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Association of Adenosine Receptor Gene Polymorphisms and *In Vivo* Adenosine A_1 Receptor Binding in The Human Brain

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Adenosine A_1 receptors (A_1ARs) and the interacting adenosine A_{2A} receptors are implicated in neurological and psychiatric disorders. Variants within the corresponding genes *ADORA1* and *ADORA2A* were shown associated with pathophysiologic alterations, particularly increased anxiety. It is unknown so far, if these variants might modulate the A_1AR distribution and availability in different brain regions. In this pilot study, the influence of *ADORA1* and *ADORA2A* variants on *in vivo* A_1AR binding was assessed with the A_1AR-selective positron emission tomography (PET) radioligand [^{18}F]CPFPX in brains of healthy humans. Twenty-eight normal control subjects underwent PET procedures to calculate the binding potential BP_{ND} of [^{18}F]CPFPX in cerebral regions and to assess *ADORA1* and *ADORA2A* single nucleotide polymorphism (SNP) effects on regional BP_{ND} data. Our results revealed SNPs of both genes associated with [^{18}F]CPFPX binding to the A_1AR. The strongest effects that withstood even Bonferroni correction of multiple SNP testing were found in non-smoking subjects ($N = 22$) for *ADORA2A* SNPs rs2236624 and rs5751876 (corr. $P_{all} < 0.05$). SNP alleles previously identified at risk for increased anxiety like the rs5751876 T-allele corresponded to consistently higher A_1AR availability in all brain regions. Our data indicate for the first time that variation of A_1AR availability was associated with *ADORA* SNPs. The finding of increased A_1AR availability in regions of the fear network, particularly in *ADORA2A* risk allele carriers, strongly warrants evaluation and replication in further studies including individuals with increased anxiety.
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INTRODUCTION

Adenosine is involved in numerous processes and pathways and plays an important role as homeostatic regulator and neuromodulator in the nervous system (Cunha, 2001; Fredholm *et al*, 2005). It exerts its functions via the G protein-coupled receptors A_1, A_{2A}, A_{2B}, and A_3, of which particularly the first one is widely distributed in the central nervous system with high expression rates in cortical, thalamic, hippocampal, and striatal neurons (Ribeiro *et al*, 2002; Wei *et al*, 2011). The adenosine A_1 receptor (A_1AR) is not only involved in physiologic conditions such as sleep, arousal, memory, cognition, and anxiety, but also in pathologic conditions like epilepsy, stroke, and neuro-inflammation (Ribeiro *et al*, 2002; Wei *et al*, 2011). Given this broad impact, an increasing number of studies focused on imaging the distribution and availability of cerebral A_1ARs *in vivo* by positron emission tomography (PET; for a recent review see Paul *et al*, 2011a) using [^{18}F]CPFPX as radioligand (Holschbach *et al*, 2002; Bauer *et al*, 2003; Meyer *et al*, 2004, 2005). Thereby changes in the binding potential of [^{18}F]CPFPX could be detected in different cerebral regions indicating, for example, pathologic conditions like glioma invasion (Bauer *et al*, 2005). Further, physiologic variation of A_1AR binding was reported, such as an age-dependent A_1AR loss (Meyer *et al*, 2007), an A_1AR upregulation after prolonged wakefulness (Elmenhorst *et al*, 2007a) as well as an influence of caffeine on [^{18}F]CPFPX binding (Elmenhorst *et al*, 2012).

Besides A_1ARs, A_{2A} adenosine receptors (A_{2A}AR) are recognized as widely distributed in the brain (Gomes *et al*, 2011). Remarkably, in several brain regions—particularly in the striatum—A_1ARs interact directly with A_{2A}ARs via coexpression and heteromerization. Thus, a functional cross-talk exists between both receptors regulating adenosinergic neuromodulation and glutamatergic neurotransmission (Cunha, 2001; Ferré *et al*, 2007; Casadó *et al*, 2010). A balanced activation of inhibitory A_1ARs and facilitatory A_{2A}ARs seems important for physiological neuromodulation, whereas deviation appears in conjunction with brain disorders such as ischemia, epilepsy, Huntington's disease, Alzheimer's disease, schizophrenia, bipolar disorders, depression, and anxiety disorders (Gomes *et al*, 2011).

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Accordingly, genetic knockout studies underline the important role of A_1AR together with A_{2A}AR in the central nervous system and particularly in relation to different neurological and psychiatric disorders (Wei *et al*, 2011). It is unknown, however, to the best of our knowledge, if small genetic variants like single nucleotide polymorphisms (SNPs) might play a role in modulating the distribution and availability of the A_1AR, as it is described to be the case for several other neurotransmitter systems such as the serotonergic, dopaminergic, and monoaminergic systems (Willeit and Praschak-Rieder 2010).

The human genes encoding the receptor types A_1AR (*ADORA1*) and A_{2A}AR (*ADORA2A*) both contain a set of SNPs, which have been intensely investigated over the years specifically in regard to pathophysiologic alterations. Association was reported, for example, with infarct size in patients with ischemic cardiomyopathy (Tang *et al*, 2007), post-traumatic seizures (Wagner *et al*, 2010), migraine with aura (Hohoff *et al*, 2007), schizophrenia (Gotoh *et al*, 2009), blood-injury phobia (Hohoff *et al*, 2009), panic disorder (Deckert *et al*, 1998; Hohoff *et al*, 2010), and autism spectrum disorder (Freitag *et al*, 2010). Aside from these findings studies in healthy subjects were conducted reporting association of several of these variants with amphetamine-induced anxiety (Hohoff *et al*, 2005), caffeine-induced anxiety (Alsene *et al*, 2003; Childs *et al*, 2008, Rogers *et al*, 2010), and caffeine effects on sleep (Rétey *et al*, 2007; Bodenmann *et al*, 2012). The strong overlap of physiologic and pathophysiologic conditions related to A_1AR and A_{2A}AR modulation on the one hand and to *ADORA1* and *ADORA2A* polymorphisms on the other hand leads to the hypothesis that genetic variants in both receptor genes might have an impact on A_1AR distribution and availability in different brain regions.

