Neurotox Res (2010) 17:435–439
DOI 10.1007/s12640-009-9125-y

Caffeine Enhances Astroglia and Microglia Reactivity Induced by 3,4-Methylenedioxymethamphetamine ('Ecstasy') in Mouse Brain

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Received: 1 September 2009 / Revised: 8 October 2009 / Accepted: 8 October 2009 / Published online: 31 October 2009

Abstract Several reports suggest that 3,4-methylenedioxymethamphetamine (MDMA) induces neurotoxic effects and gliosis. Since recreational use of MDMA is often associated with caffeinated beverages, we investigated whether caffeine interferes with MDMA-induced astroglia and microglia activation, thus facilitating its neurotoxicity. MDMA (4 × 20 mg/kg) was acutely administered to mice alone or in combination with caffeine (10 mg/kg). CD11b and GFAP immunoreactivity were evaluated as markers of microglia and astroglia activation in the substantia nigra pars-compacta (SNc) and striatum. MDMA was associated with significantly higher CD11b and GFAP immunoreactivity in striatum, whereas only CD11b was significantly higher than vehicle in SNc. Caffeine potentiated the increase in CD11b and GFAP in the striatum but not in the SNc of MDMA-treated mice. The abuse of MDMA is a growing worldwide problem; the results of this study suggest that combination of MDMA plus caffeine by increasing glial activation might have harmful health consequences.

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Keywords Glial cell · Adenosine · Drug of abuse · Striatum · Psychostimulants

Introduction

The use of psychostimulant drugs is often combined with beverages containing a high quantity of caffeine in order to amplify their stimulant properties and reduce drowsiness and fatigue. 3,4-Methylenedioxymethamphetamine (MDMA)—known as ecstasy—is one of the most popular psychostimulant whose neurotoxic effects on the central nervous system are often debated. Specific neurotoxic damage to 5-hydroxytryptamine and dopaminergic nerve endings lasting for months in rodents and years in primates has been demonstrated both biochemically and histologically (Green et al. 2003; Granado et al. 2008). These neurotoxic effects appear to result from free radical formation which in turn induces oxidative stress process and from hyperthermia (Green et al. 2003; Goni-Allo et al. 2008).

Evidence for the occurrence of MDMA-induced neurotoxic damage in human users, however, remains ambiguous since such evidence is complicated by the fact that many users often take other substances concomitantly, either intentionally or due to impurities in ecstasy tablets. The most popular of these associated substances is caffeine, which, when given acutely, enhances the acute toxicity and lethality of MDMA (McNamara et al. 2006).

Several findings have suggested that neuroinflammation may play an active role in the pathogenesis of neurodegenerative diseases. Microglial and astroglial activation appear to play an important role in neurotoxicity and MDMA-induced toxicity is associated with microglial activation, which generates many reactive species (e.g., nitric oxide, superoxide, cytokines) favouring neurodegeneration

(Thomas et al. 2004). In this context it becomes of great importance to investigate whether MDMA when given in combination with caffeine, enhances astroglia or microglia reactivity in experimental rodents.

The results of this study may bring important insights to our knowledge of the toxic effects that may be caused by the use of caffeine together with psychostimulant drugs.

Materials and Methods

Drugs

MDMA–HCl (synthesized by Prof. Plumitallo Department of Pharmaceutical Chemistry and Technology, University of Cagliari) was dissolved in saline. Caffeine (Sigma–Aldrich, Milan, Italy) was dissolved in water.

Animals

Adult male C57BL/6J mice, 3 months old (20–25 g; Charles River, Milan, Italy) were maintained at a constant temperature (21 ± 1°C) in 12-h light/dark cycles (lights on at 08:00), and given food and water ad libitum. All experimental procedures were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and approved by the ethics committee of the University of Cagliari.

Treatment

Mice were treated with repeated administration of vehicle ($N = 6$) or MDMA (4 × 20 mg/kg, intraperitoneally, i.p.) ($N = 12$) at 2-h intervals alone or in combination with repeated administration of vehicle or caffeine (2 × 10 mg/kg, i.p.) ($N = 16$) 30 min before the first and third administration of MDMA. On second day mice received two administrations of vehicle or caffeine (10 mg/kg, i.p.) at 12 h interval and one administration on third day. Mice were killed 48 h after the last administration of MDMA. This dosing protocol of MDMA has been shown to produce activation of microglial and astroglial cells (Thomas et al. 2004).

