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ORIGINAL ARTICLE

The influence of the *rs6295* gene polymorphism on serotonin-1A receptor distribution investigated with PET in patients with major depression applying machine learning

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Major depressive disorder (MDD) is the most common neuropsychiatric disease and despite extensive research, its genetic substrate is still not sufficiently understood. The common polymorphism rs6295 of the serotonin-1A receptor gene (*HTR1A*) is affecting the transcriptional regulation of the 5-HT_{1A} receptor and has been closely linked to MDD. Here, we used positron emission tomography (PET) exploiting advances in data mining and statistics by using machine learning in 62 healthy subjects and 19 patients with MDD, which were scanned with PET using the radioligand [*carbonyl*-^{11}C]WAY-100635. All the subjects were genotyped for rs6295 and genotype was grouped in GG vs C allele carriers. Mixed model was applied in a ROI-based (region of interest) approach. ROI binding potential (BP_{ND}) was divided by dorsal raphe BP_{ND} as a specific measure to highlight rs6295 effects (BP_{Div}). Mixed model produced an interaction effect of ROI and genotype in the patients' group but no effects in healthy controls. Differences of BP_{Div} was demonstrated in seven ROIs; parahippocampus, hippocampus, fusiform gyrus, gyrus rectus, supplementary motor area, inferior frontal occipital gyrus and lingual gyrus. For classification of genotype, 'RandomForest' and Support Vector Machines were used, however, no model with sufficient predictive capability could be computed. Our results are in line with preclinical data, mouse model knockout studies as well as previous clinical analyses, demonstrating the two-pronged effect of the G allele on 5-HT_{1A} BP_{ND} for, we believe, the first time. Future endeavors should address epigenetic effects and allosteric heteroreceptor complexes. Replication in larger samples of MDD patients is necessary to substantiate our findings.

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INTRODUCTION

Major depressive disorder (MDD) is the most common neuropsychiatric disease with a lifetime prevalence of about 16%. Severely limiting all aspects of life and causing fatal outcomes as suicide, it ranks among the most burden-heavy diseases. MDD also poses a threat to health care systems, having caused 3.8% of global disability-adjusted life years (DALY) in 2010. More strikingly, MDD is expected to increase in disability-adjusted life years until 2030, topping the ranking in the developed world.[1,2]

Consequently, affective disorders and especially MDD have been studied extensively over the past decades. The decisive role of serotonin (5-HT) has been validated by postmortem, pharmacologic challenge, tryptophan depletion as well as imaging studies.[3,4] Overall, a disequilibrium of 5-HT has been reported for MDD, while the precise etiological mechanisms are still speculative, shrouded by contrarious findings and uncertainty concerning the role of genetics, epigenetics and environmental factors.[5–9] On one hand, the importance of genetic contribution to MDD is universally accepted and twin studies have shown a moderate heritability of about 40%. On the other hand, the role of specific genes and single-nucleotide polymorphisms is still dubious.[10] In fact, common polymorphisms might explain only about 0.05% of heritability.[11]

The serotonin-1A receptor (5-HT_{1A}) is the most important inhibitory receptor of the serotonergic system and has been studied extensively in MDD.[12–16] The 5-HT_{1A} receptor is prevalent in two configurations. Both are inhibitory Gi/Go coupled receptors that mediate their influence through cAMP and calcium channel inhibition. Presynaptical autoreceptors are located in the dorsal and median raphe nuclei of the midbrain, the hive of serotonergic neuronal activity. Serotonergic neurons project to most parts of the brain, exhibiting postsynaptic heteroreceptors active in the cortex, limbic regions, hypothalamus as well as the spinal cord. Overexpressed 5-HT_{1A} autoreceptors and diminished heteroreceptors in MDD have been demonstrated in animal studies, *in vivo* by PET studies as well as in postmortem studies.[17–20] Thereby, increased inhibition by presynaptic autoreceptors could decrease overall serotonergic activity and contribute to MDD.[21] A common hypothesis is that antidepressants require 5-HT_{1A} autoreceptors to be downregulated and desensitized before a treatment effect can be achieved.[22–25] Consequently, as phosphorylation, internalization as well as downregulation are all adaptive mechanisms acting within days, transcriptional effects leading to decreased receptor synthesis remain as a possible explanation for this delay.[26]

The 5-HT_{1A} receptor gene *HTR1A* has been one of the most studied candidate genes in MDD. Although many polymorphisms have been considered, most of them are too rare to be of

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significant relevance or lacked consistency regarding association with MDD and treatment response. The probably most prominent polymorphism linked to MDD is the rs6295 single-nucleotide polymorphism, a common variation at the 1019 site upstream of the basal promoter area, hence also known as C(-1019)G polymorphism.[27] The more common C allele of this single-nucleotide polymorphism is recognized as a binding site for the transcriptional factors Deaf1 or NUDR, Hes1 and Hes5.[28–30] On the other hand, the G allele disables binding of the transcriptional factors. These factors repress transcription of 5-HT_{1A} receptors, however, only Deaf1 is also active in mature neuronal cells. More interestingly, Deaf1 shows a divergent effect in presynaptic 5-HT_{1A} autoreceptors of the raphe and postsynaptic heteroreceptors. Deaf1 knockout mice exhibit an increase in transcription of 5-HT_{1A} autoreceptors of about 50%, while heteroreceptors are repressed by up to 30%.[31] Based on this solid preclinical foundation, the G allele of rs6295 was associated with a higher occurrence of MDD, bipolar disorder and completed suicide as well as poor response to selective serotonin-reuptake inhibitors.[32–35] Two functional magnetic resonance imaging studies demonstrated altered reactivity of the amygdala of GG carriers with MDD and healthy subjects, respectively, in emotionally valanced faces as well as threat-related stimuli.[36,37] However, some studies also reported opposite results.[38–40]