Therefore, the present pilot study investigated whether or not an influence of *ADORA1* and *ADORA2A* SNPs exists on *in vivo* A_1AR binding assessed with [^{18}F]CPFPX PET in brains of healthy humans.

MATERIALS AND METHODS

Subjects

Altogether 28 normal control individuals were included in the study (mean age ± SD 31.8 ± 10.7, range 21–63, all males) after giving written informed consent for imaging procedure as well as for blood collection and genetic testing. Subjects completed standardized questionnaires regarding their medical history, current health status, and sleeping behavior before they underwent medical examination. Candidates were excluded if they fulfill any of the following criteria: neurological or psychiatric disorders, head trauma, systemic diseases, substance abuse, and drugs interfering with adenosine receptors (eg, theophylline). All procedures were approved by the Ethics Committee of the Medical Faculty of the University of Duesseldorf and the German Federal Office for Radiation Protection.

Participants were asked to avoid caffeine consumption 24 h prior to PET scanning. In order to provide a measure of caffeine consumption, we estimated the number of cups of caffeinated coffee per day. Other caffeinated drinks, such as caffeine-containing soft drinks (1 liter corresponding to 1.5 cups) and black tea (2 cups corresponding to 1 cup), were also taken into account (Mandel, 2002). Information on average sleep duration per night and the sleep duration of the night preceding PET scanning was obtained. Each subject underwent also a high-resolution three-dimensional T1-weighted magnetic resonance imaging (MRI; Magnetom VISION 1.5T, Siemens, Erlangen, Germany). MR images were used to exclude structural abnormalities and to define volume-of-interest (VOI).

PET Procedures

PET acquisitions were performed following a previously described protocol (Meyer *et al*, 2004), on a Siemens ECAT EXACT HR+ scanner (Siemens-CTI, Knoxville, TN, USA). For the analysis, only the first 60 min of dynamic acquisitions, starting with [^{18}F]CPFPX injection, were used. [^{18}F]CPFPX was synthesized as previously described (Holschbach *et al*, 2002). Experiments were performed at tracer doses, with a mean specific radioactivity at injection time was 161.9 ± 81.0 GBq/μmol (range 46.4–333.2 GBq/μmol) and mean injected radioactivity was 288.8 ± 26.9 MBq (224–317 MBq), resulting in a mass of injected CPFPX of 3.8 ± 3.0 nmol on average (0.9–14.7 nmol). Blood samples were taken for blood volume correction of brain ROIs by subtracting 5% of the total blood activity from the time activity curves (TACs).

MR images were oriented to the anterior commissure/posterior commissure line using the VINCI software (Cologne, Germany). In order to coregister the PET to the MRI, a summed PET image (5.5–60 min p.i.) was used and the derived parameters applied to all individual frames of the dynamic acquisition.

For masking of gray matter in PET images the MRI was segmented (SPM5, Statistical Parametric Mapping, Wellcome Department of Cognitive Neurology, London, UK). Next, the coregistered MRI scan was used to delineate VOIs by an automatic procedure based on probability maps (PVElab-20080317; Svarer *et al*, 2005). Based on these VOIs, side-averaged, decay- and blood-volume-corrected TACs were extracted from the dynamic gray matter-masked PET sequences (PMOD, Version 2.6, PMOD Group, Zurich, Switzerland, www.pmod.com) for the following 19 cerebral regions: amygdala, anterior cingulate gyrus, caudate, dorsolateral prefrontal cortex, entorhinal cortex, hippocampus, insula, middle and inferior frontal gyrus, middle and inferior temporal gyrus, occipital cortex, orbitofrontal cortex, parietal cortex, posterior cingulate gyrus, putamen, sensorimotor cortex, superior frontal gyrus, superior temporal gyrus, thalamus, and ventrolateral prefrontal cortex. The binding potential BP_{ND} of [^{18}F]CPFPX in cerebral tissue was assessed by Logan's non-invasive graphical analysis (Logan *et al*, 1996) as previously described (Meyer *et al*, 2007). The cerebellum was used as a reference region in the present study because of the low abundance of A_1ARs in this region in humans (Bauer *et al*, 2003). The slope was calculated from 20 min onwards, as previous analyses revealed that this time point corresponds to the time of linearization in [^{18}F]CPFPX non-invasive graphical analyses (Meyer *et al*, 2007). For an illustration of main image analysis steps see Figure 1.

