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Regional-specific changes in rat brain BDNF in a model of methamphetamine abuse

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Abstract

Brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family, plays key roles in neuronal protection and synaptic plasticity. Changes in BDNF are associated with various pathological conditions, including methamphetamine (meth) addiction, although the effects of meth on BDNF expression are not always consistent. We have previously demonstrated region-specific effects of a chronic meth regime on *BDNF* methylation and expression in the rat brain. This study aims to determine the effect of chronic meth administration on the expression of BDNF protein using immunohistochemistry in the rat frontal cortex and hippocampus. Novel object recognition (NOR) as a measure of cognitive function was also determined. Male Sprague Dawley rats were administered a chronic escalating dose (0.1-4 mg/kg over 14 days) (ED) of meth or vehicle; a subgroup of animals receiving meth were also given an acute "binge" (4x6mg) dose on the final day before NOR testing. The results showed that hippocampal CA1 BDNF protein was significantly increased by 72% above control values in the ED-binge rats, while other hippocampal regions and frontal cortex were not significantly affected. Meth-administered animals also demonstrated deficits in NOR after 24 hours delay. No significant effect of the additional binge dose on BDNF protein or NOR findings was apparent. This finding is consistent with our previous results of reduced DNA methylation and increased expression of the BDNF gene in this region. The hippocampal BDNF increase may reflect an initial increase in a protective factor produced in response to elevated glutamate release resulting in a neurodegenerative excitotoxicity.

Keywords: methamphetamine; brain-derived neurotrophic factor; drug dependence; hippocampus; novel object recognition

Introduction

Methamphetamine (meth) is an addictive psychostimulant drug widely misused. Long-term meth abuse can induce cognitive impairment [1], behavioral abnormalities and psychosis [2]. The effect of meth in reducing cognition is related to induction of neuronal cell death [3]. Furthermore, previous studies have reported that meth-induced cognitive dysfunction is a consequence of its effects on the structural and functional plasticity of prefrontal cortical [1] and hippocampal neurons [4,5].

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family which plays critical roles in the neuronal protection and neuroplasticity of the brain. BDNF expression in the hippocampus has been implicated in regulation of synaptic plasticity and recognition memory [6,7,8]. BDNF is involved in the stabilization of the long-term potential (LTP). It is an important molecule supporting memory formation and promoting synaptic consolidation [9, 10]. Changes in BDNF expression are associated with various conditions including psychiatric diseases, deficits in learning and memory, and addiction [10]. Administration of meth has an effect on BDNF, while alterations of DNA methylation and expression of the BDNF gene have also been observed after meth administration to rats [11].

However, although there are several reports of BDNF changes following meth administration, the results are not always consistent [4,12,13]. BDNF expression in the brain after meth administration is likely to be dependent on dose, duration and pattern of meth exposure. Also, the effect of the current protocol of an escalating dose regime of meth administration, which aims to mimic the pattern of meth abuse in humans, on BDNF protein expression has not previously been reported. Therefore, this study aims to determine the effect of different patterns of meth administration on the expression of BDNF protein in the rat frontal cortex and hippocampus, and how the findings might relate to a measure of cognitive dysfunction.

Materials and Methods

Animals and grouping

Tissues used in this study were taken from the animals of the previous experiment by Veerasakul et al (2016) [14]; these animals had also undergone novel object recognition testing as described below. The study used 18 male Sprague Dawley rats (200-250 g body weight, age 6-8 weeks) from National Animal Center, Salaya, Nakhon Pathom, Thailand. The rats were housed under a 12:12 h light/dark cycle with access to water and food ad libitum. All animal procedures followed compliance with Mahidol University Code of Practice and the National Institutes of Health (USA) guidelines for treatment of laboratory animals. The protocols were approved

by the Animal Research Committee of Naresuan University, Phitsanulok, Thailand (56 04 0057) and Burapha University, Thailand (IACUC 014/2564).

The rats were divided randomly into a control group (n=6) receiving vehicle (0.9% saline i.p.) 3 times a day (3 h interval) for 14 days; and an escalating dose (ED) group (n=12) in which the rats received gradually increasing doses of meth from 0.1 to 4 mg/kg 3 times a day for 14 days (0.1, 0.2, 0.3, …4 mg/kg at day 14, accumulative dose of meth is 90 mg/kg) and were injected with vehicle 4 times at 3-hour intervals on day 15. A subgroup of this ED group (ED binge, n=6) received a multiple high-dose of METH of 4 x 6mg instead of vehicle on day 15 (accumulative dose of meth is 114 mg/kg).

