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Dose-dependent effects of binge-like methamphetamine dosing on dopamine and neurotrophin levels in rat brain

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Abstract:

This study investigated the acute effect of a dose range of low to moderate binge-like methamphetamine treatments on the regional expression of neurotrophin proteins in brain and serum two hours after last dose, in addition to assessing behavioural effects and dopamine neurotransmitter changes produced.

Male Sprague Dawley rats received four doses of methamphetamine, (0.5, 1, 2 or 4 mg/kg s.c. or saline for control) 2 hours apart.

Methamphetamine had a dose-dependent stimulatory effect on locomotor activity over the 8 hour duration of observation. A significant increase in dopamine concentration was observed in the frontal cortex with the highest dose of methamphetamine two hours after the last dose. This effect was dose and region specific, as no significant increase was observed with lower doses of methamphetamine, nor was a significant change observed in any other brain region tested. A similar dose and region specific increase in BDNF was observed in the frontal cortex with the highest dose regime. No significant change occurred with lower doses of methamphetamine nor in any other brain region tested. A reduction in BDNF levels in serum was also observed with the highest concentration, but not with lower doses.

Collectively, this data highlights the frontal cortex as an important region in methamphetamine-induced effects, and highlights the similar dose-response effect of methamphetamine on dopamine and BDNF expression.

Keywords: Methamphetamine; Dose-response; Dopamine; Neurotrophin; Brain derived neurotrophic factor; Frontal cortex

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1.1 Introduction

Methamphetamine is an addictive, amphetamine-like psychostimulant drug of abuse that exerts profound effects on monoamine neurotransmitter systems, increasing synaptic levels of monoamines and inducing feelings of euphoria, high energy and increased alertness¹. Methamphetamine is thought to exert its effects principally through dopaminergic neurons,² It causes dopamine release from presynaptic neurons, and inhibits the action of the dopamine transporters (DAT) responsible for re-uptake, thus resulting in a high extracellular concentration of dopamine³. The increase of dopamine promoted by methamphetamine stimulates the reward circuitry, producing the psychoactive effects.

Dopamine depletion follows the period of heightened dopamine release. Although basal dopamine levels and presynaptic dopaminergic function recover gradually over time⁴, high levels and repeated exposure to methamphetamine can cause long-term damage to monoaminergic neurons, particularly dopaminergic nerve terminals⁵. A persistent decrease in dopamine transporter level is commonly used as an indicator of neurotoxicity. In monkeys, methamphetamine reduced synaptosomal uptake of neurotransmitter in dopaminergic and serotonergic terminals that was shown to persist for up to 4 years⁶. Methamphetamine users have significantly reduced dopamine transporter density in the striatum that persists after recovery from drug abuse^{7,8}, although there is evidence that levels can gradually recover with protracted abstinence⁹.

Neurotrophins are endogenous proteins that exert a range of effects on the developing and mature central and peripheral nervous systems¹⁰. Survival signals emanating from neurotrophins are mediated by tropomyosin receptor kinase (trk) receptors¹¹, whereas the p75 neurotrophin receptor (p75NTR) mediates mainly apoptotic signals in response to pro-neurotrophins¹². Neurotrophins are known to promote neuronal growth and survival during development and to convey neuroprotection against the effects of toxic agents¹⁰. Brain derived neurotrophic factor (BDNF) is the most commonly occurring neurotrophin¹³. BDNF has been shown to enhance the development of dopamine neurons in the substantia nigra, to increase the survival of mesencephalic cells¹⁴ and to protect against toxicity, including that produced by drugs of abuse. For example, BDNF has been shown to protect primary cortical neurons against methamphetamine-induced cell death¹⁵. A recent study demonstrated a change in BDNF mRNA levels from 1 hour after repeated administration of a high dose (10 mg/kg) of methamphetamine in rat¹⁶.

Glial derived neurotrophic factor (GDNF) is a member of a distinctly different family of neurotrophic factors. The common signaling receptor for all GDNF family ligands is the Rearrangement during Transformation tyrosine kinase receptor (RET)¹⁷. GDNF activates several signaling cascades responsible for regulation of cell survival, differentiation, proliferation, neurite outgrowth and synaptic plasticity via

the GDNF Family Receptor- α 1 (GFR- α 1) that can signal through RET¹⁸. Like BDNF, GDNF has also been shown to protect rat dopaminergic neurons, for example against 6-hydroxydopamine toxicity¹⁹ and intracerebral GDNF administration has also been shown to reduce the dopamine-depleting effects of neurotoxic doses of methamphetamine in rats²⁰.