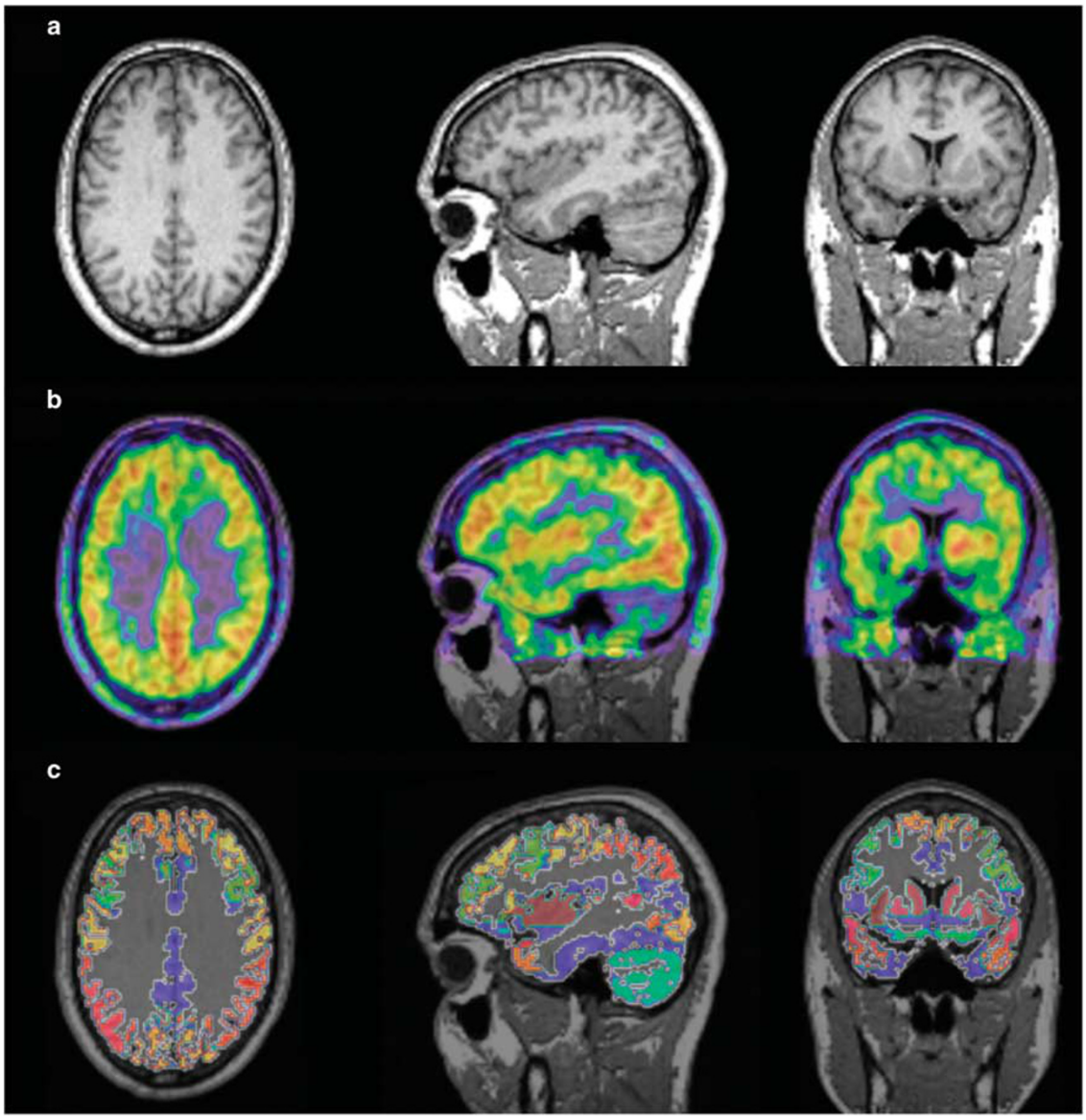

Figure 1 The figure represents the main image analysis steps: the individual MRI (panel (a)) and the summed PET image (fused with the MRI in panel (b)) were aligned and coregistered. Gray matter-masked VOIs (overlaid on the MRI in panel (c)) were used to extract regional TACs, to which Logan's non-invasive graphical analysis was applied to calculate regional BP_{ND} values (see Materials and Methods section for details).

Genotyping

Blood samples were taken from all subjects for DNA isolation using standard methods (FlexiGene DNA Kit, Qiagen). Altogether 18 SNPs were selected to cover *ADORA1* and *ADORA2A* based on tagging information (International HapMap Project, www.hapmap.org), functional potential (UCSC Genome Browser, http://genome.ucsc.edu) and own previous association studies (eg, Deckert *et al*, 1998; Alsene *et al*, 2003; Hohoff *et al*, 2005, 2007, 2009, 2010; Childs *et al*, 2008, Freitag *et al*, 2010; Rogers *et al*, 2010; Bodenmann *et al*, 2012), as illustrated in Figure 2. Genotypes were determined as described previously (refer Deckert *et al*, 1998; Freitag *et al*, 2010) and as summarized in Supplementary Table S1. To control for the risk of genotyping errors, about one-third of randomized subjects were genotyped additionally by independent assays (single-strand conformation analysis, direct sequencing, or custom TaqMan SNP genotyping assays by Life Technologies, Darmstadt, Germany) resulting in concordance rates of 100% (primer/probe sequences and assay conditions available on request). Overall genotyping resulted in completion rates of 100% and genotypes were assigned blind with respect to the phenotypic characteristics of the subjects.

Statistical Analyses

Hardy–Weinberg equilibrium of individual SNPs as well as pairwise linkage disequilibrium (LD) of *ADORA1* and *ADORA2A* SNPs, respectively, were assessed by Haploview version 4.1 (Barrett *et al*, 2005; www.broad.mit.edu/mpg/haploview). Genotype groups were formed for each SNP to