Meth preparation

The D-methamphetamine hydrochloride preparation of meth (Lipomed AG, Arlesheim, Switzerland) was used. Usage of meth was permitted by the Thai Ministry of Public Health. The meth was dissolved in 0.9% normal saline. The drug solution was freshly prepared daily based on the body weight of rats.

Novel object recognition test

The novel object recognition test (NOR) was performed after final drug or vehicle administration. NOR was performed in an open field apparatus size 40x50 cm with 50 cm high wall. The rats were allowed 5 min to freely explore the empty apparatus for habituation. 24 h after habituation, the rats were placed in the same apparatus which contained 2 objects (A1 and A2) and allowed to freely explore for 5 min (training phase). Recognition memory was assessed 24 h after training. The rats were placed in and freely explored the apparatus containing the familiar object A1 and novel object B for 5 min. All objects used in the study were different in shape, but not in size, texture or color. The apparatus was cleaned with 10% ethanol between animals.

The total time of exploration behaviors including sniffing and touching the object with nose or forepaws were recorded for recognition index calculation. The formula for the recognition index is expressed below [15]:

$$\text{Recognition (novelty) index} = TB/(TA+TB)$$

Where TA and TB are the amounts of time the rat spent exploring the familiar and novel objects respectively.

Tissue preparation and BDNF immunohistochemistry

After completion of the animal procedures, the rats were anaesthetized by CO2 and sacrificed by cervical dislocation. The brain was removed and fixed in 10% phosphate-buffered formalin. After embedding the tissue in wax, 5 µm transverse sections of the brain were placed on a slide. The prefrontal cortex section at bregma +2.28 and hippocampus section at bregma range -3.60 to -3.80 as

determined by a rat brain atlas [16] were selected and stained for BDNF, adapting the method of Lee et al (2021) [17]. Briefly, following deparaffinization in xylene and rehydration in graded alcohols, the tissue sections were immersed into citrate buffer (pH 6.0) and underwent high-temperature microwave heating (800W, 2 x 5 min) for antigen retrieval. Sections were incubated in 3% hydrogen peroxide for 15 min at room temperature (RT) to block endogenous peroxidase activity. Following washing in 1xPBS, sections were blocked with 5% normal goat serum in 1xPBS for 1h at RT. The sections were then incubated with a primary antibody rabbit anti-BDNF (mature) (Ab108319, Abcam) at a dilution 1:100 in 1% normal goat serum at 4°C overnight. After washing in 1xPBS, the goat anti-rabbit IgG H&L (HRP) (Ab6721, Abcam) at a dilution 1:200 in 1% normal goat serum was added and incubated for 1h at RT. Sections were visualized for the BDNF immunoreaction by incubation with DAB (3, 3'-diaminobenzidine tetrahydrochloride) substrate kit (Ab64238) for 4 min at RT in the dark. After stopping the reaction with distilled water, the sections were then dehydrated in graded alcohol, cleared in xylene and finally mounted in a histological mounting medium.

Image analysis

The BDNF protein immunoreactivity in the brain was captured at 10x magnification using a Nikon upright microscope (Hollywood International Ltd., BKK, Thailand) with NIS-Elements software. BDNF immunopositivity in the left and right Cg1 in frontal cortex and CA1, CA2/3 and dentate gyrus (DG) subregions in hippocampus was determined. Three non-overlapping visual fields of approximately 300um x 300um were randomly selected in the area of interest from each of 2-3 sections, except for the DG in which a single field of approximately 325um x 500um was used. The intensity of BDNF immunopositive protein/area was measured using Image J software version 1.48h3 (imagej.nih.gov/ij/.). The picture was adjusted to 8 bits for background subtraction. The mean grey value of BDNF immunopositive cells in each field, hemisphere and section for each animal was calculated and recorded for statistical analysis.

Statistical analysis

NOR performance, including the recognition index and exploration time during the training and testing phases, and the intensity of BDNF protein in the rat frontal cortex and hippocampus, were expressed as mean ± SD. Statistical significance was analyzed using independent sample t-test for control vs. total ED meth or ED vs. ED binge with SPSS 17.0 (SPSS Inc., USA). Significant differences in exploration time between familiar and novel objects in each group were also analyzed using paired sample t-tests. All p-values of $\leq$ 0.05 were considered statistically significant.

