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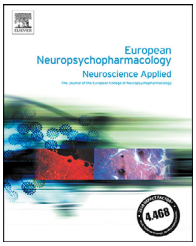

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DNA-methylation of the dopamin receptor 2 gene is altered during alcohol withdrawal

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Abstract
The dopaminergic neurotransmission is known to be of crucial importance in addictive behavior. Epigenetic regulation like methylation of DNA influences the function of dopaminergic transmission. The present study investigated alterations of DNA methylation in the dopamine D2 receptor (DRD2)-gene in patients suffering from alcohol dependence. The study sample consists of 99 alcohol dependent males admitted for alcohol withdrawal treatment and a control group of 33 healthy participants. Blood samples underwent bisulfite sequencing to determine levels of DNA-methylation of the promoter region of the DRD2 gene. Mixed linear modeling was used to test differences between patients and controls, course of methylation during detoxification. While DRD2-gene methylation did not differ significantly between patients and controls, we found a significant increase of DRD2-gene methylation during alcohol withdrawal/early abstinence. Craving, measured with the Obsessive Compulsive Drinking Scale (OCDS), was significantly associated with DRD2-gene methylation. Furthermore, smoking significantly

Abbreviations: AD, alcohol dependence; DAT, dopamine transporter; DRD2, dopamine receptor D2; DNA, deoxyribonucleic acid; mRNA, messenger ribonucleic acid; CpG, cytosine-phosphate-guanine; DSM-4, diagnostic and statistical manual of mental disorders, 4th edition; PCR, polymerase chain reaction; EMM, estimated marginal means; SE, standard error; OCDS, obsessive compulsive drinking scale.

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influenced DRD2-gene methylation in both, patients and controls. As in other types of addictive disorders, DRD2-gene methylation is altered during alcohol withdrawal/early abstinence. The findings regarding an association with alcohol craving and tobacco consumption point towards a crucial role of DRD2-gene methylation in the neurobiology of addictive behavior.

1. Introduction

Dopaminergic circuits, particularly in the mesolimbic-striatal-prefrontal pathways, are known to play a crucial role in the genesis and in the maintenance of addictive behavior. This applies for different addictive disorders, including alcohol dependence and tobacco dependence as well as non-substance use disorders like pathologic gambling (Balfour, 2015; Boileau et al., 2013; Charlet et al., 2013). However, the mechanisms that influence the alterations in the central dopaminergic neurotransmission are still subject to intensive research. One important factor influencing these pathways may be the altered availability of dopamine receptors due to epigenetic changes in gene expression. The role of epigenetic changes has been studied not only in the neurobiology of addictive disorders but in many other psychiatric diseases, many of them occurring as frequent comorbidities in alcohol dependence. This includes affective disorders, anxiety disorders, non-substance dependent addictive behaviors, psychotic diseases as well as personality disorders (Domschke et al., 2013; Hannon et al., 2016; Hillemacher et al., 2015; Tadic et al., 2014; Teschler et al., 2016). Possible epigenetic alterations include changed DNA-methylation patterns in specific gene regions like particularly the promoter region but also histone modification processes, which are able to influence chromatine structure and subsequent transcription processes in specific genes (Robison and Nestler, 2011; Rodenhiser and Mann, 2006). In alcohol dependence, different studies have focused on the role of changes in DNA-methylation in dopaminergic genes. Thus, DNA-methylation of the dopamine transporter (DAT) gene was shown to be significantly increased at the beginning of alcohol withdrawal, compared to healthy controls (Hillemacher et al., 2009). Also, this study found a negative association between methylation of the DAT-gene and alcohol craving, measured with the Obsessive Compulsive Drinking Scale (OCDS). A recent study of Jasiewicz and colleagues described methylation changes in a particular Cystosine-phosphate-Guanine-(CpG)-position in the DAT promoter in alcohol dependent patients versus healthy controls (Jasiewicz et al., 2015). Another study found no differences in DAT promoter methylation in alcohol dependent patients compared to healthy controls but a trend towards an association between lower DAT methylation levels and elevated craving (Nieratschker et al., 2014). However, the authors state that regarding their results, this finding may be influenced by age, as older patients showed higher methylation levels. Most recently, we were able to replicate the association found between DAT methylation and alcohol craving and furthermore showed that DAT methylation was associated with amygdala activation (BOLD response) during a cue reactivity task in the fMRI (Wiers et al., 2015).

