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PhD Thesis

Jan Kalbitzer

The Serotonin Transporter and Behavior

Gene*Environment Interactions

For my family

Institute: Neurobiology Research Unit

Department: Department of Neurology

Institution: University of Copenhagen and Rigshospitalet

Author: Jan Kalbitzer

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Principal supervisor: Professor Gitte M. Knudsen, Neurobiology Research Unit, Department of Neurology, Rigshospitalet, Denmark

Supervisor: Professor Erik L. Mortensen, Department of Environmental Health, Institute of Public Health, University of Copenhagen, Denmark

Supervisor abroad: Professor Morten L.Kringelbach, Department of Psychiatry, University of Oxford, United Kingdom (10/2007 – 12/2007 and 8/2008 – 2/2009)

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Jan Kalbitzer

Summary

The association between the cerebral serotonin system and human behavior is only incompletely understood. Great emphasis has been placed on the serotonin transporter, which is responsible for the regulation of the interstitial serotonin levels in the brain. The aim of this thesis was to uncover the association between the serotonin transporter and serotonin 2A receptors, as measured *in vivo* with [^{11}C]DASB/[^{18}F]altanserin and positron emission tomography, and genetic, environmental, and personality characteristics. The studies were conducted in large cohorts of healthy participants who underwent personality assessments with a well-known self-administered questionnaire: the Revised Neuroticism, Extraversion, Openness Personality Inventory (NEO-PI-R). Furthermore, blood samples from the participants were used for determination of a common polymorphism in the promoter region of the serotonin transporter gene, the 5-HTTLPR, and of a common single-nucleotide polymorphism in the CLOCK gene, 3111T/C, which is involved in the regulation of circadian rhythm.

Participants with lower serotonin transporter binding, and therefore presumably higher interstitial serotonin levels, had a more open personality; this finding was not directly associated with specific alleles of the 5-HTTLPR. A circannual fluctuation in serotonin transporter binding was observed, with highest levels during the winter, and lower levels in summertime. In the putamen, a season*genotype interaction was observed: serotonin transporter binding only varied with season in carriers of the short 5-HTTLPR allele. I then explored if a genetic polymorphism related to the circadian rhythm, the 3111T/C, was associated with serotonin pre- or postsynaptic binding *in vivo* but no effect on circannual fluctuations in serotonin transporter binding or serotonin 2A receptor binding was observed.

In conclusion, the *in vivo* cerebral serotonin transporter binding is related to the openness versus rigidity of the personality. The serotonin system in carriers of the short 5-HTTLPR allele is more sensitive to a particular environmental stressor, seasonal fluctuations in daylight times.

Dansk Resumé

Forbindelsen mellem det cerebrale serotonin system og menneskelig adfærd er uklart belyst. Der har været meget fokus på serotonin transporteren, som er ansvarlig for reguleringen af de interstitielle serotonin niveauer i hjernen. Formålet med denne afhandling var, at forstå forbindelsen mellem serotonin tranporteren og serotonin 2A receptorer gennem in vivo [^{11}C]DASB/[^{18}F]altanserin og positron emission tomografi, samt genetiske, miljømæssige og personligheds karakteristikker. Undersøgelserne blev udført i store kohorter af raske kontrolpersoner, som blev personlighedstestet med et velkendt selvrapporterings spørgeskema: The Revised Neuroticism, Extraversion, Openness Personality Inventory (NEO-PI-R). Derudover blev deltagernes blodprøver brugt til at bestemme en almindelig polymorfi af serotonin transporter genet, 5-HTTLPR, samt en gængs enkelt-nukleutid polymorfi i CLOCK genet, 3111T/C, som er involveret i reguleringen af døgnrytme.

Deltagere med lav serotonin transporter binding og derfor formentlig højere interstitielle serotonin niveauer, havde en mere åben personlighed; dette fund var ikke direkte relateret til specifikke 5-HTTLPR alleler. Der blev observeret en cirkannual fluktuation i serotonin transporter bindingen, i form af højere niveauer om vinteren og lavere niveauer om sommeren. I putamen blev der observeret en sæson*genotype interaktion: serotonin transporter bindingen varierede kun i forhold til sæson blandt bærere af det korte 5-HTTLPR allel. Derefter undersøgte vi om en genetisk polymorfi, som er relateret til døgnrytme, 3111T/C, var relateret til serotonin pre- eller postsynaptisk binding *in vivo*, men fandt ingen effekt på cirkannual fluktuationer i serotonin transporter binding eller serotonin 2A receptor binding.

Konklusion: in vivo cerebral serotonin transporter binding er relateret til åbenhed vs. rigiditet i personligheden. Serotonin systemet i bærere af det korte 5-HTTLPR allel er mere sensitivt over for en bestemt miljømæssig stressor, nemlig sæsonmæssig fluktuatione i mængden af dagslys.

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I. Jan Kalbitzer, Claus Svarer, Vibe G. Frokjaer, David Erritzoe, William F. C. Baaré, Jacob Madsen, Steen G. Hasselbalch ,and Gitte M. Knudsen. A probabilistic approach to delineate functional brain regions. J Nucl Med Technology, accepted

II. Jan Kalbitzer, Vibe G. Frokjaer, David Erritzoe, Claus Svarer, Paul Cumming, Finn Å. Nielsen, Sayed H. Hashemi, William F. C. Baaré, Jacob Madsen, Steen G. Hasselbalch, Morten L. Kringelbach, Erik L. Mortensen, and Gitte M. Knudsen. The personality trait openness is related to cerebral 5-HTT levels. NeuroImage, in press

III. Jan Kalbitzer, David Erritzoe, Klaus K. Holst, Finn Å. Nielsen, Lisbeth Marner, Szabolcs Lehel, Tine Arentzen, Terry L. Jernigan, and Gitte M. Knudsen. Seasonal changes in brain serotonin transporter binding in short 5-HTTLPR-allele carriers but not in long-allele homozygotes. Submitted to Biological Psychiatry on February 19 2009. Preliminary results are available at Nature Preceedings

IV. Jan Kalbitzer, David Erritzoe, Klaus K. Holst, William F. C. Baaré, Szabolcs Lehel, Erik L. Mortensen, and Gitte M. Knudsen. 3111T/C Clock SNP does not regulate responsiveness of the serotonin system to environmental changes. Manuscript in preparation for submission

List of Abbreviations

5-HT = 5-Hydroxytryptamine = Serotonin

5-HTT = 5-Hydroxytryptamine transporter = Serotonin transporter

5-HTTLPR = 5-Hydroxytryptamine transporter linked polymorphic region

ATD = Acute tryptophan depletion

ATP = adenosine triphosphatase

BBB = Blood brain barrier

BP_{ND} = Non-displaceable tracer binding

CSF = Cerebrospinal fluid

CNS = Central nervous system

[^{11}C]DASB = 3-amino-4-(2[^{11}C]methylaminomethylphenylsulfanyl)benzonitrile

DRN = Dorsal raphé nucleus

GABA = gamma-Aminobutyric acid

GDP = Guanosine-5'-diphosphat

GTP = Guanosine-5'-triphosphate

(f)MRI = (functional) Magnetic resonance imaging/image

OtExperience = Openess to experience

OtActions = Openness to action

OtValues = Openness to values

PET = Positron emission tomography

SNP = Single-nucleotide polymorphisms

SPM = Statistical parametric mapping

SAD = Seasonal affective disorder

S-/L-Allele = Short / long 5-HTTLPR allele

SSRI = Selective serotonin-reuptake inhibitor

TAC = Time activity curve

TRP = Tryptophan

VNTR = Variable number of tandem repeats

VOI = Volume of interest

Introduction

The aim of this thesis was to deepen understanding of how the status of the cerebral serotonin system affects human personality. My personal interest in this question began with several visits to northern Sweden between 2000 and 2005, while I was still a medical student. I then became interested in the question of how the strong seasonal variations in geographical regions of high latitude affect the biological system and how they may influence personality with regard to vulnerability to stress. I also became interested in the behavioral strategies used to compensate for the strong environmental effects of high latitude.

The serotonin system and circannual changes in environmental variables, such as minutes of daylight, provide an ideal model to test theories about gene*environment interaction effects on personality. In my thesis, the main focus was on the interaction effect of a genetic polymorphism upstream to the transcription start site of the serotonin transporter gene and seasonal variations in daylight time on cerebral serotonin transporter binding and the association between serotonin transporter binding and personality.

The first half of the present introduction to the original research manuscripts and publications consists of a review of the scientific literature (including the most recent findings) that led to the formulation of the specific scientific problems, or hypotheses and aims, which were to be tested. The second half of this introduction focuses on the methods I used, gives a short overview of the results of my studies, and concludes with a general discussion of the outcomes and interpretations drawn from the various results.

To make this thesis as accessible as possible to readers unversed in the particulars of neuroscience, I try to explain important principles in detail. Furthermore, I employ abbreviations only where they are absolutely necessary, for example, in the case of *5-hydroxytryptamine transporter linked polymorphic region* (abbreviated as 5-HTTLPR). I provide a list (above) of the terms which are often abbreviated in scientific articles (including my own), for the sake of brevity, but at the expense of clarity.

The Serotonin System

Serotonin was first identified (but not isolated) by Vittorio Erspamer in his laboratory in Pavia, Italy, in the first half of the twentieth century. Erspamer and a colleague found a physiologically-active substance in the enterochromaffin cells of the gastrointestinal mucosa, which Erspamer called enteramine (Erspamer and Asero 1952). A few years later, in the late 1940s, Maurice Rapport, Arda Green and Irvine Page isolated what proved to be the same substance from peripheral blood; not knowing that it was identical to enteramine, they called it serotonin, referring to its peripheral vasoconstrictive effects (Rapport, Green et al. 1948; Rapport, Green et al. 1948). In the early1950s, Betty Twarog, in collaboration with Irvine Page, suggested that serotonin acted as a neurotransmitter (Twarog and Page 1953).

Since then the field of serotonin research has developed enormously; a keyword search of Pubmed with serotonin and brain has nearly 40,000 results, with half of the studies being published since 1992. Neuroimaging techniques such as positron emission tomography made it possible to visualize biochemical aspects of the serotonin system in the living human brain. Genetic studies of large populations have linked variations in genes coding for transporters, receptors and enzymes involved in serotonergic neurotransmission to physiological and pathological variations of human phenotypes and intermediate phenotypes (endophenotypes) (Gottesman and Gould 2003; Meyer-Lindenberg and Weinberger 2006).

I will begin this review with an overview of the physiology and structure of the serotonin system, and of the ways to study the association between serotonin and human behavior. Subsequently, I will discuss the spectrum of behaviors which are generally postulated to be associated with serotonin, the serotonin transporter, and the 5-HTTLPR, a common polymorphism in the promoter region of the serotonin transporter gene.

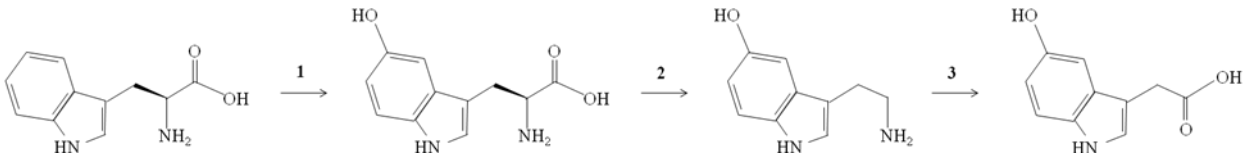
Serotonin: Chemical Structure, Synthesis, and Transport

Serotonin, also known as 5-hydroxytryptamine, is classically classified as a biogenic monoamine. In common with other biogenic monoamines, which also include catecholamines

such as dopamine, as well as histamine and melatonin, serotonin contains an ethylamine moiety linked to an aromatic ring system. All biogenic monoamines are derived from aromatic amino acid precursors. As illustrated in Figure 1, serotonin is synthesized in serotonergic cells from the aromatic amino acid tryptophan by the successive actions of tryptophan hydroxylase and aromatic amino acid decarboxylase.

Serotonin is then itself metabolized by monoamine oxidase, which yields the only known serotonin metabolite, 5-hydroxyindoleacetic acid. Serotonin has low affinity for monoamine oxidase B, which is nonetheless present inside the serotonergic neurons (Vincent 1989), such that it is assumed that serotonin is mainly metabolized by monoamine oxidase A, following the release to the extracellular space, and transfer to some other cellular compartment. Upon being synthesized inside the serotonergic cell, cytosolic serotonin it is taken up into synaptic vesicles by a vesicular monoamine transporter, and is stored within vesicles until exocytotic release of the vesicular contents. The synaptic action of serotonin is terminated by re-uptake into the cell by the serotonin transporter or by oxidative deamination, as catalyzed by monoamine oxidase (Stahl 2008).

The large neutral amino acid *L*-tryptophan, but not serotonin itself, can pass the blood brain barrier. *L*-Tryptophan is transported through the blood brain barrier by a large neutral amino acid transporter (Pardridge and Oldendorf 1975), by a facilitated diffusion process in competition with perhaps ten other large neutral amino acids, including valine, isoleucine, leucine, tyrosine, and phenylalanine (Stahl 2008). The rate of tryptophan uptake to the brain is consequently dependent on the concentration of tryptophan relative to the other large neutral amino acids in blood. This principle can be exploited by scientists for the experimental depletion of tryptophan, and thereby serotonin (discussed below).



L-Tryptophan 5-Hydroxy-*L*-Tryptophan Serotonin 5-Hydroxyindoleacetic

Figure 1: Fischer projections of serotonin synthesis through the action of tryptophan hydroxylase (1) and amino acid decarboxylase (2) and metabolization to 5-hydroxyindoleacetic acid through the action of monoamine oxidase (3).

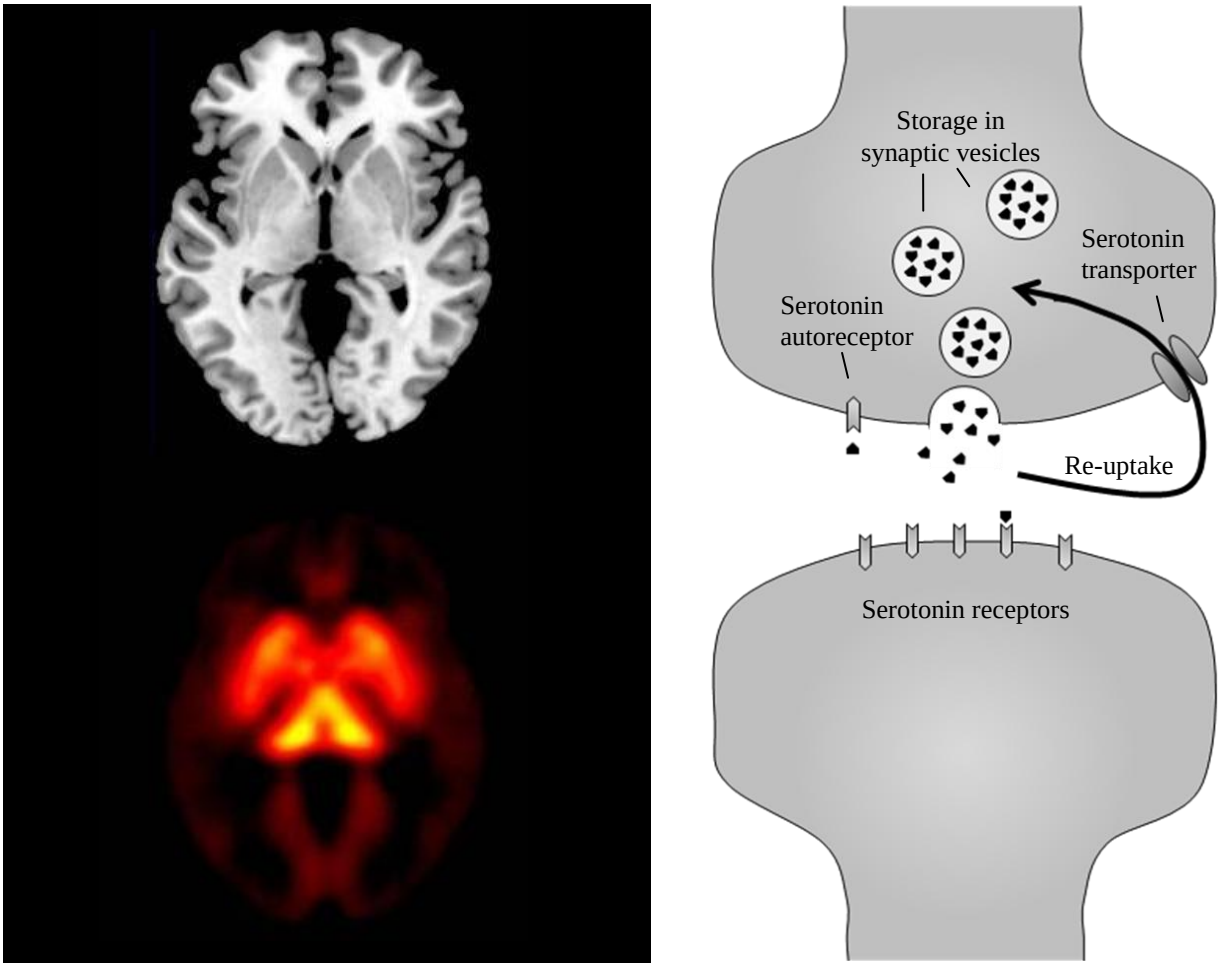


Figure 2: The left image shows a structural brain image and a positron emission tomography image representing specific binding to the serotonin transporter. For illustration purposes, the images are warped to a standard space. The right figure illustrates the role of the serotonin transporter in serotonergic neurotransmission: Serotonin is stored in vesicles until it is released to the extracellular space; here it affects presynaptic and postsynaptic serotonin receptors. The serotonin transporter terminates the action by re-uptake into the serotonergic cell.

Serotonergic Neurons / the Raphé Nuclei

The location of the serotonergic cell bodies in the brain is strictly limited to the brain stem. However, serotonergic fibers project to nearly every part of the brain. Most of the serotonergic cell bodies (soma) are clustered around the midline of the brain stem (Jacobs and Azmitia 1992; Hornung 2003). These clusters are referred to as the raphé nuclei (Greek: ραφή = seam, referring

to the location of the nuclei around the midline of the brainstem). The raphé nuclei are divided in two groups, the superior brain stem group and the inferior brain stem group.

The superior raphé group is divided into four main clusters of neurons: the *caudal linear nucleus*, the *median raphé nucleus*, the *nucleus pontis centralis oralis*, and the *dorsal raphé nucleus*. The superior group sends ascending and descending innervations of nearly the entire brain, subserving some 85% of the total serotonin production in the central nervous system (Jacobs and Azmitia 1992; Hornung 2003). The inferior group consists of five nuclei: the *nucleus raphé obscurus*, the *nucleus raphé pallidus*, the *nucleus raphé magnus*, the *lateral paragigantocellular nucleus*, and the *intermediate reticular nuclei*. From this group arises a descending innervation of the medulla and spinal cord, accounting for the remaining 15% of the total serotonin production in the central nervous system (Jacobs and Azmitia 1992; Hornung 2003). With respect to the role of serotonin in aspects of behavior and personality, the superior group of the raphé nuclei is of primary interest in the present dissertation.

The serotonergic fibers arising in the superior group of the raphé nuclei project through the medial forebrain bundle to the cortex, the basal ganglia, the limbic system and the diencephalon (Tork 1990). Serotonin is released from vesicles into the extracellular space, where it can bind to the 14 subtypes of serotonin receptors known to be expressed in the brain (Barnes and Sharp 1999). The more rostral division of the superior group, which includes the *dorsal raphé nucleus* and the *caudal linear nucleus*, innervates the basal ganglia motor system and the cerebral cortex. The more caudal division of the superior group, which includes the *median raphé nucleus* and interfascicular parts of the *dorsal raphé nucleus*, innervates the amygdala. The raphé nuclei send extensive efferent fibers to the hypothalamus. Different pathways have been described in different species, including the medial forebrain bundle and the dorsal raphé cortical tract (Jacobs and Azmitia 1992).

The superior group of the raphé nuclei receives afferents in the form of recurrent collaterals of ascending serotonergic axons. Furthermore, the superior group receives acetylcholinergic afferents from the medial longitudinal fasciculus, adrenergic afferents from the dorsolateral medulla, noradrenergic afferents from the locus coeruleus and subcoeruleus, dopaminergic

afferents from the substantia nigra, and various other inputs from the periaqueductal gray. The raphé nuclei also receive afferents from the limbic forebrain, with direct innervation of the serotonergic neurons, and also via gamma-Aminobutyric acid (GABA)ergic interneurons.

Serotonin Receptors

Serotonin receptors are, with a single exception, guanine nucleotide-binding protein (G-protein) coupled receptors. G-protein coupled receptors are integral membrane proteins that sense molecules outside the cell and activate a specific signal transduction pathway inside the cell, with a key step mediated by the agonist-induced exchange of Guanosine-5'-diphosphate (GDP) for Guanosine-5'-triphosphate (GTP), and subsequent dissociation of the GTP-bound G-protein. Affinity state of G-protein receptors is influenced by the equilibrium between GTP- and GDT-bound G-protein, which is in turn, influenced by the inherent GTP-ase activity of the G-protein-GTP complex. The exception is the serotonin 3 receptor, which is a ligand-gated cation-channel (Barnes and Sharp 1999).

In anatomical terms, serotonin receptors can be divided in two groups: presynaptic receptors and postsynaptic receptors. Once serotonin is released to the extracellular space, presynaptic receptors allow a direct self-regulatory feedback of the serotonergic cell through mechanisms such as blockade of further serotonin release (serotonin 1B/D receptors) and reduced firing rate of the serotonergic cell (serotonin 1A receptors) (Barnes and Sharp 1999; Stahl 2008).

The number of known postsynaptic receptors currently stands at 14; their function, and the need for such diversity, is scarcely understood. Postsynaptic serotonin 1A receptors, like their presynaptic counterparts, have an inhibitory action at cortical pyramidal neurons, whereas serotonin 2A receptors are excitatory to pyramidal neurons. Serotonin 2A receptors enhance glutamate release from cortical projection fibers, and inhibit dopamine release from nigrostriatal fibers. These receptors have been implicated in sleep and cognition. Serotonin 1A receptors, although not abundant in the striatum, tend to enhance dopamine release. Roughly speaking, serotonin 1A receptors and 2A receptors have opposing effects on dopaminergic transmission. The other G-protein linked serotonin receptors, serotonin 4, 5, 6, 7 receptors, have several

subtypes (Barnes and Sharp 1999), and rather poorly-defined functions. Among these receptors, the serotonin 4 receptor has recently received attention (Duman 2007), because of the rapid increase of motor activity in animals exposed to stress after treatment with a selective serotonin 4 receptor agonist (Lucas, Rymar et al. 2007). Furthermore, serotonin 4 receptor binding is altered in several animal models of depression (Licht, personal communication). The recent development of a novel radio-labeled ligand that binds specifically to the serotonin 4 receptors (Kornum, Lind et al. 2009; Marner personal communication) will allow further *in vivo* investigations in humans and other animals.

The Serotonin Transporter

The serotonin transporter is an integral membrane protein with 12 transmembrane domains. The transporter mediates the facilitated exchange-diffusion of serotonin. In the brain, serotonin transporter concentration is highest in the midbrain, the basal ganglia, and the thalamus, intermediate in the limbic system and lowest in the cortex (Cumming and Gjedde 1993; Houle, Ginovart et al. 2000; Frankle, Slifstein et al. 2006). It has a crucial role in the regulation of the serotonin tonus and function. While monoamine oxidase catalyzes the eventual breakdown of serotonin, the serotonin transporters normally transport the preponderance of interstitial serotonin back into the serotoninergic neuron, where it can be re-sequestered in vesicles (Figure 2). As such, the local activity of the serotonin transporter probably influences the temporal and spatial extent of serotonin release events.

The driving force for the uptake of serotonin by the serotonin transporter is a co-transport with one sodium and one chloride ion (Figure 3), which is permitted due to the concentration gradient of these ions between the extracellular and intracellular space, which is established by the enzyme sodium/potassium adenosine triphosphatase (Na+/K+ ATPase) (Gu, Wall et al. 1994; Torres, Gainetdinov et al. 2003). In exchange, the serotonin transporter transports one potassium ion outwards, which makes the net charge transport stoichiometrically neutral (Figure 3).