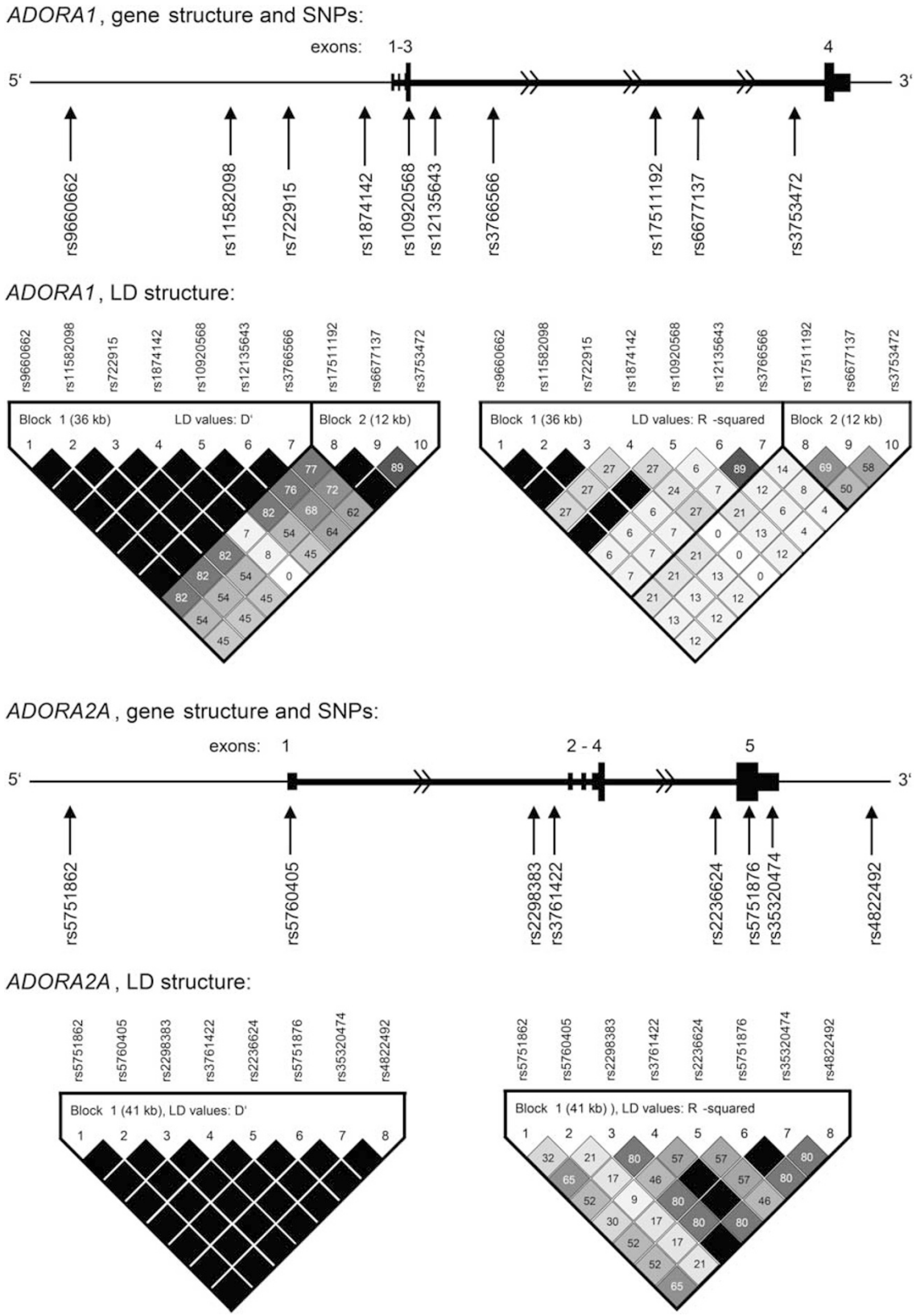

Figure 2 Organization of adenosine receptor genes *ADORA1* (NM_000674, located on Chr. 1q32.1) and *ADORA2A* (NM_000675, located on Chr. 22q11.23) with position of selected SNPs and pairwise linkage disequilibrium (LD) structure. Gene bodies were illustrated by bold black horizontal lines on the 5′ to 3′ DNA sense (+) strand, both containing 5′ and 3′ untranslated exons/exon-parts displayed as flat black blocks. The two coding exons per gene are displayed as higher black blocks and SNP positions relative to genes and exons are indicated by arrows. In the LD plots shades of gray/numbers in boxes represent the extent of LD (darker gray/higher numbers = higher LD and black/empty box = complete LD) assessed with statistics D' and *R*-squared together with the resulting haploblock assignment using the solid spine of LD method as implemented in Haploview.

increase statistical power: carriers of minor alleles of a given SNP (subjects homozygote or heterozygote for the rare allele) were combined to one group and further analyzed *vs* the major allele carriers of the same SNP (subjects homozygous for the frequent allele). Demographic and descriptive data such as age, coffee and/or cigarette consumption, sleep duration, injected activity, and specific activity at scantime (for a complete list see Supplementary Table S2) were tested for deviation from normal distribution (one-sample Kolmogorov–Smirnov and Shapiro–Wilk tests). Next, these data were checked for potential confounding effects in both categories, specific BP_{ND} data of all brain regions and genotype groups of all SNPs, by parametric (analysis of variance, Pearson's correlation analysis) or nonparametric statistics (Pearson's chi-square test, Fisher's exact test, Mann–Whitney *U*-test, and Spearman's rank correlation analysis) as appropriate. Exploratory analysis of genotype group effects of *ADORA1* or *ADORA2A* SNPs on regional A_1AR binding was performed using general linear models (GLM univariate analysis of variance) including genotype group (minor allele carriers *vs* major allele homozygotes) as independent factor, BP_{ND} data as dependent variables and demographic or descriptive data as confounder (factor or covariate) if relevant. Statistical tests were computed using SPSS Statistics software (version 22, IBM) with the alpha level set at $P<0.05$. Adjustment for multiple SNP testing was done in gene-wise manner (*ADORA1*: 7 SNPs; *ADORA2A*: 5 SNPs) using the conservative Bonferroni correction, which resulted in adjusted alpha levels of $P<0.007$ for *ADORA1* SNPs and $P<0.01$ for *ADORA2A* SNPs. Exploratory correction for comparisons of multiple (19) brain regions was performed in a SNP-wise manner based on nominal *P*-values by controlling the false discovery rate. This followed the Benjamini–Hochberg procedure (Benjamini and Hochberg, 1995) resulting in adjusted alpha levels of $P<0.0026$ for the best (smallest) nominal *P*-value, $P<0.0053$ for the second best, $P<0.0079$ for the third best, and so forth.

