Methamphetamine-Induced Early Increase of IL-6 and TNF-α mRNA Expression in the Mouse Brain

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The mechanisms by which methamphetamine (METH) causes neurotoxicity are not well understood. Recent studies have suggested that METH-induced neuropathology may result from a multicellular response in which glial cells play a prominent role, and so it is plausible to suggest that cytokines may participate in the toxic effects of METH. Therefore, in the present work we evaluated the effect of an acute administration of METH (30 mg/kg in a single intraperitoneal injection) on the interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α mRNA expression levels in the hippocampus, frontal cortex, and striatum of mice. We observed that METH did not induce changes in the IL-1β mRNA expression levels in both hippocampus and striatum, with immeasurable levels in the frontal cortex. Regarding IL-6, METH induced an increase in the expression levels of this cytokine in the hippocampus and striatum, 1 h and 30 min post injection, respectively. In the frontal cortex, the increase in IL-6 mRNA levels was more significant and remained high even after 2 h. Moreover, the expression levels of TNF-α were increased in both hippocampus and frontal cortex 30 min post METH administration, with immeasurable levels in the striatum. We conclude that the pro-inflammatory cytokines IL-6 and TNF-α rapidly increase after METH administration, providing a new insight for understanding the effect of this drug of abuse in the brain.

Key words: **methamphetamine; cytokines; inflammation; hippocampus; frontal cortex; striatum**

Introduction

Methamphetamine (METH) is an addictive stimulant drug that has steadily gained popularity, and its consumption can cause serious psychiatric and neurologic symptoms.[1] Studies with human METH abusers have reported a selective pattern of cerebral deterioration that contributes to impaired memory performance.[2] Moreover, acute METH intoxication can result in stroke, cardiac arrhythmia, malignant hyperthermia, cardiovascular collapse, and, because of the long duration of action of METH, these acute medical complications can present serious management challenges.

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It is believed that the pathogenesis of METH-induced neurotoxicity is in part identical to that of other neurodegenerative disorders such as Alzheimer's and Parkinson's disease. Although a number of factors appear to contribute to METH-induced neurotoxicity, the exact mechanisms are still unknown. Many of the hypotheses have focused on intra-neuronal events such as dopamine oxidation,[3,4] oxidative stress, and excitotoxicity.[5] Indeed, METH's noxious effects on the brain have long been associated with dopamine (DA) and serotonin (5-HT),[6] but over the last decades the focus has been shifting towards glutamate (Glu). Moreover, we have very recently demonstrated that METH induces significant alterations on hippocampal *N*-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) ionotropic glutamate receptor subunit levels.[7] Despite this evidence, recent studies have suggested that METH-induced neuropathology may also result from a multicellular response in which glial cells play a prominent role.[8–11] Several observations suggest that conversion of glial cells to their "reactive" state, and the associated increase in expression of cytokines and chemokines, may play a role in neurodegeneration. Thus it is plausible to suggest that other molecules, such as cytokines, might also participate in the toxic effects of METH.

Cytokines, a diverse group of polypeptides that are generally associated with inflammation, immune activation, and cell differentiation or death, have diverse actions, and most have little or no known function in healthy tissues, being rapidly induced in response to tissue injury or inflammation.[12] Indeed, cytokines have beneficial or detrimental effects on cell survival, as we have previously observed that interleukin-1beta (IL-1β) and tumor necrosis factor-alpha (TNF-α) act as biphasic modulators of AMPA-induced neurodegeneration.[13] Thus, changes in cytokine levels may reflect an attempt to counteract the degeneration by initiating protective and/or regenerative mechanisms. It is known that IL-1β is among the first cytokines to be synthesized by microglia, in moderate levels, in response to an insult, which in turn stimulates the proliferation of astrocytes and the consequent release of interleukin-6 (IL-6),[14,15] which is capable of modulating the differentiation and survival of neurons. Similarly, IL-1β can induce TNF-α production, which in turn acts synergistically with IL-1β to induce IL-6 expression,[16,17] and then IL-6 inhibits the synthesis of TNF-α.