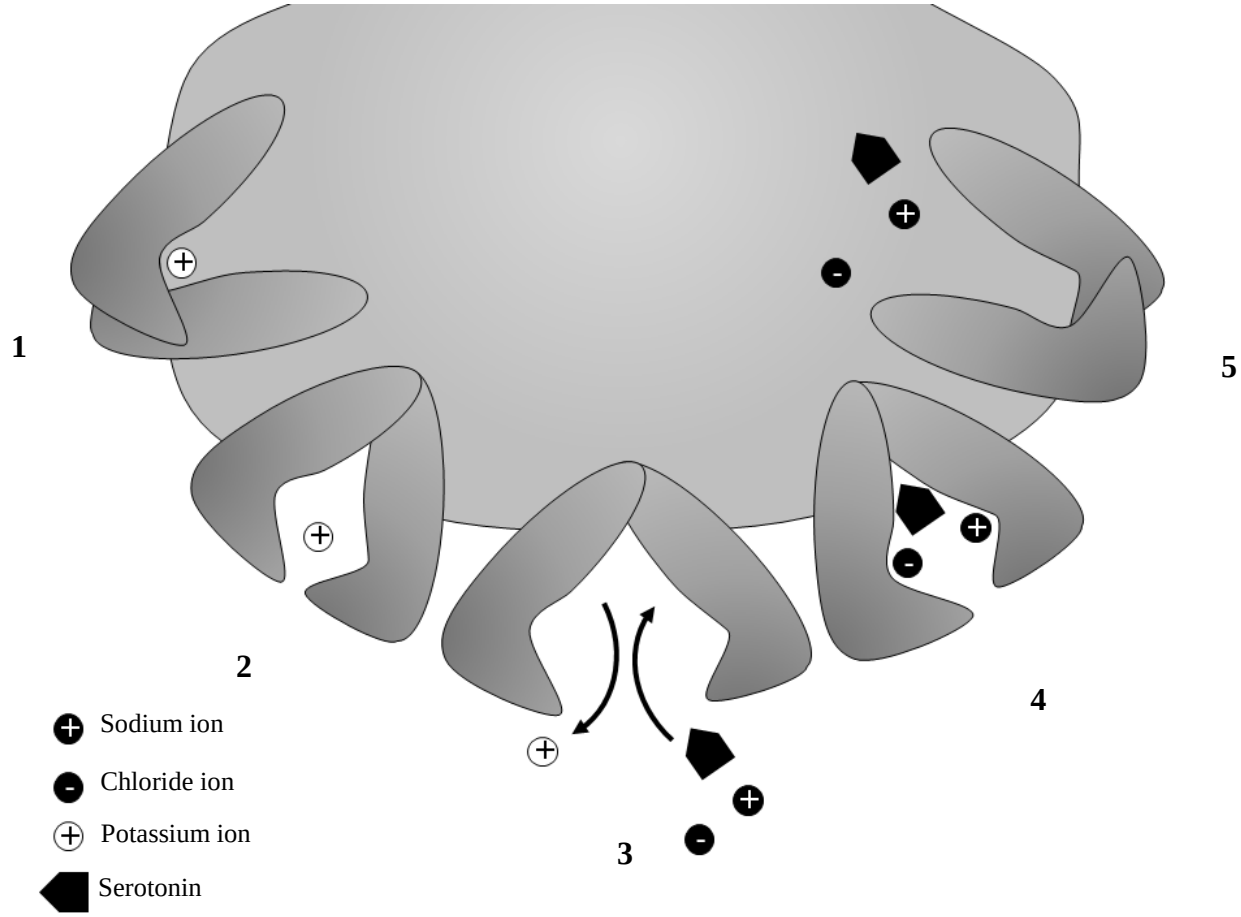


Figure 3: Schematic illustration of a possible mechanism of serotonin transport by the serotonin transporter. (1) The serotonin transporter is open at the intracellular side of the plasma membrane, where a potassium ion is able to bind to it. Once the potassium ion is bound, the serotonin transporter undergoes a series of conformational changes, closing access to the intracellular space (2). Subsequent release of the potassium ion exposes the binding site to the extracellular space (3). (4) Serotonin, together with a sodium (Na^+) and a chloride ion (Cl^-) binds to the serotonin transporter. Only when the binding site is occupied with serotonin, Na^+, and Cl^- (5), can occur a sequence of conformational changes causing the transporter to close in a manner exposing the binding site to the intracellular space. Then, all three bound entities (serotonin, sodium, and chloride) can dissociate and diffuse to the intracellular side of the plasma membrane. This 1:1:1:1 process is stoichiometrically neutral with the transport of two positive charges ($serotonin^+$ and Na^+) and one negative charge (Cl^-) into the cell, and efflux of one positive charge (K^+).

The Serotonin Transporter Gene and the 5-HTTLPR

The serotonin transporter gene (SLC6A4) is composed of 14 exons that span approximately 40,000 base-pairs, and maps to the human chromosome 17q11.2. Transcription of this gene leads to a protein composed of 630 amino acids (the serotonin transporter). Transcriptional activity of the SCL6A4 is modulated by a variation polymorphic region in the gene's major control region [serotonin (= 5-hydroxytryptamine = 5-HT) transporter (5-HTT)-linked polymorphic region = 5-HTTLPR].

The 5-HTTLPR is located upstream to the transcription start site, and contains or is located close to two single-nucleotide polymorphisms (SNPs): rs25531 (Nakamura, Ueno et al. 2000) and rs25532. The rs25531 single-nucleotide polymorphism (an A > G variation) has a minor allele frequency of 9-15% in Caucasians and 24% in African Americans and interacts with the 5-HTTLPR, so as to influence the rate of SLC6A4 transcription (Wendland, Martin et al. 2006). Additional variants in the SLC6A4 include a variable number of tandem repeats (VNTR) polymorphism in the functional second intron (Hranilovic, Stefulj et al. 2004) and a number of additional SNPs (Murphy and Lesch 2008).

The 5-HTTLPR occurs in short and long alleles, which differ with respect to inclusion of 44 base-pairs within the promoter sequence. The short 5-HTTLPR allele has an inherently lower rate of mRNA transcription and consequently lower expression of serotonin transporter in lymphoblast cell lines (Lesch, Bengel et al. 1996). Nonetheless, molecular imaging studies conducted in large cohorts have not identified lower serotonin transporter binding in the brain of short 5-HTTLPR allele carriers (Willeit, Stastny et al. 2001; Shioe, Ichimiya et al. 2003; van Dyck, Malison et al. 2004; Parsey, Hastings et al. 2006). More recent findings suggest that the rs25531 SNP is also associated with the amount of mRNA expression (Nakamura, Ueno et al. 2000). In two independent molecular imaging studies of healthy, non-depressed participants, cerebral serotonin transporter binding was higher among homozygotic carriers of the long 5-HTTLPR allele who also had the A variation at rs25531 (Praschak-Rieder, Kennedy et al. 2007; Reimold, Smolka et al. 2007).

Serotonin and Behavior

Studying Associations between Serotonin and Human Behavior

A number of methods are available for studying associations between serotonin and behavior. Animal experiments can consist of invasive tests, such as direct electrophysiological recordings from serotonergic neurons, or neurochemical lesions of sites of interest. Historically, research options for human studies have been limited. Early studies have measured serotonin metabolites in cerebrospinal fluid, for example in the studies by Ashcroft and colleagues (Ashcroft, Crawford et al. 1966), but this requires spinal puncture, and can yield variable results (Wilk and Green 1972). The most widely-used research techniques used today include (1) *post mortem* analyses, (2) acute tryptophan depletion, (3) treatment with selective serotonin re-uptake inhibitors, and (4) positron emission tomography studies.

Post Mortem Studies

Before the advent of positron emission tomography and other modern techniques for molecular imaging, *post mortem* neurochemical studies were one of the few ways to achieve direct knowledge about the serotonin system in the human brain. However, results from these studies often prove to be inconsistent. Uncontrollable factors include cause of death, and the necessary time delay, often 24 hours, between death and autopsy. Nonetheless, *post mortem* assays have the advantage of making possible the study of multiple receptors and chemical substances in the same person; positron emission tomography studies are restricted to a maximum of one or two molecular targets within an individual. Consequently, *post mortem* techniques can be used to map so-called neuroreceptor fingerprints, which refers to the abundances of diverse receptors in a particular anatomical site (Palomero-Gallagher, Mohlberg et al. 2008; Palomero-Gallagher, Vogt et al. 2008). Furthermore, the proportion of specific and non-specific binding of receptor ligands can be measured with some precision in *post mortem* autoradiographic blocking studies. In positron emission tomography studies, it is generally required to define a brain region devoid of specific binding (so called reference region). While this can be accomplished using

displacement methods *in vivo*, it is not always possible to administer a sufficient dose of blocking agents to humans for obtaining complete displacement of specific binding. Instead, a reference region can sometimes be identified on the basis of prior knowledge, obtained from *post mortem* studies. Therefore, *post mortem* studies remain a gold standard for the interpretation and standardization of data acquired with positron emission tomography.

Acute Tryptophan Depletion

Acute tryptophan depletion is used to induce acutely lower levels of serotonin in the brains of living humans. Tryptophan is a chemical precursor of serotonin, and, as noted above, competes with other large neutral amino acids for transport at the blood-brain-barrier. Consuming a liquid mixture of all the large neutral amino acids except tryptophan transiently lowers the relative tryptophan concentration in blood (Young, Smith et al. 1985). Given the nature of the competition at the blood-brain-barrier, tryptophan uptake to the brain is thus suppressed for a time, such that serotonin concentration is acutely depleted in the brain of non-human primates (Young, Ervin et al. 1989). It is assumed that behavioral changes induced by acute tryptophan depletion are caused by acutely lower serotonin concentrations in the brain.

Treatment with Selective Serotonin Re-Uptake Inhibitors

Selective serotonin re-uptake inhibitors (SSRIs) acutely increase interstitial serotonin levels by blocking the reuptake at the plasma membrane serotonin transporter. This phenomenon can be exploited for evoking increases in the extracellular serotonin concentration in the brain, as measured by cerebral microdialysis in rodent brain (Gartside, Umbers et al. 1995). It might be supposed that selective serotonin re-uptake inhibitors may rectify a deficiency in interstitial serotonin, purportedly underlying clinical depression. However, the antidepressant effect of selective serotonin re-uptake inhibitors cannot without qualification be attributed to the elevation of extracellular serotonin levels *per se*, since antidepressant response can be delayed for several weeks, while administration of selective serotonin re-uptake inhibitors leads to an immediate increase of extracellular serotonin concentration (Fuller, Wong et al. 1991). It has been argued

that the key to antidepressant response is derived from eventual desensitization of presynaptic autoreceptors on the serotonergic neurons, resulting in a salubrious change in the dynamic tone/phasic responsiveness of serotonin release. Thus, a serotonin 1A receptor antagonist has been touted as an adjunct treatment for accelerating the clinical response to selective serotonin re-uptake inhibitors (Beique, Blier et al. 2000), but this poly-pharmaceutical approach has not found its way to the clinic.

Positron Emission Tomography

In recent years, direct measurement of serotonin or its metabolite in blood, or cerebrospinal fluid, or neurochemical studies of *post mortem* brain, are being supplanted by *in vivo* imaging techniques such as positron emission tomography. The underlying principle of molecular imaging with positron emission tomography is that a radio-labeled substance (radioligand), upon intravenous injection, can bind to its receptor, or be trapped by a specific metabolic process in living brain, in direct analogy to *in vitro* and *ex vivo* autoradiographic methods. By recording the emitted radioactivity, images of its distribution in the living organism can be obtained. In the present context, positron emission tomography allows direct measurement of the density of serotonin receptors and the serotonin transporter in the living human brain. The principles of positron emission tomography are presented in some detail in the method section below.

General Theories

There is consistent evidence that serotonin is involved in the regulation of how environmental stimuli are processed, and in selection of behavioral responses (Jacobs and Azmitia 1992; Spoont 1992; Lucki 1998): Lower interstitial serotonin concentrations and reduced firing rate of serotonergic neurons are characteristic of a low arousal state (Rueter, Fornal et al. 1997), while increased serotonin concentration and release facilitates motor response and the suppression of competing (e.g. sensory) neural processes (Brodie and Shore 1957; Jacobs and Fornal 1995; Jacobs and Fornal 1999).

In animals, decreased or impaired serotonin neurotransmission increases the reactivity to environmental stimuli. For example, electrical stimulation in the area of the dorsal raphé nucleus results in reduced signaling of optic-tract fibers in the lateral geniculus, an effect that can be reversed by neurotoxic lesions of the serotonin system (Marks, Speciale et al. 1987). Furthermore, reduced serotonin in the telencephalon decreases the threshold for the startle response (Davis, Strachan et al. 1980), which is a classic behavioral paradigm for assessing the immediate response to stressful environmental cues. Lower cerebral serotonin concentration is also associated with other aspects of the behavioral response to environmental cues: decreased serotonin transmission releases the suppression of behavior that had earlier been learned by aversive conditioning, as, for example, assessed by key-pecking behavior of pigeons maintained by food presentation in combination with punishment by electric shocks (Graeff and Schoenfeld 1970; Leone, de Aguiar et al. 1983). Furthermore, a decreased cerebral serotonin concentration leads to a discount of large delayed rewards in favor of small immediate rewards, for example in rats (Wogar, Bradshaw et al. 1993; Bizot, Le Bihan et al. 1999; Mobini, Chiang et al. 2000). Conversely, increased serotonergic transmission specifically enhances satiation (McGuirk, Muscat et al. 1992), and thereby leads to reduced food intake (Simansky 1996).

A similar effect was observed in humans who chose small but immediate rewards over larger but delayed rewards after acute tryptophan depletion (Schweighofer, Bertin et al. 2008). Furthermore, like in other animals, acute tryptophan depletion attenuates impulsive and aggressive behavior in humans (Cleare and Bond 1995), while administration of an indirect serotonin agonist leads to less impulsive behavior (Poulos, Parker et al. 1996). In conjunction with increased activity of sensory processing, this can lead to faster, but less differentiated behavioral responses. In a social behavior test, acute tryptophan depletion resulted in faster and stronger negative response to behavior of other people that is regarded as unfair, with less consideration or foresight for the consequences with respect to future interaction with that person (Crockett, Clark et al. 2008).

Post mortem studies and measures of the serotonin metabolite in jugular blood have consistently shown that the concentration of serotonin is low in winter as compared to summer (Carlsson, Svennerholm et al. 1980; Sarrias, Artigas et al. 1989; Lambert, Reid et al. 2002). These

observations may be relevant to well-known behavioral changes commonly occurring in winter, such as reduced activity, higher food-intake, and increased urge to sleep (Kasper, Wehr et al. 1989; Wehr and Rosenthal 1989), all of which have been linked to low serotonin concentrations (Brodie and Shore 1957; Simansky 1996; Rueter, Fornal et al. 1997). Furthermore, molecular imaging studies have shown that binding to the serotonin transporter also fluctuates substantially over the year, with highest binding during winter and lowest binding in summer (Praschak-Rieder, Willeit et al. 2008; Willeit, Sitte et al. 2008). Thus, serotonin concentration in human brains *post mortem* (Carlsson, Svennerholm et al. 1980) and in blood from jugular veins (Lambert, Reid et al. 2002) is lowest, when availability of serotonin transporters is highest (Praschak-Rieder, Willeit et al. 2008), in the winter season when the typical symptoms of winter depression can occur.

In some people, these seasonal changes can provoke a particular form of affective disorder, termed seasonal affective disorder (Wehr and Rosenthal 1989), manifesting with depressive symptoms in winter. Importantly, the symptoms of seasonal affective disorder are substantially different from major depression. For example, winter-depressed people are not anhedonic, but crave carbohydrate-rich food. Interestingly, it has been suggested that light therapy, which can reverse the symptoms of winter depression (Rosenthal, Sack et al. 1985; Winkler, Pjrek et al. 2006), may also induce weight-loss in obese patients (Bylesjo, Boman et al. 1996; Friedman, Even et al. 2002). Consistent with the loss of punishment induced inhibition - linked above to reduced serotonin transmission - patients with seasonal affective disorder have more anger attacks with stronger vegetative symptoms and behavioral outbursts (Winkler, Pjrek et al. 2006).

In summary, lower serotonin concentration in the brain facilitates a more rigid behavioral mode, focusing on energy-preservation and orientation to the direct context with increased sensory processing, a preference of faster rewards of lower value, and generally increased food-intake, all in association with reduced inhibition of impulsive and aggressive behavior. Higher serotonin transmission facilitates more open and complex behavioral responses, which are oriented on long-term outcomes and higher but delayed rewards, and with increased motor activity and less emphasis on processing sensory information in the current environment.

Serotonin and Depression

As noted above, many behavioral changes observed in depression are linked to the serotonin system. Feeding (Simansky 1996) and sleeping (Rueter, Fornal et al. 1997), which are often altered in depression (Gelder, Cowen et al. 2006), are notable examples. The early emphasis placed on the putative role of serotonin in depression was based in part upon the finding of reduced levels of the serotonin metabolite in cerebrospinal fluid of patients with major depression, for example by Ashcroft and colleagues (Ashcroft, Crawford et al. 1966), which was a key finding leading to the hypothesis that depression resulted from a deficiency of monoamines, noradrenalin and serotonin, in much the same way that Parkinson´s disease is a dopamine deficiency syndrome (Birkmayer and Hornykiewicz 1961; Cumming 2009). However, Ashcroft and colleagues soon came to question the initial monoamine hypothesis, based on contradicting findings, and methodological problems (Ashcroft, Eccleston et al. 1972). A broad review of the literature did not reveal a straight-forward or consistent association between serotonin in cerebrospinal fluid and depression (Asberg 1997). Nonetheless, the monoamine hypothesis of depression is still widely postulated, and is implicitly accepted as a deficiency to be rectified with appropriate antidepressant medications.

Ashcroft and colleagues were unable to adhere to a model in which mood is directly related to serotonin concentration in the brain because they did not observe lower serotonin concentrations during depressive episodes in bipolar patients, as distinct from depressive episodes in unipolar depressed patients (Ashcroft, Eccleston et al. 1972). This early negative finding proved to be consistent with results of a recent large meta-analysis showing that acute tryptophan depletion (or depletion of other monoamines) does not induce depression or depressive mood in healthy controls, but only in presently healthy people with a personal or family history of depression (Ruhe, Mason et al. 2007). Lower serotonin concentrations may have a more simple association with more aggressive-impulsive behavior across a wide range of psychiatric disorders (Brown and Linnoila 1990).

The serotonin transporter has drawn considerable interest from neuroscientists and biological psychiatrists because it regulates serotonin tonus in the brain by re-uptake from the interstitial

space (Torres, Gainetdinov et al. 2003). Furthermore, blocking the serotonin transporter with selective antidepressants of the SSRI class, increases serotonin concentration in the interstitial space, predicting increased agonism at post-synaptic receptors. A review of *post mortem* measurement of the serotonin transporter in brain (Purselle and Nemeroff 2003) shows the evidence to be mixed: different studies showed serotonin transporter binding to be lower, higher, or unchanged in depressed patients who committed suicide. Positron emission tomography studies comparing patients with depression and healthy controls have also had inconsistent results. Some report lower serotonin transporter binding in patients with major depression (Parsey, Hastings et al. 2006; Oquendo, Hastings et al. 2007; Reimold, Batra et al. 2008), others report elevated serotonin transporter binding in patients with major depression (Reivich, Amsterdam et al. 2004; Cannon, Ichise et al. 2006; Cannon, Ichise et al. 2007), or no difference between patients and healthy controls (Meyer, Houle et al. 2004). A positron emission tomography study found serotonin transporter binding to be substantially reduced in patients with Parkinson´s disease, but this change was linked to specific motor symptoms, rather than mood changes (Kerenyi, Ricaurte et al. 2003).

Although impressive improvement of mood and general functioning has been reported in individual cases (Kramer 1993), results of meta-analyses have frequently called into question the antidepressant efficacy of SSRIs (Kirsch, Moore et al. 2002; Whittington, Kendall et al. 2004; Barbui, Furukawa et al. 2008; Kirsch, Deacon et al. 2008). This has been a highly charged issue, since annual sales of selective serotonin re-uptake inhibitors are probably several billion dollars per year.

Despite the inconsistent clinical evidence of efficacy, potential support for the monoamine hypothesis of depression has been derived from animal models. Neurobiologists model the human condition of despair in rodents using, for example, the learned helplessness paradigm, in which animals are exposed to unavoidable aversive stimuli via mild electric shocks, or in the forced swimming test, in which the animals swim until they seemingly abandon hope of finding a submerged platform. These stress-inducing situations increase extracellular serotonin concentration in microdialysis samples from rat brains (Kirby, Chou-Green et al. 1997). Delay to onset of behavioral despair, such as the interval before ceasing the attempt to avoid foot shocks,

or the duration of efforts to find the platform in the forced swimming test, is used in the screening of novel antidepressants, many of which are pharmacologically identified as acting via serotonin transmission (Lucki 1997; Sun and Alkon 2003). Operationally, antidepressant action is attributed to drugs prolonging the period between exposure to stress and termination of motor-behavioral output (Wong, Sonders et al. 2000; Lucas, Rymar et al. 2007). However, in light of persistent doubt about the association between serotonin and mood, and controversial claims that serotonin selective antidepressants may actually be rather ineffective, the validity of behavioral screening can be called into question. The behavioral patterns of despair in rodents could also be explained by increased serotonin transmission simply facilitating motor output (Brodie and Shore 1957), such as swimming or running away from foot shocks.

Serotonin and Personality

While behavior describes actions or reactions of any kind of thing by itself or in relation to another thing (e.g., a person might behave differently in a stressful situation than otherwise, a piece of wood may behave differently in water and in fire), personality refers to persistent traits, usually in humans, which describe a set of somewhat organized behavioral patterns, but also patterns of cognition and motivation. There were several attempts to try to relate serotonin transmission to the personality dimension Neuroticism, since Neuroticism, as assessed with different kind of personality models and questionnaires (e.g. the NEO-PI-R or the Eysenck Personality Questionnaire), may reflect an individual's propensity to develop mood disorders (Christensen and Kessing 2006). However, there is no consistent evidence for an association between the personality dimension Neuroticism and the status of the cerebral serotonin system (discussed in the next section). Instead, there is some evidence that intrinsic aspects of serotonin transmission may manifest in the personality trait Openness to Experience, which is one out five independent personality dimensions of the widely accepted five-factor model of human personality (Digman 1990). Openness to Experience reflects the openness versus rigidity of an individual's personality structure and describes, for example, how flexible a person is when dealing with environmental stimuli or adapting the own concept of the environment in response to changes in the environment (Costa and McCrae 1992). Scores on Openness to Experience can

be psychometrically measured with the Revised Neuroticism, Extraversion, Openness Personality Inventory (NEO-PI-R) (Costa and McCrae 1992), the details of the NEO-PI-R will be discussed in the method section below. In general, the trait Openness to Experience is associated with increased biological response to stress. For example, people with higher Openness to Experience scores react with higher secretion of cortisol in response to stress (Oswald, Zandi et al. 2006). On the other hand, Openness to Experience has been associated with increased cognitive and emotional flexibility. For example, Openness to Experience is also associated with more adequate coping strategies. Thus, elderly people who face traumatic life changes, such as loss of a spouse, are less at risk to commit suicide, if they have high scores in openness, presumably because they are better at coping with strong emotional stressors (Duberstein 1995).

In the context of a gene*environment interaction, Openness to Experience has consistently been linked to an increased risk of developing winter depression, a classical environment-induced mood disorder, termed seasonal affective disorder (Bagby, Schuller et al. 1996; Jain, Blais et al. 1999). Consistent with the present theme, carriers of the short 5-HTTLPR allele have been found to have higher Openness to Experience scores (Stoltenberg, Twitchell et al. 2002) and also to be more at risk to develop seasonal affective disorder (Rosenthal, Mazzanti et al. 1998; Willeit, Praschak-Rieder et al. 2003). Due to this duality, Openness to Experience may be the personality dimension that reflects the net effects of serotonin on both behavioral flexibility and sensitivity to stressful environmental cues, in the manner of a double-edged sword.

Positron emission tomography has also been used to correlate specific indices of the serotonin system with human personality. However, there have hitherto been few positron emission tomography studies probing the association between serotonin and rigid versus adaptive behavior. Borg and colleagues observed in a group of 15 healthy Swedish men that serotonin 1A receptor binding was inversely correlated with the rather obscure trait of Openness to Spiritual Experience (Borg, Andree et al. 2003). The only study relating serotonin transporter binding and personality in healthy controls reported a positive correlation between Neuroticism scores and serotonin transporter binding in a group of 31 healthy Japanese men (Takano, Arakawa et al. 2007).

*5-HTTLPR*Environment Interactions*

The occurrence of genotypes with effects on the serotonin system and on behavior has been identified as a matter of key importance for understanding the genetic nature of human personality. A well-known polymorphism in the promoter region of the serotonin transporter gene (5-HTTLPR, see above) has been associated with scores on the personality dimension Neuroticism, anxiety-related personality traits, and Harm Avoidance (Lesch, Bengel et al. 1996). In their paper, Lesch and colleagues reported that the short 5-HTTLPR allele was associated with higher scores on Neuroticism, anxiety-related personality traits, and Harm Avoidance. This association was challenged a few years later, in a study which had the opposite finding to that of Lesch *et al*., reporting a slight association between the long 5-HTTLPR allele and anxiety-related personality traits (Flory, Manuck et al. 1999). On the basis of a more recent large study (Willis-Owen, Turri et al. 2005) and a meta-analysis of published reports, it was concluded that the observed association between the short 5-HTTLPR allele (which is associated with lower mRNA expression) and Neuroticism and anxiety-related personality traits is likely to be based on false positive findings (Munafo, Freimer et al. 2008). A positron emission tomography study (Takano 2007) reported a positive correlation between serotonin transporter binding and Neuroticism. In light of the initial observation of an association between the short 5-HTTLPR allele, with lower mRNA and therefore presumably lower serotonin transporter expression, in more neurotic people, the finding by Takano an colleagues adds further inconsistency.

Other studies have claimed that the short 5-HTTLPR allele is associated with a higher prevalence of mood disorders. While some studies observed a higher frequency of the short 5-HTTLPR allele carriers in patients with mood disorders (Lotrich and Pollock 2004; Hoefgen, Schulze et al. 2005) large multi-center studies and meta-analyses did not find such an effect (Mendlewicz, Massat et al. 2004; Willis-Owen, Turri et al. 2005). A more consistent observation is that carriers of the short 5-HTTLPR allele have an increased propensity of developing mood disorders in response to stressful environmental cues (Caspi, Sugden et al. 2003; Gotlib, Joormann et al. 2008). Interestingly, carriers of the short allele are at particular risk to develop seasonal affective disorder (Rosenthal, Mazzanti et al. 1998; Willeit, Praschak-Rieder et al. 2003). By definition, seasonal affective disorder occurs only in response to specific environmental changes, related to

reduced sunlight or other aspects of the winter climate. Therefore these genetic associations further support the claim that the allele of 5-HTTLPR determines the sensitivity of the serotonin system to environmental changes, such that its effect on the phenotype is best understood in terms of a gene*environment interaction effect. This is in line with the observation by Hariri and colleagues that carriers of the short 5-HTTLPR allele show a stronger blood-oxygen-level dependent (BOLD)-signal change, as measured with functional magnetic resonance imaging, in the limbic system in response to viewing images of fearful faces (Hariri, Mattay et al. 2002). This finding has been reproduced and extended in independent studies (Pezawas, Meyer-Lindenberg et al. 2005; Heinz, Smolka et al. 2007; Dannlowski, Ohrmann et al. 2008) and remains significant in a meta-analysis of all the published results (Munafo, Brown et al. 2008).

