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Andreia Moreira da Silva Santos[1,2], Adrienne M. Gorman[3], John P. Kelly[1], Karen M. Doyle[1]

[1]School of Medicine and Galway Neuroscience Centre, National University of Ireland, Galway, Ireland

[2]Centro Universitário Unievangélica. Anápolis - GO CEP: 75083-515, Brasil

[3]School of Natural Sciences and Apoptosis Research Centre, National University of Ireland, Galway, Ireland

Corresponding Author details: Karen Doyle: karen.doyle@nuigalway.ie

Abstract

Methamphetamine (MA), a synthetic derivate of amphetamine, has become a major drug of abuse worldwide. This study investigated the effect of binge-like MA dosing (4 x 4 mg/kg, s.c., 2 hours (h) apart) at a range of different time points (from 2 h to 7 days after treatment) on brain-derived neurotrophic factor (BDNF) levels and its receptors, TrkB and $p75^{NTR}$. BDNF levels were significantly increased in the frontal cortex from 2 to 36 h after treatment, returning to normal within 48 h after treatment. In the striatum, BDNF expression was increased at 12 and 24 h after binge-like MA treatment and had returned to normal at 36 h. Increased expression of the TrkB receptor was observed in the frontal cortex at 2, 24 and 48 h after MA treatment and in the striatum at 24 and 48 h after the MA regimen. A significant increase in the $p75^{NTR}$ receptor was also noted in the striatum but not the frontal cortex, and it was less pronounced than the effect on TrkB receptor expression. These findings show that the binge-like regimen of MA affects expression of BDNF and its receptors, particularly the TrkB receptor, in a time and region dependent manner, and highlights the importance of the frontal cortex and the striatum in the response following MA binge-like dosing.

Key-words

Methamphetamine, Brain derived neurotrophic factor, TrkB receptor, $p75^{NTR}$

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Introduction

Methamphetamine (MA), a synthetic derivative of amphetamine, has become a major drug of abuse worldwide. It is a very potent psychomotor stimulant (1) Although MA and other amphetamines can have similar effects, differences also exist. Goodwin and colleagues (2) have shown that MA induces the release of 5 times more dopamine than amphetamine, which is responsible for the euphoric and addictive properties of MA. The effects of MA last longer and it causes more toxic effects than amphetamine. The N-methyl group that differentiates MA from amphetamine decreases the polarity of the molecule, enabling it to better penetrate the blood brain barrier (3).

Drug abuse, such as that associated with MA, leads to long-lasting alterations in neurons in the brain reward circuitry, that may be responsible for the development of addiction and the vulnerability to relapse (4). Furthermore, there is evidence that, when ingested repetitively at high doses, such as that which occurs with binge-like drug-taking behaviour, MA produces measurable neurotoxicity in animal models and humans (5-7). Some of the well-established effects of high dose and chronic MA in the brain include increased dopaminergic activity (8-10), formation of reactive oxygen species (11, 12) and induction of endoplasmic reticulum stress (13, 14).

Many studies have shown that dopamine has an important role in MA induced neuronal damage (15-18). In normal physiological conditions dopamine is stable in the nerve terminal but excessive levels of dopamine in the cytosol can damage dopamine neurons (15, 19). The toxicity is thought to be mediated by the generation of reactive oxygen species (ROS) and cytotoxic quinones (20, 21). Additionally, damage and degeneration of serotonergic terminals has been observed (17).

Dopamine receptors are positively or negatively coupled to adenylate cyclase. D1- like (G_s) and D2- like (G_i) receptors have opposing effects, whereby they stimulate or inhibit adenylyl cyclase respectively (22). Activation of adenylate cyclase by D1 receptors can lead to cAMP-dependent activation of protein kinase (PKA) which is responsible for phosphorylation of the transcription factor cAMP response element-binding protein (CREB) (23). pCREB is the major transcription factor involved in the expression of

neurotrophins, including Brain derived neurotrophic factor (BDNF) (24, 25). Neurotrophin functions pre- and post- synaptically are well established (26-29) but how they mediate their diverse responses in neurons is yet to be fully clarified.

