Molecular Imaging in Adult
Attention Deficit/Hyperactivity Disorder

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submitted by

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Declaration

This thesis project was conducted at the NEUROIMAGING LABS (NIL) - PET, MRI, EEG & Chemical Lab (head: Assoc. Prof. PD Dr. med. Rupert Lanzenberger) of the Department of Psychiatry and Psychotherapy (head: o.Univ.-Prof. Dr. h.c.mult. Dr. med. Siegfried Kasper), Clinical Division of Biological Psychiatry, Medical University of Vienna, Austria (www.meduniwien.ac.at/neuroimaging).

Positron emission tomography measurements were performed at the Department of Biomedical Imaging and Image-guided Therapy, Division of Nuclear Medicine, former Department of Nuclear Medicine, Medical University of Vienna, Austria. Synthesis of radioligands was done by the working group of Radiopharmaceutical Sciences (head: Prof. Dr. Wolfgang Wadsak and Prof. Dr. Markus Mitterhauser), Department/Division of Nuclear Medicine, Medical University of Vienna, Austria.

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Fig. 2 Region specific SERT binding in the human brain in vivo. High SERT binding is found in the midbrain. The image depicts triplanar structural images (axial, sagittal and coronal view) and superimposed SERT availability using \([{}^{11}\text{C}]\text{DASB}\). Mean SERT distribution maps have been generated by using imaging data from the study “The Serotonin Transporter in Attention Deficit Hyperactivity Disorder investigated with Positron Emission Tomography”, related to this thesis.
ABSTRACT

Molecular Imaging in Adult Attention Deficit/Hyperactivity Disorder (ADHD)

Attention deficit/hyperactivity disorder (ADHD) is a highly prevalent and heritable neurodevelopment disorder, with 40 to 60% of affected children suffering from ADHD also in adulthood. The ADHD pathophysiology is linked to dysfunctional connectivity within and between brain regions, which are modulated by neurotransmitters systems. Frequently prescribed treatments for ADHD, including stimulants and non-stimulants, alter norepinephrinergic and dopaminergic signaling in the central nervous system and thereby alleviate ADHD symptoms. In addition, serotonergic signaling is associated with hyperactivity, impulsivity and cognitive-emotional processes, which all represent symptoms that are present in ADHD. The causal complex neuronal mechanisms of ADHD and the way psychopharmacological therapy unfolds its efficacy are until now not entirely disclosed, thus it is of critical and public interest to gather more information of neurotransmitter signaling in ADHD.

This thesis project was designed to investigate proteins as the norepinephrine and serotonin transporter (NET, SERT) in vivo in patients with ADHD. To evaluate NET and SERT expression, we used positron emission tomography (PET) and the radioligands (S,S)-[^18]FMeNER-D2 for NET quantification and [^11]C]DASB for SERT quantification. In our first study we investigated NET binding in ADHD for the first time worldwide. There was no significant difference in NET binding in patients with ADHD compared to healthy control subjects. In the second publication, we showed an effect of genotype on NET binding, implicating a genetic influence on the expression of the NET in ADHD. Furthermore in the third study, I quantified SERT levels in patients with ADHD and found no difference in SERT binding between patients and healthy controls in specific brain regions. However, I applied interregional molecular correlational analysis of the SERT binding, and I revealed significant differences in interregional correlations between the hippocampus and precuneus in patients with ADHD compared to healthy control subjects.

The results of these imaging investigations provide needed information on the NET and SERT distribution in ADHD. The revealed lack of difference in NET and SERT binding between groups suggest that neither NET nor SERT availability is of critical relevance for the pathophysiology of ADHD. In addition, we found a genetic impact on NET binding in ADHD patients compared to healthy subjects, thus supporting previous genetic findings and underling a biological component in ADHD. To assess associations of SERT BPND between brain regions, I expanded conventional
PET imaging analysis through performing interregional molecular correlational analysis of SERT binding. I aimed to capture the complexity of brain interactions rather than higher or lower SERT levels in a specific region and found differences of interregional associations in patients with ADHD. The results help to develop a much broader understanding of the basic neurochemical constitution of ADHD.
ABSTRACT (Deutsch)

Molekulare Bildgebung in der Aufmerksamkeitsdefizit- und Hyperaktivitätsstörung (ADHS)


Hippokampus und Prekuneus zwischen Patienten mit ADHS und gesunden Kontrollprobanden zeigen konnte.

Publications arising from this thesis

The Norepinephrine Transporter in Attention Deficit/Hyperactivity Disorder Investigated with \((S,S)-[^{18}F]FMeNER-D_2\)

Effects of norepinephrine transporter gene variants on NET binding in ADHD and healthy controls investigated by PET

Altered interregional molecular associations of the serotonin transporter in attention deficit/hyperactivity disorder assessed with PET.
Acknowledgments and project funding

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**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>5-HT</td>
<td>5-hydroxytryptamine, serotonin</td>
</tr>
<tr>
<td>AAL</td>
<td>automated anatomical labeling</td>
</tr>
<tr>
<td>ADHD</td>
<td>attention deficit/hyperactivity disorder</td>
</tr>
<tr>
<td>BP&lt;sub&gt;ND&lt;/sub&gt;</td>
<td>binding potential non-displaceable</td>
</tr>
<tr>
<td>HC</td>
<td>healthy control subjects</td>
</tr>
<tr>
<td>NET</td>
<td>norepinephrine transporter</td>
</tr>
<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
</tr>
<tr>
<td>PET</td>
<td>positron emission tomography</td>
</tr>
<tr>
<td>ROI</td>
<td>region of interest</td>
</tr>
<tr>
<td>SERT</td>
<td>serotonin transporter</td>
</tr>
<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
</tr>
<tr>
<td>SPECT</td>
<td>single photon emission tomography</td>
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<tr>
<td>SPM</td>
<td>statistical parametric mapping</td>
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1. INTRODUCTION

1.1. General introduction - ADHD

Attention deficit/hyperactivity disorder (ADHD) is one of the most frequent neurodevelopmental disorders in children and adolescents. Prevalence rates of ADHD in school-aged children range between 8 and 12% (Biederman & Faraone, 2005b) and long-term follow-up studies show that 40 to 60% of children with ADHD suffer from typical ADHD symptoms in adulthood (Volkow & Swanson, 2013). While an age-dependent decline of ADHD symptoms as hyperactivity and impulsivity has been described previously, ADHD is a chronic and often life-long disorder where predominantly inattentive symptoms seem to persist into adulthood (Barbaresi et al, 2013). The core symptoms of ADHD include inattention, motoric hyperactivity and impulsivity (Association, 2013). In addition, specifically emotional dysregulation is also frequently observed in patients with ADHD (Shaw et al, 2014). Symptoms have to start during childhood, before puberty, and have to exist in at least two domains of functioning (as education or work, relationship and/or family and/or social contacts and free time/hobbies) (Association, 2013).

Adult patients with full-scale or partial ADHD symptoms are often confronted by a high personal and social burden (Kessler et al, 2006), since they get divorced more often, get terminated by their employee and switch labor significantly more often, are more frequently in sick leave and generate a lower income (Biederman et al, 2006; de Graaf et al, 2008). Epidemiological studies show that 50 % of all patients with ADHD develop psychiatric comorbidities during lifetime, including mood disorders, anxiety disorders, substance abuse disorders (SUD), oppositional defiant disorders and conduct disorders (Baird et al, 2012; Biederman, 2005; Kadesjo & Gillberg, 2001). Furthermore, the rate of ADHD in inmates varies from 10 to 70 %, whereas ADHD has been implicated as a risk factor for incarceration (Ghanizadeh et al, 2011).

Diagnostic criteria is based on the Diagnostic and Statistical Manual of Mental Disorders (DSM 5; (Association, 2013) and International Statistical Classification of Diseases and Related Health Problems (ICD-10) where ADHD is classified by the predominance of symptoms of inattention (the inattentive type; DSM 5: 314.00, ICD-10: F90.0), hyperactivity (the hyperactivity-impulsivity type; DSM 5: 314.01, ICD-10: F90.1) or a combination of inattention and hyperactivity (the combined type; DSM 5: 314.01, ICD-10: F90.0).

The psychopharmacological treatment for ADHD consists of methylphenidate (MPH) and amphetamine (AMPH), which are stimulant medication, and of atomoxetin (ATX), a
psychopharmaca that belongs to the group of the selective norepinephrine reuptake inhibitors. Both stimulants and non-stimulant medication enhance dopaminergic and noradrenergic signaling by blocking the neurotransmitter transporter in specific brain areas (Berridge et al, 2006; Bymaster et al, 2002). Randomized controlled studies have displayed that stimulant and non-stimulant medication significantly alleviate ADHD symptoms in children and adult patients with ADHD in comparison to placebo (Adler et al, 2009; Faraone et al, 2004; Volkow & Swanson, 2013). The clinical improvement to psychopharmacological therapy suggests that the mechanism through which it achieves an effect is of importance to the neuro-pathophysiology and the resulting symptomology.

1.2. Neurobiology of ADHD

Until this day, the underlying myriad and complex neuronal mechanisms of ADHD are not entirely revealed. One reason for this is, as it is the case for other psychiatric disorders, that the different state of the art methods in use to investigate morphological and functional brain alterations and accompanying behavior in ADHD are most probable not sensitive enough to detect specific neuronal correlates. Another and not less important reason is that ADHD represents a neurodevelopmental disorder, which comprises a wide range of symptoms with a divergent clinical phenotype, complicated by the circumstance that certain behaviors do not exist exclusively for ADHD. For instance cognitive deficits and more specific, executive functions such as working memory, attentional and inhibitory control are present in ADHD as well as in affective disorders and schizophrenia (Gallo & Posner, 2016). Scientific achievements of the past decades suggest that the etiology of ADHD is more likely explained through a multifactorial model, including biological, social and psychological factors.

Genetic data from twin studies from different countries on different continents project heritability to be 0.76 (Faraone et al, 2005). The genetic influence plays a main role in ADHD, whereas unrevealed common variants with small effects and gene-environment as well as gene-gene interactions are suggested to be involved in the genetics of ADHD (Archer et al, 2011b). Though few genes have been associated with ADHD. Non-genetic factors comprise environmental and psychological factors, whereas among others pregnancy and delivery complications, prematurity, low birthrate and exposure to alcohol and nicotine during pregnancy are counted to the former and low social class, low maternal education and single parenthood are examples to the latter (Biederman & Faraone, 2005b).
Neuroimaging studies investigating brain structure and the function, predominantly using magnetic resonance imaging, in children and adult patients with ADHD revealed dysfunctional fronto-parietal, dorsal fronto-striatal, and meso-cortico-limbic circuits, as well as abnormal default mode and cognitive control networks (Brennan & Arnsten, 2008b; Gallo & Posner, 2016; Volkow et al, 2007b). The neurobiological basis of ADHD symptoms has been associated to connectivity between cortical and subcortical brain region, which are modulated by numerous neurotransmitters and especially norepinephrine (NE) and dopamine (DA) (Del Campo et al, 2011).

Meta-analyses of structural magnetic resonance imaging (MRI) studies consistently found reduced gray matter volume in the basal ganglia. The basal ganglia receive input from the neo cortex and from midbrain regions and are involved in motor control, motivation and reward processing, and goal-directed behavior (Gallo & Posner, 2016). In addition to subcortical alterations, structural abnormalities in the frontal, parietal and temporal lobe have also been demonstrated (Bush, 2010). Longitudinal studies and meta-analysis, including studies investigating children as well as adult patients with ADHD, demonstrated abnormal developmental processes (Rubia, 2007; Shaw et al, 2013). Meta-analysis of task-related functional imaging studies, using the activation likelihood estimation method to compare and objectify huge data sets, found hypoactivity in patients with ADHD in frontoparietal and ventral attentional networks and hyperactivation within the default mode, ventral attention, and somatomotor networks (Cortese et al, 2012). Further, modern analysing modalities allow investigating huge amounts of imaging data in terms of large-scale brain networks. Thus, the emphasis has been put on detecting differences in brain networks between ADHD and healthy subjects in recent years. Abnormalities have been found in several brain networks in ADHD, including fronto-parietal, dorsal attentional, motor, visual and default mode networks (Castellanos & Proal, 2012).

1.3. Monoaminergic neurotransmitter systems in ADHD

Dysfunctional dopaminergic and noradrenergic neurotransmission is widely suggested to the pathophysiology of ADHD (Del Campo et al, 2011). This thesis was primarily generated by pharmacological findings showing that the mechanism by which psychotropic drugs that are prescribed for ADHD elevate DA and NE in the synaptic cleft. Subsequently performance levels will be raised, clinical symptoms and social interaction problems commonly seen in ADHD are
reduce. Investigations with animal models on the other hand revealed that dysregulations in neurotransmitter pathways affect normal behavior, leading to deficits in attention and to hyperactive and impulsive behaviors (Schneider et al, 1994; Shaywitz et al, 1978). The noradrenergic transmitter system is critically involved in higher cognitive and affective functions, modulating arousal states and state dependent processes. Reciprocal tonic and phasic discharge of the locus coeruleus (LC) to cortical and subcortical regions are related to attentiveness and the sensory to salient stimuli, modulating neuronal and behavioral activity states that are necessary to capture and process sensory information (Aston-Jones et al, 1999; Berridge & Waterhouse, 2003). The majority of noradrenergic cells in the brain are located in the midbrain nucleus, the LC (Barnes, 1991). Axons of the neurons in the LC are distributed virtually throughout the entire brain, except for main parts of the basal ganglia, which is nearly devoid of noradrenergic transmission (Berridge & Waterhouse, 2003; Gerfen & Clavier, 1979; Morrison et al, 1982; Morrison et al, 1979). Three noradrenergic receptor subtypes have been described, including $\alpha_1$, $\alpha_2$- and $\beta$-receptors. The specific cellular functions across diverse terminal brain regions that transmit into behavior are mediated by $\alpha$- and $\beta$-receptors have been only elucidated to a certain extent. The second messenger systems of $\alpha$- and $\beta$-receptors are different, where the former is coupled to the Gs/cAMP intracellular messenger system and the latter to the phosphoinositol and Gi/cAMP systems (Dohlman et al, 1991). Furthermore, $\alpha_1$- and $\beta$-receptors are located mainly postsynaptically, $\alpha_2$-receptors are found both pre- and postsynaptically (Berridge & Waterhouse, 2003). Since the NE system modulates arousal states and attentional processes and dysfunctional NE signaling has been shown to cause deficits in attention and impaired executive functions, NE has been associated with ADHD (Aston-Jones & Gold, 2009; Chamberlain et al, 2007; Frank et al, 2007). Preclinical studies in animals and humans have demonstrated that a moderate increase in NE transmission improves prefrontal brain function through the $\alpha_2$-receptors, while high levels of NE, distributed during stress, worsens performance via the $\alpha_1$-receptors in the prefrontal cortex (PFC) (Brennan & Arnsten, 2008b). Guanfacine, an $\alpha$-2A agonist in use to treat ADHD, has been shown to improve ADHD symptomology and enhance performance of PFC functioning in children and adult patients with ADHD (Arnsten et al, 2007). Studies investigating the DA system demonstrate a dopaminergic involvement in motivational and movement coordination processes as well as in the pathophysiology of ADHD (Frank et al, 2007; Glimcher, 2011). Phasic discharge of midbrain dopaminergic cells is suggested to lead reward prediction error that guides learning throughout the frontal cortex and the basal ganglia (Zhang et al, 2009).
neurons are centralized in the ventral tegmental area (VTA) and in the substantia nigra pars compacta (SNc) from where long axons project to the basal ganglia and to the frontal cortex, while little to no projections is sent to parietal, temporal and occipital regions (Glimcher, 2011). Dopamine enfolds action via the D_1 receptor family, that includes the D_1 and the D_5 receptor subtype as well as via the D_2 receptor family, including the D_2, D_3 and the D_4 receptor subtype (Brennan & Arnsten, 2008b). In particular, a moderate stimulation of the D_1 receptors, which are vastly represented in the PFC, leads to an inverted “U”-shaped response, where a processing of irrelevant information is inhibited and extensive D_1 receptor stimulation produces nonspecific suppression of cell activation. On a behavior level, D_1 receptors produce an inverted “U”-shaped dose-dependent functioning level in regard to attention regulation and working memory processes of the PFC (Arnsten, 2011).

The prefrontal cortex, a highly involved brain region in cognitive processes as attention, inhibiting processing of irrelevant stimuli, sustaining attention over a long delay of time and dividing and coordinating attention, is typically affected in ADHD (Willcutt et al, 2005) and displays distinct noradrenergic and dopaminergic innervation from midbrain regions (Arnsten & Li, 2005). Investigations with animal models revealed that neurotransmitter depletion in the PFC causes detrimental effects on working memory and is as impactful as the ablation of nerve tissue (Brozoski et al, 1979). Clinical symptoms as impairments of motivation and working memory have been attributed to the dopaminergic signaling, while noradrenergic alterations may play a fundamental role in vigilance, inattention and response inhibition (Bymaster et al, 2002; Frank et al, 2007).