Few PET studies investigated the effect of rs6295 on 5-HT_{1A} binding, showing greater binding of G-allele carriers in the dorsal raphe nuclei of MDD patients, whereas no significant effects were found in other areas.[41–43] However, a replication analysis in a bigger sample by the same group failed to demonstrate any associations.[44] In line with the positive results, G-allele carriers have been suggested to show decreased response to treatment in clinical studies. However, another recent PET study linked higher radioligand binding to 5-HT_{1A} receptors in the raphe nuclei to more pronounced treatment response to selective serotonin-reuptake inhibitor, which is conflicting with higher raphe binding reported in G-allele carriers.[45]

Based on these ambiguous findings, we conducted a PET study using [*carbonyl*-^{11}C]WAY-100635 to shed more light on the role of rs6295 in MDD. Due to the molecular architecture of this polymorphism, we further hypothesized that the diverse effect of rs6295 on pre- and postsynaptical receptors should be more refined when applying multivariate machine learning tools for classification. New statistical methods and especially machine learning have been implemented in psychiatric research over the last years as they offer advantages over conventional univariate statistics. They are suitable for large data sets with high number of predictors and allow classification by pattern recognition instead of main and interaction effects.[46] As both 'RandomForest' (RF) and 'Support Vector Machines' (SVM) have been shown to produce strong results in classification, we applied these techniques to test our hypothesis that G-allele carriers will show higher binding to [*carbonyl*-^{11}C]WAY-100635 in the raphe nuclei while showing lower binding in the projection areas.[47] We expected a successful distinction of G homozygotes and C carriers based on the PET data even if effects of rs6295 should not be demonstrable with classical statistical approaches.

MATERIALS AND METHODS

Subjects

This is a pooled sample derived from previous studies, however, all genetic data regarding rs6295 are unpublished.[48–53] Eighty-one subjects aged 18–65 years were enrolled in this neuroimaging genetics study with a cross-sectional design. Sixty-two healthy subjects (40 female) and 19 acutely depressed patients (6 female) diagnosed according to Structured Clinical Interview for DSM-IV type disorders (SCID I+II) were included. Severity was assessed for a subsample of patients using the Hamilton Depression Rating Scale ($n = 5$, HAMD: 19.6 ± 3.4, mean ± s.d., all $\geqslant 16$) and four patients suffered from generalized anxiety disorder as well. Baseline characteristics of the sample can be found in Supplementary Table 1. All the subjects were measured with [*carbonyl*-^{11}C]WAY-100635 and underwent a thorough physical and neurological examination, assessment of clinical history, ECG, routine laboratory analysis, urinary drug and pregnancy screening. All the subjects were required to be free of any psychotropic medication at least 3 months before enrollment and no severe somatic condition nor other neuropsychiatric diagnose except anxiety disorders were tolerated. Lifetime administration of neuropsychiatric medication was not registered. Written informed consent after detailed oral information concerning all the study procedures was mandatory for all the subjects. The study and all related procedures were approved by the Ethics Committee of the Medical University of Vienna.

Genotyping

Genotyping was performed as previously described.[48] Shortly, 9 ml ethylene-diamine-tetraacetic-acid blood samples were collected from each subject and DNA was isolated from whole blood via QiaAmp DNA blood maxi kit (Qiagen, Hilden, Germany). Genotyping was performed using the iPLEX assay on the MassARRAY MALDI-TOF mass spectrometer as described previously.[54] Allele-specific extension products were identified and genotypes allocated by Typer 3.4 Software (Sequenom, San Diego, CA, USA). For genotyping quality criteria, a single-nucleotide polymorphism call rate over 99% was required. Blood samples for genotyping were anonymized to ensure blinding.

Radiochemistry and PET procedures

Radiosynthesis of [*carbonyl*-^{11}C]WAY-100635 and all scans were performed at the Division of Nuclear Medicine of the Department of Biomedical and Image-guided Therapy of the Medical University of Vienna. One PET scan (General Electric Medial Systems, Milwaukee, WI, USA) was conducted per subject using the tracer [*carbonyl*-^{11}C]WAY-100635, which has high affinity and selectivity for the 5-HT_{1A} receptor. For a detailed description of the synthesis, please see ref. 55. Concerning measurement procedures, first a 5 min transmission scan using a retractable ^{68}Ge rod source for tissue attenuation correction was performed. Subsequently, the dynamic emission scan was acquired in three-dimensional mode. Mean injected dose was 312.04 ± 105.84 MBq, specific activity at the time of injection was 285.47 ± 251.22 GBq μmol^{-1} and radiochemical purity was above 95%. Reconstruction of the data was performed for 35 transaxial sections (128×128 matrix) using an iterative filtered back projection algorithm (FORE-ITER). The spatial resolution was 4.36 mm full-width at half maximum 1 cm next to the center of the field of view. Magnetic resonance images were acquired from all the participants for co-registration using a 3-Tesla Philips scanner (Achieva) and three-dimensional T_1 FFE-weighted sequences, yielding 0.88 mm slice thickness and in-plane resolution of 0.8×0.8 mm.[56]

For better image quality, during the PET scans, subjects were placed with their head parallel to the orbitomeatal line guided by a laser beam system to ensure full coverage of the neocortex and the cerebellum in the field of view. A polyurethane cushion and head straps were used to minimize head movement and to guarantee a soft head rest during the whole scanning period.

