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Alcohol-Induced Changes in Methylation Status of Individual CpG Sites, and Serum Levels of Vasopressin and Atrial Natriuretic Peptide in Alcohol-Dependent Patients during Detoxification Treatment

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Key Words
Alcohol withdrawal · Atrial natriuretic peptide · Epigenetic regulation · Vasopressin

Abstract
Disturbances of volume-regulating peptides like vasopressin (AVP) and atrial natriuretic peptide (ANP) have been described in early abstinent alcohol-dependent patients. In a longitudinal approach, we investigated whether changes in AVP and ANP serum levels correlated to cytosine-phosphatidyl-guanine (CpG) methylation of the respective gene promoters on days 1, 7 and 14 of alcohol withdrawal. We analyzed the blood samples of 99 patients suffering from alcohol dependence alongside age- and BMI-matched controls. Concerning AVP promoter methylation, we observed an interaction between time of measurement and CpG loci with CpG 2 showing a significant increase in methylation from day 1 to 14. Serum levels of AVP were significantly decreased in the patient group. Compared to healthy controls, promoter-related DNA methylation of the ANP promoter was significantly reduced on days 7 and 14. Moreover, we detected a significant interaction between CpG position and group. In both cases the difference was mainly observed at CpG 1. The present study shows significant changes in the methylation status of individual CpG sites of AVP and ANP. Observing respective alterations of AVP serum protein levels in alcohol-dependent patients during detoxification treatment, we consider methylation as a possible mode of regulation for these proteins during alcohol detoxification.

Introduction

In alcohol dependence, several recent studies have described alterations not only in neurotrophins [1], but also in the levels of volume-regulating hormones, such as the neuropeptides atrial natriuretic peptide (ANP) and vasopressin (AVP) [2–4]. Referring to alcohol dependence, both these neuropeptides have received the most exten-

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sive interest as they are known to exert direct effects on central nervous endocrinological pathways [5]. ANP is known to have strong anxiolytic effects and specific actions on different levels of the hypothalamus-pituitary-adrenocortical (HPA) axis [6, 7], such as inhibition of corticotropin-releasing hormone (CRH), adrenocorticotropin (ACTH) and cortisol secretion [8]. Contrary to that, AVP is known to enhance memory function and has been proposed to exert anxiogenic effects [9, 10] as well as stimulate CRH and ACTH expression at both the hypothalamic and pituitary levels [11]. Furthermore, studies have demonstrated that AVP is implicated in appetite regulation [12] as well as depression and obsessive behavior [13, 14]. AVP with nucleus paraventricularis hypothalami origin is a direct adrenocorticotrophin secretagogue through its V1b receptor. AVP seems to have special importance under prolonged stress conditions, which are known to be strong predictive factors of depressive disorder and can induce depressive-like symptoms [15]. Moreover, recent studies have demonstrated a role for appetite- and volume-regulating neuropeptides in alcohol dependence, particularly in association with alcohol craving. The peptides leptin, ghrelin, adiponectin, AVP and ANP have been of particular interest because of their central effects on various brain circuits, including the HPA axis [16]. Recent research has also demonstrated that the expression of these peptides in alcohol dependence is, at least partially, regulated by genetic and epigenetic mechanisms. These peptides and their associated circuits provide an intriguing new field for future pharmacological approaches for treating depression, anxiety and, potentially, addictive disorders such as alcohol dependence [16].

Knowledge on the role of AVP and ANP in alcohol-dependent patients is limited. Kiefer et al. [4] investigated changes in the plasma levels of ANP during acute alcohol withdrawal and observed reduced ANP levels in the patient group compared to healthy controls. In contrast to this study, Döring et al. [5] reported an ANP elevation and AVP suppression in recently abstinent alcoholics. Mutschler et al. [17] presented the first data on alcohol-drinking behavior in mice lacking a functional natriuretic peptide (NPR)-A receptor. Natriuretic peptides act within the central nervous system via the natriuretic peptide receptors NPR-A, NPR-B and NPR-C [18]. In that study, NPR-A mutants showed an increased stress-induced alcohol intake and aggravated neurobehavioral symptoms of alcohol withdrawal [17]. Despite the limited number of studies on the alteration of volume-regulating peptides, these studies provided the first evidence for the relevant alterations in alcohol-seeking behavior.