However, next to the well-established association between noradrenergic and dopaminergic neurotransmission and ADHD, lines of evidence from genetic, pharmacological and animal studies support the involvement of serotonin in the pathophysiology of ADHD (Dalley & Roiser, 2012). Serotonin is highly involved in human behavior, in multiple psychiatric disorders and in the arrangement of cognitive-emotional processes (Archer et al, 2011a; Hoyer et al, 2002; Lowry et al, 2008; Murakami et al, 2009).

Modification of the serotonergic neurotransmission has been implicated in the pathophysiology of ADHD, which is mainly based on the association between impulsivity and hyperactivity, two key traits of ADHD, and serotonergic signaling (Castellanos et al, 1994; Dalley & Roiser, 2012). Impulsive behavior represents performance where action is taken without previous conscious reflection, and has long been proposed to be a central clinical symptom for different
neuropsychiatric disorders and in particular ADHD. ADHD patients regularly respond and behave inappropriately, prematurely and maladaptive in daily action (Connor et al, 2010; Doerfler et al, 2011). The inability for adequate impulsive behavior is related to emotional instability and dysfunctional motor control and is expressed in aggressive impulsivity (Robinson et al, 2008; Sobanski et al, 2010).

Tricyclic and dual antidepressants, such as modanifil and bupropion show clinical efficacy in ADHD patients and have been used within this indication for decades, though existing evidence show that selective serotonin reuptake inhibitors are not effective (Faraone & Glatt, 2010). MPH does not inhibit the serotonin transporter (Volkow et al, 2000) and therefore takes no direct action in serotonin signaling. However, AMPH enhances serotonergic release (Kuczenski & Segal, 1997) and ATX inhibits serotonin (5-HT) reuptake (Bymaster et al, 2002), implicating that serotonin might also be to some extent of importance in the neuropathology and treatment of ADHD (Gainetdinov et al, 1999).

1.4. Principles of Molecular Imaging

Positron emission tomography (PET) is a non-invasive imaging method from nuclear medicine that utilizes ionizing radiation to visualize flow metabolism, metabolic turnover or specific proteins in vitro and in vivo (Cherry. S.; Sorenson, 2012; Innis et al, 2007). PET is a useful method to gain more knowledge of the underlying pathophysiology of diseases, is used to determine clinical diagnosis and prognosis and being additionally used to predict treatment effects. To conduct a PET measurement, a positron-emitting radionuclide has to be produced in a cyclotron, where a tracer is labeled with an isotope (Wadsak & Mitterhauser, 2010). After synthesis, the radiotracer has to be administered to the blood stream where it distributes according to its inherent characteristics in the tissue, binding to molecular structures such as transporters or receptors, or imitating substances to image metabolic rate or flow processes. The constantly emitting positrons expand for a short distance of few millimeters into the adjacent tissue and are thereby decelerated until they interact with an electron building high-energy photons. This interaction leads to annihilation, an event where two \( \gamma \) gamma photons move in opposite directions (Turkheimer et al, 2014). A scintillation detection ring with a photomultiplier detects two photons simultaneously getting captured at opposite ends, while those photons, which do not coincidently hit the detection ring, are neglected. Many annihilation processes are recorded
throughout a PET measurement resulting, after reconstruction through back projections, in tomographic images that allow the interpretation of the quantity and distribution of the tracer.

There are three basic principles for PET imaging of the human brain in vivo (Turkheimer et al, 2014). First, the radioactive compound should be administrated in small dosages in order to not alter or disturb the investigated system. Second, the radioactive compound has to be reliable, valid and objectifiable and lastly, the concentration of the radioligand ought to be measured quantitatively.

The process of generating positron-emitting radionuclides starts with charged particles accelerated to a high velocity by a cyclotron, leading to the production of isotopes. The main radioisotopes are $^{11}$carbon with a half-life of 20 min and $^{18}$fluorine with a half-life of 110 min, whereas $^{18}$fluorine is easily being obtained and transported due to its rather long half-life (Wadsak & Mitterhauser, 2010). To detect the target density and distribution, the radioactive compound has to reach equilibrium between the binding to and the dissociation from the target molecule. An increased affinity to the target leads to slower dissociation, thus requiring a longer scanning time to reach equilibrium. Further, the positron-emitting isotopes will be attached to the selected substrate during a series of chemical reactions. To visualize a specific molecule in a certain tissue in vivo, the radio tracer needs to fulfill chemical requirements as high specificity and affinity for the particular molecule that intended to visualize, in order to keep non-specific binding as low as possible (Heiss & Herholz, 2006; Kung, 1991). Hence, selectivity ratio for an optimal tracer has to be larger or equal to 100 for targets over non-targets so that the non-specific binding is low and the signal-to-noise ratio is high (Innis et al, 2007). Furthermore, the tracer has to have little toxic power and steady labeling as well as rapid uptake and distribution in the tissue and few, rapidly cleared, and preferably unlabeled metabolites (Heiss & Herholz, 2006; Kung, 1991).

The binding potential (BP) of a radiotracer was introduced by Mintun more than three decades ago (Innis et al, 2007; Mintun et al, 1984). The BP is the main parameter to observe the expression of a certain molecule, such as the norepinephrine transporter (NET) or the serotonin transporter (SERT) in vivo and is defined as the ratio of the target density $B_{\text{max}}$ to dissociation constant $K_D$ at equilibrium.
The $K_D$ is the concentration of the free ligand ($F$) occupying 50% of the target at equilibrium, representing the inverse tracer affinity to the target structure.

\[ K_D = \frac{1}{affinity} = \frac{k_{OFF}}{k_{ON}} = \frac{B_{avail} \cdot F}{B} = \frac{(B_{max} - B) \cdot F}{B} \]

The equilibrium is defined as the state in which the fraction of the free ligand ($B_{avail}$) equals the fraction of the target bound ($B$) tracer.

\[ F \cdot B_{avail} \cdot k_{ON} = B \cdot k_{OFF} \]

To warrant accurate modeling and elude physiological effects on the investigated system, administered tracer has to be, as mentioned above, of small volume and thus of high sensitivity (Innis et al, 2007). Once arrived at the target tissue, the tracer will be present in three different conditions: bound to the target, non-specifically bound and free, non-bound fraction. Reciprocal changes of the target condition are defined by rates of constants. A radiotracer attains equilibrium when no net transfer happens between two adjacent conditions.

1.5. Molecular imaging with PET and SPECT of the dopaminergic, noradrenergic and serotonergic neurotransmitter system

Since 1999 a number of PET and single photon emission tomography (SPECT) studies have conducted to observe the involvement of neurotransmitter systems in patients with ADHD in vivo (Fusar-Poli et al, 2012; Zimmer, 2009). Encouraged by the continuous increasing rates of prescribed psychopharmacological treatments for ADHD that are suggested to cause symptoms
improvement based on modulating with the dopaminergic and noradrenergic signaling, pharmacological and genetic studies where followed by imaging studies focusing on molecular structures within these neurotransmitter systems (Del Campo et al, 2011).

1.5.1. PET and SPECT imaging of the dopaminergic neurotransmitter system

Especially, molecular imaging studies conducted in adults patients with ADHD focused on exploring the contribution of dopaminergic systems to the neurobiology of ADHD, given its well known role in the regulation of motivational processes and motoric activation as well as attention, inhibitory and timing and functions that are mediated by fronto-striatal circuits and found dysfunctional in ADHD (Fusar-Poli et al, 2012). Structural and functional imaging findings, derived from MRI, EEG, PET and SPECT studies, have pointed towards DA associated striatal deficits (Konrad & Eickhoff, 2010; Rubia, 2011; Valera et al, 2007). The dopamine transporter (DAT) as well as dopamine receptors represent primary targets for PET and SPECT investigations. The DAT is a cell membrane bound protein, is located presynaptically at dopaminergic axons and clears dopamine and noradrenalin actively from the synaptic cleft back into the presynaptic cytosol (Piccini, 2003). The active transport via the DAT represents the primary mechanism of clearance of dopamine from the synapse. Highest levels of DAT are localized in the striatum, lower levels of DAT are found in the brainstem nuclei VTA and SNc and very low levels are detected in cortical regions and cerebellum (Piccini, 2003).

Several radiotracers are available to quantify the DAT binding potential, for PET and SPECT imaging, including $^{11}$C-altropane and $^{11}$C-PE2I for PET (Elsinga et al., 2006) and $^{123}$I-IPT and $^{99}$mTc-TRODAT for SPECT (Booij & Knol, 2007). Findings of DAT binding potential in patients with ADHD compared to healthy controls have been inconsistent. Early imaging studies in the late 90’s found DAT binding lower in the striatum in patients than in healthy controls and two studies found no differences between groups (Zimmer, 2009). In the largest investigated study sample Volkow and colleagues found lower levels of DAT in 53 drug naïve patients with ADHD in the nucleus accumbens and midbrain (Volkow et al, 2009b). Hesse et al. also found attenuated DAT binding in 17 drug naïve patients (Hesse et al, 2009).

A meta analysis found significant higher DAT density in the striatum in patients (Fusar-Poli et al, 2012). DAT binding was increased in patients with previous medication use and decreased medication-naïve patients. No significant correlation was found for age, comorbidity, gender, or
imaging technique. Nevertheless, heterogeneity across PET and SPECT studies was large and significant. Reasons for these inconsistencies are uncertain, though they are associated with clinical factors as the inherent heterogeneity of the disorder, previous exposure to stimulants or drugs, smoking status as well as age and gender and methodological factors as using different reference regions or different radio ligands (Zimmer, 2009). Investigations on the D2/D3 receptor by Volkow et al. showed significantly attenuated D2/D3 receptor binding in the nucleus accumbens and midbrain regions in patients (Volkow et al, 2009b). Though, another PET study quantifying D2/D3 receptor levels found no differences between groups (del Campo et al, 2013).

1.5.2. PET and SPECT imaging of the noradrenergic neurotransmitter system

Neuroimaging studies exploring the noradrenergic system in vivo in the healthy human brain or in neuropsychiatric disorders are rare. This is due to the difficulties to produce a specific radiotracer that fulfills requirements for a reliable NET investigation in vivo (Ding, 2014). The NET exemplifies a treatment target with several detriments as a target molecule (Ding, 2014). In comparison to other neurotransmitter transporters as the DAT or the SERT, NET levels are lower in general and there is a lesser contrast between NET-rich (e.g. LC, thalamus) and NET-poor brain regions (e.g. caudatus, neocortex). Further, the NET displays a widespread distribution throughout the brain.

In 2003 Wilson et al. showed that (S,S)-[11C]MeNER was one of the first and most promising radioligands for NET quantification (Wilson et al, 2003), facilitating radiolabeling with high selectivity and affinity for the NET (Ding et al, 2003). Nevertheless, specific binding equilibrium was not reached within the timeframe of the PET-measurement (Schou et al, 2003). Due to five-fold higher half-life, fluorine-18 labelled reboxetine analogues exhibit extended measurement time, reaching binding equilibrium within PET scan (Schou et al, 2004). For the production of [18F]FMeNER (Schou et al, 2004) or [18F]FERB (Ding et al, 2003), the aryl methoxy group of (S,S)MeNER is replaced with a fluoromethoxy or fluoroethoxy group, respectively. To further optimize the radioligand, in-vivo de-fluorination can be decreased greatly by using the deuterated homologues [18F]FMeNER-D2 and [18F]FERB-D4.

Among others, (S,S)-[18F]FMeNER-D2 has been successfully applied in vivo in healthy human to explore NET (Ding et al, 2013; Rami-Mark et al, 2013). PET studies using (S,S)-[18F]FMeNER-D2 in
healthy humans in vivo found high NET availability in the thalamus and in the midbrain region, representing the noradrenergic neurons in the LC (Takano et al, 2008b).

![PET images showing (S,S)-[18F]FMenR-D2 distribution in the human brain in vivo. High NET binding is found in thalamus and midbrain, low NET binding in the basal ganglia. High NET uptake is found in the skull bones, a phenomenon that is related to tracer deflourination. The image depicts triplanar structural images (axial, sagittal and coronal view) and superimposed NET availability. Mean NET distribution maps have been generated by using imaging data from the study “The Norepinephrine Transporter in Attention Deficit Hyperactivity Disorder (ADHD) investigated with PET”, related to this thesis.](image)

**Fig. 1**

Occupancy studies in the healthy human brain, found significant binding to the NET for nortriptyline, clomipramine as well as for atomoxetine (Logan et al, 2007a; Sekine et al, 2010). Nortriptyline and clomipramine are both tricyclic antidepressant, while atomoxetine is a selective reuptake inhibitor. A PET study by Ding et al. found dose dependent occupancy of the NET as well to a less extent to the SERT of atomoxetine in the rat brain (Ding et al, 2013). Methylphenidate has also shown to occupy the NET in a dose dependent order in the healthy brain in vivo (Hannestad et al, 2010b).

There is sparse data on NET levels and distribution in patients with psychiatric disorders. In obese patients, Li et al. found reduced NET availability in the thalamus, though not in other NET-rich regions (Li et al, 2014). Observations by the same group demonstrated reduced NET binding in the LC in patients with posttraumatic stress disorder and NET availability in the LC was associated with anxious arousal symptoms (Pietrzak et al, 2013). In a post-mortem autoradiographic study, NET binding has shown to be attenuated in the thalamus as well as in
the LC in patients with Alzheimer’s disease (Gulyas et al, 2010). In opposite, thalamic and midbrain NET levels have shown to be increased in patients with cocaine dependency (Ding et al, 2010b). In patients with depression, an occupancy study by (Nogami et al, 2013) et al. found a dose dependent reduction of \( (S,S)\text{-}[^{18}\text{F}]\text{FMeNER-D}_2 \) levels in the thalamus subsequent to milnacipran administration.

Since \( (S,S)\text{-}[^{18}\text{F}]\text{FMeNER-D}_2 \) exhibits both high affinity and specificity to the NET and enable specific binding equilibrium within PET measurement and provides acceptable de-fluorination, it is currently one of the best suited PET tracer for the \textit{in vivo} quantification of the NET (Schou et al, 2004; Takano et al, 2008b). Although there exists an extensive literature that associates the noradrenergic signaling, and in specific the NET, in the biologic mechanisms of ADHD, there have been no investigations carried out regarding the quantity and distribution of the NET in this group.

1.5.3. PET and SPECT imaging of the serotonergic neurotransmitter system

Numerous PET and SPECT studies have been performed on the SERT in the last decades to gain more insights of the main treatment target for affective disorders (Houle et al, 2000; Wong et al, 1995). Selective serotonin reuptake inhibitors (SSRIs) are potent antidepressants and are frequently prescribed. They modulate serotonergic transmission in cortical and subcortical brain regions by blocking the presynaptically bound SERT, thus increasing serotonin in the synaptic cleft and modulating SERT expression. The serotonin neurotransmission is suggested to be highly involved in the regulation of mood, emotion, appetite (Haleem, 1993), sleep (Franco-Perez et al, 2012), as well as in the processing of anxiety (Akimova et al, 2009). Further, serotonin is linked to reward and motivation (Kranz et al, 2010), to impulsivity and attentional processes (Seo et al, 2008).

So far, of all the possible molecules capable of binding to SERT only the following were selected for human \textit{in vivo} investigations with PET (Paterson et al, 2013; Saulin et al, 2012). To this day \[^{11}\text{C}]\text{McN5652}, \[^{11}\text{C}]\text{DASB} \) and \[^{11}\text{C}]\text{MADAM} \) are the three possible radioligands that fulfill PET imaging criteria for \textit{in vivo} in human investigations with PET, whereas \[^{11}\text{C}]\text{DASB} \) and \[^{11}\text{C}]\text{MADAM} \) bear higher selectivity, lower non-specific binding and faster brain uptake than \[^{11}\text{C}]\text{McN5652} \) (Frankle et al, 2004; Houle et al, 2000; Lundberg et al, 2005; Suehiro et al, 1993). In addition, studies observing occupancy of the SERT by SSRIs demonstrated displaceable
[^11]C-DASB and[^11]C-MADAM by SSRIs, which is why these two tracers are predominantly used for reliable SERT quantification (Lundberg et al, 2012; Meyer et al, 2004).

High SERT concentration has been revealed in the brainstem and midbrain regions, while a moderate binding potential has been shown in basal ganglia and diencephalic structures. Limbic brain regions as the subgenual anterior cingulate cortex and insular cortex as well as parts of the temporal cortex display high SERT binding, shown by human in vivo and post mortem studies (Saulin et al, 2012; Savli et al, 2012a). These findings are replicated by post mortem data (Varnas et al, 2004). Inconsistencies, which have been assigned to clinical and methodological reasons, have been observed in SPECT and PET studies, showing either increased, attenuated or a lack of differences of SERT levels between patients with depression and healthy control subjects (Herold et al, 2006; Ichimiya et al, 2002; Parsey et al, 2006a).

![Fig. 2 Region specific SERT binding in the human brain in vivo. High SERT binding is found in the midbrain. The image depicts triplanar structural images (axial, sagittal and coronal view) and superimposed SERT availability using[^11]C-DASB. Mean SERT distribution maps have been generated by using imaging data from the study “The Serotonin Transporter in Attention Deficit Hyperactivity Disorder investigated with Positron Emission Tomography”, related to this thesis.