Results

During the experiment, one animal receiving the meth regime died. Where tissue damage during brain removal or tissue processing impacted the region of brain studied, the sample was removed from the study. This resulted in sample sizes of 9-11 in the meth group and 5-6 in the control group.

Novel object recognition

The recognition index and exploration times in the control and meth groups are provided in table 1 and figure 1A-C. After a 24 h delay, reductions in the recognition index were seen following chronic meth administration. Comparison of ED animals that received a meth binge or vehicle dosing on day 15 showed no significant differences and the two groups were combined for further comparisons (Fig. 1).

BDNF immunohistochemistry

The BDNF immunopositive protein in the sections is represented by brown staining. BDNF protein is located in the cytoplasm and on the cell membrane of neurons (Fig. 2). Results of BDNF immunoreactivity in the four brain regions studied are shown in figure 3. The mean intensity of BDNF in the frontal cortex and hippocampus regions in each group of rats is shown in table 2. The results found that each meth administration regime showed a tendency to increase BDNF intensity in CA1. Moreover, no significant differences between the meth animals receiving a binge (ED binge) or vehicle dosing (ED) was apparent and were combined for further analysis. The meth administration regime resulted in an increase in BDNF immunoreactivity in CA1 by 72% above control values ($p=0.002$). A smaller BDNF increase in this group was observed in the CA2/3 region which was not significant ($p=0.281$), nor were significant differences in BDNF for meth-administered rats ($p>0.5$ in each case) seen in the DG, frontal cortex when compared to controls (Fig.4).

Discussion

In this study we demonstrated a significant effect of a chronic regime of meth in the rat on NOR and a specific elevation of BDNF protein in the CA1 region of the hippocampus. No significant additional influence of an additional binge dosing following the ED course was apparent in any of the results obtained.

A chronic escalating dose of meth administration to the rat has been proposed as a valuable model of meth abuse in humans by mimicking the pattern of drug use and has been shown to replicate many of its neurochemical, behavioural and neurodegenerative effects [18,19]. Using this model, we have previously observed binding profound deficits in calcium proteins as markers for GABAergic interneurons [14] as well as changes in both the epigenetics (DNA methylation) – seen also in human meth users – and gene expression of BDNF [11]. The latter findings in the rat hippocampus – a decrease in DNA methylation and an increase in expression of the BDNF gene – were not observed in the frontal cortex and are thus highly consistent with the current observation of a selective increase in hippocampal BDNF in the CA1 region. DNA methylation is an epigenetic process potentially controlling gene

and protein expression. Increased methylation can inhibit transcription factor binding to the promoter region of the gene, often leading to a reduction in gene transcription and protein expression [20], consistent with what we observe here.

The NOR test demonstrated deficits in recognition memory associated with chronic meth administration. This is a consistent finding following meth exposure in various rat models of abuse [15, 21] and reflects the cognitive deficits seen following human meth misuse [22]. The findings after 24 h indicated a deficit in recognition memory following chronic meth administration. This inter-trial delay period is relatively specific for hippocampal (particularly CA1) dysfunction while other regions are also implicated in NOR deficits with shorter inter-trial intervals [23].

Why then does a neurotoxic regime that eventually results in cognitive impairment and neurodegeneration demonstrate an elevation in the neuroprotective neurotrophin BDNF? Others have shown similar effects on BDNF; previous studies have reported that several weeks' repeated meth self administration induced an increase of BDNF expression in the hippocampus of rats [4, 24]. The former study showed the effect was also associated with a decrease in the pro-apoptotic protein Bax and increased anti-apoptotic protein Bcl2, indicative of a potential reduction in cell death. Administration of 5mg/kg meth to rats for 7 days also resulted in an increase in hippocampal BDNF, while 10mg/kg reduced BDNF below control values [13]. These effects on BDNF had equivalent consequences on apoptotic cells in hippocampal CA1, with 5mg showing reductions, and 10mg/kg showing increases, in apoptosis revealed by TUNEL staining [13]. This indicates a dose-dependent effect on the BDNF response and its acute effects on neuroprotection or degeneration.