A recent investigation from our group described alterations in DNA methylation of the dopamine-2 receptor gene (DRD2) in subjects with pathologic gambling continuing with gambling behavior compared to subjects able to abstain, which points towards an involvement of DRD2 methylation changes in addictive behavior (Hillemacher et al., 2015). Also, we have found differences in DRD2 methylation between women with eating disorders and healthy controls and in Gilles-de-la-Tourette-syndrome (Frieling et al., 2010; Muller-Vahl et al., 2017).

Recent studies describe an association of DRD2 methylation and alcohol problem severity in a community sample (Hagerty et al., 2018). Also DRD2 methylation was associated with striatal activation in response to alcohol-associated reward cues (Bidwell et al., 2019). Recent studies also show that epigenetic aging and specifically DNA methylation is accelerated in subjects suffering from alcohol dependence (Rosen et al., 2018). Also, a recent investigation showed that genome-wide methylation changes in alcohol dependence (compared to healthy controls) at least partially reverse during short-term abstinence (Philibert et al., 2014). In a further recent approach, Philibert and co-workers were able to show the discriminative ability of two small methylation based marker sets to quantify alcohol use status in patients (Philibert et al., 2018).

Thus, changes in DNA methylation of specific genes in alcohol dependence are an interesting target to identify specific mechanisms in the neurobiology of addictive behavior and deserve further attention. Aim of the present investigation was to analyze changes in DNA-methylation in the DRD2 the promoter region compared to healthy controls.

2. Experimental procedures

2.1. Alcohol-dependent patients and control group

The present study is part of a large prospective research project on neurobiological mechanisms in alcohol dependence (NENA: Studies in Neuroendocrinology and Neurogenetics in Alcoholism), which was approved by the local Ethics Committee of the University of Erlangen-Nuremberg and complies with the declaration of Helsinki. Written, informed consent was obtained from every participant. All patients suffered from alcohol dependence according to the International Classification of Diseases (ICD-10) and the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) and were included in the study after admission for detoxification treatment to the Hospital for Psychiatry, Psychotherapy and Psychosomatics, Obermain, Germany. A detailed physical examination as well as a routine laboratory testing and urine drug screening were assessed in all patients. Patients with concomitant psychiatric illnesses, other substance abuse apart from alcohol or nicotine, cerebral ischemia, cerebral hemorrhage, epilepsy, cardiovascular and renal diseases were not included in the study. The extend of alcohol craving was

Table 1 Demographic characteristics of the study population.

	Patients ($n = 99$)		Controls ($n = 33$)		T	df	P
	Mean	SD	mean	SD			
Age [years]	43.15	8.24	44.06	11.46	−0.412	40.18	0.683
BMI [kg/m²]	25.02	4.26	25.06	2.82	−0.066	72.59	0.948
Years of Drinking [years]	9.54	8.00					
Dayli intake [g]	190.65	82.24					
OCDS day 1	19.76	6.90					
OCDS day 7	10.49	7.30					
OCDS day 14	9.72	6.55					
BDI day 1	16.51	8.93					
BDI day 7	7.85	7.42					
BDI day 14	5.50	6.85					
% smoker	83.5%		27.3%				<0.001*

t-test (two-sided, Levene's correction for inequal variances.
* Fisher's exact test.

assessed using the Obsessive Compulsive Drinking Scale (OCDS) and depressive symptoms were monitored using the Becks Depression Inventory (BDI) at the beginning of in-patient withdrawal treatment (day 1), after seven (day 7) and 14 days (day 14) of treatment. The OCDS is a self-rating questionnaire (Anton et al., 1995, 1996) which allows to calculate a total score for obsessive-compulsive craving, ranging from 0 to 40 points. In the present study we included 99 male patients and 33 healthy controls. Recruited healthy controls were all male, not-abstinent, social drinkers. Alcohol dependence or risky drinking in the healthy control group was excluded by clinical interviewing and assessment of the Alcohol Use Disorder Identification Test (AUDIT), using an AUDIT-score <8 as reference for absence of risky drinking/dependence. Sociodemografic data and statistical differences between groups are given in Table 1.