The SERT has also been investigated in numerous other neuropsychiatric disorders as anxiety disorders, obsessive-compulsive disorder and eating disorders to name a few, though results have been likewise inconsistent (Spies et al, 2015). Two molecular imaging studies have investigated SERT concentration in adult, medication naïve patients with ADHD. A SPECT study
conducted by Hesse and colleges observed SERT availability with $^{[123]}$FP-CIT and SPECT in 17 patients with ADHD (Hesse et al, 2009). $^{[123]}$FP-CIT is a radiotracer that has moderate affinity to the SERT in the midbrain regions and low affinity to the SERT in subcortical and cortical brain regions. They found no significant alteration between patients and HC. Karlsson et al. assessed SERT binding with $^{[11]}$C]MADAM and PET and found no changes in between patients with ADHD and HC. Though, findings are preliminary and should be interpreted with caution, since Karlsson et al. investigated SERT binding potential ($\text{BP}_{\text{ND}}$) in only eight patients. A sample size that is insufficient in power to detect putative differences (Karlsson et al, 2013).

### 1.6. The genetic role in ADHD

As a highly heritable disorder estimated to be 76% (Faraone et al, 2005), several neurotransmitter-related genes have been associated to ADHD, which is a neurodevelopmental spectrum disorder underlying in the interplay between nature, representing genetic composition, and nurture, environmental influence on development. Although genetic studies have failed to verify a distinct gene or genetic variations accountable for a distinct neurobiology of ADHD, complex polygenetic pathways as gene-gene interactions as well as gene-environment interactions are implicated in its pathophysiology (Banaschewski et al, 2010).

The majority of candidate gene studies in ADHD observed monoaminergic system, including genes of the dopaminergic, serotonergic and noradrenergic transporters and receptor systems. Single nucleotide polymorphisms (SNPs) within as well as transcription variations of the NET gene have been linked to ADHD specific behavior (Faraone et al, 2005; Kim et al, 2008). Clinical response to MPH may be sensitive to noradrenergic (Kim et al, 2008; Yang et al, 2004) or dopaminergic gene polymorphisms (Froehlich et al, 2011; Tharoor et al, 2008), while possible dependence on Cathechol-O Methyltransferase (Kereszturi et al, 2008) or SERT genotype implicates the serotonergic system (McGough et al, 2009; Tharoor et al, 2008). The SERT gene (SERT; SLC6A4) and the genes encoding for serotonergic receptors comprise SNPs were found to be implicated in influencing the susceptibility to ADHD (Faraone & Khan, 2006; van der Meer et al, 2014).

To conclude while considering the finding described above, profound evidence exists that the noradrenergic as well as the serotonergic neurotransmission, and in particular the NET and SERT, contribute to the complex pathogenesis of adult ADHD. Molecular imaging and imaging genetics
based on PET data represents a promising research field to observe alterations in molecules as well as association of genes and psychiatric pheno- and endophenotypes in the healthy human brain as well as in neuropsychiatric disorders \textit{in vivo}. At present, there is a lack of imaging studies targeting molecular structures as the NET or the SERT in patients with ADHD, hampering the interpretation of the involvement of these structures in ADHD.
1.7. Aims of this thesis

The main aims of this thesis were to objectify the availability of NET and SERT binding in a priori selected brain regions in patients with ADHD and healthy control subjects. The first and the third publication listed in the results section deals with this scientific question. Furthermore, we performed an interregional molecular association of SERT BP_{ND} to display possible SERT patterns, characteristic for patients compared to controls. Finally, the second publication investigates an effect of SNPs on the NET BP_{ND} in patients with ADHD and healthy control subjects.

This thesis will focus on quantifying NET and SERT BP_{ND}, an index for density in the investigated tissue, in patients with ADHD and healthy control subjects based on PET. The specific aims can be summarized as:

- To test whether NET BP_{ND} is significantly different in patients with ADHD compared to healthy control subjects using PET and the radioligand (S,S)-[^18F]FMeNER-D_{2}.

- To test whether SERT BP_{ND} is significantly different in patients with ADHD compared to healthy control subjects using PET and the radioligand [^{11}C]DASB.

- To test whether interregional molecular association of SERT BP_{NDx} is significantly different in patients with ADHD compared to healthy control subjects.

- To investigate the relationship between the effects of SNPs on the NET BP_{ND} in patients with ADHD and healthy control subjects.
The Norepinephrine Transporter in Attention-Deficit/Hyperactivity Disorder Investigated With Positron Emission Tomography

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IMPORTANCE Attention-deficit/hyperactivity disorder (ADHD) research has long focused on the dopaminergic system's contribution to pathogenesis, although the results have been inconclusive. However, a case has been made for the involvement of the noradrenergic system, which modulates cognitive processes, such as arousal, working memory, and response inhibition, all of which are typically affected in ADHD. Furthermore, the norepinephrine transporter (NET) is an important target for frequently prescribed medication in ADHD. Therefore, the NET is suggested to play a critical role in ADHD.

OBJECTIVE To explore the differences in NET nondisplaceable binding potential (NET BP_{ND}) using positron emission tomography and the highly selective radioligand \((S,S)-\left[{ }^{18}F\right]FMeNER-D_2\) \([\left(S,S\right)-2-(\alpha-(2-\left[{ }^{18}F\right]fluoro\left[2H_2\right]methoxyphenoxy)benzyl)morpholine]\) between adults with ADHD and healthy volunteers serving as controls.

DESIGN, SETTING, AND PARTICIPANTS Twenty-two medication-free patients with ADHD (mean [SD] age, 30.7 [10.4] years; 15 [68%] men) without psychiatric comorbidities and 22 age- and sex-matched healthy controls (30.9 [10.6] years; 15 [68%] men) underwent positron emission tomography once. A linear mixed model was used to compare NET BP_{ND} between groups.

MAIN OUTCOMES AND MEASURES The NET BP_{ND} in selected regions of interest relevant for ADHD, including the hippocampus, putamen, pallidum, thalamus, midbrain with pons (comprising a region of interest that includes the locus coeruleus), and cerebellum. In addition, the NET BP_{ND} was evaluated in thalamic subnuclei (13 atlas-based regions of interest).

RESULTS We found no significant differences in NET availability or regional distribution between patients with ADHD and healthy controls in all investigated brain regions \((F_{1,41} < 0.01; P = .96)\). Furthermore, we identified no significant association between ADHD symptom severity and regional NET availability. Neither sex nor smoking status influenced NET availability. We determined a significant negative correlation between age and NET availability in the thalamus \(R^2 = 0.29; P < .01\) corrected) and midbrain with pons, including the locus coeruleus \(R^2 = 0.18; P < .01\) corrected), which corroborates prior findings of a decrease in NET availability with aging in the human brain.

CONCLUSIONS AND RELEVANCE Our results do not indicate involvement of changes in brain NET availability or distribution in the pathogenesis of ADHD. However, the noradrenergic transmitter system may be affected on a different level, such as in cortical regions, which cannot be reliably quantified with this positron emission tomography ligand. Alternatively, different key proteins of noradrenergic neurotransmission might be affected.

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Attention-deficit/hyperactivity disorder (ADHD), which is characterized by inattention, impulsivity, and hyperactivity, affects between 8% and 12% of children, persists into adulthood in approximately 30% of cases, and exhibits rising prevalence rates. Attention-deficit/hyperactivity disorder is often associated with detrimental comorbidities as well as with a large personal and social burden. As a result, many individuals with ADHD routinely receive psychopharmacologic treatment.

Patients with ADHD often receive methylphenidate hydrochloride and amphetamine sulfate, which are stimulant medications that enhance dopaminergic and noradrenergic signaling. Alternatively, atomoxetine hydrochloride, which is a nonstimulant drug that blocks the norepinephrine transporter (NET), is used. By blocking the NET, atomoxetine affects noradrenergic signaling and, particularly in brain regions lacking the dopamine transporter, increases dopaminergic transmission. Treatment using methylphenidate, amphetamine, and atomoxetine is associated with improvement of clinical symptoms and performance in controlled tasks eliciting executive functions, such as inhibitory control, and of cognitive functions, such as working memory and attention.

Although amphetamine and methylphenidate have been suggested to exert therapeutic efficacy via an increase in extracellular dopamine, they also have been shown to modulate noradrenergic neurotransmission, which may be therapeutically relevant. Methylphenidate may dose-dependently block the NET, thereby regulating noradrenergic and dopamine reuptake. In a similar manner, atomoxetine has been shown to facilitate therapeutic response by binding the NET. In addition, quetiapine fumarate, which is not used as an ADHD medication but has been shown to improve cognitive function in patients with psychosis, was shown to bind to the NET. Ultimately, facilitation of therapeutic response by catecholamines in general and the NET in particular suggests that these systems may be relevant to ADHD.

Furthermore, ADHD symptoms have long been attributed to abnormalities within the frontostriatal and frontoparietal networks implicated in executive functions modulated by catecholaminergic systems. The noradrenergic system, which originates in the locus coeruleus and exerts virtually ubiquitous influence within the brain, modulates, among other cortical regions, the prefrontal cortex through dynamic adaption of tonic and phasic firing. Studies displaying improvement of such symptoms by application of α₂ agonists further link noradrenergic influence on prefrontal cortex-mediated cognitive functions to ADHD.

More assertive investigation of underlying neurobiological correlates is made possible through positron emission tomography (PET) imaging studies, which have focused on ADHD-related changes in the dopaminergic system. Although changes in dopamine transporter and dopamine D₂ and D₃ receptor levels and distribution as well as dopamine release have been investigated, the results remain inconclusive. However, the proposition that methylphenidate, amphetamine, and atomoxetine may induce therapeutic response via NET modulation suggests that noradrenergic factors, and more specifically changes in the NET, may play a role in ADHD pathogenesis.

Therefore, we proposed a thorough investigation of ADHD-related NET distribution, as has been performed for the serotonergic transporter and dopamine transporter. To address this issue, we used the recently developed NET-specific radiotracer ([S,S]-2-((2-[18F]fluoro-[2H₂]methoxyphenoxyl)benzyl)imorpholine), which has been successfully applied in healthy control groups. To investigate the role of noradrenergic changes within ADHD, NET imaging was carried out in a region of interest (ROI) approach focusing on brain areas integral to the noradrenergic system.

Table 1. Epidemiologic and Clinical Characteristics of Participants

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>ADHD Group (n = 22)</th>
<th>Control Group (n = 22)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean (SD), y</td>
<td>30.7 (10.4)</td>
<td>30.9 (10.6)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>15 (68)</td>
<td>15 (68)</td>
</tr>
<tr>
<td>Female</td>
<td>7 (32)</td>
<td>7 (32)</td>
</tr>
<tr>
<td>Current smoker</td>
<td>7 (32)</td>
<td>11 (50)</td>
</tr>
<tr>
<td>Handedness</td>
<td>20 (91)</td>
<td>17 (77)</td>
</tr>
<tr>
<td>Left</td>
<td>2 (9)</td>
<td>5 (23)</td>
</tr>
<tr>
<td>CAARS score, mean (SD)²</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inattentiveness</td>
<td>18.8 (5.2)</td>
<td>0.1 (0.4)</td>
</tr>
<tr>
<td>Hyperactivity/impulsivity</td>
<td>19.6 (5.6)</td>
<td>0.2 (0.6)</td>
</tr>
<tr>
<td>Past psychopharmacologic treatment⁴</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Stimulants</td>
<td>4 (18)</td>
<td></td>
</tr>
<tr>
<td>SNRIs</td>
<td>2 (9)</td>
<td></td>
</tr>
<tr>
<td>Stimulants and antidepressants</td>
<td>1 (4)</td>
<td></td>
</tr>
<tr>
<td>Past comorbidities</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Depression, currently in remission</td>
<td>7 (32)</td>
<td></td>
</tr>
<tr>
<td>Drug abuse</td>
<td>2 (9)</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: ADHD, attention-deficit/hyperactivity disorder; CAARS, Conners Adult ADHD Rating Scale; NA, not applicable; SNRIs, selective norepinephrine reuptake inhibitors.

Methods

Participants

Written informed consent was obtained from all participants after detailed explanation of the study protocol, and the participants received financial reimbursement. The study was approved by the ethics committee of the Medical University of Vienna and the General Hospital of Vienna (EK 552/2010).

Twenty-two adults with ADHD (mean [SD] age, 30.7 [10.4] years; 15 [68%] men) and 22 age- and sex-matched healthy individuals serving as controls (30.9 [10.6] years; 15 [68%] men) (Table 1) were recruited through an ADHD outpatient clinic at Vienna.
the Department of Psychiatry and Psychotherapy, Medical University of Vienna, Vienna, Austria, and from the local community via advertisement. Patients had not received psychopharmacologic treatment for at least 6 months before the screening visit; all control participants were naive to all psychopharmacologic treatment. Of the original 51 study participants, 2 (4%) were excluded because of substance abuse, 2 (4%) because of somatic disorders, and 3 (6%) because of radiosynthesis difficulties.

Medical Examination and Clinical Exploration
Participants underwent standard medical examination including general physical and neurologic status evaluation, electrocardiography, and routine laboratory tests at the screening and final visits to ensure their physical health. Women underwent a urine pregnancy test at the screening visit and before PET measurement. A multidrug urine test was performed at the screening visit to exclude current substance abuse. Participants were interviewed by experienced psychiatrists using the Conners Adult ADHD Diagnostic Interview for DSM-IV to evaluate current and childhood attentional and hyperactivity/impulsivity symptoms and confirm the ADHD diagnosis. The Conners Adult ADHD Rating Scale (CAARS)-Observer Screening Version (Table I) was applied to assess the presence and severity of inattentive and hyperactivity/impulsivity symptoms, and third-party–reported and self-reported symptoms were determined with the CAARS-Observer Screening Version and the CAARS-Self-Report Screening Version. The Structured Clinical Interview for DSM-IV Axis I and Axis II disorders was performed to exclude comorbid psychiatric disorders. Hand edness was evaluated with the Edinburgh Inventory and IQ was determined with the Viennese Matrices Test. Patients with ADHD did not differ significantly from the controls in IQ (ADHD, 92.86 [15.22]; controls, 98.77 [12.89]; P = .16, 2-tailed, t test). Participants were subdivided into groups best describing their smoking status according to the quantity of consumption, which was assessed in an open-question format (nonsmokers, 5 cigarettes/d, 5-10 cigarettes/d, 10 cigarettes/d, 10-15 cigarettes/d, 15 cigarettes/d, and 20 cigarettes/d; ranks were 1-8, respectively). The ADHD group did not significantly differ in smoking status compared with the control group (median rank: ADHD, 0; control, 0.5; z = −0.48, P = .63, Mann-Whitney test). Individuals with PET- or magnetic resonance imaging (MRI)-incompatible implants or in pregnancy or breastfeeding were not included in this study.

Data Acquisition
All PET was carried out at the Department of Biomedical Imaging and Image-Guided Therapy, Division of Nuclear Medicine, Medical University of Vienna, using a full-ring scanner (GE Advance; General Electric Medical Systems) in a 3-dimensional acquisition mode. We applied (S,S)-[18F]FMeNER-D2, which is among the most suitable PET tracers for in vivo NET quantification as described previously for the following reasons: (1) fluorine F 18–labeled reboxetine analogues enable specific binding equilibrium to be reached within a reasonable time frame for PET measurement owing to their 5-fold higher half-life; (2) in vivo defluorination can be reduced considerably, and the interpretability of regions in proximity to bone thereby increased, through the use of deuterated homologues; and (3) (S,S)-[18F]FMeNER-D2 has shown both high affinity and selectivity to the NET. A 5-minute transmission scan using retractable germanium Ge 68 rod sources for tissue attenuation correction was performed before the emission scan. Data acquisition started 120 minutes after a bolus intravenous injection of 4.7 MBq/kg of body weight (ADHD, 395.1 [98.7] MBq; controls, 379.0 [62.2] MBq; P = .53, 2-tailed, paired t test) of (S,S)-[18F]FMeNER-D2. Mean (SD) specific radioactivity of (S,S)-[18F]FMeNER-D2 was 589.4 (399.7) GBq/μmOL (ADHD) and 440.4 (233.7) GBq/μmOL (controls) (P = .15, 2-tailed, paired t test). Brain radioactivity was measured in a series of 6 consecutive time frames lasting 10 minutes each in the interval of 120 to 180 minutes after administration of the bolus. Acquired data were reconstructed in volumes consisting of 35 transaxial sections (128 × 128 matrix) using an iterative filtered back-projection algorithm with a spatial resolution of 4.36 mm full-width at half of the maximum 1 cm next to the center of the field of view. For coregistration, MRIs were acquired from all participants on a 3-T scanner (Achieva; Philips) using a 3-dimensional Ti fast field echo-weighted sequence, yielding 0.88-mm section thickness and in-plane resolution of 0.8 × 0.8 mm.

