Effects of adenosine A2a receptor agonist and antagonist on cerebellar nuclear factor-kB expression preceded by MDMA toxicity

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Abstract
Background: Adenosine is an endogenous purine nucleoside that has a neuromodulatory role in the central nervous system. The amphetamine derivative (+)-3,4-methylenedioxymethamphetamine (MDMA or ecstasy) is a synthetic amphetamine analogue used recreationally to obtain an enhanced affiliated emotional response. MDMA is a potent monoamineergic neurotoxin with the potential of damage to brain neurons. The NF-kB family of proteins are ubiquitously expressed and are inducible transcription factors that regulate the expression of genes involved in disparate processes such as immunity and ingrowth, development and cell-death regulation. In this study we investigated the effects of the A2a adenosine receptor (A2a-R) agonist (CGS) and antagonist (SCH) on NF-kB expression after MDMA administration.

Methods: Sixty three male Sprague–Dawley rats were injected to MDMA (10 and 20mg/kg) followed by intraperitoneal CGS (0.03 mg/kg) or SCH (0.03mg/kg) injection. The cerebellum were then removed for cresyl violet staining, western blot and RT-PCR analyses. MDMA significantly elevated NF-kB expression. Our results showed that MDMA increased the number of cerebellar dark neurons.

Results: We observed that administration of CGS following MDMA, significantly elevated the NF-kB expression both at mRNA and protein levels. By contrast, administration of the A2a-R antagonist SCH resulted in a decrease in the NF-kB levels.

Conclusion: These results indicated that, co-administration of A2a agonist (CGS) can protect against MDMA neurotoxic effects by increasing NF-kB expression levels; suggesting a potential application for protection against the neurotoxic effects observed in MDMA users.

Keywords: N-Methyl-3,4-methylenedioxymphentamine (MDMA), Adenosine , Nuclear factor-kB, Cerebellum.


Introduction
Use of 3,4- methylenedioxymethamphetamine (MDMA), also known as ecstasy, has increased rapidly in the last decade. The long-term consequences of such abuse have not been understood. As there are several cases where abusers of amphetamine deriv-
a rapid release of numerous neurotransmitters that include serotonin (5-HT), dopamine, and norepinephrine (4). Although, the acute effects of MDMA involve multiple neurotransmitters, the neurotoxic profile of MDMA is selective to the serotonergic system and is characterized by long-term decreases in 5-HT tissue content, the 5-HT transporter (SERT), and tryptophan hydroxylase (TPH), as well as damage to the 5-HT nerve terminals and fibers (5,6). MDMA is also accompanied by an increase in glial fibrillary acidic protein, an astrocyte protein that serves as a marker of injury-induced gliosis (7).

Adenosine, besides its role in intermediate metabolism, also mediates its physiological functions by interacting with four receptor subtypes (A1, A2a, A2b and A3). These receptor subtypes belong to the superfamily of G protein-coupled receptors that represent the most widely targeted pharmacological protein class (8). It has recently been recognized that responses to adenosine are a balance between inhibitory A1 and facilitatory A2 receptors (9). A2 receptors are located throughout the central nervous system (CNS) (10). Activation of adenosine A2a receptors can enhance the release of several neurotransmitters such as acetylcholine, glutamate, and noradrenaline. A2a receptor activation also modulates neuronal excitability and synaptic plasticity, as well as locomotor activity and behavior (8). The ability of A2a receptors to interact with other receptors, neurotransmitters and neuromodulators such as dopamine D2 and D1 receptors, adenosine A1 receptors, metabotropic glutamate receptors and nicotinic autoregulatory receptors expands the range of possibilities used by adenosine to interfere with neuronal function and communication. To date, the majority of evidences that substantiates adenosine involvement in opiate reward focuses on conditions of opiate withdrawal. Adenosine agonists reduce whereas their antagonists increase symptoms associated with precipitated opiate withdrawal (11).