The present study is consistent with previous research by Yang et al. (2022) [25], which investigated the effect of ginsenoside Rb1 on attenuating meth-induced neurotoxicity. In their study, the authors observed a significant elevation of BDNF protein in corticolimbic regions (hippocampus, nucleus accumbens, and prefrontal cortex) in rats injected with 2mg/kg of meth. However, BDNF levels reduced in meth-treated rats when treated with ginsenoside Rb1. Therefore, the present study supports the evidence suggesting that BDNF is involved in meth-induced neurotoxicity [25]. Moreover, an increase in BDNF was observed in the hippocampus of rats that received 5mg/kg (subcutaneous injection) meth postnatally [26]. These authors suggested that BDNF, which is known to contribute to the brain's adaptive response to chronic stress, participates in the hypothalamic-pituitary-adrenal (HPA) axis's reaction to stress. Therefore, the observed rise in hippocampal BDNF in the current study may also reflect an adaptive response to the chronic stress or brain toxicity induced by meth. Additionally, 20 mg/kg i.p. meth was previously reported to damage the blood-brain barrier (BBB) in the hippocampus, resulting in brain edema and neuronal injury [27]. However, these neurotoxic effects of meth were mitigated by curcumin, which boosting the levels of BDNF and dopamine in the brain [27]. Given these findings, the significant increase in BDNF following meth injection likely indicates a response to meth-induced toxicity, with the BDNF rise serving a neuroprotective role.

We previously reported that these meth-administered rats also had losses of the hippocampal GABAergic neuronal markers calbindin and, particularly, parvalbumin [14]. Such a dysfunction of inhibitory interneurons controlling glutamatergic neuronal cell activity would be expected to be associated with increased glutamate release. A dynamic interaction between BDNF and glutamate

release has been reported [28, 29, 30, 31]. Intracellular BDNF overexpression could increase the frequency of excitatory post-synaptic currents (EPSCs), indicating glutamate release, in hippocampal neurons in culture [31]. However, glutamate can increase the expression of BDNF mRNA and BDNF peptide in fetal hypothalamic neurons [28]. Therefore, these in turn are likely to result in elevated BDNF, expression of which can increase in response to glutamate [28,29]. However, an overactivity of glutamatergic neurons from a loss of GABAergic inhibition and increased glutamate release may subsequently result in an excitotoxic neurodegenerative process resulting after some time in neurodegeneration, presumably overcoming any protective effect of elevated BDNF. An excitotoxic challenge can result in an increase in hippocampal BDNF [32]. Glutamate dysfunction subsequent to loss of parvalbumin-containing GABAergic neurons has been implicated in schizophrenia (e.g. Gaspar et al (2009) [33]), where hippocampal parvalbumin immunoreactivity is also lost [34], and may therefore also underlie the development of methamphetamine psychosis [35]. Kuczenski et al (2007) [19] demonstrate that after an ED-binge meth administration to rats, hippocampal neurodegeneration occurs following a delay of 30 days, but not 3 days, without drug.

There are also reports of differences in BDNF concentrations in plasma from human subjects exposed to meth, to some extent consistent with our findings. Increases are observed in meth dependence that are suggested to be a potentially protective factor in meth-induced neuronal toxicity [36]; the increases appear unrelated to neurocognitive deficits in such subjects [22]. This may relate to the relationship with chronic stress as discussed above [26]. However, a reduction of BDNF to control levels is found after a 40-day period of meth withdrawal [37], indicating a potential late emergence of vulnerability to neurotoxic effects and subsequent neurodegeneration [19].

There are substantial limitations to the current study, notably in the small sample sizes which were restricted for ethical and pragmatic reasons; a larger sample would inevitably have resulted in more robust findings. However, this sample was still associated with substantial and highly significant deficits in hippocampal and frontal cortical calcium binding proteins [14] so strong effects were to be expected. Additionally, a similar sample size was able to demonstrate significant, and region-specific, differences in hippocampal methylation and expression of the BDNF gene in an ED-binge group [11], results consistent with our current findings. The study was limited to investigating BDNF protein determined at the cellular level; analysis of the TrkB receptor for BDNF would be a valuable addition in future studies, as would an assessment of the time course of BDNF protein expression following the drug regime and its relationship with the development of neurodegenerative changes. These were beyond the scope and limited funding available for the current study. Nevertheless, this small investigation provides results consistent with other reports and both epigenetic and gene expression findings, as well as generating further hypotheses relating to the development of cognitive and psychiatric consequences of meth abuse.

