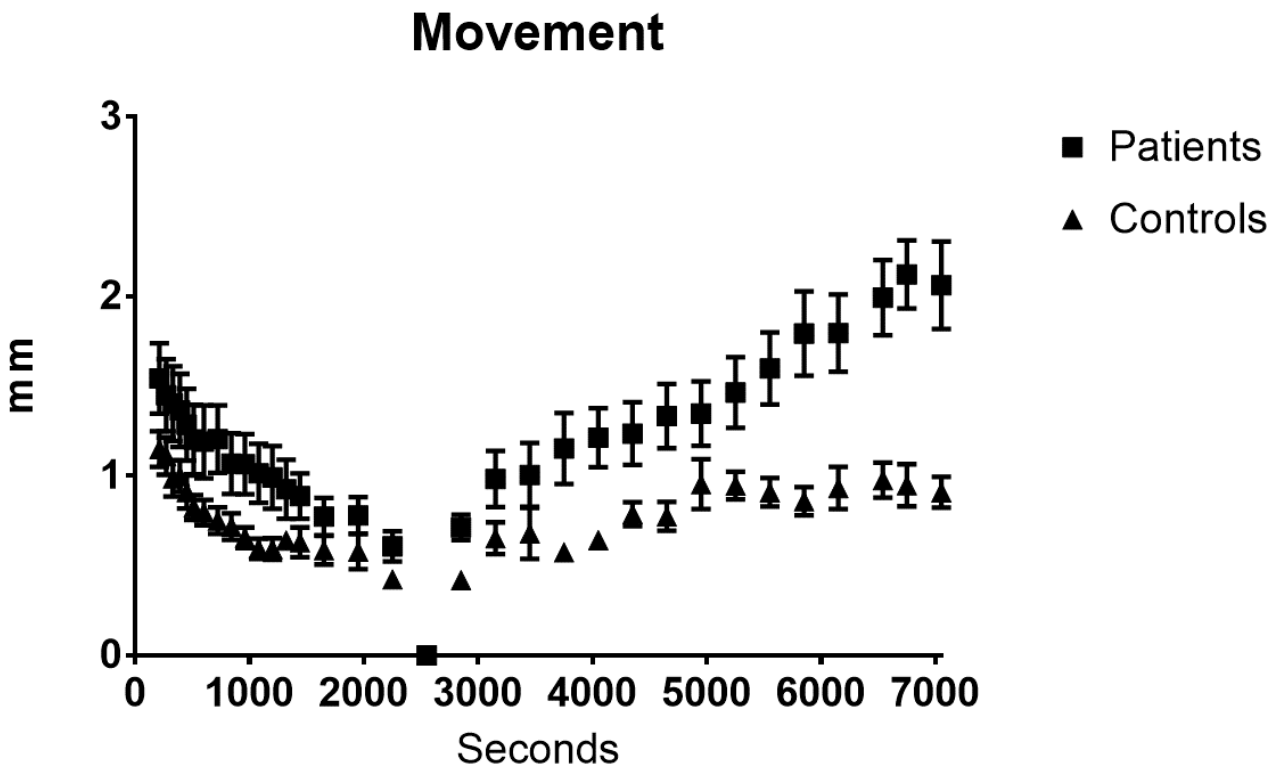

Figure A. Average ± SD median movement in each frame 1-45 of the two groups normalized to frame 30, where DBS was turned off in PD patients.

Finally, the PET frames were realigned producing the final motion corrected images. However, re-reconstruction, did not change the BP_{ND} in the cortical ROIs. The PET images were coregistered and aligned to the subject's T2-weighted MRI image and ROIs were automatically delineated[6]. Each co-registration was verified by visual inspection before extraction of time-radioactivity curves (TACs) from the gray matter of the regions of interest (ROIs), and adjusted if needed, using Hammer's atlas[7] and PVElab[6] (https://nru.dk/pveout).

Quantification of [^{11}C]AZ10419369 Binding

The TACs were fitted and visually inspected in Matlab using the Extended Reference Tissue Model (ESRTM)[8] with the DBS intervention time of 45 minutes to estimate binding potentials and coefficients of variances for each ROI (Figure B). $BP_{ND}0$ and $BP_{ND}1$. Frames acquired at time interval 0-44 min formed the basis for calculation of $BP_{ND}0$ and frames acquired at time interval 45-rest of scan for $BP_{ND}1$ corresponding to the two conditions: DBS-ON and DBS-OFF.