The nuclear factor k-B (NF-kB) family of transcription factors is composed of several members (NF-kB1, NF-kB2, RelA, c-Rel, and RelB). The classic NF-kB transcription factor is composed of p50 and p65 subunits. This transcription factor interacts with an inhibitory protein (IκB) to maintain an inactive complex in the cytosolic compartment until an appropriate intracellular signal causes dissociation of the inhibitory protein. Activation of NFκB involves IκB phosphorylation by IκB kinase followed by its ubiquitination and translocation to the nucleus where NF-kB binds to the kB-response elements in the promoter/enhancer regions of various genes. This process leads to up-regulation of inflammatory cytokines and chemotactic proteins, as well as cellular adhesion molecules (12-13). Several inducers that include interleukin la (IL-la), tumor necrosis factor α (TNF-α), UV light, and double-stranded RNAs cause dissociation and degradation of IκB and promote rapid translocation of NF-kB into the nucleus, where it directly regulates gene expression (14). NF-kB has been shown to be present in the mammalian nervous system, both in the cytoplasm (24) and nucleus (16) of rat neurons. Activation of NF-kB DNA binding activity can occur rapidly (within minutes) upon cellular stimulation and appears to be an important mechanism for the relay of stress responses (17). Neurons in the brain contain activated NF-kB in the nucleus; those in the hippocampus contain the highest amount of this constitutive activity (16). Several studies have demonstrated that MDMA neurotoxicity can be mediated, in part, via activation of neuroinflammatory responses (18-19). The oxidative stress created by MDMA in these studies can promote NF-kB activation, modulate downstream genetic expression, and induce an inflammatory response (20). Oxidative stress has been proposed as a potential mechanism of ethanol-induced neurodegeneration in the developing, mature, and aging cerebellum (21).

There is no publication in the literature concerning the direct effects of A2a receptors on NF-kB regulation and there are no
data on the effects of the A2a receptor on dose distribution for MDMA. From the current available evidence, it was our hypothesis that MDMA may activate NF-kB in the cerebellum and adenosine A2a receptors can alter its expression.

**Methods**

**Drugs and chemicals**

MDMA hydrochloride and other reagents for this study were purchased from Sigma Chemical (Sigma Chemical, La Jolla, CA, USA). The A2a-R agonist, CGS 21680 (2-[p-(2-carboxyethyl) phenethylamino]-5′-N-ethylcarboxamido adenosine), and antagonist, SCH 58261 [7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4 triazolo[1,5-a]pyrimidine], were purchased from Tocris Cookson (Ballwin, MO, USA). CGS 21680 (CGS) and SCH 58261 (SCH) were dissolved in 10% dimethylsulfoxide (DMSO), then injected intraperitoneally (i.p.) into the animals at a dose of 0.03 mg/kg of body weight.

**Animals**

This study used 63 adult male Sprague–Dawley rats (Pasteur Institute, Tehran, Iran) that weighed 200–250 g each. Animals were maintained in an animal house under controlled conditions that included a 12h light-and-dark cycle at 21°C temperature with laboratory chow and water provided ad libitum. All protocols that involved animal use were approved by the Iran University of Medical Sciences (IUMS) Animal Care Committee.

**Experimental protocol**

Animals were allowed to acclimate to the environment for seven days prior to the beginning of the experiments. After acclimatization, animals were randomly divided into nine groups (n=7 per group) as follows:

1. Control group received daily i.p. saline (1cc/kg) injections
2. MDMA10 group received daily i.p. MDMA (10mg/kg) injections
3. MDMA20 group received daily i.p. MDMA (20mg/kg) injections
4. MDMA10 + CGS group received daily i.p. MDMA (10mg/kg) + CGS (0.03 mg/kg) injections
5. MDMA20 + CGS group received daily i.p. MDMA (20mg/kg) + CGS (0.03 mg/kg) injections
6. CGS group received daily i.p. CGS (0.03 mg/kg) injections
7. MDMA10 + SCH group received daily i.p. MDMA (10mg/kg) + SCH (0.03 mg/kg) injections
8. MDMA20 + SCH group received daily i.p. MDMA (20mg/kg) + SCH (0.03 mg/kg) injections
9. SCH group received daily i.p. SCH (0.03 mg/kg) injections

**Stereological analysis**

Drug administrations were performed for seven straight days. Animals were killed seven days after the last injection. Based on preliminary findings, we selected the dose of MDMA that achieved sub-maximal serotonin neurotoxicity and avoided hyperthermia-induced death.