Data Quantification
Each time frame of the dynamic PET scan was realigned to the mean of frames with no head motion, identified by visual inspection. Subsequently, each summed image (PET integral image from realigned data) was coregistered (rigid body transformation) to each participant’s MRI using a mutual information algorithm implemented in SPM8 (Wellcome Trust Centre for Neuroimaging; http://www.fil.ion.ucl.ac.uk/spm/). Parametric images of nondisplaceable binding potential (BPND) values were calculated using the c audate as the reference region because it was devoid of NET. According to nomenclature, the BPND values were defined as follows:

\[
BP_{ND} = \frac{\int_{120}^{180} C_{target} \, dt}{\int_{120}^{180} C_{reference} \, dt} - 1,
\]

where \( C_{target} \) indicates radioactivity concentration of the target region and \( C_{reference} \) radioactivity concentration of the reference region. Caudate ROIs were delineated on MRIs in individual-participant space using image analysis software (PMOD, version 3.1; PMOD Technologies Ltd; http://www.pmod.com), which were subsequently transferred to coregistered summed PET images. Individual MRIs were spatially normalized to the Ti-weighted MRI template provided in SPM8. Resulting transformation matrices were applied to the coregistered parametric images warping them into Montreal Neurological Institute (MNI) standard space.
Regions of Interest
The ROIs selected included NET-rich regions, based on post-mortem and in vivo human brain studies, and show a good signal to noise ratio and an acceptable bone spillover due to (S,S)-(18F)FMoNER-D2 defluorination. Binding potential values were extracted from parametric maps from either atlas-generated ROIs or manually delineated ROIs. Atlas-generated ROIs were identified from the Hammers Maximum Probability Atlas including 6 regions: the hippocampus, putamen, pallidum, thalamus, midbrain with pons (including the locus coeruleus), and cerebellum. Since the NET concentration in the thalamus is not homogeneous, 13 thalamic subnuclei were generated with the Wake Forest University Pickatlas Tool (Table 2). To verify atlas-generated ROIs, 4 atlas ROIs were delineated on the MNI T1 single-participant brain: the midbrain (dorsally located including raphe nuclei, excluding pons), locus coeruleus, and hypothalamus. In addition to the above-mentioned atlas ROIs, further ROIs, specifically the locus coeruleus and thalamus, which are brain regions highest in NET concentration, were delineated manually for each participant for confirmatory purposes. Atlas ROIs match the MNI standard space.

Statistical Analysis
Data were analyzed using linear mixed models for the outcome measure NET BPND with the ROI as a repeated factor; participant groups, sex, and ROI as fixed factors; and participants and matched participant pairs as random factors. A separate model was calculated for the 6 ROIs based on the Hammers Maximum Probability Atlas and for the 13 thalamic subnuclei. Likewise, manually delineated ROIs were assessed in 2 additional models: one using the 4 atlas-based ROIs and the other using the 2 individual-based ROIs. Fixed effects were included in the model in a multifactorial approach, whereas interaction effects were dropped in instances of nonsignificance. In cases of significant interactions or main effects, post hoc pairwise comparisons were computed and Bonferroni correction was performed for multiple comparisons. In a second exploratory approach to examine the effects of handedness, smoking status, and age, a mixed model was calculated using a stepwise procedure with backward elimination, starting with all candidate variables (including participant groups and ROIs) and followed by a stepwise deletion of interactions and variables with the largest P values. Finally, mixed-models analyses were also applied to investigate the effects of the clinical variables inattentiveness and hyperactivity/impulsivity, which were assessed with the CAARS-Investigator Screening Version. According to the Akaike information criterion, repeated measurements were modeled using a compound symmetric covariance structure. As an exploratory analysis, we also compared NET BPND between patients and controls at the voxel level using SPM8 (paired t test); SPSS, version 19.0 for Windows (SPSS Inc), was used for statistical computations. The 2-tailed significance level was set at P < .05. Region of interest and voxel-wise analysis results were corrected for multiple comparisons using Bonferroni and false discovery rate analysis, respectively.

Results
Linear mixed-models analysis revealed an expected main effect of ROI (F[5,215] = 117.71; P < .001) but no main effects of participant group (F[1,41] <0.01; P = .96) (Table 2 and Figure 1) or sex (F[1,41]<0.01; P = .98) and no interaction effects (all P > .10). Post hoc pairwise comparisons revealed significant NET BPND differences between the 6 tested brain regions (atlas-generated ROIs; P < .05, corrected) except for the
comparisons of midbrain with pallidum and putamen with cerebellum, which had similar binding values (Table 2 and Figure 2). Analogous results were obtained from the 2 mixed models for the manually delineated ROIs, which showed main effects of ROI but no main effects of group and sex and no interaction effects. Similarly, the linear mixed model for NET binding within the thalamic subnuclei revealed a main effect of ROI ($F_{12,516} = 105.53; P < .001$) but no main effect of group ($F_{1,41} = 0.08; P = .78$) or sex ($F_{1,41} = 0.39; P = .54$) and no interaction effects (all $P > .10$). In addition, there was no significant difference in NET binding between patients with ADHD and the controls in any brain region at the voxel level (all $P > .05$, corrected).

When investigating the potential effects of handedness, smoking status, and age, mixed-models analysis for ROI \( \text{NET BP}_{\text{ND}} \) based on the Hammers Maximum Probability Atlas revealed an interaction effect between ROI and age ($F_{5,190} = 9.94; P < .001$) in addition to a main effect of ROI but no main effect of age. Post hoc correlation analyses between regional \( \text{NET BP}_{\text{ND}} \) and age revealed strong negative correlations in the thalamus ($R^2 = 0.29; P < .01$ corrected) and midbrain ($R^2 = 0.18 P < .01$ corrected) (Figure 3), but these correlations did not differ significantly between the control and ADHD groups. Handedness and smoking status had no effect on \( \text{NET BP}_{\text{ND}} \), nor did they lead to any significant interactions. Comparable results were observed for manually delineated ROIs, which showed strong negative correlations between \( \text{NET BP}_{\text{ND}} \) and age in the midbrain ($R^2 = 0.28; P < .01$ corrected), locus coeruleus ($R^2 = 0.26; P < .01$ corrected), and hypothalamus ($R^2 = 0.26; P < .01$ corrected).
rected). In addition, no main or interaction effects were observed for clinical variables (CAARS-Inattentiveness and CAARS-Hyperactivity/Impulsivity) and ROI BPND. Finally, exclusion of 3 patients with previous methylphenidate intake in childhood (intake duration was 4, 5, and 7 years) and 2 patients with previous atomoxetine consumption in adulthood (intake duration was 5 and 6 months) did not change NET binding results. We further excluded 2 patients exhibiting predominantly inattentive symptoms and 1 exhibiting predominantly hyperactivity/impulsivity symptoms and, in a separate analysis, 2 patients with past drug abuse. Exclusion of these participants did not change the results.

Discussion

To our knowledge, this is the first PET study to investigate the differences in brain NET distribution and availability in adults with ADHD. We found no significant differences in the BP\textsubscript{ND} of (S,S\textsuperscript{-18F})\textsuperscript{FMeNER-D\textsubscript{2}} between the patients with ADHD and the controls. Furthermore, exclusion of patients exhibiting either predominantly inattentive or predominantly hyperactivity/impulsivity subtypes and patients with previous ADHD pharmacotherapy or past drug abuse did not change the results. Our findings validate previous studies\textsuperscript{53} showing an age-related decrease in brain NET availability in the healthy human brain and show an age-related decrease in brain NET availability in adults with ADHD.

Randomized placebo-controlled studies\textsuperscript{54-56} have repeatedly shown that methylphenidate, amphetamine, and atomoxetine significantly decrease symptoms in adult ADHD patient cohorts. The clinical efficacy of a pharmaceutical agent implies that the mechanism of action through which it attains a response is relevant to the neurobiology and resulting symptoms of a particular disease. Therefore, modulation of the noradrenergic system by these 3 drugs suggests noradrenergic abnormalities in ADHD.

Executive functions, such as response inhibition, vigilance, working memory, and planning, are typically impaired in ADHD.\textsuperscript{57,58} The association of these functions with the prefrontal cortex, which exhibits pronounced noradrenergic innervation, once again implicates, more generally, the noradrenergic system in ADHD.\textsuperscript{59}

However, investigations into the involvement of other neurotransmitter systems in ADHD are similarly inconclusive. First, current data available on the dopaminergic contribution to ADHD are wrought with inconsistency. As is the case with the NET, therapeutic doses of methylphenidate have been shown\textsuperscript{60,61} using PET to reduce radiotracer striatal dopamine transporter binding in a dose-dependent manner in healthy individuals. Methylphenidate-induced dopamine transporter blockade has been causally linked to an

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Figure 3. Negative Correlation of Norepinephrine Transporter Nondisplaceable Binding Potential (NET BP\textsubscript{ND}) and Age in the Thalamus and Midbrain/Pons

A significant negative correlation existed between the NET BP\textsubscript{ND} (a unitless measure) and age in the thalamus ($R^2 = 0.29; P < .01$ corrected) (A) and midbrain/pons ($R^2 = 0.18; P < .01$ corrected) (B). Regions of interest were extracted from Hammers Maximum Probability Atlas. The significance level was set at $P < .05$ and the results were Bonferroni corrected for multiple comparisons. ADHD indicates attention-deficit/hyperactivity disorder; PET, positron emission tomography. Please note the different NET BP\textsubscript{ND} ranges on the y-axis.
increase in striatal extracellular dopamine in the human brain,14 and this effect has been associated with therapeutic responses to methylphenidate in ADHD.62 Moreover, striatal dopamine transporter availability in patients with ADHD was correlated with improvement of clinical symptoms after methylphenidate treatment.63 Brain imaging studies,31,63-65 however, have reported an array of partially contradictory results ranging from dopamine transporter increases to a lack of change66 to decreases29,67 in the brain of adults with ADHD. Although methodologic factors (eg, tracer choice) and patient characteristics (including the presence of prior medication, comorbidities, and differing sample sizes) have been suggested29,32 to account for this variability in results, investigations of other components of the dopaminergic system, such as the D2 and D3 receptors, are similarly inconsistent.29,32 In addition, serotonergic alterations have been discussed in the context of ADHD68 and are primarily based on the relationship between serotonergic innervation and impulsivity and hyperactivity, which are 2 core ADHD symptoms.69 However, serotonergic involvement in ADHD is contradicted by data showing the limited clinical efficacy of selective serotonin reuptake inhibitors in the improvement of ADHD symptoms. Furthermore, serotonin transporter imaging studies67,70 showed no difference in serotonin transporter distribution between patients with ADHD and healthy controls. Therefore, although existing evidence neither affirms nor disproves the neurotransmitter systems and healthy controls. Therefore, although existing evidence

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Norepinephrine Transporter in ADHD

ARTICLE INFORMATION

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REFERENCES


Abstract: Attention deficit hyperactivity disorder (ADHD) is a heterogeneous disorder with a strong genetic component. The norepinephrine transporter (NET) is a key target for ADHD treatment and the NET gene has been of high interest as a possible modulator of ADHD pathophysiology. Therefore, we conducted an imaging genetics study to examine possible effects of single nucleotide polymorphisms (SNPs) within the NET gene on NET nondisplaceable binding potential (BPND) in patients with ADHD and healthy controls (HCs). Twenty adult patients with ADHD and 20 HCs underwent (S,S)-[18F]FMeNER-D2 positron emission tomography (PET) and were genotyped on a MassARRAY MALDI-TOF platform using the Sequenom iPLEX assay. Linear mixed models analyses revealed a genotype-dependent difference in NET BPND between groups in the thalamus and cerebellum. In the thalamus, a functional promoter SNP (−3081 A/T) and a 5′-untranslated region (5′UTR) SNP (−182 T/C), showed higher binding in ADHD patients compared to HCs depending on the major allele. Furthermore, we detected an effect of genotype in HCs, with major allele carriers having lower binding. In contrast, for two 3′UTR SNPs (*269 T/C, *417 A/T), ADHD subjects had lower binding in the cerebellum compared to HCs depending on the major allele. Additionally, symptoms of hyperactivity and impulsivity correlated with NET BPND in the cerebellum depending on genotype. Symptoms correlated positively with cerebellar NET BPND for the major allele, while symptoms correlated negatively to NET BPND in minor allele carriers. Our findings support the role of genetic influence of the NE system on NET binding to be pertubated in ADHD.
INTRODUCTION

Attention deficit hyperactivity disorder (ADHD) is the most frequent neurodevelopmental disorder diagnosed in children. It is characterized by inattention, hyperactivity, and impulsiveness which frequently leads to severe social, academic, and vocational dysfunction [De La Fuente et al., 2013]. In around 30% of ADHD cases, the symptoms persist through adolescence into adulthood [Barbaresi et al., 2013]. Symptoms differ in adults compared to children, such as hyperactivity decreases while problems with inattention persist [Volkow and Swanson, 2013]. ADHD has a strong genetic component with a heritability estimated to be around 0.77 [Curatolo et al., 2009]. Though the heritability is rather high in ADHD, studies have failed to indicate a single gene responsible for the course of ADHD, suggesting complex polygenic mechanisms and gene environment interactions to be of importance [Banaschewski et al., 2010].

Norepinephrine (NE) neurotransmission has been hypothesized to be altered in various disorders, such as depression, PTSD, Alzheimer’s disease, and ADHD [Biederman and Spencer, 1999; Gulyas et al., 2010; Klimek et al., 1997; Pietrzak et al., 2013]. NE has long been discussed to be dysregulated in ADHD since frequently prescribed psychopharmacata such as methylphenidate (MPH) and atomoxetine (ATX) target the dopaminergic and NE systems by increasing the extracellular neurotransmitter levels through inhibition of the respective reuptake transporters [Hannestad et al., 2010; Logan et al., 2007]. MPH and ATX, a selective NE reuptake inhibitor, have been proven clinically effective in improving core symptoms in ADHD [Asherson et al., 2014], although up to 40% of patients being ascribed to stimulant and nonstimulant medication do not respond [Newcorn et al., 2008, 2009]. Recently, guanfacine, an alpha-2 adrenergic receptor agonist, has also been used as an effective treatment option for patients with ADHD [Newcorn et al., 2013]. It is, therefore, likely that alterations in the NE system may predispose to ADHD and thus, the norepinephrine transporter (NET) gene is suspected to play a major role in ADHD pathogenesis. The gene encoding for the NET (SLC6A2) contains certain single nucleotide polymorphisms (SNPs) that have been investigated in pathological conditions [Hahn and Blakely, 2007]. In association and linkage studies, various SNPs have been found to be involved throughout the ADHD population [Kim et al., 2006a; Sengupta et al., 2012]. Results, however, have varied, and there is some contradictory results confounding this theory [Barr et al., 2002; Xu et al., 2005].

As for in vivo brain quantification of the NET, specifically in ADHD, literature is quite scarce until now. In a recently published study, our group demonstrated no differences in NET nondisplaceable binding potential (BP_{ND}) in patients with ADHD compared to healthy controls (HCs) [Vanicek et al., 2014]. It is of high interest to examine whether genetic variants in the NE system have an effect on NET BP_{ND} which could shed light on individual differences in susceptibility to ADHD. Additionally, to the best of our knowledge, no positron emission tomography (PET) study is available so far investigating polymorphisms in the NE system on the NET binding, neither in HCs nor in patients with ADHD.

Thus, the aim was to examine the relationship between the effects of SNPs in the NE system and the NET BP_{ND} in a cohort comprising of ADHD subjects and HCs matched for age and sex. We hypothesized that ADHD subjects carrying either major or minor alleles will have higher binding compared to their healthy matched controls. High binding subcortical regions believed to be principal areas in behavioral and attentional control were selected [Arnsten and Rubia, 2012], whereas cortical regions were dismissed due to the defluorination and bone spillover of the radioligand (S,S)-[18F]FMoNER-D2. Moreover, we hypothesized that the symptoms of hyperactivity and impulsivity would correlate to with NET BP_{ND} in areas related to motoric activity (putamen, cerebellum, midbrain) whilst symptoms of inattention would correlate with NET BP_{ND} in the thalamus.

MATERIALS AND METHODS

Subjects

Twenty adult ADHD patients (age ± SD: 30.8 ± 10.9, 14 males) and 20 HCs (age ± SD: 30.4 ± 10.9, 14 males) were recruited through ADHD outpatient clinic at the Department of Psychiatry and Psychotherapy, Medical University of Vienna, and from the local community via advertisement as previously published elsewhere [Vanicek et al., 2014]. All patients had been free from psychopharmacological treatment for at least 6 months prior to screening visit. During the prescreening, medical examinations including withdrawal of blood samples were performed to ensure physical well being of participants. All participants underwent a multidrug urine test to assess current substance abuse. For inclusion, patients had to have a current ADHD diagnosis as well as a history of childhood ADHD. Five of the 20 patients had their first diagnosis in childhood. Subjects were interviewed using the Conners’ Adult ADHD Diagnostic Interview for DSM-IV (CAADID, Connors, 1999), Conners’ Adult ADHD Rating Scale Investigator-Screen Version (CAARS-Inv:SV), Conners’
Eleven SNPs (Fig. 1) were considered for inclusion, which were selected upon previous association studies [Bobb et al., 2005; Sengupta, et al., 2012; Thakur et al., 2014a]. Three SNPs were included in genotyping to extend the 5′ flanking region (rs15534, rs40615, rs7188230). Furthermore, three SNPs were chosen to extend the 5′ region of NET (rs2397771, rs168924, rs2242246). Haploview version 4.2 (http://www.broad.mit.edu/mpg/haploview/) was used to test whether frequencies were according to Hardy–Weinberg equilibrium. Furthermore, the tag function in Haploview was used to identify SNPs in high-linkage disequilibrium. It refers to the nonrandom association of alleles at different loci, allowing an identification of genetic variations by the information derived from one or more SNPs [Hu et al., 2004; Stram, 2004]. Thus, these tagged SNPs were used for further analysis. For final analysis, the following four SNPs were used as identified using the tag function in Haploview: −3081 A/T (rs28386840) and −182 T/C (rs2242446) \((r^2 = 0.869)\), and *269 T/C (rs15534) and *417 A/T (rs40615) \((r^2 = 0.866)\). For simplicity’s sake, they will be referred to by their rs number in this article.

