UNIVERSITY OF COPENHAGEN
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PhD thesis

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PET investigations of brain serotonin receptor binding in migraine patients

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Preface

This thesis consists of four studies, which were conducted during my appointment as a research fellow from 2014 to 2018 at the Danish Headache Center, Rigshospitalet Glostrup and the Neurobiology Research Unit, Department of Neurology, Rigshospitalet, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark.

My faculty supervisor was Prof. Messoud Ashina, and my primary co-supervisor was Prof. Gitte Moos Knudsen. My project supervisors were Dr. Anders Hougaard and Dr. Hanne Demant Hansen.

List of papers

This thesis is based on the following manuscripts:

1. **Deen M**, Hansen HD, Hougaard A, Nørgaard M, Eiberg H, Lehel S, Ashina M, Knudsen GM. High brain serotonin levels in migraine between attacks: A 5-HT_4 receptor binding PET study. *NeuroImage: Clinical,* 2018;18: 97-102.
2. **Deen M**, Hougaard A, Hansen HD, Svarer C, Eiberg H, Lehel S, Knudsen GM, Ashina M. Migraine is associated with high brain 5-HT levels as indexed by 5-HT_4 receptor binding. *Submitted.*
3. **Deen M**, Hansen HD, Hougaard A, da Cunha-Bang S, Nørgaard M, Svarer C, Keller S, Thomsen C, Ashina M, Knudsen GM. Low 5-HT_{1B} receptor binding in the migraine brain: A PET study. *Cephalalgia,* 2018; 38(3): 519-527.
4. **Deen M**, Hougaard A, Hansen HD, Schain M, Dyssegaard A, Knudsen GM, Ashina M. Sumatriptan crosses the blood-brain barrier in migraine patients and binds to central 5-HT_{1B} receptors. *Submitted.*

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Abbreviations

5-HT	5-hydroxytryptamine, serotonin
5-HIAA	5-hydroxyindoleacetic acid
CM	Chronic migraine
EM	Episodic migraine
HRRT	High Resolution Research Tomography
ICHD-3	International Classification of Headache Disorders, 3rd edition
MAO-A	Monoamine Oxidase-A
MNI	Montreal Neurological Institute
MRI	Magnetic Resonance Imaging
MRTM2	Multilinear Reference Tissue Model 2
PDE3	Phosphodiesterase-3
PET	Positron Emission Tomography
SERT	Serotonin Transporter
SPM	Statistical Parametric Mapping
SRTM	Simplified Reference Tissue Model
SSRI	Specific Serotonin Reuptake Inhibitor

Summary

Migraine is the most prevalent and disabling neurological disorder worldwide. The underlying pathophysiology is still unknown and treatment options are inadequate. Since the 1960s serotonin (5-HT) has been implicated in migraine pathophysiology with findings of lower plasma serotonin levels in migraine patients between attacks and increases during migraine attacks. However, the exact role of brain serotonin levels in migraine pathophysiology is still unknown. The overall goal of the thesis was to investigate the serotonergic system in the migraine brain using positron emission tomography imaging of two different serotonin receptors.

In study 1 we investigated 5-HT_4 receptor binding in interictal episodic migraine without aura patients and compared them to healthy controls. 5-HT_4 receptor binding is inversely related to stable brain serotonin levels and can be used as a biomarker of brain serotonin levels. Based on the serotonin deficiency theory in migraine, we hypothesized that migraine patients would have higher 5-HT_4 receptor binding compared to controls. Instead, we found that patients had lower 5-HT_4 receptor binding indicating higher brain serotonin levels. In study 2 we investigated whether brain serotonin levels were involved in the conversion from episodic to chronic migraine. We found that chronic migraine patients had lower 5-HT_4 receptor binding compared to controls but did not differ from episodic migraine patients, and that 5-HT_4 receptor binding were not associated with attack frequency. This suggests that a high level of brain serotonin is an inherent trait of the migraine brain and that brain serotonin levels are not related to attack frequency and cannot be used as a biomarker to distinguish between episodic and chronic migraine patients.

In study 3 we investigated 5-HT_{1B} receptor binding in interictal episodic migraine without aura patients. Compared to controls, migraine patients had lower 5-HT_{1B} receptor binding across pain-modulating brain regions. This could either be an inherent trait of the migraine brain,

leading to dysfunctional pain modulation, or a consequence of repeated activation of the pain-modulating regions during migraine attacks. To explore changes in brain serotonin levels during attacks and to investigate the occupancy of sumatriptan, a migraine specific 5-HT_{1B} receptor agonist, on central 5-HT_{1B} receptors, we, in study 4, investigated 5-HT_{1B} receptor binding during experimentally induced migraine and after treatment with sumatriptan. We found that compared to the interictal state, the 5-HT_{1B} receptor binding was reduced during the ictal state, indicating ictal increases in brain serotonin levels. After treatment with sumatriptan, the 5-HT_{1B} receptor binding decreased further, indicating that sumatriptan binds to central 5-HT_{1B} receptors.

In conclusion, the present thesis supports the hypothesis that the serotonergic system plays a role in migraine pathophysiology, with migraine patients exhibiting high brain serotonin levels between attacks, which increase further during attacks. Further, we show that the 5-HT_{1B} receptor density is altered in migraine patients and provide evidence that sumatriptan may act on central 5-HT_{1B} receptors. Future studies are warranted to explore the exact mechanisms by which high brain serotonin levels lead to migraine. Further, interventions aiming at lowering brain serotonin levels may prove efficient as a treatment of migraine in the future.

Summary in Danish

Migræne er den mest invaliderende neurologiske lidelse på verdensplan, men vores viden om de underliggende sygdomsmekanismer er stadig utilstrækkelig. Man har siden 1960'erne ment at serotonin (5-HT) spiller en afgørende rolle i udviklingen af migræneanfald, og tidligere studier har fundet lavere serotoninniveauer i plasma hos migrænepatienter mellem migræneanfald samt stigninger i plasmaniveauerne under migræneanfald. Imidlertid ved vi ikke ret meget om serotoninniveauerne i hjernen hos migrænepatienter. Det overordnede formål med denne afhandling var derfor at undersøge det serotonerge system i migrænehjernen ved brug af positron-emissions-tomografi (PET). PET er en billedannende teknik som anvendes til at visualisere f.eks. receptorer i hjernen. I denne afhandling undersøgte vi to forskellige serotoninreceptorer, som ikke tidligere er blevet undersøgt i en migrænepopulation.

I det første studie undersøgte vi 5-HT_4 receptorbinding i patienter med migræne uden aura og sammenlignede dem med raske kontroller. 5-HT_4 receptorbinding er invers relateret til hjernens serotoninniveau og kan derfor anvendes som en biomarkør for dette. Baseret på hypotesen om, at migrænepatienter har et lavt niveau af serotonin i hjernen, var vores hypotese, at migrænepatienter ville have højere 5-HT_4 receptorbinding sammenlignet med kontroller. I stedet fandt vi, at patienter havde lavere 5-HT_4 receptor binding, hvilket indikerer, at de har højere serotoninniveauer. I det andet studie anvendte vi den samme teknik til at undersøge, hvorvidt serotonin er involveret i udviklingen fra episodisk til kronisk migræne. Vores resultater viste, at kroniske migrænepatienter havde lavere 5-HT_4 receptor binding sammenlignet med kontroller, men at de ikke adskiller sig fra episodiske migrænepatienter. Dette tyder på, at høje serotoninniveauer i hjernen er et grundlæggende træk ved migrænehjernen uafhængig af anfaldsfrekvens. Ligeledes viser

resultaterne, at serotoninniveauerne i hjernen ikke kan anvendes som biomarkør til at skelne mellem episodiske og kroniske migrænepatienter.

I det tredje studie undersøgte vi 5-HT_{1B} receptorbinding i patienter med episodisk migræne uden aura. Sammenlignet med kontroller havde patienter lavere 5-HT_{1B} receptor binding i regioner i hjernen, der er involveret i smertemodulation. Dette kan enten være en grundlæggende, genetisk bestemt karakteristika ved migrænehjernen, hvilket fører til dysfunktionel smertemodulation eller en konsekvens af gentagen aktivering af de smertemodulerende regioner under migræneanfald. For at undersøge ændringer i hjernens serotoninniveauer under anfald samt om sumatriptan, et migrænespecifikt lægemiddel, binder til central 5-HT_{1B} receptorer, undersøgte vi i det fjerde studie 5-HT_{1B} receptorbinding under eksperimentelt induceret migræne og efter behandling med sumatriptan. Vores resultater viste, at 5-HT_{1B} receptorbindingen var reduceret under anfald sammenlignet med uden for anfald. Dette tyder på, at serotoninniveauet stiger i hjernen under anfald. Efter behandling med sumatriptan faldt 5-HT_{1B} receptorbindingen yderligere, hvilket indikerer, at sumatriptan til en vis grad binder til centrale 5-HT_{1B} receptorer.

Samlet set understreger og bekræfter resultaterne fra den foreliggende afhandling hypotesen om, at det serotonerge system i hjernen spiller en rolle for udviklingen af migræneanfald. Vi viser, at migrænepatienter udviser høje serotoninniveauer i hjernen mellem anfald, der stiger yderligere under anfald. Endvidere viser vi, at mængden 5-HT_{1B} receptorer er lavere hos migrænepatienter. Slutteligt tyder resultaterne på, at sumatriptan kan udøve en effekt på 5-HT_{1B} receptorer i hjernen. Fremtidige studier er nødvendige for at undersøge de nøjagtige mekanismer, hvormed høje serotoninniveauer i hjernen fører til migræne. Endvidere kan interventioner, der sigter mod at sænke serotoninniveauet i hjernen, vise sig at være effektive som behandling af migræne i fremtiden.

Introduction

Migraine – a disabling brain disorder

Migraine is a common neurological disease currently ranking second highest of all causes of disability worldwide[1]. Around 16% of the population is affected globally[1] and it is a disease with immense, not only personal, but also socioeconomical impact. The yearly cost of migraine in Europe exceeds 18 billion euros[2] and migraine patients, especially chronic migraine patients, have a high degree of absenteeism and loss of productivity[3,4].

Migraine is divided into two major types: migraine with or migraine without aura. This thesis focuses on migraine without aura. Migraine without aura is characterized by recurrent attacks of headache with specific features, i.e. throbbing, unilateral pain, and associated symptoms such as photo- and phonophobia and nausea and vomiting. Despite many decades of research, the pathophysiological mechanisms underlying migraine are not yet fully clarified and there is currently no biomarker for migraine. The diagnosis is based on the patient's headache history in combination with a set of criteria defined by the International Classification of Headache Disorders, ICHD-3[5]. Identifying a biomarker for migraine would increase our understanding of the underlying neurobiological mechanisms and could possibly improve treatment of this disabling disorder.

Depending on migraine frequency, migraine is defined as either episodic or chronic. Chronic migraine patients have at least 15 headache days pr. month of which 8 must fulfill the criteria for migraine[5]. Yearly, around 3% of migraine patients convert from episodic to chronic migraine[6,7]. However, over a 2-year's course, 26% convert back to episodic migraine[8]. The neurobiological mechanisms underlying the conversion from episodic to chronic migraine are still elusive, but several factors contribute to the transformation. These include medication overuse, obesity

and a high baseline frequency of migraine attacks[9]. Further studies are warranted to unravel the mechanisms underlying the in- and decreases in frequency of migraine.

Neurobiology of the serotonergic system

Serotonin is a monoamine neurotransmitter involved in a wide range of physiological processes such as sleep, mood, cognition, and pain[10]. It is synthesized in neurons of the raphe nuclei from the amino acid tryptophan in a two-step process. Firstly, tryptophan is converted into 5-hydroxy-tryptophan by tryptophan hydroxylase. Secondly, 5-hydroxytryptophan is decarboxylated to form 5-hydroxytryptamine, serotonin (5-HT). After synthetization serotonin is transported in synaptic vesicles to presynaptic terminals where it is released into the synaptic cleft by fusion of the vesicles with the presynaptic membranes. After exerting its actions, serotonin is transported back into the neurons by the serotonin transporter (SERT), which is located presynaptically. In the neurons, serotonin is then catabolized by monoamine oxidase A (MAO-A) to its main metabolite, 5-hydroxy-indoleacetic acid (5-HIAA).

From the raphe nuclei serotonergic neurons project to nearly every region of the brain, including somatosensory cortex, thalamus, the trigeminal nuclei and the dorsal horn of the spinal cord. In the projection areas, synaptic serotonin exerts its action on available pre- or postsynaptic receptors. The serotonin receptors consist of 14 different receptors which are divided into seven receptor families. Except for the 5-HT_3 receptor, which is an ion channel, all serotonin receptors are G-protein coupled receptors[10]. These receptors are located postsynaptically, where they are involved in modulation and release of other neurotransmitters such as acetylcholine, GABA and glutamate[11]. In addition, the 5-HT_{1A} and 5-HT_{1B} receptors are located presynaptically on serotonergic neurons, acting as autoreceptors controlling the release of serotonin. Thus,

the level of serotonin in the synaptic cleft is regulated both by activation of autoreceptors as well as by activity of the serotonin transporter.

Serotonin and migraine

Several decades ago it was hypothesized that migraine is fundamentally a syndrome of interictally low serotonin levels with transient increases ictally[12]. This hypothesis emerged when it was discovered that infusion of serotonin alleviated the headache in migraine patients[13] and that migraine patients had excessive urinary excretion of 5-HIAA, the main metabolite of serotonin, during migraine attacks[14]. Further, pharmacological challenges, increasing endogenous, synaptic serotonin in the brain, were able to induce migraine attacks in migraine patients[15,16]. Later it was also shown that migraine patients had lower plasma levels of serotonin between attacks compared to controls and that these levels increased during attacks[17].

Further evidence for the role of serotonin in migraine is provided by the mode of action of several anti-migraine drugs. Both the previously used preventatives, pizotifen and methysergide, are 5-HT_2 antagonists, whereas the acute abortive treatments, ergotamine and triptans, are 5-$HT_{1B/D}$ receptor agonists[18]. The first triptan, sumatriptan, was developed and launched in the early 1990s[19]. Interestingly, the development of sumatriptan was derived from the studies on serotonin and migraine. At that time, it was hypothesized that the migraine pain was caused by vasodilation, and the headache-relieving effect of intravenously administered serotonin was attributed to its ability to constrict bloodvessels[18]. Besides sumatriptan, six other triptans are now available on the market showing similar rates of efficacy and adverse events[20] and to date, triptans are still the most efficient acute migraine treatment available. Further, triptans are migraine specific and do not have an effect in e.g. tension-type headache. Evidently, the 5-HT_{1B} receptor

plays an important role in migraine pathophysiology, but the exact location and mode of action of triptans are still unknown. Whether sumatriptan crosses the blood-brain-barrier and binds to central 5-HT_{1B} receptors also remains elusive.

The early studies on serotonin and migraine focused on peripheral levels of serotonin in migraine. It is however unknown whether peripheral levels reflect central brain serotonin levels, and serotonin does not readily cross the blood-brain-barrier. Thus, less is known about the brain serotonin levels in migraine. Several studies have investigated this using either electrophysiological surrogate markers of serotonin levels, such as visual and auditory evoked potentials, or positron emission tomography (PET) imaging of different components of the serotonergic system in the brain (reviewed in Deen et al., Cephalalgia, 2016)[12]. However, these studies have given equivocal results, indicating both increased and decreased interictal brain serotonin levels as well as ictal alterations. Thus, the role of serotonin in migraine pathophysiology still remains to be unraveled.

The overall aim of this PhD thesis was, therefore, to investigate the serotonin system in the migraine brain using PET imaging of two different serotonin receptors, which have not previously been investigated in migraine patients, the 5-HT_{1B} and the 5-HT_4 receptor. The 5-HT_{1B} specific radioligand [^{11}C]AZ10419369 is sensitive to acute changes in endogenous brain serotonin levels[21–24]. In contrast, the specific 5-HT_4 receptor ligand, [^{11}C]SB207145, is not sensitive to acute changes in brain serotonin levels[25] but 5-HT_4 receptor binding shows an inverse relationship with long-term changes in brain serotonin levels, i.e. if brain serotonin levels increase over time, the 5-HT_4 receptor is downregulated and vice versa. 5-HT_4 receptor binding assessed with PET imaging can thus be used as a biomarker of brain serotonin levels[26].

Specific aims

1. To investigate interictal brain serotonin levels in episodic migraine patients compared to controls using 5-HT_4 receptor binding as a proxy.
2. To investigate whether chronic migraine patients differ from controls and episodic migraine patients with regard to brain serotonin levels, as indexed by 5-HT_4 receptor binding, and to assess whether frequency of migraine attacks is related to brain serotonin levels.
3. To investigate whether migraine patients differ from controls with regard to brain 5-HT_{1B} receptor density in pain-modulating regions.
4. To investigate 5-HT_{1B} receptor binding during experimentally induced migraine attacks and after administration of sumatriptan.

Methods

Participants

Both patients and healthy volunteers were recruited from a Danish website for recruitment of volunteers to health research (www.forsøgsperson.dk), a local database and through online advertisements. Inclusion criteria for episodic migraine patients were a verified diagnosis of migraine without aura according to the 3rd edition of The International Classification of Headache Disorders (beta version)[27], at least one migraine attack every other month and less than five migraine days per month and self-reported efficacy of treatment of migraine with sumatriptan. Inclusion criteria for chronic migraine patients were a verified diagnosis of chronic migraine according to the 3rd edition of The International Classification of Headache Disorders (beta version)[27]. Inclusion criteria for

healthy controls were no history of migraine including probable migraine and no first-degree relatives with migraine. All subjects had to be between 18 and 65 years old and they were not allowed to have a history of any other primary headache disorder (except for tension-type headache on less than five days pr. month), any cerebrovascular, cardiac or psychiatric diseases, any contraindications for magnetic resonance imaging (MRI), any daily intake of medication including migraine prophylaxis or be pregnant or nursing. All studies were approved by the regional ethics committee of The Capital Region of Denmark (H-6-2014-057). In accordance with the Declaration of Helsinki of 1964, with later revisions, all subjects gave written consent after receiving oral and written information about the study and before initiation of any study related procedures.

Clinical assessments

All subjects had a general medical history taken and underwent a physical and neurological examination. In addition, all episodic migraine patients had their ECG taken. Blood samples were drawn to assure normal biochemistry and all fertile women performed a pregnancy test. All subjects also filled out the Major Depression Inventory questionnaire. Headache history was obtained for all patients using a standardized interview including questions on duration of disease, number of migraine days per month, clinical characteristics of the migraine attacks, number of days with intake of triptans, number of days with non-migraine headache and date of last migraine attack.

Provocation of migraine

In study 4, we investigated 5-HT_{1B} receptor binding during migraine attacks in episodic migraine patients. Since the timing of spontaneous migraine attacks is hard to predict, we chose to use an

experimental human migraine model to induce migraine attacks on the day of the PET scan. Human migraine models have been used for decades to investigate pathophysiological mechanisms in migraine and several substances can be used to trigger migraine[28,29]. Here we used cilostazol, a PDE3 inhibitor, which has previously proven to be a powerful and reliable migraine inducer[30,31]. All episodic migraine patients went through a screening for induction of migraine attacks by cilostazol before inclusion in the study. The procedure was the same for the screening and for the day of the scan: 200 mg cilostazol, oral tablets, were ingested with a glass of milk where after the subjects filled out a headache diary hourly. On the day of the scan, the diary was filled out every 30 minutes after arrival to the hospital and every 10 minutes after initiation of the first scan. The diary included questions on headache intensity (0-10), location and quality as well as associated and premonitory symptoms. On the day of the screening subjects were allowed to treat a possible migraine with 100 mg sumatriptan. On the day of the scan, all subjects were treated with sumatriptan, 6 mg sc. immediately after the first scan.

Since, for ethical reasons, participants in a provocation study cannot be denied treatment for a possible headache, a modified version of the definition of migraine without aura (according to ICHD-3[5]) omitting the need for the attack to last at least 4 hours, was used. Thus, the cilostazol-induced headaches only had to fulfill criteria C and D to be classified as migraine attack:

C. Headache has at least two of the following four characteristics:

1. unilateral location
2. pulsating quality
3. moderate or severe pain intensity
4. aggravation by or causing avoidance of routine physical activity (e.g. walking or climbing stairs)

D. During headache at least one of the following:

1. nausea and/or vomiting

2. photophobia and phonophobia

Study design

Study 1 and 3 are cross-sectional case-control studies, comparing episodic migraine patients to healthy controls. In study 2 chronic migraine patients are compared to healthy controls and to episodic migraine patients. Study 4 uses a within-subject design where episodic migraine patients are investigated interictally, ictally and after treatment with sumatriptan. For study 1 and study 3 all episodic migraine patients had been migraine-free for 48 hours before the day of the scan. If they reported a migraine attack within 48 hours after the scan they were excluded from the data analysis. This consideration was taken to ensure that all PET data for these two studies were obtained in truly interictal migraine patients. Chronic migraine patients were allowed to have headache on the day of the scan. Type of headache and pain intensity were registered. All healthy volunteers were headache free on the day of the scan. The study design for study 4 is shown in figure 1.

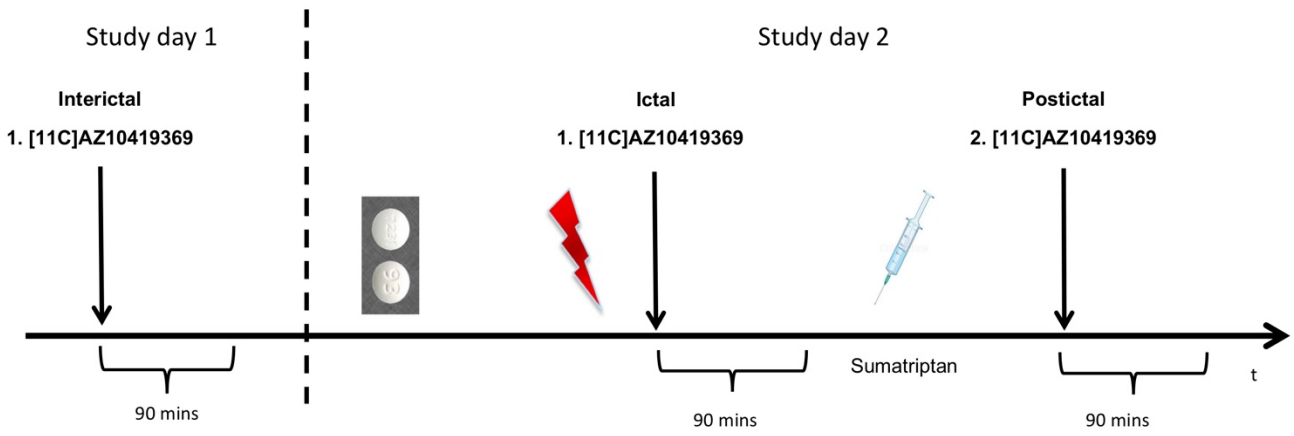

Figure 1. In study 4, subjects were first scanned between two migraine attacks to obtain data from the interictal phase. On a separate study day, they ingested 200 mg cilostazol (depicted as tablets) to induce a migraine attack. Then, when a migraine attack occurred (red arrow), the subjects were scanned again to obtain PET data from the ictal phase. Finally, they were treated with sumatriptan 6 mg sc., and then scanned to obtain data from the postictal phase.