BDNF mediates its pro-survival effects through the tropomyosin receptor kinase B (TrkB) receptor (30, 31). Pro-BDNF mediates mainly apoptotic signaling through $p75^{NTR}$ (32). BDNF can influence synaptic plasticity (26) and has been reported to be involved in drug cravings and addiction (33, 34). In this context, BDNF has been suggested to potentiate the dopaminergic activity promoted by psychostimulants and sensitization of dopamine neurons after withdrawal (35, 36). BDNF has also been suggested to serve a neuroprotective function in response to chronic cocaine exposure (33).

In humans, studies have shown that the concentration of BDNF in plasma of MA users is raised (37). Prior studies have shown that MA increased brain BDNF mRNA in a time-dependent manner in adult rats (38). Increased BDNF protein levels have also been previously reported in the hippocampus and striatum of rat pups following MA exposure (39). Additionally, TrkB mRNA has previously been shown to be transiently increased in the brain following MA exposure (38). Furthermore, BDNF signaling through TrkB receptors in striatal areas has been linked to withdrawal symptoms (40) and cravings (41).

Previously in our laboratory, we demonstrated that MA treatment leads to a dose- and region- specific increase in dopamine and BDNF expression in rat brain 2 h after a binge-dose regimen of MA (42, 43). To further increase our understanding of how MA affects BDNF expression and activity, in this study we investigated the longer-term effect of MA on BDNF expression over time, using a range of time points from 2h to 7 days. To investigate the functional implications of changes in BDNF expression, we also investigated the changes in expression of TrkB and $p75^{NTR}$.

Material and methods

Animals

Sprague Dawley rats (250 – 350 g) were used in this study and were purchased from Charles River laboratories, UK. Procedures were carried out under the guidelines of the Animal Welfare Committee of the National University of Ireland, Galway and in accordance with the EU Directive (2010/63/EU). Rats (n = 6-8 per time point) were housed singly and maintained on a 12 h light/dark cycle (lights on at 08:00h and off at 20:00h). The housing facility was temperature ($20^{o}C \pm 2^{o}C$) and humidity (40-60%) controlled. Food and water were available *ad libitum.*

Drugs

(+) Methamphetamine-HCl was purchased under license from Sigma-Aldrich, USA. Repeated dosing of MA at 2 h intervals is commonly used to mimic binge-like drug taking behaviour (8, 38, 44). Rats were administered four doses of MA, 4 mg/kg as freebase or saline (control), 2 h apart, according to our protocol previously described (42, 43). Room temperature was controlled throughout the experimental procedure and body temperature was measured by insertion of a rectal thermometer (42). Rats were euthanized by decapitation at 2 h, 24 h, 72 h or 7 days after the last dose and BNDF expression was measured. To further expand the results and the time points, another experiment was carried out and rats were euthanized at 2, 12, 24, 36 or 48 h after MA treatment. All drug treatments were administered during daytime hours. The wide array of timepoints in the second experiment meant that some tissue was harvested during the day (2, 24, 48 h after the last MA dose) and some at night (12, 36 h after the last MA dose). It has been shown previously (45, 46) that the levels of neurotrophins vary during the 24 h circadian cycle. To account for diurnal variation, two controls were employed – one control for tissue harvested during the day and another for tissue harvested at night. TrkB and $p75^{NTR}$ receptor expression was also assessed in this study.

Protein analysis

Expression of BDNF was measured by ELISA (R&D, DY248). Expression of TrkB and $p75^{NTR}$ receptors was assessed by Western blotting. Briefly, brain regions were dissected on ice into regions of interest (frontal cortex, striatum, amygdala, hippocampus, cerebellum and olfactory bulb). Brain tissue was homogenized in lysis buffer (137 nM

NaCl, 20 mM Trizma HCl, 10% v/v Glycerol and 1% v/v Igepal) on ice during the procedure. Following centrifugation at 4^0C, the supernatant was stored at -80^0C until further analysis.