The short 5-HTTLPR allele does not manifest in specific personality traits, and does not have a higher frequency of occurrence in depressed patients. Instead, it is linked, for example, to a specific response of the limbic circuit, which means that it manifests as an intermediate phenotype (Meyer-Lindenberg and Weinberger 2006), also known as an endophenotype (Gottesman and Gould 2003). Endophenotypes fall somewhere between genotype and phenotype: They are a distinct manifestation of genotypes, but not visible with the bare eye (such as increased BOLD-signal changes). The introduction of the concept of endophenotypes into psychiatric research acknowledges that gene effects are often subtle, and cannot explain an aspect of personality, or a symptom of psychiatric disorders, as if these were phenotypes such as hair color. In light of the observations of Caspi and colleagues (Caspi, Sugden et al. 2003), it is likely that the endophenotype of the short 5-HTTLPR allele is represented by a more sensitive homeostasis of cerebral circuits and transmission systems, resulting, for example, in greater vulnerability to stressors.

The Scientific Problem

As reviewed, there is no consistent model to describe the association between serotonergic neurotransmission and human behavior. In particular, there is no consistent evidence for the presumed association between mood and serotonin levels or serotonin transporter binding. Insofar as the genotype of the 5-HTTLPR influences the expression of the serotonin transporter, it should be expected to affect in some way the disposition of interstitial serotonin, somehow in terms of a gene*environment interaction. However, from an evolutionary point of view, it seems counter-intuitive that the short 5-HTTLPR allele, which is carried by 2/3 of the (Caucasian) population, constitutes a risk factor *per se*. It is more likely that the endophenotype of the short 5-HTTLPR allele constitutes a trade-off between the benefits of sensitivity to the environment, and the propensity to adapt to environmental changes on the one hand, and the cost of increased sensitivity to stress, associated with a higher propensity to develop mood disorders on the other.

Given that the endophenotype of short 5-HTTLPR allele is most likely to appear in a gene*environment setting, it can be assumed that the higher stress response brought about by a more sensitive homeostasis of the serotonin system leads to a faster and stronger behavioral reaction to environmental changes. The veracity of this more sensitive homeostasis has, for example, been established by using acute tryptophan depletion: As noted above, the selective decrease of the availability of tryptophan and thereby decreased cerebral serotonin concentrations, only had an effect on the mood of people with a predisposition for mood disorders (Ruhe, Mason et al. 2007), and in carriers of the short 5-HTTLPR allele (Nugent, Neumeister et al. 2008). The associations between genetics and the serotonin system, in terms of rigidity of behavioral responses, and the stability of serotonin homeostasis, have been scantily investigated using molecular imaging techniques. There is a particular lack of positron emission tomography studies of the serotonin system in relation to behavioral rigidity, and effects of environmental stress in carriers of different alleles of the 5-HTTLPR.

Aims of this PhD

The focus of my work was on gene*environment interaction effects on serotonin transporter function and behavior. The aim of my PhD was to

1. Develop an observer independent approach to delineate a volume of interest for positron emission tomography for the raphé nuclei (methodological study)

And to test the following hypotheses:

2. Genetically determined serotonin transporter availability is associated with openness versus rigidity of human behavior

3. The 5-HTTLPR determines the sensitivity of the serotonin system to environmental changes, in particular seasonal changes in daylight times

Post hoc I tested the hypothesis that a polymorphism in the CLOCK gene, which is part of the human biological clock system, determines responsiveness of the serotonin system to environmental changes (study IV).

Experimental Procedures

Principles of Positron Emission Tomography

Positron emission tomography is a non-invasive technique for measuring the distribution in living organisms of molecules that are labeled with a positron emitting isotope. Short-lived isotopes such as [^{11}C]carbon (half-life 20 minutes) and [^{18}F]fluorine (half-life 120 minutes) are prepared using a cyclotron, and rapidly integrated into the structure of the molecule of interest. Once purified, the radiolabeled substance, frequently called a tracer, is injected intravenously (or sometimes inhaled) and distributes throughout the body; physiological specificity of its distribution is imparted by the molecule in question, which may bind to a neuroreceptor, or enter into some metabolic pathway. Inexorably, the isotope decomposes, releasing a positron. The emitted positron travels for a relatively short distance before it encounters an electron, which results in mutual annihilation. The distance depends upon the kinetic energy of the positron, and the electron density of the medium, but is approximately 1 mm. Since this path results in uncertainty about the source of the decay event, a practical limit is imposed on the spatial resolution of the positron emission tomography image. The annihilation process leads to the formation of two gamma photons – each with the energy of 511 kilo electron Volts, corresponding to the rest mass energy of the electron-positron pair. The two photons radiate at nearly 180 degrees from the point of annihilation. Simultaneous activation of two gamma ray detectors, located in the detector ring which is positioned - outside the body - around the anatomical site of interest, registers as a decay event. Millions of such events can be transformed into a two- or three-dimensional image, using the technique of filtered back-projection. Some gamma radiation is necessarily deposited in the brain and skull, which is the topic of radiation dosimetry, and is also an issue for quantitation. In practice, emission recordings are supplemented with an attenuation scan, in which a source of known radioactivity concentration rotates around the head. A look-up table for the gamma attenuation in each image element is calculated, and used to correct the measured emission-density map for the effects of attenuation.

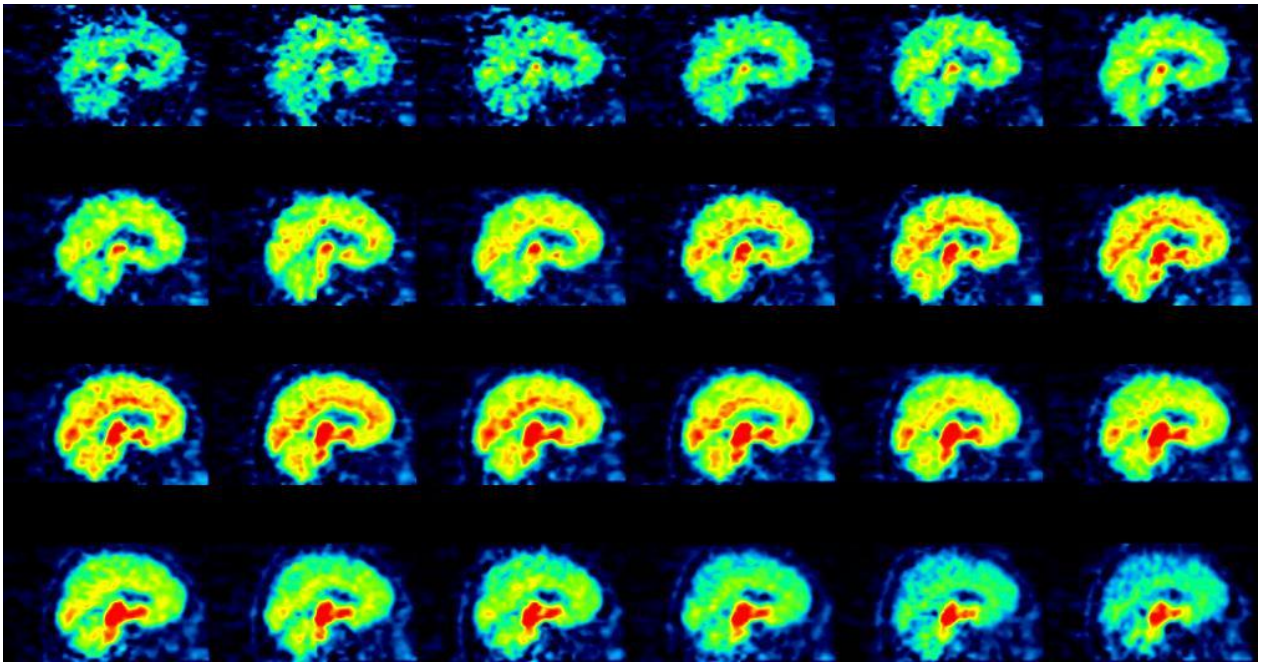


Figure 4: Sequential time frames of a dynamic [^{11}C]DASB emission recording. The frames recording in the first minutes after tracer injection (top row) mainly represent blood flow, i.e. the delivery of tracer to the brain. In later time frames, there emerges a pattern specific binding in subcortical areas, including the striatum, the thalamus and the midbrain, due to the selective retention of the tracer in regions richly endowed with binding sites, and due to the washout of the tracer from non-binding regions.

Acquisition and Co-Registration of Magnetic Resonance Images

Analysis and comparison of positron emission tomography images is best accomplished through co-registration to an image with structural information, usually a magnetic resonance image. For studies I, II, and IV, structural brain scans were acquired on a Siemens Magnetom Trio 3T magnetic resonance scanner with an eight-channel head coil (In vivo, FL, USA). All magnetic resonance images were acquired at the Danish Research Center for Magnetic Resonance.

In the case of the [^{11}C]DASB images, all time-frames of the attenuation-corrected emission recording were automatically aligned to time-frame 26 using the AIR algorithm (http://bishopw.loni.ucla.edu/AIR5/). In a next step, I used a mean positron emission tomography image, averaging time frames 10-36 for co-registration to the individual magnetic resonance

image using the AIR algorithm; the quality of each co-registration was controlled visually. In three cases, co-registration between positron emission tomography and magnetic resonance image was corrected manually. [^{18}F]altanserin images were co-registered manually by trained academic staff and cross-checked by a second colleague.

Volume of Interest Analysis

The volumes of interest were delineated automatically as described in Svarer *et al.* (Svarer, Madsen et al. 2005) in order to identify the volumes in a user-independent fashion. For each of the ten template volume of interest sets, a 12-parameter affine transformation and a warping field was calculated between each of the ten template magnetic resonance images and the individual magnetic resonance image for a participant. Having obtained the co-registration between the two image modalities for the same individual as described above, the template volume of interest sets were then transferred to the dynamic positron emission tomography image space for each participant, using the calculated transformation matrix. From the volume of interest sets, a probability map was created for each participant, and a common volume of interest set was threshold-generated. These volumes of interest sets were then used for automatic extraction of time activity curves for midbrain and volume-weighted (left-right) averages for the bilateral thalamus, caudate, putamen and cerebellum for all participants. The midbrain, the caudate, the putamen and the thalamus were selected as representative brain regions of homogenous, high serotonin transporter binding (Houle, Ginovart et al. 2000) since I expected that a genetic predisposition would be associated with a global effect on the brain. The time activity curve extracted for the cerebellum (excluding the vermis) was used as the reference tissue input for kinetic modeling. Positron Emission Tomography Scans

[^{11}C]DASB: Characteristics

The first successfully applied and currently most widely used tracer to image the serotonin transporter is the single photon emission tomography tracer Iodine-123-labeled 2β-carbomethoxy-3β-(4-iodophenyl)tropane ([^{123}I]β-CIT). The advantage of [^{123}I]β-CIT is that it

can be measured with single-photon emission computed tomography which is cheaper and more widely available than is positron emission tomography. Also, the long half-life of ^{123}I is such that the agent can be distributed commercially. However, [^{123}I]β-CIT does not bind specifically to the serotonin transporter but also to the dopamine transporter, which is far more abundant in the striatum. Furthermore, quantitation of cortical serotonin transporter binding measured with [^{123}I]β-CIT is unreliable (Hesse, Barthel et al. 2004).

Positron emission tomography yields more accurate measures than single-photon emission computed tomography due to double-photon emission coincidence registration and electronic photon collimation. The first positron emission tomography tracer selective for the serotonin transporter was [^{11}C]McN5652 (Suehiro, Scheffel et al. 1993). Later, (N,N-dimethyl-2-(2-amino-4-cyanophenylthio) benzylamine ([^{11}C]DASB) was developed (Houle, Ginovart et al. 2000). This compound is not only specific for the serotonin transporter, but has superior signal-to-noise properties *in vivo*, which allows for better statistic effects and even allows quantitation of the serotonin transporter in cortical areas of the brain, comma where specific binding is relatively low (Frankle, Huang et al. 2004). Further radioligands with a similarly high selectivity for the serotonin transporter, such as [^{11}C]AFM and [^{11}C]MADAM, have been developed. However, [^{11}C]DASB allows the measurement of serotonin transporter availability with faster equilibrium (37 minutes) and wash-out, and thus allows shorter scanning times (Hesse, Barthel et al. 2004).

[^{11}C]DASB: Procedure

Positron emission tomography scans were performed with an 18-ring GE-Advance scanner (General Electric, Milwaukee, WI, USA), operating in 3D acquisition mode, and producing 35 image slices with an interslice distance of 4.25 mm. Following a 10 minutes transmission scan, a dynamic 90 min long emission recording was initiated upon intravenous injection during 12 seconds of mean [^{11}C]DASB. The emission recording consisted of 36 frames, increasing progressively in duration from 10 s to 10 min. The attenuation and decay corrected recordings were reconstructed by filtered back projection using a 6 mm Hann filter.

[^{11}C]DASB: Quantification

The outcome parameter of the [^{11}C]DASB binding within a brain region is the non-displaceable binding potential, designated BP_{ND}, which is a dimensionless quantity, proportional to the abundance of binding sites (B_{max}) as is typically measured *in vivo*. The BP_{ND} was calculated for the four volumes of interest using the cerebellum (excluding vermis) input as a reference region, assumed to contain non-specific binding only. As such, the reference region serves as a surrogate index of the unbound tracer available for binding in regions with specific binding. I used a modified reference tissue model designed specifically for quantification of [^{11}C]DASB (MRTM/MRTM2) as described and evaluated by Ichise *et al.* (Ichise, Liow et al. 2003), and used the PKIN tool of the software PMOD Version 2.9. The MRTM yields three output parameters: (1) R_1, the ratio of the tracer permeability in the ROI to that in cerebellum, which is related to the relative blood flows, (2) k_2',(min^{-1}) the washout rate constant for the tracer in the ROI, and (3) BP_{ND}, as defined above. I first calculated the magnitude of k_2' in a pooled set of high-binding regions, comprising the caudate, the putamen, and the thalamus. This was then used as a constrained input parameter for the calculation of BP_{ND} in the four VOIs relative to cerebellum.

For the voxel-based analysis, parametric images representing BP_{ND} for each image element (voxel) were calculated for all participants from their dynamic positron emission tomography recordings. Using the PXMOD tool of the PMOD software, I imported the volumes of interest (generated as described above), and re-calculated k_2' relative to cerebellum using MRTM. As described elsewhere (Ichise, Liow et al. 2003), I applied a threshold of $R_1 > 0.3$, so as to exclude noisy voxels from the analysis, which were particularly seen in regions with low tracer clearance. Additionally, I restricted the BP_{ND} outcome to range between 0 and 10, that is physiologically plausible magnitudes. The resulting parametric images were filtered with a 3 mm median filter in their native space. SPM5 (http://www.fil.ion.ucl.ac.uk/spm/software/spm5/) was used to normalize the parametric images to Montreal Neurological Institute (MNI) space using a warping field estimated by normalization of each participant's co-registered MR image. Subsequently, a 12 mm Gaussian filter was applied to all normalized images.

[^{18}F]altanserin: Characteristics

Different radioligands are available to measure binding to the serotonin 2A receptor. As discussed in detail by Erritzoe *et al*. (Erritzoe, Rasmussen et al. 2008): [^{18}F]setoperone and [^{11}C]N-methylspiperone are older radioligands with a relatively poor selectivity for the serotonin 2A receptor. At present, two other specific the serotonin 2A receptor radioligands are available: [^{11}C]MDL 100,907 and [^{18}F]altanserin. [^{18}F]altanserin has a 200- to 500- selectivity in binding to the serotonin 2A receptor relative to dopamine 2 receptors, making it between 8 and 50 times more selective for the serotonin 2A receptor than [^{18}F]setoperone. In addition, the affinity of [18F]altanserin for the serotonin 2A receptor is at least 20-fold higher than for other serotonin receptor (sub)types.

[^{18}F]altanserin: Procedure

[^{18}F]altanserin was administrated as a combination of a bolus injection followed by continuous infusion to obtain steady state of the tracer in blood and tissue. The bolus-infusion ratio was 1.75 h, as previously described (Pinborg, Adams et al. 2003). Participants received the maximum dose of 3.7 MBq/kg body weight [^{18}F]altanserin. Reconstruction, attenuation, and scatter correction procedures were conducted as described elsewhere (Pinborg, Adams et al. 2003). Ninety minutes after the bolus injection of [^{18}F]altanserin, participants were placed in the scanner.

Five venous blood samples were drawn at mid-scan times 4, 12, 20, 28, and 36 minutes after starting the dynamic scanning sequence. The samples were immediately centrifuged, and 0.5 ml of plasma was counted in a well counter for determination of radioactivity. Three of the five blood samples drawn at 4, 20, and 36 minutes were also analyzed for percentage of parent compound ([^{18}F]altanserin) using reverse-phase High-performance liquid chromatography following the procedure described by Pinborg *et al*. (Pinborg, Adams et al. 2003). In addition, the free fraction of [^{18}F]altanserin in plasma, f_1, was estimated using equilibrium dialysis, following a modified procedure by Videbaek et al (Videbaek, Friberg et al. 1993). The dialysis was performed using Teflon-coated dialysis chambers (Harvard Bioscience, Amika, Holliston, MA, USA) with a cellulose membrane that retains proteins >10,000 Da. A small amount of

[^{18}F]altanserin (approximately 1 MBq) was added to 10 ml plasma samples drawn from the participants. A 500 µl portion of plasma was then dialyzed at 37°C for 3 hours against an equal volume of buffer, since pilot studies had shown that 3 hour equilibration time yielded stable values. The dialysis buffer consisted of 135 mM NaCl, 3.0 mM KCl, 1.2 nM CaCl2, 1.0 mM MgCl2, and 2.0 mM phosphate (pH 7.4). After the dialysis, 400 µl of plasma and buffer were counted in a well counter, and f_1 of [^{18}F]altanserin was calculated as the ratio of DPM(buffer)/DPM(plasma).

[^{18}F]altanserin: Quantification

The outcome parameter was the binding potential of specific tracer binding, relative to the free concentration in plasma (BPp). The cerebellum was used as a reference region, since it represents nonspecific binding only (Pinborg, Adams et al. 2003). In steady state, BPp is defined as:

$$BP_P = (C_{VOI} - C_{Reference}) / C_{Plasma} = fp * (B_{max}/K_d) \text{ (mL/mL)},$$

where C_{ROI} and $C_{Reference}$ are steady-state mean count density in the volume of interest and in the reference region, respectively, C_{Plasma} is the steady-state activity of non-metabolized tracer in plasma, f_1 is the free fraction of radiotracer, B_{max} is the density of receptor sites available for tracer binding, and K_d is the affinity constant of the radiotracer to the receptor.

Discussion of Psychometric Tools to Measure Personality

In western culture, efforts to categorize human personality can be traced back to antiquity, when there was codified the theory of the four humors. Analogous theories had developed (presumably independently) in ancient oriental culture. Humor theory, as presented by Galen, remained the prevalent medical theory in the late Middle Ages, and until the early modern era. Contemporary efforts to measure personality, based upon responses to questionnaires, can be considered as modern manifestations of an ancient perspective. In 1961, Tupes and Christal identified five recurring factors or personality dimensions, in a study of eight large population samples (Digman

1990). Norman replicated this observation in a large set of personality data (Norman 1963). In the early 1980s, prominent personality researchers reviewed available personality tests and agreed on the five factor model (Digman 1990), which was followed by the publication of the NEO-PI, and later the revised version, the NEO-PI-R, by Costa and McCrae. Since then, the NEO-PI-R has become a well-established and standardized instrument to assess personality traits, using either self-reports or observer ratings, and has steadily replaced other questionnaires, such the Eysenck Personality Questionnaire, which measures Neuroticism, Extraversion and Psychoticism, or Cattel's complex system with a minimum of 16 primary and eight second order factors (Digman 1990).

The NEO-PI-R incorporates the five broad traits of the five-factor model (FFM) of personality, and includes six facets or specific traits for each of the five broad factors based on 240 items (Costa and McCrae 1992). Each facet score is derived by adding the scores on eight items in 0-4 Likert format, and the personality trait score calculated as the composite scores on its six facets. Thus, the possible range of facet scores is 0 - 32 and the range of trait scores from 0 - 192. For my experiments, I used the Danish translation of the NEO-PI-R, which has been psychometrically evaluated and standardized in a sample of 600 people (Hansen and Mortensen 2004). In the NEO-PI-R, the personality dimension (trait) Openness to Experience includes the six facets, Openness to Fantasy, Openness to Aesthetics, Openness to Feelings, Openness to Actions, Openness to Ideas, and Openness to Values. Typical Openness to Experience items are (RS = reverse scored items):

"I have a very active imagination" (Openness to Fantasy)

"I am intrigued by the patterns I find in art and nature" (Openness to Aesthetics)

"I seldom pay much attention to my feelings of the moment" (Openness to Feelings, RS)

"Once I find the right way to do something, I stick to it" (Openness to Action, RS)

"I often enjoy playing with theories or abstract ideas" (Openness to Ideas)

"I believe that laws and social policies should change to reflect the needs of a changing world" (Openness to Values)

While the facets Openness to Fantasy, Openness to Aesthetics, Openness to Feelings, and Openness to Ideas represent intellectual openness and curiosity, the two facets Openness to Action and Openness to Values reflect a more active approach: Openness to Actions reflects the willingness to choose from a variety of approaches to solve problems, and also to try new experiences such as eating novel food as opposed to sticking to habitual ways of doing things; Openness to Values reflects the readiness to re-think and change one's own political, social, and religious values. Openness is strongly related to intellect (Costa and McCrae 1992; DeYoung, Peterson et al. 2005), and, in particular Openness to Values, inversely correlated with conservative cultural beliefs (Van Hiel and Mervielde 2004).

Genotyping

The principle of genotyping as used in these studies is based on the technique of polymerase chain reaction. The principle of the polymerase chain reaction is that deoxyribonucleic acid (DNA) polymerase is used to amplify a specific sequence of DNA, present in a blood or sputum sample. Heat-stable DNA polymerase is used in a process of thermal cycling, characterized by alternately heating and cooling the sample in a defined series of temperature steps, in the presence of primer sequences, enzymes, and other specific reagents. In this cycle the DNA is first denaturated at a temperature of 94-98°C for 20-30 seconds. This causes melting of DNA template and primers by disrupting the hydrogen bonds between complementary bases of the DNA strands, and thus releases two single-strands of DNA. The temperature is then lowered to 50-65°C for 20-40 seconds, thus allowing the primers to anneal to the single-stranded DNA template. The DNA polymerase enzyme then binds to the primer-template hybrid and starts synthesis of a new DNA strand complementary to the DNA template strand delineated by the primers. Repeated cycling of this step leads to an exponential amplification of the DNA piece of interest; in ideal conditions, a single specimen of the desired sequence can be amplified endlessly.

5-HTTLPR

Blood samples for DNA analysis taken during the positron emission tomography scanning were immediately frozen and stored at -20°C for subsequent analysis. DNA was extracted from the blood using a Qiagen Mini kit according to the protocol recommended by the manufacturer (Qiagen, Valencia, CA, USA). 5-HTTLPR (SLC6A4; 17q11.1-q12) genotyping was performed using a TaqMan 5'-exonuclease allelic discrimination assay, also performed according to the instructions provided by the manufacturer (Applied Biosystems, Foster City, California, Assay-on-Demand). The ABI 7500 multiplex polymerase chain reaction machine (Applied Biosystems, Foster City, California, USA) was used for this analysis. The 5-HTTLPR long (A/G) polymorphism was detected by MspI restriction enzyme digestion of the polymerase chain reaction products, following resolution of the generated fragments by gel electrophoresis. Product sizes for the digest were in line with the literature reports: L_A=340 bp, L_G=166 bp+174 bp, S=297 bp (Praschak-Rieder, Kennedy et al. 2007).

CLOCK (3111 T/C)

Blood samples for DNA analysis were taken during the scanning and immediately frozen and stored at -20°C until further analysis. DNA was extracted from the blood using a Qiagen Mini kit using the guidelines included in the kit (Qiagen, Valencia, CA, USA) CLOCK (rs1801260) genotyping was performed using a TaqMan 5'-exonuclease allelic discrimination assay (assay ID: C___8746719_20) according to the instructions provided by the manufacturer (Applied Biosystems, Foster City, California, Assay-on-Demand). The ABI 7500 multiplex polymerase chain reaction machine (Applied Biosystems, Foster City, California, USA) was used for this analysis.

Results and Discussion

Study I

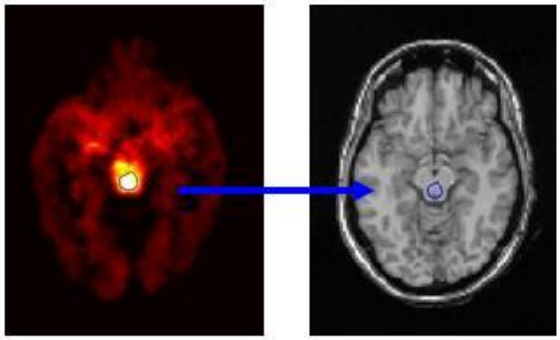
The objective of this methodological study was to develop a reliable observer-independent approach to delineate volumes of interest for functional brain regions which are not readily identifiable on structural magnetic resonance images. In the present context, the raphé nuclei are the main target. The raphé nuclei are located in the brainstem and constitute the core of the serotonin system (Jacobs and Azmitia 1992; Hornung 2003). Alterations in general morphology and serotonin transmission in this region have been reported in mood disorders and degenerative diseases (Becker, Becker et al. 1995; Drevets, Frank et al. 1999; Rub, Del Tredici et al. 2000). So far, the raphé nuclei region has been defined manually either directly on the functional image or on a co-registered magnetic resonance image (Drevets, Frank et al. 1999; Borg, Andree et al. 2003; Lundberg, Odano et al. 2005; Cannon, Ichise et al. 2006; Jovanovic, Cerin et al. 2006) . These approaches are highly user-dependant, are prone to bias arising from pathophysiological changes, and do not fully take into account individual brain morphology, since co-registration methods are optimized for matching of telencephalic structures.