Recently, it was shown that the IL-6 null genotype affords protection to DA and 5-HT terminal damage, apoptotic cell death, and reactive gliosis induced by METH.[18] Other authors have shown an increase of IL-1β mRNA in METH-treated animals, which may be partly involved in the activation of the IL-6 cascade and consequently astrogliosis.[19] Clearly, astrogliosis play a role in METH-induced hippocampal dysfunction,[7] and microglia are activated in response to METH administration.[8] However, attenuation of microglia activation is not by itself enough to afford neuroprotection.[9] Moreover, recent findings suggest that TNF-α plays a neuroprotective role in METH-induced drug dependence and neurotoxicity by activating plasmalemmal and vesicular DA transporter, as well as by inhibiting METH-induced increase in extracellular DA levels.[20]

In the present study, we sought to discover whether an acute administration of METH induces an inflammatory process by analyzing the mRNA alterations of the pro-inflammatory cytokines IL-1β, IL-6, and TNF-α in the mice hippocampus, frontal cortex, and striatum.

Material and Methods

Animals and Drug Treatment

Four-month-old male wild-type C57BL/6J mice (Charles River Laboratories, Barcelona, Spain) were housed under a standard 12:12-h light-dark schedule with *ad libitum* access to food

and water. Mice were intraperitoneally (i.p.) given METH (30 mg/kg body weight) (Faculty of Pharmacy, University of Porto, Porto, Portugal) dissolved in a maximum volume of 100 μL of sterile 0.9% NaCl solution, and were sacrificed 30 min, 1 h, and 2 h post injection. The control group was given 100 μL of sterile 0.9% NaCl and sacrificed 2 h post injection. Moreover, to induce inflammation (positive controls), mice were injected (i.p.) with 5 mg/kg lipopolysaccharide (LPS) (Sigma, St Louis, MO, USA) and were sacrificed 4 h post administration.

Experimental procedures were performed accordingly to the guidelines of the European Community for the use of animals in the laboratory. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Isolation of Total RNA

Total RNA was isolated from the hippocampus, frontal cortex, and striatum of the mice using TRI REAGENT (Sigma-Aldrich, St Louis, MO, USA) according to manufacturer's instructions. Tissue was homogenized in guanidium thiocyanate and phenol. Chloroform was added and after centrifugation (12,500 rpm, 15 min, 4°C), the RNA was isolated in the aqueous phase and precipitated with isopropanol. The pellet was then washed with 75% ethanol, dried, and redissolved in diethylpyrocarbonate (DEPC)-treated water (Fluka, Sigma-Aldrich, Switzerland). The total amount of RNA was quantified by optical density (OD) measurements at 260 nm, and the purity was evaluated by measuring the ratio of OD at 260 and 280 nm using RNA/DNA calculator GeneQuant II (Pharmacia Biotech Amersham Biosciences AB, Uppsala, Sweden).

RT-PCR Analyses

Pro-inflammatory cytokines IL-1β, IL-6, and TNF-α mRNA levels were determined by reverse transcriptase-PCR (RT-PCR). cDNA was transcribed from 2 μg RNA of the hippocampus, frontal cortex, and striatum, using Oligo dT(15) primers (Bioron, Ludwigshafen, Germany). Afterwards, PCR was performed in a 50-μL reaction volume containing 5 μL (IL-1β, IL-6 and β-actin) or 10 μL (TNF-α) cDNA, 1 μL dNTP mix, 5 μL 10× complete buffer for Taq DNA polymerase, 0.25 μL Taq DNA polymerase (Bioron, Ludwigshafen, Germany), 0.2 μL of both forward and reverse primers, and a variable volume of water. Forward and reverse primers used in PCR reactions were as follows (all presented as 5′-3′): IL-1β, ATGGCAACTGTTCCTGAACTCAACT and AGGACAGGTATAGATTCTTTCCTT; IL-6, CAAGAGACTTCCATCCAGTTGCCT and TTTCTCATTTCCACGATTTCCCAG; TNF-α, CTGTAGCCCACGTCGTAGCA and CGGCAGAGAGGAGGTTGACT; and β-actin, GACTACCTCATGAAGATCCT and ATCTTGATCATGGTGCTG (MWG-Biotech AG, Ebersberg, Germany). The PCR cycling profile for IL-1β and β-actin was 1 min at 95°C for denaturation, annealing at 58°C for 1 min, extension at 72°C for 1 min for 38 cycles, and a 10-min final extension period at 72°C. For IL-6 and TNF-α, the PCR cycling profile was 1 min at 95°C for denaturation, annealing at 60°C (IL-6)/58°C (TNF-α) for 30 sec, extension at 72°C for 30 sec for 38 cycles, and a 10-min final extension period at 72°C. The mRNA levels of β-actin were used as a loading control, and negative controls were performed to ensure that PCR products result from RNA transcription (data not shown).