Conclusion

The elevation of hippocampal BDNF, observed here in an animal model of meth abuse and addictive behaviour, is consistent with our previous findings relating to BDNF expression and gene methylation, as well as with other reports of BDNF changes following meth

exposure. This finding may reflect a potentially excitotoxic release of glutamate which in the longer term could contribute to neurodegenerative changes underlying the cognitive and psychotic consequences of meth abuse.

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Author contributions

Sri-arun Iamjan: Conceptualization, Methodology, Formal analysis and investigation, Writing - original draft preparation, Funding acquisition, Resources. **Siriluk Veerasakul:** Methodology. **Gavin P Reynolds:** Writing - review and editing, Supervision, data analysis. **Samur Thanoi:** Resources, Supervision. **Sutisa Nudmamud-Thanoi:** Conceptualization, Writing - review and editing, Funding acquisition, Resources, Supervision.

Declaration of Competing Interest

The authors have no competing interests that could influence the work reported in this paper.

Declaration of Generative AI and AI-assisted technologies in the writing process

The authors did not use any AI tools for writing this work.

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Table 1 **Recognition index and exploration time in each group of rats**

Groups (n)	Recognition index (mean ± S.D.)				Exploration time (sec.) (mean ± S.D.)					
	Training phase	p value	Recognition memory phase	p value	Training phase			Recognition memory phase		
					Familiar object	Novel object	P value	Familiar object	Novel object	P value
Control (n=6)	0.510±0.073	-	0.700±0.054	-	27.67±10.26	30.13±12.95	0.505	16.43±4.45	39.67±13.85	**0.004**
Total ED meth (n=11)	0.563±0.070	0.167[a]	0.462±0.109	**0.000[a]**	23.15±8.72	28.99±6.77	**0.019**	35.45±12.97	29.67±9.34	0.171
ED (n=5)	0.572±0.071	-	0.440±0.153	-	22.24±10.75	28.14±5.47	0.118	38.40±14.08	29.84±10.85	0.353
ED Binge (n=6)	0.555±0.076	0.712[b]	0.480±0.065	0.574[b]	23.90±7.62	29.70±8.14	0.129	33.00±12.72	29.53±8.96	0.317

Recognition index; P^a comparison between total ED meth vs. controls, P^b comparison between ED vs. ED binge, by independent sample T-test

Exploration time; P values were from paired sample t-test comparison between the familiar and novel object exploration

Table 2 Mean intensity of BDNF/area in frontal cortex and hippocampus of each group of rats

Groups (n)	Mean intensity of BDNF/area (mean ± S.D.)							
	Frontal cortex		Hippocampus					
	Cg1	p value	CA1	p value	CA2/3	p value	Dentate gyrus	p value
Control (n=5-6)	11.80±4.13	-	8.83±1.90	-	24.73±3.12	-	12.76±2.91	-
Total ED meth (n=11)	13.45±4.57	0.481[a]	15.18±4.86	**0.002** [a]	27.64±6.10	0.281[a]	12.18±4.20	0.743[a]
ED (n=5)	12.53±2.86	-	13.74±6.33	-	25.15±8.00	-	10.92±3.71	-
ED Binge (n=6)	14.60±6.45	0.582[b]	16.38±3.35	0.397[b]	30.13±2.47	0.308[b]	13.23±4.63	0.392[b]

