
Article

Joint Effects of the Epigenetic Alteration of Neurotrophins and Cytokine Signaling: A Possible Exploratory Model of Affective Symptoms in Alcohol-Dependent Patients?

Annemarie Heberlein^{1,*}, Rilana Schuster⁸, Alexandra Kleimann¹,
Adrian Groh¹, Andreas Kordon^{6,7}, Birgitt Opfermann⁵,
Ralf Lichtinghagen³, Michael Gröschl⁴, Johannes Kornhuber²,
Stefan Bleich¹, Helge Frieling¹, and Thomas Hillemacher¹

¹Department of Psychiatry, Social Psychiatry and Psychotherapy, Medical School Hanover, Carl-Neuberg-Str. 1, 30625 Hannover, Center for Addiction Research (CARe), Germany, ²Department of Psychiatry and Psychotherapy, Friedrich-Alexander-University of Erlangen-Nuremberg, Schwabachanlage 6-10, 91052 Erlangen, Germany, ³Institute of Clinical Chemistry, Medical School Hanover, Carl-Neuberg-Str. 1, 30625 Hannover, Germany, ⁴Bioanalytical Laboratory, Celerion Switzerland AG, Allmendstr. 32, 8320 Fehraltorf, Switzerland, ⁵Medical Service of the Health Funds of Lower Saxony, Hildesheimer Str. 202, 30519 Hannover, Germany, ⁶Oberbergklinik, Oberberg 1, 78132 Hornberg, Germany, ⁷Department of Psychiatry and Psychotherapy, Albert-Ludwigs-University of Freiburg, Fahrenbergplatz, 79085 Freiburg im Breisgau, Germany, and ⁸Central Institute of Mental Health, University Mannheim/Heidelberg, Mannheim, Deutschland

*Corresponding author: Fachklinikum Uchtspringe, Klinik für Gerontopsychiatrie Emil-Kraepelin-Str. 5, 39576 Stendal, Germany. Tel.: +49 39325702203; Fax: +49 39325 70-208; E-mail: heberlein.annemarie@mh-hannover.de

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Abstract

Aims: Neurotrophins have been linked to the symptomatology of alcohol dependence. We aimed to investigate a possible association between the methylation of the promoters of both neurotrophins, the serum levels of the cytokines and core symptoms of alcohol dependence as withdrawal severity and anxiety.

Methods: In this study we investigated a possible association between alterations in the methylation of the BDNF IV/NGF I gene promoter and the cytokines tumor necrosis factor α (TNF- α) and interleukin-6 (IL-6) in 55 male alcohol-dependent patients.

Results: Mean methylation of the promoter of the BDNF gene was significantly associated with the TNF- α serum levels and the CIWA-score during withdrawal ($P < 0.001$). Moreover, mean methylation of the NGF I promoter was significantly associated with the IL-6 serum levels and STAI-I score during withdrawal ($P < 0.001$).

Conclusion: Our results suggest an association between the epigenetic regulation of both neurotrophins, BDNF and NGF, cytokine release and the symptomatology of alcohol dependence. They imply that changes in the methylation of neurotrophins may contribute to the symptomatology of alcohol dependence by affecting relevant downstream signaling cascades.

INTRODUCTION

Various forms of stress and consecutive affective symptoms such as depression and anxiety have been associated with an increased risk of alcohol consumption (Pandey, 2003; Uhart and Wand, 2009). Alterations in proinflammatory cytokines as well as in neurotrophic growth factors have been associated with core symptoms of alcohol dependence (Heberlein *et al.*, 2014b). In our previous studies, we observed possible links between the cytokines interleukin-6 (IL-6), tumor necrosis factor α (TNF- α) and the neurotrophins nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF): we found an increase of the NGF and the IL-6 serum levels following alcohol consumption (Heberlein *et al.*, 2013, 2014a) as well as an association of BDNF, TNF- α serum levels and the history of alcohol consumption (Kiefer *et al.*, 2002; Heberlein *et al.*, 2014a).

There is broad literature that demonstrates direct interplay between cytokine and neurotrophin signaling. For example, expression of NGF has been reported to be stimulated by cytokines such as TNF- α (Ryan *et al.*, 2008) and IL-6 (Risbud and Shapiro, 2014) in different kinds of cell types (Hattori *et al.*, 1993; Peeraully *et al.*, 2004; Rezaee *et al.*, 2010). Regarding BDNF expression a reciprocal association between cytokine release and BDNF expression was noted (Song *et al.*, 2013).