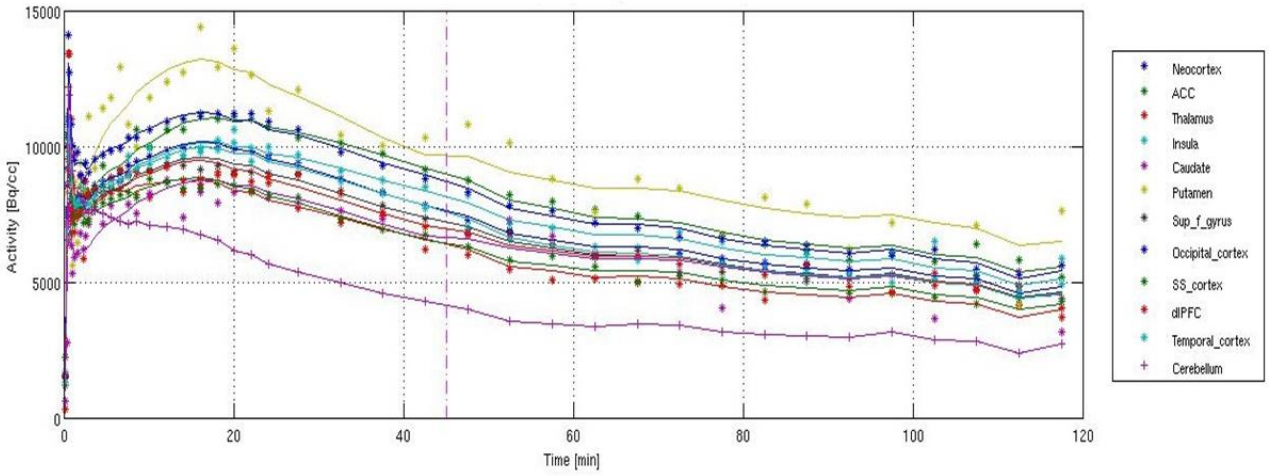

Figure B. Example of fitted TACs in a PD patient with DBS, ESRTM modeling. The DBS was turned off at 45 minutes (dotted vertical line) and modelled for $BP_{ND}0$ (0-44 min) and $BP_{ND}1$ (45 – rest of scan) with cerebellum as the reference region.

The cerebellum was used as an reference region, as the level of 5-HT_{1B} receptors in the cerebellum is insignificant[9]. For occupancy modeling, the effect of the intervention at 45 min ($BP_{ND}1 - BP_{ND}0$) relative to $BP_{ND}0$ was calculated for the ROIs.

The ROIs from caudate, putamen and thalamus included both gray and white matter, while in the remaining ROIs, BP_{ND} was extracted from grey matter only.

Since five controls were PET-scanned for less than 120 minutes, we investigated the time stability of [^{11}C]AZ10419369 ESRTM modeling by truncating the dataset to simulate shorter scans (90 min). This did not change the $BP_{ND}1$, so the full dataset was used in all participants, regardless of scan time.

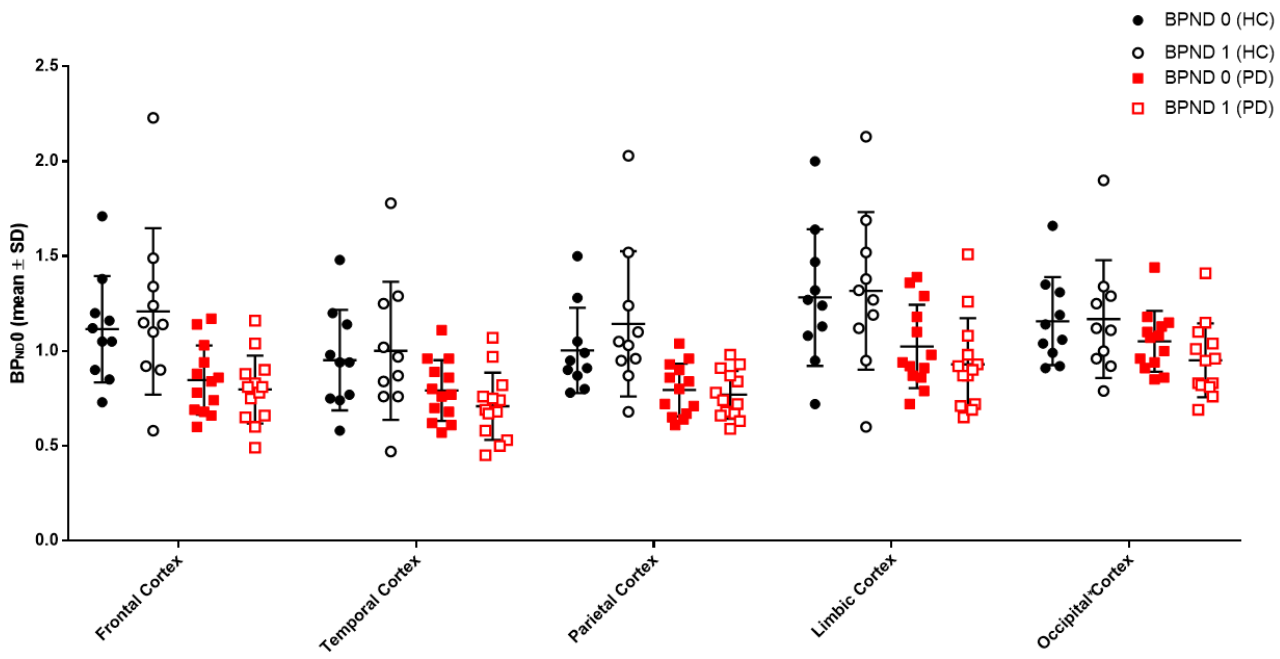

Figure C. BP_{ND} in selected brain regions. $BP_{ND}0$ (0-44 min) and $BP_{ND}1$ (45 min – end of scan) of PD patients (red) and age-matched controls (black) corresponding to the two conditions DBS-ON and DBS-OFF.

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