For Western blot analysis, protein samples were diluted (1:5) in Laemmli's SDS-PAGE sample buffer (62.5 mM Tris-HCl pH 6.8, 2% w/v SDS, 5% v/v β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.05% v/v bromophenol blue) and heated to 95^0C for 5 minutes. Approximately 40 µg protein was then resolved on 12% SDS-PAGE gel. A broad-range (6.5-175 kDa) pre-stained protein ladder was run alongside the protein samples. Gels were electrophoresed at 70 V for 30 minutes, followed by 100 V for 1-1.5 h in gel running buffer (25 mM Trizma base, 2M glycine, 3 mM SDS). SDS-PAGE gels were electrophoretically transferred onto nitrocellulose membrane for 1.5 h at 110V in transfer buffer (10 mM CAPS, pH 11, 20% v/v methanol). Membranes were blocked for 1 h in blocking buffer (phosphate buffered saline (PBS) containing 0.05% v/v Tween 20 and 5% v/v non-fat milk). The membrane was then incubated overnight at 4^0C with the anti-TrkB antibody (1:500, Abcam ab18987, glycosylated) or anti-$p75^{NTR}$ antibody (1:1000, Abcam ab8874), diluted in blocking buffer containing 0.1% v/v sodium azide. Unbound antibody was removed by 3 x 10 minute washes with PBS, containing 0.05% v/v $Tween_{20}$, at room temperature. After the washings, the membrane was incubated with secondary antibody (1:10000) for 1.5 h at room temperature, Goat Anti-Rabbit IgG H&L (ab205718) for TrkB and Goat Anti-Rabbit IgG H&L (ab205718) for $p75^{NTR}$. This was followed by 3 x 10 minute washings to remove unbound antibody. Membranes were incubated in 2 ml supersignal West Pico Western blot detection solution for 5 minutes at room temperature. Blots were stripped and re-probed for TrkB or $p75^{NTR}$ and protein bands were then visualized using X-ray film in the dark and quantified using the ImageJ analysis tool. Values were normalised against actin expression.

Statistical Analysis

Statistical comparisons were made by performing analysis of variance (one-way or two-way ANOVA, as appropriate) and Dunnett's multiple-comparisons *post hoc* test. For one-way ANOVA analysis, the independent variable was time, and for two-way ANOVA, the independent variables were time and treatment. All results were expressed as mean ± standard error of the mean (SEM). Results were considered significant when $p<0.05$.

Results

In the first experiment, tissue was harvested 2, 24, 72 h and 7 days after the last dose of MA. BDNF levels were significantly increased by MA in the frontal cortex ($F_{1,40}$=19.30, p<0.0001). There was a significant effect of time, with levels raised at 2 and 24 h and reverting to normal levels at 72 h ($F_{3,40}$=18.35, p<0.0001) (Fig.1). In the striatum BDNF expression was also significantly increased by MA ($F_{1,40}$=12.66, p<0.001). There was a significant effect of time ($F_{3,40}$=51.73, p<0.0001) (Fig.1) with raised BDNF expression at 24 h and reverting to normal levels at 72 h. In the other brain regions, no significant changes were observed (Table 1).

Figure 1. MA administration induces elevation of BDNF levels in the frontal cortex and striatum. BDNF expression measured by ELISA in (A) frontal cortex and (B) striatum following a binge-like regimen of MA at 4 mg/kg or saline (control). Results (n=8) were analysed by two-way ANOVA analysis and are expressed as mean ± S.E.M., *p<0.05

Table 1. Effect of MA treatment on BDNF levels (pg/mg of protein) in brain regions at different time points

BDNF expression in brain regions following binge like regimen of MA 4 mg/kg or saline (control). Results (n=8) were analysed by Two Way ANOVA followed by Dunnett's *post hoc* analysis and are expressed as mean ± S.E.M.; *p<0.05. Results in bold represent control values.

To confirm our findings and to further elucidate the time response relationship, in a further experiment we introduced more early time points (2, 12, 24, 36 and 48 h). A significant increase in BDNF in frontal cortex at 2, 12, 24 and 36 h was found ($F_{6,42}$ = 11.92, p<0.0001) (Fig. 2) reverting to normal levels at 48 h in comparison to the control day. Similarly, a significant increase in BDNF in the striatum was observed at 12 and 24

h ($F_{6,42}$ = 37.41, p<0.0001) (Fig. 2), reverting to normal levels at 36 h in comparison to the control night.

