Levels of BDNF and NGF in Adolescent Rat Hippocampus Neonatally Exposed to Methamphetamine Along With Environmental Alterations

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Summary

Neurotrophins are proteins included in development and functioning of various processed in mammalian organisms. They are important in early development but as well as during adulthood. Brain – derived neurotrophic factor (BDNF) and nerve growth factor (NGF) have been previously linked with many psychiatric disorders such as depression and addiction. Since during postnatal development, brain undergoes various functional and anatomical changes, we included preweaning environment enrichment (EE), since enrichment has been linked with improved function and development of the several brain structure such as hippocampus (HP), in which we monitored these changes. On the other hand, social isolation has been linked with depression and anxiety-like behavior, therefore postweaning social isolation has been added to this model as well and animal were exposed to this condition till adolescence. We examined if all these three factors had impact on BDNF and NGF levels during three phases of adolescence – postnatal days (PDs) 28, 35 and 45. Our results show that EE did not increase BDNF levels neither in control or MA exposed animals and these results are similar for both direct and indirect exposure. On the other side, social separation after weaning did reduce BDNF levels in comparison to standard housing animals but this effect was reversed by direct MA exposure. In terms of NGF, EE environment increased its levels only in indirectly exposed controls and MA animals during late adolescence. On the other hand, social separation increased NGF levels in majority of animals.

Key words

Methamphetamine • Critical developmental period • Adolescence • Social isolation • Enriched environment • BDNF • NGF • Hippocampus

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Introduction

Neurotrophins are important regulators of neural survival, development, function, and plasticity [1]. Nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) are proteins which serve as potential therapeutic options to increase neural repair and recovery as they promote neuroprotection and regeneration. These proteins are abundantly expressed in the neocortex and hippocampus (HP) during development, but their expression continues in adulthood, as reported from animal studies [2,3]. For many decades it has been suggested that alteration of neurotrophins is involved in several pathophysiologies of psychiatric disorders (depression, anxiety, addiction etc.). Development of addiction is associated with alteration of structural and functional changes which together belong to processes of neuronal plasticity. Neuronal plasticity, as ability of brain to change through the growth is necessary for successful information storage and memory formation as well as for adaptive responses resulting in various modifications of behavior. These processes are facilitated by increased BDNF synthesis and release [4]. Link between BDNF and neuronal plasticity is well established and play important role in mediating synaptic changes involved in learning and memory, which underlies behavioral and structural adaptations associated with drug addiction [5-7].

HP is part of the limbic system, where these grow factors are massively expressed [4]. Rat neurogenesis of HP occurs prenatally (embryonal days 16-19) and peaks [3] during early stages (first three postnatal weeks). Interneurons are generated postnatally, and neurogenesis of dentate gyrus continues till senescence [8,9]. Adolescence is a critical period of development when organism transits from childhood to adulthood. In humans, adolescence is considered to be around 12 to 20 years of age, and postnatal days (PD) 28 to 42 in rodents [10]. Alteration in BDNF, during development, when continuous modeling of brain occurs through adolescence into adulthood may lead to disruption of development, especially by drug exposure [9], because it has been shown that drugs may disrupt neurogenesis resulting in long-lasting changes in adulthood [8].

Our laboratory is focused on addiction research for decades, especially to neurotoxic effect of methamphetamine (MA). MA is highly addictive psychostimulant abused worldwide, but especially in central Europe (Czech Republic, Slovak Republic, etc.). It gained its popularity due to quick onset of desirable effects such as increased alertness, energy burst as well as decreased appetite and need for sleep. Main issue with MA abuse is its potential neurotoxic effects on human abusers. Experimental studies show that it causes long-term damage to various brain regions of laboratory animals resulting in long-term changes in behavior [11,12]. Our previous studies demonstrated, that prenatal, as well as early postnatal MA exposure impair cognitive functions that are associated with HP [13-15]. These alterations may be also associated with compromised behavior of mothers exposed to MA. Rat mothers exposed to MA during gestation and lactation exhibit impaired maternal behavior, which is manifested by spending less time on maternal activities and more time on self-care [12,16,17]. Early postnatal stress in humans leads to significant memory impairments in adulthood [18] and it is associated with reduced BDNF levels [19]. In rats, it leads to decreased synaptogenesis of HP, as well as decreased levels of BDNF, and long-term potentiation and memory defects [20]. According to clinical and experimental studies, any separation of the developing individuals from their mother causes significant changes in developmental patterns, such as an increased risk for addiction later in life [21,22], since it causes disruption in the hypothalamus-pituitary-adrenal axis [23,24].

Rat's brain development peaks during first two postnatal weeks. This period corresponds to third trimester of human pregnancy, when similar brain maturation occurs [13,25]. Therefore, it is very important to look on possible effect of MA exposure of pups by breastfeed of exposed mothers, since it is only source of nutrition till weaning. It has been reported that MA exposure during early gestation stages may not affect fetal brain development, but during later stages of prenatal and early postnatal development may be harmful [12,25]. During early postnatal period neurotransmitter systems mature and therefore this period could be potentially more sensitive to drug exposure [26]. Also, MA affects development of neuronal endings and alters maturation of the brain structures which begins during second half of gestation and continues till weaning [12,27]. Other study from our laboratory revealed that early postnatal administration of MA to pups between PD 1-11 affected performance during Morris water maze task, examining learning and memory function dependent on HP, such as worsened spatial learning and the ability to remember the position of a hidden platform [13].