2.2. Laboratory measurements

Fasting blood samples for DNA extraction were drawn at day 1, day 7 and day 14 of withdrawal treatment. Genomic DNA was extracted from whole frozen EDTA-blood with the NucleoMag® 96 Blood Kit (Macherey Nagel, Dueren, Germany) according to the manufacturer's protocol. Afterwards, 500 ng of genomic DNA were modified by sodium-bisulfite using the EpiTect® Bisulfite Kit (QIAGEN AG, Hilden, Germany). Sodium-bisulfite deaminates cytosines in CpG dinuleotides to uracils, whereas methylated cytosines are protected from alteration.

Primers were designed to amplify a region upstream and within exon 1 of DRD2 gene. PCR was conducted using HotStarTaq Master Mix Kit (Qiagen, Hilden, Germany). Cycle conditions, primer sequences as well as fragment sizes and chromosomal position are listed in supplementary Tables 1 and 2. Subsequently each PCR product was visualized on a standard 2.0% agarose gel, followed by purification using Agencourt AMPure XP beads (Beckman Coulter GmbH, Krefeld, Germany). Sequencing was performed using a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. A maximum concentration of 30 ng purified PCR product was applied for the extension reaction. After dye-terminator removal with Agencourt CleanSEQ System (Beckman Coulter), the products were analyzed on an Applied Biosystems® 3500×L DNA Analyzer (Applied Biosystems).

The obtained sequences were analyzed using the ESME software package that determines the DNA methylation levels from the sequence trace files. ESME performs quality control, normalizes the signals, corrects for incomplete bisulfite conversion, aligns generated bisulfite sequence and reference sequence to compare *C* to *T* values (forward sequence) and *G* to *A* values (reverse sequence) peaks at CPG-sites (Lewin et al., 2004).

2.3. Statistical analysis

Bisulfite sequencing yielded methylation values of 60 single CpG sites in the DRD2 gene. We performed initial quality checks to exclude potentially unreliable measurements: (a) all obtained sequences were screened for sequencing quality using ABI sequence scanner (Applied Biosystems). Samples with a QV-value below 20 were measured again. For the final analysis, all sequences were above the QV threshold of 20. (b) Individual CpG sites with more than 5% missing values were excluded from the analysis, leaving 51 CpG sites, which furthermore all showed an inter-individual variance above 0.001. All study participants had less than 10% missing values. Mixed linear modeling (REML) was performed computing methylation as dependent and (I) group (patients vs. controls), CpG position, group × CpG interaction and age and smoking status (yes/no) as fixed effects. CpG position was entered as repeated measurement assuming a scaled identity covariance structure.

To test the effect of withdrawal on DNA methylation, we used mixed linear modeling (II) in patients only, computing methylation as dependent variable and CpG, timepoint, CpG × timepoint interaction and age and smoking as fixed effects. CpG and timepoint were computed as repeated measurements assuming a scaled identity covariance structure.

Estimated marginal means were calculated for group (model I) or timepoint (model II) and compared by Bonferroni-corrected post-hoc test. Parameter estimates were calculated for all factors and manually inspected. We found that most of the significant differences in the parameter estimates at single CpG level were found in CpGs within exon 1. Therefore, we calculated all models for the whole fragment and for the promoter region and the exon 1 separately. Model I was repeated for all three time points, re-using the control information for every time point; to handle this aspect, we adapted the significance level for these comparisons to $\alpha = 0.05/3 = 0.016$.

To test for a possible association between DRD2 methylation and alcohol craving (measured with the OCDS) we calculated mixed linear models (III) computing OCDS score as dependent and CpG and methylation within CpG (methylation (CpG)) as fixed effects. To prevent inflation of covariate effects, we did not include age or smoking into this model. CpG position was entered as repeated measurement assuming a scaled identity covariance structure. Model III was repeated for all three timepoints. As we analyzed patients only, we did not adapt the significance level in this case.

