The Epigenetic Regulation of GATA4-Dependent Brain Natriuretic Peptide Expression during Alcohol Withdrawal

Alexander Glahn^a Mathias Rhein^a Annemarie Heberlein^a Marc Muschler^a
Johannes Kornhuber^b Helge Frieling^a Stefan Bleich^a Thomas Hillemacher^a

^aCenter for Addiction Research (CARE), Department of Psychiatry, Social Psychiatry and Psychotherapy, Hannover Medical School, Hannover, and ^bClinic for Psychiatry and Psychotherapy, Erlangen, Germany

Keywords

Alcohol dependence · Alcohol withdrawal · Brain natriuretic peptide · Epigenetic regulation · GATA4 · Hypothalamus-pituitary-adrenocortical axis

Abstract

Objective: Natriuretic peptides participate in the collection of metabolic effects during alcohol withdrawal. Having witnessed modulation of other natriuretic peptides in alcohol-dependent patients during alcohol withdrawal, we were interested in the relation of brain natriuretic peptide (BNP) methylation with protein expression and craving in this longitudinal study. **Methods:** Ninety-nine male patients were compared to 101 healthy controls concerning epigenetic regulation and protein expression during detoxification treatment. **Results:** With BNP expression being GATA4 dependent, we observed a correlation of GATA4 binding site methylation and protein expression. BNP serum levels and Obsessive Compulsive Drinking Scale scores are significantly decreased during withdrawal. Focusing on the two CpGs that are between GATA transcription factor binding sites, statistical analysis revealed a reversely proportional methylation pattern, significantly increasing with ongoing detoxification and thereby supporting the observed serum level

changes. **Conclusion:** Without the functional knowledge about regulation of BNP expression via the GATA transcription factor, it would have been easy to take the mean results of the global CpG data and propose a direct relationship between methylation and expression. Thus, these findings are a voice for functionally and mechanistically approved results. There was no causal relationship between protein expression levels and epigenetic changes. Further research is needed which includes protein expression and other approaches.

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Introduction

In alcohol dependence, several recent studies describe alterations not only in hypothalamus-pituitary-adrenocortical (HPA)-dependent stress regulation, but also in the levels of volume-regulating hormones such as the neuropeptides atrial natriuretic peptide (ANP) and vasopressin (AVP) [1–6]. Referring to alcohol dependence, both neuropeptides have received extensive interest as

A.G. and M.R. contributed equally to this work.

they are known to exert direct effects on central nervous endocrinological pathways [1, 2, 4]. ANP has strong anxiolytic properties specifically on different levels of the HPA axis, such as inhibition of corticotropin-releasing factor, adrenocorticotrophic hormone, and cortisol secretion. In contrast, vasopressin is known to enhance memory function and has been proposed to exert anxiogenic effects as well as stimulate corticotropin-releasing factor and adrenocorticotrophic hormone expression at both the hypothalamic and pituitary levels [3]. Brain natriuretic peptide (BNP) is a further member of the natriuretic peptide family and known to control cardiovascular and body fluid homeostasis. BNP is released predominantly during cardiac ventricular dilatation and pressure overload [7, 8]. Although predominantly produced by the heart muscle cells, BNP has also been found in neurons of discrete brain regions where its expression was shown to be 13 times higher compared to ANP [9]. This suggests that central BNP may also take part in the neuromodulatory system that controls addictive and emotional behavior such as craving during withdrawal and the HPA axis [10].

In the last decade, considerable evidence has emerged that indicates a role not only for ANP and AVP, but also for BNP in the regulation of HPA response to different stress responses such as craving during withdrawal of alcohol-dependent patients [11, 12]. Surprisingly, the association of epigenetic BNP regulation in the context of alcohol consumption has not been investigated although, in recent studies, epigenetic mechanisms regarding volume-regulating/natriuretic peptides like ANP and AVP have been investigated in the context of alcohol dependence. Our group demonstrated that epigenetic control and gene transcription of AVP and ANP were altered in the blood of alcohol-dependent patients compared to healthy controls [1, 3, 13]. While observing a significant hypermethylation of the gene promoter region for vasopressin, mRNA transcription levels were unaltered. 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, we describe 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) [1, 3, 13]. Also, genome-wide association studies recently showed alcohol dependence to be associated with a single-nucleotide polymorphism (rs13273672) located on a gene (GATA4) that encodes a transcription factor of ANP [14].

These findings emphasize the importance of epigenetic alterations in several genes in alcohol dependence, in-

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

	Patients	Healthy controls
<i>n</i>	99	101
Age, years	43.15±8.24	36.35±15.95
BMI	25.01±4.25	24.34±3.75
Number of smokers	81	14
Years of alcohol dependence	9.53±7.95	–
Daily intake of ethanol, g/day	190.64±81.83	–

Values are presented as *n* or means ± SD.

cluding not only candidate genes of the dopaminergic pathways or cellular chaperone dysregulation [15–18], but also potential epigenetic regulation of volume-regulating hormones like AVP, ANP, and BNP.