Figure 2. Expanded time course showing elevation of BDNF levels in response to MA in the frontal cortex and striatum. BDNF level in the (A) Frontal cortex *p<0.05 vs control day, $^{\#}$p<0.05 vs control night and (B) Striatum *p<0.05 vs control day, $^{\#}$p<0.05 vs control night following a binge-like regimen of MA 4mg/kg or saline (control). Results were analyzed by one-way ANOVA (vs control day or control night as appropriate) followed by Dunnett post hoc analysis and are expressed as mean±S.E.M.

Changes in the BDNF receptors, TrkB and $p75^{NTR}$, were evaluated in the frontal cortex and striatum and results show that expression of TrkB receptor in the frontal cortex was increased at 2, 24 and 48h ($F_{3,8}$= 51.87, p<0.0001 vs control). Expression of TrkB receptors in the striatum was also increased at 24 and 48h ($F_{3,8}$= 44.41, *p<0.0001 vs control) (Figure 3) after the MA regimen (4mg/kg, 4 doses, 2 h apart). No difference was noted in the levels of $p75^{NTR}$ receptor in the frontal cortex ($F_{3,8}$ = 2.74, p=0.113), but in the striatum, there was a significant main effect ($F_{3,8}$ = 4.86, p=0.033), but post-hoc analysis did not identify significance at any particular timepoint (Figure 4).

Figure 3. TrkB receptor in (A) the frontal cortex and (B) striatum following a binge-like regimen of MA or saline (control). The figure presented here is representative of independent Western Blot (n=6). Actin was used as loading control. Values are normalised levels of TrkB/actin ratios. Results were analysed by One Way ANOVA followed by Dunnett's post hoc analysis and are expressed as mean ± S.E.M.; p<0.05.

Figure 4. $p75^{NTR}$ receptor in (A) the frontal cortex and (B) striatum, following a binge-like regimen of MA 4mg/kg or saline (control). The figure presented here is representative of independent Western Blot (n=6). Actin was used as loading control. Values are normalised levels of TrkB/actin ratios. Results were analysed by One Way ANOVA followed by Dunnett's post hoc analysis and are expressed as mean ± S.E.M.

Discussion

In this study, we show a region-dependent increase in the expression of BDNF over time, most prominent in the frontal cortex and striatum. These findings build on a previous study by our group in which we observed a region- and dose-dependent elevation of BDNF expression 2 h after MA treatment (42). Other studies, have reported that BDNF protein levels in brain (39) and BDNF mRNA levels in brain (38) can be increased by binge-like regimens of MA administration. A transient increase in serum BDNF after MA withdrawal in human addicts, which decreases over time has also previously been reported (47).

BDNF is not only important in the development of dopaminergic neurons (48) but it also has a role in the function and plasticity in the mature brain (26). We have previously shown that raised BDNF expression correlates with enhanced dopamine levels in the frontal cortex of rats treated with MA (42). Deficiency in the dopamine transporter (DAT) in rats has been shown to reduce BDNF mRNA expression in the frontal cortex (49). It has been suggested that changes in expression levels of BDNF in the frontal cortex could also influence striatal levels through anterograde transportation mechanisms (49).

The data shown here shows MA regulation of BDNF expression in the frontal cortex and striatum at different time points. With the advent of neuroimaging studies additional brain areas, especially the frontal cortex (50), have been implicated in drug addiction while the role of striatum is much more studied in the literature (51-53).

Previous work has shown that BDNF mRNA is increased by MA in an array of brain regions, including hippocampal, brain stem and cortical regions (38). MA has also been shown to increase BDNF levels in the hippocampus 24 hours post-binge administration (54). In the present study, we also observed a slight increase in the hippocampus at 24h, but the effect did not reach significance. Our data highlights a more pronounced effect of MA on the striatum and the frontal cortex. Interestingly, cocaine (55) and MDMA (56) have also been shown to increase BDNF mRNA expression in the prefrontal cortex after drug administration.