Our studies so far show that exposure of MA can alter development of young organism which may have long-lasting serious consequences, but question remains as to whether there are factors that might repair or reverse these consequences. One of the tools can be animal model of positive modulation and stimulation of neural plasticity *via* exposing to enriched environment (EE) [28]. However, there is lack of knowledge about appropriate timing of EE exposure. Studies reported that effect of EE on brain plasticity and behavior in adolescent rodents were more significant compared with those before weaning due to the more complex neural circuits and the approaching maturity of the nervous system [29]. The mechanism involved is inseparable from the secretion of BDNF [29]. Previous studies have also shown that EE can enhance the growth factors that promote neurogenesis, including an NGF [28,29], and that EE significantly induced neurogenesis of HP in adult mice [28]. Other study reported that rats housed in group exposed 30 days to EE had significantly higher levels of NGF mRNA than rats housed individually in single cages without stimulus-enrichment [30]. Studies have shown that EE has beneficial effects on these diseases [31]. Moreover, EE exposure can also reduce the strengthening and seeking of psychostimulants and reduce the risk of relapse [32].

EE has been found to have anti-anxiety and antidepressant effects in animal models induced by social isolation. Social isolation elicits chronic stress since rats naturally live in groups and preventing them of social contacts and interaction for a longer time deprives them

of important stimuli and represents a significant stressor. Chronic social isolation induces a variety of symptoms in rats, including depression, anxiety, and psychosis-like behaviors. There has been reported an altered expression of BDNF in the brain of rats housed in social isolation. Majority of studies across all age groups (post-weaning, adolescent, adult) reported a decreased expression of BDNF in HP. This supports the evidence that chronic stress downregulates hippocampal BDNF expression in rats, in line with the findings from other chronic stress paradigms [33].

Based on the above, the aim of the present study was to examine the effect of early postnatal MA exposure, either directly (daily injection) or indirectly (by breastfeed of exposed mothers) on levels of BDNF and NGF in rat's HP with respect of different preweaning (EE vs. standard) and postweaning (grouped vs. separated) housing conditions. Adolescent male rats were analyzed at 3 ages: postnatal day (PD) 28 that corresponds to juvenile (early adolescence), PD 35 – adolescence, PD 45 – late adolescence.

Methods

Methods

The procedures used in this study were reviewed and approved by the Institutional Animal Care and Use Committee and meet the Czech Government Requirements put forth under the Policy of Humans Care of Laboratory Animals (No. 86/609/EEC) and with the subsequent regulations of the Ministry of Agriculture of the Czech Republic.

Animals

Adult female and male Wistar rats were purchased from Velaz (Prague, Czech Republic) and bred by Charles River Laboratories International, Inc. Rats were housed in a temperature-controlled (22-24 °C) room using a standard 12 h light/dark cycle. Animals were left undisturbed for one week before fertility determination. Food and water were available *ad libitum* during that period. For determination of estrous cycle phase female rats were smeared using vaginal lavage. At the estrous phase females were housed overnight with sexually matured males. Determination of fertilization was performed by smearing of females for presence of sperm. Cage [13,34]. The day after birth, the number of pups in each litter was adjusted to 12. Pups were randomly assigned to MA-treated (MA) groups and saline (SA)-treated control groups.

Chemicals

Physiological saline (0.9 % NaCl) and d-Methamphetamine hydrochloride were purchased from Sigma-Aldrich (Czech Republic).

Experimental design

In this experiment, we studied the effect of two different methods of postnatal MA administration:

- direct – subcutaneous administration to pups on postnatal days (PD) 1-12,
- indirect – subcutaneous administration to mothers on PDs 1-12, so that pups received the drug *via* maternal breast milk.

MA was injected at a 5 mg/ml/kg dose, and control SA rats were given the same volume of SA.

During the period before weaning (PD 1-21), pups were exposed to a standard environment (i.e. standard cages; L: 39×W: 24×H: 18) used in our laboratory or to an enriched environment (EE) with larger cages (L: 51×W: 42×H: 41) containing various toys. Pups were divided into groups according to the environment in which they were raised. On PD 21, the pups were weaned from mothers and divided into two different groups:

- housed together – housed in groups of 4 (natural for rats as social animals) (L: 51×W: 42×H: 41)
- housed separately – rats were housed separately, one animal per cage (L: 39×W: 24×H: 18) which is thought to be a stressor.

The experimental timeline is shown in Figure 1.

In total, 384 male rat pups were used in this study, 8-10 per group, divided according to:

- age when sacrificed – PD 28 – early adolescence, PD 35 – mid-adolescence, PD 45 – late adolescence
- treatment – MA vs. control
- drug application – direct vs. indirect
- housing before weaning – standard cage vs. EE.
- housing postweaning – group vs. separate

Eight rats were used in each group. The animals were left undisturbed until the day of sacrifice. In the present experiment, only males were tested because of the complications linked with the hormonal cycle in females.