Immunohistochemistry

Animals were anaesthetized with chloral hydrate and perfused with 4% paraformaldehyde in phosphate buffer 0.1 M (pH 7.4). Coronal sections (50 μm thick) were cut on a vibratome and immunostained for glial fibrillary acidic protein (GFAP) and CD11b in order to analyze astroglial and microglial activation, respectively. Immunohistochemistry was carried out in free-floating sections with standard avidin–biotin (ABC; Vector Laboratories, UK) immunohistochemical protocols. Sections were treated with hydrogen peroxide to block endogenous peroxidases and then incubated overnight with the following specific primary antisera: monoclonal mouse anti-GFAP (1:400; Sigma–Aldrich) and monoclonal rat anti-CD11b (1:1000; Serotec, UK). For visualization it was used 3,3′-diaminobenzidine as chromogen. Images were captured under constant light conditions using a PixeLink PL-A686 camera at 10× magnification for the SNc and at 20× magnification for the striatum. For each animal, three sections from the SNc ($A = -2.92$, -3.28, -3.64 mm from bregma, accordingly to Mouse brain Atlas by Paxinos and Franklin 2001), and three sections from striatum ($A = 1.10$, 0.74, 0.38 mm from bregma), were analyzed for each protein evaluated in the study.

GFAP immunoreactivity in the SNc was quantified by counting the number of positive cells in the entire left and right part of this structure. For quantification of GFAP immunoreactivity in the striatum, one dorsolateral and one ventromedial portion were analyzed from both the left and right parts using PixeLink image analysis software. Analysis of CD11b immunoreactivity in the SNc and striatum was done with the analysis software Scion Image. Within each frame, the area occupied by gray values above the threshold was automatically calculated.

In order to obtain averages for GFAP and CD11b, values were normalized with respect to vehicle.

All values were expressed as mean ± SEM and were statistically analyzed with a one-way ANOVA followed by the Newman–Keuls post hoc test.

Results

Previous studies have shown that expression of CD11b and GFAP, markers of activated microglia and astroglia, respectively, reached their maximum at 48 and 72 h after MDMA administration (Granado et al. 2008; Thomas et al. 2004). Therefore, in our study, mice were killed 48 h after last administration of MDMA in order to examine the activation of both microglia and astroglia.

GFAP Immunoreactivity in the Striatum and SNc

GFAP immunoreactivity was low in both the striatum and SNc in the basal condition (Fig. 1). It was significantly higher in the striatum after treatment with MDMA (20 mg/kg, i.p.) and MDMA plus caffeine (10 mg/kg, i.p.) compared with vehicle ($P < 0.0001$). Post hoc analysis indicated that administration of caffeine in MDMA-treated mice was associated with significantly higher GFAP immunoreactivity in the striatum (Fig. 1a) compared with