TrkB, the high affinity receptor for mature BDNF, exhibited substantial increases in expression following MA exposure in the brain regions with the greatest increases in BDNF expression, i.e., the frontal cortex and striatum. Through the TrkB receptor, BDNF activates signal transduction cascades leading to neural plasticity, neurogenesis, stress resistance, cell survival (57) and intercellular signalling (58). BDNF-TrkB signalling also supports differentiation, maturation and survival of neurons in the nervous system (59, 60). Unlike TrkB signalling, $p75^{NTR}$ activation is able to induce either survival or apoptosis depending on the ligand that binds and downstream signalling activated (61). Prior studies have linked BDNF with a neuroprotective effect following a neurotoxic insult (62-65). The functional effect of the increases we have observed in BDNF and TrkB expression following MA exposure is not fully understood. We suggest that raised BDNF and TrkB expression may be a response to protect neurons within the first 48 h following MA binge-like drug treatment.

Previous studies have demonstrated differences in regional TrkB receptor expression in brain (66). The results in the present study suggest that control rats may express more TrkB and $p75^{NTR}$ in the striatum than the frontal cortex, but further work would be required to confirm this finding.

The ELISA kit used in this study contained a primary antibody that was raised against the mature form of BDNF, however, it is likely that pro-BDNF expression was also measured in this study. It is known that the precursor neurotrophin proteins, including proBDNF, bind with high affinity to the receptor $p75^{NTR}$, while the mature form of BDNF binds with weak affinity to $p75^{NTR}$ (57). Activation of the $p75^{NTR}$ receptor in neurons is linked with adverse outcomes, such as apoptosis (32). Here we observed a slight, but significant increase in $p75^{NTR}$ receptor expression in the striatum, but no significant change in expression in the frontal cortex, where the most pronounced change in BDNF expression occurred. This finding suggests that mature BDNF probably accounts for the majority of the raised BDNF expression we observed, although it would be very interesting to confirm this using more specific ELISAs for mature- and pro-forms of BDNF in the future.

Studies have shown that plasma BDNF is raised in chronic MA abusers (37) and serum BDNF levels decrease during MA withdrawal (67). We have shown here that that the increase in BDNF expression in the brain is also transient. We suggest that raised BDNF and TrkB receptor expression may likely influence the plastic changes underlying drug craving and addiction, in addition to a neuroprotective response to drugs of abuse.

Conclusion

Taken together, our findings show that a binge-like regimen of MA affects BDNF and its receptor TrkB in a time- and region-dependent manner and highlights the importance of MA-induced effects in the frontal cortex and striatum in particular. More studies are needed to clarify the functional effects of the raised BDNF levels and what this elevation means in terms of MA neurotoxicity and drug addiction.

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Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

Ethical statement

All animal experiments were carried out in accordance with EU Directive 2010/63/EU. Formal approval to conduct the experiments described was obtained from the Animal Research Ethics Committee in NUI, Galway. I attest that all efforts were made to minimize the number of animals used and their suffering.

Conflict of interest: The authors declare that they have no conflict of interest.