Regulation of BNP expression is mainly triggered by the transcription factor GATA4, which is known to regulate expression of a number of genes including troponin C, rat ANP [19], and BNP [20]. GATA4 motif modulation strongly affects BNP expression levels [21]. The zinc finger GATA motif transcription factor family is part of a complex regulatory network that induces DNA transcription upon binding to conserved recognition sites, leading to gene expression [14, 21]. Known largely for the regulation of lineage-specific regulation of target genes such as cardiac gene expression, GATA4 enhances transcription upon binding to its respective motif in the enhancer/promoter region of target genes [21]. Also, epigenetic influence on the binding properties has already been suggested in the context of pancreatic cancer [22].

The purpose of our study was to investigate whether mean or individual methylation of the BNP gene promoter is altered in alcohol-dependent patients and whether there is an association with alterations in BNP serum levels on days 1, 7, and 14 of alcohol withdrawal. To our best knowledge, this is the first longitudinal investigation on CpG-island promoter methylation of the BNP gene in the blood of alcohol-dependent patients during alcohol withdrawal.

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 (NENA: Studies in Neuroendocrinology and Neurogenetics in Alcohol-

Table 2. Sequences of oligonucleotides used as bisulfite primers

Primer name	Sequence	Fragment size
109_BNP-Fwd	GAA GAG AGT AGT TTT TGA GAG TTT GTT TT	640 bp
107_BNP-Rev	CCA AAA AAA CCA AAT ACA AAA	
108_BNP-Rev-Seq	CTT CCC ACC TAC CCT CAA CCT A	

Fwd, forward; Rev, reverse.

Molecular Analysis

Serum Levels of BNP

BNP serum levels were assessed using the DuoSet enzyme-linked immunosorbent assay (ELISA) Development System (BNP: EIA-3122 DSL; Beckman Coulter, Brea, CA, USA). The data were checked for extreme outliers (values >300 ng/μL, see also online suppl. Fig. S1; see www.karger.com/doi/10.1159/000456011 for all online suppl. material) or detection errors, and excluded where necessary (Fig. 1).

Analysis of DNA Methylation

Fasting blood samples were drawn from alcohol-dependent patients on days 1, 7, and 14 between 8:00 and 10:00 am. Ethylenediaminetetraacetic acid (EDTA) blood samples were aliquoted and stored at –80°C promptly after collection. The subsequent preparation steps for the analysis of CpG-methylation were the same for all blood samples. Extraction from blood and cleanup of genomic deoxyribonucleic acid (DNA) was performed using the Nucleo-Mag® Blood 200 μL DNA Kit (Macherey-Nagel, Düren, Germany).

Bisulfite Conversion of Genomic DNA and Subsequent Amplification of BNP Target Region

DNA samples were then bisulfite converted using the EpiTect® 96 Bisulfite Kit (QIAGEN AG, Hilden, Germany) according to the manufacturer's protocol. Amplification of BNP target sequences of the purified bisulfite-converted DNA was done through (semi-) nested touchdown polymerase chain reaction (PCR). Sequences of oligonucleotides used as bisulfite primers can be found in Table 2.

(Semi-)Nested Reaction Components

- 0.4 μL (20 μmol) of the regarding forward primer (F1/F2)
- 0.4 μL (20 μmol) of the regarding reverse primer (RC1/RC2)
- 1 μL DNA
- 3.2 μL H₂O
- 5 μL HotStarTaq® Master Mix Kit (QIAGEN)
- Σ 10 μL total volume

The T_m for the amplification of the bisulfite-primers was set at 64°C in the first and second round of the seminested PCRs. One microliter of amplified product of the first PCR was used as template for the second PCR.

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

The bisulfite primer No. 108_BNP was used for sequencing the respective genes (Table 2). The sequences of our oligonucleotides used for seminested PCR contained 1 (forward-primer-109_BNP-Fwd) and 3 (reverse-primer-108_BNP-Rev-Seq) single-nucleotide polymorphisms, respectively. Because of extremely rare allele frequencies and the patient's Caucasian ancestry, none of these SNPs can disturb amplification of our desired fragment. Products of the sequencing PCR were purified using the Agencourt® CleanSeq® XP magnetic beads (Beckman Coulter, Krefeld, Germany) and then used for sequencing. Electropherograms and sequences, respectively, detected by the Genetic Analyzer were analyzed using the specialized Epigenetic Sequencing Methylation Analysis Software to determine methylation rates for every CpG locus. All PCRs were performed in a C1000™ Thermal Cycler (Bio-Rad, Hercules, CA, USA) using the regarding protocols as described above. A Biomek® NXP (Beckman Coulter, Krefeld, Germany) was used for pipetting, transferring steps, and purification of DNA and amplified oligonucleotides.