P[a] comparison between total ED meth and control by independent sample T-test

P[b] comparison between ED and ED binge by independent sample T-test

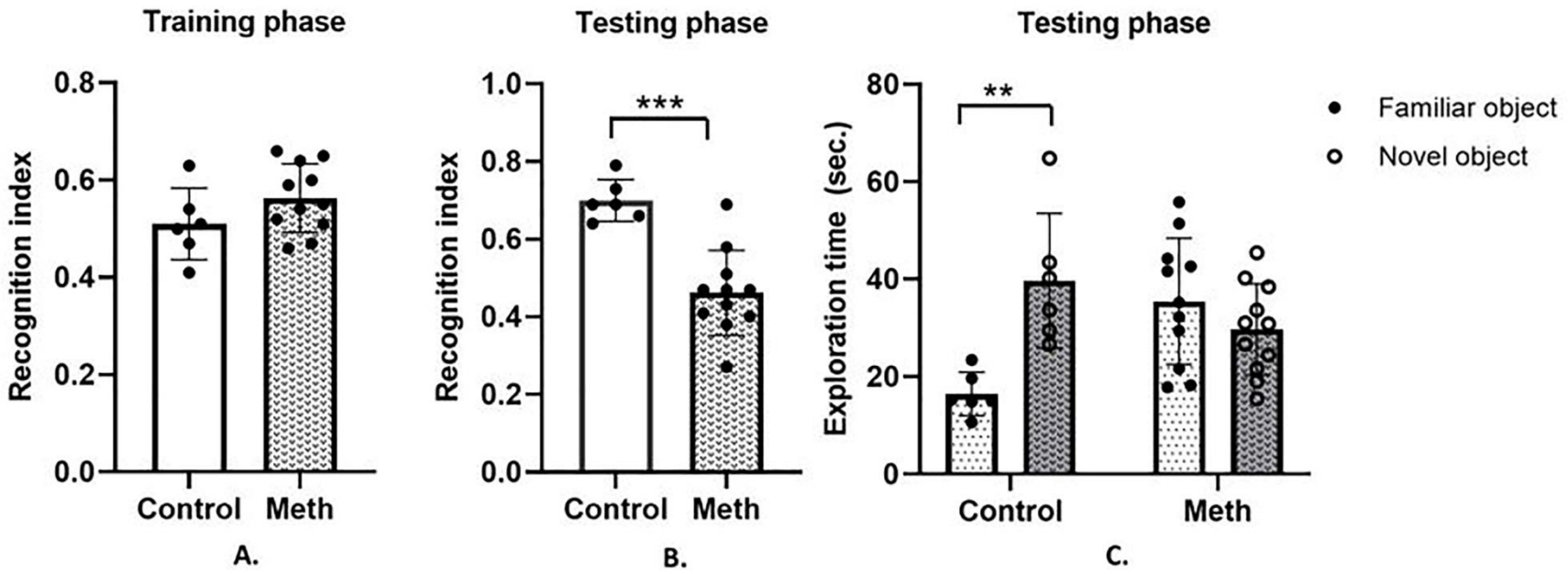

Figure 1. Illustration of novel object recognition performance between control and total ED meth. (A) Recognition index in training phase, (B) recognition index in testing phase, (C) exploration time (sec.) in testing phase. Data expressed mean±S.D. Statistical significances were analyzed using an independent sample t-test for the recognition index and a paired sample t-test for the exploration time, ***P<0.001 compared to control, **P<0.01 compared between the familiar and novel object exploration.