The mechanisms of long-term regulation of ANP and AVP expression may underlie epigenetic mechanisms. In this context, particularly methylation of cytosine-phosphatidyl-guanine (CpG) sites within the promoter region 5′ before the gene is known to be a common regulative mechanism that alters gene expression and may be important for the cellular expression of these peptides [19]. Regarding volume-regulating peptides, only ANP and AVP have been investigated in terms of epigenetic mechanisms in alcohol dependence so far. There is the general observation of hormone-dependent epigenetic regulation of AVP in the rat brain, indicating that the general mechanism at least involves epigenetic influences [20]. Hillemacher et al. [3] demonstrated that epigenetic control and gene transcription of AVP and ANP were altered in the blood of alcohol-dependent patients compared to healthy controls. Here, a significant hypermethylation of the gene promoter region for AVP was observed that led to no change of mRNA transcription. In contrast, ANP mRNA transcription was significantly elevated in the patient group and was associated with a significant hypomethylation of the corresponding promoter region. Moreover, a significant correlation between promoter-related DNA methylation of the ANP precursor gene and the extent of craving, as measured with the Obsessive Compulsive Drinking Scale, OCDS, was observed. These findings emphasize the importance of epigenetic alterations for several genes in alcohol dependence, including not only candidate genes of the dopaminergic pathways [21] or cellular chaperone dysregulation [22], but also epigenetic regulation of volume-regulating hormones like AVP and ANP.

Hillemacher et al. [3] measured promoter-specific DNA methylation status using a methylation-specific endonuclease digestion followed by real-time PCR. Blood samples were taken only once on admission to hospital for detoxification treatment.

The purpose of our study, taking a longitudinal approach, was to investigate whether mean and individual methylation of the CpG islands of the AVP and ANP gene promoter are changed in alcohol-dependent patients, and whether there is an association between alterations in AVP and ANP serum levels and the investigated changes of methylation of the CpG sites on days 1, 7 and 14 of alcohol withdrawal. To our best knowledge, this is the first investigation on CpG island promoter methylation (mean and methylation of individual CpG sites) of the ANP and AVP genes in the blood of alcohol-dependent patients during alcohol withdrawal via bisulfite sequencing. In comparison to the methylation-specific digestion applied

Table 1. Sociodemographic and disease-related data for alcohol-dependent patients and healthy controls

	Patients (n = 99)	Healthy controls (n = 101)
Age, years	43.15 (8.24)	36.35 (15.95)
BMI	25.01 (4.25)	24.34 (3.75)
Smokers, n	81	14
Alcohol dependence, years	9.53 (7.95)	–
Daily intake of ethanol, g/day	190.64 (81.83)	–

Data are either number (n) or mean values with SD in parentheses.

before, which is a technique limited to a global CpG methylation readout per analysis [22], bisulfite sequencing provides the means of sensitively, measuring different levels of individual methylation rates for a multitude of CpG loci. It is advantageous to reconfirm the results already obtained, thereby broadening the view on the epigenetics of AVP and ANP in the context of alcohol dependence.

Materials and Methods

Study Design

Alcohol-Dependent Patients and Control Group

This investigation is part of a large prospective research project on neurobiological mechanisms in alcohol dependence (Studies in Neuroendocrinology and Neurogenetics in Alcoholism, NENA) approved by the local ethics committee of the University of Erlangen-Nuernberg. All participants included in the study gave written informed consent. Each patient suffered from alcohol dependence (as defined by the International Classification of Diseases-10 and Diagnostic and Statistical Manual of Mental Disorders-IV) and was admitted for detoxification treatment to the Hospital for Psychiatry, Psychotherapy and Psychosomatics, Obermain, Germany. Exclusion criteria were concomitant psychiatric illnesses, other substance abuse apart from alcohol or nicotine, cerebral ischemia, cerebral hemorrhage, epilepsy, cardiovascular and renal diseases. All patients underwent a detailed physical examination, a routine laboratory testing and urine drug screening. In the present analysis we included 99 male patients and 101 sex-matched controls. Additional information about the sociodemographic and disease-related data, such as previous daily intake of ethanol and years of drinking, are summarized in table 1.

