

# Early Alcohol Withdrawal Reverses the Abnormal Levels of proBDNF/mBDNF and their Receptors

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## Primary research

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# Abstract

## Objective

Prolonged excessive ethanol intake impairs learning, memory and also causes brain atrophy. Brain-derived neurotrophic factor (BDNF) plays pivotal roles in the pathology of alcohol dependence. Our previous work found that chronic ethanol exposure altered the metabolism of BDNF, leading to the imbalance of proBDNF and mature BDNF (mBDNF). In this study, we hypothesized that early alcohol withdrawal would reverse the abnormal levels of proBDNF, mBDNF and their receptors.

## Method

30 male alcohol dependence patients were recruited. Peripheral blood was sampled from all the subjects before and one week after alcohol withdrawal. The lymphocyte protein levels of proBDNF, p75NTR, sortilin and TrkB were analyzed by western blots and the serum level of mBDNF and TrkB was assayed by sandwich enzyme-linked immunosorbent assay (ELISA) at two different time points.

## Results

The levels of mBDNF and its receptor (TrkB) increased, oppositely the levels of proBDNF and its receptors (p75NTR and sortilin) decreased one week after alcohol withdrawal.

## Conclusions

Early alcohol withdrawal reversed the abnormal levels of proBDNF, mBDNF and their receptors. The shift levels of proBDNF and mBDNF were both taken in the pathology of alcohol withdrawal.

# Introduction

Alcohol is a common psychoactive substance. After prolonged heavy drinking, the physical and the mental state can be significantly impaired, leading to serious damage to the occupational functions and social adaptability. The gradual growth in alcohol consumption leads to the changes in the brain structure and function, and impairs the control of behaviors, which further promote the alcohol abuse and neurodegenerative diseases [1].

Accumulating evidences indicate that decreased adult neurogenesis is involved in hippocampal integrity and behaviors which correlate with psychiatric disease [2, 3], such as alcohol dependence. Chronic excessive ethanol intake can induce neuronal cell death, impair differentiation, reduce neuronal numbers and weaken neuronal plasticity. Though there are different, even opposite conclusions drawn from many investigations on neurogenesis in adults [4, 5, 6], the clinical improvements still can be found after alcohol withdrawal, such as the amelioration of atrophy brain and the cognitive deficits [7-12]. However, the molecular mechanism about these changes remains unclear. Brain-derived neurotrophic factor (BDNF) is associated with adult neurogenesis. The pathophysiology process of alcoholism and

withdrawal is complex and BDNF has always been considered as a pivotal factor involved in the whole process. Though mature BDNF (mBDNF) is generated from the cleavage of the precursor (proBDNF), they play different, even the opposite roles in neuronal survival, differentiation and plasticity [13-15]. mBDNF binds predominantly with TrkB and proBDNF binds with p75NTR and sortilin to form complexes individually to activate the subsequent cascade system [16, 17, 18]. Our previous paper reported that chronic ethanol exposure can alter the metabolism of BDNF, leading to the imbalance of proBDNF versus mBDNF and alterations in the expressions of proBDNF/p75NTR/sortilin and mBDNF/TrkB [18].

Although previous studies showed that BDNF can significantly reverse the ethanol-induced neuronal toxicity [19-21], and the alcohol abstinence can reverse the decreased levels of BDNF and TrkB and the increased level of p75NTR [22], they have not reported the precise changes of proBDNF and mBDNF in the early of alcohol withdrawal. We speculated that alcohol withdrawal might restore the expressions of proBDNF and mBDNF. In the present study, we depicted the changes of proBDNF, mBDNF and their receptors in male alcohol dependent patients at baseline and one week after alcohol withdrawal.

## Methods

### *Sample*

The study was approved by the Ethics Committee of Yunnan Mental Hospital Institution and all the experimental procedures were conducted in accordance with the Helsinki Declaration of 1975, as revised in 1983. Studies suggested that gender differences influenced the BDNF level [23-25], so we included only male patients in our study.