I created a template set for the raphé nuclei based on regions of high serotonin transporter binding in parametric [^{11}C]DASB BP_{ND} maps obtained from ten healthy controls (Figure 5 A). I used these 10 templates in conjunction with the Svarer algorithm, described above, to create observer-independent probabilistic volumes of interest based on an intensity threshold (details described in Study I and in Figure 5B). This approach for quantitation was tested in a different group of ten participants, and compared with a manual delineation approach which was used by other groups (Drevets, Frank et al. 1999; Lundberg, Odano et al. 2005). The probabilistic map approach resulted in a volume for the probabilistic volume of interest for the raphé nuclei which was more than twice as large as the volume of interest derived from the manual approach (1.41 ± 0.12 ml vs. 0.58 ± 0.04 ml). Time activity curves derived from the observer-independent approach showed less noise (Figure 5C) resulting in a better model fit, as revealed by a significantly lower Chi^2, in comparison to the corresponding results arising from manual

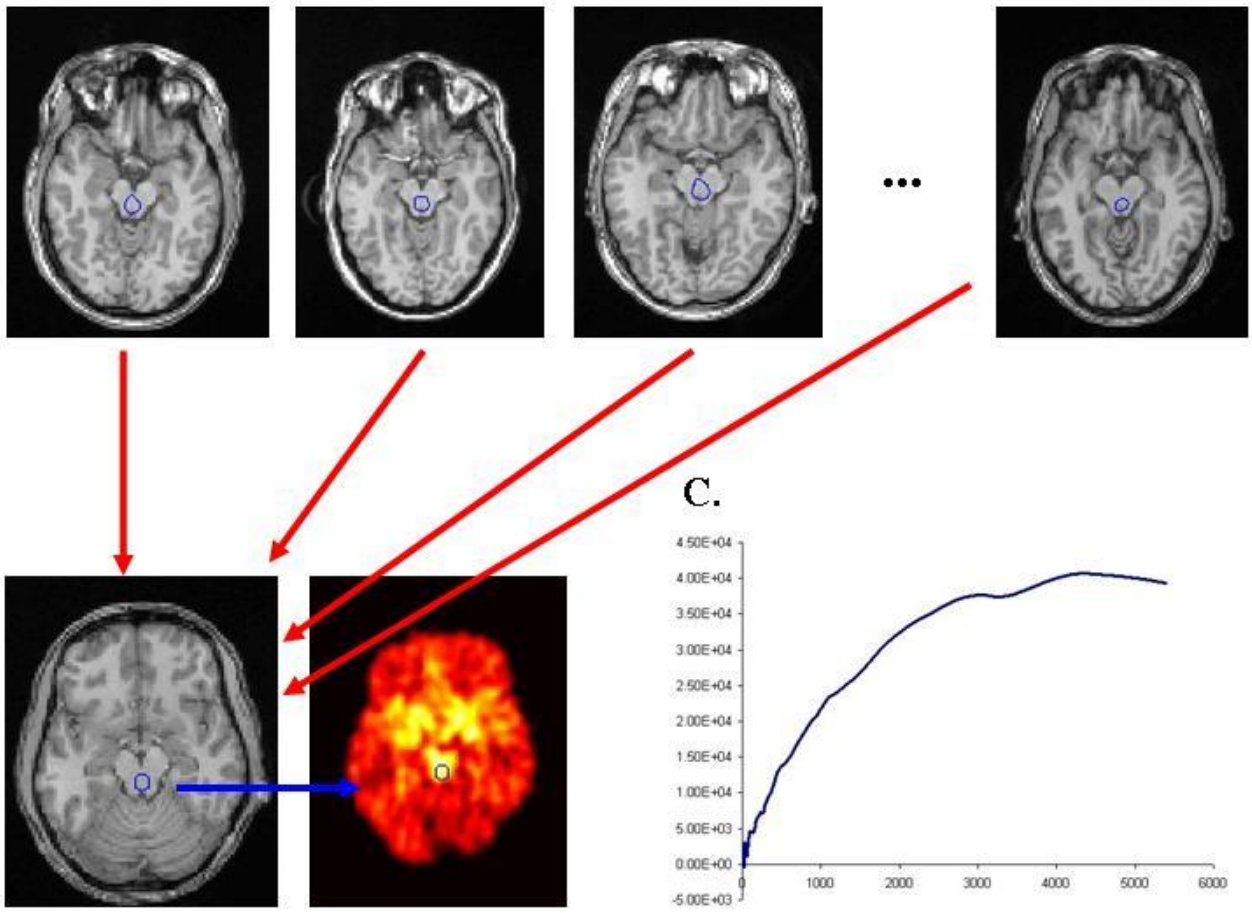
delineation, as performed by three different observers. In particular, the Chi^2 values were 12.6 ± 4.3 (probabilistic volume of interest), 15.3 ± 5.4 (observer 1), 14.5 ± 4.7 (observer 2), and 14.5 ± 5.5 (observer 3). The intercorrelation coefficient for the observer-dependent delineation approach was only 0.47. Also, probabilistic volume of interest delineation resulted in significantly lower Chi^2 as compared to manual delineation, $p<0.001$, 2-way ANOVA. The [^{11}C]DASB BP_{ND} calculated from time-activity curves extracted with the probabilistic map approach (4.40 ± 0.44) was significantly higher than the operator based delineations: 3.65 ± 0.65 (observer 1), 3.58 ± 0.62 (observer 2), and 3.45 ± 0.57 (observer 3) ($p<0.001$, 2-way ANOVA). Besides providing an observer-independent solution, the probabilistic map approach returned a higher specific binding determined in a larger brain region and this provided better data fitting in kinetic modeling. As a general rule for quantitation in small structures, high specific binding with lower relative standard deviation is indicative of greater accuracy, without penalty in precision, which is a precondition for sensitive detection of differences between groups, or specific pathophysiology. I conclude that the new approach presented here is a fast, observer-independent reliable method to delineate regions that can only be identified by functional imaging, here exemplified by the raphé nuclei.

Despite the positive results of this study, I did not use the raphé region in the studies II-IV. The article resulting from the raphé project was submitted to several scientific journals but rejected, mainly because delineation of a single volume of interest was not regarded worth being published as a research article on its own. However, the feedback of the reviewers led to improvements of the method and further analyses, and, in the current form, the manuscript not only describes a new approach to delineate a volume of interest for the raphé nuclei but also a general approach to delineate regions that cannot be identified on magnetic resonance images. I believe this makes the article worth being published on its own. Due to these changes and additions, this project was not completed before the other studies, so that the raphé region was not ready to be used when the data analysis for the other studies was performed. I therefore decided to use a volume of interest for the whole midbrain instead, which has been used by other groups (Reimold, Smolka et al. 2007; Praschak-Rieder, Willeit et al. 2008; Reimold, Batra et al. 2008) and can reliably be delineated on magnetic resonance images.

Figure 5. A: The raphé nuclei were delineated on a parametric map of a positron emission tomography image with the radioligand [^{11}C]DASB representing under visual control on a co-registered magnetic resonance image.



A threshold was set for specific binding such that only binding in the area of the raphé nuclei was visible and then the volume of interest was transferred to the co-registered magnetic resonance image. B: Warping from several template magnetic resonance images (n=10) onto the magnetic resonance image of the test person creates a probabilistic volume of interest and, in a second step, this volume of interest is transferred from the magnetic resonance image of the test person to the co-registered positron emission tomography image. C: a typical time activity curve resulting from measurements in this volume of interest



Study II

In this study, I tested the association between 5-HTTLPR, serotonin transporter measured with positron emission tomography and the trait Openness to Experience with all six facets. The rationale was to find *in vivo* evidence for an association between the human cerebral serotonin system and flexible versus rigid behavior patterns represented by scores in the personality trait Openness to Experience. Out of the 50 participants in this study, 18 were homozygotic carriers of the long 5-HTTLPR allele (36%), 11 were homozygotic carriers of the short 5-HTTLPR allele (22%), and 21 heterozygotes (41%). These frequencies did not differ significantly (Pearson's Chi^2 test with one degree of freedom) from the frequencies expected according to the Hardy-Weinberg principle or expected according to the observed frequencies in a group of 847 Caucasian non-Maori participants (Caspi et al., 2003). Of the 18 homozygotic carriers of the long 5-HTTLPR allele, 14 were homozygotic L_A-allele carriers, and 4 carried L_AL_G. The finding that homozygotic L_A-allele carriers generally had higher serotonin transporter binding than carriers of at least one S-allele or one L_G-allele (Praschak-Rieder, Kennedy et al. 2007; Reimold, Smolka et al. 2007) was also present in this sample, but this difference was only statistically significant in the caudate nuclei ($p=0.042$). There was no difference in openness scores between carriers of different alleles of the 5-HTTLPR polymorphism.

Serotonin transporter binding in the midbrain was inversely correlated with Openness to Experience ($r=-0.325$, $p=0.024$), and its facets Openness to Action ($r=-0.413$, $p=0.003$) and Openness to Values ($r=-0.371$, $p=0.009$). The correlation between serotonin transporter binding and Openness to Values was also significant in the putamen ($r=-0.328$, $p=0.023$), in the thalamus ($r=-0.427$, $p=0.002$), and approaching significance in the caudate nuclei ($r=-0.282$, $p=0.053$). These findings for Openness to Values were confirmed by voxel-based analysis. I did not find associations between 5-HTT binding and any of the other four Openness facets.

These results indicate that the score for Openness to Experience, primarily driven by the facets Openness to Action and Openness to Values, shows a consistent association with the 5-HTTLPR genotype and cerebral serotonin transporter binding. In this sample, carriers of the short 5-HTTLPR allele (combined with carriers of L_G) had lower serotonin transporter binding, and

lower serotonin transporter binding was observed in the more open individuals. The direct association between the short 5-HTTLPR allele and Openness to Experience (Stoltenberg, Twitchell et al. 2002) was not reproduced in this sample.

This finding that Openness to Action and Openness to Values correlate with serotonin transporter binding measured with positron emission tomography is the first *in vivo* evidence for an association between cognitive flexibility versus rigidity and the human cerebral serotonin system. These findings add to the indirect observations by other groups using tryptophan depletion in conjunction with behavioral tests or animal models (Murphy, Smith et al. 2002; Crockett, Clark et al. 2008). Insofar as the personality dimensions of Openness to Action and Openness to Values reflect an individual's ability to re-consider and change accustomed ways of thinking or doing things and thereby adapt to new circumstances, it is also notable that higher openness is associated with general cognitive ability and more efficient higher intellectual processes (DeYoung et al., 2005). Given that lower serotonin transporter availability is associated with higher extracellular serotonin concentrations (Mathews, Fedele et al. 2004), the finding in the present sample is in line with the observation that disturbed serotonergic function is associated with impaired reversal learning (Robbins and Roberts 2007). Reversal learning is essential for adaption to the environment (Kringelbach and Rolls 2003) and also general quality of life (Kringelbach 2004; Kringelbach 2005).

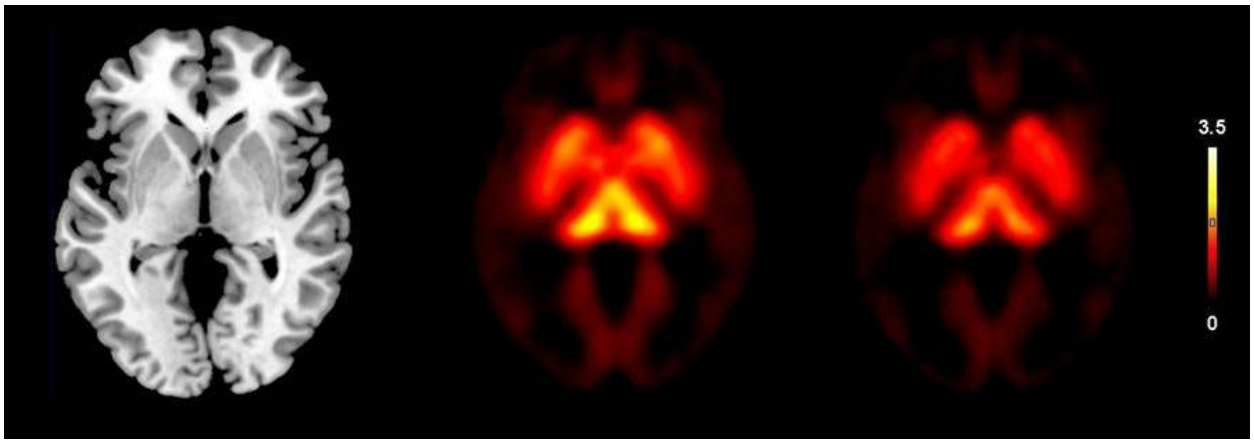


Figure 6: Averaged parametric maps representing specific binding of [^{11}C]DASB to the serotonin transporter and a structural image at same axial level (left image). Parametric maps (averaged) of the ten participants with least openness to values (middle image) and the ten participants most open to values (right image) show a global higher scaling in less open individuals.

Study III

In this study, I tested the hypothesis that the endophenotype of the short 5-HTTLPR allele is represented by a cerebral serotonin system that reacts more sensitively to the stress evoked by environmental changes. Out of 54 participants who were scanned with positron emission tomography using the radioligand [^{11}C]DASB, 19 were homozygotic carriers of the long 5-HTTLPR allele and 35 participants were carriers of the short 5-HTTLPR allele. There was a significant inverse correlation between daylight time in minutes at the day of the scan and serotonin transporter binding in the putamen ($r = -4.38$, $p < 0.001$), and bilaterally in the caudate nucleus ($r = -3.63$, $p < 0.05$). There was a non-significant trend towards a negative correlation in the thalamus ($r = -2.99$, $p < 0.1$), but no such association in the midbrain ($r = 1.92$, $p > 0.1$). The calculated day of peak serotonin transporter binding was in mid-December +/- 21 days, coinciding with the winter solstice. Similar results were obtained when the data were modeled to a harmonic function (Figure 7) that allows for a time delay in the seasonal effect.

In the putamen, there was a significant gene*daylight effect ($p < 0.05$, with correction for age and gender), with a negative correlation between daylight time and serotonin transporter binding in carriers of the short 5-HTTLPR allele, but no such correlation in homozygotic carriers of the long 5-HTTLPR allele. There was no seasonal variation of absolute or relative plasma tryptophan concentrations in this sample that could explain these variations. Also, there was no correlation between cerebral serotonin transporter binding and plasma tryptophan, either in terms of absolute concentration or relative to the (individual or composite) large neutral amino acids competing with tryptophan for transport at the blood-brain barrier. The gene*environment effect was only observed in the putamen, the region with the strongest association between serotonin transporter binding and daylight. This probably reflects a stronger gene-effect in that brain region (Praschak-Rieder, Kennedy et al. 2007), in conjunction with the stronger seasonal serotonin transporter binding fluctuations in putamen, as observed in the present experiment. This phenomenon might be explained by the high and homogenous binding in this region (Houle, Ginovart et al. 2000), which makes detection of a significant effect in a smaller sample more likely. Alternately, it might be that serotonin transmission in the putamen subserves a specific

role in the circannual adaptations of the nervous system. Such a phenomenon has not hitherto been described, and would seem to merit further exploration.

This is the first observation of temporally dynamic molecular changes in an endophenotype of the short 5-HTTLPR allele in the human brain *in vivo*. I interpret these results to reveal that homeostasis of the cerebral serotonin system is less stable in carriers of the short 5-HTTLPR alleles, and is more apt to be more affected by stressful environmental changes, for which amount daylight may be *sui generis*. However, when daylight minutes in the harmonic model (Figure 7) were replaced by time of the year, I observed the same correlation. It is therefore not possible to distinguish whether the relationship arises as a direct effect of daylight, or of any other factor with a similar circannual distribution, such as physical activity, consumption of domestic fruit, etc. However, the results suggest a testable hypothesis, in which phototherapy, as used for the treatment of seasonal affective disorder, could be used as an intervention to test the effect of light exposure *per se* on expression of the serotonin transporter.

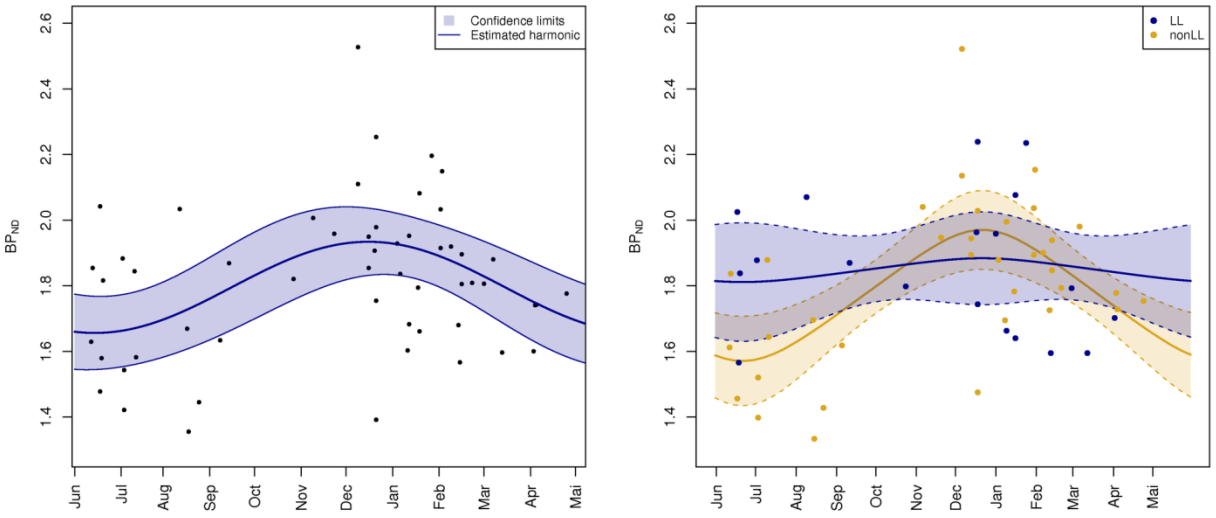


Figure 7: Left figure illustrates the seasonal effect on [^{11}C]DASB BP_{ND} (putamen) with point-wise confidence limits, modeled as a harmonic function with period one year (estimated peak in the middle of December, SE = 21 days, in good agreement with the model using daylight minutes as a predictor),and adjusted for age and gender. The plotted points are the partial residuals relative to BP_{ND} for a male of mean age). The functional form was validated by including additional frequency components and by comparison with estimates from an additive model. The right figure displays the interaction between number of daylight minutes and HTTLPR-allelic status, adjusted for age and gender. For comparison with the left figure the estimated linear response as a function of daylight minutes was transformed to a function of calendar time.

Study IV

In this study, I tested whether a polymorphism in the CLOCK gene has a similar effect on the serotonin system as the 5-HTTLPR. The CLOCK gene is an essential element in the positive-negative feedback loop of the human biological clock that is responsible for the regulation of circadian rhythms (Herzog 2007). Impaired CLOCK function substantially affects circadian rhythm-related behavior. For example, mice carrying a loss of function mutation of the CLOCK gene exhibit mania-like behavior with hyperactivity, decreased sleep and less depression-like behavior (Roybal, Theobold et al. 2007). Recently, the 3111 T/C SNP (rs1801260) in CLOCK has received increasing attention due to its effect on sleep disturbances in psychiatric disorders and on the outcome of SSRI treatment (Benedetti, Serretti et al. 2003; Serretti, Benedetti et al. 2003; Serretti, Cusin et al. 2005; Benedetti, Dallaspezia et al. 2007). Given the role of serotonin as a neurotransmitter in regulation of sleep, and its proposed involvement in different elements of mood disorders, I assumed that the effects of the CLOCK SNP are likely to be mediated by the serotonin system. I tested this claim by using the paradigm established in study III, in which nature seasonal variations were used as a surrogate for longitudinal design so as to study dynamic responses of the serotonin system in carriers of different genotypes. The effect of the CLOCK SNP was tested both on the serotonin transporter, using positron emission tomography and the radioligand [^{11}C]DASB and a post-synaptic marker, the serotonin 2A receptor, measured with positron emission tomography and the radioligand [^{18}F]altanserin.

Since I expected a global effect on the serotonin system, I chose to investigate regions of high and homogenous binding for both tracers. For the serotonin 2A receptor, these regions included the frontal cortex, the global neocortex (including the frontal cortex minus the anterior cingulated cortex) and the bilateral striatum. For the serotonin transporter, these regions included the bilateral putamen, the bilateral caudate nuclei, the bilateral thalamus, the midbrain, and a combined region for the global neocortex (defined as above). There was no statistical significant interaction effect ($p > 0.05$) between length of daylight-time in minutes and the CLOCK SNP on serotonin transporter binding or on serotonin 2A receptor binding. Also, there was no significant association between CLOCK SNP and serotonin transporter binding, serotonin 2A receptor binding, NEO-PI-R scores, or perceived stress measured with the Cohen's perceived stress scale.

These negative findings were unaffected by correction for age and gender. Among the 50 comparisons (radioligand binding in various regions, NEO-PI-R scores, PSS scores) one uncorrected p-value (for the effect of the CLOCK SNP on serotonin transporter binding in the caudate nuclei) fell just below 0.05, just as expected for a chance finding.

In spite of this negative result, this constitutes the first study to investigate the association between a functional polymorphism in the human clock system, the serotonin system, environmental changes and personality. Results failed to support the hypothesis that the CLOCK SNP might directly affect the serotonin system or modulate the responsiveness of the serotonin system to seasonal changes. Furthermore, the CLOCK SNP was not associated with any personality dimension measured with the NEO-PI-R or perceived stress as measured with Cohen's perceived stress scale. Since Openness to Experience reflects sensitivity to environmental changes (reviewed above), it seems unlikely that the effect of the CLOCK SNP has a profound effect on this aspect of personality.

A strength of this study consists of the use of highly-specific radioligands and well-validated quantification methods. A further strength is provided by the large sample size relative to many positron emission tomography studies of healthy controls, in a population including both genders and extending over a wide age range. The study is limited by the relatively small sample-size for detecting a gene effect for the low abundance SNP. In conclusion, I did not find any discernible effect of the CLOCK SNP on pre- and post-synaptic markers of the serotonin system, not even when I analyzed for gene*environment interaction effects.

Overall Comments

Overall, the results of the studies presented in this dissertation support a theoretical model in which the serotonin system is responsible for the regulation of the flexibility versus the rigidity of behavioral responses and the adaption of self-concepts and ways of doing things to the environment (Study II). Furthermore, the studies provide evidence supporting the claim that the 5-HTTLPR predicts stability of the adaptive changes of the cerebral serotonin system in response to environmental stress (Study III). I did not confirm the hypothesis that a polymorphism in the CLOCK-gene has similar effects (Study IV); this latter hypothesis was based on the assumption that the biological clock system has an upstream function in mediating circannual changes on the serotonin system. In consideration of all of these studies, it must be recalled the samples sizes are relatively large for positron emission tomography studies, but still relatively small to detect gene effects, given the low abundance of the relevant alleles and SNPs.

Most solid are the observations that serotonin transporter binding is inversely correlated with Openness to Actions and Openness to Values (Study II), and the observation that serotonin transporter binding fluctuates over seasons (Study III). The latter result was recently confirmed in a large independent study (Praschak-Rieder, Willeit et al. 2008), and can therefore be reliably proposed as a consistent effect. However, the effect of the 5-HTTLPR in interaction with seasonal changes on serotonin transporter binding barely reached statistical significance, and in only one of four brain regions (Study III), as did the effect of the L_A/L_G single-nucleotide polymorphism on serotonin transporter binding (Study II). The latter was confirmed by two independent studies (Praschak-Rieder, Kennedy et al. 2007; Reimold, Smolka et al. 2007).

In the context of the reviewed literature and my results, one might speculate that there exists a distinct endophenotype of the 5-HTTLPR (figure 8). While the homeostasis in carriers of the long 5-HTTLPR allele would seem to be more stable (study III), the lesser seasonal fluctuation is associated with a generally higher availability of serotonin transporters (study III), which predicts more rapid phasic signaling of serotonin transmission. This was associated with more rigid behavior that seems less adaptive and flexible, being more focused on individual survival in the current situation (small immediate rewards, less suppression of socially unaccepted

behavior). In contrast, the homeostasis of the serotonin system in carriers of the short 5-HTTLPR allele may impart the advantage of facilitated shifting between more open and adaptive behavior, while facilitating less adaptive survival-oriented behavior in stressful situations. Thus, increased flexibility of the serotonin system may constitute a trade-off between the advantages adaptive behavior, and a higher risk to develop mood (or other psychiatric) disorders in response to environmental stress.