Neuroimaging

Positron Emission Tomography

PET is a powerful imaging tool widely used in neuroscience due to its specific properties of *in vivo* neuroreceptor mapping. To map a specific target of interest, e.g. a serotonin receptor, a radiotracer is produced by attaching a radioisotope, e.g. ^{11}C or ^{18}F, to a tracer which binds to the target of interest. In neuroreceptor mapping, the tracer is typically an agonist or antagonist for the receptor in question. A specific property of a radiotracer is that it does not elicit a physiological or pharmacological response. To avoid this, the radiotracer is administered in very low doses, so called tracer doses. After injection of the radiotracer to the blood stream the tracer is distributed throughout the body where it binds to the target of interest. The signal detected by the PET camera is generated when the positron emitted from the decaying radioisotope annihilates with an electron in the surrounding tissue. This causes emission of a pair of photons at a 180 degrees angle which are detected by the PET camera. Thus, the exact location of the annihilation and thereby the location of the radiotracer can be determined. Images can then be generated by reconstruction in combination with correction for absorption and random events.

Radiotracers used in this study

In this study we investigated two different targets, the 5-HT_{1B} and the 5-HT_4 receptor, using two different radiotracers, [^{11}C]AZ10419369 and [^{11}C]SB207145, respectively. [^{11}C]AZ10419369 is a partial agonist for the 5-HT_{1B} receptor, whereto it binds with high specificity, i.e. it has a 47-fold selectivity for the 5-HT_{1B} receptor as compared to the 5-HT_{1D} receptor[32]. Since this radiotracer is sensitive to acute changes in endogenous brain serotonin levels, it can not only be used to investigate the density and distribution of 5-HT_{1B} receptors in different study populations, but also to detect whether certain physiological states or interventions induce alterations in brain serotonin levels. Further, it can be used to determine occupancy of drugs at central 5-HT_{1B} receptors[33].

[^{11}C]SB207145 is a 5-HT_4 receptor antagonist which binds with high selectivity and affinity to the 5-HT_4 receptor. [^{11}C]SB207145 binding is not affected by acute changes in endogenous brain serotonin levels[25]. However, there is now ample evidence of an inverse relationship between 5-HT_4 receptor density and endogenous brain serotonin levels. In both animals and humans, 5-HT_4 receptor density was decreased by long-term (2-3 weeks) SSRI administration[26,34]. Further, tryptophan depletion, which decreases brain serotonin levels, upregulated the 5-HT_4 receptor[34]. Lastly, carriers of the short allele of the serotonin transporter gene – which is related to higher synaptic serotonin levels – exhibited lower neocortical 5-HT_4 receptor binding compared to carriers of the long allele[35].

Acquisition of PET and MRI data

Dynamic PET data were acquired with a high-resolution research tomography (HRRT) PET scanner (CTI/Siemens, Knoxville, TN, USA). To minimize head movement, all subjects had their head stabi-

lized in a specialized head holder. For all scans the radiotracer was administered as an intravenously 20 seconds bolus injection into a cubital vein. Scan time was 90 minutes for [^{11}C]AZ10419369 and 120 minutes for [^{11}C]SB207145.

Structural T1 and T2 weighted images were recorded for each subject using a Siemens Prisma 3T scanner (Siemens, Erlangen, Germany) with a 64-channel head coil. These images were used for co-registration with PET, delineation of regions of interest (ROI), and for segmentation in SPM8 into grey matter, white matter and cerebrospinal fluid.

Analysis and quantification of PET data

During preprocessing, single-subject PET images were corrected for intra-scan movement by aligning frames to a reference frame using a scaled least-squares cost function in AIR 5.2.5. To avoid bias, we used an automated process to co-register and align the PET images to the corresponding MR image and to delineate the regions of interest (ROIs)[36]. Time activity curves (TAC) and grey matter volumes for each ROI were also extracted during this process. Correct co-registration and delineation of ROIs were ensured by visual inspection.

The time activity curves represent the concentration of radioactivity in tissue over time, which is used to estimate the binding potential (BP). This is done by use of kinetic modeling where the TACs are fitted to a function to estimate the BP of the tracer. *In vivo,* the BP is estimated by specific binding relative to a reference concentration such as plasma concentration or concentration of radioactivity in a reference region. For a region to be used as a reference region it needs to be devoid of the target in question. If a reference region is not available, other kinetic

models can be used for quantification of PET data such as Logan invasive or 1- or 2-tissue compartment models. However, these all require arterial cannulation in order to obtain arterial input functions.

Here, we used the simplified reference tissue model (SRTM) with cerebellar grey matter as reference region to quantify both [^{11}C]AZ10419369 and [^{11}C]SB207145 PET data. This approach has been validated for both radiotracers[37,38]. The SRTM yields non-displaceable binding potentials, BP_{ND}, which is a measure of the ratio of specifically bound radiotracer relative to that of non-displaceable radiotracer in the tissue. The non-displaceable uptake comprises the non-specifically bound and the free radiotracer in the tissue.

Voxel-based analysis

In study 1 and 3 we applied a voxel-based analysis in order to detect regional group differences in BP_{ND} not captured by our region-wise analysis. For this purpose, parametric BP_{ND} maps were generated in FreeSurfer (http://surfer.nmr.mgh.har- vard.edu, version 5.3) (see figure 2 for an example). Firstly, the structural MR images were normalized to Montreal Neurological Institute (MNI) space and then applied to the co-registered PET images. Lastly, these were smoothed with a 6 mm full-width half-maximum 3D Gaussian kernel. The Multilinear Reference Tissue Model 2 (MRTM2)[39], with cerebellum as reference region, was used to estimate voxel-level BP_{ND}s.

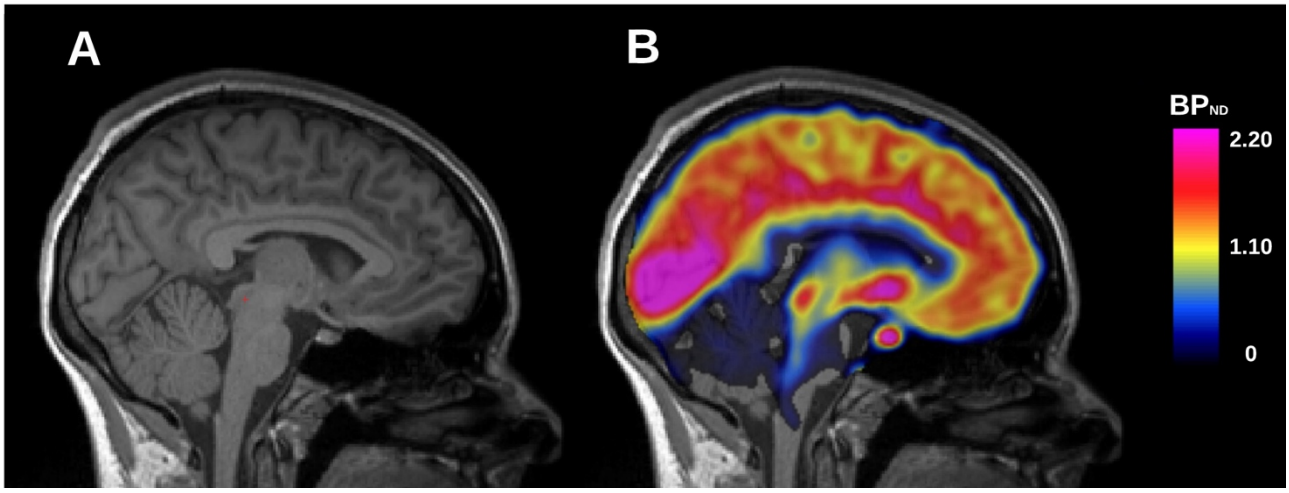

Figure 2. An example of a parametric BP_{ND} map. **A.** A structural T1-weighted MRI image from a single subject. **B.** Parametric BP_{ND} maps of [^{11}C]AZ10419369 superimposed on the corresponding structural image, highlighting the 5-HT_{1B} system.

Regions of interest

For study 1 and 2 we chose to evaluate group differences in brain serotonin levels using a large neocortical ROI. A mean neocortical BP_{ND} was estimated based on 11 neocortical brain regions (occipital cortex, orbitofrontal cortex, superior, medial and inferior frontal gyri, insula, superior, medial and inferior temporal gyri, sensory motor cortex and parietal cortex) by volume weighting grey matter segmented brain region BP_{ND}s:

$$\text{Neocortical receptor binding} =$$

$$\Sigma[BP_{ND}(\text{region}_x) * \text{volume}(\text{region}_x)] / [\Sigma(\text{volume}(\text{region}_x))]$$

In study 3 and 4 the *a priori* ROIs were amygdala, dorsolateral prefrontal cortex, ventrolateral prefrontal cortex, orbitofrontal cortex, sensorimotor cortex, anterior cingulate cortex and insula, which are all involved in pain modulation. In study 3 the individual regional BP_{ND}'s were used,

whereas in study 4 we estimated a volume weighted mean BP_{ND} for the seven regions as described above.

Statistical analysis

Study 1

Group differences in neocortical 5-HT_4 receptor binding were evaluated using a general linear model. 5-HTTLPR-status (S or L_G carriers vs. L_A homozygotes), sex and age were added as covariates. Associations between attack frequency, years with migraine and time since last migraine attack and neocortical 5-HT_4 receptor binding were also evaluated using a general linear model including 5-HTTLPR-status, age and sex as co-variates. All analyses were carried out in R Studio version 3.2.3.

In SPM8, a whole brain voxel-wise multiple regression was performed using the same linear model as above to detect regional group differences in 5-HT_4 receptor binding. A voxel level p-value threshold of $p < 0.001$ was used. To correct for multiple comparisons, only clusters at $p < 0.05$ corrected using the family wise error rate (FWE) were assumed significant.

Study 2

Group differences in neocortical 5-HT_4 receptor binding were evaluated using log-transformed BP_{ND} values. For the comparison between chronic migraine patients and healthy controls, we fitted a model allowing for unequal variance for the two groups using generalized least square. 5-HTTLPR status, sex, and age were added as covariates. Differences in neocortical 5-HT_4 receptor binding between chronic and episodic migraine patients were evaluated using a general linear model adjusting for 5-HTTLPR status, sex, and age. Associations between the number of migraine

days and 5-HT_4 receptor binding were also analyzed using a general linear model including 5-HTTLPR status, sex, and age as covariates. All analyses were carried out in R Studio.

All covariates were motivated by previous studies showing that they all affect 5-HT_4 receptor binding. For 5-HTTLPR genotype, it has been shown that individuals with one of the two low-expressing alleles (S or Lg) have lower 5-HT_4 receptor binding compared to homozygotes of the long La-allele[35,40]. Age and sex were included since both a negative association between 5-HT_4 receptor binding and age as well as a difference between men and women have been demonstrated[41].

Study 3

In R Studio, group differences in 5-HT_{1B} receptor binding across regions involved in pain modulation were evaluated using a latent variable model (LVM). This structural equation model is used to model associations with shared information across observations, e.g., receptor binding across different brain regions. The latent variable can be defined as a construct which captures something which is common for the observations, but which cannot be directly measured. An advantage of using this approach is that we omit the need for correction for multiple comparisons by only doing one test instead of testing each region separately.

In SPM8, we evaluated group differences in 5-HT_{1B} receptor binding at voxel level using multiple linear regressions. Further, we investigated associations between measures of clinical severity and 5-HT_{1B} receptor binding using whole-brain voxel-wise multiple regressions. We corrected for multiple comparisons by using the Monte Carlo simulation method to determine a cluster extent threshold unlikely to have occurred by chance (α<0.05) given a voxel-level of $p < 0.005$, uncorrected.

All analyses were adjusted for age since 5-HT_{1B} receptor binding is reduced by age[42]. Sex does not affect 5-HT_{1B} receptor binding and was not included as a covariate. Further, there is no association between injected mass of the radioligand and 5-HT_{1B} receptor binding (unpublished data), accordingly we did not include injected mass as a covariate.

Study 4

To detect within-subject differences in 5-HT_{1B} receptor binding between conditions (interictal vs. ictal, ictal vs. postictal) we used a Student's paired t-test. Since we had a clear hypothesis of the direction of the changes in BP_{ND}, a decrease, the hypothesis testing was one-tailed.

Results

Study 1

For this study, we excluded three of the 18 migraine patients who completed the study, since they reported a migraine attack within 48 hours after their PET scan. Sixteen age- and sex-matched controls completed the study and were included in the analysis. When comparing the two groups, we found that migraine patients without aura have lower neocortical 5-HT_4 receptor binding compared to healthy controls (figure 3). When looking at the individual regions of neocortex, we found that the group difference was most evident within the orbitofrontal cortex (p=0.009), insula (p=0.018), superior temporal gyrus (p=0.019), parietal cortex (p=0.026), medial and inferior temporal gyrus (p=0.032), and superior frontal gyrus (p=0.040). No significant clusters were found with the voxel-based analysis.

In the migraine group, there were no associations between 5-HT_4 receptor binding and frequency of attacks (slope estimate −0.017, CI: [−0.081;0.047], p=0.56), years with migraine

(slope estimate 0.003, CI: [−0.007;0.012], p=0.53), or days since last migraine attack (slope estimate 0.002, CI: [−0.003;0.007], p=0.40).

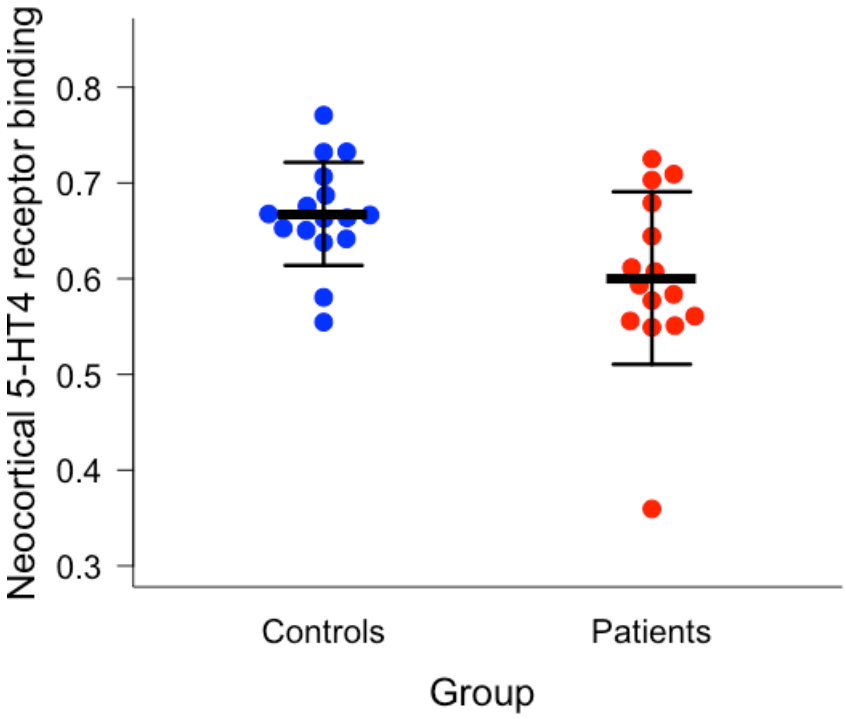

Figure 3. Compared to controls, episodic migraine patients have lower neocortical 5-HT_4 receptor binding (0.60±0.09 vs. 0.67±0.05, p = 0.024). Exclusion of the patient with the lowest BP_{ND} did not affect the results significantly (0.63±0.06 vs. 0.68±0.05, p = 0.038). Black bars indicate mean±SD.

Study 2

Sixteen chronic migraine patients completed the study. Data from all subjects were included in the analysis. Further, data from 15 episodic migraine patients and 16 controls from study 1 were included for comparison. When comparing chronic migraine patients to healthy controls, we found

that chronic migraine patients had 9.1% lower neocortical 5-HT_4 receptor binding (95% CI: [-17%; -1.0%], p = 0.039). Mean BP_{ND}±SD was 0.62±0.09 in the CM group and 0.67±0.04 in the HC group (figure 4).

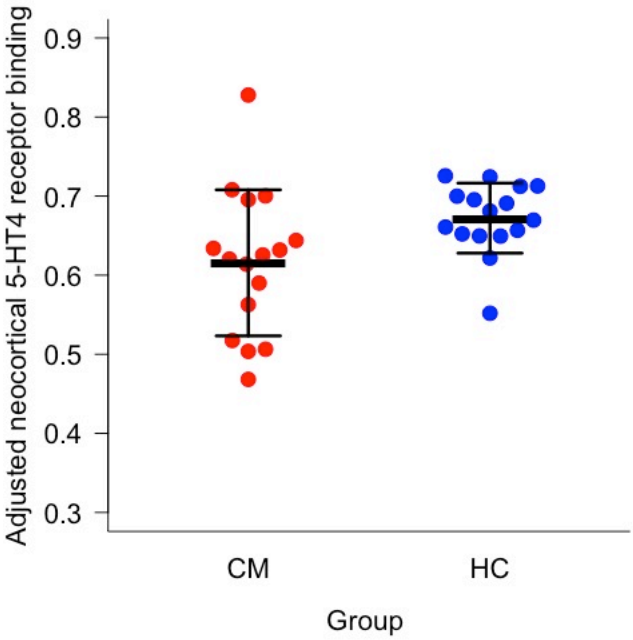

Figure 4. Compared to controls, chronic migraine patients have lower neocortical 5-HT_4 receptor binding (mean±SD: 0.62±0.09 vs. 0.67±0.04). Black bars represent mean±SD. CM = Chronic migraine patients. HC = Healthy controls.

There was no difference in 5-HT_4 receptor binding between CM and EM patients (4.8% higher BP_{ND} in CM vs. EM, 95% CI [-8.5%; 20%], p = 0.48) and we found no association between the number of migraine days and neocortical BP_{ND} (slope estimate 0.003, 95% CI: [-0.004;0.715], p = 0.393) (figure 5B) as well as no associations between BP_{ND} and years with migraine or time since last attack.

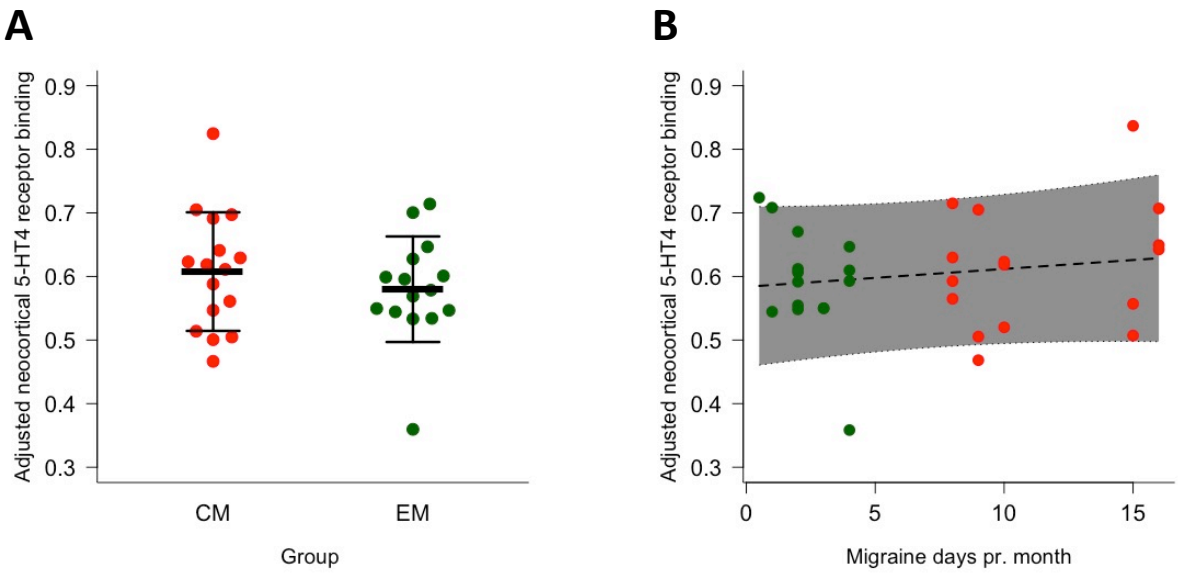

Figure 5. A. Chronic and episodic migraine patients have similar neocortical 5-HT_4 receptor binding (mean±SD: 0.61±0.09 vs. 0.58±0.08). Black bars represent mean±SD. CM = Chronic migraine patients. EM = Episodic migraine patients. **B.** We found no association between number of monthly migraine days and neocortical 5-HT_4 receptor binding in the sample of 31 migraine patients (slope estimate 0.003, 95% CI: [-0.004;0.715], p = 0.393). Red dots represent chronic migraine patients and green dots represent episodic migraine patients. Dashed line represents estimated regression line. Grey shade represents 95% confidence interval.

Study 3

Eighteen episodic migraine patients completed the study. Four subjects were excluded; three due to migraine within 48 hours after the scan and one due to excessive head movement. When fitting our latent variable model, we found that there was a high correlation between 5-HT_{1B} receptor binding across regions which were captured by the latent variable. Further, group status, migraine patient or control, significantly predicted the latent variable, with migraine patients having lower

5-HT_{1B} receptor binding across regions involved in pain modulation compared to age- and sex-matched controls (figure 6).