Male patients (n=30) with alcohol dependence were recruited from Yunnan Mental Hospital detoxification treatment unit in China from September 2010 to May 2011. Patients were enrolled to participate in the study after they had signed the informed consents for participation. The Chinese version of the Mini International Neuropsychiatric Interview (MINI) and the alcohol use disorders identification test (AUDIT) were used to screen their psychiatric and drinking conditions to confirm that they met the diagnosis of alcohol dependence according to the International Statistical Classification of Diseases and Related Health Problems 10th Revision (ICD-10) criteria by senior psychiatrists. Once the patients were in hospital, they stopped drinking completely. Excluded criteria were those: who had comorbidity with other current non-nicotine substance abuse or dependence, including sedatives; who had significant physical illnesses; who had other psychiatric disorders, such as schizophrenia, bipolar disorder, or major depressive disorder and suffered from severe cognitive impairment with difficulty in understanding the study content. All the alcohol dependence patients were hospitalized, and all received alcohol detoxification treatment with oral Oxazepam 30 - 60 mg for one to two doses with gradual tapering thereafter to prevent severe withdrawal manifestations. Multivitamins were also given. All smoking patients simultaneously received aided nicotine patches, with dose administration adjusted by the individual's average smoking amount, for smoking cessation program. (The same group of patients we recruited also participated our previous research at the same time [18].)

### ***Blood sampling***

After having obtained the permission, the coagulant and anti-coagulant vacuum blood collection tubes with Complete Proteinase Inhibitor Cocktail (cat.#04693116001, Roche Diagnostics, Indianapolis, IN, USA), plasminogen activator inhibitor 1 (PAI-1) (cat. A8111, Sigma-Aldrich) and the furin inhibitor I (Lot.#D00078005, Calbiochem) were used to get 2-5 ml peripheral blood immediately before starting the detoxification treatment (baseline) and on the eighth day after the detoxification treatments (alcohol withdrawal after 7 days).

### ***Isolation of serum***

Human blood obtained from the antecubital vein was collected in coagulant tubes and kept on ice for 4 hours, and then centrifuged for 15 min at 3000 rpm, 4°C. The top layer with light yellow color was collected and kept at -80°C before further experiment.

### ***Isolation of lymphocytes***

Blood collected in anti-coagulant tubes were kept at 4°C. The human lymphocytes separation medium (cat.# P8610-200, Solarbio, China) was used to isolate the lymphocytes from the blood. The isolated lymphocytes were kept in -80°C before protein extraction.

### ***Protein isolation from lymphocytes***

The protein isolation method was described in our previously paper (Zhou et al., 2013). Briefly, the lymphocytes were homogenized in RIPA buffer (containing 50mM Tris, 150mM NaCl, 1.0% Triton x-100, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with Complete Proteinase Inhibitor Cocktail (cat.#04693116001, Roche, USA), Plasminogen Activator Inhibitor 1 (PAI-1) (cat. A8111, Sigma-Aldrich, USA) and furin inhibitor I (Lot.#D00078005, Calbiochem, USA). After centrifugation at 13,000 rpm for 10 min, the supernatant was collected and the total protein was quantified using BCA Protein Assay kit (cat. CW0014, Cwbiotech, China).

### ***The western blotting with lymphocytes protein***

The western blotting with lymphocytes protein methods were interpreted in detail in our previous paper [26]. Briefly, 50µg lymphocyte protein was separated by running an SDS-PAGE gel, and transferred onto a PVDF membrane (400mA, 2 hours). The membrane was blocked with 5% skimmed milk dissolved in 0.1% Tween-20/Tris-buffered saline (TBST) at room temperature for 1 hour. Followed by incubation of primary antibodies (sheep anti-human proBDNF 1:200, from professor Xin-Fu Zhou's laboratory; goat anti-human p75NTR 1:250, #sc-6188, Santa Cruz, USA; rabbit anti-human sortilin 1:1000, cat.ab16640, Abcam, USA; rabbit anti-human TrkB 1:1000, cat.#07-225, Millipore, USA; mouse anti-human beta-tubulin 1:1000, #T5168, Sigma, USA) at 4 °C, overnight. After washed in TBST, the membranes were further incubated with horseradish peroxidase-conjugated anti-rabbit/mouse/sheep/goat specie-specific secondary antibodies (cat.sc-2020, Santa Cruz, USA / cat.CW0102, Cwbiotech, China / cat.CW0240, Cwbiotech,

China /cat.CW0103, Cwbiotech, China) at room temperature for 1 hour. After washed again in TBST, enhanced chemiluminescent (ECL) substrate (cat. cw0049, Cwbiotech, China) was applied to the membrane before imaging using the ChemiDoc™ XRS+ System (Bio-Rad, USA). For quantify the protein level in lymphocytes, beta-tubulin was blotted as loading control. The blots were analyzed using ImageJ software (NIH, USA).