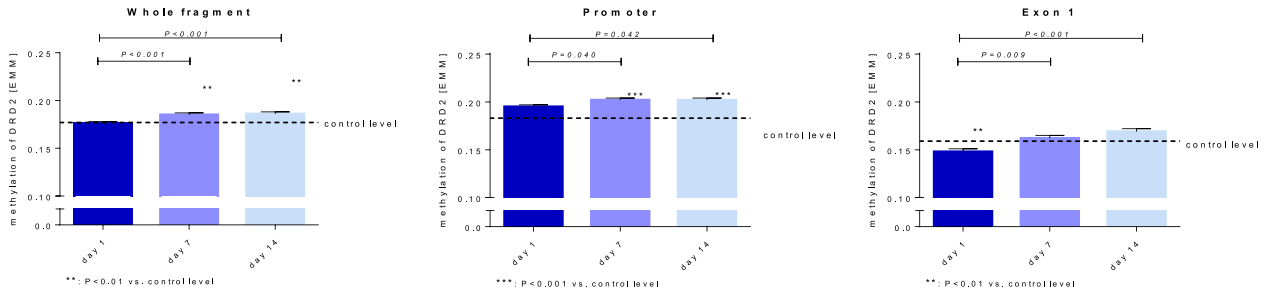

Fig. 1 Alteration of DRD2-gene methylation during withdrawal/early abstinence.
Control level: DRD2-gene methylation in the healthy control group; EEM: estimated marginal means; details of the statistical analysis are described in the results section.

Parameter estimates were calculated. Again, the majority of CpGs showing an association with craving was located in exon 1 therefore we repeated the models for promoter and exon separately. To further analyze possible association we performed multiple linear regressions (ENTER method) using craving (OCDS) as dependent variable and mean methylation or promoter or exon 1 mean methylation, respectively as predictor as well as age, BDI sum score and daily alcohol intake as further predictors, as these were correlated with alcohol craving. As a correlation between methylation and OCDS was only present at day 1, we did not repeat the linear regression for the other timepoints.

To further investigate the influence of smoking, we performed another set of mixed linear models (IV) computing methylation as dependent variable and CpG, group, smoking, CpG × group interaction, CpG × smoking interaction and group × smoking interaction as well as age as fixed effects. Repeated measurements, estimated marginal means and parameter estimates were calculated as described above. As no specific distribution of smoking related differences across CpGs was found, we performed model IV for the whole fragment only. For the last mixed linear model (V) we computed, again, methylation as dependent variable and CpG, age, number of substance use disorders (SUD) (0 = no substance use, 1 = either alcohol or tobacco use, 2 = alcohol and tobacco use) and CpG × number of SUD interaction. For all analyses if not otherwise stated, a *P*-value below 0.05 was considered significant. All analyses were performed using IBM SPSS™ 24 for Windows and GraphPad Prism 7.0.

3. Results

3.1. Differences between patients and controls

Comparing baseline methylation levels between patients and healthy controls (model I), we found no effect of diagnosis ($F_{(1,5100)} = 0.56$; $P = 0.45$), but significant effects of age ($F_{(1,5100)} = 145.83$; $P < 0.001$), tobacco consumption ($F_{(1,5100)} = 26.22$; $P < 0.001$), CpG position ($F_{(46,5100)} = 49.90$; $P < 0.001$) and CpG * diagnosis interaction ($F_{(46,5100)} = 2.79$; $P < 0.001$). When repeating the analysis for promotor and exon 1 harbored CpGs separately, we found no significant effect of diagnosis in the promotor region ($F_{(1,3479)} = 2.13$; $P = 0.145$). In exon 1, patients had a significantly lower methylation than controls ($F_{(1,1619)} = 9.84$; $P = 0.002$).

3.2. Effect of withdrawal on DRD2 methylation

During withdrawal (model II), DRD2 methylation significantly increased in the patients ($F_{(2,10,668)} = 10.71$; $P < 0.001$) and a significant CpG*timepoint interaction occurred ($F_{(92,10,668)}$=2.09; $P < 0.001$). Testing for differences between patients methylation and control levels (repeating model I at every timepoint, see statistical analysis section), alcohol dependent subjects had significantly higher methylation levels after 7 days as well as at the end of detoxification treatment (day 7: $F_{(1,4951)}$=9.43; $P = 0.002$; day 14: $F_{(1,4819)}$=7.38; $P = 0.007$). Analyzing the promoter and exon part of the fragment separately, we found that on day 7 and day 14 significant differences between patients and controls were only present in the promoter region, while no significant differences were found for exon 1 (Fig. 1).

