

Methylenedioxymethamphetamine (MDMA; ‘Ecstasy’) suppresses antigen specific IgG_{2a} and IFN- γ production

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Abstract

Methylenedioxymethamphetamine (MDMA; ‘Ecstasy’) is a widely abused amphetamine derivative. In the present study, we examined the effect of acute MDMA administration on an antigen specific immune response. Responsiveness to an *in vivo* challenge with the soluble protein antigen keyhole limpet haemocyanin (KLH) was examined in rats following MDMA administration (2.5, 5 or 10 mg/kg; *i.p.*). KLH-specific serum IgM concentrations were measured 7 days following challenge, and serum IgG concentrations were measured 14 days following the KLH challenge. In addition, antigen-specific IFN- γ and IL-6 production was measured in KLH-stimulated splenocytes. MDMA did not alter the KLH-specific IgM response. In contrast, MDMA (5 and 10 mg/kg) provoked a significant suppression of KLH-specific IgG production. Thus, MDMA administration did not alter the initial generation of the antibody response but rather inhibited antibody class switching from IgM to IgG. Two pathways for the genetic switch from IgM to IgG production were investigated. One pathway requires the Th₁ type cytokine IFN- γ to stimulate IgM-secreting cells to switch to IgG_{2a}-secreting cells. Another pathway requires the Th₂ type cytokines IL-4 and IL-6 to stimulate IgM-secreting cells to switch to IgG₁-secreting cells. IgG₁ and IgG_{2a} levels were measured to determine if these two pathways were differentially affected. The results indicate that only IgG_{2a} levels were decreased following MDMA administration. Furthermore, this decrease in IgG_{2a} was accompanied by decreased KLH-specific IFN- γ production 14 days post KLH administration. In conclusion, these data indicate that MDMA alters the ability to switch from IgM to IgG_{2a} production, possibly by reducing IFN- γ . Potential health consequences for MDMA users are discussed. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Amphetamine; Antibody; Humoral immunity; IFN- γ ; IL-6; IgG_{2a}; KLH; MDMA

1. Introduction

Methylenedioxymethamphetamine (MDMA; ‘Ecstasy’) is a widely abused amphetamine derivative in today’s youth culture. MDMA abuse has been associated with a number of serious side effects, such as cardiac arrhythmias, hyperthermia, renal failure, seizures and intracranial haemorrhage [1,2]. In addition, it has been suggested that the long term neurotoxic effects of MDMA on central serotonergic neurons may represent a predisposing factor to psychological disturbances/psychiatric disease [3]. In addition to the aforementioned adverse effects of MDMA, we have recently demon-

strated that acute MDMA administration reduces circulating lymphocyte numbers, suppresses concanavalin A-induced lymphocyte proliferation and Th₁ and Th₂ type cytokine secretion [4–6]. MDMA also suppresses the proinflammatory cytokine response to an *in vivo* challenge with bacterial lipopolysaccharide (LPS) [7]. However, to date, the effect of MDMA administration on antigen specific immunity has not been evaluated.

In rodents, administration of the soluble protein antigen keyhole limpet haemocyanin (KLH) provokes an antigen specific humoral immune response, which can be monitored by measuring serum IgG and IgM concentrations at intervals following the KLH challenge [8,9]. KLH represents a novel antigen in the rat and the antibody responses provoked by KLH require T- and B-lymphocyte collaboration as well as antigen presenting cells [8]. Thus an *in vivo* KLH challenge is a

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useful tool in assessing immunocompetence, because development of immunity to KLH reflects those *in vivo* immune processes that occur during the host response to an *in vivo* antigenic challenge.

In the present study we examined the effect of dose-related effects of acute MDMA administration on responsiveness to an *in vivo* antigenic challenge with KLH in rats. KLH-specific serum IgM concentrations were measured 7 days following challenge, and serum IgG, IgG₁ and IgG_{2a} concentrations were measured 14 days following the KLH challenge. In addition, antigen-specific IFN- γ and IL-6 production was measured in KLH-stimulated splenocytes in order to examine the effect of MDMA on antigen specific Th₁ (IFN- γ) and Th₂ (IL-6) type cytokine production.

2. Materials and methods

2.1. Subjects and procedures

Female Sprague–Dawley rats (220–250g; 12–14 weeks old) were obtained from a departmental breeding colony and housed four per cage. The rats were maintained on a 12 h:12 h light:dark cycle (lights on at 08:00 h) in a temperature controlled room (22–24 °C), with food and water available *ad libitum*. Female rats were used because our previous studies concerning the immunological effects of MDMA were conducted in female rats [4–7]. In addition, previous data generated within our laboratory demonstrated that male and female rats respond in a similar fashion to MDMA [10,11]. The experimental protocol was in compliance with the European Communities Council directive (86/609/EEC).

2.2. MDMA administration

MDMA (NIDA, Research Triangle Institute, NC) was dissolved in 0.89% NaCl to give concentrations of 2.5, 5 or 10 mg/ml; 0.89% NaCl was administered alone as a vehicle to the control group. Both MDMA and vehicle were administered in an injection volume of 1 ml/kg using the intraperitoneal (i.p.) route.