Laboratory Analysis

Serum Levels of ANP and AVP

AVP (EIA-3122 DSL; Beckman Coulter, Krefeld, Germany), ANP (Uscn Life Science Inc., Wuhan, China) and cortisol plasma levels were assessed using the DuoSet ELISA Development System.

Analysis of DNA Methylation

Fasting blood samples were drawn from alcohol-dependent patients on days 1, 7 and 14 between 08:00 and 10:00 h. Ethylenediaminetetraacetic acid blood samples were aliquoted and stored at –80°C promptly after collection.

The following preparation steps for the analysis of CpG-methylation were the same for all blood samples. Extraction from blood and cleanup of genomic DNA was performed using the NucleoMag® Blood 200 µl DNA Kit (Macherey-Nagel, Düren, Germany).

Bisulfite Conversion of Genomic DNA and Subsequent Amplification of the AVP and ANP Target Region

DNA samples were bisulfite converted using the EpiTect® 96 Bisulfite Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. Amplification of AVP and ANP target sequences of the purified bisulfite-converted DNA was done through a (semi) nested touchdown PCR. The sequences of oligonucleotides used as bisulfite primers can be found in table 2.

(Semi) Nested Reaction Components

The (semi) nested reaction components used were as follows: 0.4 µl (20 µmol) of the regarding forward primer (F1/F2), 0.4 µl (20 µmol) of the regarding forward primer (RC1/RC2), 1 µl of DNA, 3.2 µl of H_2O, 5 µl HotStarTaq® Master Mix Kit (QIAGEN). This represents a total volume of 10 µl.

The T_m for the amplification of the bisulfite-primers was set at 70°C (ANP)/60°C (AVP) in the first and second round of the semi-nested polymerase chain reaction. One microliter of amplified product of the first PCR was used as a template for the second PCR.

Amplification products of the second PCR were purified using the Agencourt® AMPure® XP magnetic beads (Beckman Coulter). Sequencing of the target fragment was performed using a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, Calif., USA) and an Applied Biosystems/HITACHI 3500xl Genetic Analyzer (Applied Biosystems) according to the manufacturer's instructions.

The bisulfite-primers #97_VP and #104-ANP were used for sequencing of the respective genes (table 2). Products of the sequencing PCR were purified using the Agencourt CleanSeq® XP magnetic beads (Beckman Coulter) and then used for sequencing. Electropherograms and sequences, detected by the Genetic Analyzer, were analyzed using the specialized epigenetic sequencing methylation analysis software to determine the methylation rates for every CpG locus. All PCRs were performed in a C1000™ Thermal Cycler (BIO-RAD, Hercules, Calif., USA) using the regarding protocols as described above. A Biomek® NXP (Beckman Coulter) was used for pipetting and transferring steps, as well as purification of DNA and amplified oligonucleotides.

Statistical Analysis

The mean differences between the ANP and AVP serum levels of the alcohol-dependent patients on days 1, 7 and 14 were calculated using a general linear model for repeated measurements with time as the independent variable and the respective serum levels as dependent variables. Confidence intervals were corrected using the Bonferroni method. Alterations in the mean and the individual methylation of the CpG sites were analyzed by mixed linear modeling using the restricted maximum likelihood method. Methylation was set as a dependent variable, and the individual CpG

Table 2. Primer list: ANP and AVP

Gene	Name	Sequence	Gene	Fragment size, bp
ANP	#101_ANP	GGG AAG GAT GTA GAA GGT ATT GAG GGA	ANP-outer-fwd	282
	#102_ANP	CTC CCA AAA ATC CAA CCC CCA	ANP-outer-rev	
	#104-ANP	AAG TTT AGG AAG GGT AGG GGG ATG T	ANP-inner-fwd_seq	
AVP	#99_VP	GAA AGT TTA GAG ATG GTT TTT AGG T	AVP-outer-fwd	329
	#98_VP	CAA CCC TAA AAT AAC CCA CAA TA	AVP-outer-rev	
	#97_VP	CAT CCT AAT ACA CAC AAA TAA ACC	AVP-inner-rev-seq	

In the first PCR of ANP gene amplification, #101_ANP and #102_ANP were used as primers. In the second PCR, #104-ANP and #102_ANP were used. For VP gene amplification, #99_VP and #98_VP were used in the first PCR, and #99_VP and #97_VP were used in the second PCR. The primers used for sequencing are marked with 'seq'. fwd = Forward; rev = reverse.