### ***Determination of mBDNF concentration with ELISA***

The ELISA method was reported first in our previous paper [26] and proved by Lim et al. [27]. The ELISA steps were as following: 2 ug/ml of the capture antibody (protein G-purified mouse anti-mBDNF monoclonal antibody, B34D10) is diluted in coating buffer (50 mM Carbonate) and 100 ul coating antibody is immediately added into every well in the microplate, incubated for 1 hour at 37 °C. Then the plate is washed for 3 times with PBS. Next, 150 ul of blocking buffer (PBS-BSA) is added into per well and incubated for 1 hour at 37 °C. Dilutions of standard mBDNF are made in a range from 2 ng/ml - 0.125 ng/ml. 100 ul of diluted serum is applied per well and the plate is incubated at 37 °C for 1 hour. Then the plate is washed for 4 times with wash buffer. The next step, 2.5 ug/ml of biotin-labelled detecting antibody is diluted in sample diluent and 100 ul is applied to every well. Then the plate is incubated at 37 °C for 1 hour. Side by side, the plate is washed 4 times with wash buffer and incubated with streptavidin-horse radish peroxidase at 37 °C for 1 hour. After washed for 4 times with wash buffer, 100 ul of freshly prepared TMB substrate is applied to every well and develop for 10 -15 minutes. At last, 1 N sulfuric acid is used to stop the color reaction. The absorbance at 450 nm was measured with a microplate reader (Model Sunrise, TECAN, Germany) to determine BDNF concentration according to the standard curve.

### ***Determination of serum level of TrkB with ELISA***

Serum level of TrkB was determined by the commercial human TrkB kit (Sino Biological, Inc., Beijing, China) according to the manufacturer's instructions.

### ***Statistical Analyses***

All the analyses were carried out using SPSS, version 19. The age, average lifetime drinking and average daily amount of ethanol consumption were expressed as mean  $\pm$  standard deviation (SD). The non-normal distribution data were presented as median + Interquartile range (IQR), others were expressed as mean  $\pm$  standard error (SE). Comparisons between baseline and one week's withdrawal were made using a Paired Sample *t*-test or the Wilcoxon test, depending on the normality of the distribution. A *P* value which is smaller than 0.05 (2-tailed) was set as statistically significant.

## **Results**

Thirty male patients diagnosed with alcohol dependence were enrolled in this study, with mean age $\pm$ SD being 43.47 $\pm$ 7.93 years old. The average duration of alcohol dependence was 16.97  $\pm$  5.80 years, and the

average daily amount of alcohol consumption in the past one month was  $392.00 \pm 171.35$  grams of pure ethanol.

### ***Effects of alcohol withdrawal on the expression of ProBDNF/p75NTR/sortilin in lymphocytes***

Fig. 1 showed the Western blots results of proBDNF, p75NTR and sortilin of alcoholic patients in two different time points. The lymphocytes protein levels of proBDNF ( $t = -4.892$ ,  $df = 25$ ,  $P < 0.001$ , Paired Sample  $t$ -test; Fig. 1A), p75NTR ( $t = 2.860$ ,  $P = 0.013$ ,  $df = 25$ , Paired Sample  $t$ -test; Fig. 1B) and sortilin ( $t = -3.436$ ,  $df = 26$ ,  $P = 0.002$ , Paired Sample  $t$ -test; Fig. 1C) were significantly decreased after withdrawal compared to baseline. Fig. 1 manifested that proBDNF/p75NTR/sortilin complex was deregulated after one week's alcohol withdrawal.