Statistical Analysis

Mean differences for the time points investigated were assessed for normality by analyzing residuals, both visually with histograms and statistically using the Shapiro-Wilk goodness-of-fit test. BNP serum levels and BNP methylation measurements deviated significantly from normality while OCDS scores matched Gaussian distribution. Therefore, we used nonparametric methods for inter-group comparison while using the appropriate statistical methods for relations within the groups. We used a 2-way ANOVA for OCDS corrected for multiple testing (Tukey's test for multiple comparisons against one control) for serum results to compare means between the 3 time points and the control cohort. Equally for methylation and serum levels, Kruskal-Wallis testing with correction for multiple comparisons (Dunn's multiple comparison test for the KW) was applied to both global and specific comparisons. For volume calculations, the total volume of alcoholic drinks was calculated. The median consumption of beer, wine, and strong alcohol in liters was used to divide the collective into 2 groups (cut-point at 3 L; low drinkers *n* = 50, high drinkers *n* = 41). All calculations were performed using either SPSS Statistics 22.0 or GraphPad Prism™ 6.0 (GraphPad Software, La Jolla, CA, USA). Further factors were added to the models to define possible confounding effects of age, BMI, OCDS scores, and smoking habits. None of these factors or their interaction led to significant results differentiating meaningful subgroups in our models (data not shown).

In silico Transcription Factor Binding Site Prediction

The transcription factor binding sites were predicted using the Alibaba 2 online package (<http://www.gene-regulation.com/pub/>

Fig. 2. **a** Core promoter methylation pattern is shown again with the respective annotated sequence similar to Figure 1. Total core promoter methylation (compared to control: day 1: **** $p < 0.0001$; day 7: *** $p < 0.001$; day 14: **** $p < 0.0001$) (**b**) is contrasted to GATA region-specific analysis (day 1: **** $p < 0.0001$; day 7: **** $p < 0.0001$, compared to controls) (**c**).

programs/alibaba2/index.html) accessing the TFsearch database. The sequence used as a template was the same Ensembl data entry for human BNP also used for primer design and analysis of methylation data (www.ensembl.org, accessionNo. ENSG00000120937). Settings were set to 64% pairsim percentage, 75% matrix conservation, and 95% similarity of sequence to matrix.

Results

BNP Serum Levels and OCDS Data

Serum levels of BNP decrease significantly from onset of detoxification to day 14 (Fig. 1a; day 7: $p < 0.01$, $t =$

3.559; day 14: $p < 0.0001$, $t = 4.649$). Baseline levels did not differ significantly from controls. Global OCDS scores decreased significantly from baseline to day 7 ($p < 0.0001$, $q = 16.63$), remaining on a comparably low level on day 14 (days 7–14: ns, $q = 2.186$) (Fig. 1b). We confirmed our previous findings concerning the relationship between volume intake and OCDS levels. OCDS scores are significantly higher in high-volume drinkers compared to low-volume drinkers in the initial testing ($p < 0.001$, $F = 19.735$), while the 2 following time points showed scores that no longer displayed a significant difference.

BNP Global Methylation

The promoter region analyzed in this study spans the core promoter region of BNP characterized elsewhere [21] (Fig. 1c). For the purpose of confirmation, we included the 5' proximity region still belonging to the CpG island. Methylation analysis of this fragment for the 3 time points assessed revealed the same core regulative promoter region as previously described, ranging from CpG 38 to CpG 46 (Fig. 1c, 2a). We therefore limited statistical analysis to this segment of the sequence.

Mean methylation for this core region decreased significantly with progression of detoxification (Fig. 2b, compared to control: day 1: $p < 0.0001$; day 7: $p < 0.001$; day 14: $p < 0.0001$).

BNP Methylation of GATA-Related Cytosines

Focusing on the two CpGs between the GATA transcription factor-binding sites, Nos. 45 and 46 (Fig. 2a), detailed statistical analysis revealed a reversely proportional methylation pattern, significantly increasing with ongoing detoxification and thereby supporting the observed serum level changes (Fig. 2, adjusted p values compared to control: day 1: $p < 0.0001$; day 7: $p < 0.0001$; day 14: ns). Analysis of CpG No. 44 revealed no significant results upon investigation between time points (see online suppl. Fig. S2) and was therefore excluded from the analysis in spite of its proximity to one of the GATA sites (Fig. 2a; online suppl. Fig. S2).