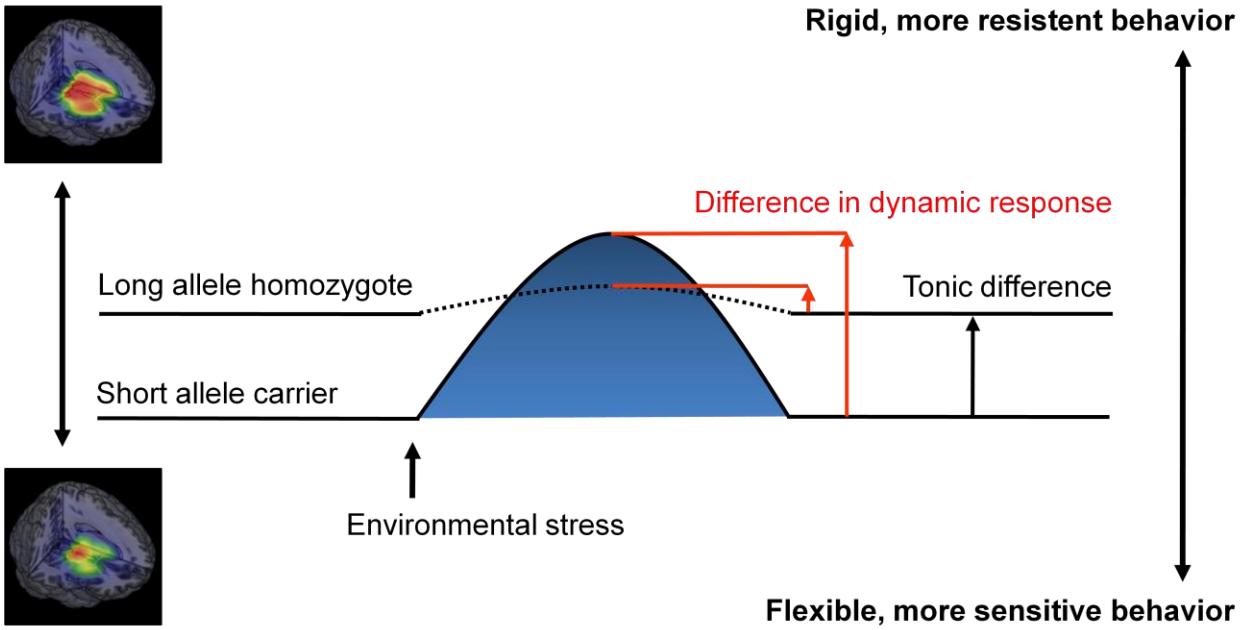


Figure 8: A diagrammatic illustration of a model integrating both the tonic (in terms of generally higher or lower binding) and the seasonal dynamic difference of the serotonin system in carriers of the short 5-HTTLPR allele and long allele homozygotes. Earlier studies, using lymphoblast cell lines (Lesch, Bengel et al. 1996), and positron emission tomography in a relatively small group of healthy controls (Heinz, Jones et al. 2000), observed that carriers of the short 5-HTTLPR allele have lower cerebral serotonin transporter binding, but larger studies have so far failed to detect this effect (Willeit, Stastny et al. 2001; van Dyck, Malison et al. 2004). Based on the observation that serotonin transporter binding fluctuates substantially over seasons, the lack of consistent evidence can, most likely, be attributed to missing control of this covariant. The combination of prior results and the results of this thesis suggest an endophenotype for the short 5-HTTLPR allele with two distinct characteristics: (1) under controlled conditions, carriers of the short 5-HTTLPR allele have lower cerebral serotonin but more importantly, (2) when exposed to stress the cerebral serotonin system in carriers of the short 5-HTTLPR allele responds stronger due to a less stable homeostasis and their behavioral patterns shift from formerly orientation on social interaction to rigid behavior with primarily orientation on small immediate rewards and other behavioral patterns typical for low levels of serotonin and, for example, seasonal affective disorder.

Outlook

The speculations and overall comments which I present at the conclusion of this thesis demand further exploration in larger populations. Some of these studies could be performed using behavioral paradigms and neurochemical challenges, such as acute tryptophan depletion, rather than positron emission tomography. This approach would allow inclusion of far more participants, and permit longitudinal measurements. Of particular interest would be to test:

- whether a sensitive homeostasis of the cerebral serotonin system, as identified with acute tryptophan depletion and behavioral paradigms, has advantages in some environmental settings and disadvantages in others. One could, for example, compare behavioral scores in a challenge paradigm in a Scandinavian population with a matched group from southern Europe.

- whether those carriers of the short 5-HTTLPR allele suffering from SAD in countries of high latitude show better adaptive skills upon migrating southward, for example retired Scandinavians moving from northern Scandinavia to Spain.

Furthermore, the model illustrated in Figure 8 could be tested combining direct and quantifiable stimuli. For example, phototherapy with an intense light source is of demonstrated effectiveness against the symptoms of seasonal affective disorder. Does winter phototherapy, while remaining at high latitude, attenuate the seasonal changes in serotonin transporter availability reported in this thesis? Another topic concerns the action of deep brain stimulation (DBS) for the treatment of Parkinson´s disease (Kringelbach, Jenkinson et al. 2007; Kringelbach, Owen et al. 2007). Successful treatment of motor symptoms by DBS is marred by emergence of depressive symptoms in some patients. Recent animal studies have revealed that DBS in the *subthalamic nucleus*, the most common target for Parkinson's disease patients, leads to an acute decrease in firing rate of the serotonergic neurons in the *dorsal raphé nucleus* (Temel, Boothman et al. 2007). Molecular imaging, in conjunction with DBS, could be brought to bare on examining the effects of DBS on pre- and post-synaptic markers of the serotonin system of genotyped Parkinson´s disease patients.

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Appendices

A probabilistic approach to delineate functional brain regions

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A probabilistic approach to delineate functional brain regions

Jan Kalbitzer[1*], Claus Svarer[1], Vibe G. Frokjaer[1], David Erritzoe[1], William F. C. Baaré[2], Jacob Madsen[3], Steen G. Hasselbalch[1] and Gitte M. Knudsen[1]

[1]Neurobiology Research Unit, Copenhagen University Hospital, Denmark

[2]Danish Research Center for Magnetic Resonance, Copenhagen University Hospital, Hvidovre, Denmark

[3]PET and Cyclotron Unit, Copenhagen University Hospital, Denmark

*Corresponding author:

Jan Kalbitzer, MD

Neurobiology Research Unit

Copenhagen University Hospital

Blegdamsvej 9, 2100 Copenhagen O, Denmark

Phone: +45 3545 6708, Fax: +45 3545 6713

Email: jan.kalbitzer@nru.dk

ABSTRACT

OBJECTIVE: To develop a reliable observer-independent approach to delineate volumes of interest (VOI) for functional brain regions which are not identifiable on structural MR images. The case is made for the raphé nuclei, a collection of nuclei situated in the brainstem known to be densely packed with serotonin transporters (5-HTT). METHODS: A template set for the raphé nuclei based on their high content of 5-HTT as visualized in parametric [^{11}C]DASB PET images, was created for ten healthy subjects. The templates were subsequently included in the region sets used by the automatic approach described by Svarer *et al*. to create an observer and activity independent probabilistic VOI map. The probabilistic map approach was tested in a different group of ten subjects and compared with a manual delineation approach. RESULTS: Besides providing an observer-independent solution, the probabilistic map approach returned a higher specific binding determined in a larger region and this ultimatively provided better data fitting in kinetic modeling. CONCLUSIONS: We developed a fast, observer-independent reliable approach to delineate regions that can only be identified by functional imaging, here exemplified by the raphé nuclei. This approach can in future studies be used to create functional VOI maps based on neuroreceptor fingerprints retrieved through in-vivo brain imaging.

Key words: Raphé nuclei, volume of interest, serotonin transporter, dasb

INTRODUCTION

The raphé nuclei are located in the brainstem and constitute the core of the 5-hydroxytryptaminic (serotonin) system [1]. Alterations in general morphology and serotonin activity in this area have been reported in mood disorders such as depression [2-5], and bipolar disorder [4, 5] and also degenerative diseases [6]. Accordingly, the raphé nuclei constitute a highly interesting brain region for positron emission tomography (PET) in neuropsychiatric research on the serotonin system. However, delineation of an area for the raphé nuclei is complicated by the region's anatomical location and structure. The nuclei consist of a heterogeneous group of cells around the midline of the brainstem which is poorly defined towards the surrounding tissue and it is therefore difficult not only to identify the raphé nuclei in structural brain images but also histologically [1].

So far, the raphé nuclei region has been defined manually either directly on the functional image or on a co-registered MR image by: 1) placing of circles with a fixed diameter on consecutive slides directly on PET scans, around the center of highest activity [2, 7], 2) placing a circle of a fixed diameter on MR images in midbrain in front of the aqueduct [5], or 3) 'direct delineation on PET images' without further description [8, 9]. These approaches are, however, prone to be biased by physiological and pathological activity alterations in this area and do not fully take into account the individual shape and size of the brain. Furthermore, they are highly user dependent and are likely to be associated with high inter- and intra-observer variability and almost unavoidable with noisy time activity curves.

Here, we describe a probability map based automatic approach for delineation of the raphé nuclei on MR- and PET images. The method is strictly observer-independent and based on a probabilistic mapping algorithm to delineate anatomical regions, as described earlier [10]. Firstly, in ten subjects

we identified the raphé region in the PET images, transferred the VOI to the co-registered MR-images and these ten MR-templates were subsequently used as templates when creating a probability-based VOI in ten new subjects.

MATERIALS AND METHODS

Subjects

Twenty volunteers were recruited by newspaper advertisement. MR- and PET-images from ten of the healthy subjects (age 22–40 years; 5 females) were used to establish templates for the raphé nuclei region, and the template was subsequently applied to images from the other ten healthy volunteers (age 25-63 years; 6 females). The protocol was approved by the Ethics Committee of Copenhagen and Frederiksberg [(KF) 01-156/04, (KF) 01-124/04, and (KF) 11-283038]. Exclusion criteria included history of medical or neuropsychiatric disorders and drug abuse.

PET- and MR imaging, co-registration

PET scans were performed with an 18-ring GE-Advance scanner (General Electric, Milwaukee, WI, USA), operating in 3D acquisition mode, producing 35 image slices with an interslice distance of 4.25 mm. The final PET voxel size was 2 mm x 2 mm x 4.25 mm. Following a 10 min transmission scan, a dynamic 90 emission scan was initiated upon intravenous injection of 246 - 590 MBq [^{11}C]DASB over 12 sec. The acquisition consisted of 36 time frames, increasing progressively in duration from 10 sec to 10 min. The attenuation and decay corrected recordings were reconstructed by filtered back projection using a Hann filter (6mm).

High-resolution 3D T1-weighted, sagittal, spoiled gradient echo scans (MPRAGE) of the head were acquired on a Siemens Magnetom Trio 3T MR scanner with an eight-channel head coil (Invivo, FL, USA). The MR voxel size was 1 mm x 1 mm x 1 mm.

The emission recording across frames 10-36 was automatically aligned to frame 26. Then the PET image (using an average of frame 10 – 36) was co-registered to the MR image using the AIR algorithm (http://bishopw.loni.ucla.edu/AIR5/); the quality of each co-registration was evaluated by visual inspection.

Quantification and Region Assessment

The radioligand [^{11}C]DASB binds with high affinity and selectivity to 5-HTT sites [11]. For quantification we used the modified reference tissue model (MRTM2) with k2' calculated for each subject individually using MRTM as described by Ichise *et al.* [12].

For the kinetic modelling PMOD Version 2.85 (PMOD Technologies) was used. PMOD returns a value, Chi^2, that describes the deviation between estimated and actual time activity curves (i.e., goodness of fit) based on a cost function.

Delineation of VOI

Firstly parametric images of specific binding (BP_{ND}) of [^{11}C]DASB were created. On [^{11}C]DASB parametric images, the rostral raphé nuclei can easily be identified as the center of highest activity (Figure 1). However, a delineation of the raphé region by the application of a fixed activity threshold directly on the PET image is prone to be biased by physiological and pathological alterations. Instead, we used an extension of the method described by *Svarer et al.* [10] where the

functional raphé nuclei region defined from BP images is included in MR VOI template sets. These templates are then used as described in [10] to create a probabilistic map of the raphé region for new subjects which is threshold independent of the BP images for these subjects.

Creating the template set

Identification of the raphe nuclei VOI on the parametric images (representing BP_{ND} for each voxel) was done on the ten template subjects by applying an individually determined threshold. First, the threshold at the level of rostral midbrain/the interpeduncular fossa, identified on the co-registered MR-image, was established, and this threshold was then fixed on consecutive slices in caudal direction. The threshold was settled on the first slices in a strictly reproducible manner by gradually increasing the threshold of BP_{ND} (using 0.01 steps) until at no level the VOI exceeded the mediolateral brainstem (Figure 2). According to histological [1] and cytoarchitectonical [13] studies, it is at this level we expected raphé activity. Usually, the last remaining activity outside the raphé were small non-confluent volumes in the vincinity of thalamus.

In this way, for all ten images, the VOI encompassed the rostral portion of the raphé nuclei, mainly the nucleus raphé dorsalis. The VOI was then transferred to each subject's co-registered MR image to be used for subsequent warping procedure (Figure 3). Additionally we used our common template set as described earlier [10] to create probabilistic VOI for cerebellum, thalamus, caudate and putamen in order to determine k2' from the extracted time activity curves for reference binding (cerebellum) and high-binding regions (thalamus, caudate, putamen).

Using the template set to create observer-independent probabilistic VOI

The resulting ten VOI templates were then used to define the raphé nuclei region **for** the remaining ten of the twenty test subjects, similar to what was done in Svarer *et al*. [10] (Figure 3). In short, the probabilistic VOI are created by estimation of a warp field from each of the ten template MR images to the individuals MR image and the warp field is then applied to the template raphé nuclei VOI. In each new subject, the ten warped VOI's constitute a unique probabilistic map that is then thresholded to define the final VOI. This threshold is automatically determined by adjusting it so the volume of the generated region has the same volume as the mean regional volume of all the transferred template regions, as described in [10]. Accordingly, the generated VOI had a volume that fits the volume of the brain region in the new subject and thereby the brain size for that subject.

Comparison between probabilistic and manual delineation

For comparison, manual delineation was also done as described by other groups [2, 7]: Starting at the level where the interpeduncular fossa was first clearly visible continuing in caudal direction, circles with a fixed diameter (6 mm) were inserted directly on 4-5 slices on the summed PET images centered around the highest activity. This task was performed independently on the same ten test subjects by three trained observers.

In order to evaluate the effect of the VOI determined by either approach (probabilistic or manual) time activity curves were extracted for all ten test subjects and the raphé nuclei specific binding **(BP_{ND})** was calculated for each subject as mentioned above. We used a 2-way ANOVA to compare BP_{ND} and Chi^2 between automatic and manual approach. Additionally, for evaluation of the inter-observer variability, we calculated the inter-class correlation coefficient (ICC) between all three observers using Matlab®-based in-house made software.

RESULTS

The probabilistic map approach resulted in a volume for the raphe VOI which was more than twice as large as the VOI derived from the manual approach (1.41 ± 0.12 ml vs. 0.58 ± 0.04 ml). The ICC for the observer-dependent delineation approach was only 0.47. Time activity curves derived from the observer-independent approach showed less noise resulting in a better fit represented by a significantly lower Chi^2 (Figure 4, panel B) as compared to the value coming from manual delineation by 3 different observers; values were 12.57 ± 4.29 (probabilistic VOI), 15.30 ± 5.41 (observer 1), 14.51 ± 4.72 (observer 2), and 14.50 ± 5.48 (observer 3). Probabilistic VOI delineation resulted in significantly lower Chi^2 as compared to manual delineation, $p<0.001$, 2-way ANOVA. As shown in figure 4, panel A, BP_{ND} resulting from the probabilistic map approach as compared to manual delineation was 4.40 ± 0.44 (probabilistic VOI), 3.65 ± 0.65 (observer 1), 3.58 ± 0.62 (observer 2), and 3.45 ± 0.57 (observer 3). Probabilistic VOI delineation resulted in significantly higher BP_{ND} as compared to manual delineation, $p<0.001$, 2-way ANOVA.

DISCUSSION

With an extension of the probability map based approach we have here shown the feasibility for objective identification of functional brain VOI that are not identified by their anatomical structure, but rather by their anatomical relationship to other brain structures. The templates were created on the basis of the activity contrast to the neighboring region, thalamus. Although this may seem to be somewhat arbitrary the resulting volumes were consistently defined and served the purpose of raphe delineation in the independent sample. Further, although the method returns volumes that are more than two times larger than a manual approach, yet the resulting BP_{ND} were significantly higher and goodness-of-fit improved. This supports that the raphé VOI is more correctly identified. In accordance with the improved goodness of fit we observed less noisy time activity curves for the

raphé, probably largely because of the inclusion of a larger volume, associated with better count statistics. The volume of the probabilistic VOI (approx. 1.4 ml) exceeds what was found in cytoarchitectonical studies in humans for the rostral raphé nuclei (approx. 70 ml) [13]. This is mainly caused by two factors: 1) the probabilistic VOI we created merged activity of all sub-nuclei of the nucleus raphe dorsalis in one region, including most of the interfascicular, ventral, ventrolateral and dorsal portion. The manually delineated VOI usually did not include activity from the interfascicular and ventrolateral subnuclei. 2) The probabilistic VOI is based on and created for activity measured with PET which suffers from low resolution and partial volume effects and thus is less precise than histological sections. On the other hand, the raphe region is difficult to accurately outline in histological sections, which further may have undergone some shrinkage. It should be underscored that we do not attempt to validate the appropriateness of the identified raphe volume. However, the probabilistic VOI approach is far superior to the current alternative, namely manual delineation on the functional image, which is both laboursome, subjective, less reprodicible, and potentially biased in disorders where the raphe activity is altered.

The large interobserver variability observed with the manual approaches was surprising, particularly since the observers are experts in anatomical delineations and they received the exact same instructions. Our finding suggests that results published from the raphé region with the manual approach are likely to be associated with considerable noise, not only because of the more noisy time activity curves, but also the larger interobserver variability and smaller BP_{ND}'s requires larger sample sizes to correctly identify group differences or associations. To build the probability map we used templates from ten healthy subjects and we did not attempt to validate the impact of including more or less subjects. We have, however, previously shown that inclusion of six subjects is enough to stably identify even small VOI correctly [10] and found that inclusion of additional

subjects/templates did not significantly improve the estimates. An alternative approach would be to create a probabilistic map in standard space and that could easily have been done. It has previously been shown, however, that this approach is inferior to a probabilistic map created on the basis of ten independent templates [10, 14].

The defined VOI encompassed the rostral portion of the raphé nuclei only, mainly the nucleus raphé dorsalis (Fig. 1). Although this may be seen as a relative limitation, the rostral group of the raphé nuclei produces more than 85% of serotonin in the entire human brain The approach would need, however, to be properly validated for assessment of, e.g., 5-HT_{1A} receptor binding.

Although in this particular case we showed the approach to be valid for 5-HTT binding in the raphé nuclei region, the approach is principally applicable for any functional brain region, no matter which imaging modality (e.g., SPECT, PET, fMRI) is used, as long as the functional image comes with a structural image of sufficient quality. Because of the large inter-subject variability in structure-function relationship, this may be more difficult for fMRI studies. However, functional delineation of the brain by neurotransmitter receptor fingerprints as described by Zilles *et al.* [15] would be an obvious way to apply the probability map based approach, by combining different templates for different tracers in order to ultimatively create a probabilistic map of the receptor fingerprint of a functional region.

CONCLUSION

We have presented a fast observer-independent probability map based method which yields less noisy data, higher binding potentials, and is more reproducible for quantification of the serotonin transporter binding in the raphé nuclei. The approach may prove useful for delineations of other

functional brain regions that lack a clear anatomical correlate, where functionality is defined either with molecular imaging, fMRI or MEG.

Templates and software for the probabilistic VOI map are freely available and included in the software package for partial volume correction pvelab provided proper reference to this paper is given. Please register at http://nru.dk/pveout for download and installation instructions.

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FIGURE LEGENDS

Figure 1. Three dimensional overlay of a MR-image and a parametric map of a [^{11}C]DASB PET image representing specific binding (BP_{ND}) to 5-HTT. BP_{ND} was thresholded until only activity in the area of the raphé nuclei was visible.

Figure 2. Creation of a template: delineation of the rostral raphé nuclei on a parametric PET image by increasing the threshold until no activity outside the raphe area is included (A) and then transferring the VOI to a co-registered MR image (B).

Figure 3. Warping from several template MR images (n=10) onto the MR image of the test person creates a probabilistic VOI (A) and in a second step the VOI is transferred from the MR image of the test person to the co-registered PET image (B).

Figure 4. Specific binding (BP_{ND}) (panel A) and resulting Chi^2 (panel B) obtained using the probabilistic VOI and the three observers VOI. Horizontal lines represent means, bars indicate one SD.

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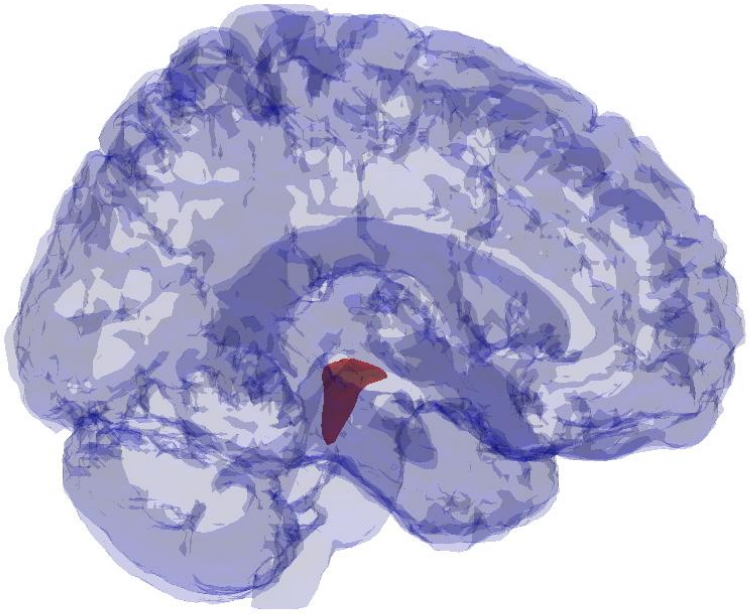