Determination of BDNF and NGF by enzyme-linked immunosorbent assay (ELISA)

On the respective days (PD 28, 35, and 45), the animals were anesthetized with an overdose of chloral hydrate (400 mg/kg, Sigma-Aldrich) and given an intracardial perfusion of heparinized saline. The hippocampi were removed, snap frozen on dry ice, and stored at -80 °C for further processing. During processing, the samples were homogenized in phosphate

buffered saline (Sigma Aldrich) containing cOmplete™ Protease Inhibitor Cocktail (Roche) for a final concentration of 100 mg/ml. The homogenates were centrifuged at 10000× g in a cooled microcentrifuge for 10 min; the supernatants were aliquoted and stored frozen at -80 °C until assayed. Levels of BDNF and NGF were measured with commercially available ELISA kits (E-EL-R1235 and E-EL-R0652, Elabscience Biotechnology Inc.). Assays used samples diluted 20times, and analyses were performed according to the manufacturer´s instructions. The absorbance was read at 450 nm on an 800™ TS microplate Absorbance Reader (BioTek).

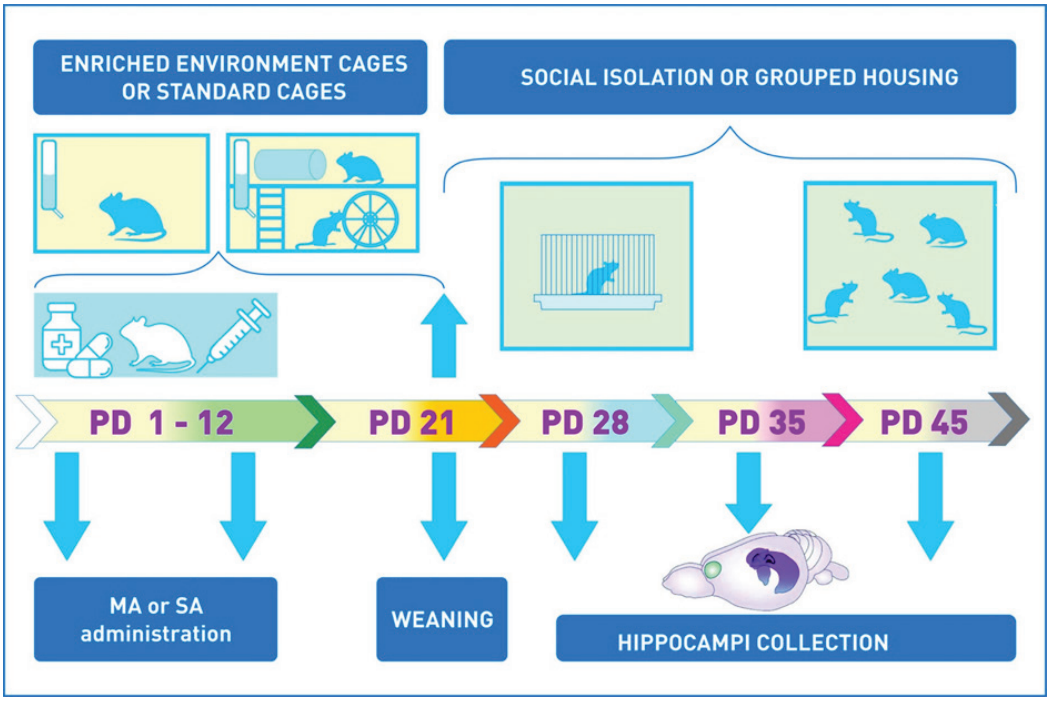

Fig. 1. Timeline of the experiment and design. Figure demonstrates time slots of respective procedures such as substance administration, experimental condition of animals as well as hippocampi collection.

Statistical analyses

A three-way ANOVA (treatment × environment × housing) was performed to measure the statistical significance, which was done separately for each of the different ages and forms of exposure. The Tukey *post hoc* test was used for multiple comparisons between groups. GraphPad Prism 9 and TIBCO Statistica™ software were used for statistical analyses.

Results

Levels of BDNF after direct exposure

On PD 28 there were no significant differences between groups (Fig. 2A).

On PD 35 several factors of significance were found: treatment [$F_{(1,56)}$=12.41, p=0.0009], environment [$F_{(1,56)}$=31.20, p=0.0001], interaction between environment and housing [$F_{(1,56)}$=5.030, p=0.0289], and interaction between all factors [$F_{(1,56)}$=6.588, p=0.0130]. In multiple comparison analysis we acquired differences between these groups: levels of BDNF in CTRL standard separate animals were significantly lower than in MA standard separate (p=0.0005). This levels in MA standard separate were significantly higher than MA standard group (p=0.0069), MA EE separate (p=0.0001) as well as MA EE group (p=0.0004) (Fig. 2C).

On PD 45 the following significant factors were: treatment [$F_{(1,51)}$=5.495, p=0.0230], treatment × housing [$F_{(1,51)}$=4.624, p=0.0360] and interaction of all three factors [$F_{(1,51)}$=5.290, p=0.0256]. Levels of BDNF in MA standard separate were significantly higher than in CTRL standard separate (p=0.0030), MA standard group (p=0.0301) and MA EE group (p=0.0481) (Fig. 2E).

Levels of BDNF after indirect exposure

According to statistical analyses, our results shows, that indirect exposure of MA did not eminently altered BDNF levels and we did not obtain significant differences (Fig. 2B, D, F).

Levels of NGF after direct exposure

On PD 28 after direct exposure, there were no significant differences between groups (Fig. 3A).