### Genotyping

Procedures were performed as previously described [Balldinger et al., 2014]. In short, 9 ml EthyleneDiamineTetraacetic Acid (EDTA) blood samples were drawn from each subject and DNA was isolated from whole blood using the QiaAmp DNA blood maxi kit (Qiagen, Hilden, Germany). Genotyping was performed using the iPlex assay on the MassARRAY MALDI-TOF mass spectrometer as described [Oeth et al., 2009]. Allele specific extension products were identified and genotypes allocated by Typer 3.4 Software (Sequenom, San Diego, CA). All applied quality criteria were met [individual call rate >80%, SNP call rate >99%, identity of genotyped of CEU trios (Coriell Institute for Medical research, Camden, NJ) with HapMap database >99%].

### Positron Emission Tomography

Scans were conducted at the Department of Biomedical and Image-guided Therapy, Division of Nuclear Medicine at the Medical University of Vienna. Each subject underwent a PET (General Electric Medical Systems, Milwaukee, WI) scan using the tracer (S,S)-[18F]FMeNER-D2, synthesized as previously described [Rami-Mark et al., 2013]. (S,S)-[18F]FMeNER-D2 is currently the most suitable radioligand for in vivo NET quantification previously described [Vanicek et al., 2014]. Briefly, fluorine-18-labelled reboxetine analogue allows, due to its long half-life \((t_{1/2} = 109.77\) min) and excellent affinity and selectivity, to reach the specific binding equilibrium within the time-frame of the PET measurement. A 5-min transmission scan using a retractive 68Ge rod sources for tissue attenuation correction was performed prior to the dynamic emission scan acquired in 3-D mode. Data acquisition started 120 min after a bolus i.v. injection of 4.7 MBq/kg body weight (ADHD patients: 393 ± 95 MBq, HC: 384 ± 61 MBq; \(P > 0.05\), t-test) of (S,S)-[18F]FMeNER-D2. Mean specific radioactivity of (S,S)-[18F]FMeNER-D2 was 537 ± 383 GBq/μmol (ADHD patients) and 473 ± 218 GBq/μmol (HC) \((P > 0.05\), t-test). Brain radioactivity was measured in a series of six consecutive time frames lasting 10 min each in the interval of 120–180 min after tracer bolus application. Acquired data were reconstructed in volumes consisting of 35 transaxial sections \((128 \times 128\) matrix) using an iterative filtered back projection algorithm (FORE-ITER) with a spatial resolution
of 4.36-mm full-width at half maximum 1 cm next to the center of the field of view. For coregistration, magnetic resonance (MR) images were acquired from all participants on a 3-Tesla Philips scanner (Achieva) using a 3-D T1 FFE-weighted sequence, yielding 0.88-mm slice thickness and in plane resolution of $0.8 \times 0.8$ mm [Vanicek et al., 2014].

**Data Preprocessing and Quantification of NET**

As described previously [Vanicek et al., 2014], each time frame of the dynamic PET scan was realigned to the mean of frames with no head motion, which was identified by visual inspection. These summed realigned images were then coregistered to each individual’s MRI scan using a mutual information algorithm in SPM8 (Wellcome Trust Centre for Neuroimaging, London, UK; http://www.fil.ion.ucl.ac.uk/spm/). Parametric images of NET BP$_{ND}$ were computed using the caudate as the reference region. The quantification was done as previously described [Arakawa et al., 2008]. Briefly, the ratio method was used to express the BP$_{ND}$ as area under the time-activity curve of the target region/area under the time-activity curve for the reference region. The ratio method was highly correlated to the golden standard used in their study, values were $r = 0.88$ ($y = 0.71x + 0.29$) in the thalamus and $r = 0.88$ ($y = 0.86x + 0.12$) for other brain regions. The integration interval of 120–180 min was used. Manual delineation of the caudate ROI was performed on individual MR images using PMOD image analysis software, version 3.1 (PMOD Technologies, Zurich, Switzerland, www.pmod.com). MRI scans were spatially normalized using SPM8 (Wellcome Trust Centre for Neuroimaging, London, UK; http://www.fil.ion.ucl.ac.uk/spm/) and the resulting transformation matrices applied to the coregistered parametric images warping them into MNI standard space.

**Regions of Interest**

Four regions of interest (ROIs) were selected including NET rich regions [Schou et al., 2005] as well as regions thought to be “core” regions in behavioral control (inattention, impulsivity, hyperactivity) [Armsten and Rubia, 2012]. These were the thalamus, midbrain with pons (including the locus coeruleus), putamen, and cerebellum. Cortical regions, such as the prefrontal cortex (PFC) were not taken into account due to the bone spill over of (S,S)-[18F]FMeNER-D$_2$ inherent to the radioligand. NET BP$_{ND}$ for each region was extracted from parametric maps from the Hammers Maximum Probability Atlas [Hammers et al., 2003].

**Statistical Analysis**

Descriptive statistics were computed and regional NET BP$_{ND}$ values were evaluated for normality using the Shapiro–Wilk test. For each analysis, subject were grouped according to their genotype, that is, minor allele carriers versus major allele homozygotes. Genotype frequencies were determined and found to be distributed according to the Hardy–Weinberg equilibrium ($P > 0.1$).

To examine the effect of genotypes on NET BP$_{ND}$, linear mixed models for each SNP were computed, using the genotype (homozygous major vs. minor allele carriers) and group (ADHD patients vs. HC) as fixed factors and ROI as a repeated factor and the NET BP$_{ND}$ as the dependent variable. Possible effects of cofactors (age and sex) were also tested for and were excluded if insignificant.

A separate model for each SNP was computed as follows; linear mixed model with the factors group, ROI, and genotype as the independent variables and the BPND as the dependent variable. For each model, main effects were tested for, and interactions among ROI, group, and genotype. If rendered significant, further analysis included testing for interaction between group and genotype, separated by ROI. Further analysis included post hoc $t$-tests.

The model prevailing the best fit was the autoregressive 1 (AR(1)). Individual slopes and intercepts were fitted for subjects and for random effects the variance components structure was used.

To test whether there was any effect of behavioural sub-scales on NET BP$_{ND}$ depending on genotypes, Pearson’s correlation coefficient was used. All analyses were computed using SPSS version 22.0 (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Armonk, NY: IBM Corp). Each model was corrected for multiple comparisons using the false discovery rate (FDR) at a significance level of $z = 0.05$ [Benjamini et al., 2001].

**RESULTS**

Demographics and allele counts of study subjects can be seen in Table I. Control and ADHD groups did not differ significantly in terms of age and sex.

<table>
<thead>
<tr>
<th>SNP</th>
<th>Minor Allele Carriers</th>
<th>Major Allele Carriers</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs28386840</td>
<td>12/8</td>
<td>10/10</td>
</tr>
<tr>
<td>rs2242446</td>
<td>9/9</td>
<td>12/8</td>
</tr>
<tr>
<td>rs15534</td>
<td>13/7</td>
<td>11/9</td>
</tr>
<tr>
<td>rs40615</td>
<td>12/8</td>
<td>10/10</td>
</tr>
</tbody>
</table>

**TABLE I. Demographics, psychological tests, past comorbidities, and allele frequencies between patients with ADHD and HCs**

<table>
<thead>
<tr>
<th></th>
<th>Controls ($n = 20$)</th>
<th>ADHD ($n = 20$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>30.4 ± 10.9</td>
<td>30.8 ± 10.9</td>
</tr>
<tr>
<td>Sex</td>
<td>M/F</td>
<td></td>
</tr>
<tr>
<td>SNP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs28386840</td>
<td>A/T</td>
<td></td>
</tr>
<tr>
<td>rs2242446</td>
<td>T/C</td>
<td></td>
</tr>
<tr>
<td>rs15534</td>
<td>C/T</td>
<td></td>
</tr>
<tr>
<td>rs40615</td>
<td>T/A</td>
<td></td>
</tr>
<tr>
<td>CAARS Total score</td>
<td>0.32 ± 0.82*</td>
<td>37.45 ± 8.23*</td>
</tr>
<tr>
<td>CAARS Inattention</td>
<td>0.11 ± 0.32*</td>
<td>18 ± 4.78*</td>
</tr>
</tbody>
</table>

Significant differences between groups are indicated with * at $P < 0.001$. Genotype frequencies are shown for major/minor allele.
For rs2242446, three-way interaction was detected among ROI, group, and genotype ($F_{2,90} = 103.57$, $P = 0.003$, $P < 0.05$, corrected). Based on different ROIs, the analysis demonstrated an interaction between group and genotype in the thalamus ($F_{10.05} = 33.90$, $P = 0.003$, $P < 0.05$, corrected). Post hoc $t$-test revealed that ADHD subjects had higher binding for the major allele (T) ($t = -3.0$, $P = 0.008$, $P < 0.05$, corrected) than controls and no difference was detected for minor allele (C) between groups (Table III and Fig. 2b). Which is likely due to difference in HCs between major and minor allele groups, which did not survive corrections ($t = -2.54$, $P = 0.022$, $P > 0.05$, corrected).

A three-way significant interaction was detected among rs15534 genotype, group, and ROIs ($F_{2.75} = 117.52$, $P = 0.004$, $P < 0.05$, corrected). After separating the analysis by each ROI to determine where the difference was, an interaction was detected between rs15534 genotypes and group in the cerebellum ($F_{7.75} = 35.63$, $P = 0.009$, $P < 0.05$, corrected). Post hoc $t$-test revealed that controls carrying the major allele (C) in rs15534 had higher binding compared to major allele carrying patients ($t = 3.19$, $P = 0.004$, $P < 0.05$, corrected) (Table IV and Fig. 3a). No difference was detected between minor allele (T) groups, and a trend between patients was detected between major and minor allele groups ($t = 2.09$, $P = 0.051$) and between minor and major allele in HCs ($t = 1.80$, $P = 0.088$).

For the SNP rs40615, a three-way interaction was also observed between genotypes, ROI and group ($F_{2.65} = 108.78$, $P = 0.006$, $P < 0.05$, corrected). Further analysis demonstrated an interaction in the cerebellum between genotypes and status ($F_{8.94} = 35.41$, $P = 0.005$, $P < 0.05$, corrected).

### TABLE II. Linear mixed model effects summary for the SNP rs28386840

<table>
<thead>
<tr>
<th>Fixed effects</th>
<th>Model rs28386840</th>
<th>df</th>
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<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
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<td>12.78</td>
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</tr>
<tr>
<td>Group</td>
<td></td>
<td>26.34</td>
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<td>0.41</td>
</tr>
<tr>
<td>ROI</td>
<td></td>
<td>83.66</td>
<td>195.59</td>
<td>&lt;.000</td>
</tr>
<tr>
<td>rs28386840</td>
<td></td>
<td>39.97</td>
<td>1.77</td>
<td>0.19</td>
</tr>
<tr>
<td>group<em>ROI</em>rs28386840</td>
<td></td>
<td>104.60</td>
<td>3.08</td>
<td>0.002</td>
</tr>
</tbody>
</table>

Separated by ROI

- Cerebellum group*rs28386840 | 33.27 | 0.74 | 0.20 |
- Midbrain group*rs28386840  | 34.31 | 4.10 | 0.049 |
- Putamen group*rs28386840   | 32.97 | 1.16 | 0.29 |
- Thalamus group*rs28386840  | 34.87 | 11.16 | 0.002 |

Values given are degrees of freedom (df), $F$ values, and $P$ values.

For the functional promoter SNP (rs28386840) a significant three-way interaction was detected between ROI, status and genotype ($F_{3,08} = 104.6$, $P = 0.002$, $P < 0.05$, corrected). On a ROI-based level, a further analysis detected an interaction between status and genotype in the thalamus ($F_{11.16} = 34.87$, $P = 0.002$, $P < 0.05$, corrected). Post hoc $t$-tests revealed that ADHD subjects had higher NET BPND than controls for the major allele (A) ($t = -3.0$, $P = 0.006$, $P < 0.05$, corrected) and no difference was detected for the minor allele (T) between groups ($t = 0.73$, $P > 0.05$). This is likely due to the difference in HCs between major and minor allele groups, with major allele having lower binding than the minor allele group ($t = -3.06$, $P = 0.007$, $P < 0.05$, corrected) (Table II and Fig. 2a).

![Figure 2](image-url)

**Figure 2.**

Differences in NET BPND in the thalamus between alleles rs28386840 (−3081 A/T) (a) and rs2242446 (−182 T/C) (b). The white bars depict the major alleles while the gray ones depict the minor alleles. Major allele (A) carriers for rs28386840 were (n = 9) for HCs and (n = 14) for ADHD subjects. Minor allele (T) carriers for rs28386840 were (n = 10) for controls and (n = 6) for ADHD subjects. Major allele (T) carriers for rs2242446 were (n = 9) for HCs and (n = 9) for ADHD subjects. Minor allele (T) carriers for rs2242446 were (n = 12) for controls and (n = 8) for ADHD subjects. Error bars indicate 95% confidence interval. Difference between groups is marked with an * in which the difference is at $P < 0.05$ corrected level significance.
Here, we report the influence of genetic variants within the NE system and its effect on in vivo NET binding using PET and the radioligand (5,5-[18F]FMeNER-D2. Our results showed significant differences in cerebellar and thalamic NET binding dependent on genotypes between patients with ADHD and HCs. These were largely due to the impact of NET gene polymorphisms on NET BPND in HCs which is not as pronounced in patients with ADHD. Strikingly, in patients with ADHD, a high correlation between specific behavioural symptoms, that is, hyperactivity/impulsivity, and NET BPND in the cerebellum was detected, an effect which was strongly moderated by genotype.

Our results for the functional promoter SNP (rs28386840) deviate from in vitro experiments which found that the minor (T) allele resulted in decreased promoter activity and the major allele (A) in higher expression [Kim et al., 2006a]. We detected high NET binding for the minor (T) allele carriers which, indicating high expression of this allele. However, a possible reason for this opposite effect are changes in gene expression based epigenetic mechanisms. The T allele was found to bind to transcriptional repressors, slug, and scratch, which result in decreased expression [Kim et al., 2006b]. Slug recruits a corepressor, which in turn recruits histone deacetylase (HDAC) [Shirley et al., 2010] resulting in tighter packing

### DISCUSSION

To test whether these effects were associated with specific ADHD symptoms, scores from CAARS-Inattentiveness and CAARS hyperactivity/impulsiveness were tested between genotype groups and NET BPND. No correlation of symptoms scores with NET binding was detected in any region in patient groups separated by SNPs rs28386840 and rs2242446 with any region. Conversely, a significant correlation was detected between the behavioral subscales CAARS hyperactivity/impulsiveness ($P < 0.05$) and CAARS total score ($P < 0.05$) with NET BPND in the cerebellum depending on genotype for rs15534 and rs40615. For the major allele in rs15534, CAARS hyperactivity/impulsivity was positively associated with NET BPND ($r = 0.664, P = 0.026$). For the minor allele group, the scale was negatively associated with NET BPND ($r = -0.729, P = 0.026$) (Fig. 4a). For the CAARS total score, in the major allele group the positive correlation was $r = 0.772, P = 0.005$ (Fig. 5a). No association was detected for the minor allele. For rs40615, differential association between CAARS hyperactivity/impulsivity was also detected depending on genotype. Depending on the major allele, the positive association detected was $r = 0.689 (P = 0.028)$. On the contrary, the negative association for the minor allele was $r = -0.669 (P = 0.034)$ (Fig. 4b). For the CAARS total score, the positive association with NET BPND was $r = 0.825 (P = 0.003)$ in the major allele group (Fig. 5b). The association for the minor allele did not reach significance.

In addition, for the minor allele group, a negative correlation was detected between NET BPND in the midbrain with CAARS hyperactivity/impulsivity ($r = -0.831, P = 0.006$, rs15534) and ($r = -0.877, P = 0.001$, rs40615).

### Effects of NET Gene Variants

<table>
<thead>
<tr>
<th></th>
<th>Model rs2242446</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
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</tr>
<tr>
<td>group</td>
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<td>ROI</td>
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</tr>
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<td>0.003</td>
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<td>Separated by ROI</td>
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<td></td>
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<tr>
<td>Cerebellum group*rs2242446</td>
<td>31.50</td>
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<td>0.46</td>
</tr>
<tr>
<td>Midbrain group*rs2242446</td>
<td>34.66</td>
<td>6.47</td>
<td>0.026</td>
</tr>
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</tr>
<tr>
<td>Thalamus group*rs2242446</td>
<td>33.90</td>
<td>10.05</td>
<td>0.003</td>
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</table>

Values given are degrees of freedom (df), $F$ values, and $P$ values

$P < 0.05$, corrected). Post hoc $t$-test revealed that controls carrying the major allele (T) in rs40615 had higher binding compared to major allele carrying patients ($t = 3.53$, $P = 0.002$, $P < 0.05$, corrected) (Table V and Fig. 3b). No difference was detected between minor allele (A) groups, nor between genotypes in patients and HCs ($P > 0.05$).