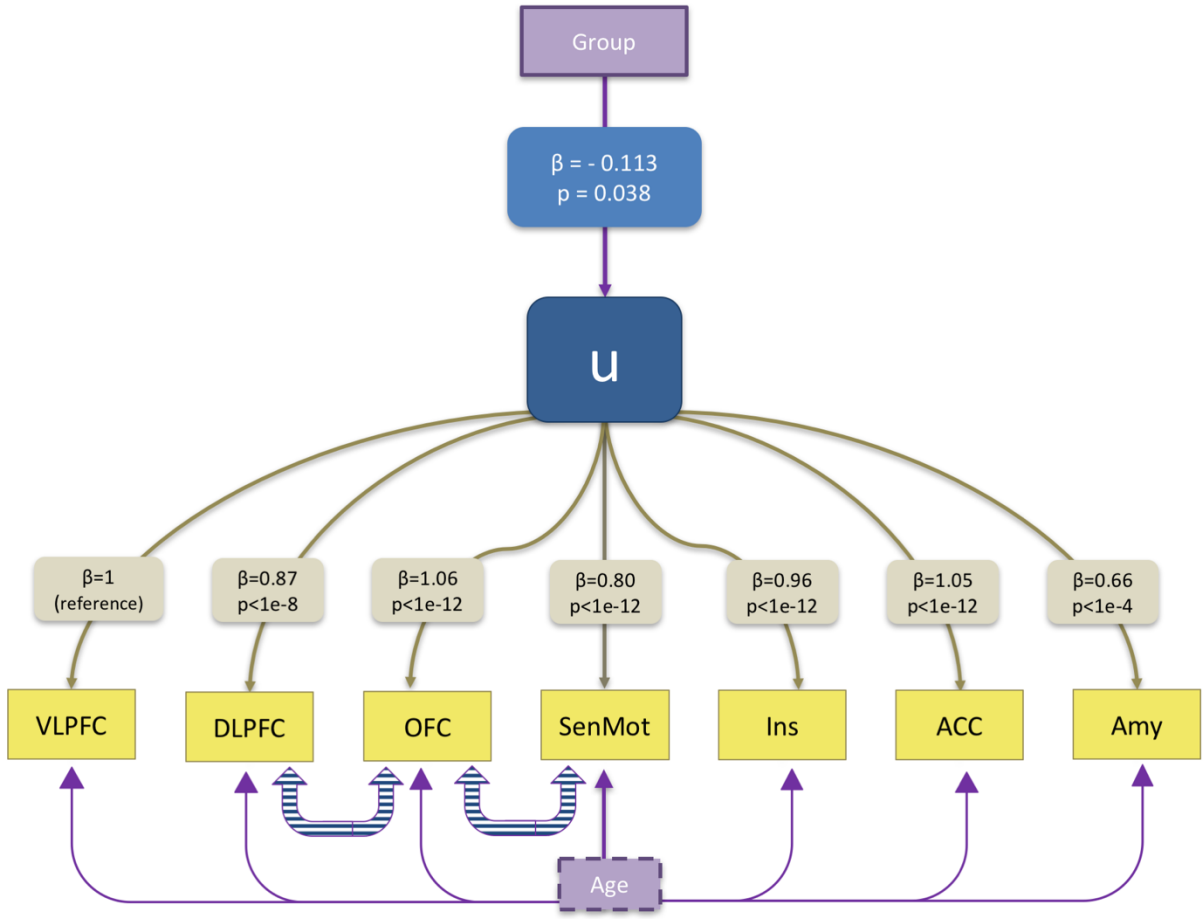

Figure 6. Group status significantly predicts a latent variable (p = 0.038), which models shared correlations in 5-HT_{1B} receptor binding across regions involved in pain modulation. Purple boxes represent observed predictors. Blue box (u) represents the latent variable. Yellow boxes represent regional BP_{ND}'s. Striped arrows indicate additional shared correlations. The parameter estimate, β, is noted for each model path as well as significance, p, of parameter estimates

In a post hoc analysis of each region included in the LVM we found that the difference in 5-HT_{1B} receptor binding was most pronounced within the anterior cingulate cortex (Mean BP_{ND}±SD in patients vs. controls: 1.64±0.15 vs. 1.78±0.20, p = 0.041) and within the sensorimotor cortex (1.24±0.13 vs. 1.34±0.14, p = 0.048).

The voxel-wise analysis showed no group differences in 5-HT_{1B} receptor binding. In the migraine group we found a positive correlation between 5-HT_{1B} receptor binding and days since last migraine attack in a cluster spanning the midline covering bilateral red nucleus, left substantia nigra, and the dorsal raphe (k = 3589, t(11) = 6.41, p < 0.05 corrected, x = 15, y = 22, z = 8) (figure 7).

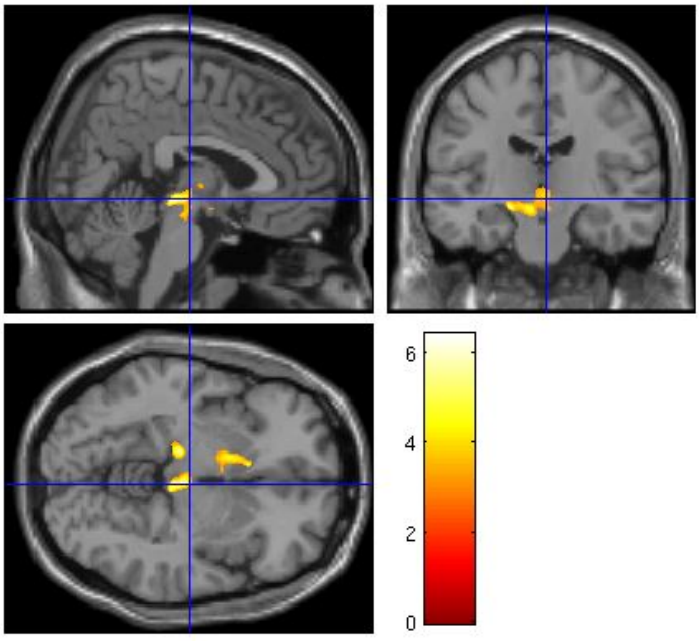

Figure 7. In the whole-brain voxel-based analysis we found a positive correlation between 5-HT_{1B} receptor binding and days since last migraine attack in a cluster encompassing the raphe nuclei. Color bar indicates t-score. Image shown at z = 5.84.

Study 4

We included eight migraine patients in this study. They were all scanned between two migraine attacks, during a cilostazol induced migraine attack and after treatment with sumatriptan.

When comparing the patients' interictal scan to their ictal, we found that BP_{ND} was significantly decreased across pain-modulating regions during the ictal condition (mean BP_{ND}±SD 1.63±0.22 vs. 1.20±0.20, p = 0.019). Further, BP_{ND} decreased even further after treatment with sumatriptan (mean BP_{ND}±SD 1.20±0.20 vs. 1.02±0.22, p = 0.0001) (figure 8). The mean occupancy ± SD of sumatriptan was 16.0±5.3%.

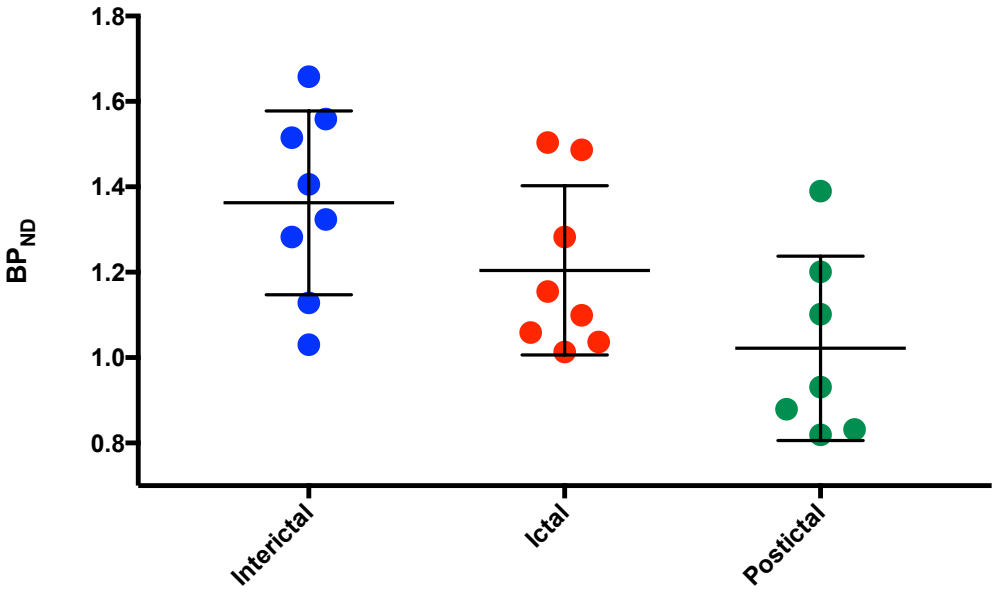

Figure 8. In migraine patients we found a significant decrease in 5-HT_{1B} receptor binding during attacks and after sumatriptan across seven regions involved in pain modulation (dorso- and ventrolateral prefrontal cortex, orbitofrontal cortex, anterior cingulate cortex, sensorimotor cortex, insula and amygdala) BP_{ND} = mean volume weighted non-displaceable binding potential. Black bars indicate mean ± SD.

Discussion

High brain serotonin levels in migraine (study 1 and 2)

Based on previous studies we hypothesized that we would find high 5-HT_4 receptor binding, indicating low brain serotonin levels, in migraine patients in study 1 and 2. In contrast, we found low 5-HT_4 receptor binding, indicating that migraine patients have higher brain serotonin levels compared to controls. This discrepancy could be due to the use of different methods or study population. Previous studies on brain serotonin levels in migraine have mostly used electrophysiological surrogates of brain serotonin levels such as visual or auditory evoked potentials[43]. These are thought to reflect brain serotonin levels, with lower levels of serotonin leading to increased intensity dependency of auditory evoked potentials[44] and a lack of habituation of visual evoked potentials[45]. However, several of these studies were unblinded, rendering the results unreliable[46]. Further, several of the studies did not follow up with patients with regard to their next migraine attack, inducing uncertainties about subjects being truly interictal.

Other PET studies have investigated brain serotonin levels using α-[^{11}C]methyl-L-tryptophan uptake as a marker of brain serotonin synthesis. These studies found both increased[47], decreased[48] and no difference[49] in serotonin synthesis rate compared to controls. It should, however, be noted that the applied method is inadequate and uptake of α-[^{11}C]methyl-L-tryptophan probably reflects tryptophan uptake rather than serotonin synthesis as such[50]. In our study we also used an indirect, but quite robust biomarker of brain serotonin levels and we investigated only migraine without aura patients, leaving our study population more homogenous than previous studies. Further, the person analyzing the data was blinded to diagnosis and all episodic migraine patients were truly interictal. Our findings of high rather than low brain serotonin levels in migraine could also help explain why SSRIs are not effective as migraine prophylaxis[51].

Serotonergic mechanisms in migraine

But how do high brain serotonin levels lead to migraine? One hypothesis states that brain serotonin levels increase during migraine attacks[12]. Further, pharmacological interventions increasing brain serotonin levels induce migraine in migraine patients[52]. Thus, if migraine patients have a constitutional increased level of serotonin in the brain, this might render them more sensitive to further increases, pushing them towards – and across - the threshold for migraine induction.

Serotonin plays an important role in central pain modulation where it is both a substrate of the descending inhibitory pain pathways[53] but also plays a facilitatory role[54,55]. This dual action of serotonin in pain modulation depends on both receptor subtype and pathophysiological state[56]. The 5-HT_{1B} and the 5-HT_7 receptors are generally considered antinociceptive, even though data for the 5-HT_7 receptor are diverging[57]. In contrast, the 5-HT_{2A} and the 5-HT_3 receptors are considered pronociceptive[53], but antinociceptive properties of the 5-HT_3 receptor have also been demonstrated[57,58]. We also know that pizotifen and methysergide, both 5-HT_2 receptor antagonists, are efficient as migraine prophylaxis[59], in contrast to 5-HT_2 receptor agonists, which induce migraine[60]. Thus, the role of 5-HT in migraine may depend on the composition of the different receptor subtypes, and the pathophysiology of migraine may include an imbalance between pain inhibition and pain facilitation due to changes in 5-HT receptor expression and increased brain 5-HT levels.

Serotonin is not involved in the conversion from episodic to chronic migraine (Study 2)

In study 2 we found that chronic migraine patients had higher brain serotonin levels compared to healthy controls, but also that there was a lack of difference between episodic and chronic migraine patients. Currently there is no available biomarker with which to objectively distinguish the two disease states. Previous studies have shown that chronic migraine patients have increased cortical excitability[61] and exhibit abnormal pain processing[62] compared to episodic migraine patients. Interestingly, serotonin is involved in both cortical excitability and pain modulation, and we therefore hypothesized that brain serotonin levels would be different between the two groups – and could possibly thereby serve as a biomarker. However, our results – showing similar brain serotonin levels in the two patient groups – indicate that this is not the case. Rather, high brain serotonin levels may be a biomarker of the migraine brain in general.

Clinically, episodic and chronic migraine are distinguished by an arbitrary limit of 15 headache days[5], but the neurobiological mechanisms underlying the conversion from episodic to chronic migraine are only poorly understood. Further, the discussion on whether episodic and chronic migraine are two ends of the same spectrum or are two distinct disease entities is ongoing[6,63]. We hypothesized that brain serotonin levels could be an underlying substrate for progression in frequency of migraine attacks. However, we found no association between 5-HT_4 receptor binding and frequency of migraine attacks across the 31 migraine patients suggesting that brain serotonin levels are not related to attack frequency. These results also indicate that brain serotonin is not a modifiable risk factor for evolution from episodic to chronic migraine. Therefore, other risk factors such as transformation of the brain structure, obesity, depression and medication overuse must be considered[9,64]. In the present study, none of the participants suffered from depression or medication overuse headache, and we found no difference in neither neocortical grey

matter volume nor BMI between groups. High baseline migraine frequency, which is also a risk factor for chronic migraine[6], might have played a role in our study group.

Low density of the 5-HT_{1B} receptor in migraine patients (Study 3)

In this study, we found that across regions involved in pain modulation, migraine patients without aura have lower binding of the 5-HT_{1B} receptor compared to controls. One interpretation of this finding is that the 5-HT_{1B} is downregulated due to ictal increases in brain serotonin levels. In animals, chronic SSRI exposure leads to downregulation of 5-HT_{1B} mRNA in raphe[65], suggestively due to higher synaptic concentrations of serotonin. However, in our study we found no association with days since last attacks in these regions or frequency of attacks, which would have been expected if the receptor was downregulated due to ictal increases in serotonin. The low 5-HT_{1B} receptor binding could also be caused by a degeneration of serotonergic neurons in the migraine brain. Previous studies have reported decreased grey matter volume of both insula, the cingulate cortex and the orbitofrontal cortex in migraine patients compared to controls[66,67]. However, we found no differences in grey matter volume between our two groups of subjects. A lower BP_{ND} in one group of subjects may also reflect changes in synaptic levels of the endogenous ligand. Since we did find higher brain serotonin levels in migraine patients in study 1 and 2, it might be that the lower binding of the 5-HT_{1B} receptor found in this study actually is a reflection of higher synaptic serotonin levels.

Another possible interpretation is that the lower BP_{ND} reflects a genetically or developmentally determined low 5-HT_{1B} receptor density. A low density of the 5-HT_{1B} receptor could thus be a trait of the migraine brain rendering it more susceptible to pain facilitation. The 5-HT_{1B}

receptor acts as both an autoreceptor, involved in regulation of serotonin release, and a hetero-receptor[68] involved in release of other neurotransmitters. We cannot with our method distinguish between the two receptor subtypes since [^{11}C]AZ10419369 binds to both, and we can only speculate whether one or both are decreased in migraine patients. A low density of the autoreceptor could lead to a disinhibition of serotonin release from serotonergic projections from raphe and a dysfunction of the regulation of synaptic serotonin in migraine patients. On the other hand, a decreased density of the heteroreceptor could lead to changes in other neurotransmitter systems. In the rat anterior cingulate cortex, activation of the 5-HT$_{1B}$ heteroreceptor inhibits glutamate release[69]. Thus, a decrease in the heteroreceptor in migraine patients could cause increased glutamate release possibly leading to increased pain facilitation.

Involvement of the brainstem in migraine pathophysiology

In study 3 we also found that 5-HT$_{1B}$ receptor binding was positively correlated with days since last attack in a region of the brainstem including the dorsal raphe nucleus. The raphe nuclei constitute the main hub of serotonin in the brain and the dorsal raphe is involved in central modulation of nociception[70]. Interestingly, several studies have implicated the brainstem – including the dorsal raphe – in migraine pathophysiology by showing activation of this region during migraine attacks[71–73]. We therefore suggest that activation of this region during migraine attacks may lead to a downregulation of the 5-HT$_{1B}$ receptor in this region, possibly due to release of serotonin, which gradually recovers with time from the last migraine attack. In contrast, another PET study found that 5-HT$_{1A}$ receptor availability was increased in the brainstem during migraine attacks. However, this finding was confined to the pontine raphe nuclei whereas our finding was located in the midbrain.

Increased brain serotonin levels during migraine attacks (Study 4)

In study 4 we found that [^{11}C]AZ10419369 binding to the 5-HT$_{1B}$ receptor decreased during migraine attacks as compared to outside of migraine attacks, and that administration of sumatriptan caused a further decrease in binding. We interpret the first finding as being caused by increases in endogenous brain serotonin levels during migraine attacks. Previous studies investigating brain serotonin levels during migraine attacks found similar changes. One study used α-[^{11}C]methyl-$_L$-tryptophan uptake to investigate changes in endogenous serotonin levels and found that uptake was increased during migraine attacks[48]. This finding was interpreted as reflecting an increase in serotonin synthesis during migraine attacks. An electrophysiological study found that visual and auditory evoked potentials, which were altered in interictal migraine patients compared to controls, normalized during migraine attacks[74]. Both visual and auditory evoked potentials are thought to reflect serotonergic neurotransmission[75,76], and the normalization was therefore interpreted as an increase in serotonin levels during migraine attacks.

Whether increases in synaptic serotonin is a cause or a consequence of the migraine attack remains elusive. We know from pharmacological studies, that agents, such as fenfluramine, causing increases in brain serotonin levels can induce migraine attacks[52]. Further, migraine patients were more likely to develop a migraine attack after administration of the 5-HT$_2$ receptor agonist, m-Chlorophenylpiperazine (mCPP) compared to controls[60]. These findings indicate that serotonin is able to induce migraine attacks when the concentration increases. One possible explanation for this mechanism is that when present in low concentrations, serotonin binds to the antinociceptive 5-HT1$_{B/D}$ receptor, for which it has higher affinity, but when concentrations increase it

binds to pronociceptive 5-HT_{2A} receptors, shifting the balance from pain inhibition to pain facilitation[77].

Occupancy of sumatriptan on central 5-HT_{1B} receptors

We found that in ictal migraine patients, administration of sumatriptan in clinically relevant doses decreased neocortical BP_{ND} of [^{11}C]AZ10419369. Occupancy rates were modest with a mean of 16.0%. These are in congruence with findings of a mean occupancy of 4.5% of zolmitriptan on central 5-HT_{1B} receptors in healthy volunteers[78]. Of note, low occupancies do not exclude a clinical significant effect – especially not for agonists. Opioids exert effects with occupancies < 10%[79] and 5-HT_{1A} receptor agonists induce adverse events compatible with a CNS effect at non-significant occupancies[80]. Efficacy of a drug is dependent not only on occupancies but also on intrinsic activity. A high intrinsic activity has been demonstrated for sumatriptan[81] and it may be speculated that the low occupancies are sufficient for the therapeutic effect of sumatriptan.

The design of our study does not allow for conclusions on, whether activation of central 5-HT_{1B} receptors is a requirement for the antimigraine efficacy of sumatriptan. A previous PET study, found that sumatriptan, given during a migraine attack, decreased α-[^{11}C]methyl-$_L$-tryptophan brain uptake, possibly reflecting serotonin synthesis, in several brain regions involved in pain modulation. This effect was, however, not related to treatment response[48]. Likewise, we found no evidence for an association between treatment response and occupancy of sumatriptan, e.g. two subjects still experienced migraine during the second scan but had occupancies of 18.6% and 20.8%. Collectively, these findings suggest that even though sumatriptan access the brain parenchyma and binds to central 5-HT_{1B} receptors, the antinociceptive effect may not, or at least only partially, be dependent on stimulation of central 5-HT_{1B} receptors.

Due to the design of our study, we cannot exclude that residual binding of endogenous serotonin in the migraine patients contribute to the estimated occupancies of sumatriptan. Serotonin levels may keep increasing in the brain even after pain relief and termination of the migraine attack. In support of this, systemic administration of sumatriptan had no effect on serotonin release in the rat brain[82]. In contrast, when applied via reverse microdialysis, sumatriptan decreased serotonin release in the mouse brain via binding to 5-HT_{1B} autoreceptors[83]. Thus, if sumatriptan crosses the BBB and binds to central 5-HT_{1B} autoreceptors, as indicated by our findings, serotonin levels may more likely decrease instead of continuing to increase. Further, we found a larger ictal decrease in binding in the pain-modulating regions compared to neocortex, whereas occupancies did not differ between these two regions. This speaks against a significant contribution of binding of endogenous serotonin to the occupancy.

It might be speculated that the finding of central occupancy of sumatriptan to central 5-HT_{1B} receptors during migraine attacks is due to an attack-related disruption of the BBB. For decades it has been hypothesized that the BBB is leaky during migraine attacks[84,85], but recently several studies have contradicted this hypothesis[86–88]. It is therefore unlikely that a BBB disruption is necessary for sumatriptan to access the brain parenchyma. BBB permeability of a drug is determined by both lipophilicity and molecular weight as well as by interactions with efflux pumps such as the P-glycoprotein (Pgp)[89]. Polymorphisms of the ABCB1 gene, which encodes the Pgp, are related to different treatment response to SSRI treatment in depression[90] and to topiramate in migraine prophylaxis[91]. However, in contrast to eletriptan[92], sumatriptan is not a substrate of the Pgp[93] and It is therefore unlikely that such polymorphisms play a role for BBB penetration of sumatriptan. Instead it may be speculated that interindividual differences in sensitivity to sumatriptan is

caused by central sensitization[94] or differences in number of 5-HT_{1B} receptor in the high-affinity state (the active state of G-protein coupled receptors[95]).

Methodological considerations

A challenge with PET imaging of neuroreceptors is that the outcome, BP_{ND}, not only reflects receptor binding, but is a combined measure of number of available (B_{avail}) receptors, affinity of the tracer to the receptor ($1/K_d$) and f_{ND}, free fraction of the tracer in non-displaceable compartment. In general, it is assumed that affinity and f_{ND} is the same across subjects, and that changes in BP_{ND} reflects changes in B_{avail}. However, this may not always hold true. In this study, we have no reason to believe that migraine patients should differ from controls with regard to either affinity or f_{ND}, but we cannot exclude that this is the case.

The project is also limited by the fact that we cannot do microdialysis in the living human brain and we, therefore, have to use indirect markers of brain serotonin levels. The inverse relationship between 5-HT_4 receptor binding and stable brain serotonin levels have so far been underlined in several studies[26,34,35]. However, since we only examine the subjects at one time point we cannot conclude on the temporal course of the changes in brain serotonin levels. Further, an alternative interpretation of our results is that migraine patients have low 5-HT_4 receptor density, which is unrelated to brain 5-HT levels. Instead it could be a specific characteristic of the migraine brain either genetically determined, congenital or acquired. So far, no studies have investigated whether the 5-HT_4 receptor is specifically related to migraine pathophysiology but taking the known physiological functions of the receptor (involvement in memory and learning, feeding and mood behavior[96]) into account a prominent role does not seem plausible, i.e. the 5-HT_4 receptor is not known to be involved in pain modulation.