Data preprocessing

PET preprocessing was done in SPM8 (Wellcome Trust Centre for Neuroimaging, London, UK, http://www.fil.ion.ucl.ac.uk/spm/) as described previously.[57] Personnel involved in data preprocessing was blinded to the subjects' genotype or diagnosis. After realignment to the motion-free mean image, scans of the entire time series were summed up and spatially normalized (affine regularization, average-sized template) to a tracer-specific template within standard MNI-space (Montreal Neurological Institute). Thereafter, the resulting transformation matrix was applied to each time frame.

We assessed *in vivo* target structure density as indexed by 5-HT_{1A} receptor binding potentials (BP_{ND}), which represent the ratio at equilibrium of specifically bound radioligand to that of non-displaceable radioligand in tissue.[58] All binding potentials were computed using the voxel-wise modeling tool in the PMOD 3.509 software package (PMOD Technologies, Zurich, Switzerland) and applying the two-parameter linearized reference tissue model (MRTM2).[15]

We modeled 5-HT_{1A} BP_{ND} as previously described by our group using the insula as receptor-rich region and the cerebellum as receptor-poor

region.[15] The cerebellar gray matter excluding cerebellar vermis and venous sinus served as reference region. Mean overall BP_{ND} and mean cerebellar BP are listed in Supplementary Table 1. Regions of interest (ROI) were taken from an automated anatomical labeling-based atlas after normalization of BP_{ND} maps to standard MNI-space, except for the dorsal raphe nuclei (DRN), which were located manually in PMOD due to known difficulties of automated detection for this ROI.[59] The values were averaged across both hemispheres. Due to inherent smoothness of PET data of the scanner and temporary smoothing during normalization, we did not smooth during statistical processing.

Statistical analysis

All statistics were performed using the statistical software 'R 3.3.3' (cran.r-project.org). Analyses were performed for the combined sample of 19 patients and 62 healthy controls with group considered as a factor for all models. If group showed a significant effect, models were also generated for the subgroups of healthy and MDD subjects only. Concerning genotype, GG allele carriers were compared with CC and CG allele carriers to maximize the sensitivity for the region-specific alterations in binding potential. Based on previous research, we expected G homozygotes to be affected by transcriptional dysregulation comparable to knockout studies of Deaf1.[31] Furthermore, we divided all normalized BP_{ND} values with BP_{ND} of the DRN, resulting in a value further referenced as BP_{Div}. As the DRN is expected to show opposite influence from rs6295 than all other ROIs, this measure was undertaken to increase sensitivity for effects in projection areas, which have not been demonstrable in previous PET studies. An additional rationale behind this procedure that we had successfully applied for a prediction model before was to get rid of interpersonal variance in 5-HT_{1A} receptor binding, which could confound genotype effects.[51]

Differences of 5-HT_{1A} BP_{Div} between *HTR1A* C(-1019)G genotype were computed using a ROI approach. Differences between genotype groups (GG vs C carriers) were calculated with a linear mixed model as provided by the 'lmne' package of 'R'.[60] Thereby, subject (between-subject factor) and ROI (within-subject factor) served as the random factors and *HTR1A* rs6295 genotype status, group, ROI, age and sex served as fixed factors. In total, 46 ROIs were integrated in the model. Significance was determined by $P < 0.05$, Bonferroni corrected. Based on these results, *post hoc* analyses were performed for all ROIs to further specify significant mixed-model effects. For *post hoc* analysis, a *P*-value threshold of 0.05 was determined.

Furthermore, we conducted a machine-learning classification using the 'randomForest' (RF) and 'e1071' (SVM) package for the statistical software 'R'.[61] For machine-learning classification, a ROI-based and a voxel-wise model were computed.

RF assigns importance values to the predictors based on their usefulness for the classification model, disregarding classical main or interaction effects. Thereby, the most helpful variables for classification of the genotype show the highest importance values measured by mean decrease in Gini. No power calculation for RF has been established so far, however, recent investigations point toward sufficient reliability even with the number of predictors outreaching the observations, with limitations regarding sufficient patient counts and missing data.[46,47] For the ROI approach, BP_{Div} was used for classification. The voxel-wise approach, however, would surpass the computational capabilities of the machine-learning algorithm. To reduce the number of features without *a priori* selection, we decided to increase the voxel ranges from $2 \times 2 \times 2$ mm to $4 \times 4 \times 4$ mm, thereby reducing the number of predictors to 18 050. To address interpersonal variation in 5-HT_{1A} binding for this approach, we normalized BP_{ND} values for all 18 050 voxels by transforming them to values within a range from 0 to 1, thereby attributing 1 to the highest and 0 to the lowest BP_{ND} of a specific subject. Finally, after variable importance was calculated, the subjects were divided into training and test sets for the prediction of genotype based on BP_{ND} using a 10-fold cross-validation design as implemented in 'rfcv' function of 'randomForest'.