Current research illustrates activation of signal cascades such as the cAMP responsive element binding protein (CREB): activation of signal cascades may underlie the interaction between cytokine release and neurotrophic growth factors (for review see Eyre and Baune, 2012). With respect to such study results one could suggest a possible association between cytokine and neurotrophin signaling underlying known associations between both neurotrophins and clinical symptoms of psychiatric diseases as depression or anxiety (Maestripieri *et al.*, 1990; Levi-Montalcini *et al.*, 1996; Cirulli and Alleva, 2009; Tadic *et al.*, 2014).

Newer research delineates the importance of the epigenetic regulation of target genes as BDNF for the symptomatology of alcohol dependence. For example, Wolstenholme *et al.* (2011) demonstrated a direct impact of epigenetic gene alterations on alcohol consumption in mice. Consistently, our studies suggest that alterations in epigenetic regulation of NGF (Heberlein *et al.*, 2011) and BDNF (Heberlein *et al.*, 2015) contribute to alcohol consumption. Since alterations in cytokine release have been associated with core symptoms of affective disorders as anxiety and depression (Schiepers *et al.*, 2005) and affective symptoms have been shown to be relevant for alcohol consumption (Uhart and Wand, 2009), we aimed to investigate a possible crosstalk between the serum levels of IL-6 and TNF- α and the epigenetic regulation of the BDNF and the NGF gene in alcohol-dependent patients in this study. With regard to study results, which suggest an association between regulatory circuits of BDNF/TNF- α (Saha *et al.*, 2006) and NGF/IL-6 (Ales *et al.*, 2008), we aimed to investigate (a) a possible association between IL-6 and the methylation of the NGF promoter, (b) a possible association between TNF- α and the methylation of the BDNF promoter and (c) possible associations between the BDNF/NGF promoter methylation, IL-6/TNF- α serum levels and affective symptoms of alcohol withdrawal.

MATERIALS AND METHODS

Patients

The present study was part of a large prospective research project (Studies in Neuroendocrinology and Neurogenetics in Alcoholism [NENA]) (Heberlein *et al.*, 2012) that was approved by the local Ethics Committee of the Friedrich-Alexander-University of

Erlangen-Nuremberg (authorization number: 3296). The investigation was conducted in accordance with the Declaration of Helsinki. Each participant gave written informed consent. In total, we investigated the methylation of the BDNF promoter (exon IV) and the NGF promoter (exon I) in 55 patients, who suffered from alcohol dependence according to ICD-10 and DSM-IV. All patients were admitted for detoxification treatment (Klinik für Psychiatrie, Psychotherapie und Psychosomatik, Bezirksklinikum Obermain, Kutzenberg, Germany). All patients who participated in this study were active smokers. Patients with concomitant psychiatric illnesses, substance abuse apart from alcohol or nicotine, existence of severe somatic illnesses (in particular patients suffering from any type of cancer), known autoimmune diseases or known HPA axis deregulations were not enrolled in the study. In addition, patients with a positive history of cerebral damage (e.g., ischemia or cerebral hemorrhage) were excluded.

Measures

Breath alcohol concentration was measured on admission and during alcohol withdrawal using the alcohol breath analyzer (Draeger, Dietikon, CH). Additional data such as age, body mass index, years of drinking and daily intake of alcohol in grams were obtained by interview.

State Anxiety was measured by the State Anxiety Inventory (STAI-I, Day 1: mean 47,89, SD 12,26; Day 7: mean 34,96, SD 9,43; Day 14: mean 34,83, SD 9,92) (Spielberger *et al.*, 1970). Severity of alcohol withdrawal was measured by the Clinical Institute Withdrawal Assessment for Alcohol (CIWA-A, Day 1: mean 15,46, SD 4,05, Day 7: mean 12,79, SD 2,69, Day 14: mean 12,13, SD 2,40) (Sullivan *et al.*, 1989). TNF- α and IL-6 were both determined by solid-phase, chemiluminescent immunometric assay. The detection levels of this assay were 1.7 ng/mL for TNF- α and 2 ng/mL for IL-6. The BDNF and the NGF serum levels were assessed using enzyme-linked immunosorbent assay (DY248, DY256, R&D Systems, Wiesbaden-Nordenstadt, Germany). All the assays were performed according to the manufacturer's directions. The lower thresholds of determination were 21 pg/mL (BDNF) and 25 pg/mL (NGF). Psychometric measurements and laboratory analyses were taken once a day on Day 1, Day 7 and Day 14.

Bisulfite-sequencing of BDNF promoter IV and NGF promoter I

Genomic DNA was extracted with the QIAamp_DNA Blood Mini Kit (QIAGEN GmbH, Hilden, Germany) from whole EDTA-blood according to the manufacturer's protocol.