As for acute changes in brain serotonin levels, we also use an indirect marker, changes in [^{11}C]AZ10419369 binding. The sensitivity of this radiotracer to acute changes in synaptic serotonin levels has been documented in several both animal and human studies[21–24]. Besides occupancy of endogenous serotonin to the 5-HT_{1B} receptor, the decrease in BP_{ND} of the radiotracer could be caused by rapid internalization of the 5-HT_{1B} receptor after exposure to serotonin as has been shown *in vitro*[97]. It is currently unknown whether [^{11}C]AZ10419369 has reduced affinity for internalized receptors.

Lastly, we investigated experimentally induced migraine attacks instead of spontaneous migraine attacks. This was primarily due to logistic reasons since PET scans have to be planned days in advance due to synthesis of the radiotracer and availability of the scanner. Human migraine provocation models have been used for decades to investigate the pathophysiology of migraine and they provide a reliable alternative to spontaneous migraine attacks[28]. In our study all subjects fulfilled the criteria for migraine attacks during the scans and all subjects reported that the induced migraine attack mimicked their normal attack. We therefore believe that our findings can be extrapolated to spontaneous migraine attacks.

Concluding remarks and future perspectives

This thesis provides new insight into the role of the serotonergic system in migraine pathophysiology. In contrast to previous findings of low brain serotonin levels, the present results suggest that migraine patients have high brain serotonin levels. Thus, migraine might be a syndrome of high rather than low brain serotonin levels, which leads to an increased susceptibility to migraine triggers and induction of migraine attacks due to dysfunctional pain modulation. Further, the fact that

brain serotonin levels are not related to attack frequency suggests that a high level of brain serotonin might serve as a biomarker of the migraine brain in general; an inherent trait rather than a risk factor associated with attack frequency or conversion from episodic to chronic migraine. However, the cross-sectional study design makes it difficult to draw firm conclusions and we cannot disentangle whether high brain serotonin levels predispose to or are a result of recurrent migraine attacks. For this purpose, longitudinal studies are warranted where subjects are scanned when they are diagnosed and then after having experienced multiple attacks or when they convert from episodic to chronic migraine. It would also be interesting to see whether different migraine prophylactics affect brain serotonin levels, since the mode of action of most of the preventative drugs are currently unknown.

The finding of lower density of the 5-HT_{1B} receptor across pain-modulating regions in migraine patients might partly explain why triptans are effective in migraine but not in other pain conditions. In this study we included only triptan responsive subjects. In the future, it would be relevant to investigate non-triptan responders or patients suffering from other pain conditions in order to elucidate whether the finding is pain related or specific for head pain or migraine patients. Lastly, we found that brain serotonin levels increase during migraine attacks. From this study we can only speculate whether increases in brain serotonin levels leads to migraine – or whether increases in brain serotonin levels are a consequence of the migraine attack. Studies investigating subjects in the premonitory phase and in the postdromal phase of the migraine attack would be beneficial in order to characterize and determine the course of changes. We can also only speculate upon the mechanisms behind the possible migraine inducing effects of increases in brain serotonin levels. These could possibly be investigated further in e.g. animal studies investigating the potential of serotonin to activate the trigeminal nociceptive pathway. To investigate a

possible difference in susceptibility to increases in brain serotonin levels between healthy controls and migraine patients, the headache inducing effects of SSRIs in these two study populations would be relevant to study. We also found that sumatriptan binds to central 5-HT_{1B} receptors but that the occupancy was not related to the clinical efficacy of the drug. Thus, even though we here provide evidence that sumatriptan accesses the brain parenchyma, it remains to be answered whether the therapeutic effect of sumatriptan is dependent on activation of central 5-HT_{1B} receptors. One possible way to assess this could be to investigate the sumatriptan response in animal migraine models using knockout mice not expressing the central 5-HT_{1B} receptor.

In conclusion, the present thesis contributes to our current understanding of the serotonergic system in the migraine brain. However, it also gives rise to new questions and further studies are warranted to shed light on the mechanisms by which changes in serotonin levels contribute to migraine pathophysiology. In the future, unraveling the relationship between migraine and serotonin might lead to better treatment for migraine sufferers.

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Appendix

Paper 1-4

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High brain serotonin levels in migraine between attacks: A 5-HT$_4$ receptor binding PET study

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ABSTRACT

Migraine has been hypothesized to be a syndrome of chronic low serotonin (5-HT) levels, but investigations of brain 5-HT levels have given equivocal results. Here, we used positron emission tomography (PET) imaging of the 5-HT$_4$ receptor as a proxy for brain 5-HT levels. Given that the 5-HT$_4$ receptor is inversely related to brain 5-HT levels, we hypothesized that between attacks migraine patients would have higher 5-HT$_4$ receptor binding compared to controls. Eighteen migraine patients without aura (migraine free > 48 h), and 16 age- and sex-matched controls underwent PET scans after injection of [^{11}C]SB207145, a specific 5-HT$_4$ receptor radioligand. An investigator blinded to group calculated a neocortical mean [^{11}C]SB207145 binding potential (BP$_{ND}$). Three migraine patients reported a migraine attack within 48 h after the scan and were excluded from the primary analysis. Comparing 15 migraine patients and 16 controls, we found that migraine patients have significantly lower neocortical 5-HT$_4$ receptor binding than controls (0.60 ± 0.09 vs. 0.67 ± 0.05, p = .024), corrected for 5-HTTLPR genotype, sex and age. We found no association between 5-HT$_4$ receptor binding and attack frequency, years with migraine or time since last migraine attack. Our finding of lower 5-HT$_4$ receptor binding in migraine patients is suggestive of higher brain 5-HT levels. This is in contrast with the current belief that migraine is associated with low brain 5-HT levels. High brain 5-HT levels may represent a trait of the migraine brain or it could be a consequence of migraine attacks.

1. Introduction

Migraine is a highly debilitating and socioeconomically costly neurological disorder, affecting 16% of the population worldwide (Olesen et al., 2012; Steiner et al., 2013). Despite intensive research during the past several decades, the neurobiological basis and pathophysiology of migraine remains largely unknown. Serotonin (5-hydroxytryptamine, 5-HT) has been directly implicated in the pathophysiology of migraine (Hamel, 2007) and studies on plasma and urinary levels of 5-HT and its main metabolite, 5-hydoxyindoleacetic acid (5-HIAA) suggest that between their migraine attacks, patients have decreased levels of plasma 5-HT (Ferrari et al., 1989; Sicuteri et al., 1961). Accordingly, although plasma levels of 5-HT do not necessarily reflect brain 5-HT levels, migraine has been considered a syndrome of chronically low brain 5-HT levels. Several studies have attempted to assess brain 5-HT levels in migraine patients, but results have been equivocal, showing both higher and lower levels compared to controls (Deen et al., 2017a). We here use a novel neuroimaging method to investigate if migraine is a syndrome associated with low 5-HT brain levels.

The 5-HT$_4$ receptor, one of 14 5-HT receptors, is inversely related to central serotonergic tonus and can thus be used as an indirect biomarker of brain 5-HT levels. In rats, brain 5-HT$_4$ receptor binding decreased after 14 days of selective 5-HT reuptake inhibitor (SSRI)

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administration (Licht et al., 2009). In humans, carriers of the short allele of the 5-HT transporter (5-HTT) gene, which is associated with relatively increased synaptic 5-HT levels, had lower neocortical 5-HT_4 receptor binding compared to carriers of the long allele (Fisher et al., 2012). Furthermore, the final support for the 5-HT_4 receptor being inversely related to brain 5-HT levels, came from a study showing that three weeks of SSRI intervention led to a significant decrease in brain 5-HT_4 receptor binding in humans (Haahr et al., 2014).

Here we investigated differences in brain 5-HT levels between migraine patients without aura and controls using PET imaging of the 5-HT_4 receptor as an in vivo biomarker of brain 5-HT levels. According to existing beliefs, we hypothesized that migraine patients had higher 5-HT_4 receptor binding compared to controls.

2. Materials and methods

2.1. Subjects

Participants were recruited through a Danish website for recruitment of volunteers to health research and from a local database. All patients fulfilled the following inclusion criteria: 1) 18–65 years old, 2) a verified diagnosis of migraine without aura according to the International Headache Society Criteria (HCC IHS, 2013). 3) at least one migraine attack every other month but less than five migraine days per month, 4) self-reported previous effect of treatment of migraine attacks with sumatriptan (a 5-$HT_{1B/1D}$ receptor agonist drug). The last criterion was applied, since the subjects were also included in a study investigating the 5-HT_{1B} receptor (Deen et al., 2017b). A standardized interview of all patients was conducted at screening including the following items: duration of disease (years), duration of attack when untreated (hours), migraine days pr. month, frequency of attack (number per month), maximum pain intensity of untreated headache as measured with the Numerical Rating Scale (NRS) (number 0–10), intake of acute pain medication including triptans (days per month), and date of their last migraine attack. Inclusion criteria for age- and sex-matched controls included: 1) no history of migraine including probable migraine and no first-degree relatives with migraine. For all participants, the following exclusion criteria were applied: 1) a history of any other primary headache (except tension-type headache < 5 days per month), 2) psychiatric, cerebro- or cardiovascular disease, 3) contraindications for magnetic resonance imaging (MRI), 4) pregnancy or nursing, 5) daily intake of medication including migraine prophylaxis.

All subjects reported to be headache free on the day of their PET scan, and no medication intake was allowed for the last 24 h prior to the scan. All migraine patients were migraine free for at least 48 h prior to the PET scan. In addition, to ensure that all included subjects were truly between two migraine attacks, headache diaries were obtained from all patients for 48 h after the scan. All included participants had a normal physical and neurological examination and unremarkable brain MRI. All participants filled out the major depression inventory (MDI) on the day of the PET scan.

The study was approved by the Ethics Committee of The Region of Copenhagen (H-6-2014-057). In accordance with the Declaration of Helsinki of 1964, with later revisions, all participants gave written consent after detailed oral and written information about the study.

2.2. PET and MR imaging

Synthesis of the radioligand, [^{11}C]SB207145, was performed using an automated radiosynthesis system as previously described (Marner et al., 2009). An intravenous bolus injection of the radioligand was given over 20 s, followed by 120-minute dynamic data acquisition with a high-resolution research tomography (HRRT) PET scanner (CTI/Siemens, Knoxville, TN, USA). To minimize head movement, all subjects had their head stabilized in a specialized head holder. The scans were reconstructed into 38 frames (6 × 5, 10 × 15, 4 × 30, 5 × 120, 5 × 300, and 8 × 600 s) using a 3D-OSEM-PSF algorithm (16 subsets, 10 iterations) with TXTV based attenuation correction (image matrix, 256 × 256 × 207; voxel size, 1.22 × 1.22 × 1.22 mm), as previously described (Hong et al., 2007; Keller et al., 2013; Sureau et al., 2008). T1 and T2 weighted MRI scans used for co-registration were acquired for each subject using a Siemens Prisma 3T scanner (Siemens, Erlangen, Germany) with a 64-channel head coil.

2.3. Quantification of 5-HT_4 receptor binding

Single-subject PET images were corrected for intra-scan movement by aligning the frames 10–38 to a reference frame (frame 26) using a scaled least-squares cost function in AIR 5.2.5. Co-registration and alignment of PET images to the corresponding T1-weighted MRI image was done using SPM8. Regions of interest (ROIs) were automatically delineated on each subject's MRI using PVElab software (www.nru.dk) as previously described (Svarer et al., 2005). Accurate co-registration and ROI placement were confirmed by visual inspection for each subject, across all planes. Time activity curves (TAC) and grey matter volumes for each ROI were then extracted.

The Simplified Reference Tissue Model (SRTM) was applied to calculate the non-displaceable binding potential (BP_{ND}) of [^{11}C]SB207145. This model has previously been validated for quantification of [^{11}C]SB207145 in the human brain (Marner et al., 2009). Cerebellum (excluding vermis) was used as a reference region since it has a negligible density of 5-HT_4 receptors (Ganz et al., 2017; Marner et al., 2009). Kinetic modeling was performed in MATLAB R2013a (8.1.0.604) 64 bit (Mathworks Inc., MA) using an in-house script and the person performing the kinetic modeling was blinded to group status (migraine patient or control). Parametric 5-HT_4 receptor binding images for voxel based analysis were generated using PETSurfer (http://surfer.nmr.mgh.harvard.edu, version 6.0), as previously described (Greve et al., 2014). In summary, a combined volumetric and surface registration algorithm was used to normalize each single-subject structural T1 to Montreal Neurological Institute (MNI) space (Postelnicu et al., 2009). After application to the co-registered PET-images, these were then volume-smoothed with a 6-mm full-width half-maximum 3D Gaussian kernel. The Multilinear Reference Tissue Model 2 (MRTM2), using cerebellum as reference region and high-binding regions (putamen, pallidum and caudate) for estimation of k_2', was applied to estimate voxel-level BP_{ND}s.

2.4. Genotyping

Participants were genotyped for the tri-allelic 5-HT transporter-linked polymorphic region (5-HTTLPR) polymorphism. Briefly, genotyping was performed by PCR amplification from forward primer 5′-TAATGTCCCTACTGCAGCCC-3′ and reverse primer 5′-GGGACTGAG CTGGACAACC-3′. The fragments were then digested by the restriction enzyme MspI and separated by gel electrophoresis. Participants were categorized into two groups: 1. Carriers of the short allele (S-carriers) or the long L_G allele (L_G-carriers). 2. Homozygotes of the long L_A allele (L_A-homozygotes). This dichotomization was based on a previous study showing that carriers of the low-expressing alleles, S and L_G have lower neocortical 5-HT_4 receptor binding compared to homozygotes of the high-expressing allele, L_A (Fisher et al., 2012).

2.5. Experimental design and statistical analysis

This study was an observational, cross-sectional study comparing interictal migraine without aura patients (> 48 hour migraine free before and after the scan) with sex- and age matched controls. Sample size was based on a previous study showing that n = 15 is sufficient to detect a 15% difference between groups with a power of 0.80 in very large brain regions (> 50 cm^3), such as e.g. neocortex (Marner et al., 2010). Differences between groups in demographics, genotypes and

PET variables were evaluated using two-sample *t*-tests.

To evaluate differences in brain 5-HT levels between migraine patients and controls we used the BP_{ND} of a large neocortical region as a proxy of central serotonergic tonus. The neocortical BP_{ND} was chosen, since previous studies investigating brain 5-HT levels in migraine mostly focused on cortical regions (for review see (Deen et al., 2017a)). The cortical brain regions receive numerous serotonergic projections from the raphe nuclei and 5-HT plays an important role in the modulation of cortical activity (Celada et al., 2013). Additionally, the relationship between the 5-HTTLPR genotype and 5-HT_4 receptor binding is most pronounced in neocortex (Fisher et al., 2012).

The mean neocortical [^{11}C]SB207145 BP_{ND} was calculated based on 11 neocortical brain regions (occipital cortex, orbitofrontal cortex, superior, medial and inferior frontal gyri, insula, superior, medial and inferior temporal gyri, sensory motor cortex and parietal cortex) by volume weighting grey matter segmented brain region BP_{ND}'s:

$$\text{Neocortical } 5-HT_4 \text{ receptor binding} = \Sigma[5-HT_4\ BP_{ND}(\text{region}_x) * \text{volume}(\text{region}_x)]/[\Sigma(\text{volume}(\text{region}_x))]$$

As our primary investigation, a general linear model including neocortical BP_{ND} as the primary dependent variable and group status (patients vs. controls) as the predictive variable was then used to model effects of group on neocortical 5-HT_4 receptor binding. To this model, 5-HTTLPR-status (S or L_G carriers vs. L_A homozygotes), sex and age were added as covariates, since all are known to affect neocortical 5-HT_4 receptor binding (Fisher et al., 2012; Madsen et al., 2015, 2011a). All subjects received tracer doses (injected mass of SB207145 < 0.024 μg/kg) obviating the inclusion of injected mass a covariate (Madsen et al., 2011b). Effects of interaction between group and genotype were evaluated and excluded unless statistically significant. To detect any regional (including subcortical) specific group differences in 5-HT_4 receptor binding, whole brain voxel-wise multiple regression was performed using the same linear model as in the primary analysis. Only voxels (sized 1 × 1 × 1 mm) with an average BP_{ND} > 0.3 were evaluated within a whole brain mask. In addition, associations between measures of clinical severity (attack frequency, years with migraine and time since last migraine attack) and neocortical 5-HT_4 receptor binding were evaluated in the patient group only using a general linear model including 5-HTTLPR-status, age and sex as co-variates.

Statistical tests were carried out using R Studio 3.2.3 and SPM8. We ensured that model assumptions were met by examination of quantile-quantile plots, distribution of the residuals, and predicted values plotted against residuals. In the ROI analyses, the significance threshold was set at $p < .05$ (two-tailed). In the voxel based analysis, a p-value threshold of $p < .001$ at voxel level was used. To correct for multiple comparisons only clusters at $p < .05$ corrected using the family wise error rate (FWE) were assumed significant. All other p-values are reported without correction for multiple comparison.

3. Results

3.1. Demographics and migraine characteristics

Out of the 18 migraine patients who completed the study, three reported to have a migraine attack within 48 h after the PET scan. These were excluded from the primary analysis. Sixteen controls completed the study. One migraine patient (subject 15) was scanned for 90 instead of 120 min, because she felt anxious in the scanner. To ensure that this patient did not affect our results, we conducted the primary analysis both with and without this patient. Thus, data from 15 migraine patients and 16 controls were included in the primary analysis.

A summary of demographics and PET variables are presented in Table 1. Clinical data of the migraine group is shown in Table 2. The regional distribution of the tracer was in concordance with previous studies with lowest binding in neocortex and highest binding in striatum. We found no differences in grey matter volume or MDI score between the two groups and no interaction between group and genotype.

Table 1
Demographics and PET variables.

	Patients	Controls	p-Value[a]
Number of subjects (men/women)	15 (2/13)	16 (3/13)	
Genotype (L_A homozygote/S or L_G carrier)	6/9	6/10	
Age (years)	29.6 ± 10.2	28.9 ± 10.2	.85
BMI (kg/m^2)	22.6 ± 1.7	23.9 ± 4.8	.33
Major depression inventory	7.87 ± 7.6	7.13 ± 4.8	.75
Injected radioactivity (MBq)	584 ± 16	591 ± 19	.34
Specific radioactivity (GBq/μmol)	567 ± 282	486 ± 217	.38
[^{11}C]SB injected mass per kg (μg/kg)	0.008 ± 0.006	0.008 ± 0.005	.90
[^{11}C]SB cerebellum AUC/specific radioactivity (GBq/μmol)	31.1 ± 23	32.6 ± 21	.85

Continuous variables are presented as mean ± SD.
[a] Two-sample *t*-test.

Table 2
Migraine history.

Subject	Migraine history (years)	Attack frequency (n/month)	Time since last migraine attack (days)
1	8	2	17
2	7	2	12
3	21	3	11
4	25	1	22
5	15	2	15
6	20	1	31
7	19	1	19
8	8	0.5	50
9	17	2	15
10	7	1	29
11	6	3	10
12	2	2	7
13	36	4	4
14	16	3	5
15	10	2	13
Median (range)	15 (2–36)	2 (0.5–4)	15 (4–50)

3.2. Differences in neocortical binding

We found that migraine patients had significant lower neocortical [^{11}C]SB207145 binding compared to controls (0.60 ± 0.09 (mean ± SD) vs. 0.67 ± 0.05 (mean ± SD), p = .024) (Fig. 1) after adjustment for covariates. This difference remained after excluding subject 15 (0.63 ± 0.06 vs. 0.68 ± 0.05, p = .038). Post hoc explorative analysis of the ROIs included in the neocortical region showed that the low binding was most pronounced within the orbitofrontal cortex (p = .009), insula (p = .018), superior temporal gyrus (p = .019), parietal cortex (p = .026), medial and inferior temporal gyrus (p = .032), and superior frontal gyrus (p = .040). The voxel-based analysis revealed no significant clusters after rigorous FWE correction.

3.3. Associations with migraine characteristics

We found no associations between neocortical 5-HT_4 receptor binding and attack frequency (slope estimate −0.017, CI: [−0.081;0.047], p = .56), years with migraine (slope estimate 0.003, CI: [−0.007;0.012], p = .53), or days since last attack (slope estimate 0.002, CI: [−0.003;0.007], p = .40).

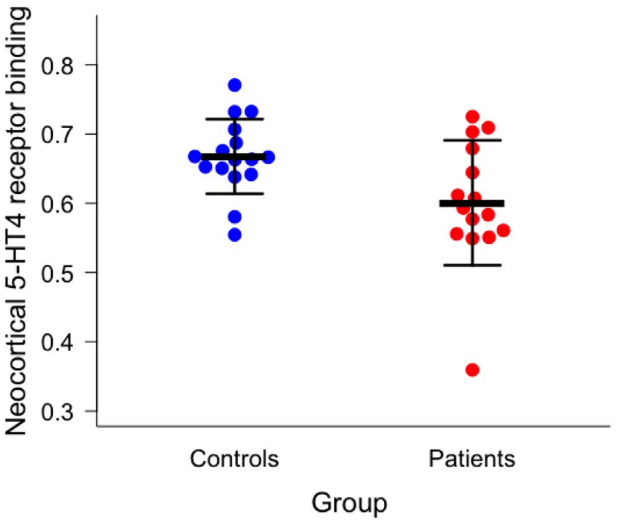

Fig. 1. Migraine patients have lower neocortical 5-HT_4 receptor binding than controls (0.60 ± 0.09 vs. 0.67 ± 0.05, p = .024) after adjusting for covariates (5-HTTLPR status, age, and sex). Black bars indicate mean ± SD. The difference remained after exclusion of subject 15 (the patient with the lowest BP_{ND}) (0.63 ± 0.06 vs. 0.68 ± 0.05, p = .038).

4. Discussion

The key finding of this study is that between attacks, migraine patients have lower 5-HT_4 receptor binding within neocortex compared to controls. Our post hoc ROI analysis showed that this difference was most pronounced within the orbitofrontal, parietal, temporal and insular cortices, but no significant clusters were found when using a whole brain voxel-based analysis. This discrepancy is probably due to the conservative statistical method applied to the voxel-based analysis (p-value threshold of $p < .001$ at voxel level, and correction for multiple comparisons using FWE) and due to the large whole-brain search volume. The latter was applied in order to detect any possible subcortical differences. Further, the detected difference between migraine patients and healthy controls was rather small, around 10%. This is, however, in line with previous studies finding significant differences in 5-HT_4 receptor binding between groups differing with regards to intervention, genes or disease. Thus, a 5.2% decrease was found after 3 weeks of fluoxetine intervention (Haahr et al., 2014), BDNF met-carriers were shown to have 7% higher neocortical binding relative to val/val-carriers, whereas 5-HTTLPR S-carriers had 7% lower binding compared to LL homozygotes (Fisher et al., 2015, 2012), and lastly, in Alzheimer's patients, PIB-positive patients had 13% higher binding compared to PIB-negative patients (Madsen et al., 2011c).