RF was chosen as the primary algorithm for this analysis as we expected highest variable importance in the DRN. Importance measurements might therefore be a useful alternative for automated or manual labeling of the DRN. However, as there is no clear recommendation as to which specific machine-learning algorithm to use for imaging genetics, we decided to also compute an exploratory model using SVM as implemented in the 'R' package 'e1071'.[62] Similar to RF, SVM is a machine-learning algorithm fit for classification and regression. Based on the construction of a discriminative hyperplane in high-dimensional space, successful classification depends on functional margin and generalization error. The implemented function 'tune.svm' was used to determine the hyperparameters cost and gamma, regulating bias and variance based on error penalty and the nonlinear kernel function. The optimal model was chosen from a range of $2^{-10 \text{ to } 1}$ for gamma and $2^{-1 \text{ to } 10}$ for cost. Other parameters were kept at default settings. A voxel-wise (normalized BP_{ND}) and ROI-based approach (BP_{Div}) similar to the RF analysis was conducted.

For the machine-learning analyses, separate models were computed for the patient and the control groups, as well as the combined sample.

RESULTS

The patients' sample showed lower age by an average of 7 years compared with the healthy subjects ($P = 0.666$) and more women were featured in the patients' group ($P = 0.037$). Allelotypes for rs6295 were in Hardy–Weinberg equilibrium for healthy as well as depressed subjects ($P < 1$). The G-allele was equally distributed in both the groups. For details on demographic parameters, please see also the Supplementary Section.

As can be seen in Figure 1, the DRN feature a higher average BP_{ND} in GG allelotype than in C allele carriers, even more so in the patients' group. Other ROIs show rather similar appearance of BP_{ND} for rs6295 genotype. Overall, the patient group exhibits slightly diminished BP_{ND} (mean BP_{ND} 2.53 ± 0.74 for patients vs 2.71 ± 0.7 for controls). For the patients' group, a graphic representation of BP_{Div} for each ROI can be found in Figure 2; for a boxplot of all ROIs and a table of mean BP_{ND} and BP_{Div} according to group and genotype, please see the Supplementary Section.

Mixed-model results

As expected, ROI showed significant effects in all mixed models ($P < 0.001$, corrected; $F = 641.663$ overall and 149.134 for the patients' subgroup). Furthermore, a three-way interaction effect could be demonstrated for ROI, group and genotype ($P = 0.019$, corrected; $F = 1.482$) as shown in Table 1, section A. No main or two-way interaction effect were found for group and genotype. Repeating the mixed model in the patients' and healthy group separately yielded no effect of genotype in healthy subjects. For the patients' group, no main effect but a significant two-way interaction effect between ROI and genotype was found ($P = 0.017$, corrected; $F = 1.511$). Please consider also Table 1, section B.

Further tracking this effect down, *post hoc* analyses produced differences between GG and C allele carriers in seven regions, also portrayed in Table 1, Section C: the fusiform gyrus ($P = 0.041$; $F = 4.920$), gyrus rectus ($P = 0.048$; $F = 4.543$), hippocampus ($P = 0.046$; $F = 4.609$), inferior occipital gyrus ($P = 0.044$; $F = 4.718$), parahippocampus ($P = 0.045$; $F = 4.679$), lingual gyrus ($P = 0.027$; $F = 5.849$) and supplementary motor area ($P = 0.049$; $F = 4.468$). Thereby, GG carriers showed diminished BP_{Div} in these regions, suggesting higher BP_{ND} in the DRN and reduced BP_{ND} in the projection areas. These effects are portrayed in Figure 3.

Machine-learning results

RF reached an accuracy around 0.725 for all samples (vs 0.750 for SVM) for classification of genotype regardless of voxel or ROI-based approach. The predictive power was severely limited by a sensitivity of only 0.2 (vs 0.1 for SVM). Therefore, no useful prediction of genotype could be achieved either with RF or SVM. For a comprehensive table of classification parameters, please see Table 2.

DISCUSSION

Applying conventional mixed model and more advanced machine-learning algorithms, namely RF and SVM, in a large sample of 62 healthy subjects and 19 MDD patients studied with PET and [*carbonyl*-^{11}C]WAY-100635, an effect of *HTR1A* rs6295