Table 3. Serum levels of ANP and AVP (in ng/μl) during alcohol withdrawal on days 1, 7 and 14, as well as serum levels of the healthy controls

	n	Minimum	Maximum	Mean	SD
Patients					
ANP T 1	86	5.50	3,713.00	160.4000	433.61388
ANP T 7	92	0.90	2,375.30	122.1467	311.55308
ANP T 14	88	2.70	3,541.60	129.5091	395.33525
AVP T 1	99	34.50	2,899.90	517.6687	630.26769
AVP T 7	93	13.50	2,181.70	218.9108	291.32950
AVP T 14	90	10.20	1,121.30	156.2511	146.21528
Valid data	74				
Controls					
ANP T 1	91	2.40	1,780.80	113.1374	294.06783
ANP T 7	91	2.40	1,780.80	113.1374	294.06783
ANP T 14	91	2.40	1,780.80	113.1374	294.06783
AVP T 1	101	14.80	4,218.90	605.1158	671.05618
AVP T 7	101	14.80	4,218.90	605.1158	671.05618
AVP T 14	101	14.80	4,218.90	605.1158	671.05618
Valid data	91				

T = Time of measurement.

sites and days of investigation were set as fixed factors. Likewise, the influence of CpG island methylation on ANP and AVP serum levels was calculated by setting ANP and AVP serum levels as a dependent variable, with methylation of individual CpG sites, mean methylation of all investigated CpG sites and days of investigation as fixed factors. Akaike's information criterion was used to assess the best-fitting covariance structure to model the repeated measurements. In all cases, scaled identity served as the best covariance model. Each patient's number was set as a random factor in order to correct the obtained results on the basis of intrapersonal changes of CpG-site methylation. Further factors were added to the models to define the possible confounding effects of age, BMI or smoking habits – none of these factors or their interactions led to significant changes in our models (data not shown). Confidence intervals were corrected using Bonferroni correction. The data was analyzed using PASW Statistics 20.0 and Graph Pad-Prism TM 5.0 (GraphPad Software, La Jolla, Calif., USA).

Results

Vasopressin

Methylation of the AVP promoter did not change significantly over the course of detoxification treatment (time: $F = 1.003$; $p = 0.367$). We observed an interaction between time of measurement and CpG loci ($F = 2.225$; $p < 0.001$), with only CpG 2 showing a significant increase in methylation from day 1 to day 14 (Bonferroni corrected t test: $t = 4.20$; $p < 0.001$).

We found no main effects of group (patients vs. controls) at any time point during treatment (day 1: $F = 0.65$; $p = 0.42$; day 7: $F = 1.75$; $p = 0.18$; day 14: $F = 3.25$; $p = 0.071$). Again, a significant interaction between CpG sites and group occurred (CpG/x: $F = 8.92$; $p < 0.001$; day 7: $F = 8.76$; $p < 0.001$; day 14: $F = 8.28$; $p < 0.001$). CpG sites 2, 10, 11, 14 and 15 differed significantly between controls and patients at baseline. At the end of the detoxification treatment, only CpG 2 differed significantly between controls and patients.

Serum levels of AVP (table 3) were not significantly different between patients and controls on day 1 ($T = 0.95$; $p = 0.34$). During detoxification treatment, AVP serum levels decreased in the patient group with significant differences visible at days 7 and 14 (p values derived from the Bonferroni post hoc test are provided in fig. 1). We found no significant relationship between AVP serum levels and methylation of the AVP gene promoter (data not shown).