Although an acute increase in brain 5-HT levels does not affect [^{11}C]SB207145 BP_{ND} (Marner et al., 2010), we took great care to ensure that all included patients were scanned during an attack-free interval (migraine free 48 h before and after scan) to exclude possible effects of attack related changes in brain 5-HT levels on 5-HT_4 receptor binding. However, inclusion of the three subjects who reported to have a migraine attack within 48 after the scan did not change our results (p = .024). Furthermore, to reduce the possibility of variability in [^{11}C]SB207145 binding due to diurnal 5-HT variations, we scanned all patients at the same time of the day (± 1 h). Since we found no difference in grey matter volume between patients and controls within neocortex this is likewise not thought to affect the magnitude of 5-HT_4 receptor binding. Lastly, the low 5-HT_4 receptor binding could reflect changes in affinity of the 5-HT_4 receptor in migraine patients. However, in colliculi neurons desensitization of the 5-HT_4 receptor is caused by a loss of binding sites after continued 5-HT exposure (Ansanay et al., 1996). Thus, we interpret our findings of low [^{11}C]SB207145 binding to reflect low neocortical density of the 5-HT_4 receptor in migraine patients.

The low neocortical 5-HT_4 receptor density in patients could be explained by repeated surges of 5-HT during migraine attacks – causing an on average higher brain 5-HT level with subsequent downregulation of the cerebral 5-HT_4 receptor. In support of this, our post hoc analysis identified lower 5-HT_4 receptor binding in several regions implicated in migraine attacks; e.g. functional neuroimaging studies reported increased activation of insula, the prefrontal cortex and the temporal lobe during attacks compared to the attack free interval (Afridi et al., 2005; Weiller et al., 1995). Moreover, both insula and the orbitofrontal cortex are involved in pain modulation (Tracey, 2008). However, we found no direct association between 5-HT_4 receptor binding and migraine history (years), attack frequency (number per month) or days since the last migraine attack. It would be interesting to investigate whether duration of attack combined with frequency – as a measure of hours with pain pr. month – is associated with 5-HT_4 receptor binding, but this analysis would have required a detailed, prospective headache diary. In addition, we cannot rule out a possible relationship between migraine severity and brain 5-HT levels in high frequency or chronic migraine. Future studies should include this patient group to investigate further whether the low 5-HT_4 receptor density is a consequence of repeating migraine attacks.

Alternatively, the low neocortical 5-HT_4 receptor density may reflect high brain 5-HT levels in migraine patients in the attack free interval. This interpretation challenges the longstanding belief that migraine patients have low brain 5-HT levels between attacks. To date, electrophysiological studies have provided the most substantial evidence for the 5-HT deficiency hypothesis (Coppola et al., 2009). Migraine patients between attacks showed a lack of habituation of visual evoked potentials (VEP) (Afra et al., 2000) and an increased intensity dependence of auditory evoked potentials (AEP) (Wang et al., 1996), both thought to reflect low brain 5-HT levels (Schoenen, 1996; Wutzler et al., 2008). However, most of these studies were unblinded and did not report time to the next migraine attack. In addition, the findings were not reproduced in other studies (Omland et al., 2013; Sand, 2013). In the current study, all included migraine patients had been migraine free for 48 h before and after the scan and all data analyses were conducted by an investigator who was blinded to diagnoses and clinical data.

In addition to electrophysiological studies, some PET studies have suggested that brain 5-HT levels are low in migraine patients, but the results have been inconsistent. In a 5-HT_{1A}-receptor PET neuroimaging study, higher cortical BP_{ND} in migraine patients compared to controls was interpreted as reflecting low brain 5-HT levels (Lothe et al., 2008). However, the 5-HT_{1A} receptor radioligand, [^{18}F]MPPF, is not convincingly sensitive to endogenous 5-HT levels in humans (Paterson et al., 2010). One study reported a higher brain 5-HT synthesis capacity in migraine patients without aura when using α-[11 C]methyl-L-tryptophan as a surrogate marker of brain 5-HT synthesis capacity (Chugani et al., 1999). This was interpreted as being consistent with a high 5-HT turnover and thus, low brain 5-HT levels. Interestingly, in another study using the same method, findings of a low cortical 5-HT synthesis capacity in migraine patients was likewise interpreted as reflecting low cortical 5-HT levels (Sakai et al., 2008). Taking the limitations of these studies (Chugani et al., 1999; Sakai et al., 2008) into account (the reliability of α-[11 C]methyl-L-tryptophan as a surrogate marker of brain 5-HT synthesis capacity has been questioned (Shoaf et al., 2000)), one might speculate whether a low 5-HT synthesis capacity could indeed reflect high 5-HT levels – as found in the current study – due to a negative feedback regulation on 5-HT synthesis. On the other hand, a high synthesis capacity may potentially lead to high 5-HT levels. In further support of our findings, high brain 5-HT levels in migraine patients between attacks may, at least partly, explain, why selective 5-HT reuptake inhibitors are not efficient as migraine prophylaxis (Banzi et al., 2015).

4.1. Possible migraine-inducing mechanisms of serotonin

Serotonergic agents such as m-chlorophenylpiperazine (m-CPP) (Leone et al., 2000), reserpine (Curzon et al., 1969), and fenfluramine (Sicuteri et al., 1976) induce migraine attacks more frequently in migraine patients than in controls, and most likely via increasing brain 5-HT levels (Panconesi and Sicuteri, 1997). Since our data suggest the presence of increased brain 5-HT levels in migraine patients between attacks, we propose that migraine patients could be more susceptible to additional acute increases in 5-HT, which lead to migraine induction.

5-HT has generally been considered an inhibiting agent of pain and is one of the major neurotransmitters of the descending pain-inhibition pathway (Stamford, 1995). In vitro, 5-HT exerts an antinociceptive effect in the trigeminal system (Kilinc et al., 2016) and in healthy animals 5-HT inhibits pain (Viguier et al., 2013). However, recent preclinical studies have shown that 5-HT may be involved in pain facilitation as well (Bardin, 2011). In 5-HT depleted rats, the acute pain reaction was intact but pain behavior in the second phase after formalin injection was attenuated. In addition, in persistent pain models 5-HT depleted rats exhibited a decrease in thermal hyperalgesia and mechanical allodynia (Wei et al., 2010). These pathophysiological-dependent properties of 5-HT may be due to the complex role of the different 5-HT receptor subtypes: The 5-HT_1 and 5-HT_3 receptors are generally considered antinociceptive, whereas the 5-HT_{2A} and the 5-HT_7 receptors are considered pronociceptive (Viguier et al., 2013). Thus, 5-HT may be both pro- and antinociceptive depending on receptor type, affinity and concentration (Sommer, 2006). Interestingly, low density of the antinociceptive 5-HT_{1B} receptor was recently found in pain modulating regions in migraine patients between attacks (Deen et al., 2017b). In light of our present findings we therefore speculate that the pathophysiology of migraine includes an imbalance in the pain modulating system caused by high interictal brain 5-HT levels and changes in expression of different 5-HT receptor subtypes, resulting in loss of inhibition and enhancement of pain facilitation.

4.2. Limitations

Even though several studies have corroborated the inverse relationship between 5-HT_4 receptor binding and brain 5-HT levels (Haahr et al., 2014; Licht et al., 2009), the method used in the present study is still an indirect measure of brain 5-HT levels. In addition, we cannot rule out that the effect of migraine status on the 5-HT_4 receptor is specific for this receptor, and not due to changes in brain 5-HT levels.

5. Conclusions

Migraine patients have low neocortical 5-HT_4 receptor binding. Since pharmacological studies of 5-HT_4 receptor binding suggest an inverse relationship with brain 5-HT levels, this most likely indicates higher brain 5-HT levels in migraine patients compared to controls. Our results, therefore, support the involvement of the serotonergic system in migraine pathophysiology, but are in contrast with the current hypothesis that migraine is a syndrome of low brain 5-HT levels. Future studies must explore whether our observation is due to high brain 5-HT levels between attacks, predisposing to migraine, or is the result of recurring migraine attacks with surges of 5-HT.

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Conflicts of interest

Dr. Knudsen has received honoraria as a board member of Brain Prize and the Elsass Foundation. She is also on the advisory board for the Kristian G. Jebsen Foundation and a field editor for Int J Neuropsychopharm. Messoud Ashina is a consultant and/or scientific adviser/speaker for the ATI, Allergan, Amgen, Alder and Eli Lilly. All other authors declare no competing financial interests.

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Migraine is associated with high brain 5-HT levels as indexed by 5-HT$_4$ receptor binding

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Running Head 5-HT$_4$ receptor binding in chronic migraine

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Abstract

Introduction

Serotonin (5-HT) plays a role in migraine pathophysiology, but whether brain 5-HT is involved in the conversion from episodic to chronic migraine is unknown. Here, we investigated brain 5-HT levels, as indexed by 5-HT_4 receptor binding, in chronic migraine patients and evaluated whether these were associated with migraine frequency.

Methods

Sixteen chronic migraine patients underwent a dynamic PET scan after injection of [^{11}C]SB207145, a specific 5-HT_4 receptor radioligand. Data from 15 episodic migraine patients and 16 controls were included for comparison. Quantification of 5-HT_4 receptor binding was used as a proxy for brain 5-HT levels, since 5-HT_4 receptor binding is inversely related to brain 5-HT levels.

Results

Chronic migraine patients had 9.1% (95% CI: [-17%; -1.0%]) lower 5-HT_4 receptor binding compared to controls (p = 0.039). There was no difference in 5-HT_4 receptor binding between chronic and episodic migraine patients (p=0.48) and no association between number of monthly migraine days and 5-HT_4 receptor binding (slope estimate 0.003, 95% CI: [-0.004; 0.715], p = 0.39).

Conclusion

The finding of low 5-HT_4 receptor binding suggests that cerebral levels of 5-HT is elevated in chronic migraine patients. This is in line with observations made in patients with episodic migraine. Elevated brain 5-HT levels may thus be an inherent trait of the migraine brain rather than a risk factor for conversion from episodic to chronic migraine.

Keywords: Headache, neuroimaging, pain, PET, CNS

Introduction

Chronic migraine (CM) affects 2% of the population worldwide[1]. Chronic migraine patients have higher levels of disability and comorbidity compared to episodic migraine (EM)[2], leading to high levels of absenteeism and reduced productivity[3]. Currently, CM is defined as a separate disease entity and is distinguished from EM by an arbitrary frequency limit of 15 headache days per month[4], while the neurobiological mechanisms underlying conversion from EM to CM are poorly understood[2]. Putative mechanisms include a combination of increased excitability of neurons in central nociceptive pathways (central sensitization) and dysfunctional pain modulation[5]. Serotonin (5-hydroxytryptamine, 5-HT) plays an important role in migraine pathophysiology[6] and, interestingly, 5-HT regulates both cortical excitability[7] and pain transmission[8].

The 5-HT_4 receptor is inversely related to brain 5-HT levels and 5-HT_4 receptor binding assessed with PET neuroimaging can thus be used as a proxy for brain 5-HT levels[9]. Using this novel neuroimaging method, we recently found that EM patients had lower 5-HT_4 receptor binding compared to healthy volunteers[10], indicating higher brain 5-HT levels. Brain 5-HT levels have not previously been investigated specifically in CM patients and it is unknown whether alterations in brain 5-HT levels could reflect migraine severity and potentially serve as a biomarker of CM. In the present study, we hypothesized that CM patients have higher brain 5-HT levels, i.e., lower 5-HT_4 receptor binding, compared to both healthy volunteers and to EM patients. In addition, we hypothesized that migraine frequency would be directly related to 5-HT_4 receptor binding.

Material and methods

Participants

All participants were recruited from a Danish website for recruitment of volunteers to health research (forsøgsperson.dk), through online adverts or from a local database. Specific inclusion criteria for CM patients were 1) A verified diagnosis of chronic migraine without aura according to the International Headache Society Criteria[11]. All data in CM patients, EM patients and controls were collected between January 2015 and May 2017. Data on EM patients and controls included in this paper have been published previously[10]. Briefly, specific inclusion criteria for EM patients were: 1) a verified diagnosis of migraine without aura according to the International Headache Society Criteria[11], 2) at least one migraine attack every other month but less than five migraine days per month, 3) reported previous successful treatment of migraine attacks with sumatriptan. Specific inclusion criteria for healthy controls were no history of any primary headache and no first-degree relatives with migraine. Furthermore, all participants were eligible for inclusion if they were between 18 and 65 years, did not suffer from psychiatric, cardio- and cerebrovascular diseases, were not pregnant or nursing, and had no daily intake of medication.

All patients underwent a standardized interview including duration of disease (years), frequency (migraine days per month), maximum pain intensity of untreated headache as measured with the Numerical Rating Scale (NRS) (number 0–10), triptan use (days per month) and time since last migraine attack. We allowed CM patients to have headache on the day of the scan; in those cases, we registered pain intensity and type of headache (migraine or tension-type headache (TTH)). Medication intake was not allowed for any participant 24 hours before the scan. All participants had a normal physical and neurological examination and a normal structural brain MRI. All participants filled out the major depression inventory (MDI)[12] on the day of the PET scan.

The study was approved by the Ethics Committee of the Capital Region of Denmark and the Danish Data Protection Agency (H-6-2014-057). The study was registered at Clinicaltrials.gov (ID: NCT01896167). In accordance with the Declaration of Helsinki of 1964, with later revisions, all participants provided their written informed consent to participate in the study after detailed oral and written information and before any study specific procedures.

Data acquisition

All participants were placed in a supine position on the scanner bed. A specialized head holder was used to reduce head movement. The radioligand, [^{11}C]SB207145, was synthesized using an automated radiosynthesis (described elsewhere[13]). The radioligand was administered as an intravenously bolus injection over 20 s. Immediately after injection, a 120-minutes dynamic scan was acquired using a high-resolution research tomography PET scanner (CTI/Siemens, Knoxville, TN, USA). Reconstruction was done as previously described[10]. Briefly, a 3D-OSEM-PSF algorithm with TXTV based attenuation correction were used to reconstruct the scans into 38 frames[14–16]. All subjects underwent a T1 and a T2 weighted structural MRI scan (Siemens Prisma 3T scanner, Erlangen, Germany), which were used for co-registration with PET and delineation of regions of interest (ROI).

Pre-processing and regions of interest

Each PET frame was aligned to the first five-minutes frame to correct for intra-scan movement using the AIR 5.2.5 software. PET images were then aligned and co-registered to the corresponding T1 weighted MR image using SPM8. Automatic delineation of ROIs was done in PVElab software (www.nru.dk) as previously described[17]. Correct co-registration and

ROI placement were ensured by visual inspection. Lastly, time-activity curves used for kinetic modeling and grey matter volumes for each ROI were extracted.

The non-displaceable binding potential (BP_{ND}) was estimated in MATLAB R2013a (8.1.0.604) 64 bit (Mathworks Inc, MA, USA) using the simplified reference tissue model (SRTM) with the cerebellum (excluding vermis) as reference tissue. This model has been validated for quantification of [^{11}C]SB207145 in the human brain[13]. The investigator performing the kinetic modeling was blinded to group status. A volume-weighted neocortical BP_{ND} based on 11 brain regions (occipital cortex, orbitofrontal cortex, superior, medial and inferior frontal gyri, insula, superior, medial and inferior temporal gyri, sensory motor cortex, parietal cortex) was calculated by volume weighting grey matter segmented brain region BP_{ND}s:

$$\text{Neocortical 5-HT}_4\text{ receptor binding} =$$

$$\Sigma[\text{5-HT}_4\ BP_{ND}(\text{region}_x) * \text{volume}(\text{region}_x)] / [\ \Sigma\ (\text{volume}(\text{region}_x))]$$

Genotyping

Neocortical 5-HT_4 receptor binding has been shown to be related to the tri-allelic 5-HT transporter-linked polymorphic region (5-HTTLPR) polymorphism[18]. Therefore, participants were genotyped and categorized into two groups: 1. Carriers of the short allele (S-carriers) or the long L_G allele (L_G-carriers) (low-expressing alleles). 2. Homozygotes of the long L_A allele (L_A-homozygotes) (high-expressing alleles). Briefly, the genotyping was performed by PCR amplification from forward primer 5'-TAATGTCCCTACTGCAGCCC-3' and reverse primer 5'-GGGACTGAGCTGGACAACC-3' and subsequently digested by the restriction enzyme MspI and separated by gel electrophoresis.

Statistical analyses

Based on a previous study[19] a sample size of n = 15 was deemed sufficient to detect a 15% difference between groups with a power of 0.80 in very large brain regions (>50 cm3), such as e.g. neocortex. Differences between groups in demographics, genotypes and migraine characteristics were evaluated using Mann-Whitney or Fisher's exact test as appropriate. For our group comparisons, our primary outcome measure was log-transformed neocortical $BP_{ND.}$ Based on the model fit (examination of quantile probability plots, distribution of the residuals, and predicted values plotted against residuals), we found that this was the appropriate way to model our data as to not violate the assumption of normality. Our first primary endpoint was to asses group effects (CM vs. HC) on neocortical 5-HT_4 receptor binding. To account for the heteroscedasticity within the data used for this analysis, we fitted a model allowing for unequal variance for the two groups using generalized least square. Group status (CM vs. HC) was included as the predictive variable. Since 5-HT_4 receptor binding is affected by 5-HTTLPR status, sex, and age, these were all added as covariates to the model[18,20]. Our second primary endpoint was to assess the difference between CM and EM patients. This was done using a general linear model including log-transformed neocortical BP_{ND} as our primary dependent variable and group status (CM vs. EM) as the predictive variable and lastly adding 5-HTTLPR status, sex, and age as covariates. No model assumptions were violated using this approach. The differences between CM and HC and CM and EM are reported as percent difference since this measure is easier to interpret than the corresponding β estimates (relative percent difference=1−exp(β)). Thirdly, to evaluate the association between the number of migraine days and 5-HT_4 receptor binding, data for both EM and CM patients were pooled and analyzed using a general linear

model including neocortical BP_{ND} as our primary dependent variable and migraine days pr. month as the predictive variable. 5-HTTLPR status, sex, and age were added as covariates. Again, no model assumptions were violated using this approach. Post hoc, the association between BP_{ND} and years with migraine and time since last migraine attack were evaluated using the same general linear model as above (changing the predictive variable accordingly). All statistical analyses were done in R Studio 3.2.3. The significance threshold was set at $p < 0.05$, and no corrections for multiple comparisons were performed.

Results

Data from 16 CM, 15 EM patients and 16 controls were assessed in this study. Table 1 summarizes relevant participant characteristics. The regional distribution of the tracer was in concordance with previous studies (Striatum > Limbic regions > Neocortex)[18].

In comparison to healthy controls, chronic migraine patients had significantly lower neocortical BP_{ND} (9.1%, 95% CI: [-17%; -1.0%], p = 0.039). This corresponded to a mean BP_{ND}±SD of 0.62±0.09 in the CM group and of 0.67±0.04 in the HC group (figure 1). We found no difference in 5-HT_4 receptor binding between CM and EM patients (4.8 % higher BP_{ND} in CM vs. EM, 95% CI [-8.5%; 20%], p = 0.48). Correlation analysis combining CM and EM data revealed no association between the number of migraine days and neocortical BP_{ND} (slope estimate 0.003, 95% CI: [-0.004;0.715], p = 0.39) (figure 2). Post hoc analyses showed no associations between BP_{ND} and years with migraine or time since last attack (table 2).

Discussion

An important novel finding of the present study was that CM patients had lower 5-HT_4 receptor binding compared to controls but did not differ from EM patients. In addition, there was no association between 5-HT_4 receptor binding and monthly migraine days. The inverse relationship between 5-HT_4 receptor binding and stable brain 5-HT levels has been corroborated in several both pre- and clinical studies, showing that pharmacological increases in 5-HT downregulates the 5-HT_4 receptor whereas the receptor is upregulated when 5-HT levels are reduced[9,21–24]. Additionally, a post-mortem study in suicidal depressed patients showed increased 5-HT_4 receptor density in caudate nucleus and frontal cortex, which were related to decreased 5-HT levels[25]. Thus, we interpret our findings of low 5-HT_4 receptor binding as reflecting high brain 5-HT levels in the migraine patients.

5-HT has for long been implicated in migraine pathophysiology[6] with the main hypothesis being that migraine patients have low brain 5-HT levels between attacks. However, we recently demonstrated increased brain 5-HT levels, as indexed by low 5-HT_4 receptor binding, interictally in EM patients[10]. Interestingly, in the current study we found that CM patients also had lower 5-HT_4 receptor binding compared to controls. The precise mechanisms of 5-HT in migraine pathophysiology have not been fully clarified. 5-HT is involved in pain modulation and is generally considered analgesic[26], but in 5-HT depleted animals pain responses are attenuated, indicating that 5-HT is also involved in central pain facilitation[27,28]. 5-HT is also present in the descending pain modulation system, and dysfunction of this system, leading to increased facilitation and decreased inhibition of pain, may contribute to persistent pain[29]. Thus, the high level of brain 5-HT found in migraine patients may contribute to the underlying pathophysiology of migraine through increases in pain facilitation. In the present study, we therefore speculated that brain 5-HT levels would be higher in CM patients compared to patients with EM. However, we found no difference in 5-HT_4

receptor binding between EM and CM patients and no evidence for frequency of migraine attacks being associated with 5-HT_4 receptor binding. Thus, a high level of brain 5-HT could be an inherent trait of the migraine-susceptible brain rather than a risk factor for conversion from episodic into chronic migraine.

Although CM patients did not differ from EM patients regarding brain 5-HT_4 receptor levels, the composition of other 5-HT receptors might be different in CM patients compared to EM patients. In a recent PET study, we demonstrated that EM patients had lower 5-HT_{1B} receptor density in pain-modulating regions compared to controls and that 5-HT_{1B} receptor binding in the raphe nuclei decreased with proximity to the last migraine attack[30]. Pontine dysfunction has been reported interictally in CM patients[31,32], while brain stem activation has been reported in EM patients during attacks[33,34]. It would be relevant to explore whether CM and EM patients differ in terms of 5-HT_{1B} receptor binding, especially in the pontine raphe nuclei. Rather than differences in brain 5-HT levels, alterations in 5-HT receptor composition could be the underlying mechanism for differences in cortical excitability between CM and EM patients[5,35]. Given that some serotonergic receptors are excitatory and some inhibitory, the action of 5-HT may have differential effects on the cortical neurons and their excitability, depending on which receptor subtype is activated[7].