On PD 35, factor of significance was environment [$F_{(1,56)}$=8.626, p=0.0048]; levels of NGF levels in standard group were significantly higher than in EE group (p=0.0040), which was more apparent in CTRL group (Fig. 3C).

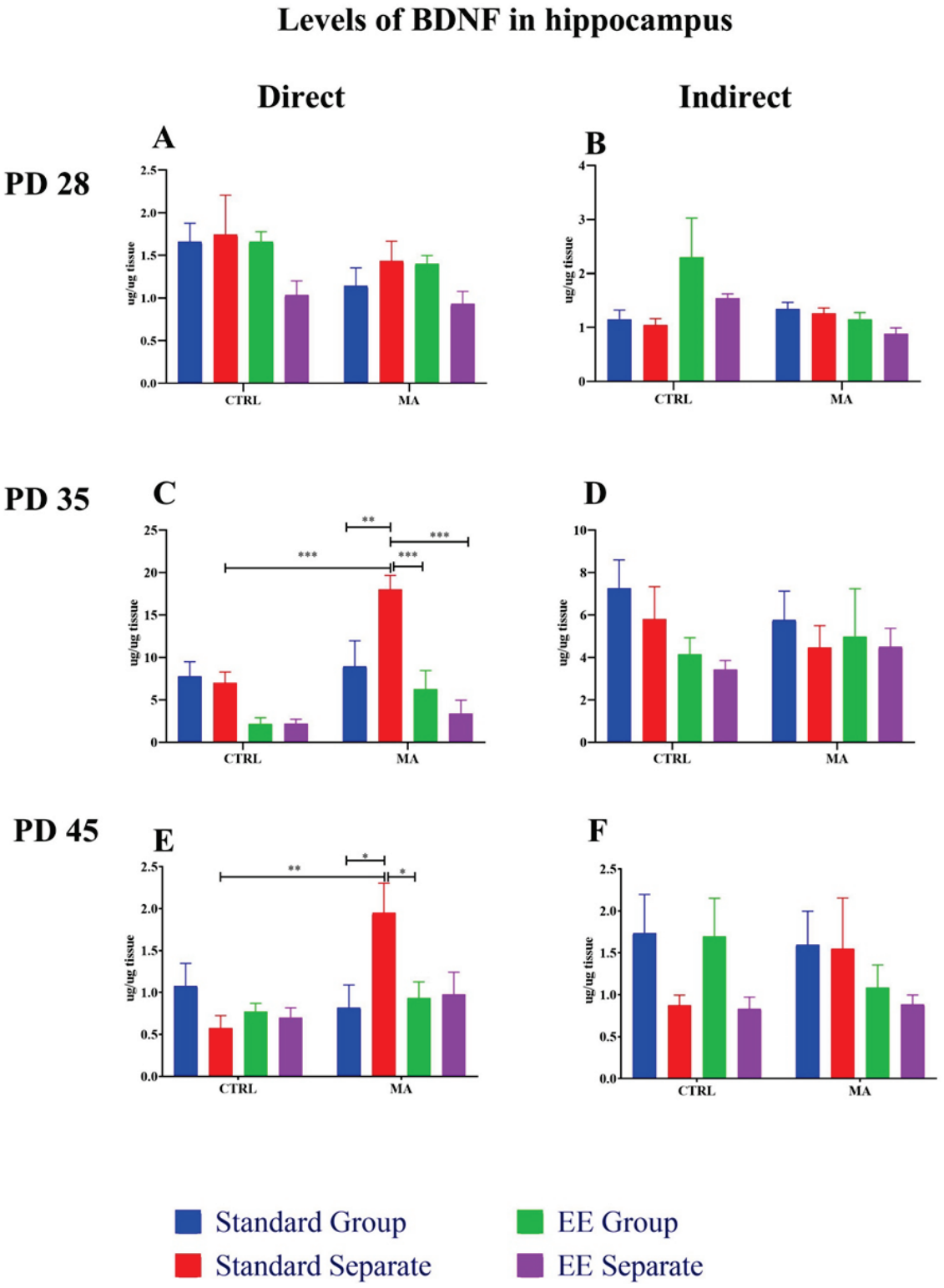

Fig. 2. Levels of BDNF in hippocampus. (**A**, **C**, **E**) levels of BDNF in HP after direct exposure of MA or SA as control. (**B**, **D**, **F**) levels of BDNF in HP after indirect exposure of MA or SA as control. Levels of BDNF are expressed per wet weight of HP. Values are ± SEM. n=8-10. * p<0.05, ** p<0.01, *** p<0.001.

On PD 45 the only factor of significance was environment [$F_{(1,55)}$=27.45, p=0.0001]; there are significantly lower NGF levels in standard group (p=0.0059) and standard separate than in EE group (p=0.0040) (Fig. 3E).