PCR products were separated by gel electrophoresis (1.5% agarose) and stained with ethidium bromide. Densitometric analysis was performed on Universal Hood II (Bio-Rad Laboratories, Milan, Italy), and mRNA expression was evaluated by the band-intensity ratio of control versus METH-injected mice using the ImageQuant 5.0 software.

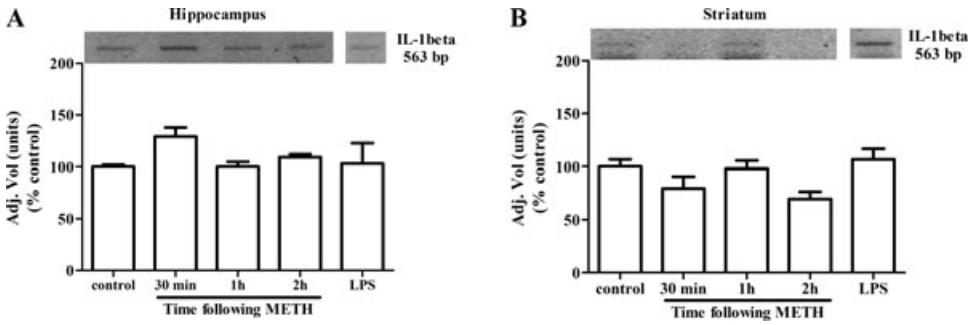

Figure 1. Expression levels of IL-1β; mRNA in the mice hippocampus (**A**) and striatum (**B**) after acute administration of METH (30 mg/kg). The mRNA levels were analyzed 30 min, 1 h, and 2 h post administration by RT-PCR. The control animals were given 0.9% NaCl solution and sacrificed 2 h later. As a positive control we used the mRNA obtained from mice injected with 5 mg/kg LPS and sacrificed 4 h later. The IL-1β mRNA expression was measured using ImageQuant 5.0 and each value represents the means ± SEM of three mice.

Statistical Analyses

The data are expressed as means ± SEM. Statistics were performed using one-way ANOVA, followed by Dunnett's post hoc, as indicated in the figure legends. The significance level was set at $P < 0.05$.

Results

The acute administration of 30 mg/kg METH did not induce statistically significant changes in the IL-1β mRNA expression levels in either hippocampus and striatum or at the different time-points analyzed (Fig. 1). In the hippocampus (Fig. 1A), IL-1β mRNA expression levels at 30 min, 1 h, and 2 h post injection were 129.1 ± 8.8%, 100.1 ± 4.5%, and 109.2 ± 2.9% of control levels, respectively. Also, the injection of 5 mg/kg LPS did not induce a significant change in the pro-inflammatory cytokine levels (103.2 ± 19.6% of control) (Fig. 1A). Moreover, in the striatum (Fig. 1B), IL-1β mRNA expression levels at 30 min, 1 h, and 2 h post injection were 79.6 ± 11.2%, 98.2 ± 7.6%, and 69.7 ± 6.9%, respectively, and again, the administration of LPS resulted in IL-1 β mRNA expression levels similar to those of the controls (106.7 ± 7.6% of control) (Fig. 1B). In contrast with the results obtained in both hippocampus and striatum, the expression of IL-1β mRNA was not detected in the frontal cortex under the same experimental conditions (data not shown).

Concerning IL-6, the changes induced by METH were highly significant in the three brain regions analyzed (Fig. 2). In the hippocampus (Fig. 2A), IL-6 mRNA values were very similar to those of the control group at 30 min post METH administration (111.2 ± 3.9% of control). Only after 1 h it was possible to observe an increase of IL-6 mRNA expression levels to 155.5 ± 28.2% of control levels ($P < 0.05$) recovering to basal levels after 2 h (115.1 ± 7.3% of control). Moreover, LPS induced a significant increase of IL-6 mRNA levels to 190.5 ± 49.9% of control (Fig. 2A). Of interest, in the frontal cortex the effect induced by METH was very significant at both 30 min and 1 h post injection (270.9 ± 18.8% and 304.8 ± 7.2% of control, respectively; $P < 0.01$), and the cytokine levels remained increased even after 2 h (211.7 ± 42.1% of control; $P < 0.05$) (Fig. 2B). Also, 5 mg/kg LPS induced a very significant increase of IL-6 levels (338.0 ± 26.6%; $P < 0.001$). In the striatum, we could observe a significant increase of IL-6 mRNA levels at 30 min and 1 h after METH administration (129.9 ± 6.7% and 147.2 ± 0.1% of control, respectively; $P < 0.05$), whereas 2 h post METH or LPS