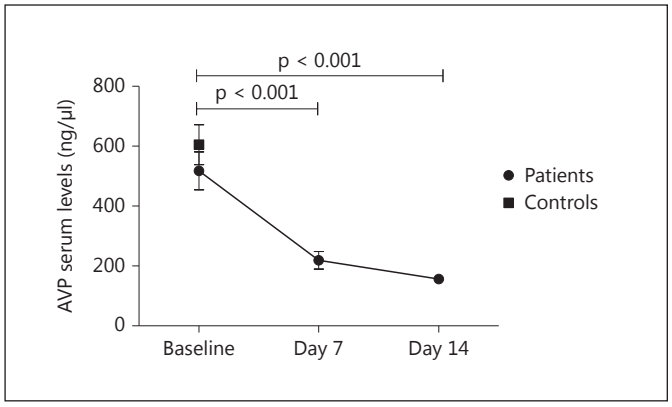

Fig. 1. Serum levels of AVP during withdrawal compared to healthy controls. Serum levels of AVP significantly decreased during detoxification treatment. Serum levels of the controls were only taken on day 1 of alcohol withdrawal.

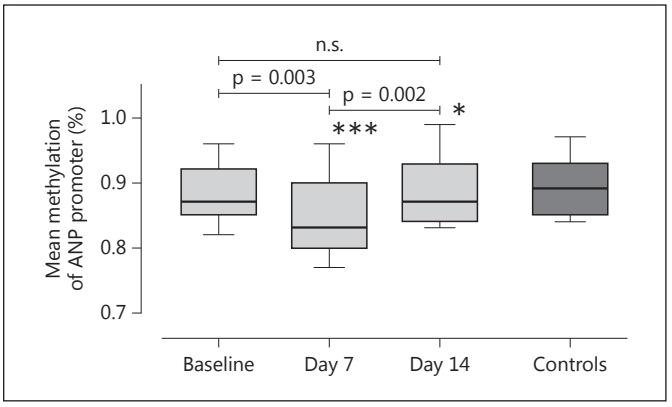

Fig. 2. Mean methylation of the ANP promoter compared to healthy controls during detoxification treatment. Controls showed slightly higher levels of ANP methylation, with the main effect being nearly significant compared to the patients' levels at the first point of measurement, and significant differences when compared to patients' levels at days 7 and 14. * $p < 0.05$ and *** $p < 0.0001$ versus controls.

Atrial Natriuretic Peptide

ANP promoter methylation significantly decreased in the first week of treatment and increased to the height of day 1 at the end of detoxification treatment ($F = 7.38$; $p = 0.001$; p values of the Bonferroni post hoc test are provided in fig. 2). There was no significant interaction between time and CpG position ($F = 1.170$; $p = 0.30$). Controls showed slightly higher levels of ANP methylation, with the main effect being nearly significant compared to the patients' levels at the first point of measurement ($F = 3.425$; $p = 0.064$) and significant differences when compared to patients' levels at day 7 ($F = 25.65$; $p < 0.0001$) and day 14 ($F = 4.007$; $p = 0.046$; fig. 2).

For time points 7 and 14, we also observed a significant interaction between CpG position and group (day 7: $F = 5.169$; $p < 0.0001$; day 14: $F = 3.061$; $p = 0.006$). The CpG-wise post hoc test revealed that in both cases the difference was mainly driven by CpG 1 (fig. 3). Serum levels of ANP in alcohol-dependent patients did not change from day 1 to day 14 of detoxification treatment (RM-ANOVA: Pilai-Spur = 0.002; $F = 0.059$; $df = 2$; $p = 0.943$).