Neurotrophins can also influence functional synaptic plasticity^{10, 21}. Evidence that BDNF can influence neuronal plasticity is most compelling and it has been implicated in the pathophysiology of many neuropsychiatric disorders¹³. BDNF has also been implicated in the behavioural sensitization and neuroadaptation to drugs of abuse in drug addiction²².

Taken together, this suggests that neurotrophins could play a role in the neuroplasticity leading to addiction and also could possibly moderate the neurotoxicity of methamphetamine if expression is stimulated. However, little is known about the acute effects of binge-like dosing regimes on neurotrophin levels. To further enhance understanding of the neurotrophin response in the effects of methamphetamine, we investigated the acute effect of a dose range of low to moderate (0.5 – 4 mg/kg) binge-like methamphetamine treatments on the regional expression of neurotrophin proteins in brain and serum two hours after last dose, in addition to assessing behavioural effects and dopaminergic neurotransmitter changes produced.

1.2 Methods

1.2.1 Animals

Male Sprague Dawley rats (250 – 350 g) were used in this study. 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. In the spirit of reduction, refinement and replacement, the numbers of animals per group were kept to a minimum. Rats ($n = 4 - 7$ per time point) were housed singly and maintained on a 12 hour light/dark cycle (lights on at 08:00h and off at 20:00h). The housing facility was temperature ($20\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$) and humidity (40-60%) controlled. Food and water were available *ad libitum*.

1.2.2 Drugs

(+) Methamphetamine-HCl was purchased under license from Sigma-Aldrich, USA.

1.2.3 Experimental Procedure

Rats received four doses of methamphetamine, (calculated as free base; 0.5, 1, 2 or 4 mg/kg s.c. or saline for control) 2 hours apart, and were killed 2 hours after the last dose ($n = 4 - 7$ per group). The effects of methamphetamine on home cage locomotor activity was measured by the EthoVision® video tracking system as described previously²³. Briefly, the apparatus consisted of four cameras suspended from the ceiling above four plastic-bottomed cages containing wood shaving bedding, dyed with black wood stain. Immediately following the first methamphetamine dosing, animals were placed individually in home cages and their motor activity was recorded for 4 hours (preliminary study) or 8 hours (main study; rats were dosed an additional 3 times, 2 hours apart during the recording period). The images were analysed using EthoVision® software to derive the total distance moved over the duration (cm). Body temperature was measured by rectal probe 2 hours after the final methamphetamine dose, immediately following completion of locomotor activity recording, at the end of the experiment.

Upon completion of each experiment, animals were killed by decapitation and each rat brain was dissected on ice into regions of interest bilaterally (amygdala (and overlying cortical tissue), striatum, and frontal cortex) and stored at $-80\text{ }^{\circ}\text{C}$ until further processing. The brain regions were chosen on the basis of their involvement in cognitive behavior (frontal cortex), emotional behavior (amygdala), motor function (frontal cortex and striatum) and reward (striatum). Concentrations of Dopamine, 1-3-4-Dihydroxyphenylamine (L-DOPA), 3,4-Dihydroxyphenyl-acetic acid (DOPAC) and, Homovanillic acid (HVA) were measured by HPLC with electrochemical detection as described previously²⁴. In brief, bilateral brain tissue was weighed and sonicated in 1 ml of mobile phase (0.1 M Citric Acid, 0.1 M NaH_2PO_4 , 1.4 mM 1-octane sulphonic acid, 0.01 mM EDTA, 10% methanol; pH 3.5) containing 2 ng/20 μl *N*-methyl 5-HT as an internal standard. Homogenates were centrifuged at $4\text{ }^{\circ}\text{C}$ for 15 min at $14,000 \times g$ and stored at $-80\text{ }^{\circ}\text{C}$ until monoamine determination. Mobile phase was circulated via a Shimadzu LC-10AT pump (Mason

Technology Dublin) through the HPLC system at a constant flow rate of 1 ml/min. A 20 µl sample of supernatant was injected onto a reverse-phase C18 column (Licrosorb RP-18 column; Phenomenex, UK) maintained at 30°C and eluting monoamines and their metabolites were detected by an L-ECD-6A Shimadzu electrochemical detector maintained at a potential of +0.8V (Mason Technology Dublin). A 20 µl solution containing a mixture of L-DOPA, DOPAC, DA, HVA, and N-methyl 5-HT, each at a concentration of 2ng/20µl was also run intermittently between samples. Standard and sample chromatograms peak heights were recorded and analysed using Shimadzu Class VP 4.2 software. Concentration of monoamines and their metabolites was determined by ratiometric analysis of standard and sample data and the results expressed as ng neurotransmitter/g of tissue.