RESULTS

Genotype frequencies of all *ADORA1* and *ADORA2A* SNPs in the PET group of healthy volunteers conformed to Hardy–Weinberg equilibrium ($P_{all}>0.1$). Pairwise LD analysis revealed moderate to strong LD between most *ADORA1* and *ADORA2A* variants (Figure 2). Complete LD was detected between *ADORA1* SNPs rs9660662-rs11582098-rs722915-rs10920568 (each pairwise comparison: D' = 1.0, *r*-squared = 1.0), as well as *ADORA2A* SNPs rs3761422-rs5751876-rs35320474 (D' = 1.0, *r*-squared = 1.0) and rs2298383-rs4822492 (D' = 1.0, *r*-squared = 1.0). Therefore we dropped the redundant SNPs (rs9660662, rs11582098, rs722915, rs2298383, rs3761422, and rs35320474) leaving seven *ADORA1* SNPs (rs1874142, rs10920568, rs12135643, rs3766566, rs17511192, rs6677137, and rs3753472) and five *ADORA2A* SNPs (rs5751862, rs5760405, rs2236624, rs5751876, and rs4822492) for further analysis.

Demographic and descriptive data analyses revealed an impact of age, sleep duration, and smoking on BP_{ND} data (Supplementary Table S2) and were therefore included as factor/covariate in all subsequent analyses. Analysis of *ADORA1* and *ADORA2A* genotype group effects on regional [^{18}F]CPFPX binding revealed association of several SNPs of both genes with a variety of different brain regions as summarized in Table 1 (for detailed data see Supplementary Table S3). Bonferroni adjustment for multiple SNP testing, however, suggested trend significance only for *ADORA2A* SNPs (rs5760405: $P_{corr}=0.063$ and 0.070; rs2236624: $P_{corr}=0.080$), but not for *ADORA1* SNPs.

Subsequent stratification to smoking further supported this difference between genes and revealed almost exclusively *ADORA2A* SNP effects on [^{18}F]CPFPX binding in the subsample of non-smoking subjects ($N=22$) as overviewed in Table 2 (for detailed data see Supplementary Table S4). These *ADORA2A* effects overall expanded and strengthened compared to the total sample analysis, now including more brain regions as well as findings per SNP. Accordingly, Bonferroni adjustment for multiple SNP testing supported several of these findings, with associations withstanding correction and remaining significant: the effect of rs2236624 on the superior frontal gyrus ($P_{corr}=0.017$) and dorsolateral prefrontal cortex ($P_{corr}=0.013$) as well as of rs5751876 on the entorhinal cortex ($P_{corr}=0.006$), hippocampus ($P_{corr}=0.040$), and whole cortical BP_{ND} data ($P_{corr}=0.031$); all illustrated also in Figure 3a–d).

DISCUSSION

The main finding of the present study is an association of SNPs of adenosine receptor genes with *in vivo* [^{18}F]CPFPX binding to the A_1AR in brains of healthy human subjects, an association that remained significant for several *ADORA2A* SNPs in the subsample of non-smoking subjects even after Bonferroni correction of multiple SNP testing. This is the first time, to the best of our knowledge, that variation of A_1AR availability was shown associated with genetic factors at all. With it we could add another important factor for interindividual variability in A_1AR availability beyond physiological or external factors such as age (Meyer *et al*, 2007), wakefulness (Elmenhorst *et al*, 2007a), or caffeine (Elmenhorst *et al*, 2012). Further, our finding is in line with recent findings of other genetic variants reported to play a role in availability of receptors of the serotonergic, dopaminergic, and monoaminergic systems (Willeit and Praschak-Rieder, 2010). Together these data support a broad-based impact of genetic factors on general receptor binding in the human brain.

Several brain regions were identified in our study as particularly prone to *ADORA2A* SNP effects: the superior frontal gyrus, the dorsolateral prefrontal cortex, the hippocampus, and the entorhinal cortex. They are important players in emotion processing and aberrations in volume, reactivity, or interconnectivity could be related to dysfunctions in emotion or mood regulation as shown to be the case in different psychiatric disorders like anxiety disorders, depression, bipolar disorders, and schizophrenia (Baiano *et al*, 2008; Moghaddam and Homayoun, 2008; Rich *et al*, 2011; Hamilton *et al*, 2012). Thus, our findings might hint to *ADORA2A* SNP effects playing an important role in emotion regulation.

In addition to the brain regions with highest sensitivity to SNP effects, also lower, statistically sub-threshold sensitivity

Table 1 Overview of SNP Effects on *In Vivo* Brain Regional A_1AR Binding Presenting *P*-Values (uncorrected) in Total Sample of Healthy Subjects

Brain region	*ADORA1* SNP genotype groups (*N*)							*ADORA2A* SNPs genotype groups (*N*)				
	rs1874142 A (8) *vs* AG+G (19)	**rs10920568 T (18) *vs* GT+G (9)**	**rs12135643 C (18) *vs* AC+A (9)**	**rs3766566 G (17) *vs* AG+A (10)**	**rs17511192 T (8) *vs* CT+C (19)**	**rs6677137 T (10) *vs* CT+C (17)**	**rs3753472 T (11) *vs* CT+C (16)**	**rs5751862 G (7) *vs* AG+A (20)**	**rs5760405 C (17) *vs* CT+T (10)**	**rs2236624 C (16) *vs* CT+T (11)**	**rs5751876 C (9) *vs* CT+T (18)**	**rs4822492 G (7) *vs* CG+C (20)**
Cortical BP_{ND}	—	—	—	(0.0507)	—	—	—	—	**0.0255**	—	(0.0939)	—
Subcortical BP_{ND}	—	—	—	—	—	(0.0830)	—	—	—	—	—	—
Mean BP_{ND}	—	—	—	(0.0557)	—	—	—	—	**0.0292**	—	(0.0908)	—
Orbitofrontal cortex	—	**0.0215**	(0.0760)	**0.0350**	—	—	—	—	—	—	—	—
Middle/inferior frontal gyrus	—	(0.0670)	—	—	—	—	—	—	**0.0444**	—	(0.0972)	—
Anterior cingulate gyrus	—	—	—	—	—	—	—	—	(0.0503)	—	—	—
Thalamus	—	—	—	(0.0586)	—	—	—	—	**0.0125**	—	(0.0974)	—
Insula	—	—	—	(0.0634)	—	—	—	—	**0.0411**	—	(0.0864)	—
Caudate	—	—	—	—	—	(0.0600)	—	—	—	—	—	—
Putamen	—	—	—	(0.0768)	—	—	—	—	—	—	—	—
Superior temporal gyrus	—	(0.0986)	—	**0.0499**	—	—	—	—	**0.0466**	—	—	—
Parietal cortex	—	—	—	**0.0307**	—	—	—	—	**0.0287**	—	—	—
Middle/inferior temporal gyrus	—	**0.0457**	—	**0.0343**	—	—	—	—	(0.0596)	—	—	—
Superior frontal gyrus	—	—	—	—	—	—	—	—	**0.0140**	**0.0209**	(0.0580)	—
Occipital cortex	—	—	(0.0904)	**0.0392**	—	—	—	—	**0.0479**	—	—	—
Sensorimotor cortex	—	—	(0.0991)	**0.0294**	—	—	—	—	**0.0240**	—	—	—
Posterior cingulate gyrus	—	—	—	—	—	—	—	—	—	—	—	—
Dorsolateral prefrontal cortex	—	—	—	—	—	—	—	—	**0.0388**	**0.0159**	(0.0938)	—
Ventrolateral prefrontal cortex	—	**0.0384**	—	—	—	—	—	—	(0.0521)	—	—	—
Entorhinal cortex	—	—	—	—	—	—	—	—	(0.0944)	—	**0.0450**	—
Hippocampus	—	—	—	—	—	—	—	—	**0.0277**	—	—	—
Amygdala	—	—	(0.0861)	**0.0218**	—	—	—	(0.0965)	**0.0359**	—	—	—