3.3. Association with alcohol craving

To test a possible influence of DRD2 methylation on alcohol craving, we computed another set of mixed linear models (model **III**) as specified in the methods section. We found a significant effect of methylation (CpG) on OCDS score ($F_{(47,3747)} = 1.657$; $P = 0.003$) on day 1. Inspecting parameter estimates, it occurred that 4 out of 5 CpG sites with a significant association with craving were located in exon 1. Therefore, we repeated the analysis for the promoter part and the exonic part of the fragment. A significant association between methylation and craving was only present in exon 1 ($F_{(15,1188)}$=2.53; $P = 0.001$, Fig. 2). No significant association of the whole fragment nor of the promoter or exon was found on days 7 and 14.

We used multiple linear regression analysis to further explore the association of DRD2 methylation and alcohol craving. For the whole fragment, we found a trend towards significance in the final model including age, daily intake of ethanol and self-rated depression (BDI). Using instead of the whole fragment's mean methylation only the mean methylation of exon 1, we found a significant and independent negative influence of methylation on alcohol craving (Table 2a and b), while the mean promoter methylation had no significant effect *(data not shown)*.

3.4. Effect of smoking

Patients and controls currently smoking had higher DRD2 methylation than non-smokers (model IV) ([*estimated marginal means* (*standard error*)]: non-smokers: 0.167(0.003) vs. smokers: 0.184(0.003)).

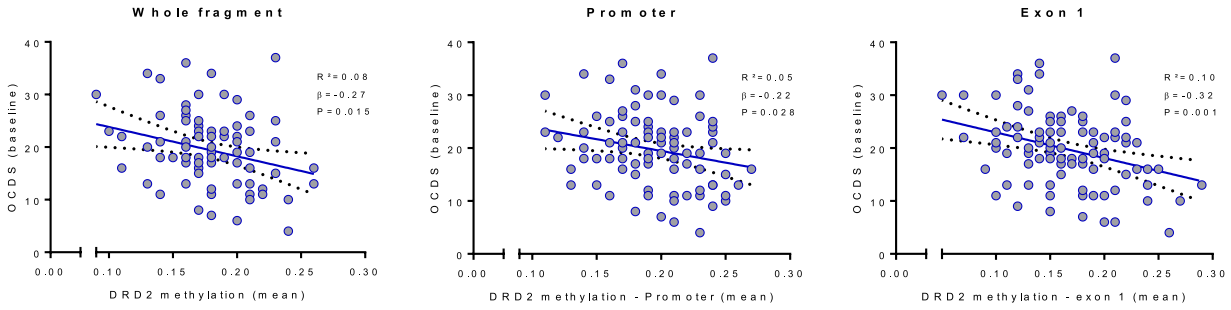

Fig. 2 Association of DRD2-gene methylation with craving.
Time point day 0 (admission for withdrawal treatment); OCDS: Obsessive Compulsive Drinking Scale; details of the statistical analysis are described in the results section.

Table 2a Regression analysis/whole gene fragment.

	Beta	*T*	*P*
Constant		3.63	<0.001
Age	−0.27	−0.25	0.801
BDI	0.28	2.81	0.006
Daily ethanol intake	0.27	2.64	0.010
Mean methylation whole DRD2 fragment	−0.20	−1.99	0.051

Dependent variable: OCDS sum score. $F_{(4)} = 6.93$; $P < 0.001$; R^2 adj. = 0.210.

Table 2b Regression analysis/whole gene fragment.

	Beta	*T*	*P*
Constant		3.75	<0.001
Age	−0.03	−0.32	0.748
BDI	0.25	2.62	0.010
Daily ethanol intake	0.25	2.55	0.013
Mean methylation whole DRD2 fragment	−0.24	−2.37	0.020

Dependent variable: OCDS sum score. $F_{(4)} = 7.47$; $P < 0.001$; R^2 adj. = 0.225.

At the end of detoxification treatment, alcohol dependent patients who were also smoking had the highest DRD2 methylation levels; non-smoking patients and controls who were smoking showed similar levels and non-smoking controls had the lowest DRD2 methylation (Fig. 3). There were no differences between the promotor and the exonic parts of the fragment.