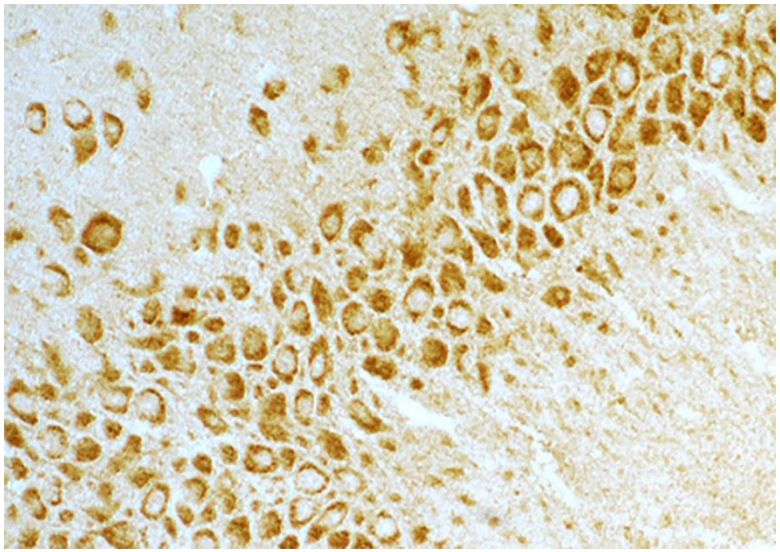

Figure 2. BDNF localization in the neuron

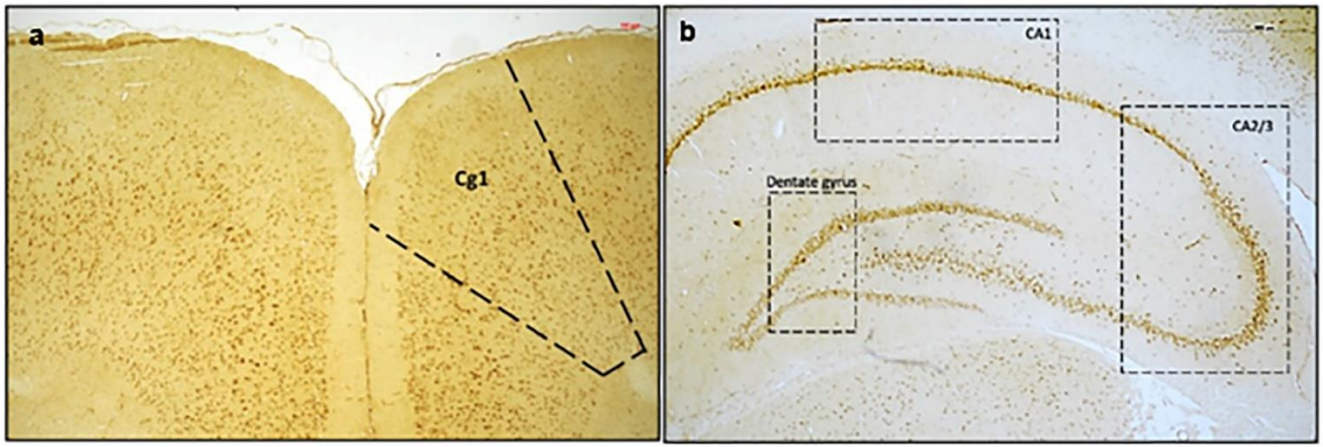

Figure 3. illustration of Cg1 of the frontal cortex (a) and CA1, CA2/3 and DG of the hippocampus (b)

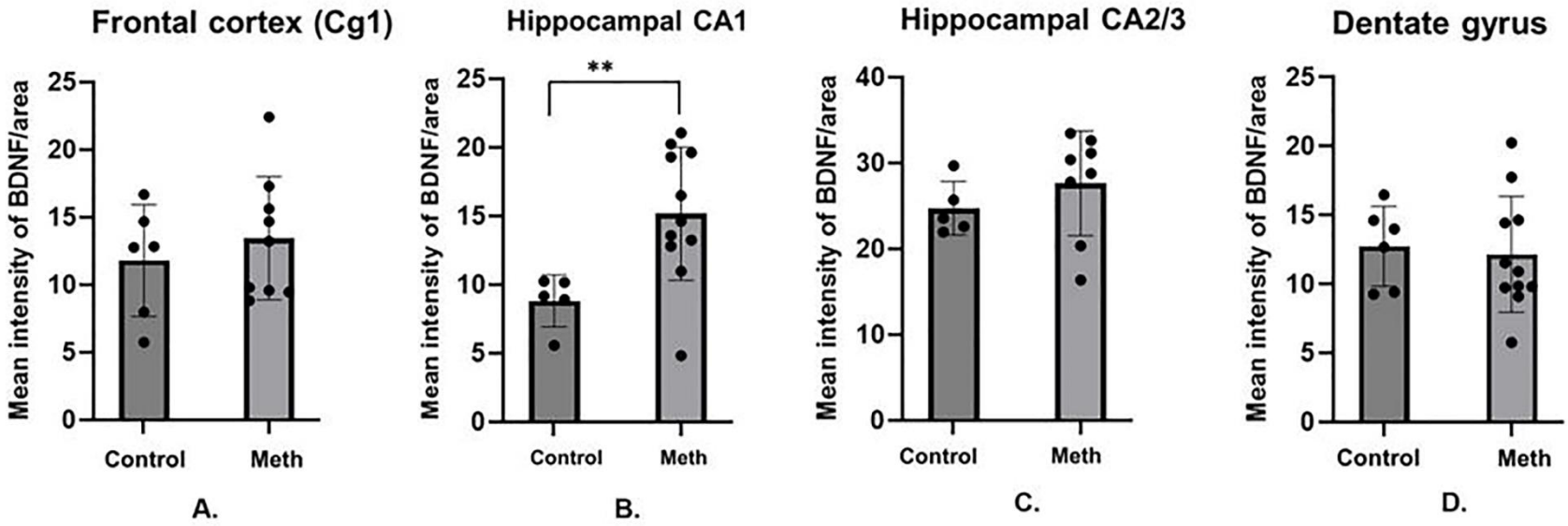

Figure 4. Illustration of mean intensity of BDNF protein comparison between control and total ED meth in rat frontal cortex (A) and hippocampus (B-D). Data expressed mean±S.D. and analyzed by independent sample T test. ** $P < 0.01$ compared to control.