Bold face indicate nominal significant *P*-values, brackets trend values.

Table 2 Overview of SNP Effects on *In Vivo* Brain A_1AR Binding Presenting *P*-Values (uncorrected) in Subsample of Non-Smoking Subjects

Brain region	*ADORA1* SNPs							*ADORA2A* SNPs				
	rs1874142 A (8) *vs* AG+G (14)	**rs10920568 T (15) *vs* GT+G (7)**	**rs12135643 C (14) *vs* AC+A (8)**	**rs3766566 G (14) *vs* AG+A (8)**	**rs17511192 T (7) *vs* CT+C (15)**	**rs6677137 T (8) *vs* CT+C (14)**	**rs3753472 T (10) *vs* CT+C (12)**	**rs5751862 G (6) *vs* AG+A (16)**	**rs5760405 C (13) *vs* CT+T (9)**	**rs2236624 C (14) *vs* CT+T (8)**	**rs5751876 C (8) *vs* CT+T (14)**	**rs4822492 G (6) *vs* CG+C (16)**
Cortical BP_{ND}	—	—	—	—	—	—	—	—	(0.0516)	**0.0304**	**0.0062***	**0.0446**
Subcortical BP_{ND}	—	—	—	—	—	—	—	—	—	—	—	—
Mean BP_{ND}	—	—	—	—	—	—	—	—	(0.0555)	(0.0534)	**0.0103**	(0.0643)
Orbitofrontal cortex	—	—	(0.0666)	(0.0666)	—	—	—	—	—	**0.0251**	**0.0288**	—
Middle/inferior frontal gyrus	—	—	—	—	—	—	—	—	(0.0906)	**0.0423**	**0.0327**	—
Anterior cingulate gyrus	—	—	—	—	—	—	—	—	—	(0.0789)	**0.0180**	**0.0317**
Thalamus	—	—	—	—	—	—	—	—	**0.0160**	**0.0428**	**0.0196**	(0.0621)
Insula	—	—	—	—	—	—	—	—	(0.0962)	—	**0.0166**	**0.0347**
Caudate	—	—	—	—	—	—	—	—	—	—	—	—
Putamen	—	—	—	—	—	—	—	—	—	—	—	—
Superior temporal gyrus	—	—	—	—	—	—	—	—	—	—	**0.0207**	—
Parietal cortex	—	—	(0.0860)	(0.0860)	—	—	—	—	(0.0622)	**0.0399**	**0.0415**	(0.0866)
Middle/inferior temporal gyrus	—	—	(0.0603)	(0.0603)	—	—	—	—	—	—	**0.0115**	(0.0633)
Superior frontal gyrus	—	—	—	—	—	—	(0.0517)	—	**0.0316**	**0.0034***	**0.0180**	**0.0343**
Occipital cortex	—	—	(0.0543)	(0.0543)	—	—	—	—	—	(0.0887)	**0.0398**	(0.0657)
Sensorimotor cortex	—	—	(0.0910)	(0.0910)	—	—	—	—	**0.0410**	**0.0162**	(0.0562)	(0.0844)
Posterior cingulate gyrus	—	—	—	—	—	—	—	—	—	—	(0.0945)	—
Dorsolateral prefrontal cortex	—	—	—	—	—	—	**0.0418**	—	(0.0782)	**0.0026***	**0.0333**	—
Ventrolateral prefrontal cortex	—	—	—	—	—	—	—	—	—	—	**0.0262**	—
Entorhinal cortex	—	—	—	—	—	—	—	—	(0.0951)	—	**0.0012***	**0.0409**
Hippocampus	—	—	—	—	—	—	—	(0.0915)	**0.0168**	—	**0.0080***	—
Amygdala	—	—	(0.0554)	(0.0554)	—	—	—	—	**0.0431**	—	(0.0605)	—

Bold face indicate nominal significant *P*-values, brackets trend values, and asterisks mark significant *P*-values that withstood Bonferroni correction for multiple testing (adjusted alpha level for *ADORA1*: $P<0.007$; for *ADORA2A*: $P<0.01$).