4. Discussion

The results of our investigation are in line with other results regarding alterations of DRD2 methylation in other addictive diseases. As stated above, a previous study from our group showed that subjects with a lifetime history of pathologic gambling able to abstain had significantly lower DRD2 methylation levels than subjects continuing gambling (Hillemacher et al., 2015). In the present study, we found that patients undergoing alcohol withdrawal showed an increase of DRD2 methylation during early abstinence, particularly in the promoter region of the DRD2 gene. Furthermore, results show a significant negative association of DRD2 methylation with obsessive-compulsive alcohol craving at the beginning of withdrawal treatment (withdrawal craving), using multivariate statistics particularly in the exon 1 fragment. While the specific regulatory function of exon 1 remains mainly unrevealed, it can be hypothesized that it may act as a negative regulatory element in the transcription process. These findings underline that alterations in dopaminergic gene methylation like DRD2 may contribute to addictive behaviors like craving, at least in specific regulatory gene fragments. Interpretation of the association of different regulatory gene fragments (exon 1, promoter region) with specific features of addictive behavior (alcohol withdrawal, obsessive-compulsive craving) however remains difficult. It can be hypothesized that the dopaminergic transmission as well as its interaction with other neurotransmission circuits may be regulated by different regulatory fragments in the DRD2 gene. Overall, a more extensive research including genome-wide methylation analysis may help to receive a more complete picture of the role and interaction of the different regulatory fragments and their association with specific behaviors in addiction. The lack of association with the OCDS at the other time points during withdrawal is not surprising as specifically so called primary craving (during abstinence) can be supposed to be an even more complex psychological feature compared to secondary craving (during withdrawal). Due to the important neurobiological alterations in the dopaminergic and glutamatergic neurotransmission during detoxification, craving during withdrawal may have a more prominent neurobiological basis (Bauer et al., 2013). Additionally, in both groups (alcohol dependent patients and healthy controls) smoking participants had significantly higher DRD2 methylation patterns than non-smoking subjects. While this finding is limited by its exploratory nature, it contributes to the hypothesis that DRD2 methylation patterns may impact addictive behavior in general, including both substance and non-substance addictions.

Our finding of “normal” DRD2 methylation during alcohol intake followed by an increase in DRD2 methylation during withdrawal fits well into the Chromatin Remodeling Model of alcohol dependence discussed in a recent seminal paper by Berkel and Pandey (2017) . In brief, they assume that acute ethanol intake blocks the DNA methyltransferases (DNMTs) leading to a *hypo*methylation and relaxed chromatine.

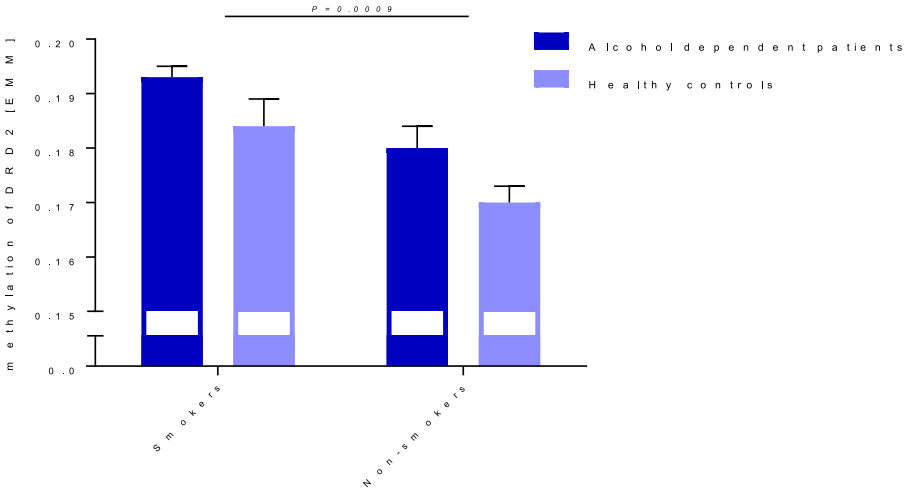

Fig. 3 Association between DRD2-gene methylation and smoking status.
Smokers vs. non-smokers after the end of detoxification treatment. *P*-value derived from mixed linear modeling (contrast: smokers vs. non-smokers; contrast: patients vs. controls: $P = 0.0177$). EMM: estimated marginal means; details of the statistical analysis are described in the results section.

Under the circumstance of chronic ethanol intake, expression of DNMTs is upregulated to counteract the direct effect of ethanol on DNMT resulting in a "normal" methylation pattern. If alcohol is withdrawn, following this model one can expect an increase in methylation due to the high expression of now functional DNMTs resulting in gene-specific *hyper*methylation like our observation in DRD2.