To identify the role of the A2a receptor with regards to MDMA cerebellar neurotoxic effects, we used cresyl violet staining for detection of the neurons in the cerebellum. Rats were deeply anesthetized with ketamine (0.1 ml/kg) then transcardially perfused with ice-cold phosphate-buffered saline (PBS) that consisted of 0.01 M phosphate and 0.15-M sodium chloride, followed by 4% paraformaldehyde in PBS. Whole brains were extracted and post-fixed overnight at 4°C in 4.0% paraformaldehyde. Tissues were processed for paraffin embedding and sectioned into 5 μm sagittal sections. Sections were subsequently dehydrated, mounted, and stained with hematoxylin and eosin; then examined under a light microscope using a 100x objective lens. Images were photographed by an Olympus microscope (BX51, Japan). The number of both purkinje and dark neurons and per unit area was determined from sagittal sections taken from the entire extent of the cerebellum, was counted using a 1000
μm² counting frame. The mean number of neurons per unit area (NA) in cerebellum was calculated using the following formula [R]:

$$N_A = \frac{\sum Q}{a/f\Sigma P}$$

In this formula “ΣQ” is the sum of counted particles appeared in sections, “a/f” is the area associated with each frame, “ΣP” is the sum of frame associated points hitting space.

Following anesthesia and craniotomy, the rats’ brains were rapidly removed and placed on an ice-cold cutting board. The meninges were carefully removed and the cerebellum dissected from the hemispheres, snapped frozen in liquid nitrogen, and stored at −70°C until RNA and protein extraction.

**Western blot analysis**

Collected tissues were homogenized in an ice-cold homogenizing buffer (50 mMTris-HCl, 150 mMNaCl, 1 mM EDTA, and 0.5 mM Triton X-100, pH 7.4) and protease inhibitor cocktail tablets (Roche, Germany) for 1 h, then centrifuged (Eppendorf, Hamburg, Germany) at 12000 g for 20 minutes at 4°C. The supernatant was removed and the protein concentration determined with a Bio-Rad assay system (Bio-Rad, San Francisco, CA, USA). The protein extracts (10 μg) were run on a 10% SDS-PAGE and electroblotted onto nitrocellulose membranes (Millipore, USA). The membranes were then stained with washable Ponceau S solution to confirm equal protein loading. After washing the membranes with distilled water, they were blocked with tris-buffered saline that contained 0.02% Tween-20 and 5% nonfat milk. Antibodies for NF-kB p50 (rabbit monoclonal, 1:1000 dilution; Beyotime Biotech) and β-actin (rabbit polyclonal, 1:10000 dilution; Sigma, St. Louis, MO, USA) were applied at 4°C. Blots were then washed and incubated with the respective alkaline-phosphatase-coupled secondary antibodies (Bio-Rad) at 1:1000 to 1:10000 dilutions. After extensive washing, the protein bands detected by the antibodies were analyzed and values compared with densitometric measurements using an image analysis system (UVIdoc, Houston, TX, USA).