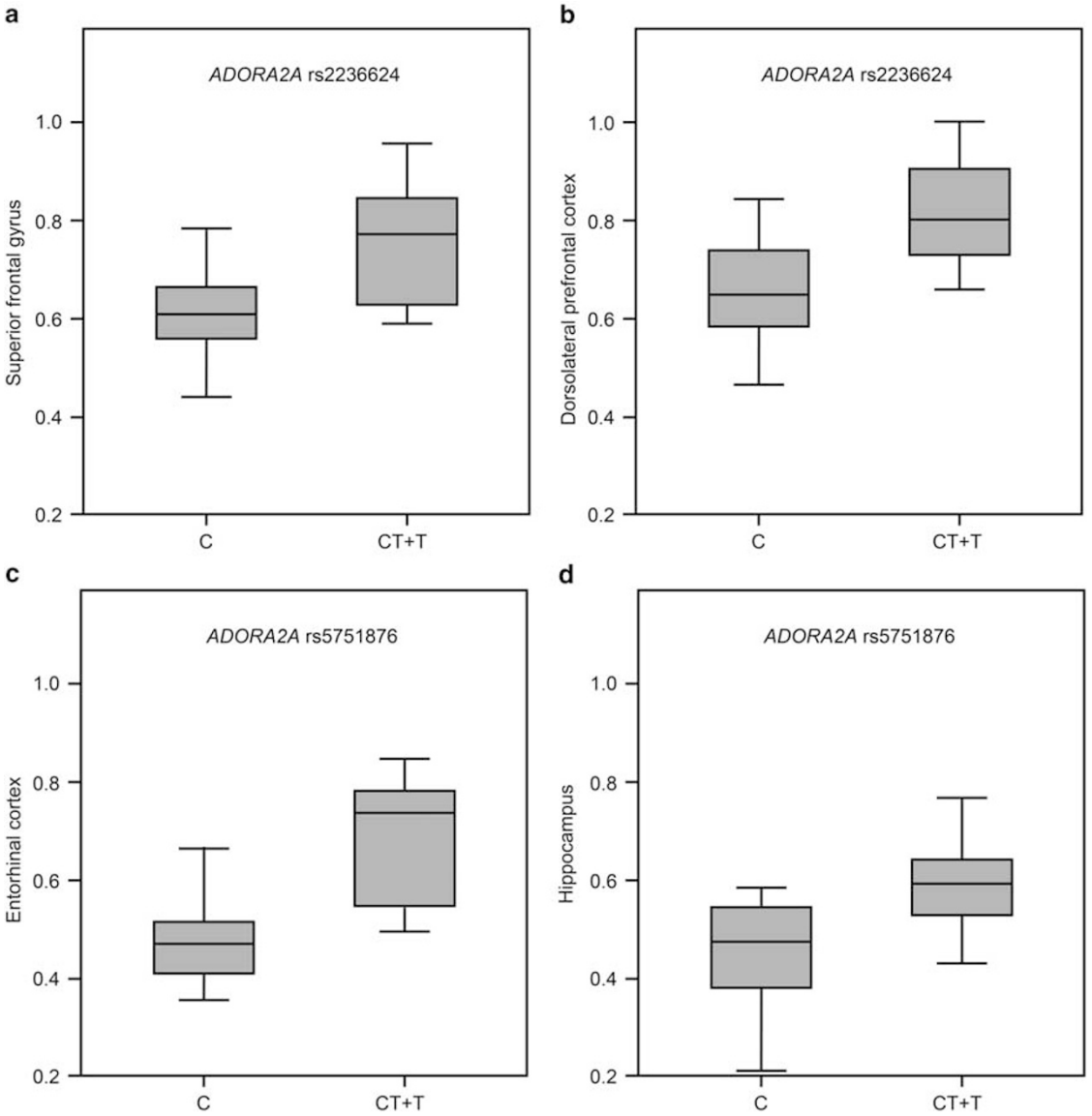

Figure 3 Boxplots showing *ADORA2A* genotype group effects on regional A_1AR binding in the subsample of non-smoking subjects. General linear models with Bonferroni adjustment for multiple SNP testing revealed following effects: rs2236624 on superior frontal gyrus ((a); $P_{corr} = 0.017$) and dorsolateral prefrontal cortex ((b); $P_{corr} = 0.013$) as well as rs5751876 on entorhinal cortex ((c); $P_{corr} = 0.006$) and hippocampus ((d); $P_{corr} = 0.04$).

to SNP effects was found in the remaining 15 investigated regions. Notably, therein the direction of SNP effects was highly consistent and in accordance to the highly sensitive regions. For example, in subjects homozygous for the rs5760405 C-allele, always higher [^{18}F]CPFPX binding to the A_1AR was detected across all 19 brain regions compared to T-allele carriers (CT or TT genotype). The same consistently higher A_1AR availability was true for carriers of the rs5751876 T-allele (CT + TT) in all brain regions and for carriers of the rs2236624 T-allele (CT + TT) in nearly all regions (except caudate). This might indicate an underlying widespread genotype group effect on A_1AR binding, which, however, overcomes the Bonferroni-corrected threshold for significance only in case of particular strong association with the above mentioned brain regions with importance for emotion regulation.

Our findings can be linked to studies reporting an association between *ADORA2A* SNPs and alterations in emotionality, especially altered anxiety. Some of the *ADORA2A* SNPs that turned out to be strongly involved in for instance anxious personality (Hohoff *et al*, 2010: rs5751862, rs5760405, and rs5751876) and panic disorder (Deckert *et al*, 1998; Hohoff *et al*, 2010: rs5751876), had also effects on A_1AR binding in our present study. That is, rs5751862 GG homozygotes, rs5760405 CC homozygotes, and particularly carriers of the rs5751876 T-allele had higher A_1AR availability in [^{18}F]CPFPX PET. Thus, our data connected increased A_1AR binding with reported risk alleles from the *ADORA2A* gene. This indirect impact of *ADORA2A* SNPs on A_1AR binding was particularly strong in the subsample of non-smokers, a group without nicotine interference. Exploratory correction for multiple

comparisons (19 brain regions) by controlling the false discovery rate supported at least the strongest *ADORA2A* SNP effects on A_1AR binding (rs2236624: $P_{corr} = 0.049$ for superior frontal gyrus, $P_{corr} = 0.032$ for dorsolateral prefrontal cortex; and rs5751876: $P_{corr} = 0.023$ for entorhinal cortex, but only a trend for hippocampus with $P_{corr} = 0.076$).