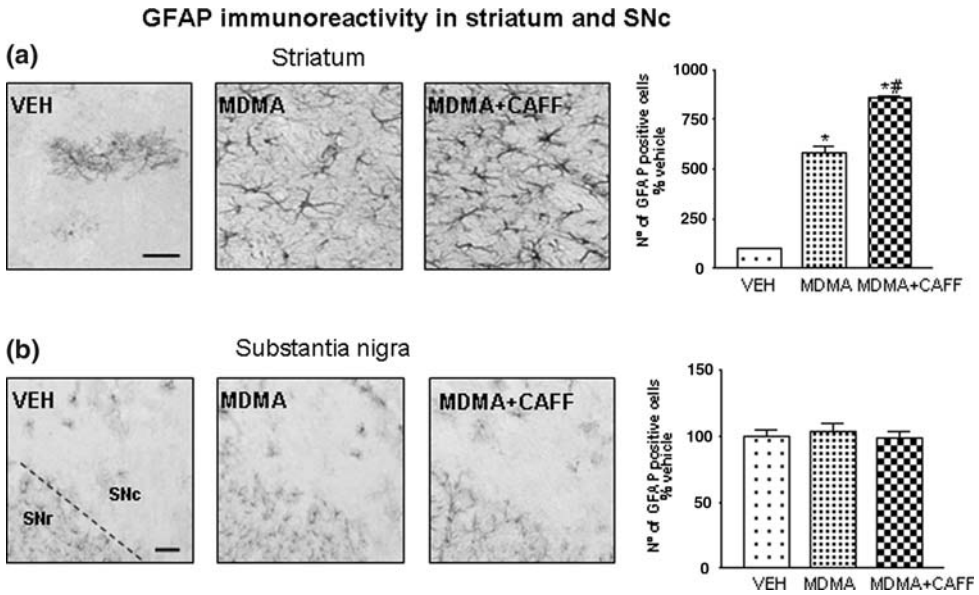

Fig. 1 Effect of repeated administration of MDMA (20 mg/kg, i.p.) and caffeine (10 mg/kg, i.p.) on astroglial activation, using GFAP immunoreactivity as a marker. Representative images and histograms from the **a** striatum and **b** SNc, immunostained for GFAP. Values are reported as a percentage of GFAP-positive cells with respect to vehicle-treated mice and are expressed as mean ± SEM. * $P < 0.0001$ versus vehicle-treated group; # $P < 0.0001$ versus the group treated with MDMA alone. *Scale bar*: 50 μm. *SNc* substantia nigra pars-compacta, *SNr* substantia nigra pars-reticulata

mice treated only with MDMA ($P < 0.0001$). Treatment with MDMA and MDMA plus caffeine did not alter GFAP immunoreactivity in the SNc compared with vehicle (Fig. 1b). Caffeine alone did not modify GFAP both in striatum and SNc (data not shown).

CD11b Immunoreactivity in the Striatum and SNc

Similarly, to GFAP, CD11b immunoreactivity was low in both the striatum and SNc in the basal condition (Fig. 2). It was significantly higher in both the striatum (Fig. 2a) and

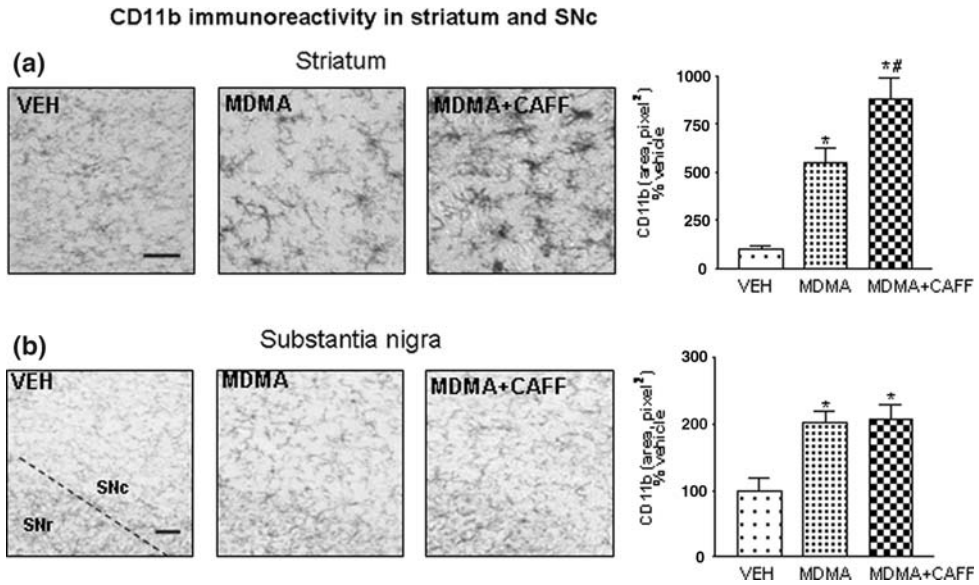

Fig. 2 Effect of repeated administration of MDMA (20 mg/kg, i.p.) and caffeine (10 mg/kg, i.p.) on microglial activation, using CD11b immunoreactivity as a marker. Representative images and histograms from the **a** striatum and **b** SNc, immunostained for CD11b. The area occupied by *gray* values above a threshold was calculated and expressed as *square pixels* and as a percentage of staining in vehicle-treated mice, and values are expressed as mean ± SEM. * $P < 0.02$ versus vehicle-treated group, # $P < 0.05$ versus the group treated with MDMA alone. *Scale bar*: 50 μm. *SNc* substantia nigra pars-compacta, *SNr* substantia nigra pars-reticulata

SNc (Fig. 2b) after treatment with MDMA (20 mg/kg, i.p.) and MDMA plus caffeine (10 mg/kg, i.p.) compared with vehicle ($P < 0.05$). Post hoc analysis indicated that administration of caffeine in MDMA-treated mice was associated with significantly higher CD11b immunoreactivity in the striatum but not in the SNc compared with mice treated only with MDMA ($P < 0.05$). Caffeine alone did not modify CD11b levels both in striatum and SNc (data not shown).