Discussion

The natriuretic peptides ANP, AVP, and BNP are tightly interwoven into side effects observed during detoxification treatment [1, 2, 13]. ANP and BNP are hormones involved in the regulation of the HPA system [1–4, 25]. This conclusion is also based on broad experimental evidence in animals and human studies [12, 26–28]. Concerning ANP, epigenetic mechanisms have been investigated in alcohol dependence by our group. We previously observed that methylation status of other volume-regulating hormones of the natriuretic peptide family can potentially serve as markers for individual treatment progression [1, 13].

This is the first study that reports changes in the methylation status of individual CpG sites of the BNP gene and BNP serum levels in alcohol-dependent patients during detoxification treatment. Moreover, we confirmed our previous findings concerning the relationship between volume intake and OCDS levels. Since detoxification side

effects correlate with alcohol volume intake [29], the observed deregulation could depict the body readapting to drastically different volume intake. In this trial, we were unable to correlate promoter methylation to volume intake or craving scale. Nevertheless, the previously made observation holds true: high-volume drinkers enter detoxification with significantly higher OCDS scores ($p < 0.001$, $F = 19.735$; see online suppl. document S1A, B). Over the course of the 2 treatment weeks, the scores document the effect of detoxification therapy by consecutively lower OCDS scores in both high- and low-volume drinkers. Splitting of the collective according to volume intake shows no significant group differences in BNP serum levels as well as global and specific methylation analysis (see online suppl. document S1C, D). The current clinical practice uses BNP levels as a biomarker for cardiac disease states. Neither the underlying regulation nor the interaction between alcohol detoxification and BNP levels has been investigated satisfactorily. Baseline serum levels of BNP were not significantly different from controls, arguing for a deregulation phenomenon during detoxification. In our study, we measured decreased BNP serum levels during withdrawal. Lower levels of BNP have also been observed in subjects with sleep deprivation due to a stressful situation [30]. The decreasing level of BNP could imply that the inhibitory effects on the HPA system are reduced during alcohol withdrawal.

According to the methylation status, the mean methylation values of the core regulative promoter region of BNP surprisingly revealed counterintuitive data until the scope of analysis was narrowed to CpG sites in immediate proximity to predicted GATA sites [21]. Function-driven site selection revealed a tendency of epigenetic regulation contributing to BNP regulation that otherwise would not have been recognized. Focusing on the two CpGs that are between GATA transcription factor binding sites, Nos. 45 and 46 (Fig. 2a), statistical analysis revealed a reversely proportional methylation pattern, significantly increasing with ongoing detoxification and thereby supporting the observed serum level changes, accordingly (Fig. 1a). Knowing that methylation usually shows an inverse relationship concerning binding of the transcriptional activator GATA in combination with SP1, the methylation findings would have been counterintuitive. Limiting the analysis to the two CpGs in proximity to GATA and SP1 regions displays the expected inverse relationship (Fig. 2c).

With BNP expression being GATA4 dependent [21], we observed a correlation of GATA4-binding site methylation and protein expression during alcohol withdraw-

al, pointing towards a mechanism of activation and potential possibility to correlate epigenetic imprinting on this site with BNP expression levels. Without the functional knowledge about the crucial regulation of BNP expression via the GATA transcription factor [21], it would have been easy to take the mean results of the global CpG data and propose a direct relationship between methylation and expression. Thus, these findings also support functionally and mechanistically sound results. One further point of interest deserves attention: the kinetics of BNP level alteration occurs on day 7, while a measurable change in methylation is only visible on day 14 (compare Fig. 1a with Fig. 2c). Thereby, we believe we have further evidence for epigenetics being a documentation rather than a prerequisite of altered metabolic requirements. BNP research in the context of alcoholism could thereby also serve as a proof-of-concept model to deepen our knowledge about kinetics of methylation pattern adaptation as a consequence of altered environmental or metabolic conditions.

There are several limitations regarding 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 or smoking. Abstaining from alcohol itself removes a big volume load from the system, leading to numerous side effects. The change in expres-

sion of natriuretic peptides is likely strongly influenced by this change. The interindividual differences in hormone regulation can lead to variances of measurements, especially concerning serum levels. BNP is not directly involved in ethanol metabolism, but instead controls the peripheral reactions on a more holistic level, being therefore influenced by a multitude of other regulative stimuli. In spite of these unavoidable shortcomings, we see significant correlations of methylation levels and protein expression.

This is the first study that reports changes in the methylation status of individual CpG sites of the BNP gene and BNP serum levels in alcohol-dependent patients during detoxification treatment. With BNP expression being GATA4-dependent, we observed a correlation of GATA4-binding site methylation and protein expression during alcohol withdrawal. Investigations of the long-term course of CpG site methylation of the BNP promoter in alcohol-dependent patients withdrawn from alcohol will be necessary to interpret these preliminary results correctly.

Disclosure Statement

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

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