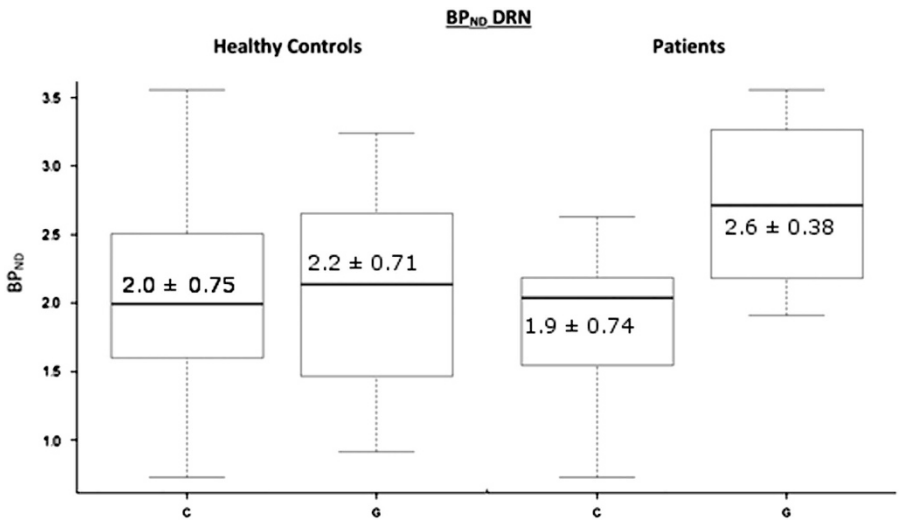

Figure 1. Boxplot for binding potential (BP_{ND}) for the dorsal raphe nuclei, showing BP_{ND} on the *y* axis. On the left, BP_{ND} for the healthy controls ($n = 62$) is portrayed. On the right side, BP_{ND} of the patients subgroup ($n = 19$) is shown. BP_{ND} is portrayed for merged CC and CG carriers as well as GG homozygotes to maximize the effect of the rs6295 polymorphism. Mean values are provided for each group and genotype. The difference in DRN BP_{ND} did not reach statistical significance. DRN, dorsal raphe nuclei.

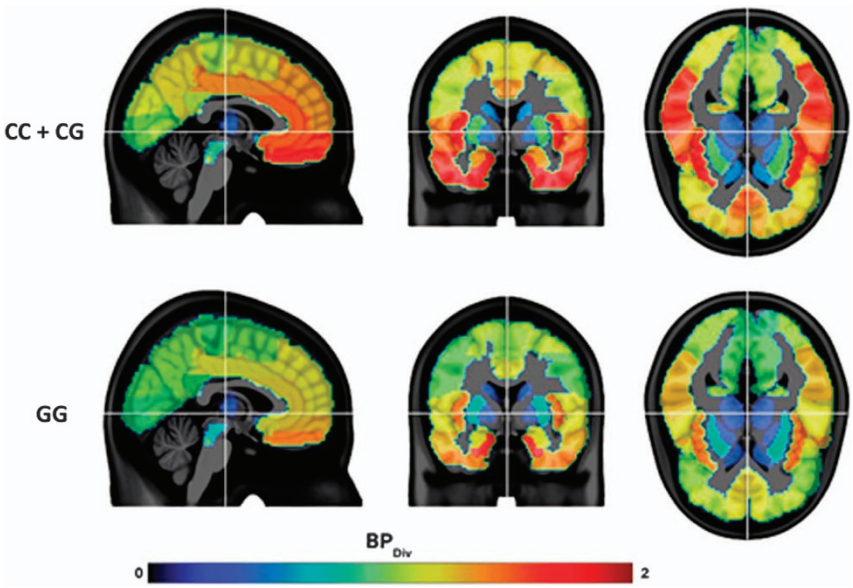

Figure 2. Average binding potential ($BP_{ND)}$ divided by BP_{ND} of the dorsal raphe ROI (BP_{Div}) for the patients' group ($n = 19$). The color bar represents BP_{Div} values ranging from 0 (blue) to 2 (red). G allele homozygotes ($n = 4$) are compared with the merged sample of C allele homozygotes and CG heterozygotes ($n = 15$). G allele homozygotes suffering from major depressive disorder show overall lower BP_{Div}.

genotype on BP_{Div} could be observed in the MDD group in seven ROI.

In contrast to many other polymorphisms, rs6295 has been extensively studied and the molecular mechanics of the C(-1019)G variation have been described in detail.[21,29] Deaf1 transcription factor is only available to the C but not G allele at the transcription site, resulting in a vastly diminished binding in GG carriers. As Deaf1 has locally divergent effects, with increased 5-HT_{1A} heteroreceptor activity in the serotonergic projection areas and decreased 5-HT_{1A} autoreceptors activity in the raphe nuclei, a robust effect of rs6295 carrier status should be demonstrable in PET imaging. Thereby, the divergent direction of 5-HT_{1A} manipulation in raphe ROI compared with other areas would be expected to highlight this effect despite interpersonal variation in 5-HT_{1A} binding, which has been shown to be a significant limitation in our previous studies.

Nevertheless, only differences in the DRN have been recognized by the two previous PET studies investigating rs6295 in MDD. A study published in 2006 suggested that DRN BP_F would increase with the number of G alleles and this finding was later confirmed in a second sample by the same group in 2012.[41,43] However, as in a recent replication study of the same group, no effect of rs6295 could be observed, they concluded that this polymorphism most likely does not affect BP_F considered separately.[44] Another study in depressed bipolar patients found higher BP_F in amygdala and hippocampus as well as DRN in G allele carriers.[63] Interestingly, the

Table 1. Mixed-model results, only significant results are shown

	DF numerator	*DF denominator*	*F-value*	P-*value*
(A) Predictor	*mixed model* (all)			
Genotype × ROI × Group	46	3404	1.482	0.005
ROI	46	3404	641.663	< 0.0001
(B) Predictor	*mixed model* (subgroups)			
Healthy subjects (n = *62)*				
ROI	46	2622	855.433	< 0.0001
Patients (n = *19)*				
ROI	46	690	149.134	< 0.0001
ROI × Genotype	46	690	1.511	0.006
(C) ROI	*post hoc analyses*			
Fusiform gyrus			4.92	0.041
Gyrus rectus			4.543	0.048
Hippocampus			4.609	0.046
Inferior occipital gyrus			4.718	0.044
Lingual gyrus			5.849	0.027
Parahippocampus			4.679	0.045
Supplementory motor area			4.468	0.049