The present study, however, suffers from some limitations limiting the interpretation and generality of the described findings. First, the follow-up time of this longitudinal study is short, covering only the first two weeks of early abstinence. A longer follow-up period would be necessary to study long-term changes in DRD2-gene methylation and its possible association with craving and relapse. Second, the present study only included male patients. Thus, the findings are not transferable for a female population suffering from alcohol dependence. Third, a broader approach including methylation and analysis of genetic variants of other dopaminergic genes like DAT or DRD3 would help to study possible interactions between genes and methylation patterns or even epigenome-wide analysis would be warranted, even though these analyses themselves come along with a plethora of unsolved statistical issues. Fourth, our analysis is limited to blood samples which do not necessarily provide a picture of the situation in the brain. According to the blood brain DNA methylation comparison tool, several CGs within the DRD2 gene including probes located in the fragment analyzed in our study show high correlation between blood and several brain regions (Hannon et al., 2015). Interestingly, not much is known on the functional relevance of DRD2 expression on blood cells. Several studies have analyzed expression and also methylation of DRD2 in blood cells in different disease contexts, among them schizophrenia. In many studies, an association of peripheral expression and/or methylation signatures with psychometric scales (indicating central nervous processes) was found, e.g. an association between DRD2 expression in lymphocytes and PANSS-scores in schizophrenic patients (Liu et al., 2013). DRD2 is also involved in inflammatory processes and is necessary for neutrophil-endothelial interaction (Niewiarowska-Sendo et al., 2018). In the context of alcohol dependence, there are no studies present analyzing the functional role of dopamine receptors on blood cells.

Fifth, even though we and others have previously shown the functional relevance of DRD2 methylation for the mRNA expression levels of DRD2 (Frieling et al., 2010; Kordi-Tamandani et al., 2013), we were not able to also assess the expression levels in this study as no blood tubes with RNase inhibiting agents were used for sample collection. Sixth, our results are derived from whole blood analysis. As methylation levels can differ between different cell types, it cannot be ruled out, that all differences found in our study are merely caused by shifts in blood cell distribution. To the best of our knowledge, no studies addressing this question regarding DRD2 have been published so far. As frozen blood samples cannot be subjected to cell sorting, we can unfortunately not answer this question in the present study.

Furthermore, it must be considered that changes in DNA methylation are only one – while important – mechanisms of epigenetic regulation. Thus, further studies including a more comprehensive laboratory analysis, including female patients and possible subjects suffering from other addictive disorders are warranted to further elucidate the role if epigenetic changes in dopaminergic genes contributing to addictive behavior.

5. Conclusion

The present study describes significant alterations in DRD2 gene methylation in alcohol dependent patients undergoing alcohol withdrawal. Furthermore, DRD2 gene methylation was associated with tobacco consumption. In the light of recent investigations on changes of DRD2 gene methylation in pathologic gambling, results of this study point towards an involvement of DRD2 gene methylation in both, substance and non-substance addictions.

Significant outcomes

1. DNA methylation of the DRD2 gene increases during alcohol withdrawal treatment.
2. After alcohol withdrawal, alcohol dependent subjects show higher DNA methylation of DRD2 than healthy controls.
3. Tobacco smoking as another substance use disorder is independently associated with higher DRD2 gene DNA methylation in alcohol dependent subjects and healthy controls.

Limitations

1. Follow up time after detoxification treatment (2 weeks) may be too short to determine long-term changes in DRD2 regulation.
2. Only a limited sample size of male patients and controls was analyzed.
3. Only DNA methylation was analyzed while other epigenetic mechanisms (i.e. Histone modification or miRNA) were not considered.

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Role of the funding source

The funders had no role in the design and conduct of the study; collection, management, analysis, and interpretation of the data; preparation, review, or approval of the manuscript; and decision to submit the manuscript for publication.

Contributors

TH, HF, SB and JK were responsible for study concept and design. TH, HF, MR, and AB performed analysis and interpretation of data. Statistical analysis was done by TH, HF and AH. The first draft of the manuscript was written by HF, TH, AG, JW and MANM, the manuscript was critically revised by KGK, SB, JK and MR. All authors critically reviewed content and approved final version for publication.

Conflict of interest

The authors report no conflict of interest regarding the present investigation.

Financial disclosure

The authors have no financial disclosures to declare.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.euroneuro.2019.09.002.

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