Figure 1

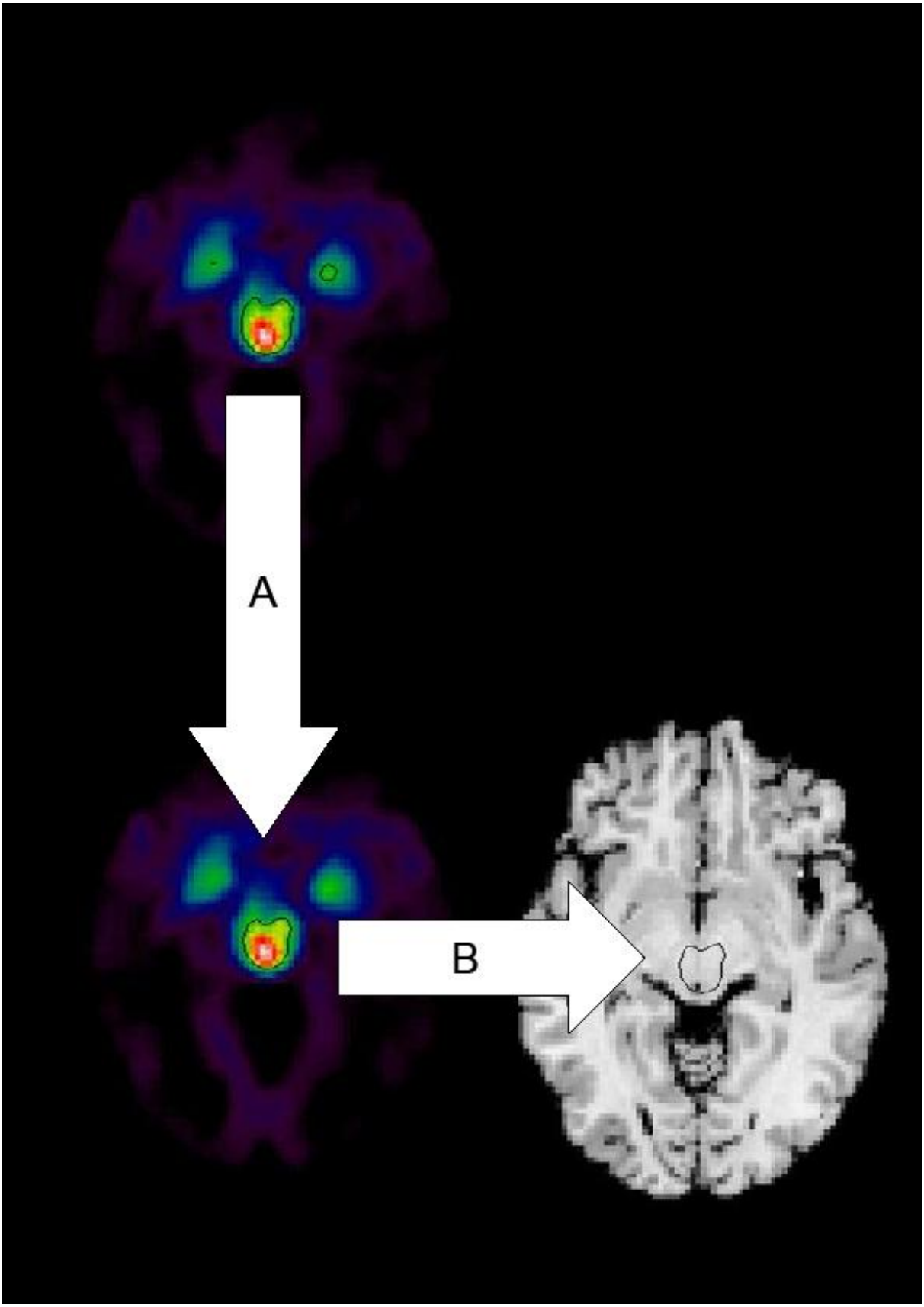

Figure 2

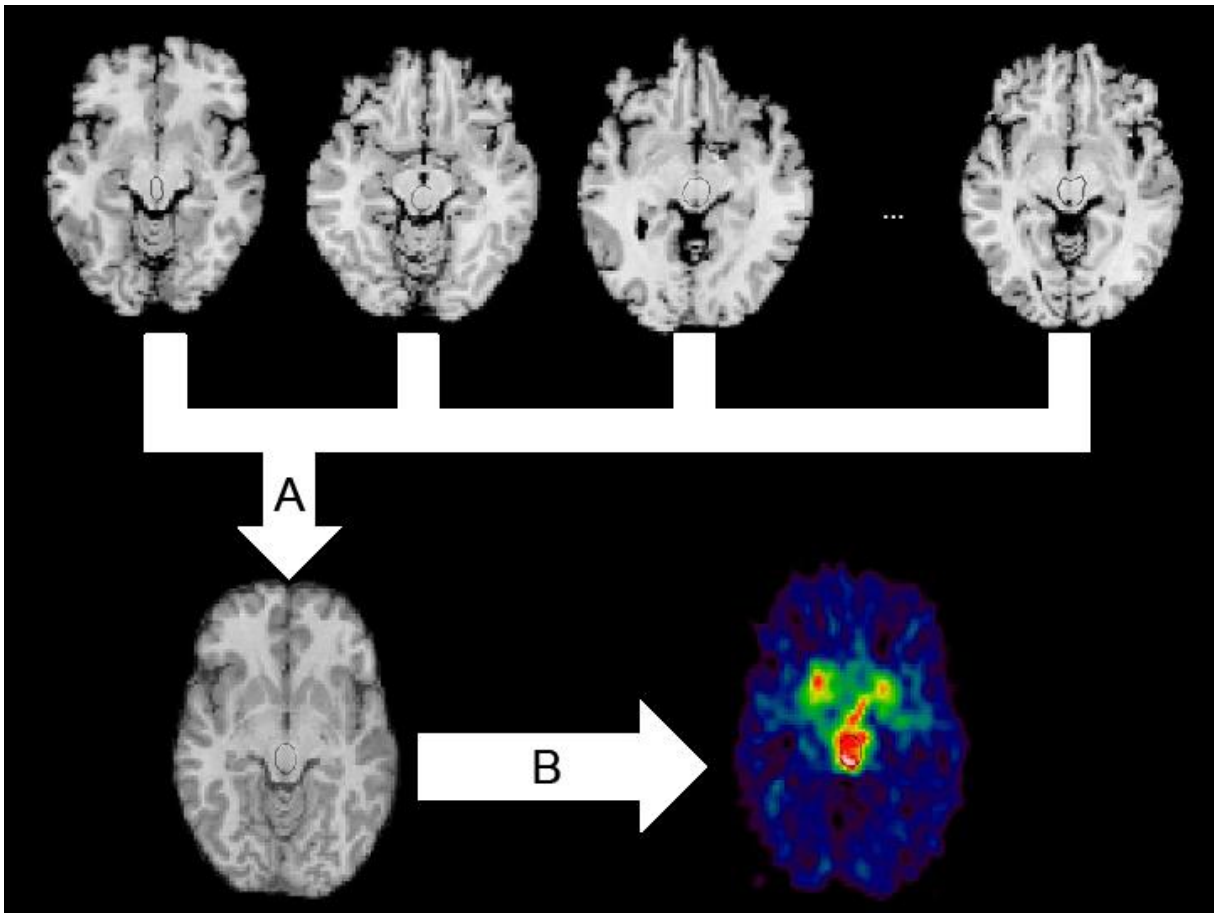

Figure 3

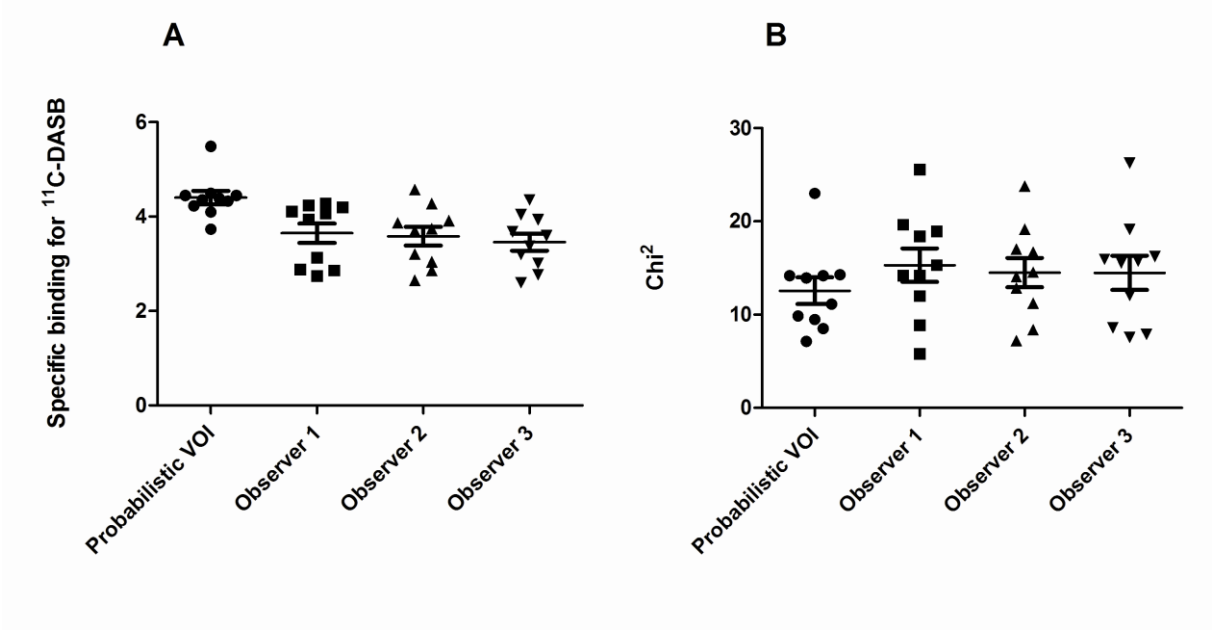

Figure 4

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The personality trait openness is related to cerebral 5-HTT levels

Jan Kalbitzer [a,b,*], Vibe G. Frokjaer [a,b], David Erritzoe [a,b], Claus Svarer [a,b], Paul Cumming [c], Finn Å. Nielsen [b,d], Sayed H. Hashemi [a,b], William F.C. Baaré [b,e], Jacob Madsen [b,f], Steen G. Hasselbalch [a,b], Morten L. Kringelbach [g,h], Erik L. Mortensen [i], Gitte M. Knudsen [a,b]

[a] Neurobiology Research Unit, Copenhagen University Hospital Rigshospitalet, Denmark
[b] Center for Integrated Molecular Brain Imaging, Copenhagen University Hospital Rigshospitalet, Denmark
[c] Department of Nuclear Medicine, Ludwig-Maximilian University, Munich, Germany
[d] Department of Informatics and Mathematical Modelling, Technical University of Denmark, Denmark
[e] Danish Research Center for Magnetic Resonance, Hvidovre Copenhagen University Hospital, Denmark
[f] PET and Cyclotron Unit, Copenhagen University Hospital Rigshospitalet, Denmark
[g] Department of Psychiatry, University of Oxford, UK
[h] CFIN, Aarhus University, Aarhus, Denmark
[i] Department of Environmental Health, Institute of Public Health, University of Copenhagen, Denmark

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ABSTRACT

Potentiation of serotonergic transmission increases cognitive flexibility, but can in other circumstances increase sensitivity to stressful environmental cues. The personality trait *Openness to Experience* reflects and is also associated with an increased risk for mood disorders. We hypothesized that the personality trait has an association with a biomarker of serotonergic transmission, the plasma membrane serotonin transporter (5-HTT). In 50 healthy volunteers, we tested for correlations between scores on the NEO-PI-R scale *Openness to Experience* and its subscales, and cerebral binding of the 5-HTT selective PET radioligand [^{11}C]DASB. Subjects were genotyped for the 5-HTT long/short polymorphism, and for a single nucleotide polymorphism in the long allele, designated L_A/L_G. Midbrain [^{11}C]DASB binding correlated negatively with scores for *Openness to Experience* and its two subscales, *Openness to Actions* and *Openness to Values*. The latter subscore was negatively correlated with [^{11}C]DASB binding in all brain regions in which [^{11}C]DASB binding was quantified. Genetic analysis showed that homozygote L_A carriers had significantly higher [^{11}C]DASB binding in the caudate nucleus, but no significant differences in openness scores. Thus, high scores in personality facets indicative of cognitive flexibility and openness to change are associated with lower [^{11}C]DASB binding. Lower abundance of 5-HTT sites may result in potentiation of serotonergic signaling, which occurs during treatment with SSRIs. We speculate that the set-point of serotonergic signaling in an individual represents a trade-off between flexibility and vulnerability when exposed to environmental stress.

Introduction

In the 60 years since its first chemical isolation (Rapport et al., 1948), serotonin has been implicated as a factor in cognitive flexibility, as well as in mood disorders. Results of studies with behavioral paradigms in humans and other animals have linked serotonin with the processing of affective stimuli (Canli et al., 2008), working memory (Robbins and Roberts, 2007), and synaptic plasticity (Celine et al., 2006). Among healthy human subjects, selective depletion of tryptophan, which reduces serotonin synthesis, led to more rigid behavior (Murphy et al., 2002) and decreased tolerance of the perceived unfair behavior of others (Crockett et al., 2008). Furthermore, reversal learning in rats, which entails flexible re-assignment of behavior in response to environmental cues, is interrupted by lesions of cortical serotonin innervations (Robbins and Roberts, 2007).

Based on these associations between the serotonin system and behavior, we asked if the broad personality dimension *Openness to Experience* (OtExperience) is related to 5-HTT function: OtExperience as measured with the Revised Neuroticism, Extraversion, Openness Personality Inventory (NEO-PI-R) is associated with increased biological response to stress (Oswald et al., 2006) and increased risk of developing winter depression, a classical environment-induced mood disorder (Bagby et al., 1996; Jain et al., 1999). Consistent with the present theme, carriers of the 44-base pair deletion (*S-allele*) in the 5-HTT-linked polymorphic region (5-HTTLPR), which is associated with *lower* 5-HTT mRNA expression (Lesch et al., 1996), have been found to have higher OtExperience scores (Stoltenberg et al., 2002)

* Corresponding author. Neurobiology Research Unit, Copenhagen University Hospital Rigshospitalet, 9 Blegdamsvej, 2100 Copenhagen O, Denmark. Fax: +45 3545 6713.
E-mail address: jan@kalbitzer.net (J. Kalbitzer).

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and also to be more at risk to develop seasonal affective disorder (SAD) (Rosenthal et al., 1998; Willeit et al., 2003). On the other hand, OtExperience has been associated with increased cognitive flexibility, more adequate coping strategies for psychiatric disorders among the elderly (Duberstein, 1995), and higher survival rate in somatic disorders (Jonassaint et al., 2007). Due to this duality, OtExperience may be the personality dimension that reflects the net-effects of serotonin on both cognitive flexibility and sensitivity to stressful environmental cues. However, despite this evidence, there has been only a single PET study linking openness (specifically to spiritual experience) and serotonin 1_A receptor availability (Borg et al., 2003).

We used positron emission tomography (PET) in conjunction with genetic analysis of the 5-*HTTLPR* to test for associations between cerebral 5-HTT availability measured *in vivo*, and OtExperience scores from the NEO-PI-R in a population of 50 healthy volunteers. Because recent findings suggest that not only the *S-allele*, but also a single nucleotide polymorphism (SNP) within the 44-base pair insertion of the *L-allele* (L_G) is associated with lower 5-HTT mRNA expression (Nakamura et al., 2000) and lower cerebral 5-HTT binding (Praschak-Rieder et al., 2007; Reimold et al., 2007), we also conducted a genetic analysis of the 5-*HTTLPR* in our sample. Individual scores on the personality test and PET end-point, the 5-HTT binding potential (BP_{ND}) for [^{11}C]DASB, were tested for associations, with the 5-*HTTLPR* carrier status taken into account.

Methods

Subjects

Fifty healthy volunteers were recruited by advertisement for a research protocol approved by the Ethics Committee of Copenhagen and Frederiksberg, DK [(KF) 01-156/04, (KF) 01-124/04, and (KF) 11-283038]. After complete description of the study to the subjects, written informed consent was obtained from 35 males with a mean age of 32 years (SD 15 years), and from 15 females with a mean age of 38 years (SD 18 years). Exclusion criteria for all test persons included a significant medical history, drug abuse or psychiatric disorders. All subjects had a normal neurological examination and their magnetic resonance (MR) brain images were without pathological findings.

PET scans

PET scans were performed with an 18-ring GE-Advance scanner (General Electric, Milwaukee, WI, USA), operating in 3D acquisition mode, and producing 35 image slices with an interslice distance of 4.25 mm. Following a 10 min transmission scan, a dynamic 90 min long emission recording was initiated upon intravenous injection during 12 s of mean 484 (SD 89) MBq (range: 246–601) [^{11}C]DASB with mean specific activity of 32 (SD 16) GBq/μmol (range: 9–82). The emission recording consisted of 36 frames, increasing progressively in duration from 10 s to 10 min. The attenuation and decay corrected recordings were reconstructed by filtered back projection using a 6 mm Hann filter.

MR scans

Structural brain scans were acquired on a Siemens Magnetom Trio 3 T MR scanner with an eight-channel head coil (In vivo, FL, USA). Thirty-five subjects underwent a high-resolution 3D T1-weighted, sagittal, magnetization prepared rapid gradient echo (MPRAGE) scan of the head (MPRAGE1: echo time (TE)/repetition time (TR)/inversion time (TI)=3.93/1540/800 ms; slice resolution=75%; Bandwidth=130 Hz/Px; Echo spacing=9.8 ms) and fifteen subjects underwent a 3D T1-weighted, sagittal, MPRAGE (MPRAGE2: TE/TR/TI=3.04/1550/800 ms; slice resolution=100%; Bandwidth=170 Hz/Px; Echo spacing=7.7 ms). Common to both MPRAGE's was a flip angle of 9°, an FOV of 256 mm, a matrix of 256×256, 1×1×1 mm voxels and 192 slices.

MR/PET co-registration

All time-frames of the attenuation-corrected emission recording were automatically aligned to frame 26 using the AIR algorithm (Woods et al., 1998). In a next step we used a mean PET image, averaging time-frames 10–36 for co-registration to the individual MR image using the AIR algorithm (Woods et al., 1998); the quality of each co-registration was controlled visually.

Volume of interest (VOI) analysis

The VOI were delineated automatically as described in Svarer et al. (2005) in order to identify the volumes in a user-independent fashion. For each of the 10-template VOI sets, a 12-parameter affine transformation and a warping field was calculated between the template MR image and the individual MR image for a subject. Having obtained the MR/PET co-registration for the same individual as described above, the template VOI sets are then transferred to the dynamic PET image space for each subject, using the identified transformation parameters. From the VOI sets, a probability map was created for each subject, and a common VOI set was threshold-generated. These VOI sets were then used for automatic extraction of time activity curves (TAC) for midbrain and volume-weighted (left–right) averages for bilateral thalamus, caudate, putamen and cerebellum for all subjects. The midbrain, the caudate, the putamen and the thalamus were selected as representative brain regions of homogenous, high 5-HTT binding (Houle et al., 2000) since we expected that the 5-*HTTLPR* status would lead to a global effect. The TAC extracted for the cerebellum was used as the reference tissue input for kinetic modeling.

Quantification of non-displaceable tracer uptake

The outcome parameter of the cerebral [^{11}C]DASB binding within a brain region is the non-displaceable binding potential, designated BP_{ND}. The BP_{ND} was calculated for the four VOIs, using the cerebellum input as reference region with non-specific binding. We used a modified reference tissue model designed specifically for quantification of [^{11}C]DASB (MRTM/MRTM2) as described and evaluated by Ichise et al. (2003) using the PKIN tool of the software PMOD version 2.9: A fixed washout constant, designated k2′, was calculated for each individual as an average of k2 in caudate, putamen and thalamus relative to cerebellum using MRTM. Subsequently, k2′ was used as a constrained input parameter for the calculation of BP_{ND} in the four VOIs relative to cerebellum.

Parametric images

For the voxel-based analysis, parametric images representing BP_{ND} for each voxel were calculated for all subjects from the dynamic PET images. Using the PXMOD tool of the PMOD software, we imported the VOI, generated as described above, and re-calculated k2′ relative to cerebellum using MRTM. As described by Ichise et al. (2003), we applied an R1 (tracer delivery to the VOI relative to cerebellum, to some extent reflecting blood flow) threshold of 0.3 to exclude noisy voxels, particularly seen in regions with low tracer clearance. Additionally, we restricted the BP_{ND} outcome to range between 0 and 10. The resulting parametric images were filtered with a 3 mm median filter in their native space. SPM5 (http://www.fil.ion.ucl.ac.uk/spm/software/spm5/) was used to normalize the parametric images to Montreal Neurological Institute (MNI) space using a warping field estimated by normalization of each subject's co-registered MR image. Subsequently, a 12 mm Gaussian filter was applied to all normalized images.

Personality assessment

OtExperience with its facets *Openness to Fantasy*, *Openness to Aesthetics*, *Openness to Feelings*, *Openness to Ideas*, *Openness to Actions* (OtAction), and *Openness to Values* (OtValues) was assessed on the day of the PET scanning with the Danish version of the Revised NEO Personality Inventory (NEO-PI-R). The NEO-PI-R is a well-established and standardized instrument to assess personality traits, using either self-reports or observer ratings. It incorporates the five broad traits of the five-factor model (FFM) of personality and includes six facets or specific traits for each of the five broad factors. Each facet score is derived by adding the scores on eight items in 0–4 Likert format, and the personality trait score is composed by the scores on its six facets. Thus, the possible range of facet scores is 0–32 and the range of trait scores from 0–192. The Danish translation of the NEO-PI-R has been psychometrically evaluated and standardized in a sample of 600 subjects (Costa and McCrae, 1992; Hansen and Mortensen, 2004).

Genotyping

Blood was drawn on the day of the PET-scanning and genomic DNA was purified from the buffy coat by standard methods. The applied procedure for polymerase chain reaction (PCR) is previously described (Klauck et al., 1997). The *5-HTTLPR* long (A/G) polymorphism was detected by MspI restriction enzyme digestion of the PCR products and the generated fragments were resolved by gel electrophoresis. Product sizes for the digest were in line with the literature: L_A=340 bp, L_G=166 bp+B174 bp, S=297 bp (Praschak-Rieder et al., 2007).

Statistics

We used SAS software (SAS Institute Inc.) version 9.1.3 for statistical analysis. To test the correlation between personality and 5-HTT binding, the scores of the NEO-PI-R dimension (trait) OtExperience and its facets were correlated to a left and -right volume-weighted average of BP_{ND} for [^{11}C]DASB in the thalamus, the caudate, the putamen, and the midbrain, with adjustment for age and gender, in a multiple linear regression analysis with OtExperience and its facets as dependent variables and BP_{ND}, genotype, age, and gender as independent variables. To test the correlation between genotype and personality, we used OtExperience and its facets as dependent variables and *5-HTTLPR* genotype, age, and gender as independent variables. We also tested the genotype effect on BP_{ND} for [^{11}C]DASB reported by other groups (Praschak-Rieder et al., 2007; Reimold et al., 2007); for this we used a general linear model with BP_{ND} for [^{11}C]DASB as dependent variable and genotype, age, and gender as independent variables. In addition to the VOI based data we tested the correlation between personality and BP_{ND} for [^{11}C]DASB using a voxel-based analysis with OtExperience and its facets as dependent variables and BP_{ND}, age, and gender, and genotype as independent variables using the Brede Toolbox (http://hendrix.imm.dtu.dk/software/brede/). We analyzed post-hoc for a gene–age interaction effect on personality (with gender as covariate), an association between *Neuroticism* and BP_{ND} for [^{11}C]DASB in the four VOIs (corrected for age and gender), as reported by another group (Takano et al., 2007), and for a correlation between *Neuroticism* and OtExperience and its facets.

For the VOI-based analysis, we report p-values lower than 0.05 as statistically significant. For the voxel based analysis we applied a mask on MNI space including the four VOIs named above. This mask was created by step-wise increasing the BP_{ND} threshold on a mean of all spatially normalized parametric maps until we obtained a distinct, mostly confluent mask encompassing all the target regions as visually identifiable on the overlaid mean of all normalized MR images. For the voxel-based analysis, we report p-values below 0.05 as significant, corrected for multiple comparisons (all voxels within this mask). To this end, we used a permutation test to identify the probability of finding random significance, as described by Nichols and Holmes (2002), applied with 10,000 iterations for each trait or facet.

Results

5-HTT binding versus personality scores

We found a significant negative correlation between 5-HTT binding in the midbrain and OtExperience (r=−0.325, p=0.024), its facets OtAction (r=−0.413, p=0.003), and OtValues (r=−0.371, p=0.009) (Fig. 1). The correlation between 5-HTT binding and OtValues was also significant in the putamen (r=−0.328, p=0.023), the thalamus (r=−0.427, p=0.002) (Fig. 1)), and approaching significance in the caudate nuclei (r=−0.282, p=0.053). These findings

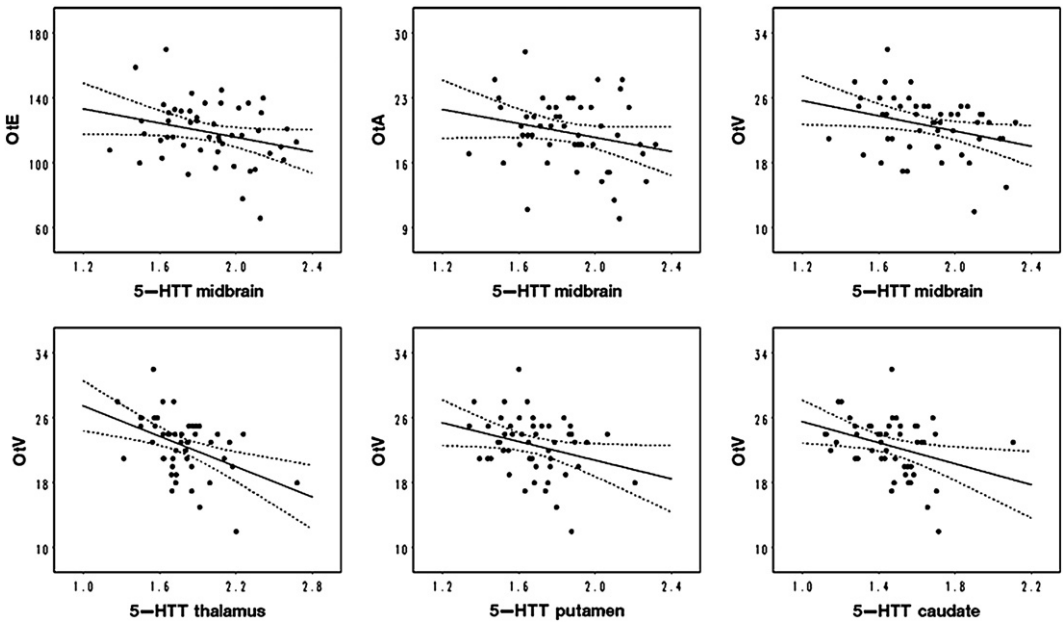

Fig. 1. Correlations between regional specific binding of [^{11}C]DASB and openness, corrected for age and gender, with 95% confidence intervals. The upper three graphs show the correlations in midbrain for OtE, OtA and OtV. The lower three graphs show the correlations between specific binding of [^{11}C]DASB and OtV in thalamus, putamen and caudate.

for OtValues were confirmed by voxel-based analysis for the following coordinates in the MNI space (all p-values corrected for multiple voxels as described above): 14;−24; 4 (1482 voxels, $r=-0.550$, $p=0.004$), 26; −12; −6 (23 voxels, $r=-0.504$, $p=0.023$), 18; −8; −6 (2 voxels, $r=-0.474$, $p=0.050$), 12; 4; 4 (2 voxels, $r=-0.478$, $p=0.043$). We did not find associations between 5-HTT binding and any of the other four openness facets. Further, post-hoc analysis revealed no significant correlation between 5-HTT and the trait *Neuroticism*, or any of its facets, including anxiety and depression. However, in our sample OtValues was inversely correlated with *Neuroticism* ($r=-0.296$, $p=0.033$) and, in particular, its facet anxiety ($r=-0.361$, $p=0.010$).

Gene-effects

Eighteen test persons where homozygote *L-allele* carriers (36%), eleven were homozygote *S-allele* carriers (22%), and twenty-one heterozygotes (41%); these frequencies did not differ significantly (Pearson's Chi^2 test with one degree of freedom) from the frequencies expected according to the Hardy–Weinberg principle or expected according to the observed frequencies in a larger group of 847 Caucasian non-Maori test persons (Caspi et al., 2003). Among the total of eighteen homozygote *L-allele* carriers, fourteen were homozygote L_A-*allele* carriers, and four carried L_AL_G. We found that homozygote L_A-*allele* carriers generally had higher 5-HTT binding than carriers of at least one *S-allele* or one L_G-*allele*, but this difference was only statistically significant in the caudate nuclei ($p=0.042$). We did not find a difference in openness scores between carriers of different alleles of the *5-HTTLPR* polymorphism. However, post-hoc analysis revealed a tendency for a gene–age interaction ($p=0.060$, adjusted for gender) with carriers of L_AL_A becoming less open to values with age, while *S-* or L_G-*allele* carriers retained a high openness to values score with age.

Regional binding and personality facet intercorrelations, age and gender

We found that OtExperience scores correlated significantly with the scores of the facets OtAction ($r=0.586$, $p<0.001$) and OtValues ($r=0.606$, $p<0.001$). 5-HTT binding in the midbrain, the caudate and the putamen was correlated to each other, e.g. 5-HTT binding in the midbrain predicted the 5-HTT binding in the putamen ($r=0.550$, $p<0.001$), in the thalamus ($r=0.502$, $p<0.001$). Men had higher 5-HTT significantly higher binding in the caudate nuclei ($p=0.008$) and significantly lower binding in the midbrain ($p=0.030$), and 5-HTT binding declined with age, statistically significant in the thalamus ($r=0.333$, $p=0.018$). Since the fixed wash-out constant, k2′, has an important influence on BP_{ND} for [^{11}C]DASB, we tested for correlations between k2′ and OtExperience and all openness facets; neither trait nor facets were correlated to k2′ (p, uncorrected for comparisons <1 in all cases). Eight of the participants were smokers, but smoking does not have any impact on BP_{ND} for [^{11}C]DASB (Erritzoe, personal communication).

Discussion

We confirmed our hypothesis that OtExperience is negatively correlated with in vivo cerebral 5-HTT binding. Since OtExperience and its facets OtAction and OtValues showed a negative correlation with the BP_{ND} for [^{11}C]DASB in all these regions, we propose that openness, in particular OtValues, is associated with global scaling in 5-HTT binding, as illustrated in Fig. 2. Furthermore, we replicated, in a group of Europeans, the finding of previous studies that in vivo 5-HTT binding is higher in the caudate nuclei in homozygote L_A-carriers (Praschak-Rieder et al., 2007; Reimold et al., 2007) in the caudate nuclei. We did not replicate the reported association between *5-HTTLPR* genotype and OtExperience (Stoltenberg et al., 2002). Interestingly, a statistical trend suggested that an effect of *5-HTTLPR* on openness was revealed when analyzing age–gene interaction; While carriers of the *S-* or L_G-*allele* remain at the same level of openness over the complete age range, homozygote L_A-*allele* carriers have similar openness scores when younger but become less open to values with age. We suggest that the direct association between *5-HTTLPR* genotype and openness should be reproduced in a larger group of test persons covering a wider age range since (only 6 of our 50 subjects were older than 40 years).