Abbreviations: DF, degree of freedom; ROI, region of interest. For mixed model, only results withstanding correction for multiple testing are shown, *post hoc* analyses are uncorrected. (A) For all the subjects, as expected, ROI showed significant results. More importantly, a three-way interaction was found between groups, ROI and genotype that withstood correction for multiple testing. (B) Mixed-model results for patients and healthy subjects respectively, showing only significant results after correction for multiple testing. No effects of genotype were found in the healthy sample. Regarding the patients sample, an interaction effect of ROI and genotype could be demonstrated. (C) *Post hoc* analysis of variance (ANOVA) results for ROI, uncorrected. Seven regions were affected by rs6295 carrier status and effects were only present in the patients' group of the sample.

results of PET studies on 5-HT_{1A} seem to be dependent on the BP parameter investigated. BP_F has repeatedly been shown to be raised in MDD patients, whereas binding potential non-displaceable (PB_{ND}) usually was found diminished.[17,41–44,64,65,66] This has been explained by differences in reference region binding that impact BP_{ND}. Convergent with these positive PET findings in MDD, but divergent from the molecular fundamentals, we also did not detect significant differences in BP_{ND} in any serotonin projection areas. However, although not significant, differences in BP_{ND} have been more distinct in the MDD group, featuring a higher average BP_{ND} in the DRN of GG carriers as we had expected.

As has been suggested by a recent review on rs6295 in MDD, trait effects in healthy subjects have been lacking except for an association of impulsivity with the G allele and increased negative emotionality in a reward-punishment paradigm.[21] On the other hand, the G allele has been shown to be overexpressed in MDD, bipolar disorder and suicide victims, to modify response to selective serotonin-reuptake inhibitor in depressed patients and to alter reactions to various paradigms in functional magnetic resonance imaging. Recent findings in mouse models suggested a stress-mediated control of Deaf1 activity, which was shown to be decreased in chronically stress-exposed mice.[67] In conformity, the GG allelotype was indicated to stunt glucocorticoid response to stress and lead to overall susceptibility to stress in MDD and anxiety patients. In synopsis, these data suggest an important lifetime contribution of rs6295 to serotonin equilibrium and risk for mood disorders. Although these effects may be present in healthy subjects as well, they seem to be mostly relevant and visible *in vivo* after transition to patient status, most likely triggered by stress and negative life events. Therefore, it seems likely that the region-specific effect initially reported by the group of Lemonde, Czesak and Albert can only be observed in patients.[27,30] Difficulties to track these effects down in a clinical sample might derive from compensational regulation of 5-HT, increasingly so as in Deaf1 knockout mice a more prominent role of other factors as Pet and Freud1 and 2 that are usually overshadowed by Deaf1 has been reported by the same group.[27,31]

Based on the overall encouraging but still incongruous results, we decided to apply the ratio of ROI/DRN as alternative measure BP_{Div}. The ratio of serotonin core to projection areas has been successfully fielded before by our group, providing a better predictor for treatment response to selective serotonin-reuptake inhibitor than original BP_{ND}.[51] Here, we could show that GG carriers suffering from MDD displayed lower BP_{Div} in seven ROI, including 5-HT_{1A} mainstays as the hippocampus and parahippocampus as well as the fusiform gyrus, gyrus rectus, inferior occipital gyrus, lingual gyrus and supplementary motor area. Thereby, our results are conformable to the original proposal of Albert and colleagues, both demonstrating a higher BP in DRN and lower BP in cortical and projection areas, meaning lower BP_{Div} in GG allelotype carriers.[21] As we did not find significant effects of rs6295 on BP_{ND} but on BP_{Div}, this methodological difference might also explain negative findings by previous investigations.[44]

However, while our results clearly support previous findings from *in vitro*, *in vivo* animal and human samples, the question remains how a seemingly distinct connection as the rs6295 polymorphism in MDD can produce divergent results as shown in the PET studies performed so far on that topic. Even more surprisingly, the multivariate machine learning approach failed at deriving a classification model for allelotypes of rs6295. Multivariate analyses as RF and SVM should be able to translate the suspected divergent changes in BP_{ND} or BP_{Div} in an accurate classification of GG and C allele carriers. Given the significant results of our mixed-model analysis, the effects attributable to genotype might still be too delicate to enable successful engagement of a predictive algorithm. Arguing that effects could only be observed in the patient sample, only 19 subjects were disposable for RF and SVM, limiting the effectiveness of these algorithms. Also, only four patients exhibiting the GG alellotype were featured, which might be too low of a number to guarantee stable classification with high numbers of predictors. On the other hand, we still achieved an accuracy above random guessing. As importance measurement by RF could potentially provide an alternative labeling method for the DRN, we advocate further