### ***Effects of alcohol withdrawal on the expression of TrkB in serum and lymphocytes***

As shown in Fig. 2A, the TrkB level after withdrawal assayed by western blot in lymphocytes protein was significantly higher than the baseline level ( $t = 3.678$ ,  $df = 26$ ,  $P = 0.001$ , Paired Sample  $t$ -test, lymphocytes western blot method). We also determined the serum level of TrkB by ELISA assays. As shown in Fig. 2B, TrkB levels were  $423.373 \pm 29.512$  pg/ml at baseline ( $n = 30$ , mean  $\pm$  SEM) and  $784.178 \pm 66.750$  pg/ml ( $n = 30$ , mean  $\pm$  SEM) after withdrawal. The serum level of TrkB after withdrawal was significantly higher than it at baseline ( $t = -5.958$ ,  $df = 29$ ,  $P < 0.001$ , Paired Sample  $t$ -test), being consistent with the Western blot results.

### ***Effect of alcohol withdrawal on the serum level of mBDNF***

The mBDNF levels were determined by ELISA. As shown in Fig. 3, the mBDNF levels were  $17.554 + 17.29$  ng/ml at baseline ( $n = 30$ , median + IQR) and  $27.003 + 12.650$  ng/ml after withdrawal ( $n = 30$ , median + IQR). The serum level of mBDNF after withdrawal was significantly higher than at baseline ( $Z = -4.782$ ,  $P < 0.001$ , Wilcoxon test).

## **Discussion**

This study assessed the levels of proBDNF and its receptors (p75NTR and sortilin), mBDNF and its receptor (TrkB) in peripheral blood. The results represented that the level of proBDNF/p75NTR/sortilin complex was declined and the mBDNF/TrkB complex was elevated after one week's alcohol withdrawal. The results were similar to previous researches in human [28, 29] and in the animals [22], though only total BDNF level was involved in the three papers. In contrast, Heberlein et al. [30] reported that BDNF serum level did not vary significantly during alcohol withdrawal. Despite the BDNF levels detected in Costa's and Heberlein's articles were all from serum after one week's alcohol withdrawal, they got quite different results. Because proBDNF and mBDNF play opposite roles in neuronal survival, differentiation and plasticity [14], unable to distinguish mBDNF from proBDNF may give some explanations to the variation of the two researches. Our ELISA kit can accurately identify mBDNF [27], which can help us to tell the exact effects of proBDNF and mBDNF during alcohol withdrawal. It is known that

proBDNF/p75NTR/sortilin complex suppresses the neurogenesis by inhibiting proliferation, migration and differentiation of neural stem cells in mice [31]. We speculate that the increased proBDNF complex may decrease neurogenesis by retarding neural stem-progenitor cells (NPC) proliferation [2] and inhibiting new born cell survival [32, 33] in the pathology of alcohol dependence. This signaling cascades as Ron et al. [34] termed 'GO pathways' which can escalate drinking from moderate to excessive levels and that contribute to the maintenance of high level of drinking. After alcohol withdrawal, elevated mBDNF binds with TrkB to promote neuroregeneration and produced axonal morphological changes through intracellular signaling cascades, including MAPK, PLC and Rho pathways, then generating protective functions against neuronal damage produced by alcoholism. Researches have reported that chronic alcohol exposure inhibited neurogenesis and after weeks of abstinence, new neurons would be flourishing in surviving, proliferating and differentiating [33] and the reversibility of brain atrophy is believed to be due to sprouting of dendrites and axons. In the clinic, we can also find that after physical withdrawal and large supplemental doses of vitamin, especially Vitamin B1 can partly reverse cognitive disturbances, especially memory deficit [35] and control of impulse behavior [1]. Furthermore, abstinence can also increase cortical gray matter and shrink the third ventricle [36].

In conclusion, this study showed that the levels of proBDNF, mBDNF and their receptors significantly reversed after one-week alcohol withdrawal in alcohol dependent patients. The findings support that the shift levels of proBDNF and mBDNF may be both involved in the pathology of alcohol withdrawal, and the restored levels of proBDNF and mBDNF may reduce neuronal apoptosis and neuroregeneration which is related to the recovery of the brain volume and functions [8].

