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Review

A significant association between *BDNF* promoter methylation and the risk of drug addiction

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ABSTRACT

As a member of the neurotrophic factor family, brain derived neurotrophic factor (BDNF) plays an important role in the survival and differentiation of neurons. The aim of our work was to evaluate the role of *BDNF* promoter methylation in drug addiction. A total of 60 drug abusers (30 heroin and 30 methylamphetamine addicts) and 52 healthy age- and gender-matched controls were recruited for the current case control study. Bisulfite pyrosequencing technology was used to determine the methylation levels of five CpGs (CpG1–5) on the *BDNF* promoter. Among the five CpGs, CpG5 methylation was significantly lower in drug abusers than controls. Moreover, significant associations were found between CpG5 methylation and addictive phenotypes including tension-anxiety, anger-hostility, fatigue-inertia, and depression–dejection. In addition, luciferase assay showed that the DNA fragment of *BDNF* promoter played a key role in the regulation of gene expression. Our results suggest that *BDNF* promoter methylation is associated with drug addiction, although further studies are needed to understand the mechanisms by which *BDNF* promoter methylation contributes to the pathophysiology of drug addiction.

Contents

1. Introduction

Heroin and methamphetamine (METH) addiction has become a serious problem in China. Although heroin is the most widely used addictive drug in China, METH, an amphetamine derivative, is becoming increasingly popular in the youth (Hart et al., 2012). Addiction to these drugs is accompanied by severe social consequences, particularly in terms of premature mortality and high morbidity (Hser et al., 2001).

Abbreviation: *BDNF*, brain-derived neurotrophic factor; DSM-IV, diagnostic and statistical manual of mental disorders, fourth edition.

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Twin studies have revealed a strong heritability in drug addiction (Tsuang et al., 1999; van den Bree et al., 1998; Xu et al., 2014). Genetic variants are associated with the onset (Li et al., 2011) and development (Peng et al., 2013; Vereczkei et al., 2013; Xie et al., 2013) of drug dependence. Meanwhile, epigenetic modifications may be important as well. For example, aberrant *OPRM1* methylation has been shown to contribute to heroin addiction (Chorbov et al., 2011; Nielsen et al., 2009, 2010).

Brain-derived neurotrophic factor (BDNF) is widely distributed in brain (Leibrock et al., 1989). METH can alter DNA methylation pattern via changing the expression of DNA methyltransferase 1 (DNMT1) (Numachi et al., 2007), which is involved in the preservation of DNA methylation patterns (Goyal et al., 2006) as well as de novo DNA methylation (Gowher et al., 2005). Addictive substances have been shown to increase the BDNF protein levels in multiple brain regions (Graham et al., 2007; Grimm et al., 2003; Le Foll et al., 2005). Increased BDNF levels are found in the hippocampus of METH self-administering rats (McFadden et al., 2014) and the plasma of human METH users (Kim et al., 2005). *BDNF* Val66Met polymorphism is implicated in the heroin and METH abuse (Greenwald et al., 2013; Hou et al., 2010; Sim et al., 2010). *BDNF* promoter methylation is associated with multiple psychiatric disorders, such as depression (Kang et al., 2013), schizophrenia (Auta et al., 2013; Ikegame et al., 2013), and Alzheimer's disease (Chang et al., 2014). Since there is a lack of evidence between *BDNF* promoter methylation and heroin or METH addiction, we perform a case–control study to evaluate the contribution of *BDNF* promoter methylation to drug addiction.

2. Methods and materials

2.1. Subjects and clinical data

We collected the blood samples of 60 drug users (30 heroin and 30 METH addicts) and 52 age- and gender-matched healthy controls from the Ningbo Addiction Research and Treatment Center. The diagnosis of the drug addicts was done according to the substance abuse and dependence in the Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV) criteria. All the controls didn't have any psychological disorders or a history of drug addiction. None of the participants in our study had serious mental illness. Diagnosis of drug users was made upon heroin or amphetamine use disorder in the DSM IV criteria. Investigators were trained to classify the patients into substance abuse or dependence with DSM-IV criteria. All the subjects met the criteria for heroin or amphetamine dependence. We judged the mood state of all the drug addicts based on the Profile of Mood State (POMS) (Shen et al., 2012). An abbreviated version of POMS contains 40 items rated on a 5 point scale from 0 (not at all) to 4 (extremely) was used. POMS was used to evaluate the mood states of drug addicts, but not for the diagnosis of serious mental illness, which were judged by well-trained investigators according to DSM-IV criteria. All the subjects were assessed for severe psychiatric symptoms, such as hallucinations and delusions, and none of drug addicts had any positive evidence of serious mental disorder. Unfortunately, only the male drug addicts but not female ones had the interview records based on POMS. Our study was approved by the ethics committees of Ningbo University and Ningbo Addiction Research and Treatment Center. Written informed consent forms were obtained from all the subjects.