Levels of NGF after indirect exposure

On PD 28 factors of significance were environment [$F_{(1,56)}$=17.55, p=0.0001] and interaction between treatment and environment [$F_{(1,56)}$=19.31, p=0.0001]. There were significantly higher levels in

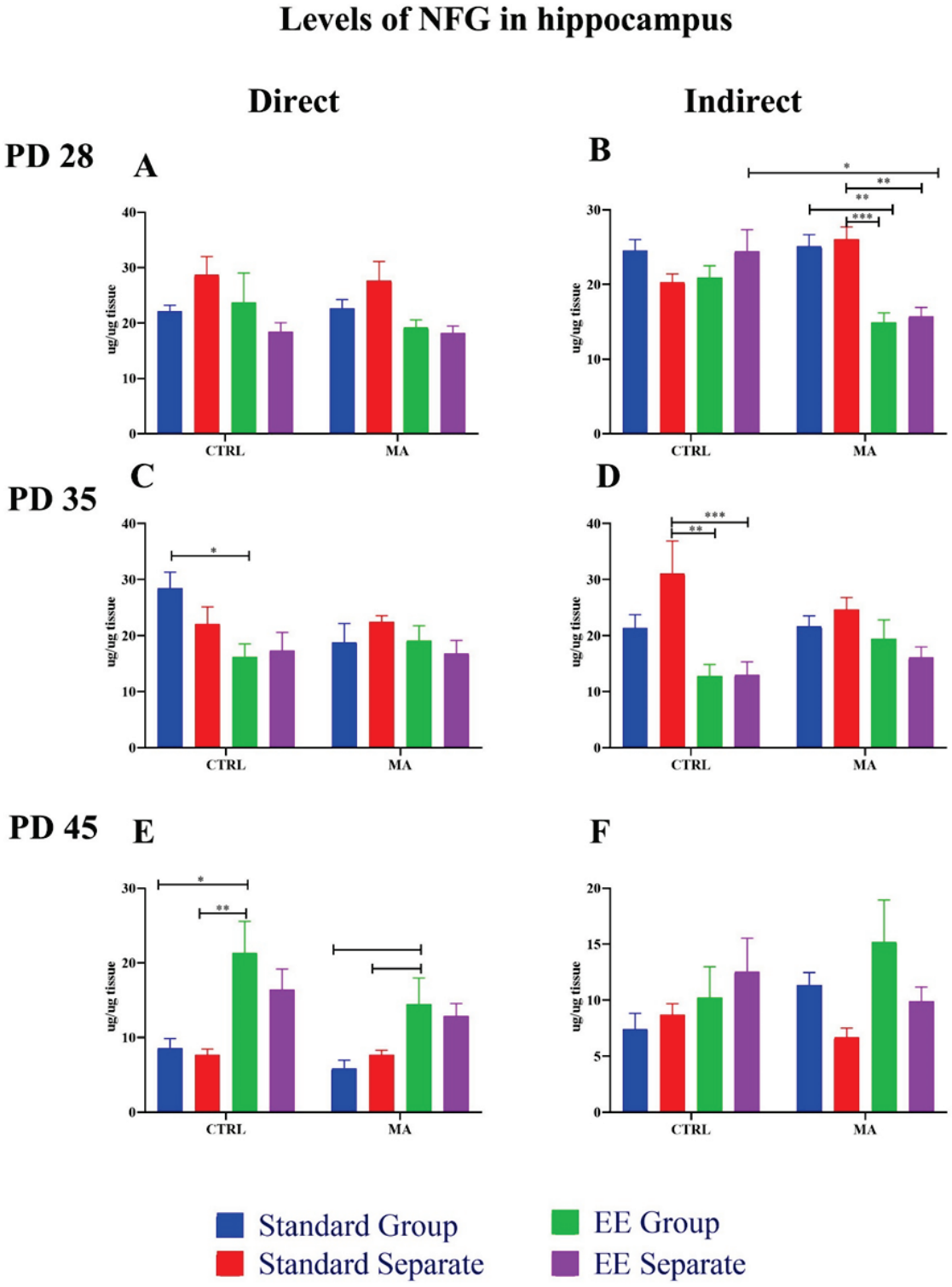

Fig. 3. Levels of NGF in hippocampus. (**A**, **C**, **E**) show levels of NGF in HP after direct exposure of MA or SA as control. (**B**, **D**, **F**) levels of NGF in HP after indirect exposure of MA or SA as control. Levels of NGF are expressed per wet weight of HP. Values are ± SEM. n=8-10. * p<0.05, ** p<0.01, *** p<0.001.

CTRL EE separate in comparison to MA EE separate (p=0.072), significantly higher levels in MA standard group in comparison to MA EE group (p=0.0009) as well as significantly higher levels in MA standard separate in comparison to MA EE separate (p=0.0008) and MA EE group (p=0.0006) (Fig. 3B).

On PD 35 the only factor of significance was environment [$F_{(1,56)}$=19.48, p=0.0001]. We obtained significantly higher levels of NGF in CTRL standard separate in comparison to CTRL EE separate (p=0.0009) as well as CTRL EE group (p=0.0018) (Fig. 3D).

On PD 45, there were none significant differences (Fig. 3F).

Fluctuations of BDNF and NGF during all stages of adolescence

In this part of analysis, we measured fluctuation of BDNF and NGF in control and MA exposed animals. We compared animals exposed to pleasant environment (EE and group) with animals in most depriving environment (standard separate).

BDNF

We obtained significant differences between groups according to PD of animals (Fig. 4).

After direct exposure, all factors were significant. Interaction [$F_{(6,56)}$=18.81, p=0.0001], PD [$F_{(3,28)}$=87.12, p=0.0001] and group [$F_{(3,28)}$=17.12, p=0.0001].