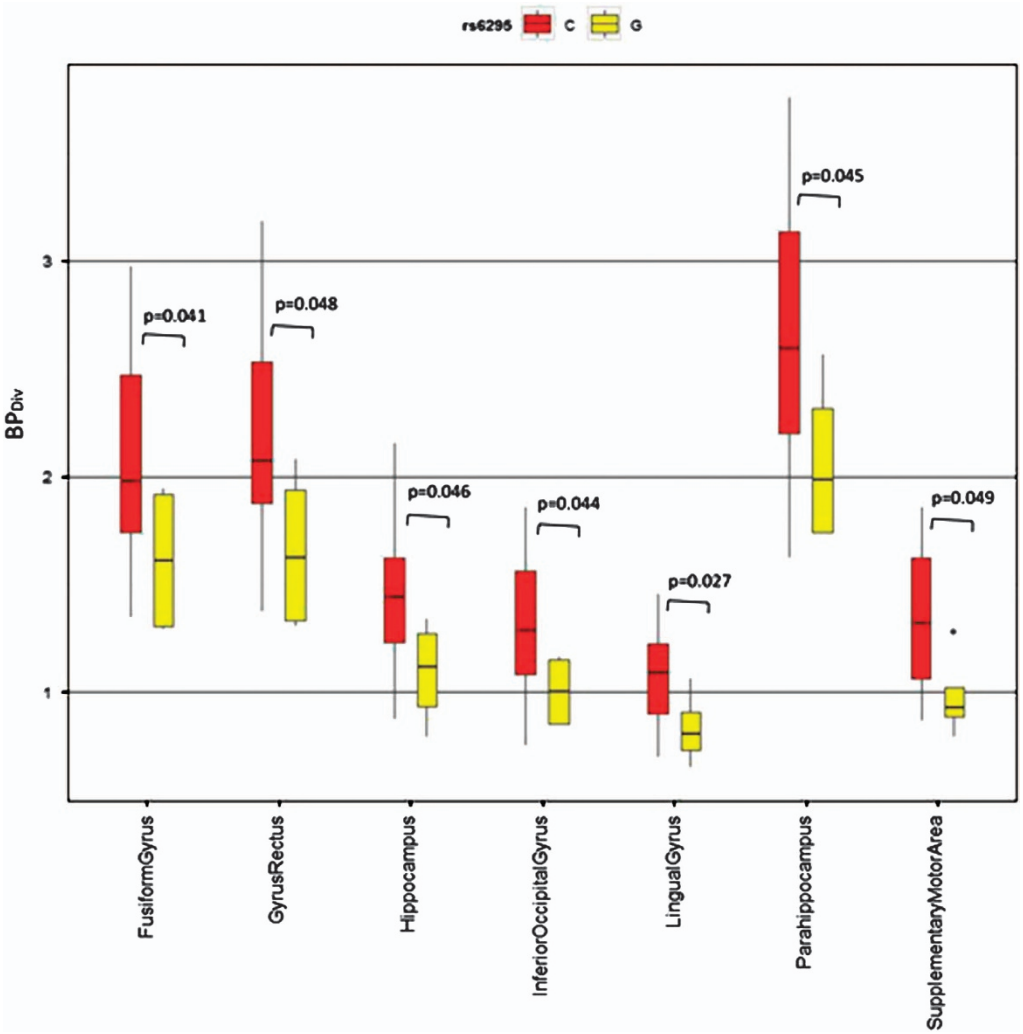

Figure 3. Boxplot showing the average binding potential ($BP_{ND)}$ divided by BP_{ND} of the dorsal raphe region of interest (ROI; BP_{Div}) for the patients' group ($n = 19$). On the *x* axis, ROIs reaching significance in *post hoc* analysis of variance (ANOVA) are shown, the *y* axis shows binding potential BP_{Div}. G allele homozygotes are colored yellow, C allele carriers red. *P*-values of the *post hoc* ANOVA (uncorrected) are shown for all ROI.

Table 2. Machine-learning results using RF and SVM

Type	*Sensitivity*	*Specificity*	*Accuracy*
RF			
ROI based ($n = 47$)	0.33	0.80	0.725
Voxel based ($n = 18053$)	0.20	0.90	0.733
SVM			
ROI based ($n = 47$)	0.10	0.945	0.750
Voxel based ($n = 18\,053$)	0.10	0.945	0.750

Abbreviations: RF, RandomForest; ROI, region of interest; SVM, support vector machines. Sensitivity is correct classification of GG allele carrier status. Classification was performed using voxel-wise as well as ROI data, only data for the combined sample analysis are shown. No useful model could be established.

research applying multivariate techniques as there is certainly a potential for future refinement.

Besides the already mentioned compensational effects that can be expected to bias results, other factors should be taken into account as well. Most importantly to our concern, epigenetic contribution as methylation status has been widely neglected so far in imaging genetics in MDD. Epigenetic variation is a distinctive feature in monozygotic twins who show discordant affection from MDD and several methylation markers were associated with MDD.[68,69] Also, methylation-dependent 5-HT_{1A} receptor upregulation was recently constituted, for example, mediated through an Sp4 site prone to stress-induced hypermethylation.[70] As Deaf1 activity has been shown to correlate with stress and life events, the C(-1019)G binding site might be inactive in some subjects, therefore resulting in erroneous grouping of genotype. Recent reviews in the field of genetics in MDD have strongly recommended to check for methylation effects to fathom the rampant ambiguity of association findings.[71,72]