Genomic DNA was modified with sodium-bisulfite to deaminate unmethylated cytosines to uracils using the Epi-Tect_Bisulfite Kit (QIAGEN). Primers were designed to amplify CpG-sites within the promoter regions of BDNF exon IV and NGF (Table 1). For amplification of the products semi-nested polymerase chain reactions (PCRs) were performed. After purification of the PCR product using AmPure (Agentcourt) each product was visualized on a standard 2.0% agarose gel. Purification of the sequencing PCR was performed with CleanSec (Agentcourt). Sequencing in reverse direction was performed with a maximum of 30 ng of purified PCR product using a Big-Dye_Terminator v3.1 Cycle Sequencing Kit (Life Technologies, Foster City, CA, USA) and the 3500xl Genetic Analyzer (Life Technologies) according to the manufacturer's instructions.

Table 1. Primers used for amplification of BDNF and NGF

NGF-Bis-Fn:	5'-TTTGGAATTTAGGGTTTTAAGTTAAG-3'
NGF-Bis-R:	5'-TCCCTAAACTACAAACCATCCTAAA-3'
NGF-Bis-seneF:	5'-TTTTGTTAGAGGAGTTGGGTATTG-3'
NGF-Bis-R:	5'-TCCCTAAACTACAAACCATCCTAAA-3'
BDNF-F1:	GGGGGAGGATTAATTGAGTTAGTTTTG
BDNF-F2:	TTTGTGGGGTTGGAAGTAAAAT
BDNF-R1:	ATATATACTCCTTCTATTCTACAACAA
BDNF-Rseq:	ACAAAAAATTTCTACTAA

Statistical analysis

All variables, except IL-6 serum levels, were normally distributed according to the Kolmogorov–Smirnov test. IL-6 serum levels were ln-transformed in order to reach normal distribution. Afterwards parametric methods were applied. Associations between the cytokine serum levels and the NGF/BDNF methylation were assessed by applying the linear mixed model.

Precisely, we did two analyses investigating the BDNF/TNF- α association and the NGF/IL-6 association.

In Model 1, the CpG methylation of BDNF was set as fixed factor and the TNF- α serum levels were set as independent factor. In Model 2, the CpG methylation of NGF was set as fixed factor and the IL-6 serum levels were set as independent factors.

Day of investigation (Day 1, 7 and 14), CpG dinucleotide location and CIWA (BDNF)/STAI (NGF) were set as further independent factors in both models. Also, the patient's number was set as a random variable in all analyses in order to alleviate the results regarding a possible high variability of methylation in individual patients.

Regarding BDNF, carbamazepine and clomethiazole dosage, regarding NGF, age and alcohol breath level were set as covariates. These covariates were chosen because of data pointing towards an association of these factors and BDNF (Chang *et al.*, 2009; Lee and Kim, 2009)/NGF (Heberlein *et al.*, 2013) expression. Furthermore, we investigated a possible association of IL-6 serum levels and CpG methylation of the NGF gene as well as TNF- α serum levels and the CpG methylation of the BDNF gene in those areas, in which relevant transcription factors as AP-2 and NF- κ B are located. Association between methylation of selected clusters of CpG-islands of the NGF/BDNF promoter and the IL-6/TNF- α serum levels were calculated by linear regression.

Mean methylation of the BDNF and the NGF promoter was calculated by repeating the analyses above without setting CpG dinucleotide location as a fixed factor. In order to calculate a possible association between the mean methylation and the cytokine levels, the same analyses were repeated without including results of psychometric measurements (STAI-I-score, CIWA-A-score) as a fixed factor.

Data were analyzed by SPSS 22 (SPSS Inc., Chicago, IL). Graphs were developed by Graph Pad Prism™ 6.0 (Graph Pad Software Inc., San Diego, CA).

RESULTS

Association between BDNF IV promoter methylation, TNF- α serum levels and CIWA-score

Mean methylation of the BDNF IV promoter was significantly associated with the TNF- α serum levels ($F = 3.083$, $P < 0.001$) during alcohol withdrawal.

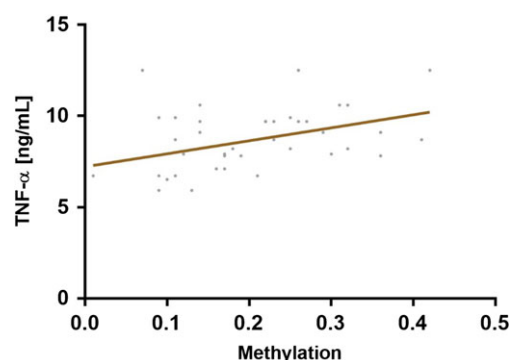


Fig. 1. Linear regression ($r^2 = 0.489$, $P < 0.001$) of the mean methylation of the BDNF IV promoter and the TNF- α serum levels restricted to the CpGs (22, -9).