Mean NET BPND of controls and ADHD group depending on genotype grouping is listed in Table VI. In this relatively small sample, no significant associations between SNPs and ADHD were detected ($P > 0.05$). Highest significance was reached with the rs28386840 SNP for the A allele with a $P$ value of 0.09.

### Behavioral Correlation

| | Model rs15534 |  |
|---|---|---|---|
| Intercept | 16.92 | 733.19 | <0.000 |
| group | 33.25 | 0.29 | 0.59 |
| ROI | 88.09 | 194.08 | <0.000 |
| rs15534 | 51.62 | 0.61 | 0.44 |
| group*ROI*rs15534 | 117.52 | 2.75 | 0.004 |
| Separated by ROI |  |
| Cerebellum group*rs15534 | 35.63 | 7.73 | 0.009 |
| Midbrain group*rs15534 | 35.97 | 0.48 | 0.56 |
| Putamen group*rs15534 | 35.62 | 2.78 | 0.89 |
| Thalamus group*rs15534 | 33.49 | 0.95 | 0.34 |

Values given are degrees of freedom (df), $F$ values, and $P$ values.

<p>| | | | |</p>
<table>
<thead>
<tr>
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</tr>
</thead>
<tbody>
<tr>
<td>TABLE III. Linear mixed model effects summary for the SNP rs2242446</td>
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<td></td>
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<td>Fixed effects</td>
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<tr>
<td>Intercept</td>
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<td>930.46</td>
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<td>group</td>
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<td>0.52</td>
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<td>ROI</td>
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<td>0.21</td>
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<td>103.57</td>
<td>2.90</td>
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<td>Separated by ROI</td>
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<tr>
<td>Cerebellum group*rs2242446</td>
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<td>0.57</td>
<td>0.46</td>
</tr>
<tr>
<td>Midbrain group*rs2242446</td>
<td>34.66</td>
<td>6.47</td>
<td>0.026</td>
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<tr>
<td>Putamen group*rs2242446</td>
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<td>1.41</td>
<td>0.24</td>
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<tr>
<td>Thalamus group*rs2242446</td>
<td>33.90</td>
<td>10.05</td>
<td>0.003</td>
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</table>

Values given are degrees of freedom (df), $F$ values, and $P$ values.

<p>| | | | |</p>
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<td>TABLE IV. Linear mixed model effects summary for the SNP rs15534</td>
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<td></td>
<td></td>
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<tr>
<td>Fixed effects</td>
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<td>$F$ value</td>
<td>$P$ value</td>
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<td>Intercept</td>
<td>16.92</td>
<td>733.19</td>
<td>&lt;0.000</td>
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<td>group</td>
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<td>0.59</td>
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<tr>
<td>ROI</td>
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<td>0.95</td>
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</tr>
</tbody>
</table>

Values given are degrees of freedom (df), $F$ values, and $P$ values.
of the DNA of thus lower transcription of the gene. A counteraction of a repressor, such as degradation, inactivation by interaction with other elements, such as small interfering RNAs or HDAC inhibitors, could lead to overexpression and thus result in a reversed effect of the polymorphism in vivo as observed in our findings [Prelich, 2012; Tuschl, 2001]. Further research is needed to determine the functional effect of the major (A) allele. No significant difference was detected between major and minor allele in ADHD subjects indicating that this effect is not pronounced in ADHD. Moreover, Kim et al. [2006b] found the T allele to be overtransmitted in ADHD, and thus concluded it to be a risk allele for ADHD. Even though no SNP reached significance for association to ADHD in our sample, the strongest effect was seen for the SNP rs28386840 with the A as the associative allele. This is compatible with a recent study by Hohmann et al. [2015] which reports homozygotic A allele carriers to have a higher rate of lifetime ADHD diagnosis. Additionally, another study reported higher response times for ADHD subjects carrying the A allele [Kim et al., 2013]. Nonetheless, one has to bear in mind that studies have been inconsistent, possibly due to confounding factors, such as medication history, individual differences, comorbidities, and differences in sample sizes [de Zubicaray et al., 2008; Leo and Cohen, 2003].

The SNP rs2242446, first determined by Zill et al. [2002] also showed this similar binding in the thalamus as for the functional promoter SNP. This SNP is located on the 5' flanking region of the NET and the functional effects of the 5' flanking region is crucial in transcription regulation [Kim et al., 1999; Meyer et al., 1998]. It has been implicated in antidepressant response to milnacipran in depressed subjects. Yoshida et al. [2004] found that major allele (T) carriers responded better to the treatment than the minor (C) allele. The comorbidity of ADHD and depression ranges from 5% to 40%. Symptoms of depression often overlap with those in ADHD, such as distractibility, poor concentration, and impulsivity [Goodman and Thase, 2009; McIntosh et al., 2009]. In addition to the antidepressant effect of milnacipran, it has also been demonstrated to alleviate symptoms of inattention and impulsivity [Hiraide et al., 2013; Kako et al., 2007]. Here, the major allele carrier ADHD group had higher binding than the major allele HC carriers. Thus, the major allele may lend support to the

**Table V. Linear mixed model effects summary for the SNP rs40615**

<table>
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</thead>
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<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>group<em>ROI</em>rs40615</td>
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</tr>
<tr>
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</tr>
<tr>
<td>Midbrain group*rs40615</td>
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<td>Putamen group*rs40615</td>
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<tr>
<td>Thalamus group*rs40615</td>
<td>34.97</td>
</tr>
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</table>

Values given are degrees of freedom (df), F values, and P values.
use of milnacipran to treat ADHD patients with comorbid depression.

Noticeable, our major novel findings was the inverse effect of genotype on NET BP ND between controls and patients for rs15534 and rs40615. Even more intriguingly, we detected correlation of scores on CAARS Hyperactivity/Impulsivity and CAARS total score scales with the NET BPND in the cerebellum for ADHD subject which was strongly modulated by genotype. For both SNPs, in patients carrying the major allele higher NET availability was associated with higher symptom scores. On the contrary, as NET availability decreased, scores increased for the minor allele. From a pharmacological point of view, these findings can only explain the higher NET binding found for the minor allele as it reflects lower NE levels. However, further influencing factors remain unclear. These results resemble the classic inverted U-shaped effect as Aston–Jones and associates (1999) established for interaction of LC and NE on task performance. The hypothesis states that ADHD symptoms are due to increased tonic activity in the LC which in turn inhibits the basal activity in the cerebellum and other areas and thus increases motor hyperactivity and impulsivity [Berridge and Waterhouse, 2003; Howells et al., 2012].

Aston–Jones and associates research on monkeys showed that with increased tonic discharge of the LC, phasic activity of LC neurons is decreased. This results in poorer performance on focusing on task stimuli. Moreover, they state that for optimal

![TABLE VI. Rounded mean ± SD for NET BPND values in selected ROIs, shown depending on genotype (major/minor allele) in patients with ADHD and controls](image)

<table>
<thead>
<tr>
<th>ROI</th>
<th>rs28386840</th>
<th>rs2242446</th>
<th>rs15534</th>
<th>rs40615</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>A/T</td>
<td>T/C</td>
<td>C/T</td>
<td>T/A</td>
</tr>
<tr>
<td>Putamen</td>
<td>0.16 ± 0.04/0.18 ± 0.04</td>
<td>0.16 ± 0.04/0.19 ± 0.05</td>
<td>0.18 ± 0.04/0.21 ± 0.03</td>
<td>0.19 ± 0.04/0.16 ± 0.02</td>
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<tr>
<td>Midbrain/pons</td>
<td>0.22 ± 0.08/0.30 ± 0.12</td>
<td>0.22 ± 0.08/0.29 ± 0.12</td>
<td>0.26 ± 0.10/0.26 ± 0.11</td>
<td>0.27 ± 0.10/0.25 ± 0.11</td>
</tr>
<tr>
<td>Thalamus</td>
<td>0.39 ± 0.07/0.52 ± 0.12</td>
<td>0.39 ± 0.07/0.52 ± 0.13</td>
<td>0.45 ± 0.13/0.48 ± 0.11</td>
<td>0.45 ± 0.13/0.48 ± 0.10</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>0.20 ± 0.06/0.26 ± 0.06</td>
<td>0.20 ± 0.06/0.26 ± 0.07</td>
<td>0.24 ± 0.07/0.21 ± 0.06</td>
<td>0.25 ± 0.06/0.20 ± 0.06</td>
</tr>
</tbody>
</table>

**ADHD**

<table>
<thead>
<tr>
<th>ROI</th>
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<th>rs2242446</th>
<th>rs15534</th>
<th>rs40615</th>
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<tbody>
<tr>
<td></td>
<td>A/T</td>
<td>T/C</td>
<td>C/T</td>
<td>T/A</td>
</tr>
<tr>
<td>Putamen</td>
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</tr>
<tr>
<td>Midbrain/pons</td>
<td>0.24 ± 0.09/0.22 ± 0.12</td>
<td>0.25 ± 0.10/0.22 ± 0.10</td>
<td>0.25 ± 0.10/0.22 ± 0.11</td>
<td>0.26 ± 0.10/0.22 ± 0.11</td>
</tr>
<tr>
<td>Thalamus</td>
<td>0.48 ± 0.08/0.48 ± 0.03</td>
<td>0.48 ± 0.08/0.47 ± 0.04</td>
<td>0.49 ± 0.05/0.47 ± 0.08</td>
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</tr>
<tr>
<td>Cerebellum</td>
<td>0.20 ± 0.09/0.21 ± 0.09</td>
<td>0.20 ± 0.09/0.21 ± 0.09</td>
<td>0.17 ± 0.06/0.24 ± 0.10</td>
<td>0.17 ± 0.06/0.23 ± 0.10</td>
</tr>
</tbody>
</table>

Association between NET BPND in the cerebellum and the CAARS hyperactive/impulsive scale depending on genotype in rs15534 (T/C) (a) and rs40615 (A/T) (b). The scatter plot shows the correlation split by major (white circles) and minor (gray circles) alleles in ADHD subjects only. The significance for the SNP rs15534, depending on major allele C was \( P = 0.026 \), and for minor allele T, \( P = 0.026 \). For the SNP rs40615, depending on major allele T, \( P = 0.028 \), for minor allele A, \( P = 0.034 \).

![Figure 4.](image)

**Figure 4.**

Association between NET BPND in the cerebellum and the CAARS hyperactive/impulsive scale depending on genotype in rs15534 (T/C) (a) and rs40615 (A/T) (b). The scatter plot shows the correlation split by major (white circles) and minor (gray circles) alleles in ADHD subjects only. The significance for the SNP rs15534, depending on major allele C was \( P = 0.026 \), and for minor allele T, \( P = 0.026 \). For the SNP rs40615, depending on major allele T, \( P = 0.028 \), for minor allele A, \( P = 0.034 \).
performance, balanced levels of tonic, and phasic activity are needed. If the levels of tonic discharge are too low or too high, attentional performance suffers vastly [Aston-Jones et al., 1999]. Extracellular levels of NE have been demonstrated to follow a positive linear relationship with tonic discharge from the LC [Berridge and Abercrombie, 1999; Florin-Lechner et al., 1996]. With that in mind, for the major allele carriers, the tonic release may be too high as NET binding is lower indicating high levels of extracellular NE. This inverted-U relationship between tonic activity and task performance may explain why we detected this inverse genotype effect. The level of this inverse effect is, therefore, likely determined by the genotype. Along these lines, studies indicate a region specific LC stimulation and LC–NE effect. The LC effect may differ in terms of interaction with receptor subtypes and sensitivity as well as for NE concentration in that region [Berridge and Waterhouse, 2003; Devilbiss et al., 2006]. A study done on healthy rats revealed that tonic stimulation of the LC had differential effects on cortical cells versus cells in the thalamus [Devilbiss and Waterhouse, 2004]. Another study revealed projections to the PFC and the motor cortex to differ [Chandler et al., 2014]. They revealed that neurons projecting from LC to the PFC show more spontaneous activity and are more excitable than those projecting to the motor cortex. The LC might have a differential effect on the thalamus and the cerebellum and this may explain why we detected opposite binding for the major alleles on SNPs located in the 5′UTR versus those in the 3′UTR region.

Another explanation involves the location of these SNPs. They are located in the 5′UTR and 3′UTR regions which have been demonstrated to play an important part in translation, stability and localization of the mRNA. The SNPs r28386840 and rs2242446 are located within the promoter regions while rs40615 and rs15534 are located downstream at the termination codon. The NET may be deregulated by changes in gene expression, mRNA translation or stability, post-translational modifications such as phosphorylation, protein trafficking, cytoskeleton interaction, and oligomerization [Chatterjee and Pal, 2009]. Substrates involved in the aforementioned processes have also shown to have a regulatory effect on the NET. An injection of the enzyme inhibitor α-methyl-p-tyrosine (AMPT), resulted in around 50% reduced levels of NE as well as lower mRNA levels in the brainstem indicating a compensatory mechanism for reduced extracellular NE levels [Xiao et al., 1995]. Furthermore, activation of protein kinase C (PKC) has also been shown to regulate the NET. Activation of PKC is believed to result in redistribution of surface NET as radioligand binding demonstrated a reduction in $B_{\text{MAX}}$ to NET without any change to $K_D$ [Apparsundaram et al., 1998]. Different location of the SNPs and function may explain why we only detected differences for one allele and why we detected opposite binding on major alleles between SNPs in the 5′UTR and the SNPs in the 3′UTR.

**Limitations and Future Directions**

A limitation of this study is that with (S,S)-[18F]FMeNER-D2 cortical areas cannot be properly assessed due to spill over from suspected bone uptake. Therefore, genetic influence in the neocortex could not be examined. Studying the cortex, specifically the frontal cortex due to its vast role in cognitive and behavioral control would be very intriguing to test whether and what effects polymorphisms in the NET gene would have on the binding. Another limitation is that we did not include the CAARS inconsistency index and, therefore, we could not assess whether there was any inconsistencies or irregularities in

**Figure 5.**

Association between NET $B_{\text{PETD}}$ in the cerebellum and the CAARS total score depending on genotype in rs15534 (*269 T/C) (a) and rs40615 (*417 A/T) (b). The scatter plot shows the correlation split by major (white circles) and minor (gray circles) alleles in ADHD subjects only. Significance was only found depending on the major alleles, for rs15534 (C, $P = 0.005$) and for rs40615 (T, $P = 0.003$).
The evidence presented in this article gives rise to a role for genetic influence of the NE system and alterations in NE signalling as a part of the pathophysiology contributing to ADHD and thus strengthening the hypothesis of imbalances in NE system in the neurobiological mechanism of ADHD. However, we did not detect any differences in the midbrain and the putamen. We can only hypothesize about the possible reasons for these distinct effects. Our results may suggest that the NET genotype effects modulate the NET availability in a region specific manner. Conversely, we cannot exclude other possibilities, such as other influential genetic or nongenetic factors that interact with these regions. In addition, we did not detect any association of any SNP to ADHD, which is probably due to insufficient power of this sample to assess these subtle effects. Due to the heterogeneous nature of ADHD further research requires larger samples for the validation and for the establishment of potential endophenotypes in ADHD. Replication in vivo and in vitro studies is needed to establish if NET genotypic influence could serve as an endophenotype for NE neurotransmission. Moreover, SNP within the NET may also be very important in relation to the dopaminergic system. The NET is also responsible for reuptake of dopamine in cortical regions [Morón et al., 2002], and therefore, SNPs could possibly affect the availability of dopamine within the cortex. Future studies could explore the possibility whether there are any effects of the NET on the dopaminergic system.

To conclude, this is the first imaging genetic study showing significant differences in NET BPND in patients with ADHD compared to HCs, depending on their genotype. We find genotypic difference in the thalamus between major and minor alleles for a functional promoter SNP in HCs only. The inverse effect of genotype which was detected in the cerebellum indicates genetic influence of NET on the binding in the cerebellum to differ between groups of ADHD subjects and HCs. The results are compatible with the theory that NE follows an inverted-U-shaped curve. Its effect on differential association of behavioral scales with binding further demonstrates a functional and neuropsychological activity to be imbalanced in ADHD.

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Altered Interregional Molecular Associations of the Serotonin Transporter in Attention Deficit/Hyperactivity Disorder Assessed with PET

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Abstract: Altered serotonergic neurotransmission has been found to cause impulsive and aggressive behavior, as well as increased motor activity, all exemplifying key symptoms of ADHD. The main objectives of this positron emission tomography (PET) study were to investigate the serotonin transporter binding potential (SERT BPND) in patients with ADHD and to assess associations of SERT BPND between the brain regions. 25 medication-free patients with ADHD (age ± SD; 32.39 ± 10.15; 10 females) without any psychiatric comorbidity and 25 age and sex matched healthy control subjects (33.74 ± 10.20) were measured once with PET and the highly selective and specific radioligand [11C]DASB. SERT BPND maps in nine a priori defined ROIs exhibiting high SERT binding were compared between groups by means of a linear mixed model. Finally, adopted from structural and functional connectivity analyses, we performed correlational analyses using regional SERT binding potentials to examine molecular interregional associations between all selected ROIs. We observed significant differences in the interregional correlations between the precuneus and the hippocampus in patients with ADHD compared to healthy controls, using SERT BPND of the investigated ROIs (P < 0.05; Bonferroni corrected). When correlating SERT BPND and age in the ADHD and the healthy control group, we confirmed an age-related decline in brain SERT binding in the thalamus and insula (R² = 0.284, R² = 0.167, Ps < 0.05; Bonferroni corrected). The results show significantly different interregional molecular associations of the SERT expression for the precuneus with hippocampus in patients with ADHD, indicating presumably altered functional coupling. Altered interregional coupling between brain regions might be a sensitive approach to demonstrate functional and molecular alterations in psychiatric conditions. Hum Brain Mapp 00:000–000, 2016.