2.2. DNA methylation assay

DNA extraction and the measurement of DNA quantity and quality were done as previously described (Cheng et al., 2013; Jiang et al., 2013; Zhang et al., 2013). Bisulfite pyrosequencing technology was used to determine the promoter methylation (Fig. 1). Primer design and the detailed procedures were described in our previous work (Cheng et al., 2013; Jiang et al., 2013; Zhang et al., 2013). Primer sequences were 5′-TTAGTATTTAAGAGGAAAAGGGAAAGTTGT-3′ for the forward primer, 5′-CCCCCATCATAACTAAAAATCT-3′ for the reverse primer, and 5′-GGGAAAGTTGTTGGG-3′ for the sequencing primer.

2.3. Cell culture

The human HEK293T cells were purchased from the cell bank of Chinese academy of sciences (Shanghai). And the cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (HyClone) with 10% fetal bovine serum (FBS) (TransGen Biotech, Beijing) in a humidified 5% CO_2 incubator at 37 °C.

2.4. Construction of recombinant plasmids

We selected one long fragment (BDNF-L) and one short one (BDNF-S) of BDNF promoter in the current luciferase assay. BDNF-L contained the pyrosequenced sequence, while BDNF-S didn't. The insertion in the BDNF-L was amplified using the forward primer (5′- CTAGCTAGCG GCTGAGGGTAGGCAAAG-3′) and reverse primer 5′- GGAAGATCTCGA TGAGGTCGTGAGTGAT -3′. And the insertion in the BDNF-S was amplified using the forward primer (5′- CTAGCTAGCCTGAGGGTAGGCA AAG-3′) and the reverse primer (5′-GGAAGATCTACCAGCCCAACAACT TT-3′). PCR products were gel purified using the Gel Extraction Kit (Omega, Atlanta, USA). The insertions and pGL3 Basic vector (Promega, Madison, WI) were both digested with NheI and BglII (New England Biolabs, Ipswich, MA), and then purified by Cycle Pure Kit (Omega, Atlanta, USA). The target DNA fragment was cloned to pGL3 Basic vector via DNA Ligation Kit (TaKaRa, Japan). The pRL-SV40 vector (Promega, Madison, WI) with Renilla luciferase gene was used as internal control in this study. All the primers were synthesized by Sangon Biotech Company (Shanghai).

2.5. Transfection and reporter gene activity assay

The human HEK293T cells in exponential growth phase were rinsed twice with phosphate buffer saline (PBS) and plated on 24-well plates at density of 0.5×10^4/well in 500 μl Dulbecco's modified eagle medium (DMEM) with 10% fetal bovine serum(FBS). After 6 hour culture, cells of 70% attachment were transfected with recombinant pGL3 vector and pRL-SV40 using the Lipofectamine® 2000 Transfection Reagent according to manufacturer's protocol (Invitrogen, Carlsbad, USA). After 4–6 h, medium was changed by fresh Dulbecco's modified eagle medium with 10% fetal bovine serum. After 24 h of human HEK293T cells transfection, Renilla and firefly luciferase activity was detected by SpectraMax 190 (Molecular Devices, Sunnyvale, USA). Reporter gene activity was measured using the Dual-Luciferase® Reporter Assay Systems according to the manufacturer's protocol (Promega, Madison, WI).

2.6. DNA methylation inhibition assay

After 24 h co-transfection with pGL3-BDNF-L and pRL-SV40, the cells were treated with 0.5 μl DNA methyltransferase inhibitor 5-aza-2-deoxycytidine at 0.5 μM (DAC; Sigma, St Louis, MO, USA). All the experiments were preformed in triplicate and repeated three times.

2.7. 2.3 statistical analysis

ASW Statistics 18.0 software (SPSS, Inc., Somers, NY, USA) was used to perform the statistical analyses in this study. Student *t*-test was used to compare the mean value between two groups. Pearson correlation test was used to detect the relationship between methylation levels and phenotypes of participators. It was considered significant when the p-value was less than 0.05.