**Analysis of gene expression by RT-PCR**

The phenol-chloroform extraction method was used to extract total mRNA from the rats’ cerebella. Chloroform (0.1 ml) was added to each tube and the contents mixed by shaking for 15 s, then incubated for 2 min at room temperature and subsequently centrifuged at 12000 g for 10 min. The aqueous supernatants that contained RNA were carefully removed and placed into new RNase-free mini-centrifuge tubes. An equal volume of 100% isopropanol was added to precipitate each RNA sample. After centrifugation at 12,000 g for 10 min, the supernatant was removed and discarded. The RNA pellet was washed with 70% ethanol and centrifuged again at 12000 g for 5 min at 4°C, after which the supernatant was removed and discarded. The RNA pellet was air dried and then reconstituted with DEPC water. A cDNA Synthesis Kit (Qiagen, Hilden, Germany) was used for cDNA synthesis according to the manufacturer’s instructions. The resultant cDNA samples were stored at −20°C. The RT-PCR mixture (final volume of 20μl) contained 3 μl of cDNA, 10 μl of Qiagen Multiplex PCR Master Mix 2x (Qiagen, Germany) and 10 pmols of each complementary primer specific for NF-kB and β-actin (as an internal control). The sequences were follows: NF-kB forward and reverse primers: 5’-GCAAAACCTGGGAATACTT CATGTGACTAAG-3’ and 5’-ATAGGC AAGGTCAGAATGACACAAGTGCC-3’, respectively; β-actin forward and reverse primers: 5’-TGGAGAACAGAGCT ATGAGC TGCCCTG-3’ and 5’-GTGCCAC CAGCAC GTGGTGTGTTT-3’, respectively.

The PCR program included 1 cycle at 94°C for 5 min, followed by 30 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, and a final elongation cycle at 72°C for 10 min using a Corbett Research.
thermocycler (Sydney, Australia). PCR products were subjected to electrophoresis on 1% agarose gels, stained with ethidium bromide for 30 min, visualized under a UV source, and photographed with a digital camera (Gel Logic 200 Imaging System, Eastman Kodak Company, Rochester, NY).

Statistical analysis
Statistical analyses were performed using the SPSS software, version 15.0. Kolmogorov–Smirnov test used to check normal distribution. Differences between groups were determined using an independent sample t-test and one-way analysis of variance (ANOVA) followed by Tukey’s test for comparison of groups. The results were expressed as mean ± SD. p<0.05 was considered statistically significant.

Results
A2a-R activity can alter NF-kB protein levels after MDMA treatment
We used Western blot analysis to determine NF-kB protein levels in the rat cerebellum. After normalization against the corresponding β-actin bands, the mean relative densities of the NF-kB bands were calculated for all groups. There was an immunoreactive band visualized at approximately 50 kDa (Fig. 1). Western blot analysis revealed that MDMA significantly increased NF-kB expression in both MDMA groups compared with the control group and between the MDMA10 compared with the MDMA20 groups (p= 0.02). NF-kB expression levels were not significantly affected by CGS or SCH alone, compared with the control group. Thus we considered these three groups as one (Fig. 1). We observed a significant increase in NF-kB protein levels in the hippocampi of the MDMA10 + CGS group compared with the MDMA10 group (p= 0.03). There was a significant increase in NF-kB protein levels in the MDMA20 + CGS group compared with the MDMA20 group (p= 0.02). However, there were no significant differences observed between the MDMA10+SCH and MDMA10 groups, nor between the MDMA20+SCH and MDMA20 groups (p>0.05).

A2a-R activity can alter NF-kB transcription following MDMA treatment
We performed PCR analysis to demonstrate NF-kB expression changes at the mRNA level. The results strongly supported Western blot findings. As seen in Figure 2, there are significant differences between the MDMA and the control group (p=0.04). We observed an increase in the MDMA10 + SCH group that was not significant compared with the MDMA10 group (p> 0.05). By contrast, the MDMA10+CGS group showed significant increase in NF-kB mRNA levels when compared to the MDMA10 group (p= 0.01). NF-kB mRNA
Effects of adenosine receptor expression significantly increased in the MDMA20+CGS group compared to the MDMA20 group (p= 0.02). This difference was not significant between the MDMA20 + SCH and MDMA20 groups.