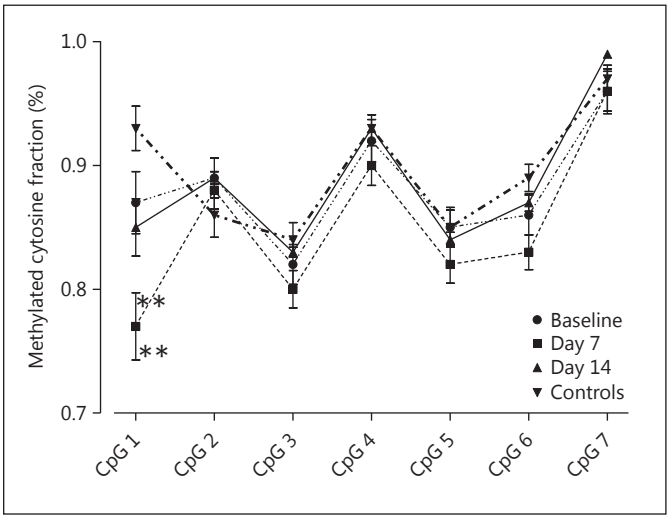

Fig. 3. Methylated cytosine fraction of the different CpG sites of the ANP gene during detoxification treatment compared to healthy controls. For time points 7 and 14, we observed a significant interaction between CpG position and group. The CpG-wise post hoc test revealed that in both cases the difference was mainly driven by CpG 1. ** $p < 0.001$ versus controls (Bonferroni post hoc test).

Discussion

This is the first study that reports changes in the methylation status of individual CpG sites of the AVP and ANP gene in alcohol-dependent patients during detoxification treatment. Although methylation status of the AVP promoter did not change significantly over the course of detoxification treatment, we observed an interaction between time of measurement and CpG loci with CpG 2 showing a significant increase in methylation from day 1 to day 14. According to group comparisons, a significant interaction between CpG sites and group occurred again with methylation of CpG2 being increased over the whole study period in the patient group. To date,

promoter-specific DNA methylation of the AVP precursor gene in alcohol dependence has only once been investigated [3]. Here, a significant hypermethylation of the AVP gene promoter region in alcohol-dependent patients has been reported. In this investigation, promoter-specific DNA methylation status was measured using a methylation-specific endonuclease digestion followed by real-time PCR. We used bisulfite sequencing due to its increased sensitivity and precise per-CpG methylation readout. Thus, our study not only corroborates the results of Hillemacher et al. [3], but also indicates that the observed altered AVP promoter methylation might be mainly driven by CpG 2.

Due to methodological reasons, Hillemacher et al. [3] were not able to obtain a reliable measurement of the protein expression of AVP in serum. Our study revealed significantly decreased serum levels of AVP in patients with alcohol dependence compared to healthy controls. During detoxification treatment, AVP serum levels were reduced at day 1 compared to healthy controls and also further declined during detoxification treatment. This confirms earlier results showing a decrease of AVP serum levels during alcohol withdrawal [5]. Here, the author reported suppressed AVP serum levels in alcohol-dependent patients even over months of alcohol abstinence. Beside a postulated epigenetic influence [3], the persistently decreased basal AVP levels in our patients might additionally be caused by a reduction of vasopressinergic neurons in the water balance-regulating nuclei of the hypothalamus as a consequence of chronic alcohol consumption [23–27]. AVP-synthesizing neurons are also centrally localized [28, 29], originating within the paraventricular nucleus, bed nucleus of the stria terminalis, medial amygdala and suprachiasmatic nucleus. Silva et al. [26] showed that prolonged alcohol intake leads to degeneration of neurons of the hypothalamic magnocellular nucleus of the paraventricular nucleus.

In our study, the significantly suppressed serum levels of AVP were not associated with changes in the methylation of CpG sites within the AVP gene promoter (data not shown). Furthermore, Hillemacher et al. [3] also reported no correlation between the changes in the hypomethylation within the AVP gene promoter and mRNA expression of AVP. This finding may be explained by the fact that, unlike ANP, AVP is exclusively secreted by the posterior pituitary gland. Hence, measurement of its mRNA-expression in peripheral blood may only be of limited relevance. However, to date, little is known about the contributions of epigenetic mechanisms on the homeostasis of AVP, an issue further studies may address.