We acknowledge some limitations of the present study. Firstly, even though no previous findings indicate that that the 5-HT_4 receptor is involved in migraine pathophysiology, we cannot exclude that low 5-HT_4 receptor density is specific for migraine patients. It could be either genetically determined or congenital and thus independent on brain 5-HT levels. However, to the best of our knowledge, the 5-HT_4 does not play a prominent role in pain modulation, and several studies have corroborated that 5-HT_4 receptor binding inversely reflects stable brain 5-HT levels. Secondly, our study is cross sectional and does not allow us to investigate the temporal course of the changes in

brain 5-HT levels. Longitudinal studies are warranted to clarify these remaining questions. Thirdly, whether a high level of brain 5-HT is specific for migraine or is present in other pain conditions is currently unknown. Increased plasma 5-HT levels have been found in complex regional pain syndrome, whereas low plasma 5-HT levels have been found in fibromyalgia[8]. However, plasma levels do not reflect brain 5-HT levels[36] and 5-HT does not cross the blood-brain-barrier[37].

Conclusion

In conclusion, the present results suggest that low 5-HT$_4$ receptor binding, indicating high brain 5-HT levels, may be a trait marker of the migraine brain rather than a risk factor for conversion from EM to CM. While the pathophysiological consequences are not clear, increased brain 5-HT levels could enhance the susceptibility to migraine attacks, while other mechanisms determine frequency and severity. Consequently, reduction of brain 5-HT levels could be effective in the treatment of migraine. Future studies involving other 5-HT receptor subtypes and modulation of brain 5-HT levels in migraine patients are needed to further clarify these mechanisms.

Key Findings

- Chronic migraine patients have lower 5-HT$_4$ receptor binding, interpreted as higher brain 5-HT levels, compared to controls
- Episodic and chronic migraine patients have similar brain 5-HT levels and migraine frequency is not related to brain 5-HT levels
- High brain 5-HT levels may be a trait marker of the migraine brain

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Conflicts of Interest

Gitte Moos Knudsen has received honoraria as a council member of The Brain Prize and the Elsass Foundation and she is on the advisory board for the Kristian G. Jebsen Foundation and field editor for Int J Neuropsychopharm. Messoud Ashina is a consultant and/or scientific adviser/speaker for Allergan, Amgen, Alder, Eli Lilly, Novartis and Teva. All other authors report no conflicts of interest.

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Tables

Table 1. Participant characteristics					
	CM (n = 16)	HC (n = 16)	EM (n = 15)	P-value (CM vs. HC)	P-value (CM vs. EM)
Age (median (range))	27 (19-49)	25 (21-60)	25 (20-59)	0.78^{+}	0.89^{+}
Sex (W/M)	16/0	13/3	13/2	0.23*	0.23*
Genotype (S or L_G/L_{AA})	15/1	10/6	9/6	0.083*	0.037*
Major depression index (median (range))	9 (0-27)	6.5 (1-17)	5 (2-32)	0.38^{+}	0.44^{+}
Monthly migraine days (median (range))	10 (8-16)	NA	2 (0.5-4)	NA	< 0.001^{+}
Total monthly headache days (median (range))	25 (15-30)	NA	1.25 (1-6)	NA	< 0.001^{+}

*Fisher's exact test. $^{+}$Mann Whitney. NA: not applicable.

Table 2. Associations between migraine characteristics and BP_{ND} after adjusting for age, sex and genotype			
Migraine characteristic	Estimate	95% CI	p-value
Years with migraine	-0.002	[-0.007;0.003]	0.37
Days since last migraine attack	0.0006	[-0.003;0.004]	0.71

Figures

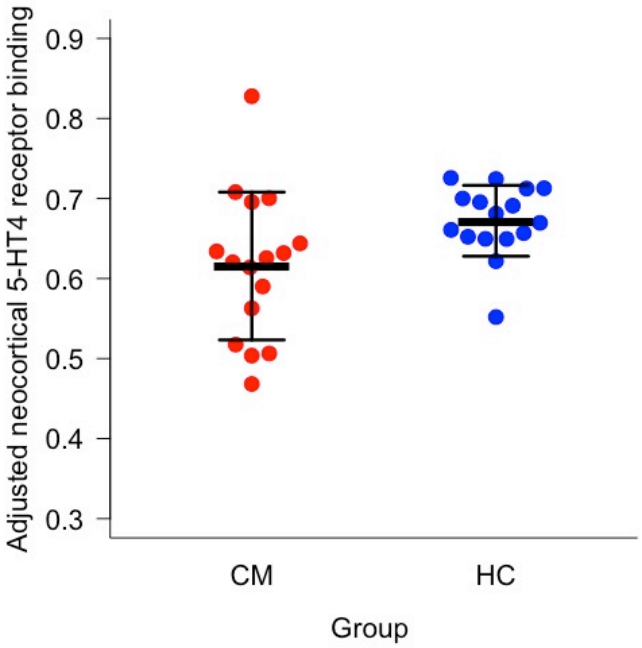

Figure 1. Decreased 5-HT$_4$ receptor binding in chronic migraine patients compared to controls

CM patients have lower neocortical 5-HT$_4$ receptor binding compared to controls after adjusting for age, sex and 5-HTTLPR genotype (mean±SD: 0.62±0.09 vs. 0.67±0.04). Black bars represent mean±SD.

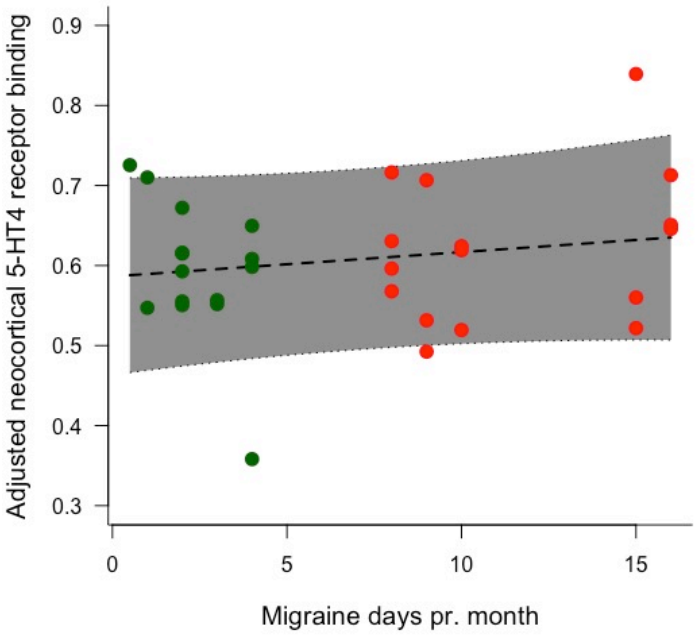

Figure 2. No association between monthly migraine days and 5-HT$_4$ receptor binding

In the sample of 31 migraine patients, frequency of migraine (migraine days pr. month) was not associated with adjusted neocortical 5-HT$_4$ receptor binding ($p = 0.39$). Green dots represent EM patients. Red dots represent CM patients. Dashed line represents estimated regression line. Grey shade represents 95 % confidence interval.

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Low 5-HT$_{1B}$ receptor binding in the migraine brain: A PET study

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Abstract

Background: The pathophysiology of migraine may involve dysfunction of serotonergic signaling. In particular, the 5-HT$_{1B}$ receptor is considered a key player due to the efficacy of 5-HT$_{1B}$ receptor agonists for treatment of migraine attacks.

Aim: To examine the cerebral 5-HT$_{1B}$ receptor binding in interictal migraine patients without aura compared to controls.

Methods: Eighteen migraine patients, who had been migraine free for >48 hours, and 16 controls were scanned after injection of the 5-HT$_{1B}$ receptor specific radioligand [^{11}C]AZ10419369 for quantification of cerebral 5-HT$_{1B}$ receptor binding. Patients who reported migraine <48 hours after the PET examination were excluded from the final analysis. We defined seven brain regions involved in pain modulation as regions of interest and applied a latent variable model (LVM) to assess the group effect on binding across these regions.

Results: Our data support a model wherein group status predicts the latent variable ($p = 0.038$), with migraine patients having lower 5-HT$_{1B}$ receptor binding across regions compared to controls. Further, in a whole-brain voxel-based analysis, time since last migraine attack correlated positively with 5-HT$_{1B}$ receptor binding in the dorsal raphe and in the midbrain.

Conclusion: We report here for the first time that migraine patients have low 5-HT$_{1B}$ receptor binding in pain modulating regions, reflecting decreased receptor density. This is either a primary constitutive trait of the migraine brain or secondary to repeated exposure to migraine attacks. We also provide indirect support for the dorsal raphe 5-HT$_{1B}$ receptors being temporarily downregulated during the migraine attack, presumably in response to higher cerebral serotonin levels in the ictal phase.

Keywords

Headache, pain modulation, raphe, neuroimaging, serotonin 1B receptors, serotonin

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Introduction

One of the longest-standing theories on migraine pathophysiology holds that this highly prevalent condition is fundamentally a disorder of serotonergic transmission, involving chronic low brain serotonin levels (1). This 'serotonin theory' of migraine emerged from biochemical studies in the 1960s (2) and is supported by more recent electrophysiological and neuroimaging studies (3). In addition, the anti-migraine drugs, such as ergotamine and triptans, act on serotonergic targets (4) and are highly selective for pain modulation of migraine, for example, triptans have no effect in tension type headache. Triptans are unlikely to exert their action through binding to vascular serotonin 1B

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(5-HT_{1B}) receptors, but rather through binding to peripheral, neuronal 5-HT_{1D} (5) and central 5-$HT_{1B/1D/1F}$ receptors in the trigemino-cervical complex (6). The relative contributions in the anti-migraine effect of the 5-HT_1 subtypes (B, D, F) is still unclear, although the selective 5-HT_{1D} receptor agonist PNU-142633 is ineffective in migraine (7). Therefore, the 5-HT_{1B} receptor seems to play an important and specific role in migraine pathophysiology.

5-HT_{1B} receptors are present in all parts of the trigeminal pain-signaling pathway, including the dural vasculature, trigeminal ganglion, trigeminal nucleus caudalis, raphe nuclei, thalamus, hypothalamus and the cerebral cortex (8). In addition to being a heteroreceptor that modulates the release of gamma-amino-butyric acid (GABA), glutamate and acetylcholine, the 5-HT_{1B} receptor is also an autoreceptor located presynaptically on serotonergic neurons, inhibiting synthesis and release of serotonin in raphe (9) and in projection areas (8).

To our knowledge, no study has yet investigated *in vivo* cerebral 5-HT_{1B} receptor binding in migraine patients. The aim of this PET study was to examine cerebral 5-HT_{1B} receptor binding in interictal migraine patients without aura and compare it to healthy volunteers. We focused our analysis on brain regions of relevance for migraine, i.e., those involved in pain modulation or emotional and cognitive aspects of pain: The prefrontal cortex, sensorimotor cortex, anterior cingulate cortex, insula, and amygdala. We subsequently evaluated with an exploratory whole-brain voxel-based analysis whether differences in 5-HT_{1B} receptor binding could be detected in any other regions. Finally, we investigated whether 5-HT_{1B} receptor binding correlated with clinical characteristics of the patients' migraine attacks.

Materials and methods

Subjects

All participants were recruited from a Danish website for recruitment of volunteers to health research (www.forsogsperson.dk), through online adverts on the intranet of the Capital Region of Denmark and from a local database. Patients were eligible for inclusion if they were 18–65 years old, had a verified diagnosis of migraine without aura according to the International Headache Society Criteria (10), had at least one migraine attack every other month but less than five migraine days per month, and reported having experienced successful treatment of migraine attacks with sumatriptan. Patients underwent a standardized interview and interview items included duration of disease (years), frequency (migraine days per month), maximum pain intensity of untreated headache as measured with the Numerical Rating Scale (NRS) (number 0–10), triptan use (days per month) and time since last migraine attack (days). Age and sex matched controls were eligible for inclusion if they did not have any history of migraine including probable migraine and had no first-degree relatives with migraine. Exclusion criteria were: A history of any other primary headache (except tension-type headache for less than five days per month), psychiatric disease, cerebro- or cardiovascular disease, contraindications for magnetic resonance imaging (MRI), pregnancy or nursing, and intake of daily medication. On the day of the scheduled PET scan, all subjects were headache free and had not had any intake of medications for the last 24 hours. Patients were also excluded if they had not been migraine free for at least 48 hours prior to the PET scan. Headache diaries were obtained from all patients for 48 hours after the scan. All included participants had a normal physical and neurological examination and brain MRI.

The Ethics Committee of the Region of Copenhagen approved the study (H-6-2014-057), which was conducted in accordance with the Helsinki II Declaration of 1964, with later revisions. All participants gave written consent after receiving detailed oral and written information prior to any study-specific procedures.

PET and MRI

[^{11}C]AZ10419369 was synthesized using an automated radiosynthesis system as previously described (11). The subjects were placed in a supine position on the scanner bed with their head in a specialized head holder to minimize movement. [^{11}C]AZ10419369 was given intravenously as a bolus over 20 seconds followed by a 90 min PET dynamic data acquisition. PET scanning was performed with the high-resolution research tomography (HRRT) PET scanner (CTI/Siemens, Knoxville, TN, USA) with an in-plane resolution of approximately 1.4 mm (12). Reconstruction was done using ordinary Poisson 3-dimensional ordered-subset expectation maximization with point spread function modeling (16 subsets, 10 iterations) (13,14) with attenuation map improvements as previously described (15).

MRI was conducted using a Siemens Prisma 3T scanner (Siemens, Erlangen, Germany) with a 64-channel head coil. Structural T1 and T2 weighted images were recorded for each subject. MR images were used to rule out structural pathology, for co-registration with PET and delineation of regions of interest (ROI), and for segmentation in SPM8 into grey matter, white matter and cerebrospinal fluid.

Region of interest analysis

PET images were co-registered and aligned to the corresponding T1-weighted MRI image using SPM8 (16).

Confirmation of accurate co-registration was done by visual inspection for each subject across all planes. Correction for intra-scan movement was performed on all PET images using the AIR 5.2 software. All frames were aligned to the first five-minute frame. Delineation of ROIs was done automatically on each subject's MRI using PVElab software (www.nru.dk) as previously described (16), and time activity curves (TAC) and grey matter volumes for each ROI were extracted. Grey matter volumes were quantified summing the grey matter voxels from the SPM8 segmentation.

The kinetic modeling was performed in Matlab using the simplified reference tissue model (SRTM), with the cerebellum (excluding vermis) as a reference region. The cerebellum is suitable as a reference region since it is almost devoid of 5-HT_{1B} receptors (17). We used the SRTM to calculate the non-displaceable binding potential (BP_{ND}), which has been validated for quantification of [^{11}C]AZ10419369 in humans (18). The person performing the kinetic modeling was blinded to group status (migraine patient or control).

Statistical analysis

Group differences in demographics, ROI specific grey matter volumes (corrected for total grey matter volume) and PET variables were compared using two-sample t-tests. We used a latent variable model (LVM) to evaluate group effects on BP_{ND} across the predefined ROIs, using the Lava package in R (19). LVM is a linear regression statistical framework used to model associations with shared information across observations, e.g., receptor binding across different brain regions. With this approach, it is possible to avoid multiple comparisons. In addition, the concept of an underlying common regulation of 5-HT_{1B} receptor binding is also captured by the latent variable model. Here, we estimated a single latent variable, modeling the shared correlation between 5-HT_{1B} receptor binding within regions involved in pain-processing including cognitive and affective aspects of pain: Anterior cingulate cortex (ACC), dorsolateral prefrontal cortex (DLPFC), ventrolateral prefrontal cortex (VLPFC), orbitofrontal cortex (OFC), sensorimotor cortex (SenMot), insula (Ins), and amygdala (Amy). An identifiable model was chosen such that covariate effects can be interpreted relative to effects on VLPFC 5-HT_{1B} receptor binding. Individual additional model paths were considered based on score tests. A score test is similar to a likelihood ratio test, and it tests whether including additional paths benefits the overall model fit. All estimates and significance values were determined simultaneously, and p-values < 0.05 (two-tailed) were considered statistically significant.

As an exploratory post hoc analysis, the group effect (patients vs. controls) in each region included in the LVM was evaluated using univariate linear regression analyses. Age was included as a covariate in all models, since cerebral 5-HT_{1B} receptor binding declines significantly with age (20). Cerebral 5-HT_{1B} receptor binding does not differ between males and females (21) and, accordingly, sex was not included as a covariate.

For all analyses the significance threshold was set at $p < 0.05$ (two-tailed) and p-values are reported without correction for multiple comparisons. Statistical tests were carried out using Prism Version 6 and R Studio 3.2.3.

Voxel-based analysis

Parametric images of 5-HT_{1B} receptor binding were generated using FreeSurfer (http://surfer.nmr.mgh.harvard.edu, version 5.3) as previously described (22–24) (Figure 1). In summary, each single-subject structural T1 was normalized to Montreal Neurological Institute

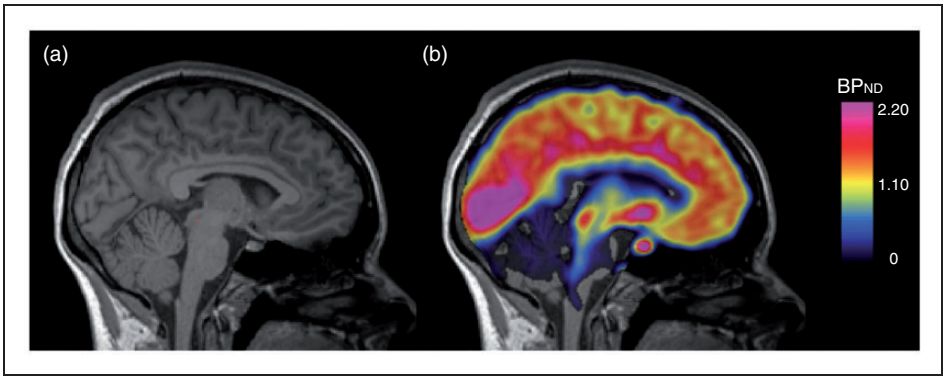

Figure 1. Parametric image of 5-HT_{1B} receptor binding. (a) Structural T1-weighted MRI image. The 5-HT_{1B} binding is not identifiable. (b) [^{11}C]AZ10419369 PET image superimposed on the corresponding structural image, highlighting the 5-HT_{1B} system.

(MNI) space using the combined volumetric and surface registration algorithm (CVS), as this has been shown to be particularly sensitive to both cortical and subcortical alignment (25). Subsequently, this was applied to the co-registered PET images. Finally, the PET images were volume-smoothed with a 6 mm full-width half-maximum 3D Gaussian kernel, and voxel-level BP_{ND}'s were estimated using the Multilinear Reference Tissue Model 2 (MRTM2), with the cerebellum as the reference region and the neocortex as the high-binding region for estimation of k_2'.

Group differences were evaluated at voxel level using multiple linear regressions. Lastly, whole-brain voxel-wise multiple regressions were performed with measures of clinical severity, including time since last migraine attack. All analyses were adjusted for age. Correction for multiple comparisons was performed using 3dClustSim, a program within AFNI (National Institute of Mental Health, Bethesda, MD; http://afni.nimh.nih.gov/afni) that uses a Monte Carlo simulation method to determine a cluster extent threshold unlikely to have occurred by chance ($\alpha < 0.05$). The cluster extent threshold for a whole-brain search volume given a voxel-level of $p < 0.005$ uncorrected, was $k > 2248$.

Results

Eighteen migraine patients and 16 age- and sex-matched controls completed the study. Three patients had a migraine attack within 48 hours after the scan and were excluded from the final analysis. All other patients were migraine free for at least 48 hours following the scan. One patient was excluded due to excessive head movement in the scanner. Hence, 14 patients and 16 controls were included in the final analysis. The clinical data of the migraine patients are presented in Table 1 and demographic data and details of the injected radioligand for both groups are shown in Table 2. The regional distribution of the tracer was in concordance with previous studies showing high binding in the occipital region and low binding in the cerebellar cortex (Figure 1) (18,26). There were no differences in grey matter volumes between patients and controls in any of the included ROIs.

Latent variable model

The basic model predicted a high correlation between regions captured by the latent variable (all factor loadings: $p < 1.6 \times 10^{-5}$). To this model we added group status (patient or control) as a predictor of the latent variable and age as a predictor of binding. Score tests revealed an additional shared correlation between $5\text{-}HT_{1B}$ receptor binding in (a) the orbitofrontal cortex and dorsolateral prefrontal cortex ($p = 0.004$) and (b) the orbitofrontal cortex and sensorimotor cortex ($p = 0.009$). No subsequent model paths were supported following an additional score test, consistent

Table 1. Migraine characteristics of included subjects.

Subject	Years with migraine	Frequency (days/month)	Side R/L (%)	Triptan intake (days/month)	Severity of migraine attack (NRS)	Days since last migraine attack
1	8	1	50/50	1	6	17
2	21	1	Bilateral	0	9	22
3	25	3	50/50	3	5	11
4	19	1	70/30	1	8	19
5	15	2	Bilateral	0	9	31
6	20	1	50/50	1	7	4
7	8	2	50/50	1	8	50
8	9	1	50/50	1	9	8
9	17	1	Bilateral	1	7	15
10	6	2	Bilateral	2	8	10
11	7	3	10/90	1	8	29
12	36	4	50/50	3	7	5
13	2	4	100/0	4	7	4
14	16	1	Bilateral	1	9	7
Median (range)	15.5 (2–36)	1.5 (1–4)		1 (0–4)	8 (5–9)	13 (4–50)

NRS = Numerical Rating Scale.

Table 2. Summary of demographics and PET variables in the two groups.

	Patients	Controls	*p*-value
Number of subjects (male/female)	14 (1/13)	16 (3/13)	
Age (years)	30.4 ± 10.0	28.9 ± 9.9	0.69
BMI (kg/m^2)	22.5 ± 1.4	24.1 ± 4.6	0.25
Injected radioactivity (MBq)	588 ± 13	580 ± 51	0.62
Specific radioactivity (GBq/μmol)	629 ± 304	492 ± 134	0.13
[^{11}C]AZ injected mass per kg (μg/kg)	0.012 ± 0.014	0.009 ± 0.003	0.35
[^{11}C]AZ cerebellum AUC/Injected dose (counts/MBq)	10.53 ± 1.91	9.71 ± 2.23	0.29

Values are given as mean ± SD. *p* values represent differences between groups evaluated using two-sample t-test. [^{11}C]AZ = [^{11}C]AZ10419369; AUC = Area under the time activity curve for cerebellum, representing the non-displaceable binding.

with sufficient model fitting ($\chi^2 = 23.54$ on 18 degrees of freedom giving a *p*-value of 0.17). In addition, the effect size of group on the latent variable was consistent throughout these model modifications (range −0.105 to −0.113). Within this final model, group significantly predicted the latent variable (parameter estimate and 95% confidence interval: −0.113 [−0.219; −0.006]), $p = 0.038$, with patients having lower binding across regions (Figure 2).