**A2a-R activity can alter dark neuron formation after MDMA treatment**

Although we observed dark neurons in all groups, they were less abundant in the groups that received CGS (p< 0.05). MDMA treatment increased dark neuron formation in all groups (p= 0.03 versus control); however there were no differences between the MDMA10 and MDMA20 groups. As seen in Figure 3, the mean number of dark cerebellar neurons were higher in the MDMA and MDMA+SCH groups (p= 0.02).

**A2a-R activity can alter purkinje neurons count after MDMA treatment**

Quantitative analyses of Purkinje cells is presented in Fig. 4. We observed a decrease in purkinje neurons in both MDMA groups compared with the control group. Also this difference between MDMA 10 and MDMA 20 groups was significant (Fig. 5).

CGS caused a significant increase in the number of purkinje neurons in MDMA10 + CGS group when compared with MDMA10 group. Increase in purkinje neurons in MDMA20+CGS group was significant in comparison with MDMA20 group.

The number of purkinje neurons decreased in MDMA10+SCH group, but it was not significant when compared with MDMA10 group; whereas it was a marked decrease in the MDMA20+SCH group compared to the MDMA20 group (p< 0.05).
significant reduction in the Purkinje neurons when compared with MDMA group.

Discussion

Our data demonstrated the neurotoxic effects of MDMA on cell death induced by this drug in the rat cerebellum. MDMA caused a significant increase in the number of dark cerebellar neurons compared to control. Dark neuron formation is one type of neuronal cell death for which the mode of death is neither necrotic nor apoptotic (22). There are at least three types of dark neurons in neuropathology: reversible, irreversible, and artifact (23). Kermanian et al. demonstrated that MDMA caused a significant increase in the number of dark neurons located in the hippocampi of rats that received MDMA for seven days (24). Neuronal death is also involved in various chronic and progressive neurodegenerative conditions, including Alzheimer's and Parkinson's disease (22, 25). The border zone separating physiological and pathological neuronal death is often undetectable. Previous studies reported that MDMA can produce long-term deficits in spatial memory as determined by the Morris water maze in probe trials (26-27). The key brain regions involved in navigation in the Morris water maze task include the striatum, the frontal cortex, cerebellum and hippocampus (28). The cerebellum has a well-established role in the fine control of motor activity and emerging data increasingly support a broader cognitive role for this structure. For example, the cerebellum plays a role in motor-associative learning (29), visio-spatial judgment (30), and influences auditory, visual, language and spatial abilities during development (31). In this study we have reported that the adenosine A2a antagonist (SCH) increased and the A2a agonist (CGS) decreased MDMA-induced dark neuron formation. A study demonstrated that SCH significantly increased extracellular levels of glutamate with approximately 100% increases in the shell of the nucleus accumbens (32). Methamphetamine neurotoxicity might also occur via excitotoxic damage following glutamate release and activation of glutamate (33). These results have suggested that A2 receptors play a protective role in MDMA-induced neural death.

This study revealed that MDMA administration can cause Purkinje cell loss, dose dependently. Cerebellar Purkinje neurons are among the most vulnerable neurons in the nervous system and neuropathological alterations in CNS are characterized by significant decrease in the number of surviving Purkinje neurons. Purkinje neurons are selectively vulnerable to toxins such as ethanol (34). Bekheet et al. in 2010 reported that morphine significantly decreased the diameter of Purkinje cells and thickness of molecular and granular layers in all treated animals (35). Co-administration of CGS with MDMA attenuated the MDMA-induced purkinje loss. Treatment with adenosine, and uracil nucleotides protected Purkinje neurons from cell death in primary cerebellar cultures (36). In particular, adenosine may play adverse role in the central nervous system, where it is involved in multiple processes via A1 and A2 receptors, e.g., pre-synaptic inhibition by opening of K' channels (16), modulation of pre-synaptic Ca\(^{2+}\) channels (37), , and regulation of cerebral blood flow (38).