In MA standard separate we obtained significantly lower levels on PD 28 than 35 (p=0.0001) as well as on PD 45 in comparison to PD 35 (p=0.0001). In control standard separate we obtained significant differences between all PDs. Levels on PD 28 were signifi-cantly lower than PD 35 (p=0.0048), significantly higher on PD 28 vs. PD 45 (p=0.0492) and significantly lower on PD 35 than PD 45 (p=0.0031). In control EE group, there were significantly higher levels on PD 28 than PD 45 (p=0.0046).

After indirect exposure, only PD factor was significant ($F_{(3,28)}$=22.12, p=0.002). In MA standard separate we obtained significantly lower levels on PD 28 vs. PD 35 (p=0.0439). In control standard separate levels on PD 28 were significantly lower vs. PD 35 (p=0.0342) and as well on PD 45 (p=0.0328).

NGF

There were no significant differences between groups after direct exposure (Fig. 5).

After indirect exposure, all factors were significant. Interaction [$F_{(6,56)}$=4.938, p=0.0004].

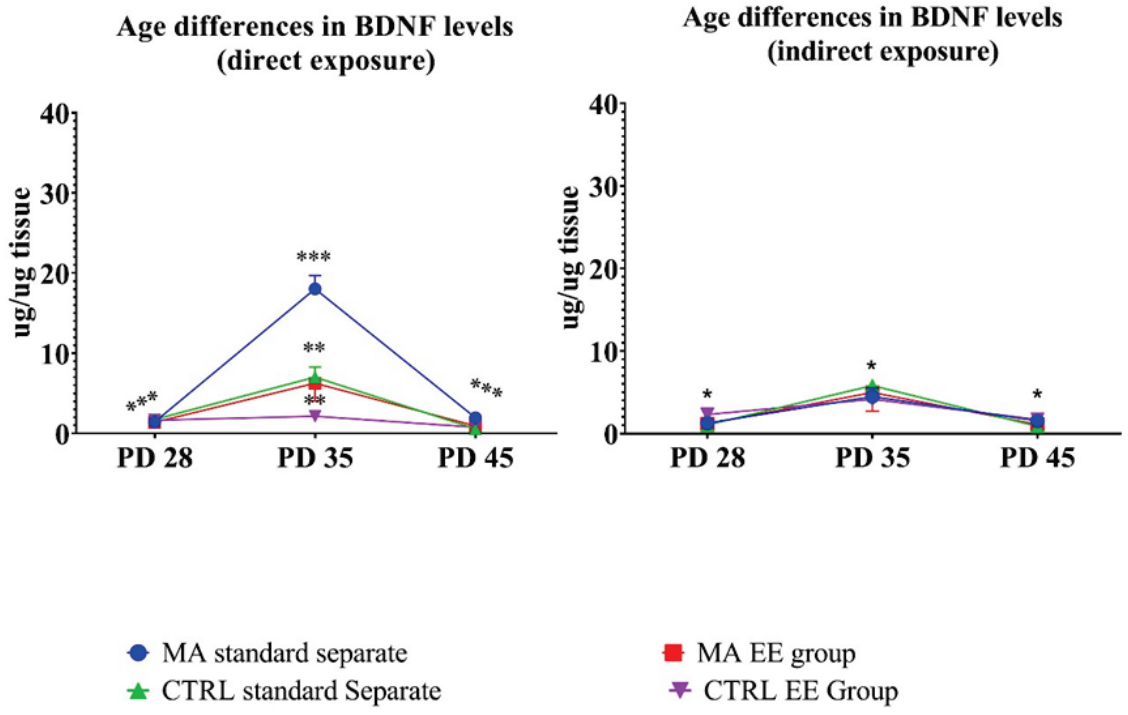

Fig. 4. Age differences in BDNF levels. Left graph shows levels of BDNF in HP after direct exposure of MA or CTRL and right graph shows these levels after indirect exposure. Figures demonstrate developmental changes in these levels of animals in standard separate environment (worst environment) vs. EE group (best environment). Levels of BDNF are expressed per wet weight of HP. Values are ± SEM. n=8-10. * p<0.05, ** p<0.01, *** p<0.001.

PD [$F_{(3,28)}$=21.87, p=0.0001] and group [$F_{(3,28)}$=3.354, p=0.0329]. In MA standard separate, levels were significantly higher on PD 28 than PD 35 (p=0.0001) and higher levels on PD 35 in comparison to PD 45 (p=0.0001). In control standard separate, there were significantly higher levels on PD 28 vs. 45 (p=0.0013) as well as on PD 35 in comparison to PD 45 (p=0.0167). In control EE group there were significantly higher levels on PD 28 in comparison to PD 45 (p=0.0006).