Linear regression analysis

Post hoc ROI analyses showed a significantly lower binding in the anterior cingulate cortex of migraine patients compared to controls (1.64 ± 0.15 vs. 1.78 ± 0.20, $p = 0.041$) and within the sensorimotor cortex (1.24 ± 0.13 vs. 1.34 ± 0.14, $p = 0.048$). There was a trend towards a lower 5-HT$_{1B}$ receptor binding in patients compared to controls within insula (1.54 ± 0.15 vs. 1.65 ± 0.17, $p = 0.071$) and ventrolateral prefrontal cortex (1.63 ± 0.14 vs. 1.74 ± 0.18, $p = 0.070$).

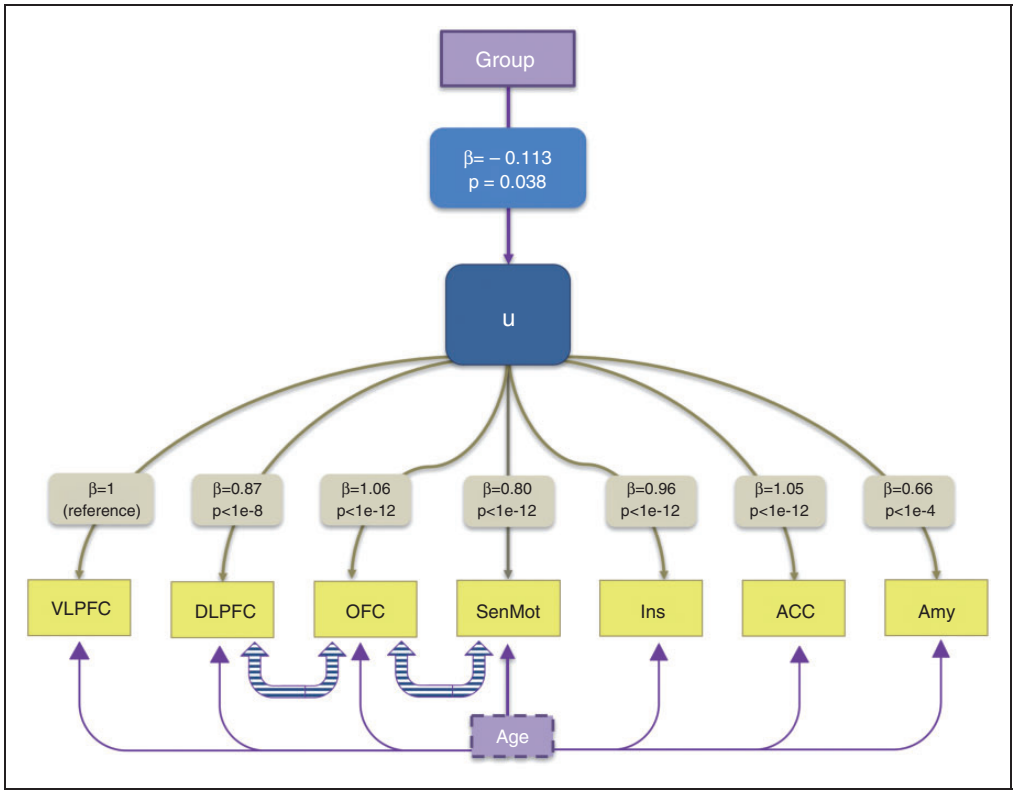

Figure 2. Latent variable model of group effects on [^{11}C]AZ10419369 BP$_{ND}$. Purple boxes represent observed predictors. The blue box (u) represents the latent variable, whereas yellow boxes represent regional [^{11}C]AZ10419369 binding potential. Striped arrows indicate additional shared correlations. The parameter estimate, β, is noted for each model path as well as significance, *p*, of parameter estimates.

Voxel-based analysis

Whole-brain voxel-wise multiple regression analysis revealed no significant group differences in 5-HT_{1B} receptor binding. The whole-brain voxel-wise multiple regression analyses with measures of clinical severity revealed a positive correlation between 5-HT_{1B} receptor binding and days since last migraine attack in a cluster spanning the midline. By visual inspection using xjView, a viewing program for SPM (http://www.alivelearn.net/xjview8/), this cluster was determined to cover bilateral red nucleus, left substantia nigra, and the dorsal raphe ($k = 3589$, $t(11) = 6.41$, $p < 0.05$ corrected, $x = -15$, $y = -22$, $z = -8$, Figure 3). No other correlations were detected.

Discussion

We found that patients with migraine without aura have lower 5-HT_{1B} receptor binding than controls across brain regions involved in pain modulation. The association between the latent variable and the large, 5-HT_{1B} receptor high-density control region (occipital cortex), was the weakest of all regions (beta = 0.65), indicating that the current finding is specific for the pain modulating regions. Hence, our data indicate that migraine patients (at least when interictal) have low 5-HT_{1B} receptor binding in brain regions involved in pain-processing including emotional and cognitive aspects of pain. In contrast to previous studies (27), we found no group difference in grey matter volume between patients and controls within any of the ROIs and thus, the lower 5-HT_{1B} receptor binding is not driven by differences in brain structure. Given that all subjects were scanned at the same time of the day, and that all migraine patients were truly interictal, we do not expect diurnal or ictal variations in brain serotonin levels to explain differences in 5-HT_{1B} receptor binding. Instead, we believe that the low binding reflects a decreased density of the 5-HT_{1B} receptor in migraine patients.

5-HT_{1B} receptor binding in pain modulating regions

There may be several alternative or possibly co-existing reasons why the 5-HT_{1B} receptor density is low in pain modulating brain regions in migraine patients.

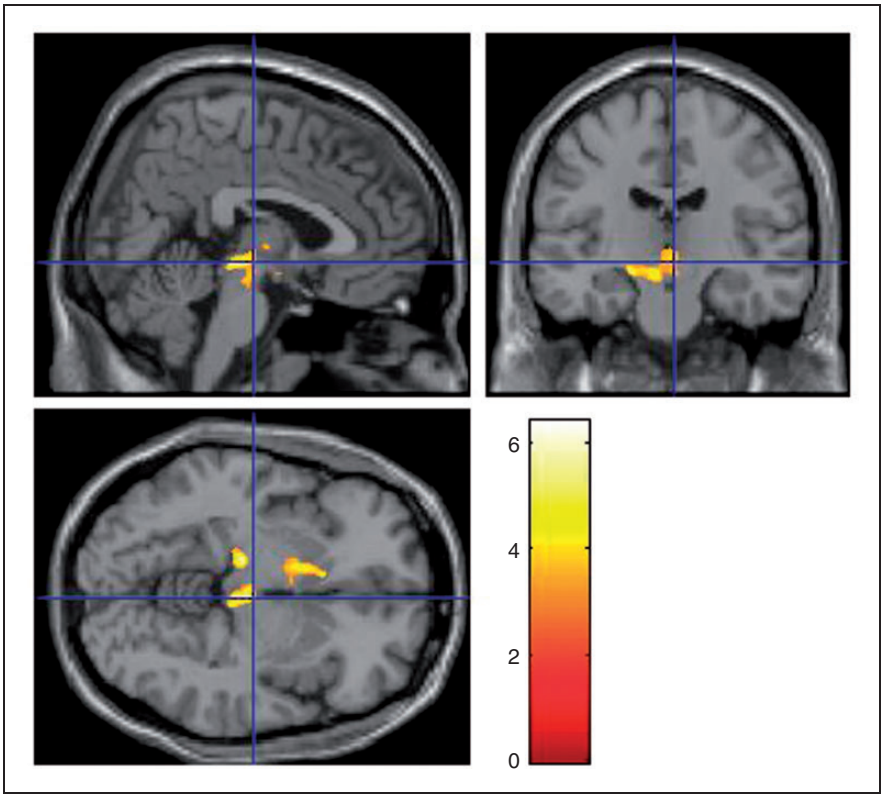

Figure 3. Voxel based analysis. Whole-brain voxel-based analysis in the migraine patients showed a positive correlation between 5-HT_{1B} receptor binding and days since the last attack in a cluster in the brainstem and midbrain (($k = 3589$, $t(11) = 6.41$, $p < 0.05$ corrected, $x = -15$, $y = -22$, $z = -8$). Color bar indicates t-score. Image shown at $z = -5.84$.

Firstly, the finding could represent a primary abnormality, e.g., a genetically determined trait marker for migraine susceptibility with an ensuing altered pain sensation and processing. This hypothesis is supported by the lack of correlation with time since last attack or duration of disease within the predefined ROIs. On the other hand, we did not observe any correlation between pain severity or headache frequency and 5-HT_{1B} receptor binding, which could have been expected if the latter is a trait marker. The 5-HT_{1B} receptor serves both as an auto- and a heteroreceptor and PET neuroimaging does not allow for a distinction between the two receptor types. Decreased 5-HT_{1B} autoreceptor density diminishes the ability to regulate synaptic serotonin levels (28). Low levels of 5-HT_{1B} autoreceptors in certain pain-related areas could thus increase the susceptibility to pain stimuli and trigger migraine attacks due to a decreased capacity to normalize serotonin levels. In the rat cingulate cortex, activation of 5-HT_{1B} heteroreceptors leads to inhibition of glutamate release (29). Low 5-HT_{1B} heteroreceptor neurotransmission could therefore lead to increased excitatory neurotransmission in ACC. In support thereof, changes in glutamatergic neurotransmission have been described in both insula and ACC interictally in migraine patients (30). In summary, our findings could reflect a functional modification of pain processing in migraine patients, caused by low 5-HT_{1B} autoreceptor and/or heteroreceptor neurotransmission.

Secondly, the low 5-HT_{1B} receptor density in the pain modulating brain regions could be a result of repeated migraine episodes, with a migraine-induced modulation of 5-HT_{1B} receptor density in brain areas involved in the pain matrix. Several studies provide support for the idea that the continuous activation of the pain related areas in migraine leads to neurochemical, metabolic or even structural modifications (30–32). In the insula and anterior cingulate cortex, the glucose metabolism is negatively correlated to duration and lifetime headache frequency (31), and similarly, a negative association between these regions' grey matter volume and headache duration and lifetime headache frequency has been found (32).

Theoretically, our observation of lower 5-HT_{1B} receptor binding could also result from higher regional serotonin levels in the pain modulating regions. From studies with acute pharmacological interventions aiming at increasing cerebral serotonin levels, there is some evidence that [^{11}C]AZ10419369 binding is reduced with acutely increased endogenous serotonin levels. This was, however, only shown in non-human primates (33), but not in humans (34). It is currently unknown if chronically elevated serotonin levels alter the 5-HT_{1B} receptor binding.

Raphe nuclei

In our supplementary voxel-based correlation analysis, we did not identify any clusters showing significant group differences. However, in migraine patients we found that time since last migraine attack correlated positively with 5-HT_{1B} receptor binding within a cluster encompassing the dorsal raphe and other parts of the midbrain. The presence of this correlation was also confirmed in a ROI based approach where we analyzed dorsal raphe separately (uncorrected $p = 0.001$, data not shown). This suggests that 5-HT_{1B} receptors in dorsal raphe, critically involved in the regulation of serotonin synthesis and serotonergic neurotransmission, are regulated in synchrony with the migraine cycle.

Some authors have suggested that brain serotonin levels are temporarily increased in the ictal phase of a migraine attack (35). Such a stimulation of the raphe nuclei could lead to a temporary downregulation of 5-HT_{1B} autoreceptors that only gradually recovered. This explanation finds support in animal studies that show downregulation of presynaptic 5-HT_{1B} autoreceptors in the raphe nuclei following an increase in synaptic serotonin (36,37). Thus, it is possible that our results reflect spontaneous increases in synaptic serotonin levels in migraine patients compared to controls.

Interestingly, the raphe nuclei, including the dorsal raphe, have previously been implicated in migraine pathophysiology. In particular, [^{15}O]H_2O PET studies reported an increase in regional cerebral blood flow during spontaneous attacks, which persisted after effective treatment with 5-$HT_{1B/1D}$ receptor agonist sumatriptan (38,39). More recently, a functional MRI study reported strong functional connectivity between the hypothalamus and the dorsal pons during a migraine attack (40). Collectively, these findings point toward an involvement of the dorsal raphe during migraine attacks. In our analysis, we only included patients that were migraine free for 48 hours before and after the scan, but in the light of our findings it would be relevant to investigate the dynamic changes in raphe related to the migraine cycle in the period from 48 hours before attack onset to 48 hours post-ictally.

Limitations

A few shortcomings of our study need to be mentioned. Since we only examined patients in their inter-ictal phase we cannot exclude that we would have had a different outcome had we studied them immediately before, after, or during a migraine attack. Further, since most of the migraine participants were occasional triptan users, we cannot exclude that the 5-HT_{1B} receptors were desensitized in response to the periodic intake of 5-HT_{1B} receptor agonists. However, the absence of a

global downregulation of 5-HT_{1B} receptors and the lack of correlation between duration of disease, frequency or triptan use and 5-HT_{1B} receptor binding speaks against this explanation.

Conclusion

We report novel evidence that patients with migraine without aura have low 5-HT_{1B} receptor binding in pain modulating regions of the brain, and we explain it as either a primary constitutive trait of the migraine brain or secondary to the repeated exposure to migraine attacks. We also provide some indirect support for the dorsal raphe 5-HT_{1B} receptors being temporarily downregulated during the migraine attack, presumably in response to higher cerebral serotonin levels in the ictal phase. Further studies are warranted to clarify the mechanisms underlying our observations.

Key findings

- We found a lower binding of 5-HT_{1B} receptors in migraine patients compared to controls in pain modulating regions of the brain.
- The lower binding is either a trait marker of the migraine or a consequence of repeated activation of the pain modulating areas.
- We found that 5-HT_{1B} receptor binding increased in the midbrain with days since the last attack, supporting the involvement of the dorsal raphe in migraine pathophysiology.

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Declaration of conflicting interests

The authors declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: Dr. Knudsen has received honoraria as a consultant/speaker for H Lundbeck and Pfizer, and as a board member of Brain Prize and the Elsass Foundation. She is also on the advisory board for the Kristian G Jebsen Foundation and a field editor for *Int J Neuropsychopharm*. Messoud Ashina is a consultant and/or scientific adviser/speaker for the ATI, Allergan, Amgen, Alder and Eli Lilly. All other authors declare no conflicts of interest and report no financial disclosures.

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Sumatriptan crosses the blood-brain barrier in migraine patients and binds to central 5-HT_{1B} receptors

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Running title: Sumatriptan and central 5-HT_{1B} receptor binding

Abstract

Background

Triptans, the most efficient acute treatment for migraine attacks, are serotonin (5-HT) $_{1B/1D}$ receptor agonists but their precise mechanism of action is not completely understood. The 5-HT_{1B} receptor is expressed abundantly throughout the human brain but to which extent triptans enter the CNS and bind to 5-HT_{1B} receptors in the brain is unknown.

Methods

We used the sensitive and validated method of PET imaging of the 5-HT_{1B} receptor radiotracer [^{11}C]AZ10419369 to determine the occupancy of sumatriptan on central 5-HT_{1B} receptors and to investigate changes in brain 5-HT levels during migraine attacks. Eight migraine patients were scanned three times: 1) during an experimentally induced migraine attack, 2) after a subcutaneous injection of 6 mg sumatriptan, and 3) on an attack-free day.

Findings

Sumatriptan significantly reduced cerebral 5-HT_{1B} receptor binding (mean BP_{ND}±SD 1·20 ± 0·20 vs. 1·02 ± 0·22, p = 0·0001), corresponding to a mean occupancy±SD of 16·0±5·3%. Further, during migraine attacks 5-HT_{1B} receptor binding was significantly reduced in pain modulating regions as compared to outside of attacks (mean BP_{ND}±SD 1·63±0·22 vs. 1·20±0·20, p = 0·019).

Interpretation

Sumatriptan crosses the blood-brain barrier and binds to central 5-HT_{1B} receptors. This may be an integral part of the antimigraine effect of sumatriptan. Further, migraine attacks are associated with an increase in brain 5-HT levels, indicating that migraine attacks may be triggered by increases in endogenous 5-HT.

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Introduction

Triptans, which were first introduced in the late 1980s,[1] have revolutionized migraine management and remain the standard abortive therapy for migraine. Triptans are 5-$HT_{1B/1D}$ receptor agonists, but the specific mechanism of action relevant to the therapeutic effects is unknown. While the anti-migraine mechanism was originally thought to be exerted exclusively through its vasoconstrictor effect,[2] preclinical studies suggest a more complex mode of action such as impeding signals between the first and second order trigeminal neurons,[3] or preventing release of vasoactive and inflammatory substances from trigeminal nerve endings.[4] In addition, the 5-HT_{1B} receptor is expressed abundantly throughout the human brain[5] and common triptan-related adverse events such as dizziness and somnolence[6] suggest a possible CNS effect. However, to which extent triptans enter the brain and bind to 5-HT_{1B} receptors in the brain parenchyma remains unclear.

PET imaging with the specific 5-HT_{1B} receptor radiotracer [^{11}C]AZ10419369 is suitable for measuring endogenous brain 5-HT release[7,8] and for assessing the occupancy of drugs binding to the 5-HT_{1B} receptor, e.g. triptans.[9,10] Using [^{11}C]AZ10419369, we recently reported low 5-HT_{1B} receptor binding across pain modulating regions in migraine patients on an attack-free day and that binding of 5-HT_{1B} receptors in the raphe correlated positively with time since last migraine attack.[11] Since brain 5-HT levels are thought to increase during migraine attacks,[12] it is possible that higher brain 5-HT levels during attacks result in a temporary downregulation of the 5-HT_{1B} receptor. However, conclusive evidence for increases in brain 5-HT during a migraine attack and for binding of sumatriptan to central 5-HT_{1B} receptors during migraine attacks is still lacking.

Here, we investigated 5-HT_{1B} receptor binding in migraine patients during migraine attacks (ictal) and after treatment with sumatriptan (postictal). Additionally, all patients underwent a PET scan on an attack-free day (interictal). We hypothesized that brain 5-HT_{1B} receptor binding would decrease after administration of a clinical relevant dose of sumatriptan. Further, we hypothesized that 5-HT_{1B} receptor binding would be lower during the ictal phase compared to the interictal phase due to competition between endogenous 5-HT and [^{11}C]AZ10419369 at the $5HT_{1B}$ receptor site.

Materials and methods

Participants

All participants were recruited via a Danish website for recruitment of participants to health research (www.forsøgsperson.dk), through online adverts, and through a local database. Data from five patients' interictal scans have been published previously.[11] The inclusion criteria were: 1) 18–65 years old, 2) Verified diagnosis of migraine without aura according to the International Headache Society Criteria, 3rd edition (beta version),[13] 3) At least one migraine attack every other month but less than five migraine days per month**,** 4) Previous experience of successful treatment of migraine attacks with sumatriptan, 5) Successful induction of migraine after cilostazol administration (assessed by pre-inclusion screening). None of the participants had a history of any other primary headache (except tension-type headache for less than five days per month), any psychiatric, cerebro- or cardiovascular diseases or daily intake of medication. Nor were any participant pregnant or nursing and all participants were eligible for a magnetic resonance imaging (MRI) scan.

The study was approved by the Ethics Committee of the Capital Region of Denmark (H-6-2014-057) and the Danish Data Protection Agency and registered at Clinicaltrials.gov (ID: NCT01896167). All participants provided their written informed consent to participate in the study after detailed oral and written information about the study. The study was conducted in accordance with the Declaration of Helsinki of 1964, with later revisions.

Experimental design

On the day of the interictal scan, all included participants had been migraine free > 48 hours. The date of their last migraine attack was registered. No medication intake was allowed for 24 hours before the scan. Patients were excluded if they reported a migraine attack <48 hours after the scan.

To ensure that participants would have a migraine attack on the day of their planned scan, we used a human migraine provocation model to investigate [^{11}C]AZ10419369 binding during migraine attacks. We used cilostazol, a phosphodiesterase 3 inhibitor, which has proven highly suitable for experimental triggering of migraine.[14,15] In the morning on the day of the scan, participants ingested 200 mg cilostazol. Intake of the drug was ensured by a video phone call from investigator (MD) to the participant. After ingestion, the participants filled out a headache diary every hour until arrival at the hospital. After arrival, the diary was filled out every 30 minutes until initiation of the first scan where after the diary was filled out every 10 minutes continuing until the end of the second scan. The diary included questions on headache intensity (0-10), associated symptoms and premonitory symptoms. We defined migraine like-attacks according to criteria C and D of the International Classification of Headache Disorders, 3rd edition (beta version)[13] definition of migraine without aura:

C. Headache has at least two of the following four characteristics:

1. unilateral location
2. pulsating quality
3. moderate or severe pain intensity
4. aggravation by or causing avoidance of routine physical activity (e.g. walking or climbing stairs)

D. During headache at least one of the followings:

1. nausea and/or vomiting
2. photophobia and phonophobia

Immediately after the first scan, all patients were treated with sumatriptan, 6 mg sc.

Data acquisition and analysis

On each scan day two venous catheters were inserted in the cubital veins, one for injection of the radiotracer and one for drawing of blood samples. All participants underwent three PET scans on two separate study days; the interictal scan was conducted on a separate day, and the ictal and the postictal scans were conducted on the same day (figure 1). Details regarding the imaging procedures have been described previously[11]. In brief, the radiotracer was synthesized using an automated radiosynthesis system. All participants were placed in a supine position on the scanner bed with the head in a specialized holder to minimize movement. The radiotracer [^{11}C]AZ10419369 was administered intravenously over 20 seconds after which emission data were acquired for 90 minutes using the High-Resolution Research Tomography PET scanner (CTI/Siemens, Knoxville, TN, USA). PET images were reconstructed using 3D-OP-OSEM including point spread function modeling and attenuation

map improvements[16–18]. On a separate day, all participants underwent a T1 and a T2 weighted structural MRI scan (Siemens Prisma 3T scanner, Erlangen, Germany), the MR images were used to delineate of regions of interest (ROI).

To compensate for head motion, each PET frame was aligned to a single PET frame (frame 27, first 5 minutes frame) using the scaled least squares cost-function in AIR 5·2·5 and filtered with a 10 mm Gaussian filter. PET images were then aligned and co-registered to the corresponding T1 weighted MR image using SPM8. Correct co-registration was ensured by visual inspection. ROIs were delineated on each subject's MRI and projected onto the PET images to extract regional time activity curves (TAC) and grey matter volumes. This was done automatically using PVElab software (nru.dk/index.php/component/jdownloads/category/37-pvelab?Itemid=-1)[19].

Quantification of [^{11}C]AZ10419369 binding

Previous studies have shown that [^{11}C]AZ10419369 binding can be quantified with the simplified reference tissue model, SRTM, using cerebellar grey matter (excluding vermis), which is devoid of 5-HT_{1B} receptors, as a reference region.[20] Therefore, we used this approach to calculate the non-displaceable binding potential, BP_{ND} for [^{11}C]AZ10419369.