Key words: neuroimaging; ADHD; positron emission tomography; PET; serotonin; SERT; interregional molecular associations

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Attention deficit hyperactivity disorder (ADHD) is characterized by inappropriate inattention, hyperactivity, impulsive behaviour and emotional dysregulation [American Psychiatric Association, 2013; Rosler et al., 2010], as well as by a certain constellation of deficits in executive functions. ADHD is considered to be the most prevalent neurodevelopmental disorder, prevalence rates are estimated to range between 8% and 12% in childhood [Biederman and Faraone, 2005]. In about 30% of children diagnosed with ADHD [Barbaresi et al., 2013], especially inattentive symptoms persist into adulthood.

Frequently prescribed stimulant and non-stimulant psychopharmacological treatment for patients with ADHD are suggested to unfold efficacy through modulation of dopaminergic (DA) and norepinephrinergic neurotransmission in cortical and subcortical brain circuits and improvement of neurocognitive deficits [Castells et al., 2011; Chamberlain et al., 2009; Retz et al., 2011]. Although the serotoninergic system is not a direct target for ADHD medication, evidence from pharmacological, genetic and animal studies suggest an involvement of the serotonergic neurotransmission in the neurobiological mechanisms of ADHD [for review see (Banerjee and Nandagopal, 2015)].

Although methylphenidate does not inhibit the serotonin transporter, amphetamines enhance serotoninergic release [Bymaster et al., 2002; Kuczenski and Segal, 1997]. A recently published positron emission tomography (PET) animal study found that atomoxetine applied at clinical dosage blocks the norepinephrine transporter as well as the serotonin transporter (SERT) [Ding et al., 2014] and atomoxetine has been shown to significantly alleviate symptoms in adult ADHD patients [Adler et al., 2009]. This has led some researchers to suggest that serotoninergic transmission might also be of relevance to ADHD treatment and neuropathology [Gainetdinov et al., 1999].

Several lines of evidence suggest that serotonin is involved in impulsive behaviour and extensive motor activity [Dalley and Roiser, 2012; Winstanley et al., 2006]. Serotonergic neurons in the medial and dorsal raphe project into the striatum, ventral tegmental area and nucleus accumbens as well as into the amygdala, hippocampus and the frontal cortex [ Muller and Jacobs, 2009]. Serotonin regulates dopaminergic neurotransmission via projections to the dopaminergic neurons in the midbrain and neuronal interactions between these neurotransmitters are found to profoundly modulate impulsive behaviour [Oades, 2008; Wood and Wren, 2008]. Furthermore, a deficit to withhold attention for an adequate time, related to a specific context, can lead to emotional dysregulation, a symptom of ADHD that affects patients markedly throughout lifetime. Brain regions implicated in emotional dysregulation comprise the striatum, amygdala and the medial prefrontal cortex, regions that are strongly modulated by serotonergic neurotransmission [Shaw et al., 2014].

Neuroimaging studies have been demonstrating that patients with ADHD display altered neural activation for inhibition and attention in frontal, parietal and thalamic brain regions as well as in the basal ganglia [Aron and Poldack, 2005; Hart et al., 2013]. In comparison to healthy control subjects (HC), the administration of fluoxetine, a selective serotonin reuptake inhibitor, prior to functional magnetic resonance imaging (fMRI) measurements, has been shown to normalize neuronal activation during a stop signal task measuring motor inhibition in the orbitofrontal cortex and in the basal ganglia in 18 patients with ADHD [Chantiluke et al., 2015]. Fluoxetine, as well as its metabolite norfluoxetine, also binds to the norepinephrine transporter, although to a far lesser extent [Wong et al., 1993]. In addition, Fluoxetine has been found to be effective to improve attention and alleviate hyperactivity in children with ADHD and non-bipolar comorbid mood-disorders [Barrickman et al., 1991; Quintana et al., 2007].

With a remarkable heritability estimated to be 77% [Faraone et al., 2005], ADHD exemplifies a spectrum disorder with behavioural and personality traits, which underlie a combination and an interaction of genetic and environmental factors [Fliers et al., 2012]. The gene encoding the serotonin transporter (SERT; SLC6A4) as well as the genes encoding certain serotonergic receptors comprise various single nucleotide polymorphisms that have been examined in ADHD and other neuropsychiatric disorders and were found to be influencing the susceptibility to ADHD [Faraone and Khan, 2006; van der Meer et al., 2014]. Thus, the SERT gene is alleged to play a main role in ADHD pathogenesis.

Studies applying PET or single photon emission tomography (SPECT) in adult patients with ADHD have explored glucose, blood flow metabolismand [for review see (Zimmer, 2009)] and especially the dopaminergic and noradrenergic neurotransmitter systems. Dysfunctional dopaminergic signaling, including investigations on the dopamine transporter [Fusar-Poli et al., 2012] and dopamine receptors [del Campo et al., 2013; Volkow et al., 2009], has been identified in different brain regions, though results remain inconsistent. In a recently published PET study, we found no difference in norepinephrine transporter in subcortical regions between patients with ADHD and HC [Vanicek et al., 2014]. A PET study has investigated serotonin transporter binding potential (SERT BP\textsubscript{ND}) in patients with ADHD [Karlsson et al., 2013], using \textsuperscript{[11]}C\textsuperscript{MADAM}, which is a frequently used tracer for estimating brain SERT levels. The results depict no

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**Abbreviations**

| AAL | Automated anatomical labelling |
| ADHD | Attention deficit hyperactivity disorder |
| HC | Healthy control subjects |
| PET | Positron emission tomography |
| SERT | Serotonin Transporter |
| SPECT | Single photon emission tomography |
differences compared to HC. However, the findings are preliminary, since the sample size is too small to exemplify reasonable size for power analysis and the tracer. As mentioned above, evidence from behavioral, neuroimaging and genetic studies suggest an involvement of the serotonergic system in ADHD. The SERT terminates serotonin from the synaptic cleft, therefore withholding a pivotal role in the regulation of serotonergic signaling. The SERT BP$_{ND}$ has been investigated in the past with [11C]DASB and PET in various neuropsychiatric disorders [Spies et al., 2015].

In the last decades neuroimaging investigations have begun to change the conceptual focus from activation paradigms towards connectivity analysis, from univariate, where activation in cue-related regions is explored, to multivariate analysis, where correlations of activation across brain regions are evaluated [Bullmore, 2012]. To disclose a possible involvement of a specific neurotransmitter system in neuropsychiatric disorders, PET imaging has predominantly been used to observe regional availability of a particular transporter or receptor in a specific brain region. Though, through performing interregional correlation analyses, PET imaging has also been applied to explore brain connectivity in HC, major depressive disorder, autism and obsessive-compulsive disorder, Alzheimer’s disease and epilepsy [Baldinger et al., 2014; Horwitz et al., 1984; Lee et al., 2008; Morbelli et al., 2013; Vanicek et al., 2016].

The serotonergic system represents one of the chief modulatory neurotransmitter systems in the human brain, where neurons from the raphe nuclei innervate nearly all cortical regions and several subcortical structures. Therefore, serotonin is associated with almost all emotional and cognitive functions. Since the SERT expression is modified via available and released serotonin [Benmansour et al., 2002], investigations on the relation of SERT expression between different brain regions may exemplify a valuable method to understand the function on a more global level of this neurotransmitter system. Studies from our group showed that molecular associations of the serotonergic neurotransmitter system (serotonin-1A receptor and SERT) differed between depressive patients and HC [Baldinger et al., 2014; Hahn et al., 2014; Lanzenberger et al., 2012], implicating that interregional molecular correlation analyses is a promising method to generate more insight to the complexity of neurotransmitter systems and their role in neuronal pathophysiology.

Therefore, we applied [11C]DASB and PET to assess SERT BP$_{ND}$ in SERT rich regions to observe differences in SERT availability between adult patients with ADHD and HC. Furthermore, we performed a correlational analysis, to examine interregional association of SERT binding as an index for interregional molecular balance of serotonergic neurotransmission. We hypothesized that SERT BP$_{ND}$ and interregional molecular associations of SERT availability across brain areas will reflect a characteristic pattern that differs between patients with ADHD and HC.

**METHODS**

**Subjects**

Twenty-five adult patients with ADHD (age ± SD; 32.39 ± 10.15; 10 females) and 25 age and sex matched HC (aged 33.74 ± 10.20) were recruited through the ADHD outpatient clinic at the Department of Psychiatry and Psychotherapy, Medical University of Vienna and from the local community via advertisement. Patients were free from psychopharmacologic treatment for at least six months prior to the screening visit while HC were naive to all psychopharmacologic treatment. Four patients used methylphenidate in the past, one patient atomoxetine and one antidepressant medication. Written informed consent was obtained from all participants after detailed explanation of the study protocol and subjects received financial reimbursement for their participation. This study was approved by the Ethics Committee of the Medical University of Vienna and the General Hospital of Vienna (EK 552/2010).

**Medical Examination and Clinical Exploration**

Subjects underwent standard medical examination including a general physical and neurological status, electrocardiography and routine laboratory tests at the screening- and final visit in order to ensure physical health. Female participants underwent a urine-pregnancy test at the screening visit and prior to PET measurement. A multidrug-urine test was performed at the screening visit in order to exclude current substance abuse. Participants were interviewed by experienced psychiatrists using Conners’ Adult ADHD Diagnostic Interview for DSM IV (CAADID, Conners 1999) to evaluate current and childhood attentional and hyperactivity/impulsivity symptoms and to attest ADHD diagnosis. (ADHD: impulsive symptoms: 20.05 ± 4.34 hyperactive symptoms: 20.05 ± 4.42; HC: impulsive symptoms: 0.55 ± 0.92 hyperactive symptoms: 0.35 ± 0.79). For five patients hyperactivity/impulsivity symptoms were not recorded, thus we excluded these patients and their matched HC from this analysis. Structured Clinical Interview for DSM IV Axis I and Axis II disorders (SCID-I, SCID-II) was performed to exclude comorbid psychiatric disorders. Smoking status was recorded and subjects were subdivided into groups best describing their smoking status according to quantity of consumption (non-smokers, five cigarettes/week, five cigarettes/day, five to ten cigarettes/day, ten cigarettes/day, ten to 15 cigarettes/day, 15 cigarettes/day and 20 cigarettes/day; ranks 1-8, respectively). ADHD patients did not significantly differ in smoking status compared to HC (Mann-Whitney U = 161.5, Z = −1.25, P = 0.30). Subjects with PET- or MRI-incompatible implants or in pregnancy or breastfeeding were also excluded.

**Data Acquisition**

All PET scans were carried out at the Dept of Biomedical Imaging and Image-guided Therapy, Division of
Data was analyzed using linear mixed models for the outcome measure SERT BP\textsubscript{ND} with group, sex, and ROI as fixed factors, with ROI as repeated factor, and subjects and matched participant pairs as random factors. Fixed effects were included in the model in a multifactorial approach whereas interaction effects were dropped in case of non-significance. In case of significant interactions or main effects, post-hoc pairwise comparisons were computed and Bonferroni corrected for multiple comparisons. In a second exploratory approach to examine the effects of age and smoking status, a mixed model was calculated using a stepwise procedure with backward elimination, i.e., starting with all candidate variables (including subject groups and ROI) followed by a stepwise deletion of interactions and variables with largest $P$-values. Finally, mixed models using the same procedure were applied to investigate the effects of clinical variables CAARS-inattentiveness and CAARS-hyperactivity/impulsivity. According to Akaike’s information criterion [Akaike, 1974], repeated measurements were modelled using the diagonal structure. SPSS version 19.0 for Windows was used for statistical computations. The two-tailed significance level was set at 0.05.

Interregional molecular association matrices were calculated between each ROI pair using Spearman’s rank correlation coefficient ($\Delta p$) for each group separately. For the assessment of statistically significant differences ($P<0.05$) in balance between patients with ADHD and HC, correlation matrices were transformed using Fisher’s $r$-to-$z$ transformation and a 10,000 fold permutation test was performed. Results were Bonferroni correction for multiple comparisons.

**RESULTS**

Linear mixed models analysis revealed a main effect of ROI ($F_{80,22} = 72.08, \ P < 0.001$) and of subject group ($F_{29,35} = 261.37, \ P < 0.001$; Table 1; Fig. 1), but no main effects for sex ($F_{1,21} = 21.26, \ P = 0.1$) and no interaction effects (all $P > 0.1$). Post-hoc pairwise comparisons revealed significant attenuated SERT BP\textsubscript{ND} in patients with ADHD compared to HC in the striatum ($P = 0.029$; uncorrected) as well as trend in the anterior cingulate cortex and insula ($P = 0.066$ and $P = 0.085$; uncorrected). After applying Bonferroni correction for multiple comparisons, we were not able to detect any significant differences (Table I).

When investigating the potential effects of age, mixed models analysis for ROI SERT BP\textsubscript{ND} based on AAL atlas revealed an interaction effect between ROI and age ($F_{7,51} = 69.79, \ P < 0.002$), in addition to a main effect of ROI main effect of age ($F_{7,15} = 21.26, \ P = 0.014$). Post-hoc
correlation analyses between regional SERT BPND and age revealed negative correlations in the thalamus and insula ($R^2 = 0.284$, $R^2 = 0.167$, $P < 0.05$; Bonferroni corrected; Fig. 2). Furthermore, negative correlations in the anterior cingulate cortex ($R^2 = 0.128$), posterior cingulate cortex ($R^2 = 0.119$) and the precuneus ($R^2 = 0.129$) were detected, however not significant after Bonferroni correction. These correlations did not differ between HC and ADHD patients. Smoking status had no effect on SERT BPND nor did they lead to any significant interactions. Additionally, no main or interaction effects were observed for clinical variables (CAARS-Inattentiveness, CAARS-Hyperactivity/Impulsivity) and SERT BPND.

When comparing interregional SERT BPND correlations between patients with ADHD and HC, we found a significant difference in the correlation of precuneus with amygdala, hippocampus, insula, DRN and ACC, of the hippocampus with insula and ACC as well as of the PCC and the ACC. Only the differences in interregional molecular correlations of precuneus with hippocampus survived Bonferroni correction for multiple comparisons ($P = 0.0324$; see Table II, Figs. 3 and 4).

**DISCUSSION**

In this cross-sectional PET study we aimed to investigate SERT availability in adult, medication free patients with ADHD. When comparing groups, we observed lower SERT availability for all ROIs pooled together in patients with ADHD compared HC. For separate brain regions and after correction for multiple comparisons, results show no significant differences in SERT BPND between patients with ADHD and HC. When comparing interregional SERT BPND correlations between groups, we found a significant increase for interregional SERT BPND correlations of the precuneus with hippocampus. In addition, we observed a negative correlation for SERT BPND and age for patients and HC in the thalamus and the insula.

Previously published PET and SPECT imaging studies found no changes in SERT availability between patients with ADHD and HC [Hesse et al., 2009; Karlsson et al., 2013]. Though, findings are preliminary and should be interpreted with caution, since Karlsson et al. investigated SERT BPND in eight patients with ADHD, a sample size insufficient in power to detect putative differences [Karlsson et al., 2013]. Another study observed SERT availability with $[123I]$FP-CIT, a SPECT radiotracer showing only moderate specificity to the SERT in subcortical regions [Hesse et al., 2009].
We found attenuated SERT binding in patients with ADHD at uncorrected \( P \)-value in the striatum, a region that has been found to exhibit ADHD specific morphological and functional alterations [Plichta et al., 2009; Qiu et al., 2009]. Elevated SERT availability has been shown to be correlated with cognitive performance in the caudate as well as in other brain regions in HC [Madsen et al., 2011] whereas a negative association has been found between SERT binding and impulsive behavior in suicide attempters [Ryding et al., 2006]. Our finding may suggest a contribution of the SERT to the pathophysiology in ADHD, which may be key for impulsive symptoms. Nevertheless, and in line with previous SERT imaging in ADHD, we found no differences in SERT BP\(_{ND}\) after correction for multiple testing in patients with ADHD in comparison to HC.