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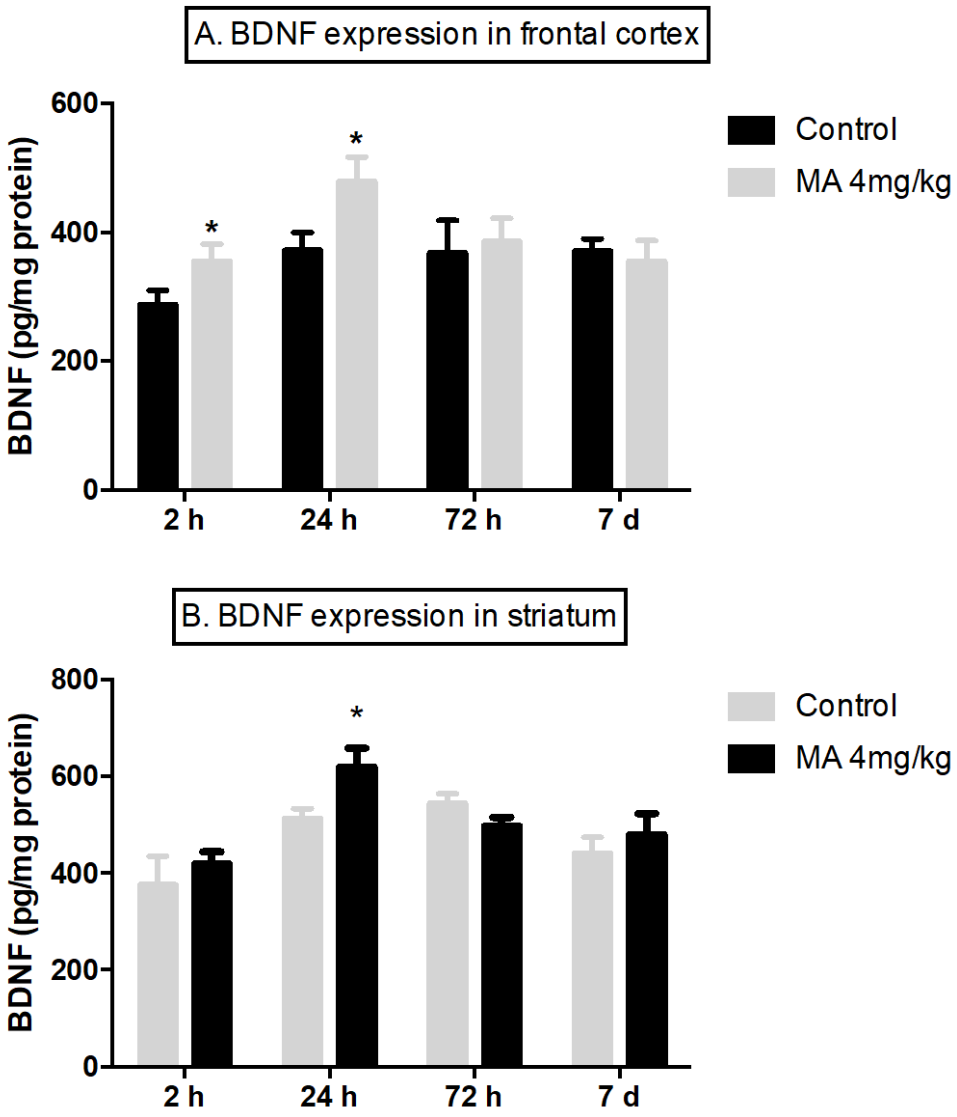
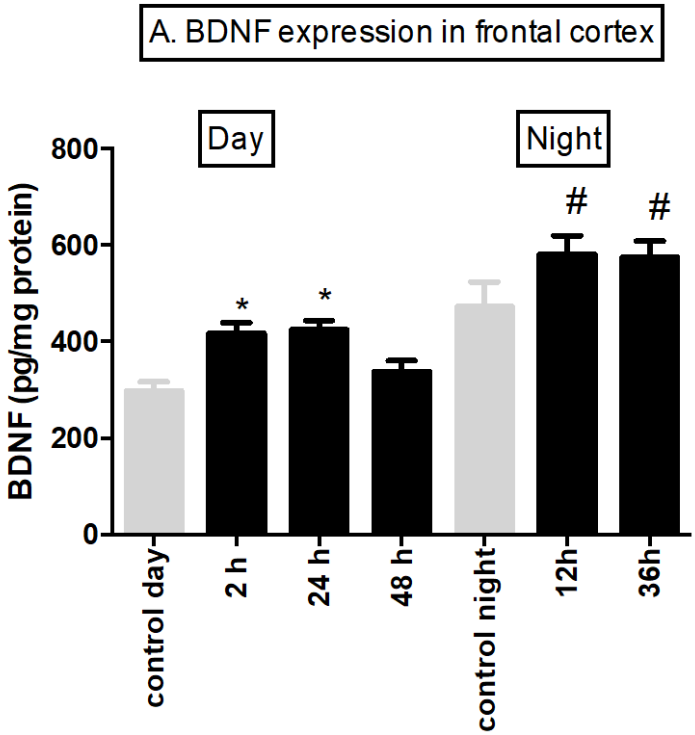