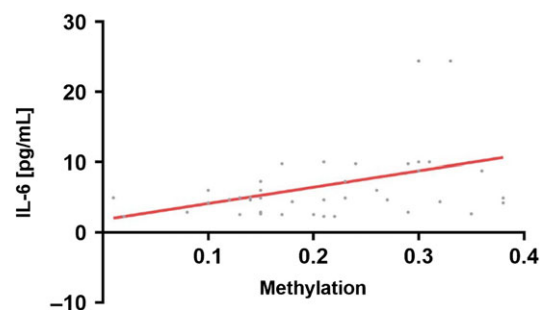


Fig. 2. Linear regression ($r^2 = 0.159$, $P = 0.008$) between the mean methylation of the NGF I promoter and the IL-6 serum levels restricted to the CpGs (54, 60, 69, 74, 76, 80).

Moreover, there was a significant association between the mean ($F = 6.755$, $P < 0.001$) and the individual ($F = 6.187$, $P < 0.001$) methylation, the TNF- α serum levels and the CIWA-score during alcohol withdrawal.

Post hoc analysis showed a prominent association between the CpG-islands (22, -9) and the TNF- α serum levels ($r^2 = 0.489$, $P < 0.001$), see Fig. 1.

Association between NGF I promoter methylation, IL-6 serum levels and the STAI-I score

The mean NGF I promoter methylation was significantly associated with the IL-6 serum levels ($F = 10.005$, $P < 0.001$).

We found a significant association between the mean ($F = 527820$, $P < 0.001$) and the individual methylation ($F = 115133$, $P < 0.001$) of NGF exon I, the STAI-I score and the IL-6 serum levels.

Post hoc analysis showed a prominent association between the cluster of CpG-islands (54, 60, 69, 74, 76, 80) and the IL-6 serum levels ($r^2 = 0.159$, $P = 0.008$), see Fig. 2.

DISCUSSION

This study addressed the question of a possible interaction of the two cytokines TNF- α and IL-6, alterations of the methylation of the NGF and the BDNF gene and symptoms of alcohol dependence in patients undergoing withdrawal. Indeed, our data show an association between the TNF- α serum levels, the methylation of the BDNF IV promoter and the CIWA-A score as well as a joint association

between the STAI-I score, the methylation of the NGF I promoter and the IL-6 serum levels. The associations found were present during the whole period of alcohol withdrawal investigated.

These results are consistent with current research which demonstrates an association between cytokine serum levels and affective symptoms as anxiety and depression. For example, Fumaz *et al.* (2012) reported about a positive correlation between self-reported anxiety, psychological stress and peripheral blood levels of IL-6 and TNF- α in human immunodeficiency virus infected patients. In our studies we found NGF and IL-6 serum levels to be significantly increased due to alcohol intoxication and to decrease significantly during alcohol withdrawal (Heberlein *et al.*, 2008, 2014a), suggesting a common mechanism of action. Consistently, as well TNF- α as methylation of the BDNF IV promoter was associated with alcohol consumption in our sample of alcohol-dependent males (Heberlein *et al.*, 2014a).

In preclinical studies, regulatory loops between neurotrophins and proinflammatory cytokines have been identified which include modulation of signal cascades as Akt phosphorylation, I κ B phosphorylation and NF- κ B binding (Prencipe *et al.*, 2014). In so far the associations between neurotrophin promoter methylation and cytokine serum levels found in this study may be reasoned by modulation of signal cascades by (de-)activation of transcription factor binding sites within the promoter region of the two genes (Otten *et al.*, 2000).

In our data we found a prominent association between the IL-6 serum levels and the methylation of a cluster of CpG-islands within exon I of the NGF gene (54, 60, 69, 74, 76, 80, see Fig. 1) that contains transcription factors as activator protein 1 which has been demonstrated to be relevant for IL-6 transcription (Irwin *et al.*, 2014). Similarly, the association between the TNF- α serum levels and the mean methylation of exon IV of the BDNF gene was most prominent in a CpG cluster (CpG-22 and -9, see Fig. 2), which contains transcription binding sites for NF- κ B and AP2 α , which have been shown to be relevant for TNF- α transcription (Koch *et al.*, 2014).

In conclusion, our results present a possible mechanism by which alterations in epigenetic neurotrophin gene regulation may affect signaling cascades that result in up- or downregulation of cytokines. By these means alteration in epigenetic regulation of neurotrophic growth factor possibly represents a mechanism which may reason affective symptoms of psychiatric disorders via activation of downstream signaling cascades. While our study design is insufficient to proof such hypotheses, further clinical and preclinical studies will be necessary in order to replicate our results and to unravel the nature of the associations found in this study.

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CONFLICT OF INTEREST STATEMENT

None declared.

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