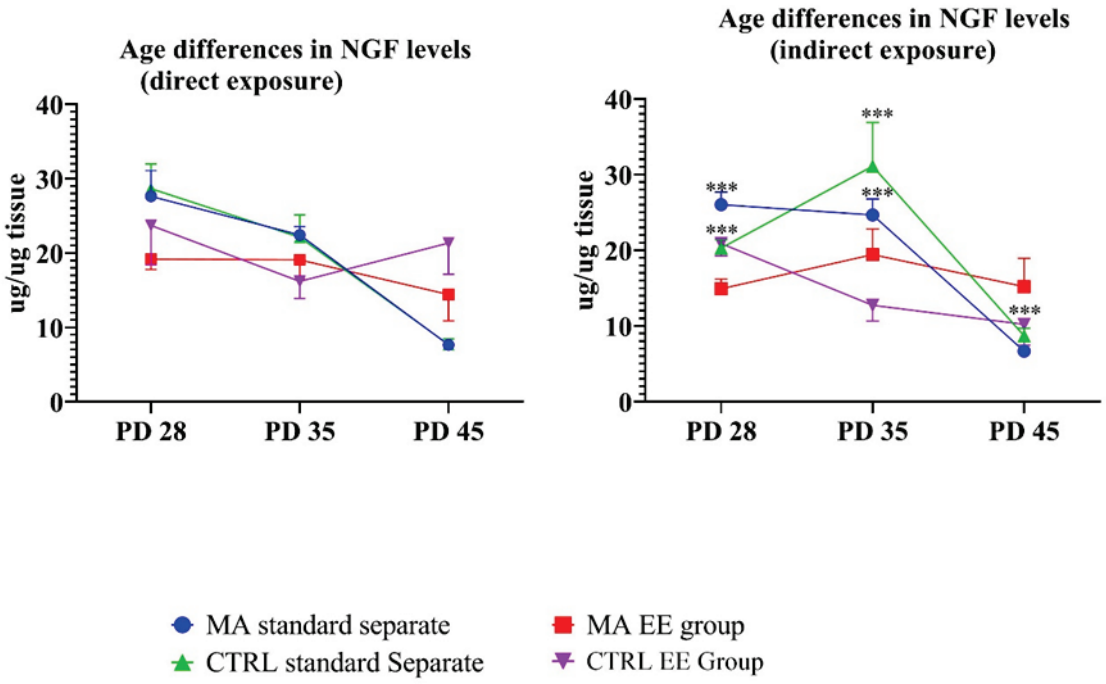

Fig. 5. Age differences in NGF levels. Left graph shows levels of NGF in HP after direct exposure of MA or SA as control and right graph shows these levels after indirect exposure. Figures demonstrate developmental changes in these levels of animals in standard separate environment (worst environment) vs. EE group (best environment). Levels of NGF are expressed per wet weight of HP. Values are ± SEM. n=8-10. * p<0.05, ** p<0.01, *** p<0.001.

Discussion

Our current study investigated influence of early postnatal exposure of MA along with preweaning EE and postweaning social isolation on BDNF and NGF levels. These neurotrophins have been targets of research in many psychiatric disorders for many decades since they have crucial role in development, survival of cells, cell death and many other functions in CNS [4].

Firstly, it is important to mention that we obtained very eminent differences in levels of these neurotrophins (primarily BDNF) according to various stages of adolescence. Some studies investigated expression of these proteins during development of rats. Study by Maisonpierre *et al.* [35] investigated levels of BDNF and NGF RNA in rat brain from postnatal stages till adulthood [35]. This study reported, that gene expression of BDNF and NGF in HP was highest in adults, but expression of BDNF fluctuated from birth to adulthood, while NGF expression remained relatively consistent. Total BDNF RNA was however highest during adolescence, while NGF RNA was again very consistent from the birth [35]. Since there is not enough research demonstrating normal levels during various developmental stages, our results can be explained by increased neuronal plasticity processes during adolescence.

Impact of MA on BDNF and NGF levels

As continuation of previous work of our laboratory, main focus of this study was investigation of possible neurotoxic effect of early postnatal exposure of MA. Effect of MA on levels of neurotrophins, has been reported by other authors and extended access to MA self-administration enhances BDNF expression in the HP of adult rats and there is evidence that early postnatal MA exposure elevated levels of BDNF, however not NGF [36].

In our current work, we did not suspect many eminent changes in terms of MA exposure, except in case of direct exposure in MA in combination with separation. MA elevated levels of BDNF in separated animals, while in indirectly exposed animals and control animals are these levels reduced in comparison to standard housing. In contrast of our results, study by Grace *et al.* [37] reported increased BDNF levels in HP after repeated MA administration during PD 11-15 [37]. Authors hypothesize that effect of MA on BDNF develops only after multiple days of exposure or it is protected from fluctuations by some mechanism, because they did not observe any changes on PD 11 in these levels. However, it is important to mention that dose in this research was two times higher than we used in current study, and these levels were measured after exposure, in contrast to our study.

Impact of social isolation and EE on BDNF and NGF levels

Secondly, we investigated consequences of social isolation stress during postweaning period, on the levels on neurotrophins in combination of previous MA exposure. BDNF as well as NGF are involved in adaptive plasticity of chronic stress and can participate in the HPA axis response to stressful stimuli [38]. It has been reported that 4-6 week of social isolation in mice eminently affected levels of these neurotrophins in HP [39,40]. Another experimental evidence brought the idea that NGF expression is regulated by behavioral activation since it has been reported that intermale aggression induced a large increase in levels of NGF in HP [41]. These findings bring us hypothesis, that NGF levels are responsive to stressors associated with fear and anxiety. It is very well established that the CNS undergoes serious developmental processes during early postnatal development in rats (critical period) and it is sensitive to various external stimuli [42,43]. NGF and BDNF play eminent role in modulating brain plasticity to better cope with them [3].