The findings further demonstrate a decrease of SERT BP\(_{ND}\) with increasing age in the thalamus, insula, precuneus and anterior and posterior cingulate cortex in patients and HC. This validates previous SERT investigations [Hesse et al., 2003; Yamamoto et al., 2002] as well as PET studies observing the noradrenergic transmitter system [Ding et al.,

**TABLE II. Significant differences in interregional molecular correlations of the SERT BP\(_{ND}\)**

<table>
<thead>
<tr>
<th>Region of interest</th>
<th>( \Delta \rho )</th>
<th>( P )-value</th>
<th>( P )-value Bonferroni corrected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precuneus—amygdala</td>
<td>0.3846</td>
<td>0.0206( ^a )</td>
<td>0.7416</td>
</tr>
<tr>
<td>Precuneus—hippocampus</td>
<td>0.74308</td>
<td>0.0009( ^a )</td>
<td>0.0324( ^b )</td>
</tr>
<tr>
<td>Precuneus—insula</td>
<td>0.52231</td>
<td>0.0072( ^a )</td>
<td>0.2592</td>
</tr>
<tr>
<td>Precuneus—dorsal raphe nucleus</td>
<td>0.68</td>
<td>0.0134( ^a )</td>
<td>0.4824</td>
</tr>
<tr>
<td>Precuneus—anterior cingulate cortex</td>
<td>0.50923</td>
<td>0.0077( ^a )</td>
<td>0.2772</td>
</tr>
<tr>
<td>Hippocampus—posterior cingulate cortex</td>
<td>0.42</td>
<td>0.0218( ^a )</td>
<td>0.7848</td>
</tr>
<tr>
<td>Hippocampus—insula</td>
<td>0.30385</td>
<td>0.0243( ^a )</td>
<td>0.8748</td>
</tr>
<tr>
<td>Anterior cingulate cortex—posterior cingulate cortex</td>
<td>0.42769</td>
<td>0.0259( ^a )</td>
<td>0.9324</td>
</tr>
</tbody>
</table>

We observed significant stronger interregional associations of SERT BP\(_{ND}\) between the listed ROIs in patients with ADHD and healthy control subjects (Spearman’s delta rho; \( P < 0.05 \); corrected for multiple comparisons).

\( ^a \) Marks significant differences between patients with ADHD and HC.

\( ^b \) Marks significant differences between patients with ADHD and HC after Bonferroni correction for multiple comparisons.

ADHD: attention deficit/hyperactivity disorder; HC: healthy control subjects; SERT BP\(_{ND}\): serotonin transporter binding potential; ROIs: regions of interest.
Molecular interregional molecular correlations of patients with ADHD and HC. Left map shows the correlation (Spearman’s ρ) of SERT BPND in HC indicating interregional differences in functional coupling. Right map denote the condition in patients with ADHD. The color table represents the strength of interregional associations, red indicates lowest and yellow highest interregional associations. ADHD: attention deficit/hyperactivity disorder, SERT BPND: serotonin transporter binding potential, ROIs: regions of interest. HC: healthy controls. [Color figure can be viewed at wileyonlinelibrary.com]
with depressive symptoms [Posner et al., 2014]. Though other structural MRI investigations have showed inconclusive data in ADHD, depicting higher or no differences in hippocampal volume between patients with ADHD and HC [Castellanos et al., 1996; Plessen et al., 2006]. Imaging and behavioral studies have demonstrated that serotonergic neurotransmission affects impulsive behavior [Dalley and Roiser, 2012], motor planning and sensory perception [Biskup et al., 2016] and modulates the default mode network [Hahn et al., 2010]. Recently, it has been found that, compared to HC, patients with ADHD show elevated functional connectivity of the default mode network and attenuated functional connectivity in a state of diminished brain serotonin levels, evoked through acute tryptophan depletion [Biskup et al., 2016]. We found a higher molecular correlation of the SERT between the precuneus and the hippocampus in patients with ADHD and in general lower correlations in HC [Castellanos et al., 1996; Plessen et al., 2006].

Imaging and behavioral studies have demonstrated that serotonergic neurotransmission affects impulsive behavior [Dalley and Roiser, 2012], motor planning and sensory perception [Biskup et al., 2016] and modulates the default mode network [Hahn et al., 2010]. Recently, it has been found that, compared to HC, patients with ADHD show elevated functional connectivity of the default mode network and attenuated functional connectivity in a state of diminished brain serotonin levels, evoked through acute tryptophan depletion [Biskup et al., 2016]. We found a higher molecular correlation of the SERT between the precuneus and the hippocampus in patients with ADHD and in general lower correlations in HC, which may reflect higher impulsivity in patients and might be explained by a more diverse, region specific modulated serotonergic system in HC and by more rigid and less variable serotonergic signaling in ADHD.

This PET study has limitations that compromise the interpretation of its results. Regarding group differences in regional SERT binding a main effect was observed, but only trends for significant differences were obtained in separate brain regions. Although the sample size of this study is common for investigations with PET [Kranz et al., 2015; Volkow et al., 2007], it is still possible that more subjects are required to identify more subtle differences. On the other hand, the significance in the main effect but not for single ROIs might be driven by a more reliable variance estimate for the former one. Next to the thalamus and the insula, we found an association between age and SERT binding in the anterior cingulate cortex, posterior cingulate cortex and the precuneus, though not significant after applying Bonferroni correction. Previous PET studies found a decline in SERT with age in the raphe nuclei, though, we did not observe an age-related decline in SERT in the dorsal raphe nuclei. The dorsal raphe nuclei is relatively small structures in the mid-brain where signal to noise ratio is rather low. Therefore, it is possible that there is an age-related decline in SERT in this region, although we did not detect an association. In addition, no blood sampling was carried out in this study. This impedes the evaluation of potential differences in the cerebellum, which was however suggested to represent an optimal reference region [Parsey et al., 2006].

**CONCLUSION**

In conclusion, we observed altered interregional SERT BPND correlation of the precuneus and the hippocampus in patients with ADHD, underlining the involvement of these brain areas in the pathophysiology of ADHD. On the other hand, SERT binding does not differ after applying correction for multiple comparisons on a regional level between patients with ADHD and HC. Given the fact that the SERT expression is modulated by regional serotonergic release, our results are compatible with alterations of inter-regional coupling within the serotonergic system in ADHD.

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**DISCLOSURE OF BIOMEDICAL FINANCIAL INTERESTS AND POTENTIAL CONFLICTS OF INTEREST**

All authors declare no competing financial interests in relation to the work described. Without any relevance to this work, M Hacker has received conference speaker honoraria from Covidian, GE Healthcare, IBA and

![Figure 4.](image-url)
Serotonin Transporter in ADHD Revealed by PET

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3. DISCUSSION

3.1. General Discussion

Within the scope of this thesis, I aimed to address fundamental neuroscientific questions by observing molecular structures of the noradrenergic and serotonergic neurotransmitter systems in ADHD. PET studies are cost and labor intensive and are methodologically highly complex. Though, PET imaging is the most sensitive and promising method to gather information on neurotransmitter systems in vivo, which are hypothesized to cause or mediate neuropsychiatric disorders. To shed light on the underlying neurochemical mechanisms I used PET and (S,S)-[18F]FMeNER-D$_2$ or [11C]DASB to quantify NET and SERT BP in patients with ADHD and healthy control subjects.

There is a lack of imaging studies targeting noradrenergic molecules and in particular the NET, which is caused by several methodological issues in producing a reliable and suitable radioligand for the NET (see section 1.5.2.). Since the NET represents a main treatment target for ADHD specific psychopharmacological treatment, expression of the NET in the cell membrane in patients is of high interest. In the first publication, patients with ADHD and matched healthy control subjects were measured once with PET and (S,S)-[18F]FMeNER-D$_2$ (Vanicek et al, 2014). This is the first PET study to explore differences in brain NET binding in ADHD. Based on post mortem and in vivo studies we a priori selected ROIs that show high levels of NET, including subcortical region as the hippocampus, the putamen, the pallidum, the thalamus, the midbrain with pons (stating a ROI which includes the LC), and the cerebellum, in adults with ADHD. We found no significant differences in NET binding between patients and healthy control subjects. Further, we revealed an age-associated decline in NET binding in the healthy human brain as well as in adult patients with ADHD. As previously described (Schou et al, 2004; Takano et al, 2008b), we found highest NET levels in the thalamus and LC and lower levels of NET in the pallidum, putamen, cerebellum and hippocampus. Lines of evidence suggest that noradrenergic signaling in cortical regions, and in particular in the prefrontal cortex, is important for regulating arousal, vigilance and executive functions (Berridge et al, 2006). Though, due to general low levels of NET in cortical brain regions and skull bound radioactivity, which is associated with (S,S)-[18F]FMeNER-D$_2$ and superimposes NET quantification in bordering cortical regions, it is currently not possible to objective NET levels in the neocortex (Ding, 2014; Rami-Mark et al, 2013). In relation to NET findings in different neuropsychiatric disorders, where lower levels were found in obese patients, patients with posttraumatic stress disorder and in Alzheimer’s
disorder and higher levels were found in cocaine dependency (Ding et al, 2010b; Gulyas et al, 2010; Li et al, 2014; Pietrzak et al, 2013), results are diverse and do not point toward an unitary up- or down-regulation in different disorders. Nevertheless, more studies on the NET have to be executed in patients with ADHD to replicate and to underline the non-findings demonstrated in our study, as well as in other neuropsychiatric disorder to gain more knowledge of the noradrenergic transmitter system.

In our second publication, we investigated the effect of genotypes on NET BP_{ND} between groups (Sigurdardottir et al, 2016). Imaging genetics is an approach that is derived from MRI investigations (Meyer-Lindenberg and Weinberger, 2006). This approach also seems suitable for PET data, since genotypes are related to measureable proteins. In our imaging genetics analysis we detected genotype-differences between groups in the thalamus and cerebellum. Furthermore, we demonstrated an effect of genotypes in healthy control subjects, with major allele carriers exhibiting lower NET binding while patients with ADHD had lower levels of NET binding dependent on major allele expression. Moreover, depending on genotype, ADHD specific symptoms as hyperactivity and impulsivity significantly correlated with NET BP_{ND} in the cerebellum. A positive correlation was found between symptoms NET BP_{ND} in the cerebellum for the major allele, whereas a negative correlation was found in minor allele carriers. Though, we did not observe any association of SNPs to ADHD. This is possibly due to insufficient power, caused by a small sample size, which is too small to assess subtle effects. The findings implicate a genetic influence on noradrenergic signaling contributing to ADHD. To establish endophenotypes for ADHD future research requires larger samples sizes as well as replication studies.

Genetic, pharmacological, as well as behavioral and imaging studies suggest an involvement of the serotonergic system in the ADHD pathophysiology. The SERT critically modulates serotonergic signaling, therefore representing a central serotonergic molecule for scientific examinations. The SERT has been inspected twice in adult patients with ADHD, though in one study methodological issues and in the other study a small sample size hamper the ability to interpret the results. In this cross-sectional PET study, we detected no differences in SERT availability between patients with ADHD and healthy subjects (Vanicek et al, 2016b). PET has primarily been used to examine a targeted transporter or receptor density. Based on interregional correlation analyses, PET imaging has also been used to investigate neuronal connectivity (Baldinger et al, 2014; Horwitz et al, 1984; Lee et al, 2008; Morbelli et al, 2013; Vanicek et al, 2016a). We performed an interregional correlation of SERT binding in every ROI.
and found significant higher correlations in patients with ADHD in numerous ROIs and after correction for multiple comparisons between the hippocampus and the precuneus. These results describe SERT associations between brain regions, thus leading to a more realistic understanding of an altered serotonergic system in ADHD. In addition and similar to the age related findings of the NET, we found a negative correlation of age and SERT binding in patients with ADHD and healthy control subjects.

3.2. Conclusion & future prospects

To summarize, this thesis intended to quantify essential transporter proteins of the noradrenergic and serotonergic neurotransmitter system in ADHD in vivo and to detect the extent of the involvement of genotypes on noradrenergic signaling. Three publications arose from this thesis, whereas PET and the radioligands \( (S,S)^{-18}\text{F} \)FMeNER-D_2 or \(^{11}\text{C}\)DASB as well as genotyping was used to test study hypothesis. With these investigations I was able to shed light on previously not studied neurochemical pathway, as demonstrated in the first publication, where NET distribution has been described for the first time in patients ADHD. In addition, a genotype effect on the expression on NET has been demonstrated, suggesting a genetic influence on the NET in ADHD. Furthermore, I was the first to apply interregional correlational analysis of the SERT binding, which represents an approach that aims to reveal alterations on more global level throughout the brain to capture a complex pattern of the SERT distribution, and demonstrate significant differences in patients with ADHD. These publications improve knowledge about fundamental neuroscientific understanding on NET and SERT availability and function.

On the one hand future research will have to replicate imaging findings in larger study samples, especially results describing the NET distribution, since the NET plays a critical role in noradrenergic and dopaminergic pathways that are highly involved in the neurobiology of ADHD and since these are the first and only data of NET in this patient group. On the other hand, studies have to expand our aims by applying a new and suitable radioligands to investigate NET levels in cortical regions. Furthermore, molecular imaging studies will have to target different receptors within the noradrenergic and dopaminergic system and genotypes of different proteins. Lastly, occupancy studies are needed to be executed in vivo in patients with ADHD to
disclose the neurobiological mechanisms of the neurotransmitters system in ADHD, influenced by psychotropic medication frequently prescribed in ADHD.
4. MATERIALS AND METHODS

This doctoral thesis was designed, organized, coordinated and executed by the “NEUROIMAGING LABS (NIL) - PET, MRI, EEG & Chemical Lab” at the Department of Psychiatry and Psychotherapy, Clinical Division of Biological Psychiatry, Medical University of Vienna and the “Doctoral Programme Clinical Neurosciences – CLINS”. The thesis project was followed through within the scope of the projects “The Norepinephrine Transporter in Attention Deficit Hyperactivity Disorder (ADHD) investigated with PET” (PI: Assoc. Prof. PD Rupert Lanzenberger, MD) and “The Serotonin Transporter in Attention Deficit Hyperactivity Disorder investigated with Positron Emission Tomography.” (PI: Prof. Dr. Markus Mitterhauser). The study protocols where approved by the Ethics Committee of the Medical University of Vienna and the General Hospital of Vienna (EK 552/2010; EK 784/2010). The project “The Norepinephrine transporter in Attention Deficit Hyperactivity Disorder investigated with PET” was funded by the Austrian Science Fund FWF (FWF Projektnr.: P 22981) and “The Serotonin Transporter in Attention Deficit Hyperactivity Disorder Investigated with Positron Emission Tomography” by the Jubiläumsfonds der Oesterreichischen Nationalbank (OeNB Projektnr.: 13675).

Patients with ADHD where recruited through the focus outpatient clinic for ADHD in adults at the Department of Psychiatry and Psychotherapy, Clinical Division of Biological Psychiatry. Healthy control subjects were recruited from the local community via advertisement.

Adult patients with ADHD and age and sex matched healthy control subjects were measured once with the GE Advance PET scanner (General Electroc Medical Systems, Milwaukee, Wisconsin, USA) at the Department of Biomedical Imaging and Image-guided Therapy, Division of Nuclear Medicine Department of Nuclear Medicine, Medical University of Vienna, in accordance to the specific study procedures with PET and \( (S,S)\cdot \left[^{18}\text{F}\right] \text{FMMeNER-D}_2 \) or \( \left[^{11}\text{C}\right] \text{DASB} \). The radiotracers have been used successfully in previous studies (Lanzenberger et al., 2007; Rami-Mark et al. 2014) and have been shown to be suitable to quantify the norepinephrine and serotonin transporter in vivo in humans. PET data analysing techniques have to be acquired in order to perform quantitative tracer kinetic modeling using reference tissue compartmental models and the kinetic modelling tools, which are implemented in the biomedical image quantification software PMOD 2.9 (Burger and Buck 1997; [http://www.pmod.com](http://www.pmod.com)).

Further Statistical Parametric Mapping (SPM) was used to co-register PET- and MRI images. Main outcome measures were either NET or SERT BP$_{ND}$ in areas of interest (region-of-interest
approach). General linear mixed model (GLM) was performed to reveal significant differences in NET and SERT binding potential according to study groups, gender and age as well as to the genotype groups and other variables. Interregional molecular associations analysis were calculated using Spearman’s rank correlation coefficient ($\Delta \rho$) for each group separately and compared between ROIs and added to the analysis of significant differences in SERT BP$_{ND}$ between groups. Interregional correlation matrices were transformed using Fisher’s r-to-z-transformation and a 10,000 fold permutation test was performed. All results where corrected for multiple testing.
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Medical University of Vienna, AKH Wien


The Norepinephrine Transporter in Attention Deficit/Hyperactivity Disorder Investigated with \((S,S)-\text{[}^{18}\text{F}]\text{FMeNER-D}_2\)

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**Poster Award of the 30th CINP Congress, Seoul, Korea** July 3-5, 2016


Interregional Correlations of SERT in Attention Deficit/Hyperactivity Disorder compared to Healthy Controls; Investigated with PET and \([^{13}\text{C}]\text{DASB}\)

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Collegium Internationale Neuro-Psychopharmacologicum (CINP)
Publication List (PL)

Original Investigations—First Author Publications (Top)

   *The Norepinephrine Transporter in Attention Deficit/Hyperactivity Disorder Investigated with (S,S)-[18F]FMeNER-D2.*

   *Insights into intrinsic brain networks based on graph theory and PET in right- compared to left-sided temporal lobe epilepsy.*

   *Differences in interregional molecular balance of the serotonin transporter in attention deficit/hyperactivity disorder revealed by PET.*

Co-Author Publications (Top)

   *Ketamine-dependent neuronal activation in healthy volunteers.*

   *Quantification of task-specific glucose metabolism with constant infusion of [18F]FDG.*
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**Co-Author Publications (Standard)**


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