Our subjects were all from in-hospital patients who received oral Oxazepam 30-60mg as a standard care to prevent severe withdrawal symptoms. Though researches reported that Benzodiazepines may suppress the BDNF level in patients receiving the drugs therapeutically [37, 38], they did not study the proBDNF level. A study with animal showed that compensatory neurogenesis, reflected by increased cell proliferation, was correlated with alcohol withdrawal severity despite the use of diazepam (10mg/day) [32]. But in our study, we did not perform further study of Oxazepam on the expression of proBDNF, we could not rule out the effect of Oxazepam on the conversion of proBDNF to mBDNF and the expression of proBDNF and its receptors.

## **Limitations**

There were still some limitations in our study. Firstly, it should be cautious during making interpretations and drawing conclusions because of the small sample size. Secondly, our study subjects were all from clinical, the oral Oxazepam 30-60 mg was used for detoxification treatment for the sake of safety. The third, some blood samples had not enough lymphocytes to complete all the experiments, which led to the variation in sample numbers in the results and figures. Further studies will be undertaken, taking these variables into account to overcome these limitations and confirm the conclusion drawn in this preliminary study.

# Declarations

## Acknowledgements

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## Disclosures

All the authors have no conflicts of interest in relation to this manuscript.

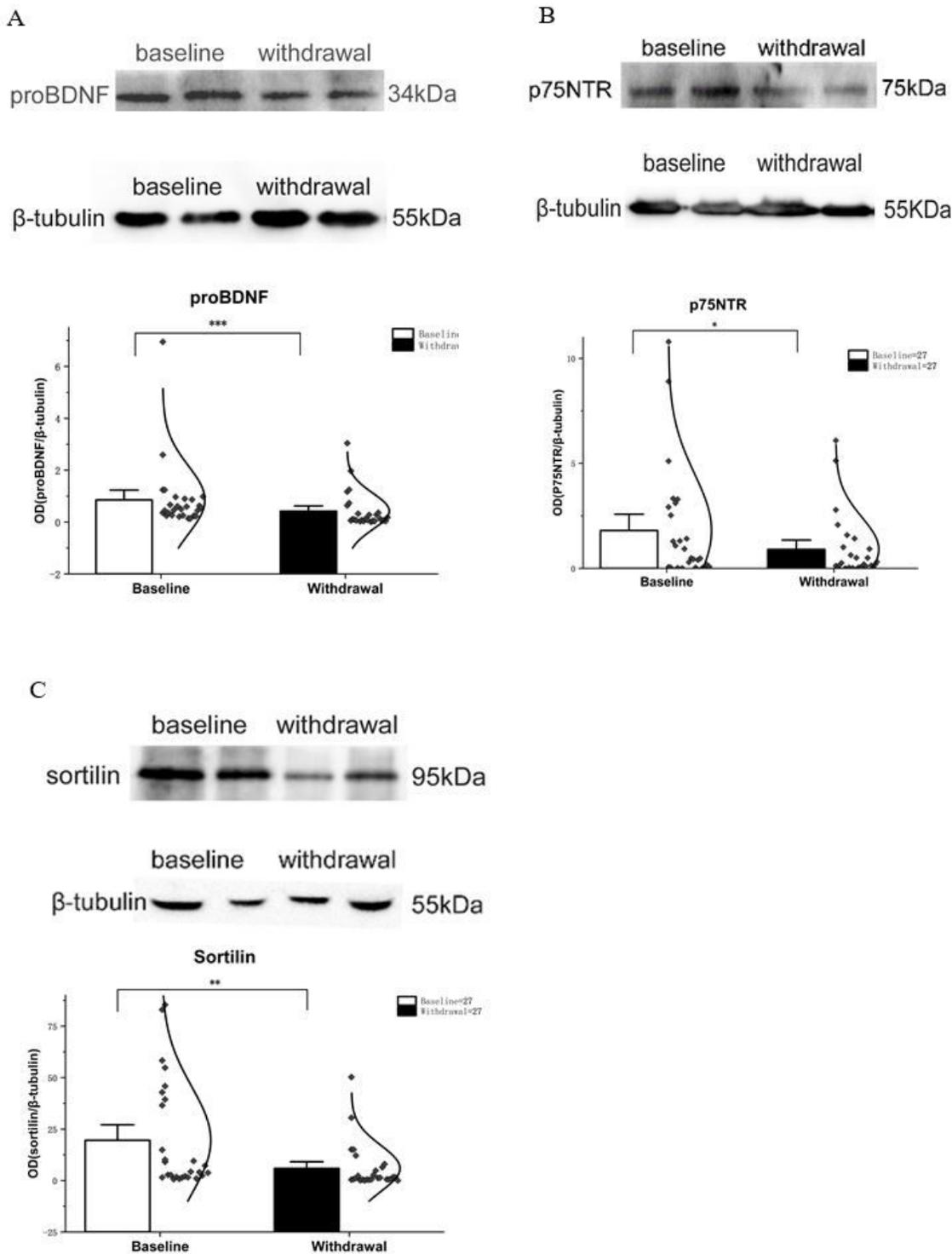
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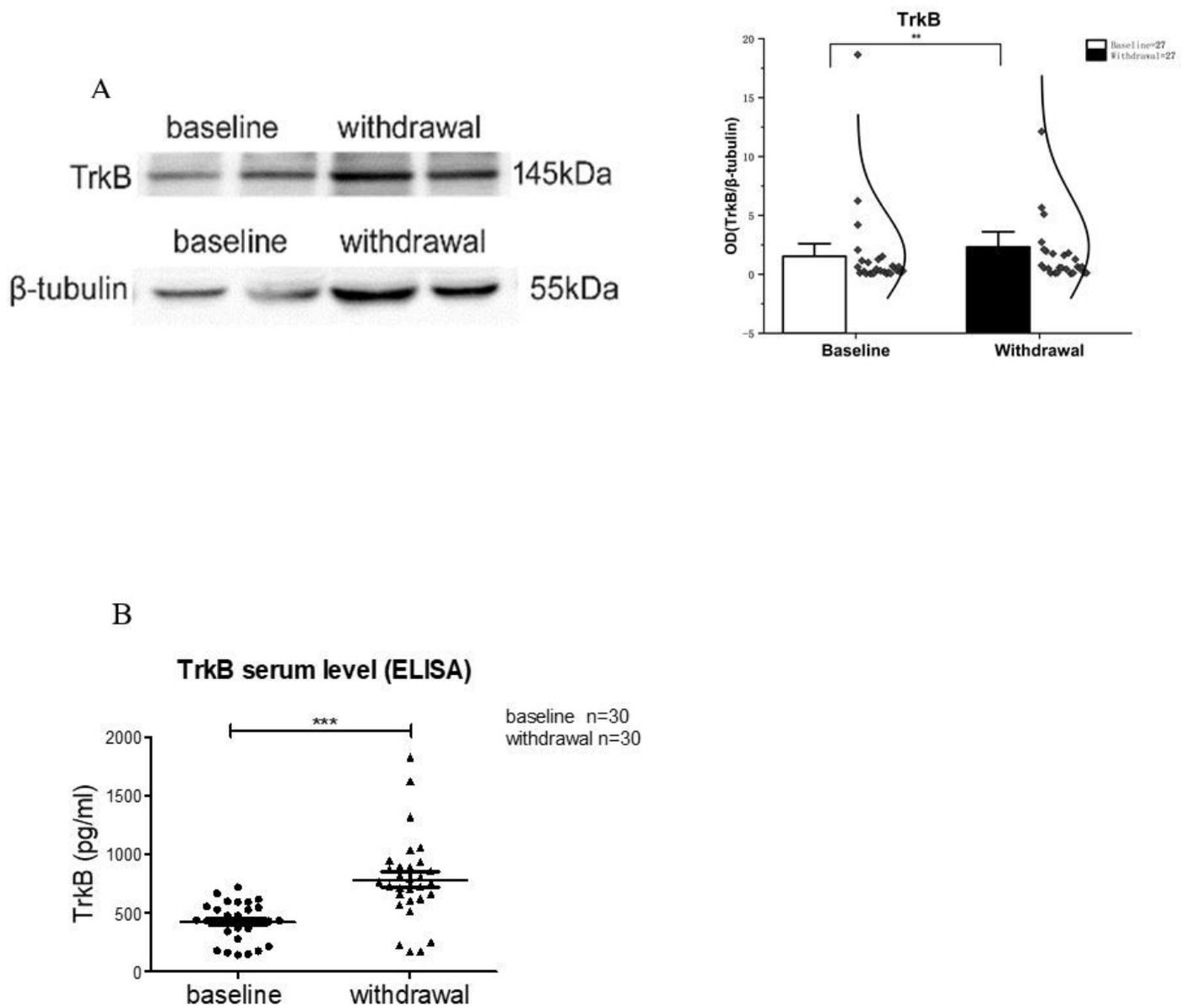
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## Figures