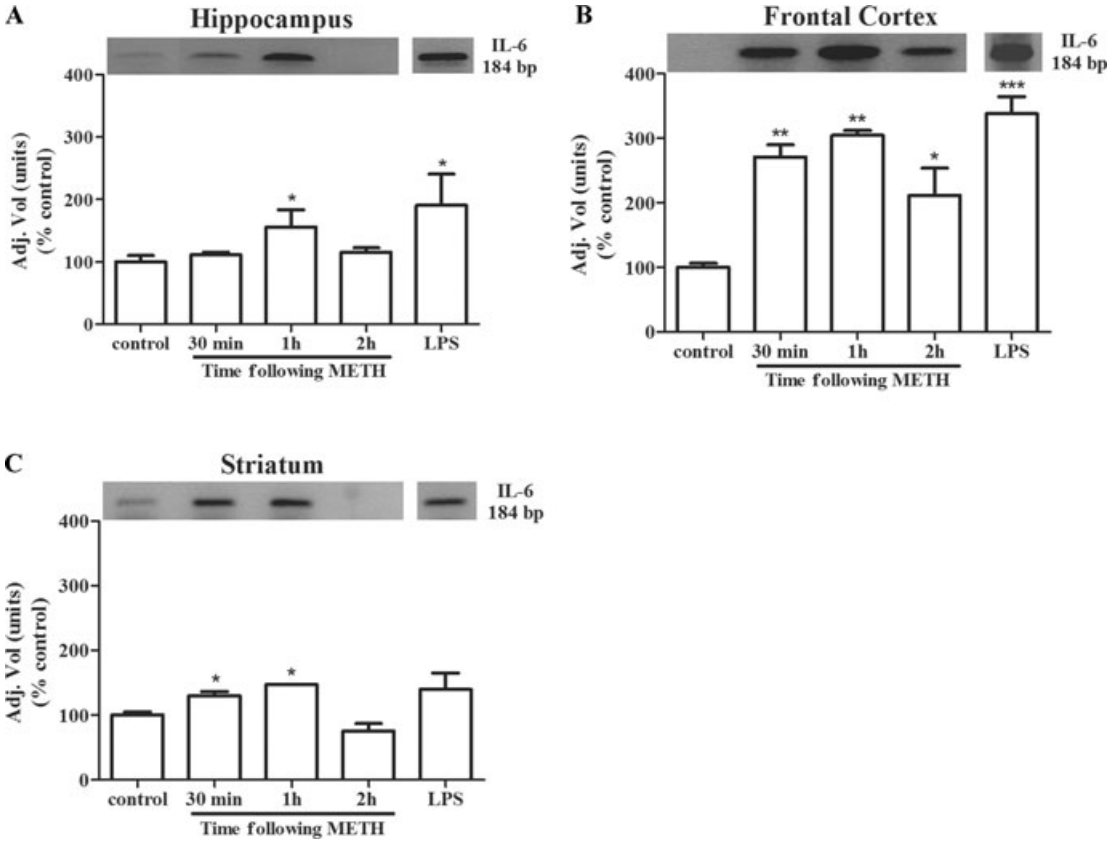

Figure 2. Acute administration of METH (30 mg/kg) induces significant alterations of IL-6 mRNA expression levels in the hippocampus (**A**), frontal cortex (**B**), and striatum (**C**) of mice. The mRNA levels were analyzed 30 min, 1 h, and 2 h post administration by RT-PCR. The control animals were given 0.9% NaCl and sacrificed 2 h later. As a positive control we used the mRNA obtained from mice injected with 5 mg/kg LPS and sacrificed 4 h later. The IL-6 mRNA expression was quantified using ImageQuant 5.0 and each value represents the means ± SEM of four mice. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ – Dunnett's post hoc; statistical significance when compared to control (NaCl administration).

administration, the values were not statistically different from those of controls (75.3 ± 11.5% or 139.7 ± 24.9% control, respectively).