Concerning ANP, a peptide repeatedly shown to be involved in the pathophysiology of withdrawal [4, 30], we detected a significant hypomethylation of the ANP gene promoter region on day 7 in alcohol-dependent patients compared to healthy controls. These results again are in line with the results of Hillemacher et al. [3], who reported a decreased promoter-related DNA methylation of ANP combined with a significantly elevated ANP mRNA expression in alcohol-dependent patients compared to healthy controls. Additionally, we observed a significant interaction between CpG position and group on days 7 and 14. In both cases the CpG-wise post hoc test revealed that the difference was mainly driven by CpG 1 (fig. 3). This result hints towards a more complex network of interactions regulating the expression of ANP since within the timespan of 2 weeks the methylation of this respective part of the promoter only shows deviation from baseline and control levels on day 7. We expect this to be an immediate response to detoxification without prolonged effects on overall protein expression. As ANP is not directly involved in the metabolism of ethanol but postulated to have a role in the pathophysiology of alcohol withdrawal [4], finding serum levels of ANP unchanged is yet another indication of a more complex regulation than proposed. This perspective is also reflected in the literature; while Kiefer et al. [4] reported reduced ANP levels in the patient group during acute alcohol withdrawal compared to healthy controls, Döring et al. [5] observed a persistent ANP elevation in recently abstinent alcoholics. These contradictory results may also be due to the involvement of different study populations – Döring et al. [5] investigated abstinent alcoholics (with a minimum of 2–3 weeks of abstinence), while Kiefer et al. [4] observed patients during alcohol withdrawal, similar to our study design.

ANP was found not only to be involved in regulating blood pressure and volume through its natriuretic and diuretic properties [29], but is also said to be a relevant component in the neuroendocrine regulation of stress. It attenuates the stress response by inhibiting the HPA system at the hypothalamic, pituitary and adrenocortical level [8, 30, 31]. Moreover, other studies have reported that a dysregulation of ANP plasma level during alcohol withdrawal contributes to symptoms of protracted withdrawal [4]. Patients with decreased ANP plasma concentrations during alcohol withdrawal suffered from more intense and frequent craving as well as from higher anxiety levels [4]. In addition, Hillemacher et al. [3] reported a significant correlation of promoter-related DNA methylation of ANP with the extent of craving.

In a recent study, Kiefer et al. [32] suggested that ANP plasma concentration is influenced by a variant in GATA-binding protein 4, an ANP transcription factor, with the AA/AG genotype of the single-nucleotide polymorphism rs13273672. In particular, the reduced variability of ANP in carriers of at least one G allele, as observed by Kiefer et al. [32], may be indicative of diminished metabolic responsiveness and consequently a reduced ANP synthesis. These interesting data suggest an effect on ANP plasma concentration depending on the allelic predisposition [32], supposedly further complementing the yet elusive mechanism of ANP regulation. Future gene expression and replication studies should examine the interactions between ANP plasma levels and the HPA axis. Aside of genetic regulation effects, ANP is also known to be regulated by selective blood brain barrier transporters, forming additional means of regulation towards the periphery [33].

There are several limitations in regard of the scope of this study. Hormone levels tend to vary in cycles depending on many factors such as age, sex, general physiology and the alcoholic subtype. The interindividual differences in hormone regulation lead to the variance of measurement observed, especially in the serum level measurements. Both proteins are not directly involved in the ethanol metabolism but rather control the peripheral reactions on a more holistic level, being therefore influenced by a multitude of other regulative stimuli.

Another crucial but unavoidable drawback in patient studies is the tissue of investigation. Since both ANP and AVP are expressed in the brain and transported to the site of effect through the blood, investigating the methylation of both peptide promoters in blood cells could potentially diverge from the neuronal context and needs to be evaluated by additional studies including both tissues. In spite of this caveat, variation of promoter methylation was found to bear unexpected similarities between blood and brain [34].

Future studies are needed to map ethanol-induced changes in the methylation status of individual CpG sites and determine the functional links between ethanol-induced changes in methylation status in specific CpG sites and transcription factor accessibility to DNA promoter in the regulation of gene expression. Moreover, investigations of the long-term course of methylation of CpG sites of the ANP and AVP promoter in alcohol-dependent patients withdrawn from alcohol, and comparison with healthy controls, will be necessary in order to interpret these preliminary results correctly.

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Disclosure Statement

All authors report no biomedical financial interests or potential personal conflicts of interest.

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