Brain regions were homogenized in lysis buffer on ice, and following centrifugation at 4 °C, the lysates were used for neurotrophin analysis (pg/ml). DuoSet® ELISA kits (R&D Systems, UK) were used to measure the concentration of BDNF and GDNF in duplicate brain tissue and serum samples, according to the manufacturers instructions. Absorbance was read using an Anthos plate reader at 450 nm, and 570 nm to correct for plastic interference. Total protein in brain tissue samples was assessed by Bradford assay (mg/ml). Neurotrophin concentration in brain tissue was expressed as pg/mg of protein.

1.2.4 Statistical Analysis

Statistical comparisons were made by performing analysis of variance (One way ANOVA with methamphetamine dose as the factor). Inter-group comparisons to control were assessed using Dunnett's multiple comparison *post-hoc* test. Tukey's *post-hoc* analysis was also employed to assess significance of differences between different doses of drug. All results were expressed as mean ± standard deviation (SD).

1.3 Results

1.3.1 Locomotor activity

The repeated dosing regime (4 doses administered two hours apart), mimics binge drug taking behavior in humans, and had a significant, dose-dependent effect on locomotor activity over the 8 hour duration of observation [$F(4, 14) = 29.47, p < 0.0001$], (Fig. 1), Locomotor activity increased dose-dependently to a maximal level with the 2mg/kg dose regime. With the highest dose (4 mg/kg), locomotor activity was also significantly higher than control, but lower than the peak locomotor activity (Fig. 1). Although not quantified in this study, we observed the emergence of stereotyped rearing behavior with the highest dose, which may have contributed to a decline in locomotor activity relative to the 2 mg/kg dose. As expected, methamphetamine caused hyperthermia. The body temperature of animals treated with the 4 mg/kg regime of methamphetamine was 39.0 ± 0.3 °C in comparison to 36.2 ± 0.5 °C in controls.

1.3.2 Dopamine, L-DOPA and dopamine metabolites

A significant increase in dopamine concentration was observed in the frontal cortex of animals treated with the highest dose of methamphetamine (4 mg/kg, 4 times, s.c.), [$F(4, 18) = 6.80, p < 0.01$]; Fig. 2). The increased dopamine concentration was dose and region specific, as no significant increase was observed in response to the lower doses of methamphetamine (Fig. 2), nor was a significant change in dopamine levels observed in the amygdala [$F(4, 21) = 1.48$] or the striatum [$F(4, 21) = 1.19$], (Fig. 2).

L-Dopa levels were not changed in any brain region tested (Table 1). There was also no change in either dopamine metabolite analysed, (DOPAC and HVA) in any brain region tested (Table 1). Furthermore, there was no change in dopamine turnover observed in any brain region tested ($[(DOPAC) + (HVA)] / [Dopamine]$) (data not shown).

1.3.3 GDNF

One way ANOVA and *posthoc* analysis showed no significant change in GDNF expression in serum [$F(4, 14) = 1.94$] or amygdala [$F(4, 14) = 0.32$] following methamphetamine treatment (Table 2).

There was an overall main effect of methamphetamine on GDNF expression in the frontal cortex [$F(4, 21) = 2.96, p < 0.05$], and the striatum [$F(4, 14) = 3.91, p < 0.05$] although Dunnett's post hoc analysis did not find an effect between controls and any specific dose. Tukey's post-hoc analysis revealed a significant difference between the 0.5mg/kg dose and the 4 mg/kg dose in the frontal cortex (Table 2).

1.3.4 BDNF

A significant reduction in BDNF levels in serum was observed following methamphetamine repeated dosing [F (4,14) = 3.07, p=0.05]. Post hoc analysis demonstrated that the highest concentration of methamphetamine significantly reduced serum BDNF levels in comparison to control (Fig. 3).