Furthermore, as the compensational capability of the neurotransmitter system has been addressed before, the still fresh area of allosteric heteroreceptor complexes might be of relevance.[73] Regarding 5-HT_{1A} receptors, brain-derived neurotrophic factor and galanin receptor heteroreceptor complexes have been highlighted as possible key targets of MDD as well as therapeutic agents. Especially brain-derived neurotrophic factor–5-HT_{1A} autoreceptor complexes in the raphe nuclei might be of paramount

importance for serotonin equilibrium as the negative feedback of autoreceptors might be absorbed in a trophic boost effect.[74,75] Interaction effects of such complexes are hardly understood at this point, therefore possibly disguising results of single receptor approaches. Regarding recent methodological advances in PET imaging that will possibly cut short scanning time significantly by relying on a bolus/constant infusion technique, multi-receptor imaging studies could pave the way to a clearer understanding of the role of 5-HT_{1A} receptors and rs6265.[76,77]

Except for these considerations, some clear limitations of our study must be discussed. First, our sample comprises different study populations, resulting in different ratios of sex and age for MDD and healthy subject subgroups as no matching for these variables could be performed. Due to the resource-intensive nature of PET studies, this synoptic approach was necessary to gain a sufficiently large sample for our analyses. Most important, however, the patients' sample is featuring four subjects among the CG heterozygotes suffering from a comorbid anxiety disorder. As involvement of 5-HT_{1A} receptors has been shown to differ between anxiety disorders and MDD, also regarding the rs6295 polymorphism and the Hes1 and 5 transcription factors, this comorbidity might impede interpretation of our results. A recent review argued for elevated 5-HT_{1A} levels in adult anxiety opposed to low 5-HT_{1A} levels in MDD, pointing towards an early affection of low 5-HT in neonatal anxiety, related to Hes1 and 5, which are mostly active at this time of neuronal development.[78] On the other hand, one of the studies focused on rs6295 in MDD and anxiety suggested strongest associations in comorbid anxiety and depressive disorder.[79] Furthermore, while all subjects featured for this analysis were free of neuropsychiatric medication for at least 3 months before scanning, lifetime records concerning drug naivety were not available. Consequently, we cannot rule out bias of our results caused by previous medication. This might be of special importance as previous studies have argued that 5-HT_{1A} receptor binding is significantly impacted by previous medication up to at least 4 years.[43] Also, depression severity and sociodemographic or other clinical predictors could not be implemented in this analysis as they were not registered for most of the subjects. Hence, we cannot rule out that differences in severity or heterogeneity in sociodemographic parameters impacts our results.

Furthermore, even though we conducted this study featuring one of the largest samples regarding PET imaging genetics in MDD, only 19 patients were disposable for analysis. Although we could track down genotype effects using BP_{Div}, statistically no significant elevation of BP_{ND} in the raphe nuclei of GG carriers opposed to reduced BP_{ND} in projection areas could be observed for the patients' subgroup. Therefore, more resounding results could be expected in a larger cohort, benefitting from higher power. This might be even more relevant for machine-learning classification, which failed to provide useful prediction in this study. Even though RF allegedly works for samples with observations largely outnumbered by predictors, this robustness is supposedly only guaranteed with sufficient overall sample size.[47,80]

Considering these limitations, we cannot rule out considerable bias by comorbidity, demographics, differing severity or lifetime medication. On the other hand, this study introduces the application of BP_{Div} to limit some of the known issues with imaging genetics as interpersonal variance. Our results suggest that refined methodical and statistical arrangements can enhance detection of complex effects. Furthermore, even though no successful application could be performed in this study, we also believe that machine learning holds great potential that we adumbrated by our rationale and findings. Keeping in mind the limitations, we provide further evidence for the important role of the rs6295 polymorphism in affective disorders using PET imaging and [*carbonyl*-11C]WAY-100635. Our results are overall in line with preclinical data, mouse model knockout studies as well as previous clinical analyses, demonstrating the two-pronged effect of the G allele on 5-HT_{1A} BP_{Div} for, we believe, the first time. Future endeavors should also address epigenetic effects and allosteric heteroreceptor complexes, possibly by scanning for multiple targets, and replication in larger samples of MDD patients is necessary to further substantiate our findings.

CONFLICT OF INTEREST

SK received grants/research support, consulting fees and/or honoraria within the last 3 years from Angelini, AOP Orphan Pharmaceuticals AG, AstraZeneca, Eli Lilly, Janssen, KRKA-Pharma, Lundbeck, Neuraxpharm, Pfizer, Pierre Fabre, Schwabe and Servier. RL received travel grants and/or conference speaker honoraria from AstraZeneca, Lundbeck A/S, Dr Willmar Schwabe GmbH, AOP Orphan Pharmaceuticals AG, Janssen-Cilag Pharma GmbH and Roche Austria GmbH. Without any relevance to this work, WW received speaker honoraria from GE Healthcare, research grants from DSD, BSM Diagnostica and ABX and is a part-time employee of CBmed GmbH (Graz, Austria). Without any relevance to this work, MM received speaker honoraria from GE Healthcare. PB-M received a travel grant from AOP Orphan Pharmaceuticals AG and speaker honoraria from Janssen. CK has received travel grants from Roche Austria GmbH and AOP Orphan. GSK received travel grants from Roche and Pfizer. The remaining authors declare no conflict of interest.

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