Some consideration regarding the issue of multiple comparisons needs to be done. To test our primary hypothesis, the association between personality and 5-HTT binding, we compared scores of the dimension itself and its six facets and 5-HTT binding in four regions. However, we found overall correlations among the personality scores as well as among the 5-HTT binding in the four VOIs. Therefore, conservative correction for multiple comparisons is inappropriate, and a global association between the dimensions OtAction and OtValues (which both contribute to the score of OtExperience) and the 5-HTT binding in all measured brain regions is likely. In the case of our second positive finding, the gene-effect was found in one out of four regions, with a p-value just reaching significance ($p=0.042$). Since two previous

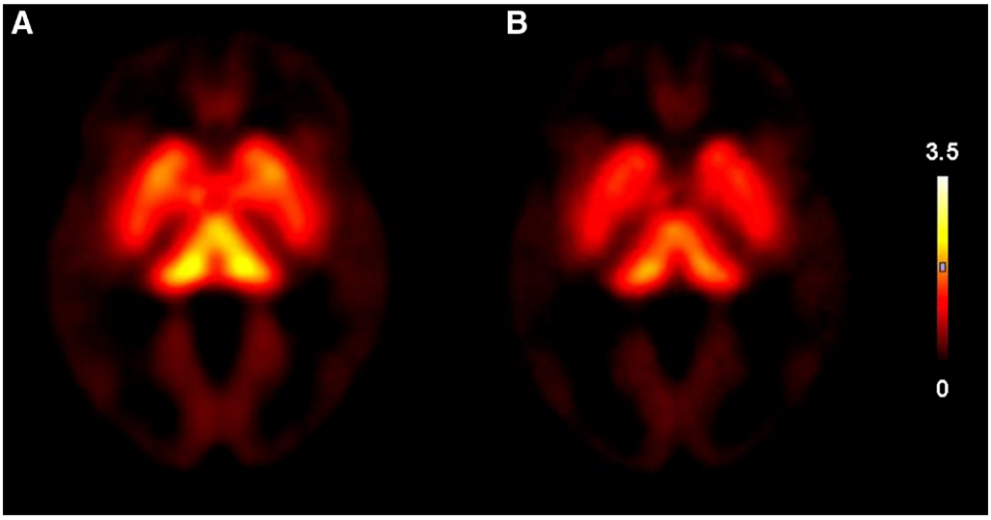

Fig. 2. Averaged parametric maps representing specific binding of [^{11}C]DASB to 5-HTT. Parametric maps (averaged) of (A) the ten subjects with least openness to values and (B) the ten subjects most open to values show a global higher scaling in less open individuals.

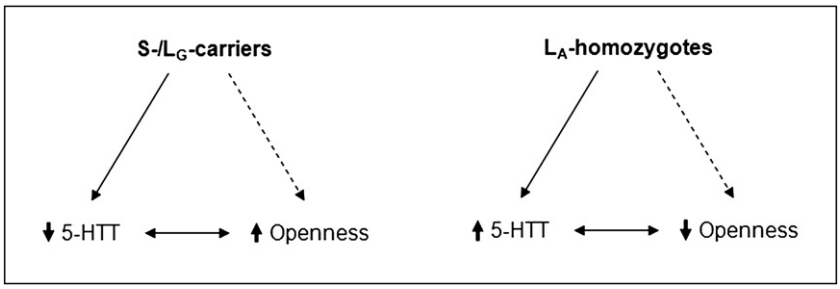

Fig. 3. A suggested model for the association between 5-*HTTLPR* genotype, 5-HTT binding and openness. The bidirectional full connecting arrow represents the negative 5-HTT personality correlation. The one-directional full connection arrow represents the gene effect on 5-HTT binding found by other groups and replicated in our study. The one-directional dashed connecting arrow represents the gene–personality effect as found in one study and only indirectly confirmed in our sample.

studies reported an effect, although in different regions than investigated in our study (i.e. the putamen (Praschak-Rieder et al., 2007) and the midbrain (Reimold et al., 2008)), we propose that 5-HTT binding is determined by a global, but rather subtle, gene-effect, possibly subject to modification by environmental factors.

In vivo imaging studies with PET have emphasized the search for aberrant serotonin markers in the brains of depressed patients. A number of PET studies have measured the abundance of 5-HTT, which clears serotonin from the interstitial space. An important class of antidepressant medications, the SSRIs, blocks this process by binding to the 5-HTT. Results of these PET studies have been inconsistent, showing 5-HTT binding to be elevated (Cannon et al., 2007), decreased in the brains of patients with major depressive disorder (MDD), (Reimold et al., 2008; Oquendo et al., 2007; Parsey et al., 2006) or unchanged (Meyer et al., 2004) in brain of depressed patients. Only one PET study of healthy subjects investigated the relationship between 5-HTT binding and *Neuroticism* scores on the NEO-PI-R and found that high 5-HTT binding was associated with *Neuroticism* (Takano et al., 2007). Conversely, the vast number of genetic studies has associated the *S-allele*, which results in *lower* 5-HTT mRNA expression, to *Neuroticism* (Canli and Lesch, 2007; Lesch et al., 1996).

Our results indicate that OtExperience, primarily driven by the facets OtAction and OtValues, shows a consistent association with the *5-HTTLPR* genotype and cerebral 5-HTT; *S-Allele* carriers had lower 5-HTT binding, and lower 5-HTT binding was found in the more open individuals (Fig. 3). Our suggested model is consistent with the findings in SAD; this mood disorder, which is characterized by winter depression with seasonal deterioration in eating and sleeping patterns, appears by definition only in response to environmental changes. As such, SAD provides an innate model for probing the neurochemical basis of sensitivity to environmental changes. SAD has been consistently linked to both openness to experience (Bagby et al., 1996; Jain et al., 1999) and the *S-allele* (Rosenthal et al., 1998; Willeit et al., 2003). In our sample, we did not find a direct association between *S-/L$_G$-allele* and openness, but rather detected a tendency for an indirect effect, mediated via a gene–age interaction, in our post-hoc analysis. This interaction with age could, if reproducible, be of particular interest, since it indicates that cognitive flexibility might not primarily be higher in carriers of the *S-allele* but remain higher during the process of ageing.

The global association between OtExperience and 5-HTT binding in our study was primarily driven by OtAction and OtValues. While the other four facets (openness to fantasy, openness to aesthetics, openness to feelings, and openness to ideas) represent intellectual openness and curiosity, the two facets OtAction and OtValues reflect an active approach of the individual to try new ways of doing things and re-define their own self-understanding, e.g. values or world-views. In particular, high OtActions reflect the willingness to use a variety of approaches to solve problems and also to try new things such as unusual food as opposed to sticking to the known ways of doing things; high OtValues reflect the readiness to re-think and change one's own political, social, and religious values, as opposed to people with low openness who tend to be conservative and "dogmatic" (Costa and McCrae, 1992). Our findings that OtAction and OtValues correlate with 5-HTT match well with previous views that serotonin contributes to cognitive and behavioral flexibility versus rigidity (Crockett et al., 2008; Murphy et al., 2002; Robbins and Roberts, 2007). Insofar as the personality dimensions of OtAction and OtValues reflect an individual's ability to re-consider and change accustomed ways of thinking and thereby adapt to new circumstances, it is also notable that higher openness is associated with general cognitive ability and more efficient higher intellectual processes (DeYoung et al., 2005). Based on our findings we therefore propose a model that links cognitive flexibility and depression risk in the personality dimension of openness: Lower [^{11}C]DASB BP$_{ND}$, as present in carriers of the *S-* or *L$_G$-allele*, is a marker for slower serotonin re-uptake at the plasma membrane, resulting in higher extracellular serotonin levels leading to a more dynamic neural responsiveness and increased plasticity. This characteristic mode of serotonin transmission would manifest in the realm of personality as higher openness to embark on new ways of living, and an enhanced capacity to re-consider one's self-understanding. But by the same token, this same mechanism also imparts higher sensitivity to stressful environmental cues, as found by population studies, in the manner of a trade-off between benefits of cognitive flexibility and risks of vulnerability.

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Seasonal changes in brain serotonin transporter binding in short 5-HTTLPR-allele carriers but not in long-allele homozygotes

Jan Kalbitzer[1,2*], David Erritzoe[1,2], Klaus K. Holst[2,3], Finn Å. Nielsen[2,4], Lisbeth Marner[1,2], Szabolcs Lehel[2,5], Tine Arentzen[1,2], Terry L. Jernigan[2,6], and Gitte M. Knudsen[1,2]

[1]Neurobiology Research Unit, Copenhagen University Hospital Rigshospitalet, Copenhagen, Denmark

[2]Center for Integrated Molecular Brain Imaging, Copenhagen, Denmark

[3]Department of Biostatistics, University of Copenhagen, Denmark

[4]Department of Informatics and Mathematical Modelling, Technical University of Denmark, Lyngby, Denmark

[5]PET and Cyclotron Unit, Copenhagen University Hospital Rigshospitalet, Copenhagen, Denmark

[6]Danish Research Center for Magnetic Resonance, Copenhagen University Hospital Hvidovre Hospital, Copenhagen, Denmark

* Corresponding author: Jan Kalbitzer, Neurobiology Research Unit, Copenhagen University Hospital, 9 Blegdamsvej, Section 9201, 2100 Copenhagen, Denmark

Summary

A polymorphism in the promoter region of the serotonin transporter gene (5-HTTLPR) has been associated with seasonality both in patients with seasonal affective disorder (SAD) and in the general population. We used *in vivo* molecular imaging to measure cerebral serotonin transporter (5-HTT) binding in healthy Scandinavians and related the outcome to season of the year, and to the 5-HTTLPR carrier status. We found that the number of daylight minutes at the time of scanning correlated negatively with 5-HTT binding in the putamen and the caudate, with a similar tendency in the thalamus, whereas this association was not observed for the midbrain. Furthermore, in the putamen, an anatomical region with relatively dense serotonin innervation, we found a significant gene*daylight effect, such that there was a negative correlation between 5-HTT binding and daylight minutes in carriers of the short 5-HTTLPR allele, but not in homozygote carriers of the long allele. Our findings is in line with S-carriers having an increased response in neural circuits involved in emotional processing to stressful environmental stimuli, but here demonstrated as a endophenotype with dynamic changes in serotonin reuptake.

Introduction

Light deprivation is a known environmental stressor in Scandinavia and other regions of high latitude; in a large fraction of the northern population, behavioral changes such as reduced physical activity, higher food-intake, and increased urge to sleep (1) are frequently noted during months with few or no daylight minutes in the depths of winter. In vulnerable people, this environmental stress can provoke a particular form of mood disorder, termed seasonal affective disorder (SAD), which is characterized by the occurrence of depressive symptoms in winter. This observation led to the successful development of the rational and efficient treatment with bright light therapy (2).

Several findings suggest that SAD is mediated through the serotonin (5-HT) system. For example, 5-HT concentrations are lowest in post mortem brain samples from people dying in the winter (3). Also, the concentration of the serotonin metabolite 5-HIAA is lower in jugular blood samples collected in the winter (4). These biomarkers may be associated with seasonal changes in the activity of plasma-membrane serotonin transporter (5-HTT), a molecular entity which serves a central role in cerebral serotonin transmission by regulating interstitial 5-HT levels (5, 6). A few studies have investigated seasonal variation in the *in vivo* cerebral 5-HTT binding as measured with molecular imaging techniques. One published study reported lower binding (7), whereas another study reported higher (8) binding in winter compared to summer. However, both of these studies suffered from small sample sizes, questionable criteria for the cut-off between summer and winter, and employed radioligands of lesser specificity (and sensitivity) for 5-HTT imaging than those available today. In a large Canadian sample of 88 healthy

participants investigated with ^{11}C-DASB and positron emission tomography (PET), a seasonal fluctuation in cerebral 5-HTT binding was observed, lowest in summer, and highest in winter (9).

A link between 5-HTT and SAD has been identified in that carriers of a 44-base pair deletion in the 5-HTT-linked polymorphic region (*short 5-HTTLPR allele = S-allele*) generally are more vulnerable to SAD than carriers of the insertion (*long 5-HTTLPR allele = L-allele*) (10). This polymorphism influences 5-HTT expression, with lower expression occurring in carriers of the *S-allele* (5). In comparison to *L-allele* homozygotes, *S-allele* carriers have increased propensity to develop mood disorders in response to stressful environmental cues (11, 12), in particular, in response to the transition to winter (10, 13). Furthermore, results of functional magnetic resonance imaging (fMRI) studies revealed that exposure to stressful and fearful environmental cues evoke greater activation of limbic brain structures in *S-allele* carriers (14, 15). However, there was no in vivo difference in cerebral 5-HTT binding between carriers of the *S-allele* and *L-allele* homozygotes within a group of 96 healthy participants (16) investigated by single photon emission computed tomography (SPECT), nor was there any difference discernible in a group of 42 healthy participants (17) investigated with PET. Two subsequent imaging studies did demonstrate an association between *in vivo* 5-HTT binding and 5-HTTLPR genotype (18, 19), but only when the participants were stratified according to an additional single nucleotide polymorphism (SNP) in the area of the *L-allele*; this triallelic stratification had not been employed in most fMRI activation studies (6, 15).

In order to attempt to resolve the discrepant findings, we investigated in a group of healthy Scandinavians if there were any seasonal effects on cerebral 5-HTT binding *in vivo* as measured with [^{11}C]DASB-PET. To accommodate a potential time delay in any seasonal effect, given that 5-HTT expression might not respond immediately to seasonal changes, we applied a harmonic statistical model. Provided that we could see a seasonal effect on 5-HTT binding in our population sample, we next tested the hypothesis that the seasonal effect should be more pronounced in *S-allele* carriers.

Materials and Methods

Participants

Fifty-four healthy participants were recruited by advertisement for a research protocol approved by the Ethics Committee of Copenhagen and Frederiksberg, DK [(KF) 01-156/04, (KF) 01-124/04, and (KF) 11-283038]. After complete description of the study to the participants, written informed consent was obtained from 37 males with a mean age of 34 yrs (SD 18 yrs), and from 17 females with a mean age of 34 yrs (SD 20 yrs). Exclusion criteria for all participants included medical history, drug abuse or psychiatric disorders. All participants had a normal neurological examination and a structural MR image of the brain was without pathological findings.

PET scans

PET scans were performed with an 18-ring GE-Advance scanner (General Electric, Milwaukee, WI, USA), operating in 3D acquisition mode, and producing 35 image slices

with an interslice distance of 4.25 mm. Following a 10 min transmission scan, a dynamic 90 minute long emission recording was initiated upon intravenous injection during 12 sec of mean 487 (SD 90) MBq (range: 246 - 601) [^{11}C]DASB with mean specific activity of 32 (SD 16) GBq/μmol (range: 9 - 82). The emission recording consisted of 36 frames, increasing progressively in duration from 10 sec to 10 min. The attenuation and decay corrected recordings were reconstructed by filtered back projection using a 6 mm Hann filter. Daylight minutes on the day of the PET-scan, at the latitude of Copenhagen were computed based on (http://aa.usno.navy.mil/data/docs/Dur_OneYear.php/), as the time between sunrise and sunset.

MR scans

Structural brain scans were acquired on a Siemens Magnetom Trio 3T MR scanner with an eight-channel head coil (In vivo, FL, USA). Thirty-five participants underwent a high-resolution 3D T1-weighted, sagittal, magnetization prepared rapid gradient echo (MPRAGE) scan of the head (MPRAGE1: echo time (TE)/repetition time (TR)/inversion time(TI)=3.93/1540/800 ms; slice resolution=75%; Bandwidth=130 Hz/Px; Echo spacing=9.8 ms) and fifteen participants underwent a 3D T1-weighted, sagittal, MPRAGE (MPRAGE2: TE/TR/TI=3.04/1550/800 ms; slice resolution=100%; Bandwidth=170 Hz/Px; Echo spacing=7.7ms). Common to both MPRAGE acquisitions was a flip angle of 9°, a FOV of 256 mm, a matrix of 256x256, 1x1x1mm voxels and 192 slices.

MR/PET co-registration

All time-frames of the attenuation-corrected emission recording were automatically aligned to frame 26 using the AIR algorithm (http://bishopw.loni.ucla.edu/AIR5/). In the next step, we calculated the mean PET image for frames 10-36 for co-registration to the individual MR image, again using the AIR algorithm. The quality of each co-registration was controlled visually. In three cases, co-registration between PET and MR image was corrected manually.

Volume of interest (VOI) analysis

The VOI were delineated automatically as described in Svarer *et al.* (20) in order to identify the brain volumes in a user-independent fashion. In brief, for each set out of a 10-template VOI sets, a 12-parameter affine transformation and a warping field was calculated between the template MR image and a participant's individual MR image. Having obtained the MR/PET co-registration for the same individual as described above, the template VOI sets were then transferred to the dynamic PET image space for each participant, using the identified transformation parameters. From the VOI sets, a probability map was created for each participant, and a common VOI set was generated according to a fixed threshold. These VOI sets were then used for automatic extraction of time activity curves (TAC) for midbrain and volume-weighted (left-right) averages for bilateral thalamus, caudate, putamen and cerebellum for all participants. The midbrain, the caudate, the putamen, and the thalamus were selected as representative brain bilateral regions of homogenous (at this resolution), high 5-HTT binding (21), with pooling of left and right, to test for a global effect.

Quantification of non-displaceable tracer binding

The outcome parameter of the [^{11}C]DASB binding within a brain region is the non-displaceable binding potential, designated BP_{ND}. The BP_{ND} was calculated for the four VOIs using the cerebellum (excluding vermis) input as a reference region. We used a modified reference tissue model designed specifically for quantification of [^{11}C]DASB (MRTM/MRTM2) as described and evaluated by Ichise *et al.* (22) using the PKIN tool of the software PMOD version 2.9. A fixed washout constant, designated k_2', was calculated for each individual as an average of k_2 in caudate, putamen and thalamus relative to cerebellum using MRTM. Subsequently, k_2' was used as a constrained input parameter for the calculation of BP_{ND} in the four VOIs relative to cerebellum.

Parametric images

For the voxel-based analysis, parametric images representing BP_{ND} for each voxel were calculated for all participants from the dynamic PET images. Using the PXMOD tool of the PMOD software, we imported the VOIs, generated as described above, and re-calculated k_2' relative to cerebellum using MRTM. R1 is defined as the relative tracer delivery to a VOI relative to that in cerebellum, the magnitude of which is to some extent related to the regional cerebral blood flow. As described by Ichise *et al.*(22), we applied a threshold of 0.3 to R1 so as to exclude voxels with noisy TACs, as was particularly seen in regions with low tracer uptake. Additionally, we constrained the BP_{ND} outcome to a physiologically plausible range between 0 and 10. The resulting parametric images were filtered with a 3 mm median filter in their native space. SPM5 (http://www.fil.ion.ucl.ac.uk/spm/software/spm5/) was used to normalize the parametric

images to Montreal Neurological Institute (MNI) space using a warping field estimated by normalization of each participant's co-registered MR image. Subsequently, a 12 mm Gaussian filter was applied to all normalized images.

Genotyping

Blood samples for DNA analysis were taken during the scanning and immediately frozen and stored at -20°C until further analysis. DNA was extracted from the blood using a Qiagen Mini kit using the guidelines included in the kit (Qiagen, Valencia, CA, USA) 5-HTTLPR (SLC6A4; 17q11.1-q12) genotyping was performed using a TaqMan 5'-exonuclease allelic discrimination assay according to the instructions provided by the manufacturer (Applied Biosystems, Foster City, California, Assay-on-Demand). The ABI 7500 multiplex PCR machine (Applied Biosystems, Foster City, California, USA) was used for this analysis.

Plasma tryptophan

Blood samples were collected in heparinized vials and put on ice until centrifuged. Plasma was stored at -80 degrees Celsius until analyzed. Immediately after the samples were thawed, sulphosalicylic acid was added to precipitate protein. Norleucine was added as internal standard. The tryptophan concentration in the plasma extracts samples was then measured with HPLC using a Li^{2+} cation exchange column (Pickering, www.pickeringlabs.com). Tryptophan competes at the blood-brain barrier with other large neutral amino acids (LNAA), such as leucine, isoleucine, valine, tyrosine, and phenylalanine. We therefore measured concentrations of all these LNAAs and calculated

both absolute plasma tryptophan measures as well as tryptophan load relative to the other LNAAs. We then searched for correlations between tryptophan levels with daylight minutes and with [^{11}C]DASB BP_{ND}.

Statistics

The [^{11}C]DASB BP_{ND} levels were analyzed with a general linear model adjusting for gender, age and the number of daylight minutes in Copenhagen at the day of the scan. The model fit was assessed by examination of scatter plots of the residuals and their empirical distribution. No signs of significant misspecification were noted. Further, the functional form of the two quantitative predictors was checked by comparison with the non-parametric estimates of a general additive model.

By using daylight minutes as a predictor, we are implicitly assuming an almost instantaneous effect of light deprivation on BP_{ND}. To allow for a time delay of any effect, or a cumulative effect of the preceding months, we modeled the seasonal effect as a harmonic function with a period of one year. This was achieved by omitting the minutes of daylight variable and including a sine and cosine term in the model. The optimized parameters of these two terms yielded the peak amplitude and calendar day for peak BP_{ND}, along with corresponding standard errors as obtained by the delta method. Inclusion of additional frequency components was employed so as to validate the functional form of the calendar effect in this model.

Due to the frequency distribution of the 5-HTTLPR genotypes, allelic status was coded as a binary variable (homozygote L-allele carrier vs. S-allele carrier). The association between BP_{ND} and 5-HTTLPR-allelic status was analyzed by including a linear interaction term, gene*daylight minutes, in the model. The estimates was comparable to the estimated interaction between gene and season; the latter being modeled using the periodic function approach. All statistical analyses were performed in R 2.7.

Results

We found that length of daylight in minutes correlates negatively with BP_{ND} for [^{11}C]DASB bilaterally in the putamen (-0.0438 BP_{ND}/(100 minutes) [-0.0689; -0.0186], $p<0.001$), and bilaterally in the caudate nucleus: -0.0363 [-0.0702; -0.0023] BP_{ND}/(100 minutes), $p<0.05$). There was a non-significant trend towards negative correlation in the thalamus (-0.0299 BP_{ND}/(100 minutes) [-0.0656; 0.00581, $p<0.1$), but no such association in the midbrain (0.0192 BP_{ND}/(100 minutes) [-0.0858; 0.1242], $p>0.1$). The day of peak BP_{ND} was in mid-December +/- 21 days. Similar results were obtained when the data were modeled to a harmonic function that allows for a time delay in the seasonal effect.

Correction for age and gender revealed the presence of higher binding in the caudate of male participants ($p<0.001$), and decreasing [^{11}C]DASB BP_{ND} with age for both genders in the putamen ($p<0.05$) and the thalamus ($p<0.05$). There was no seasonal variation of absolute or relative plasma tryptophan concentrations in our sample. Furthermore, no correlation was found between [^{11}C]DASB BP_{ND} and plasma tryptophan, neither in terms

of absolute concentration nor relative to large neutral amino acids which at the blood-brain barrier compete with tryptophan for facilitated transport (23).

Nineteen participants were homozygotic *L-allele* carriers and 35 participants were *S-allele* carriers. In the putamen, we found a significant gene*daylight effect ($p<0.05$, with correction for age and gender), with a negative correlation between the [^{11}C]DASB BP_{ND} and daylight time in carriers of the *S-allele*, but no such correlation in carriers of the *L-allele* (Figures 1 and 2).

Discussion

We observed the strongest seasonal effect on 5-HTT binding in the putamen, an anatomical region with a dense serotonin innervation (21), which is implicated in motor functions as well as in processing of aversive stimuli (24, 25). We did not identify a seasonal variation in the midbrain (9), where the serotonergic cell bodies are located (26). Effects in this small brain region, were they present, would have been inherently more difficult to detect than in the putamen, due to lower and more variable estimates of BP_{ND} (21). Likewise, the gene*environment effect was only observed in the putamen. It is possible that the serotonergic innervation of the putamen is particularly disposed to an allelic effect (18); this would imply a mechanism by which 5-HTT sites in putaminal fibers are subject to a regulation distinct from those of other telencephalic structures. A more parsimonious (and likely) explanation is that the high and homogenous 5-HTT binding in putamen (21) facilitates detection of an effect.

Prior studies have shown *S-allele* carriers to be over-represented among SAD patients (10, 13), who are reported to have elevated platelet 5-HTT binding during winter depressive episodes, which normalizes during natural remission in summer (27). Thus, our finding of a winter zenith and summer nadir in brain 5-HTT binding, most prevalent in *S-allele* carriers, contributes to a model in which the *S-allele* imparts particular lability to 5-HTT expression, even in healthy participants.

Seasonal factors other than daylight could potentially also underlie seasonal variations in 5-HTT binding in our *S-allele* carriers. Others have reported a weak correlation between 5-HTT binding and relative humidity, and no correlation with daily air temperature (9). It might be proposed that seasonal changes in food availability and in food preferences might affect cerebral serotonin levels through variation in the intake of the 5-HT precursor tryptophan. However, since we did not find any seasonal variation in absolute or relative plasma tryptophan concentrations, and no correlation between cerebral 5-HTT binding and plasma tryptophan.

Our use of a harmonic statistical model allowed for a potential time delay in any seasonal effect, such that 5-HTT binding sites need not responding immediately to seasonal changes. We anticipated that the daylight effect on cerebral 5-HTT binding is likely associated with a time delay because internalization and degradation of 5-HTT takes up to two weeks in experimental animals (28). The zenith in cerebral 5-HTT binding occurred around mid-December, coincident with the winter solstice. This suggests that

there is only a limited delay in the seasonal effect. When employing the harmonic model, we defined time of the year rather than daylight minutes as the independent variable, which yielded a similar correlation to cerebral 5-HTT binding in putamen. Our data do thus not allow for a distinction between amount of daylight, for which the number of minutes between sunrise and sunset serves as a surrogate, and time of year. It follows that any factor varying in phase with the time of year might be causative for the observed entrainment of 5-HTT availability.