A significant increase BDNF in the frontal cortex of animals treated with the highest dose (4mg/kg) was observed (Fig. 4), [F (4, 18) = 16.54, p<0.001]. The increased BDNF concentration was dose and region specific, as no significant change was observed in response to the lower doses of methamphetamine (Fig. 4), and no significant change in BDNF in the amygdala [F (4, 14) = 1.03], or the striatum [F (4, 14) = 1.85] was observed (Fig. 4).

It was of interest to investigate if there was a correlation between changes in dopamine and BDNF levels in the frontal cortex in response to methamphetamine treatment. A Pearson's correlation coefficient analysis showed there was a significant positive correlation between dopamine and BDNF levels in the frontal cortex with increasing doses of methamphetamine treatment ($r = 0.878$, $p = 0.025$ (one-tailed analysis)).

1.4 Discussion

The main findings in this paper relate to changes observed with the repeated administration of the highest (4 mg/kg) dose of methamphetamine, mimicking binge-like drug exposure in an addict. At this dose, but not at lower doses, methamphetamine increased dopamine concentration and BDNF expression in the frontal cortex and reduced BDNF level in serum. The effect on dopamine and BDNF was brain region specific and dose specific.

Several groups of investigators have used either single large doses or repeated doses of methamphetamine to investigate behavioural and biochemical changes in response to the drug in rats. Administration of single large doses, of up to 15 mg/kg, s.c. have been reported²⁵. However, repeated administration of lower doses is a more favoured approach by researchers in the area, as there is reduced risk of mortality and the dosing regime more closely resembles typical binge drug-taking behaviour. In particular, a regime using four injections of methamphetamine at 2 hour intervals is a popular approach^{16, 26-29}. The highest dose used in this study (4mg/kg) has been identified as the highest safe level in our hands as higher doses increase mortality (data not shown). As expected, we observed a dose-dependent effect of methamphetamine on locomotor activity. Peak locomotion was observed with the 2 mg/kg regime. We observed the emergence of rearing behavior with the highest dose, which is in line with the literature³⁰, and which may have contributed to the relative decline in locomotor activity from the peak with the 2 mg/kg dose. Although the

stereotypy was not quantified in the present study, it would be of interest to quantify fully the stereotypy that develops following repeated methamphetamine administration in future work.

In this study, we were interested in the short-term effects on dopamine, assessed 2 hours following binge-like methamphetamine treatment, and how changes in neurotransmission might correlate with changes in neurotrophin expression. We found a significant positive correlation between the increase in dopamine and BDNF levels observed in the frontal cortex. We observed significantly raised dopamine levels in the frontal cortex, 2 hours after the last dose of methamphetamine, but only with the highest dose. Dopamine (D1) receptor activation leads to an increase in cAMP, triggering intracellular cascades activating several kinases that phosphorylate CREB. Phosphorylation of CREB increases the transcription of many genes involved in cell survival and plasticity, including the BDNF gene³¹. It is highly likely that the increase in BDNF expression was directly related to the increased dopamine levels through this mechanism as dopaminergic dysfunction profoundly influences BDNF expression in the frontal cortex³². Whilst our data suggests a direct relationship between high dose methamphetamine treatment, raised dopamine and BDNF levels in the frontal cortex, further work is required to examine the relationship more explicitly. In future work, it would be of interest to measure dopamine and BDNF release by microdialysis in freely moving animals. The effect of methamphetamine on dopamine release and BDNF levels in animals pretreated with D1 and D2 antagonists could also yield valuable insight in the mechanism of effect^{33,34}.

The specificity of the increase in BDNF expression in the frontal cortex with the highest dose used in this study is a very interesting finding. We found an increase in BDNF expression in the frontal cortex 2 hours after the last dose of methamphetamine, but only with the highest dose regime, and only in the frontal cortex. Our results concur with the work of Braun et al (2011), who demonstrated that BDNF mRNA was increased in frontal, parietal and entorhinal rat cortex following repeated dosing (4 times, 2h intervals) of a higher dose of methamphetamine (10 mg/kg) at time points from 1h after last administration¹⁶. It is possible that raised mRNA levels 1 hour after last administration could cause the increase in protein that we observed 2 hours after last dose, but further studies would be required for confirmation. Our results contribute evidence of the regional specificity of the rapid increase in BDNF protein expression, and our findings also demonstrate the lower end of the dose regime necessary to induce the increased expression of BDNF. Interestingly, an increase in BDNF mRNA has previously been reported in the frontal cortex in response to another amphetamine derivative, MDMA³⁵ and the psychostimulant cocaine has also been shown to increase BDNF mRNA in the frontal cortex after drug administration³⁶.