In this study, administration of MDMA once daily led to NF-kB activation. Our data showed that the uptake of MDMA led to activation of NF-kB in the rat cerebellum. In line with this observation, two previous studies have reported that increased NF-kB expression in the myocardium and liver following MDMA administration appeared to activate NF-kB following MDMA exposure, which in turn upregulated the expression of cytokines and cellular adhesion molecules that have important roles in inflammatory responses (20). The activation of these genes by MDMA suggests that the cells are subjected to and that a cellular compensatory response is initiated to counteract the exposure and promote survival (14,20).There is some evidence
 linking MDMA exposure to increased lymphocyte infiltration and inflammatory damage to the myocardium (39). The cellular and molecular mechanisms involved in causing these effects have not been fully elucidated. Most published work show that NF-kB plays a protective role, as its inhibition enhances the cell death rate and/or sensitizes cells towards apoptotic conditions (40). Cadet and Brannock have reviewed the evidence that oxidative stress is involved in methamphetamine-induced neurotoxicity (41). MDMA has been shown to simultaneously increase hydroxyl radical formation in rat hepatic stellate cells (42) and the rat brain (43). Numerous in vitro studies demonstrated that diverse neurotoxic and pro-apoptotic stimuli including high concentrations of glutamate (44), cytokines (45) and H2O2 (46) are potent activators of NF-kB in neuronal cells.

Here, we have characterized the effects of A2a-R on the NF-kB expression in the rat cerebellum after i.p. injections of MDMA. Our findings indicated that CGS, an A2a-R agonist, increased NF-kB expression in the rat cerebellum that previously received MDMA injections. This result was consistent with our previous study (47). The adenosine A2a receptor subtype is one of the four adenosine receptors that have been identified in mammals and are abundant in the CNS. The recent development of selective A2a receptor ligands makes it possible to elucidate the function of A2a receptors under normal and altered conditions. It is well established that in the CNS endogenous adenosine plays a pivotal role in neurodegeneration. Although low, nanomolar concentrations of adenosine are normally present in the extracellular fluid; however there is a dramatic increase during enhanced nerve activity, hypoxia or ischemia. Under these pathological conditions, adenosinergic transmission-potentiating agents, which elevate adenosine level by either inhibiting its degradation or preventing its transport, offer protection against ischemic or excitotoxic neuronal damage. Some authors have suggested that the neuroprotective properties of A2a-R may be related to the inhibitory effects of these drugs on oxidative stress (48). Some evidence has shown that A2a-R could inhibit inflammation in the lung, at least in part, by suppressing NF-kB activation and downstream gene expression (49). At the molecular level adenosine modulates the release of many neurotransmitters (dopamine, glutamate, GABA, serotonin) with the inhibition of excitatory neurotransmitter release (glutamate) being the most pronounced (50-51). Consistent with this idea is the finding that overexpression of p65 prevents cell death. Inhibition of apoptosis by p65 has been demonstrated in many cell types and mice that lack p65 show increased apoptosis in the liver and die early during embryogenesis (52). Activation of A2A receptors can promote glial proliferation after brain injury (53). Since these cells produce anti-inflammatory cytokines as well as pro-inflammatory compounds, the net balance of these may influence neuronal survival. Therefore, understanding the mechanism/s involved could provide clues for devising new strategies for drug therapy and clinical applications.

Conclusion
Our results suggest that the adenosine receptor might regulate NF-kB activation. We also showed that the selective A2a receptor agonist (CGS) attenuates cerebellar neuronal death after MDMA.

A2a receptors in CNS may exert complex actions on neuronal cell death (both, potentially deleterious as well as neuroprotective) and possibly other functions such as modulation of synaptic transmission. This existence of multiple and apparently conflicting hypothesis illustrate how little we actually know about the biology of A2A receptors. Thus further investigation of the molecular mechanisms triggered by A2a-R activation would increase our understanding of its actions in different tissues.

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Conflicts of interest
The authors declared no competing interests.

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