The main outcome measure was a volume weighted BP_{ND} average across seven brain regions involved in pain modulation: dorso- and ventrolateral prefrontal cortex, orbitofrontal cortex, anterior cingulate cortex, sensorimotor cortex, insula and amygdala brain regions. This was calculated according to:

$$\text{Mean } BP_{ND} =$$

$$\Sigma[\text{5-HT}_{1B}\ BP_{ND}(\text{region}_x) * \text{volume}(\text{region}_x)] \,/\, [\ \Sigma\ (\text{volume}(\text{region}_x)]$$

Receptor occupancy was estimated from the two scans preceding and following sumatriptan administration as:

$$\text{Occupancy (\%)} = 100 * (BP_{ND,\ attack} - BP_{ND,\ drug}) / BP_{ND,\ attack}$$

Statistical analysis

Differences in demographics and PET variables between scans were assessed with Student's paired t-test. Within-subject changes in 5-HT_{1B} receptor binding between conditions (ictal vs. postictal, interictal vs. ictal) were assessed using a Student's paired t-test. Since we had a clear hypothesis of the direction (decrease) of the changes in BP_{ND} and powered the study accordingly, we performed one-tailed hypothesis testing. Difference in area under the curve (AUC) for headache intensity scores was analyzed using Wilcoxon matched-pairs signed rank test. P-values below 0.05 were considered significant. No corrections for multiple comparisons were done.

Results

Demographics and PET variables

Eight migraine patients (7 women) were included in the study. Their mean age±SD was 29·5±9·2 years on study day 1 and 30·0±8·9 years on study day 2. The mean injected dose was similar across scans (593±11, 576±14, and 590±14 MBq for the ictal, postictal and interictal scan, respectively). Postictal data from one subject were excluded because of intake of Buventol, a β_2 agonist, which was not allowed according to the protocol. Details on injected mass of AZ10419369 per kg and time-normalized area under curve (AUC) for the cerebellar TAC can be found in supplementary table S1.

Changes in 5-HT_{1B} receptor binding

Compared to the ictal state, 5-HT_{1B} receptor binding decreased after sumatriptan administration (mean BP_{ND}±SD 1·20±0·20 vs. 1·02±0·22, p = 0·0001) (figure 2). This decrease in binding corresponded to a mean drug occupancy±SD of 16·0±5·3%. Migraine patients had reduced 5-HT_{1B} receptor binding during migraine attacks (ictal state) compared to the interictal state (mean BP_{ND}±SD 1·63±0·22 vs. 1·20±0·20, p = 0·019).

Headache data

All patients fulfilled criteria for migraine during the ictal scan and the cilostazol-induced migraine attacks mimicked the patients' usual headache characteristics (Supplementary Table S1). AUC for the headache intensity was significantly lower during the sumatriptan scan compared to the baseline scan ($AUC_{0\text{-}90\ min}$, p = 0·008) (figure 3). For two participants (subject 53832 and 55063), the headache still fulfilled the criteria for migraine during the postictal scan.

Discussion

A novel key finding of the present study is that administration of clinical relevant doses of sumatriptan during a migraine attack significantly reduces 5-HT_{1B} receptor binding corresponding to an occupancy of 16% at central 5-HT_{1B} receptors. These data suggest that sumatriptan crosses the blood-brain barrier (BBB) and binds to central 5-HT_{1B} receptors. Further, we demonstrate that during pharmacologically induced migraine attacks, migraine patients have lower 5-HT_{1B} receptor binding in pain-modulating regions as compared to

outside of migraine attacks. These data suggest ictal increases in endogenous brain 5-HT levels.

Brain 5-HT_{1B} receptor occupancy of sumatriptan

When given during the migraine attack, subcutaneously administered sumatriptan in clinically relevant doses was associated with a 16% reduction in 5-HT_{1B} receptor binding. This suggests that sumatriptan crosses the blood-brain barrier and binds to central 5-HT_{1B} receptors during migraine attacks. Previously, drug occupancies of triptans have only been investigated for zolmitriptan in healthy volunteers.[9] Of note, modest receptor occupancies do not exclude a significant clinical effect, particularly not when dealing with agonist compounds. For example, opioids exert clinical effects at occupancies < 10 %[21] whereas 5-HT_{1A} receptor agonists induced central, serotonergic side effects without significant occupancies.[22] Since a high intrinsic activity has been demonstrated for sumatriptan,[23] the occupancies found in this study may be sufficient for antimigraine effects. A previous PET study found that administration of 6 mg sumatriptan sc. decreased 5-HT synthesis rate in the brain. An effect which was not related to reduction of pain intensity.[24] In the present study, both pain intensity and 5-HT_{1B} receptor binding were reduced in all participants after sumatriptan administration, but the migraine attack was only terminated in six out of eight patients. We can only speculate whether the therapeutic effect of sumatriptan was related to activation of the central 5-HT_{1B} receptors during attacks and to which extent our results also apply to healthy individuals or outside migraine attacks. Thus, even though we here provide evidence that sumatriptan accesses the brain parenchyma and binds to central 5-

HT_{1B} receptors, the antimigraine efficacy of sumatriptan may be mediated through other sites of action than central 5-HT_{1B} receptors.

We cannot completely exclude that the demonstrated drug occupancy could be partly caused by residual, elevated levels of endogenous 5-HT. A continuous increase in endogenous 5-HT even after sumatriptan administration could explain the relatively high occupancies (18·6 and 20·8 %) in the two patients that still experienced migraine during the postictal scan. On the other hand, two participants did not exhibit reductions in 5-HT_{1B} receptor binding during attacks but had a large decrease after sumatriptan (supplementary figure S1). Further, the ictal reductions in 5-HT_{1B} receptor binding were larger for the pain modulating regions compared to neocortex whereas the occupancy rates after sumatriptan administration were similar in these two regions (data not shown). Collectively, this speaks against a significant contribution of residual binding of 5-HT to the occupancy.

Brain serotonin levels during migraine attacks

We interpret the decrease in 5-HT_{1B} receptor binding during the migraine attack as being caused by a migraine-initiated acute increase in brain 5-HT levels, which leads to displacement of [^{11}C]AZ10419369 binding. This interpretation is consistent with a previous PET study demonstrating an increase in brain 5-HT synthesis during attacks.[24] Another study found attack-related normalization of visual and auditory evoked potentials compared to the interictal state which was interpreted as increases in central serotonergic activity.[25] The question remains, whether an increase in brain 5-HT levels is a cause or a consequence of migraine pain. Pharmacological interventions promoting release of 5-HT in the brain[26] as well as administration of m-chlorophenylpiperazine (mCPP),[27] a 5-HT_2 receptor agonist, is

known to provoke migraine attacks, supporting that an increase in brain 5-HT can elicit a migraine attack. Since 5-HT has higher affinity for the 5-$HT_{1B/1D}$ receptor compared to the 5-HT_{2A} receptor, it has been hypothesized that when present in low concentrations 5-HT binds to the antinociceptive 5-$HT_{1B/1D}$ receptor but as 5-HT concentrations increase, 5-HT exerts its actions on the pronociceptive 5-HT_{2A} receptor.[28] We suggest that migraine attacks are partially initiated by increases in endogenous 5-HT which leads to a shift in pain modulation with decreased pain inhibition and increased pain facilitation.

Methodological considerations

When using a reference tissue model for quantifying the BP_{ND} in occupancy studies, it is important to assess whether drug administration affects the reference region (cerebellum) used for quantification of binding of the radiotracer.[29] We detected borderline changes in cerebellar radiotracer uptake in migraine patients during migraine attacks as compared to outside of migraine attacks (p = 0·06, supplementary table S2). However, uptake in cerebellum did not change after sumatriptan administration (p = 0·29, supplementary table S2). Based on this, we have no reason to believe that the change in uptake seen in migraine patients is due to specific binding in cerebellum. Instead, it could be caused by a global effect of cilostazol. In animal models, cilostazol enhances BBB integrity.[30,31] An increase in BBB integrity could potentially reduce uptake of the radiotracer and thus the non-displaceable distribution volume, V_{ND}. However, if V_{ND} decreases between the two conditions (interictal and ictal), we would overestimate the BP_{ND} for the ictal scan and thus underestimate the change in BP_{ND} between the two conditions. We acknowledge that there may be differences between normal attacks and those emulated in this study. However, all

migraine attacks mimicked the patients' normal migraine attacks, and we believe it plausible to extrapolate our findings to spontaneous migraine attacks.

Conclusion

We provide evidence that sumatriptan, when given in clinical relevant doses, crosses the BBB and binds to central 5-HT_{1B} receptors. Due to the relatively low lipophilicity of sumatriptan compared to the other triptans,[32] we find it plausible that this can be extrapolated to all triptans. Whether activation of central 5-HT_{1B} receptors is necessary for the antimigraine efficacy of triptans remains to be determined. Further, we demonstrate that brain 5-HT levels are increased during migraine attacks, indicating that migraine attacks may be partly triggered by increases in endogenous brain 5-HT.

Panel: Research in context

Evidence before this study

It is a matter of ongoing debate whether triptans, 5-$HT_{1B/1B}$ receptor agonists, exert their antimigraine effects inside or outside of the CNS. The presence of 5-HT_{1B} receptors in the human brain and CNS side effects reported by patients using triptans indicate possible central mechanisms. While the triptans were originally thought to act mainly through vasoconstriction, several animal studies have shown that sumatriptan in the CNS can elicit pharmacological responses compatible with an antimigraine effect. However, to which extent sumatriptan has access to brain parenchyma in the human living brain remains unknown.

We searched PubMed for human studies published in English any time before January 1st 2016, with the terms “sumatriptan and blood-brain barrier”; “sumatriptan and CNS”; “sumatriptan and brain”; “sumatriptan and central effects”; “sumatriptan and central binding”. Articles from the reference lists were also included. Previous studies investigating central effects of sumatriptan in migraine patients found that administration of clinical relevant doses reversed an attack-related increase in brain 5-HT synthesis, whereas no effect of sumatriptan was detected on cerebral vessel diameter when administered during a spontaneous migraine attack. Additionally, sumatriptan had no effect on migraine attack-related activation in the brainstem and hypothalamus.

In healthy volunteers, sumatriptan reversed pain-induced activation in several regions of the brain. Further, sumatriptan was shown to inhibit functional trigemino-cortical connectivity and to activate regions of the brain involved in the affective dimension of pain.

No previous study has assessed the occupancy of sumatriptan to central 5-HT_{1B} receptors in either migraine patients or healthy volunteers, but the brain penetrance of zolmitriptan, a more lipophilic triptan, has been assessed with findings of rapid brain uptake and an occupancy of 4·5%.

Added value of this study

This is the first study to provide direct evidence that sumatriptan crosses the blood-brain barrier, enters the brain parenchyma and binds to central 5-HT_{1B} receptors in migraine patients. We find that clinical relevant doses of sumatriptan significantly decrease 5-HT_{1B} receptor binding in migraine patients with an occupancy of 16%. This is in line with the occupancy needed for an agonist to exert central effects.

Implications of all the available evidence

We here show that sumatriptan binds to 5-HT_{1B} receptors in the CNS. In combination with the findings from previous studies, this indicates a central mechanism of action of sumatriptan. Future studies should investigate whether activation of central 5-HT_{1B} receptors is needed for the antimigraine effects of sumatriptan.

Contributors

MD contributed to study design, protocol development, participant enrolment, data acquisition, processing, and analysis, statistics, interpretation of results, and drafting and revision of the paper. AH contributed to study design, protocol development, statistics, interpretation of results, and critical review of the paper. HDH contributed to study design, protocol development, data acquisition, interpretation of results, and critical review of the paper. MS contributed to data analysis, interpretation of results, and drafting and revision of the paper. AD contributed to data acquisition and analysis, and critical review of the paper. GMK contributed to study design, protocol development, interpretation of results, and drafting and revision of the paper. MA initiated the study and contributed to study design, protocol development, interpretation of results, and drafting and revision of the paper.

Conflicts of interest

Messoud Ashina is a consultant and/or scientific adviser/ speaker for the ATI, Allergan, Amgen, Alder and Eli Lilly. All other authors declare no conflicts of interest and report no financial disclosures.

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Figures

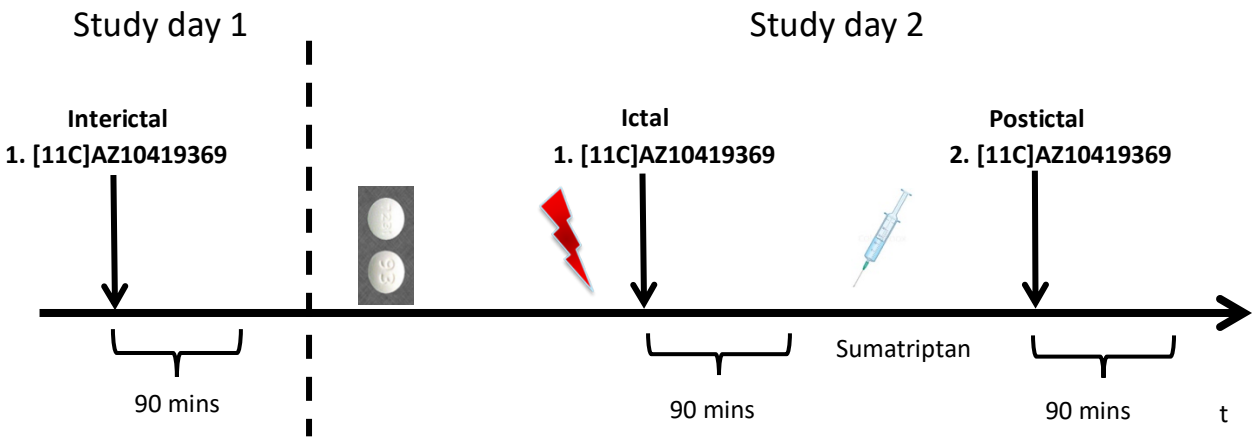


Figure 1. Study design

The median time between study day 1 and 2 was 219·5 days (range 2-326 days). On study day 2, the median time from cilostazol ingestion to scan 1 was 5 hours (range 3·5 – 7·5 hours). The median time between sumatriptan administration and scan 2 was 43 minutes (range 33 – 48 minutes). The pills illustrate cilostazol, whereas the red flash indicates migraine attack. t = time.

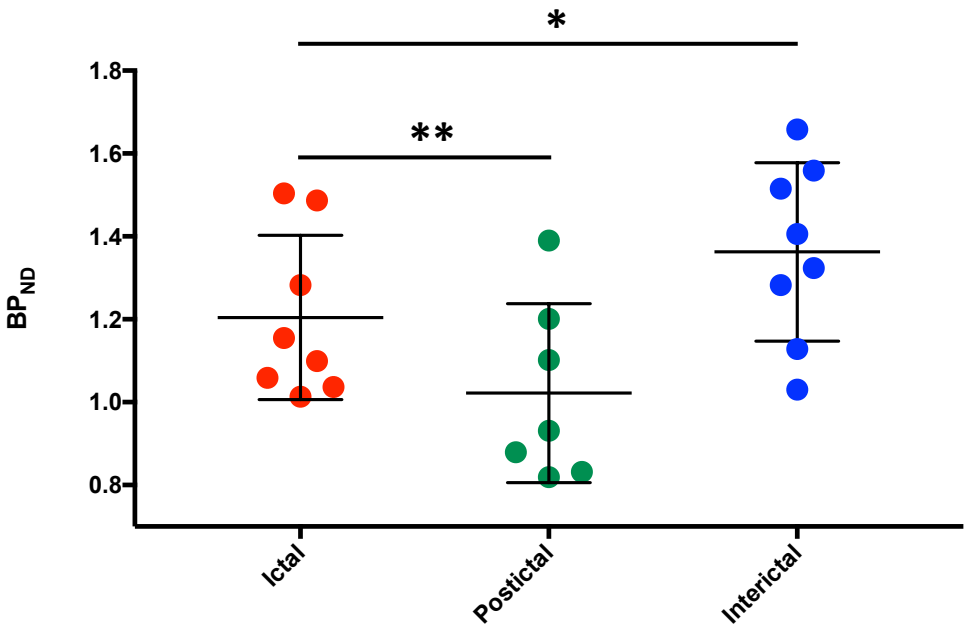


Figure 2. Changes in 5-HT$_{1B}$ receptor binding in migraine patients

5-HT$_{1B}$ receptor binding decreased across seven pain modulating regions (dorso- and ventrolateral prefrontal cortex, orbitofrontal cortex, anterior cingulate cortex, sensorimotor cortex, insula and amygdala) after sumatriptan administration. During migraine attacks, binding was decreased compared to on a migraine-free day. BP$_{ND}$ = mean volume weighted non-displaceable binding potential. Black bars indicate mean±SD. *p < 0·05. **p < 0·001.

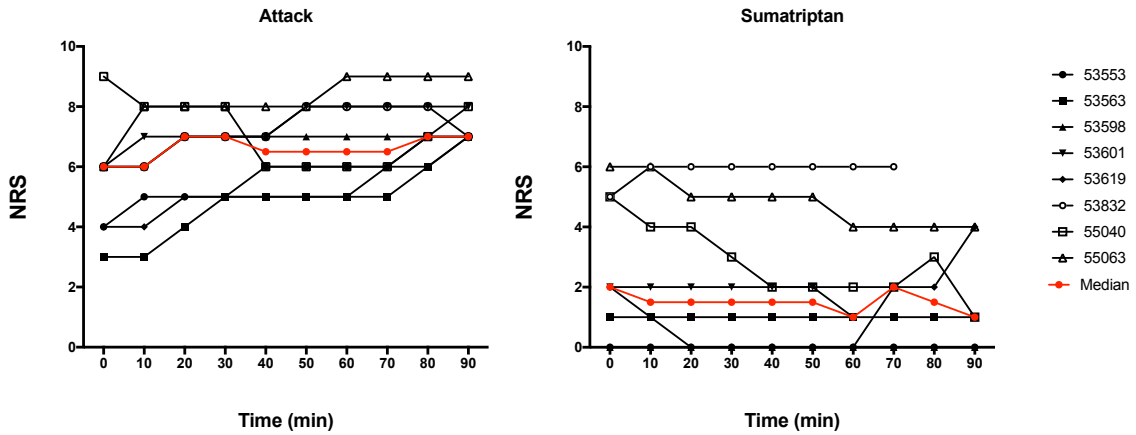
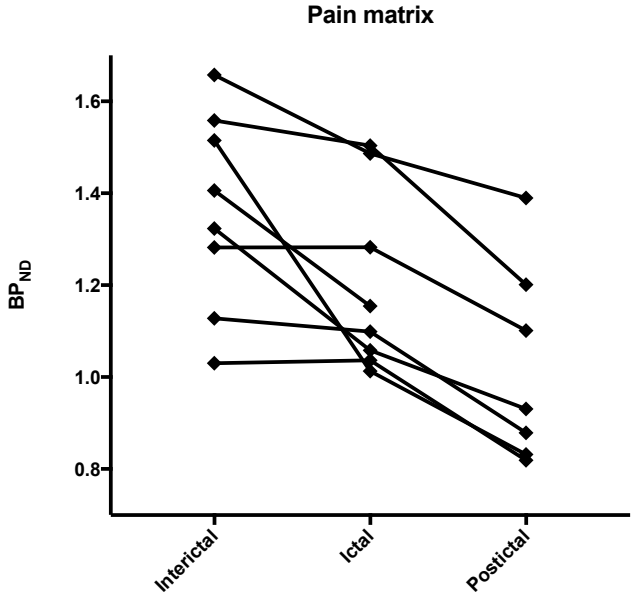


Figure 3. Headache scores measured from radioligand injection and throughout the PET scan

Black bars indicate individual headache scores for each subject. Red line indicates median. NRS = Numerical Rating Scale.

Supplementary

Supplementary figure S1. Individual changes 5-HT_{1B} receptor binding.



Supplementary table S1. PET variables

Migraine patients (n=8)					
	Interictal	**Ictal**	**Postictal**	**Ictal vs. interictal**	**Ictal vs. postictal**
AZ injected mass per kg (microgram/kg)	0.01±0.01	0.01±0.004	0.02±0.02	p = 0.48	p = 0.31
AZ Time-normalized AUC-CB (Bq/ml)/AZ Inj. Dose (MBq)	10.1±2.08	8.8±1.6	8.5±1.5	p = 0.06	p = 0.29

AUC = Area Under the Curve.

Supplementary table S2. Clinical characteristics of spontaneous and cilostazol induced migraine attacks

Subject	Migraine	Unilateral/ bilateral	NRS	Pulsating	Worsens with activity	Nausea	Vomit	Photo	Phono
53553	Spon	U	7	1	1	1	1	1	1
	Cilo	U	7	1	1	0	0	1	1
53563	Spon	U	8	1	1	1	0	1	0
	Cilo	B	7	1	1	1	0	1	0
53598	Spon	B	7	0	1	1	0	1	1
	Cilo	B	7	0	1	1	1	1	1
53601	Spon	U	8	1	1	1	1	1	1
	Cilo	B	8	1	1	1	0	1	1
53619	Spon	B	8	1	1	1	0	1	1
	Cilo	B	7	1	1	1	0	1	1
53832	Spon	B	9	1	1	1	1	1	1
	Cilo	B	8	1	1	1	0	1	1
55063	Spon	U	8	1	1	1	0	1	1
	Cilo	U	9	1	1	0	0	1	1
55040	Spon	U	8	0	1	0	0	1	1
	Cilo	U	8	0	1	0	0	1	1

Spon = Spontaneous. Cilo = Cilostazol induced. U = Unilateral. B = Bilateral. 1 = Yes. 0 = No.

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Title of PhD thesis:
PET investigations of brain serotonin receptor binding in migraine patients

This declaration concerns the following article:
High brain serotonin levels in migraine between attacks: A 5-HT4 receptor binding PET study

The PhD student's contribution to the article: *(please use the scale (A,B,C) below as benchmark*)*	(A,B,C)
1. Formulation/identification of the scientific problem that from theoretical questions need to be clarified. This includes a condensation of the problem to specific scientific questions that is judged to be answerable by experiments	B
2. Planning of the experiments and methodology design, including selection of methods and method development	C
3. Involvement in the experimental work	C
4. Presentation, interpretation and discussion in a journal article format of obtained data	C

**Benchmark scale of the PhD student's contribution to the article*		
A. refers to:	*Has contributed to the co-operation*	*0-33 %*
B. refers to:	*Has contributed considerably to the co-operation*	*34-66 %*
C. refers to:	*Has predominantly executed the work independently*	*67-100 %*

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This declaration concerns the following article:
Low 5-HT1B receptor binding in the migraine brain: A PET study

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PET investigations of brain serotonin receptor binding in migraine patients

This declaration concerns the following article:
Sumatriptan crosses the blood-brain barrier in migraine patients and binds to central 5-HT1B receptors

The PhD student's contribution to the article: *(please use the scale (A,B,C) below as benchmark*)*	(A,B,C)
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