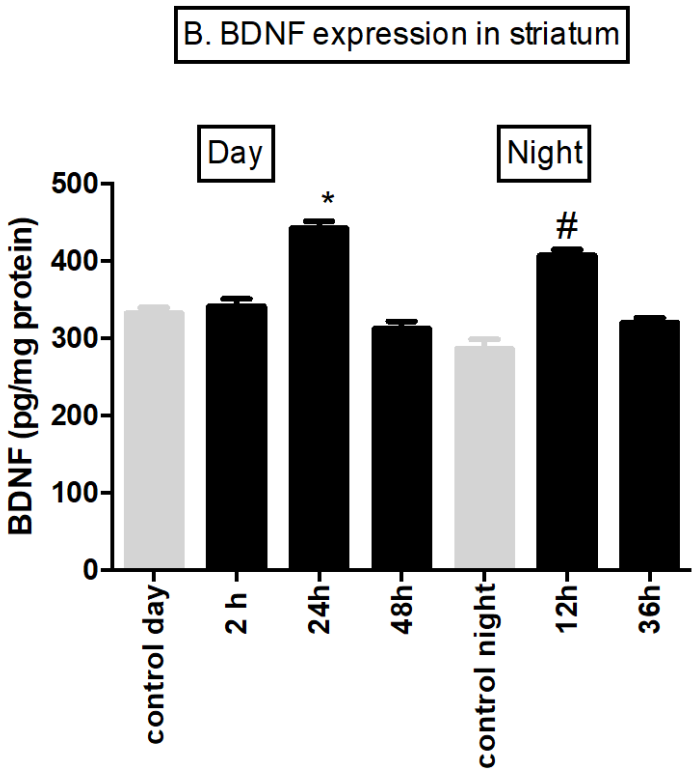
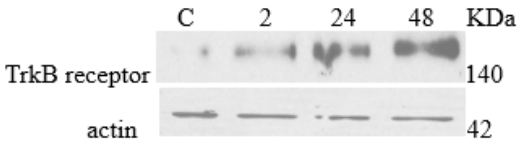
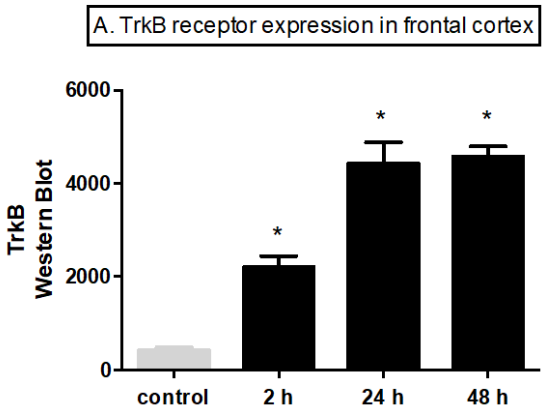