A higher 5-HTT binding, representing a greater abundance of 5-HTT, predicts more efficient clearance of interstitial serotonin into the cytosol, thus attenuating the duration and amplitude of phasic serotonin signaling. Assuming that abundance (5-HTT density) is predictive of neuronal 5-HT flux, the clearance of extracellular serotonin seems to be enhanced in carriers of the *S-allele* during the winter months. Irrespective of the causal mechanism, the present results suggest a less stable regulation of serotonin transmission in response to seasonal changes among carriers of the *S-allele*. Another aspect is that earlier failures to detect differences in 5-HTT binding between *S-allele* carriers and *L-allele* homozygotes (16, 29) might be due to the omission of taking the season into account. The participants included in the present study were carefully screened for history and presence of mood disorders, and so may not be entirely representative of the population at large. However, cognitive activation (14) and population-based epidemiology studies (12), in consistency with the present observations, which indicate that the *S-allele* primarily manifests as a gene*environment interaction, predicting a more sensitive homeostasis of the cerebral serotonin system in carriers of the *S-allele*.

In summary, we investigated the relationship between the number of daylight minutes at the time of the PET scan, on the availability of 5-HTT receptors in brain. We identified carriers of the short 5-HTTLPR-allele as neurobiological endophenotype, characterized by dynamic seasonal changes in 5-HTT binding in the putamen, with the zenith occurring in mid-winter. This neurobiological endophenotype directly links cognitive activation studies, showing responses in neural circuits, with dynamic changes in serotonin transporter expression measured *in vivo*. Future *in vivo* imaging studies, particularly those carried out in countries with large seasonal variation in amount of daylight may benefit from taking season into consideration in the data analysis. It remains to be established if the mid-winter peak in 5-HTT is predictive for vulnerability to depression or SAD, or responsiveness to light therapy.

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Figure 1

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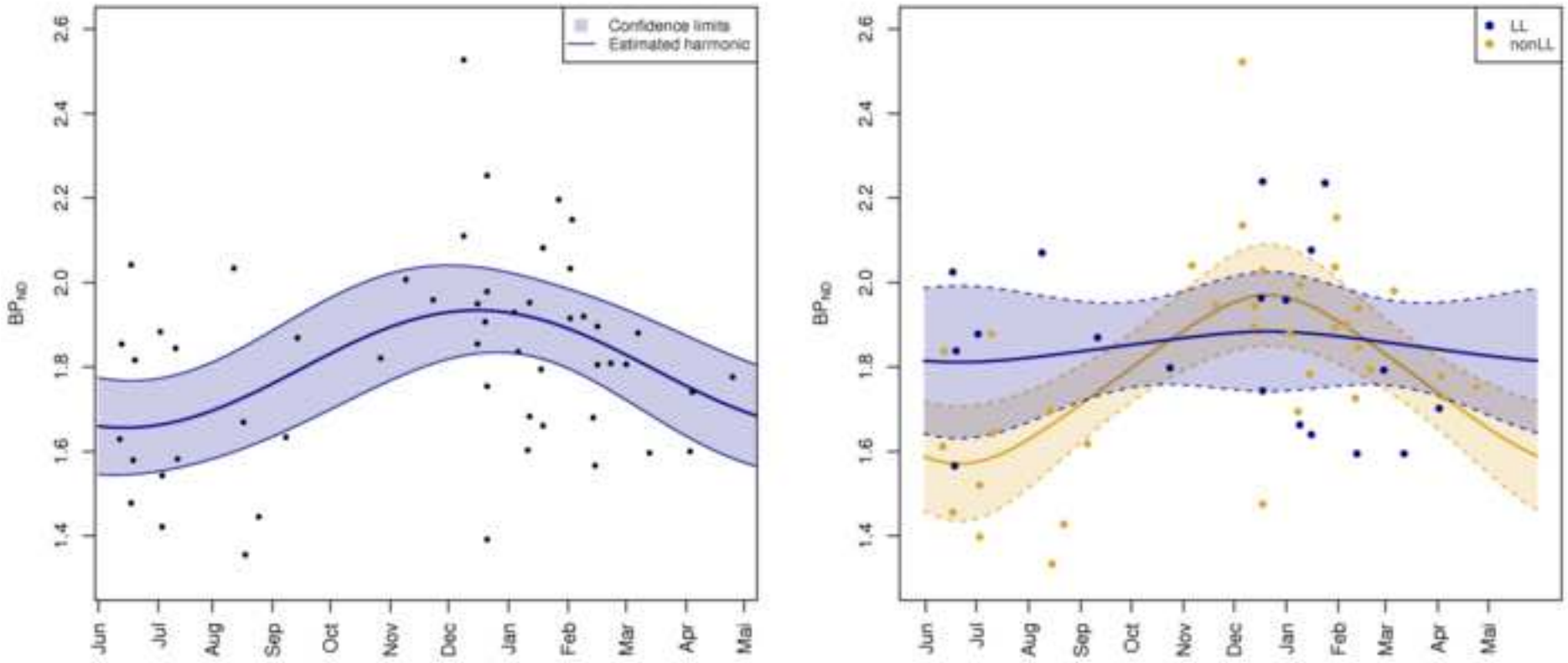

Figure 2

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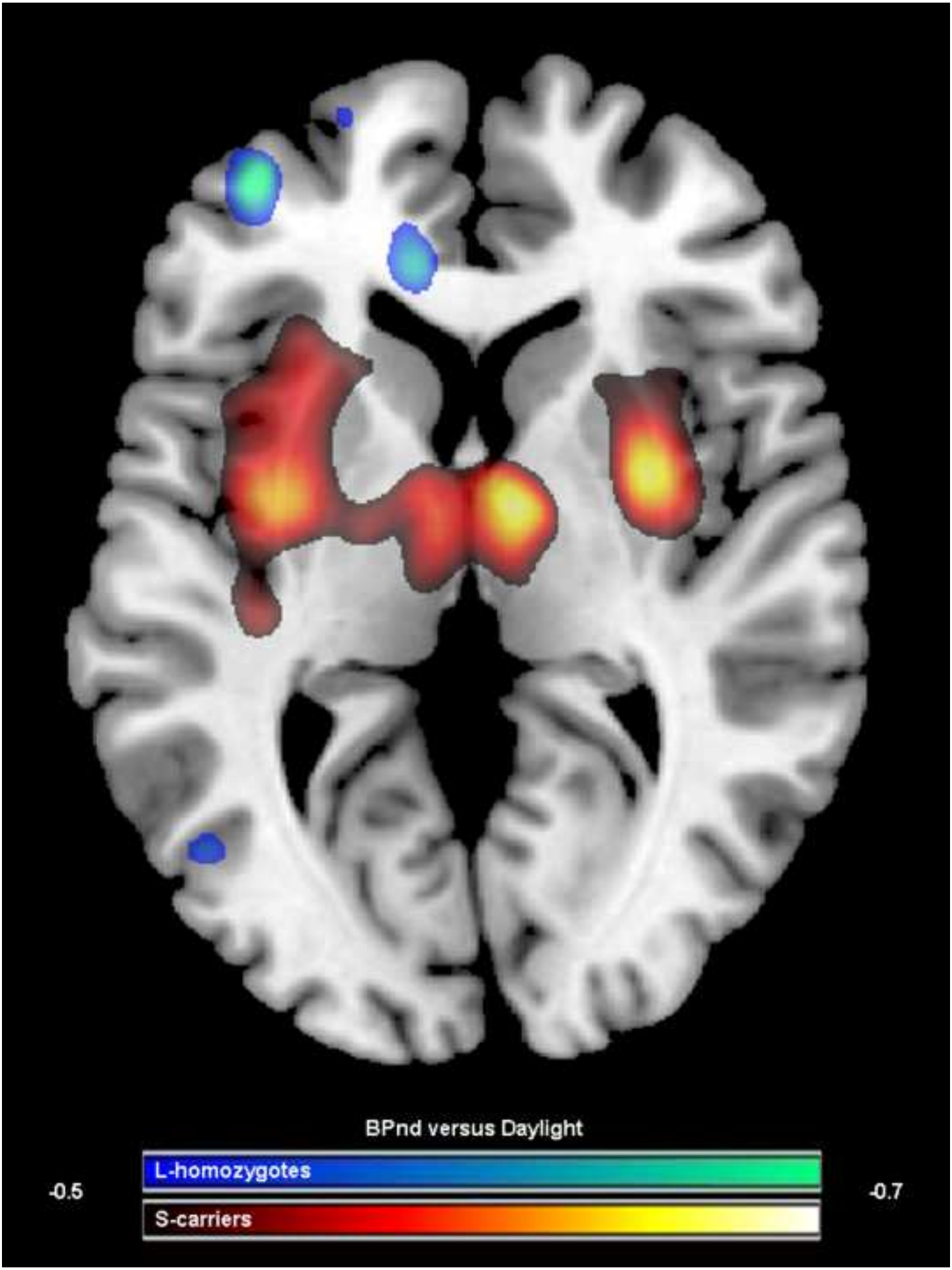

Figure legends

Figure 1. Left figure illustrates the seasonal effect on [^{11}C]DASB BP_{ND} (putamen) with pointwise confidence limits, modeled as a harmonic function with period 1 year (estimated peak in the middle of December, SE = 21 days, in good agreement with the model using daylight minutes as a predictor) adjusting for age and gender. The plotted points are the partial residuals (male of mean age). The functional form was validated by including additional frequency components and by comparison with estimates from an additive model. The right figure displays the interaction between number of daylight minutes and HTTLPR-allelic status adjusting for age and gender. For comparison with the left figure the estimated linear response as a function of daylight minutes was transformed to a function of calendar time.

Figure 2. Results of voxel-based analysis using parametric images representing specific 5-HTT binding, all normalized to Montreal Neurological Institute (MNI) space. Correlations between [^{11}C]DASB BP_{ND} (adjusted for age and gender) and amount of daylight on the day of the scan in Copenhagen.

3111T/C Clock SNP does not regulate the response of the serotonin system to environmental changes

Jan Kalbitzer[1,2]*, David Erritzoe[1,2], Klaus K. Holst, William F. C. Baaré[2,3], Szabolcs Lehel[2,4], Erik L. Mortensen[5] and Gitte M. Knudsen[1,2]

[1]Neurobiology Research Unit and Center for Integrated Molecular Brain Imaging, Copenhagen University Hospital Rigshospitalet, Denmark [2]Center for Integrated Molecular Brain Imaging [3]Danish Research Center for Magnetic Resonance, Hvidovre Copenhagen University Hospital, Denmark [4]PET and Cyclotron Unit, Copenhagen University Hospital Rigshospitalet, Denmark [5]Department of Environmental Health, Institute of Public Health, University of Copenhagen, Denmark

Abstract

It has been proposed that the CLOCK 3111 T/C single-nucleotide polymorphism (SNP) (rs1801260) affects diurnal activity and nocturnal sleep in healthy and depressed humans. Seasonal variations in mood are most likely regulated through the cerebral serotonin system. CLOCK is a central element of the circadian clock and might thus be an upstream regulator of seasonal variations in serotonergic neurotransmission. We tested the hypothesis that variations in the CLOCK 3111 T/C SNP affect the sensitivity of the cerebral serotonin system to seasonal variations. In 140 participants we investigated binding to the serotonin 2A receptor (5-HT2A) - as presynaptic marker - and the serotonin transporter (5-HTT) – as postsynaptic marker - with positron emission tomography and sought for a gene*environment interaction effect with minutes of daylight on the day of the scan as environmental measure. We also investigated the gene*daylight effect on perceived stress on the day of the scan. Additionally, all participants replied to the Revised Neuroticism Extraversion Openness Personality Inventory (NEO-PI-R). There was neither a gene*environment effect on 5-HT2A and 5-HTT binding nor an effect of the CLOCK SNP on perceived stress or personality. We conclude that the effect of CLOCK on

nocturnal preference and mood disorders is most likely not mediated through the serotonin system.

Introduction

24h rhythms in humans and other animals are controlled by a biological clock, based on a transcription-translation negative-feedback loop (Herzog, 2007). Recently, the 3111 T/C SNP (rs1801260) in the CLOCK gene has received increasing attention due to its effect on sleep disturbances in psychiatric disorders and the effect on SSRI-treatment outcome (Benedetti et al., 2003; Benedetti et al., 2007; Serretti et al., 2003; Serretti et al., 2005). Given the role of serotonin as a neurotransmitter in regulation of sleep (Jacobs & Fornal, 1999) and its proposed involvement in different elements of mood disorders, we assumed that the effects of the CLOCK SNP are likely to be mediated by the cerebral serotonin system. We tested this claim using a paradigm we established earlier (Kalbitzer, Erritzoe et al., 2008), using seasonal variations to study dynamic responses of the serotonin system in carriers of different genotypes.

We tested the effect of the CLOCK SNP both on the serotonin transporter, using positron emission tomography and the radioligand [^{11}C]DASB, and a post-synaptic marker, the serotonin 2_A receptor, measured with positron emission tomography and the radioligand [^{18}F]Altanserin. We accessed the cimbi database (http://cimbi.org) and received data and DNA samples for 142 healthy human participants of which 140 had been scanned with positron emission tomography and [^{18}F]Altanserin, a radioligand highly specific for serotonin (5-HT)2_A receptors scan and 56 with [^{11}C]DASB, a radioligand highly specific for serotonin transporters (5-HTT). Since we expected a global effect on the serotonin system, we chose regions of high and homogenous binding for each tracer. For the serotonin 2_A receptor, these regions included the frontal cortex, the global neocortex (including the frontal cortex minus the anterior cingulate cortex) and the bilateral striatum. For the serotonin transporter, these regions included the bilateral putamen, the bilateral caudate nuclei, the bilateral thalamus, the midbrain, and a combined region for the neocortex. 137 of the participants had replied to the revised Neuroticism Extraversion Openness Personality Inventory (NEO-PI-R) and 34 had filled in the Cohen's perceived stress scale (PSS) on the day of examination. All participants were genotyped for the CLOCK SNP. First we

sought for a gene*environment (daylight minutes) effect on specific binding (BP_{ND}) for 5-$HT2_A$ receptors and BP_{ND} for 5-HTT according to our primary hypothesis. We then analyzed for main effects of the polymorphism on BP_{ND} for 5-HT2A receptors, BP_{ND} for 5-HTT, NEO-PI-R scores, and PSS scores.

Methods

Participants

Fifty-four healthy participants were recruited by advertisement for a research protocol approved by the Ethics Committee of Copenhagen and Frederiksberg, DK [(KF) 01-156/04, (KF) 01-124/04, and (KF) 11-283038]. After complete description of the study to the participants, written informed consent was obtained from 37 males with a mean age of 34 yrs (SD 18 yrs), and from 17 females with a mean age of 34 yrs (SD 20 yrs). Exclusion criteria for all participants included medical history, drug abuse or psychiatric disorders. All participants had a normal neurological examination and a magnetic resonance (MR) image of the brain without pathological findings.

Genotyping

Blood samples for DNA analysis were taken during the scanning and immediately frozen and stored at -20°C until further analysis. DNA was extracted from the blood using a Qiagen Mini kit using the guidelines included in the kit (Qiagen, Valencia, CA, USA) CLOCK (rs1801260) genotyping was performed using a TaqMan 5'-exonuclease allelic discrimination assay (assay ID: C___8746719_20) according to the instructions provided by the manufacturer (Applied Biosystems, Foster City, California, Assay-on-Demand). The ABI 7500 multiplex PCR machine (Applied Biosystems, Foster City, California, USA) was used for this analysis.

[11C]DASB scans

PET scans were performed with an 18-ring GE-Advance scanner (General Electric, Milwaukee, WI, USA), operating in 3D acquisition mode, and producing 35 image slices with an interslice distance of 4.25 mm. Following a 10 min transmission scan, a dynamic 90 minute long emission recording was initiated upon intravenous injection during 12 sec of mean 487 (SD 90) MBq

(range: 246 - 601) [^{11}C]DASB with mean specific activity of 32 (SD 16) GBq/µmol (range: 9 - 82). The emission recording consisted of 36 frames, increasing progressively in duration from 10 sec to 10 min. The attenuation and decay corrected recordings were reconstructed by filtered back projection using a 6 mm Hann filter.

The outcome parameter of the [^{11}C]DASB binding within a brain region is the non-displaceable binding potential, designated BP_{ND}. The BP_{ND} was calculated for the four VOI using the cerebellum input as reference region with non-specific binding. We used a modified reference tissue model designed specifically for quantification of [^{11}C]DASB (MRTM/MRTM2) as described and evaluated by Ichise *et al.* (Ichise et al., 2003) using the PKIN tool of the software PMOD version 2.9 : A fixed washout constant, designated k2', was calculated for each individual as an average of k2 in caudate, putamen and thalamus relative to cerebellum using MRTM. Subsequently, k2' was used as a constrained input parameter for the calculation of BP_{ND} in the four VOIs relative to cerebellum.

[^{18}F]Altanserin scans

[^{18}F]altanserin was administrated as a combination of a bolus injection followed by continuous infusion to obtain steady state of the tracer in blood and tissue. The bolus-infusion ratio was 1.75 h, as previously described (Pinborg et al., 2003). Participants received the maximum dose of 3.7 MBq/kg body weight [^{18}F]altanserin. Reconstruction, attenuation, and scatter correction procedures were conducted according to Pinborg et al (Pinborg et al., 2003). Ninety minutes after the bolus injection of [^{18}F]altanserin, the participants were placed in the scanner.

Five venous blood samples were drawn at mid-scan times 4, 12, 20, 28, and 36 min after starting the dynamic scanning sequence. The samples were immediately centrifuged, and 0.5 ml of plasma was counted in a well counter for determination of radioactivity. Three of the five blood samples drawn at 4, 20, and 36 min were also analyzed for percentage of parent compound ([^{18}F]altanserin) using reverse-phase HPLC following the procedure described by Pinborg et al (Pinborg et al., 2003). In addition, the free fraction of [^{18}F]altanserin in plasma, f_1, was estimated using equilibrium dialysis, following a modified procedure by Videbaek et al (Videbaek et al., 1993). The dialysis was performed using Teflon-coated dialysis chambers (Harvard Bioscience,

Amika, Holliston, MA, USA) with a cellulose membrane that retains proteins >10,000 Da. A small amount of [^{18}F]altanserin (approximately 1 MBq) was added to 10 ml plasma samples drawn from the participants. A 500 µl portion of plasma was then dialyzed at 37°C for 3 hours against an equal volume of buffer, since pilot studies had shown that 3 hour equilibration time yielded stable values. The dialysis buffer consisted of 135 mM NaCl, 3.0 mM KCl, 1.2 nM CaCl2, 1.0 mM MgCl2, and 2.0 mM phosphate (pH 7.4). After the dialysis, 400 µl of plasma and buffer were counted in a well counter, and f_1 of [^{18}F]altanserin was calculated as the ratio of DPM(buffer)/DPM(plasma).

The outcome parameter was the binding potential of specific tracer binding, relative to the free concentration in plasma (BPp). The cerebellum was used as a reference region, since it represents nonspecific binding only (Pinborg et al., 2003). In steady state, BPp is defined as:

$$BP_P = (C_{VOI} - C_{Reference}) / C_{Plasma} = fp * (B_{max}/K_d) \text{ (mL/mL)}$$

where C_{ROI} and $C_{Reference}$ are steady-state mean count density in the VOI and in the reference region, respectively, C_{Plasma} is the steady-state activity of non-metabolized tracer in plasma, f_1 is the free fraction of radiotracer, B_{max} is the density of receptor sites available for tracer binding, and K_d is the affinity constant of the radiotracer to the receptor.

MR scans

Structural brain scans were acquired on a Siemens Magnetom Trio 3T MR scanner with an eight-channel head coil (In vivo, FL, USA). Thirty-five participants underwent a high-resolution 3D T1-weighted, sagittal, magnetization prepared rapid gradient echo (MPRAGE) scan of the head (MPRAGE1: echo time (TE)/repetition time (TR)/inversion time(TI)=3.93/1540/800 ms; slice resolution=75%; Bandwidth=130 Hz/Px; Echo spacing=9.8 ms) and fifteen participants underwent a 3D T1-weighted, sagittal, MPRAGE (MPRAGE2: TE/TR/TI=3.04/1550/800 ms; slice resolution=100%; Bandwidth=170 Hz/Px; Echo spacing=7.7ms). Common to both MPRAGE's was a flip angle of 9°, a FOV of 256 mm, a matrix of 256x256, 1x1x1mm voxels and 192 slices.

MR/PET co-registration

All time-frames of the attenuation-corrected emission recording were automatically aligned to frame 26 using the AIR algorithm (http://bishopw.loni.ucla.edu/AIR5/). For the [^{11}C]DASB-analysis, the mean PET image, averaging time-frames 10-36 for co-registration to the individual MR image using the AIR algorithm; the quality of each co-registration was controlled visually. [^{18}F]Altanserin images were co-registered manually by trained academic staff and cross-checked by a second colleague.

VOI analysis

The VOI were delineated automatically as described in Svarer *et al.* (Svarer et al., 2005) in order to identify the volumes in a user-independent fashion. For each of the 10-template VOI sets, a 12-parameter affine transformation and a warping field was calculated between the template MR image and the individual MR image for a participant. Having obtained the MR/PET co-registration for the same individual as described above, the template VOI sets are then transferred to the dynamic PET image space for each participant, using the identified transformation parameters. From the VOI sets, a probability map was created for each participant, and a common VOI set was threshold-generated. These VOI sets were then used for automatic extraction of time activity curves (TAC) for midbrain and volume-weighted (left-right) averages for bilateral thalamus, caudate, putamen and cerebellum for all participants. The midbrain, the caudate, the putamen and the thalamus were selected as representative brain regions of homogenous, high 5-HTT binding[12] since we expected that a genetic predisposition would lead to a global scaling effect. The TAC extracted for the cerebellum was used as the reference tissue input for kinetic modeling.

Personality Assessment

All participants replied to the Danish version of the Revised NEO Personality Inventory (NEO-PI-R). The NEO-PI-R is a well-established and standardized instrument to assess personality traits, using either self-reports or observer ratings. It incorporates the five broad traits of the five-factor model (FFM) of personality (Neuroticism, Extraversion, Openness, Agreeableness, Conscientiousness) and includes six facets or specific traits for each of the five broad factors. Each facet score is derived by adding the scores on eight items in 0-4 Likert format, and the

personality trait score is composed by the scores on its six facets. Thus, the possible range of facet scores is 0 - 32 and the range of trait scores from 0 – 192. The Danish translation of the NEO-PI-R has been psychometrically evaluated and standardized in a sample of 600 participants (Costa & McCrae, 1992; Hansen & Mortensen, 2004).

Statistical Methods

Hardy-Weinberg equilibrium of the 3111-CLOCK SNP was checked with Fishers Exact Test. Only 10 individuals were carriers of the rare homozygote variant (CC) and as a consequence we pooled this group with the heterozygotes. The association between each of the outcomes and the CLOCK SNP (binary) was examined with a Welch t-test taking possible variance heterogeneity into account. Next, to achieve a potential gain in efficiency, we adjusted all outcomes in a linear regression model for the effects of gender and age at examination. For the serotonin markers we further adjusted for number of daylight minutes at the day of examination. Finally for the serotonin outcomes the main hypothesis was analysed by including an interaction term between the amount of daylight minutes and the 3111 CLOCK SNP. The interaction was tested using a F-test with 2 degrees of freedom. All analyses were performed in R version 2.7 (http://www.r-project.org).

Results

Since we expected a global effect on the serotonin system, we chose regions of high and homogenous binding for each tracer. For 5-HT2A these regions included the frontal cortex, the global neocortex (including the frontal cortex minus the anterior cingulated cortex) and the bilateral striatum. For 5-HTT these regions included the bilateral putamen, the bilateral caudate, the bilateral thalamus, the midbrain, and a combined region for the neocortex. There was no statistical significant interaction effect ($p > 0.05$) between length of daylight-time in minutes and 3111 T/C on BP_{ND} for 5-HT2A receptors or BP_{ND} for 5-HTT. Also, there was no significant association between the 3111 T/C variations and BP_{ND} for 5-HT2A receptors, BP_{ND} for 5-HTT, NEO-PI-R scores, or PSS scores (results summarized in tables 1-4). These findings were also

reproduced after correction for age and gender. Among our 50 comparisons one uncorrected p-value (for the effect of the polymorphism on BP_{ND} for 5-HTT in caudate) were just below 0.05 which covers the expected likelihood of chance findings.

Discussion

This is the first study to investigate the association between a functional polymorphism in the human clock system, the serotonin system, environmental changes and personality. We falsified our hypothesis that the 3111 CLOCK SNP might directly affect the serotonin system or modulate the responsiveness of the serotonin system to seasonal changes. Furthermore, the 3111 CLOCK SNP was not associated with any personality dimension. Since openness to experience reflects sensitivity to environmental changes (Kalbitzer, Frokjaer et al., 2008), it seems unlikely that the effect of the 3111 CLOCK SNP has a profound effect on this aspect of personality.

The strength of our study includes the use of highly-specific radioligands and well-validated quantification methods. A further strength is the relatively large sample size for a PET study of healthy controls including both genders across a wide age range. The limitation of our study is the relatively small sample-size to detect a gene effect. Further limitations are the relatively unspecific question for a broader effect and the inherent problem of the potential side-effects of radioligands that made it impossible to measure the same individuals repetitively over the course of seasons.

In conclusion, we did not find a any mentionable effect of the 3111 CLOCK SNP on pre- and post-synaptic markers of the serotonin system, not even when we analyzed for gene*environment interaction effects. Furthermore, we did not identify a personality dimension that reflects the reported vulnerability of C-carriers. Improved radiotracers and even larger PET samples will be needed to further evaluate whether the proposed effects of the 3111 CLOCK SNP on mood, sleeping patterns, and sensitivity seasonal changes is mediated by the serotonin system and even larger samples will be needed to evaluate the effect on personality in healthy humans.

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