Discussion

The results of the present study show that acute repeated administration of MDMA induced a neuroinflammatory process in mice, characterized by microgliosis in the striatum and SNc and astrogliosis in the striatum. Moreover, of great importance in terms of possible consequences in humans is the finding that caffeine, when given together with MDMA, potentiated the activation of microglia and astroglia in the striatum.

Caffeine is a safe substance present in beverages such as coffee, tea, soft drinks, and energy drinks. However, as shown by the present study, caffeine taken with MDMA at doses similar to those that may be taken for recreational use with energy drinks may exacerbate MDMA toxicity posing health consequences for consumers of this drugs of abuse.

Neuroinflammation is one of the factors responsible for the pathogenesis of neurodegenerative disorders (Kerschensteiner et al. 2009). Studies have reported that microglial and astroglial activation represent an early step in MDMA-induced neurotoxicity and that within the amphetamine class of drugs only those causing neurotoxicity result in microglia activation (Thomas et al. 2004; Granado et al. 2008).

Moreover, it is well-known that activation of glial cells, particularly microglia, may contribute to neuronal damage by the release of proinflammatory cytokines such as tumor necrosis factor-α, interleukin-1β, and neurotoxic factors that include reactive nitrogen species, reactive oxygen species, and excitatory amino acids (Kerschensteiner et al. 2009), although a neuroprotective role of glial cells has also been hypothesized (Merrill and Benveniste 1996). It has also been shown that proinflammatory molecules such as neuronal nitric oxide synthase (NOS) and inducible NOS are increased 1 day after MDMA administration and that their inhibition provides protection against MDMA-induced dopamine loss in the striatum (Green et al. 2003). Therefore, by showing higher activation of both microglia and astroglia after MDMA plus caffeine compared with MDMA alone, our results suggest that this drug combination may facilitate the neurodegenerative processes induced by MDMA in mouse brain. Interestingly, studies on MPTP model of Parkinson's disease have shown that striatal dopaminergic terminals are more vulnerable than SNc cell bodies (Schmidt and Ferger 2001), being in line with the higher glial activation in striatum observed in our study.

One of the mechanisms implicated in MDMA neurotoxicity is a hyperthermic response induced by the drug. Consistent hyperthermia has been observed in rats and a hyperthermic response, though much more variable, has been seen in mice (Green et al. 2003; McNamara et al. 2006). Moreover, promotion of temperature elevation by caffeine was observed (McNamara et al. 2006), suggesting a possible mechanism that may potentiate MDMA effects, contributing to the increase in astroglia and microglia reactivity observed after MDMA plus caffeine administration.

Caffeine affects the absorption of MDMA in intestinal epithelial cells and increases the area under the plasma concentration curve of MDMA (Kuwayama et al. 2007). This effect, as well as hyperthermia, may have a role in caffeine's potentiation of MDMA effects (Green et al. 2003; Goni-Allo et al. 2008). A further consideration that should be taken into account is that caffeine and MDMA are extensively metabolized, and caffeine metabolites have a long half-life. Therefore, the possibility that their metabolites may have a role in glial activation cannot be ruled out. Interestingly, however, it has been established that caffeine does not inhibit CYP2D6, the enzyme responsible for MDMA metabolism, and it did not alter the half maximal inhibitory concentration for MDMA (Downey and O'Boyle 2007).

The results of the present study might seem to contradict the results of studies showing that caffeine counteracts other forms of toxicity. On the other hand, several studies have shown that the role of adenosine can be neuroprotective or neurotoxic depending on the type of insult and the specific cellular conditions (Cunha 2001). Therefore, it is reasonable to hypothesize that endogenous adenosine may counteract MDMA-induced glial activation. Adenosine may in fact decrease blood pressure, heart rate and induce hypothermia (Jonzon et al. 1986) as well as regulate the actions of neurotrophic factors through adenosine A_{2A} receptors (Sebastião and Ribeiro 2009). By blocking both adenosine A_1 and A_{2A} receptors, caffeine may facilitate neuroinflammatory processes induced by MDMA. It should be, however, emphasized that our study relates to acute caffeine treatment and that due to the rapid tolerance developed to the drug, different results might be obtained in a chronic protocol of caffeine administration.

In conclusion, by activating astroglia and microglia cells, the combination of MDMA plus caffeine enhanced inflammatory brain processes induced by MDMA. Since MDMA and energy drinks are very popular among young

people, particular attention should be paid in the harmful health consequences of this combination of drugs.

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