Methamphetamine has been shown to induce toxicity in vivo²⁷ and in vitro^{37,38}, There is evidence that BDNF may play a neuroprotective role against drug toxicity³⁹. BDNF has been shown to suppress dopamine release and dopamine related-behaviour in methamphetamine treated rats⁴⁰. BDNF may also be involved in the neuroplasticity related to drug addiction²² and it has been suggested that increased brain

BDNF may be a risk factor for drug addiction ⁴¹. Alcohol has also been shown to increase BDNF expression in rodent brain and it has been suggested that this may reduce further alcohol consumption ⁴². In the rat dorsolateral striatum, raised BDNF was shown to reduce alcohol intake, while downregulation of BDNF using viral-mediated siRNA increased further ethanol consumption ⁴³. BDNF was elevated within the mesolimbic dopamine system after cocaine withdrawal in rats, and has been suggested to promote craving and relapse after prolonged abstinence from cocaine ^{44, 45}. It is possible that our observation of increased BDNF expression 2 hours after the last binge-dose of methamphetamine may be related to a homeostatic neuroprotective feedback mechanism and to the neuroplasticity underlying cravings and drug addiction, but further research is required in this area. We found no significant change in BDNF or dopaminergic neurotransmission in the striatum in the present study. In future work it would be of interest to study the effect of methamphetamine on sub-regions of the striatum, specifically the caudate/putamen and nucleus accumbens, to separate out the effect of methamphetamine on the motor and reward functions of the striatum.

It has been suggested that elevated plasma BDNF in methamphetamine users may protect against neural damage ⁴⁶. Some studies have shown that plasma BDNF levels are increased in drug addicts ⁴⁶, and that serum levels decline with withdrawal ⁴⁷. However, other studies have also shown that BDNF levels in serum of methamphetamine addicts during withdrawal are lower than controls ⁴⁸. A factor in these conflicting conclusions is time of sampling. The vast majority of circulating BDNF is stored in platelets ⁴⁹, which have a lifespan of about 10 days, so timing of sampling over a protracted period could impact on findings. Serum BDNF levels have been shown to be at least ten times higher than plasma as platelets release BDNF from α -granules when activated, such as during blood clotting ⁵⁰. Animal studies investigating short-term changes in BDNF expression caused by methamphetamine have primarily focused on the changes in brain regions, rather than serum. Here, we show that serum BDNF was reduced 2 hours after binge dosing with the highest concentration of methamphetamine, the same time point as when levels increased in the frontal cortex. Previously, a correlation between serum and cortical BDNF levels in rat has been demonstrated ⁵¹, although the timeframe of changes were in the order of weeks and months, not hours as in the current study. The capacity of BDNF to cross the blood brain barrier from the periphery has also previously been demonstrated ⁵². Interestingly, that study showed that within minutes of i.v. injection, the labeled BDNF was detected in the parenchyma of the cerebral cortex ⁵². It is possible that platelet-derived BDNF crossed the blood brain barrier in the present study in response to methamphetamine treatment, and may have contributed to the increased BDNF levels in the frontal cortex 2 hours after treatment. This is worthy of further study.

GDNF has also been proposed as a potential target to treat addiction ⁵³. Behavioural effects of drugs of abuse such as cocaine and alcohol are negatively regulated by GDNF ⁵⁴. GDNF infusion in the rat VTA decreased ethanol self-administration acutely and also long-term ⁵⁵. GDNF may also be neuroprotective

against the toxicity caused by drugs of abuse. GDNF has been shown to attenuate the dopamine depletion promoted by methamphetamine when administered one day in advance⁵⁶. In addition to GDNF, other members of the GDNF family, neurturin, and artemin, also protected the rat brain against methamphetamine induced depletion of dopamine when injected into the striatum, 1 day before methamphetamine^{20, 57}. We observed an overall main effect of methamphetamine on GDNF expression in the frontal cortex and striatum in the present study, however, post-hoc analysis did not demonstrate clear differences to controls. Due to the variability of samples, it would be useful to repeat this study with larger numbers to confirm the positive results, and even more so, the negative findings observed, which commonly require larger numbers to confirm that no difference exists⁵⁷.