Study by Alleva (2001) reported that communal nest of mice, where three mothers kept their pups together increased levels of BDNF and NGF in HP as well as newly generated cells in this brain area. However as mentioned above, the psychosocial stress also increases NGF levels in HP. This fact leads to hypothesis that NGF have role in the emotional status caused by psychosocial stress and physiological need of the organism to remember the events leading to an appropriate coping with stressor [44]. Cirulli and his team [45] brought the hypothesis that milder manipulation could promote neural plasticity, while chronic stressful condition could lead to sensitization of limbic system to stress, decrease brain plasticity which can lead to higher susceptibility to psychopathology. In rodents, separation of infant from mother for brief period of time increased NGF levels in HP in a time-dependent manner while longer periods of separation led to increased rate of cell-death in neocortex [3,45].

Our results agree with the observations that social separation stress can elevate NGF levels. We demonstrate increased levels of this protein after social separation in almost all experimental group not dependent of drug exposure. BDNF levels were mostly decreased after social separation in all PDs, but as mentioned above, within direct MA exposure, there is significant increase in these levels.

As mentioned above, several studies reported that MA induce BDNF signaling, so we hypothesize that this decrease induced by separation, was reversed by MA exposure. Most evident effect is visible on PD 35. However, this effect was not visible within indirectly exposed MA animals, suggesting that this form of exposure, did not cause same effect as direct. This result correlates with our previous experiments which demonstrated differences between direct vs. indirect MA administration on rat performance on various behavioral tests [13,46,47]. Explanation for different action of direct and indirect MA exposure may be hypothesis, that direct early postnatal MA injection exhibits an instantaneous effect on the body while MA injected through breast milk is slowly absorbed into the body of pups. During indirect MA exposure, MA is partly metabolized in the mother's body. The half-life of MA in rats is reported to be around 70 min [48,49]. Mothers exposed to MA often display more self-care activities and that led to paying less attention to the litter after drugs exposure [50]. Therefore, pups may not have a chance to suck until the effect of the drug on the mother has diminished, which may expose the pups to lower MA doses than after direct MA injections.

Thirdly, we tracked if EE during development can reverse negative impact of MA exposure. Since both stressors (MA and separation) could have the negative effect of development, we hypothesized that EE could reverse possible negative effect of MA exposure and produce different results in comparison to exposure of stress. Adding some positive enrichment to environment could have significant impact of developmental pattern.

Other studies also reported that EE may have positive influence on neuronal development. In terms of BDNF, on mice model of EE there was enhanced exploratory behavior, memory performance as well as increased BDNF levels in HP were observed [51]. Also, it has been shown that showed that EE led to stronger dorsal HP BDNF response and higher serum BDNF levels. Animals exposed to EE showed higher brain weight compared to isolated rats and increased BDNF profile of enriched animals might represent the neurobiological correlate of resilience phenotype under a stressful situation [52]. In terms of NGF response to EE, it has been found by another study that levels of NGF were higher in HP of rats exposed to EE in comparison to standard housing. Researchers explained this fact by possible higher activity of cholinergic neurons caused by the stimulus-rich environment because of the fact that combined action NGF and acetylcholine levels most effectively facilitates neuronal plasticity, and activity of noradrenergic neurons most probably results in higher levels of neutrophins [30]. Studies mentioned above suggested, that there is questionable mechanism of response in BDNF and NGF to external stimuli.

Interestingly, in our study EE decreased levels of BDNF in most experimental groups as well as separation, so there is mystery how this protein truly reacts to positive and negative environmental stimuli. Effect of EE on NGF levels was less consistent.

It has been reported that EE caused an elevated conversion of pro BDNF to BDNF within the HP and EE increased the expression BDNF protein levels in the rat HP. Alteration of environment could facilitate or impair the conversion of pro BDNF to BDNF in the rodent HP. BDNF acts on systems involved with intracellular signaling and synaptic transmission. In contrast, pro BDNF bound the p75NTR receptor and its action had been related to neuronal cell death and synaptic withdrawal [53]. Decreased BDNF levels after EE exposure can be however sex specific. In this experiment we used only male rats. Study by Sadegzadeh *et al.* reported significant increase in these levels in prefrontal cortex of female rats exposed to EE, however not male rats [54]. In male rats, these levels were slightly lower in comparison to control animals [54]. According different experimental model, link between these similarities may be only speculation.

Our results of decreased BDNF levels after separation as well as EE (which should induce opposite effects) can possibly be explained by not sufficient sensitivity of ELISA method to BDNF and NGF and cross reactivity with their pro forms. These issues were previously reported before [55,56].

The number of animals in this study was the key reason for choosing this method for BDNF and NGF determination, since ELISA enables to determine huge number of samples on one plate in comparison to methods such as Western blot. Nevertheless, our results indicate that environment alterations along with social separation and partially treatment as well has significant impact on these levels, whether it is the BDNF, NGF or their pro forms.

Conclusions

Our results indicate different consequences of direct MA exposure in comparison to indirect *via* breastfeeding, which agrees with our previous studies. According to the present results we may claim that mainly BDNF is elevated after exposure to severe stressors, which was presented by early postnatal l MA administration and social separation after weaning. Surprisingly separation as well as EE (opposite phenomenon) did decrease levels of neurotrophins, independently from substance exposure, which may indicate various biological roles of neurotrophins and their pro forms in brain of developing rodents.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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