Increased A_1AR availability has been observed after different negative physiological conditions like prolonged wakefulness (Elmenhorst *et al*, 2007a) or acute exposure to ethanol (Paul *et al*, 2011b). Increased A_1AR binding was also detected in the hippocampus of two different rat models of stress, using unpredictable chronic mild stress or chronic restraint stress (Crema *et al*, 2013). Crema *et al* discussed a possible relationship between the hypothalamic–pituitary–adrenal (HPA) axis and A_1AR modulation, maybe via positive regulation by endogenous corticosteroids and suggested that an upregulation of A_1AR could be involved in protecting hippocampal cells from insults induced by chronic stress. Our finding of increased A_1AR availability in risk allele carriers is in line with these findings and suggestions. Stress response regulation including HPA axis activation is altered in panic disorder patients (recent review by Ising *et al*, 2012) and sleep deprivation occurs with negative mood and increased anxiety up to catastrophizing in them (Kahn *et al*, 2013). Notably, these consequences of sleep deprivation are greater after the presentation of a mild stressor, possibly due to a lowered psychological threshold to estimate a situation as stressful (Minkel *et al*, 2012). Increased sensitivity to mild stressors or stimuli combined with anxiety (anxiety sensitivity) was shown also as a risk factor in the development of anxiety disorders (Schmidt *et al*, 2006). It might correspond to a hyperarousal state that is assumed in *ADORA2A* risk allele carriers potentially due to transcriptional inhibition and decreased amounts of A_{2A}AR molecules with functional consequences comparable to A_{2A}AR blocking by receptor antagonists such as caffeine (Hohoff *et al*, 2010). As a consequence A_1AR binding might be upregulated and serve as a compensatory mechanism, eg to protect brain cells from possibly negative effects of chronic stress (hyperarousal) as proposed by (Crema *et al*, 2013). This is in line with chronic caffeine intake leading to increased cerebral A_1AR binding (Rudolphi *et al*, 1989; Johansson *et al*, 1993) and exhibiting neuroprotective effects (Rudolphi *et al*, 1989; Maia and de Mendonça 2002; Ascherio and Chen 2003).

However, at present no data are available on A_{2A}AR receptor binding modulation by genetic variation in *ADORA1* and *ADORA2A* genes. Increased A_1AR binding might therefore not be the consequence of A_{2A}AR availability and might alter the normally balanced system of inhibitory A_1AR and facilitatory A_{2A}AR necessary for physiological functioning as mentioned in the introduction. Thereby such misbalance might serve itself as an underlying risk factor contributing to the development of particularly anxiety disorders. Though intriguing, these considerations are highly speculative until studies on A_{2A}AR binding and further studies with functional examinations are available. Obviously there are several limitations to our study. First of all, our study has a pilot character with exploratory analysis of SNP effects on A_1AR binding in a sample of 28 individuals. Therefore statistical power is only moderate and might mask several associations between SNPs and brain regions. However, our results have to be considered with caution since we only performed correction for multiple SNP testing with no systematic correction for testing of multiple brain regions that might have turned out overly stringent in this exploratory study. Further, given the mutual dependence of brain regions as detected in our sample (Supplementary Table S5), future GLM multivariate procedures could provide an additional analysis tool of global SNP effects on brain A_1AR binding. Such multivariate models allow simultaneous analysis of multiple dependent and highly inter-correlated variables, but need bigger sample sizes than 28 individuals, particularly if additional confounders have to be included as it is the case in our sample with age, sleep, and cigarette consume. Therefore replication studies in independent, larger samples of healthy subjects are strongly warranted. Further limitation might result from using the cerebellum as reference region. Former studies with the radiotracer [^{18}F]CPFPX demonstrated that the outcome parameter BP_{ND} is stable and reliable. As the cerebellum contains a small portion of A_1ARs (Bauer *et al*, 2003) there is a theoretical possibility that receptor polymorphisms might differently affect A_1ARs in the reference region and the regions of interest, which would result in an over- or underestimation of BP_{ND} values. However, previous simulations showed that changes in the specific binding of [^{18}F]CPFPX in the reference region (cerebellum) were alleviated in the resulting BP_{ND} (Elmenhorst *et al*, 2007b). Furthermore, additional comparisons of area under the curve values of normalized cerebellar TACs between major and minor allele carriers of all SNPs did not show any significant difference in A_1AR BP_{ND} in any region. Another limitation might derive from the sample composition itself, as our sample included subjects ranging from 21 to 63 years and age turned out to confound brain-specific BP_{ND} data. An effect of aging on cerebral A_1AR binding was observed also in a sample of comparable size ($N = 36$) aged 22–74 years (Meyer *et al*, 2007). However, although we included age as covariate in the present sample analysis it should be considered for future recruitment of replication samples. Further, we aimed at a rather homogeneous sample for this pilot study (male European of young to medium age), which prevented analysis of potentially existing gender or race-/ethnicity-related effects. Similarly, we have no data on personality features like anxiety in our subjects that might be a possible confounding factor. A comparison of *ADORA1* and *ADORA2A* risk-allele effect on A_1AR binding in male and female individuals with increased anxiety or even panic disorder is therefore of particular interest in replication studies.

In summary, our results revealed variation of regional A_1AR availability associated with *ADORA1* and particularly *ADORA2A* SNPs, several of which had previously been shown to be associated with increased anxiety and panic disorder. Future studies are strongly warranted to replicate our pilot findings of increased A_1AR in risk alleles carriers, to provide data on modification of A_{2A}AR binding by variation in *ADORA1* and *ADORA2A* genes and to evaluate the mechanistic considerations by adding studies in individuals with increased anxiety and panic disorder.

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