1.5 Conclusions

Collectively, these findings highlight the similar effect of methamphetamine on dopamine and BDNF, and also highlight the frontal cortex as an important brain region in methamphetamine-induced acute effects. Future work is needed to assess the longer-term effects of binge-like methamphetamine treatment on neurotrophin expression, particularly at dose levels that cause neurotoxicity.

Figure legends:

Fig. 1 Dose-response effect of methamphetamine binge dosing on distance travelled over an 8 hour period (mean \pm SD). Sprague-Dawley rats received methamphetamine 0.5, 1, 2 or 4 mg/kg, 4 times 2 hours apart, or saline (control). Results were analysed by One Way ANOVA followed by Dunnett's post hoc analysis, **p<0.01, ***p<0.001, ****p<0.0001 vs control

Fig. 2 Mean (\pm SD) dopamine concentration in the frontal cortex (A), amygdala (B) and striatum (C) following binge-dosing of methamphetamine. Sprague-Dawley rats (n=4-7) received methamphetamine (0.5, 1, 2 or 4 mg/kg, s.c.), 4 times 2 hours apart, or saline (control). Brain tissue was harvested 2 hours after last dose. Results were analysed by One Way ANOVA followed by Dunnett's post hoc analysis, **p<0.01. vs control

Fig. 3 BDNF concentration in serum (pg/ml) following a range of doses of methamphetamine administered 4 times 2h apart or saline (control). Results are expressed as mean \pm SD and were analysed by One Way ANOVA, followed by Dunnett's post hoc analysis, *p<0.05 vs control

Fig. 4 BDNF concentration in frontal cortex (A), amygdala (B) and striatum (C) (pg/mg protein), following a range of doses of methamphetamine administered 4 times 2 hours apart or saline (control). Results are expressed as mean \pm SD and were analysed by One Way ANOVA followed by Dunnett's post hoc analysis, ***p<0.001

Table Headings:

Table 1. L-Dopa and dopamine metabolite levels (ng/g fresh tissue) in brain regions following a range of doses of methamphetamine administered 4 times, 2h apart.

Table 2. GDNF concentration in serum (pg/ml) and brain regions (pg/mg protein) following a range of doses of methamphetamine administered 4 times 2 hours apart, or saline (control).

Statement on the welfare of animals

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 at which the studies were conducted.

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	Frontal cortex	Amygdala	Striatum
<i>L-Dopa</i>			
Control	177±49	1518±356	198±90
0.5 mg/kg	212 ±36	1899±340	219±74
1 mg/kg	178±26	1760±226	232±49
2 mg/kg	220±42	1689±338	193±61
4 mg/kg	222±36	1899±763	210±53
<i>DOPAC</i>			
Control	75±38	2440±340	5181±149
0.5 mg/kg	65±26	2838±450	6021±1201
1 mg/kg	89±34	2678±432	5976±987
2 mg/kg	75±32	2896±479	5286±929
4 mg/kg	63±41	2872±520	5533±187
<i>HVA</i>			
Control	110±24	376±73	1267±149
0.5 mg/kg	145±48	486±89	1268±217
1 mg/kg	161±59	472±79	1167±123
2 mg/kg	108±37	369±47	1379±243
4 mg/kg	146±38	434±54	1147±227

Biogenic amine levels (ng/g fresh tissue) are expressed as mean ± SD. Results were analysed by One Way ANOVA followed by Dunnett's post hoc analysis. No significant change was observed

	Serum	Frontal cortex	Amygdala	Striatum
Control	88±28	409±36	376±69	369±38
0.5 mg/kg	68±18	346±34	289±64	298±39
1 mg/kg	48±13	434±77	340±67	398±39
2 mg/kg	64±28	452±80	329±98	398±48
4 mg/kg	80±15	487±94*	296±106	304±78

Results are expressed as mean ± SD and were analysed by One Way ANOVA followed by Dunnett's and Tukey's post hoc analysis. $p < 0.05$ vs 0.5 mg/kg dose regime, Tukey's post-hoc test.