Despite the fact that no expression levels of TNF-α were detected in the striatum (data not shown), we could observe a significant increase of TNF-α mRNA expression levels in the hippocampus 30 min post METH administration (151.3 ± 8.6% of control; $P < 0.05$) (Fig. 3A), and in the frontal cortex 30 min and 1 h after METH administration (147.0 ± 6.0% and 174.3 ± 26.6% of control, respectively; $P < 0.05$) (Fig. 3B). Moreover, the administration of LPS also induced a significant increase of TNF-α levels in the hippocampus (172.0 ± 27.7% of control; $P < 0.05$) (Fig. 3A), but not in the frontal cortex (136.7 ± 11.0% of control) (Fig. 3B). At 1 h and 2 h post METH administration the TNF-α levels in the hippocampus were 86.0 ± 1.1% and 89.8 ± 9.3% of control levels, respectively (Fig. 3A), and in the frontal cortex, 2 h after METH administration, the level of the pro-inflammatory cytokine was 110.2 ± 0.7% of control (Fig. 3B).

Discussion

It has been suggested that cytokines are involved in the effects of psychostimulants in the

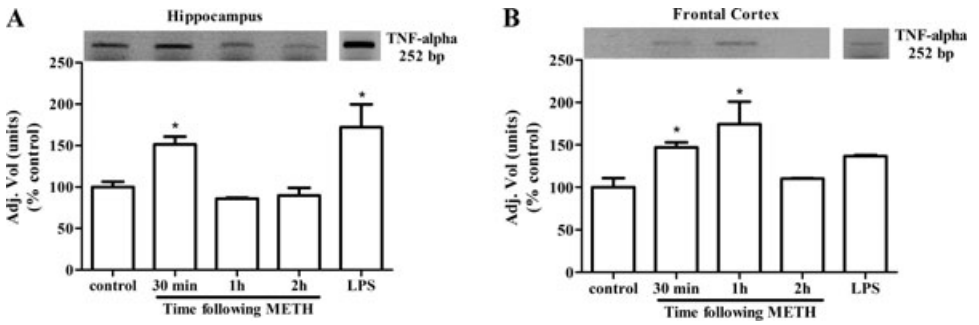

Figure 3. Alterations of total mouse hippocampal (**A**) and frontal cortex (**B**) TNF-α mRNA expression levels after METH (30 mg/kg) administration. The mRNA levels were examined 30 min, 1 h, and 2 h post injection by RT-PCR. The control animals were given 0.9% NaCl solution and sacrificed 2 h later. As a positive control, we used the mRNA obtained from mice injected with 5 mg/kg LPS and sacrificed 4 h later. Quantification of mRNA levels was performed by ImageQuant 5.0 and each value represents the means ± SEM of at least three mice. $^{*}P < 0.05$, Dunnett's post hoc; statistical significance when compared to control (NaCl administration).

central nervous system. Indeed, a high single dose of METH induces TNF-α mRNA and protein expression in the mouse brain,[21] whereas others showed that chronic low doses, but not acute treatment, increases TNF-α expression.[20] Moreover, the neurotoxicity induced by a repeated METH administration was attenuated in mice with a null mutation for IL-6,[18] and a single METH injection causes a marked induction of hypothalamic interleukin-1β mRNA in mice.[19,22,23] Here we investigated the effect of an acute high dose of METH (30 mg/kg) on the pro-inflammatory cytokines levels in the hippocampus, frontal cortex, and striatum. We showed that this drug of abuse does not induce changes in IL-1β mRNA levels in both hippocampus and striatum; the mRNA expression levels are very low under physiological conditions. Nonetheless, a small, but not statistically different increase was observed in hippocampal IL-1β mRNA levels at 30 min post METH administration. Several studies have demonstrated that when this cytokine is released in modest concentrations, it enhances the capacity to modulate the differentiation and survival of neuronal cells by activating astrocytes.[24–26] Indeed, some authors have shown that the production of IL-1β by microglial cells, after a brain insult, stimulates the proliferation of astrocytes and thereby the consequent release of IL-6.[14,15] Moreover, our group has previously observed that IL-1β and TNF-α act as biphasic modulators of AMPA-induced neurodegeneration since either potentiation of excitotoxicity or neuroprotection was observed, depending on the concentration of the cytokines and the timing of exposure.[13] In the present study, we also demonstrated that administration of LPS did not induce an increase of IL-1β mRNA levels, at least between 1 h and 4 h post injection. In accordance with our results, Li and collaborators[27] showed that LPS-activated microglia result in significant upregulation of IL-1β expression only after 8 h of exposure, and the level of expression was not so high as that of other pro-inflammatory cytokines, such as IL-6 and TNF-α.