A. BDNF expression in frontal cortex
BDNF (pg/mg protein)
600
400
200
0
*
*
2 h
24 h
72 h
7 d
Control
MA 4mg/kg
B. BDNF expression in striatum
BDNF (pg/mg protein)
800
600
400
200
0
*
2 h
24 h
72 h
7 d
Control
MA 4mg/kg


A. BDNF expression in frontal cortex
Day
Night
BDNF (pg/mg protein)
800
600
400
200
0
*
*
#
#
control day
2 h
24 h
48 h
control night
12h
36h

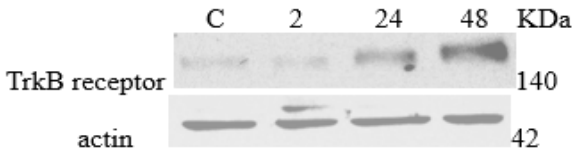

B. BDNF expression in striatum
Day
Night
BDNF (pg/mg protein)
500
400
300
200
100
0
*
#
control day
2 h
24h
48h
control night
12h
36h

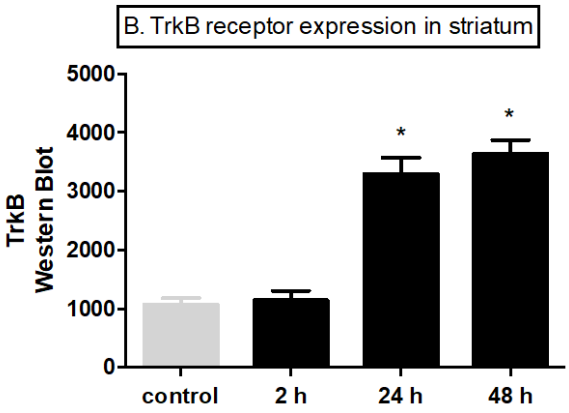
A. TrkB receptor in frontal cortex



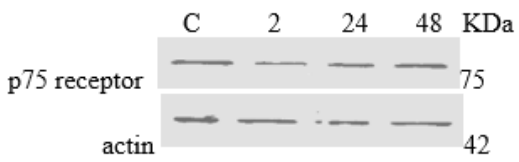


B. TrkB receptor in striatum

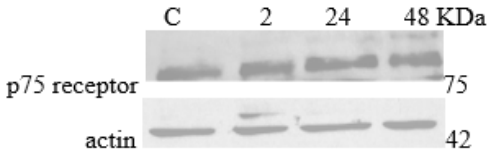


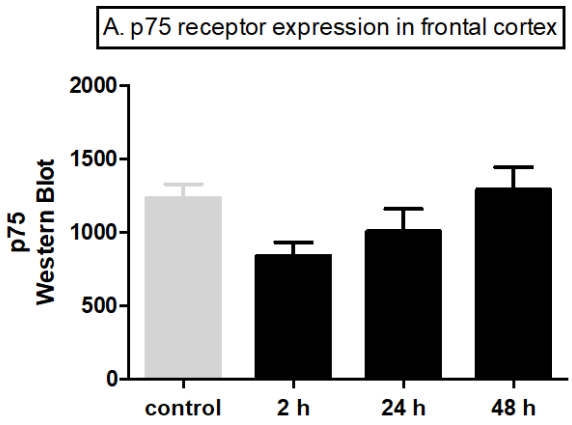


A. p75 ^{NTR} receptor in frontal cortex



B. p75 ^{NTR} receptor in striatum





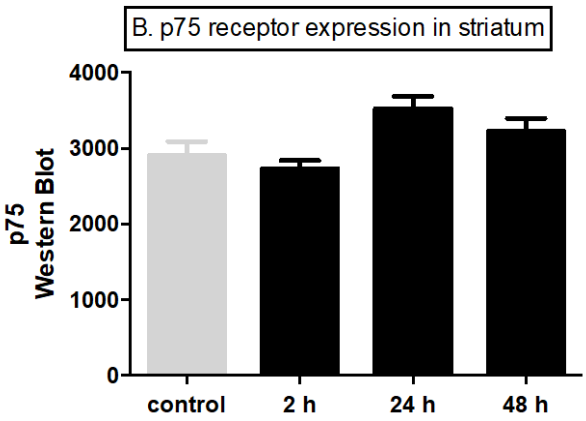


Table 1. Effect of MA treatment on BDNF levels (pg/mg of protein) in brain regions at different time points

Group	2 h	24h	72h	7 days	
Cerebellum	**633±30**	**570±47**	**631±98**	**559±94**	$F_{1,40}$ =2.65
	474±18	522±90	599±50	630±143	p=0.11
Amygdala	**398±33**	**307±80**	**549±38**	**344±13**	$F_{1,40}$ = 0.72
	432±80	336±74	450±194	279±16	p=0.40
Hippocampus	**505±104**	**558±91**	**637±31**	**684±11**	$F_{1,40}$= 1.32
	545±35	621±42	553±61	590±30	p=0.26
Olfactory Bulb	**617±78**	**663±58**	**659±54**	**501±27**	$F_{1,40}$ = 1.45
	639±53	627±34	665±50	581±39	p=0.24

BDNF expression in brain regions following binge like regimen of MA 4 mg/kg or saline (control). Results (n=8) were analyzed by Two Way ANOVA followed by Dunnett's *post hoc* analysis and are expressed as mean±S.E.M.; *$p<0.05$. Results in bold represent control values.