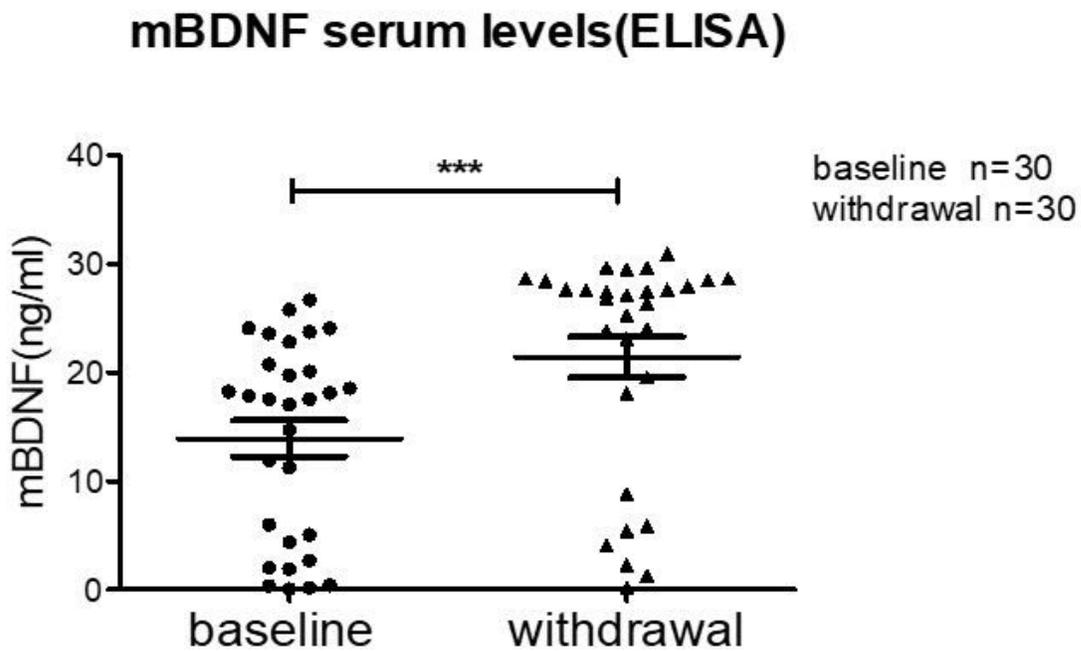
**Figure 1**

Effect of alcohol withdrawal on the protein levels of proBDNF/p75NTR/sortilin in lymphocytes. The lymphocytes protein levels of proBDNF/p75NTR/sortilin were quantified by Western blots (A, B and C). Representative Western blotting images and relative levels of proBDNF, p75NTR and sortilin in lymphocytes. Densitometric values from all western blots were normalized to  $\beta$ -tubulin (n = 27, Pared Sample t-test, \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001).



**Figure 2**

Effect of alcohol withdrawal on the protein levels of TrkB in lymphocytes as determined by Western blots and Elisa assays. Representative Western blotting images and relative levels of TrkB in lymphocytes (A). Densitometric values from the western blots were normalized to  $\beta$ -tubulin (A, n = 27). The data of TrkB assayed by the ELISA method was presented as mean  $\pm$  SEM (B, n = 30). All the data was analyzed by Pared Sample t-test (\*\*P < 0.01, \*\*\*P < 0.001).



**Figure 3**

Effect of alcohol withdrawal on the serum level of mature BDNF. The serum levels of mBDNF was assayed in the patients at baseline and after one week's alcohol withdrawal. The serum level of mBDNF were elevated in withdrawal group (n = 30, ELISA method). The data of mBDNF was presented in median + IQR and analyzed by Wilcoxon test.

## Supplementary Files

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