The precise role of IL-6 in response to injury remains unclear. In some instances IL-6 appears to exert neuroprotective effects,[28–30] but in other studies it has been shown to promote degenerative mechanisms.[31] Regarding this pro-inflammatory cytokine, we observed that mRNA expression levels were very rapidly increased in all brain regions analyzed, being, however, more prominent in the frontal cortex. Thus, the augmentation of IL-6 by administration of METH can lead us to different interpretations. Ladenheim and collaborators[18]

demonstrated that METH-induced damage of DA and 5-HT terminals, apoptotic cell death, and reactive gliosis are attenuated in IL-6 knockout mice, suggesting that IL-6 might be an important component of the toxic cascade caused by METH. In addition, others have reported that IL-6 deficiency conferred protection from disruption in working memory induced by LPS[32] and attenuated sensitivity to the depressing effects of LPS and IL-1 on social exploration and body weight.[33] Furthermore, circulating IL-6 is positively correlated with deficits in cognition in humans.[34] Indeed, Balschun and collaborators[35] suggested that IL-6 may sit in a pivotal position and serve as an important catalyst because administration of IL-6 neutralizing antibodies prolonged long-term potentiation (LTP) and improved spatial alternation behavior. Several studies have, indeed, demonstrated that cytokines have important normative functions in learning and memory and that perturbation of steady-state levels may alter cognitive processes. On the other hand, it was demonstrated that IL-6, as well as IL-1β, is required for a cytotrophic response to support sprouting of dopaminergic neurons, because glial response induced by these cytokines is essential in the regulation of axonal sprouting after injury.[25] A more recent study also provides strong evidence that IL-6 promotes axonal regrowth and network repair of CNS tissue after lesioning, using an *in vitro* model of lesion in hippocampal slice cultures.[36] Regarding our results, they suggest an important role for IL-6 under conditions of METH consumption, since mRNA levels were significantly increased in the brain. However, the source and the consequence(s) of such increase remain unknown.

Brain TNF-α levels are typically increased in a variety of CNS disorders, including trauma,[37] ischemia,[38] multiple sclerosis,[39] and temporal lobe epilepsy.[40,41] Moreover, several studies demonstrated that treatment with METH increases TNF-α mRNA.[20,21,42] Indeed, Nakajima and collaborators[20] demonstrated that, although acute treatment at a dose of 2 mg/kg did not change TNF-α gene expression, a repeated treatment with METH (2 mg/kg for 5 days) induces a significant increase in TNF-α mRNA and protein expression in the rat brain. In contrast, we presently show that a single high dose of METH induces a significant increase in TNF-α mRNA levels in both hippocampus and frontal cortex, just after 30 min and 1 h, respectively. Although TNF-α has been suggested to be toxic to neurons and glia, and to be correlated with neurotoxicity induced by METH,[42] recent studies have also demonstrated neuroprotective effects of this cytokine. Indeed, the upregulation of TNF-α has been associated with a neuroprotective role in METH-induced drug dependence and neurotoxicity by activating plasmalemmal and vesicular DA transporter as well as by increasing extracellular DA levels.[20] Moreover, the same authors clearly showed that exogenous TNF-α blocked locomotor-stimulating and rewarding effects of METH, as well as METH-induced dopaminergic neurotoxicity in mice following a chronic administration (4 mg/kg; four times at 2-h intervals).[20] The dual role (i.e., toxic and protective) of TNF-α will be explored by us under conditions of acute METH administration.

In conclusion, we demonstrated that an acute high dose of METH induces an early increase in the expression levels of IL-6 mRNA in the hippocampus, frontal cortex, and striatum, and TNF-α mRNA only in the hippocampus and frontal cortex. Studies are under way in our laboratory to understand the beneficial/detrimental effects of IL-6 and TNF-α on cell survival under conditions of METH administration.

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Conflicts of Interest

The authors declare no conflicts of interest.

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