2.3. *In vivo* KLH challenge

KLH (Calbiochem, U.K.) was dissolved in sterile 0.89% NaCl at a concentration of 100 μ g/ml. Animals were challenged with KLH (100 μ g/kg; i.p) at the same time as MDMA/vehicle, as we have previously observed that MDMA-induced suppression of IL-1 β and TNF- α in response to *in vivo* LPS challenge is maximal when MDMA is administered in close proximity to the immune challenge [7]. KLH was administered in a 1 ml/kg injection volume by the i.p. route. Rats were killed by

decapitation 7 or 14 days following the KLH challenge and trunk blood was collected for immunoglobulin analysis. Following collection, blood samples were centrifuged ($800 \times g$ at 4 °C for 15 min) and aliquots of serum were removed for determination of immunoglobulin concentrations. Serum samples were frozen and stored at –20 °C until the assays were performed. Previous studies conducted in this laboratory have found that this dose and route of administration of KLH produces quantifiable increases in circulating IgM and IgG concentrations [12]. KLH-specific IgM concentrations peak at 7 days and IgG concentrations reach a peak 14 days following an *in vivo* KLH challenge [12]. Therefore in the present study, KLH-specific serum IgM was measured 7 days following the KLH challenge, whereas KLH-specific IgG, IgG₁ and IgG_{2a} were measured 14 days following KLH administration.

2.4. Antibody capture ELISA

KLH was diluted to a working concentration of 100 μ g/ml with bicarbonate coating buffer (15 mM NaHCO₃, 35 mM Na₂CO₃; pH 9.6) and 100 μ l of this solution was added to wells of flat bottomed 96-well maxisorp immunoplates. The plates were covered and incubated at 4 °C for 16–24 h. Following the incubation period the plates were washed three times by filling each well with 400 μ l of wash buffer (137 mM NaCl, 1.46 mM KH₂PO₄, 8.1 mM Na₂HPO₄·12H₂O, 2.7 mM KCl, 0.05% Tween 20; pH 7.4) using an automated strip-washer (Tecan) and the plates were blotted dry on a paper towel. Bovine serum albumin (100 μ l; 1% BSA) solution in bicarbonate coating buffer was added to each well, the plates were covered and incubated at 37 °C for 1 h. The plates were emptied and washed three times. The serum samples were thawed to room temperature and diluted to the desired concentration in wash/diluent buffer. Each sample (100 μ l) was added per well and plates were incubated at 37 °C for 3 h. Blanks were also ran with each assay, i.e. 100 μ l of wash/diluent buffer per well. The plates were emptied and washed three times. HRP-conjugated antibody (100 μ l) was added to each well and plates were incubated at 4 °C overnight (16–24 h). HRP-conjugated anti-IgM and -IgG antibodies (Southern Biotechnology Associates, USA) were used at a 1:5000 dilution in sample/wash buffer and the anti-IgG₁ and IgG_{2a} antibodies (Zymed Laboratories, USA) were used at a 1:500 dilution in sample/wash buffer. The plates were emptied and washed three times. TMB substrate reagent (100 μ l) was added to each well for 10–15 min. 1M H₂SO₄ (100 μ l) was added to each well, to stop the enzyme reaction and facilitate colour development. Absorbance was measured at 450 nm using a microtitre plate reader. In the calculation of results the blank reading was subtracted from all samples.

2.5. IFN- γ and IL-6 production from KLH-stimulated splenocyte cultures

Splenocytes were used to examine the effect of MDMA treatment on KLH-specific cytokine production, as following i.p. injection of KLH, antigen is transported to the mesenteric lymph nodes and the spleen [13,14]. In addition, it was recently demonstrated that when restimulated in vitro, splenocytes from mice and rats administered KLH by the i.p. route produce KLH-specific Th₁ and Th₂ type cytokines [15,16].

The spleen was aseptically removed from each rat and placed into a sterile Stomacher 80 bag and dissociated by applying pressure to the spleen. RPMI 1640 (5 ml; Life Technologies, Scotland) was added to the Stomacher 80 bag and placed into the Stomacher (Seward, UK) for 240 sec at top speed. The contents of the Stomacher bag were passed through a cell filter (Becton Dickinson, UK) into a sterile 50 ml conical tube (Starstedt, Ireland) in order to remove cell debris. The tubes were centrifuged at 1600 RPM for 15 min at 4 °C and the supernatant was discarded. The erythrocytes were removed by hypotonic lysis and the remaining splenocytes were washed twice in RPMI 1640 medium. The splenocytes were resuspended in complete RPMI 1640 medium (RPMI 1640 supplemented with 10% foetal bovine serum and 1% penicillin/streptomycin), counted using a veterinary haematology counter (ABC counter; Roche Diagnostics) and adjusted with complete RPMI 1640 medium to a final concentration of 2×10^6 cells/ml. Aliquots (1 ml) of splenocytes were pipetted into wells of a sterile flat bottomed 24 well plate (Starstedt, Ireland). To each well was added either 100 μ l of RPMI for unstimulated cultures or endotoxin free KLH (Calbiochem, UK) at a working concentration of 50 μ g/ml. Cultures were incubated for 72 h at 37 °C in a 5% CO₂ atmosphere. We have previously found these conditions to be optimal for the production of IFN- γ and IL-6 in KLH-stimulated cultures (unpublished data). At the end of the culture period the contents of each well was transferred into Eppendorf tubes and centrifuged at 12,000 rpm for 15 min at 4 °C. Following centrifugation the supernatants were stored at –80 °C until cytokine assays were performed.

2.6. IFN- γ and IL-6 measurements

IFN- γ concentrations were measured using antibodies and standards from a commercially available rat IFN- γ DuoSet (R&D systems, UK). The assay was performed as per the manufacturers instructions and results were expressed as pg IFN- γ /ml.

IL-6 concentrations were determined using a specific rat IL-6 ELISA sandwich assay performed using antibodies and standards obtained from Dr S. Poole (NIBSC, Potters Bar, Herts, UK). Briefly, 96 well

Maxisorp microtitre plates (Nunc, Belgium) were coated with sheep anti-rat IL-6 polyclonal antibodies (2 μ g/ml in bicarbonate coating buffer; 0.1 M NaHCO₃, 0.1 M NaCl, pH 8.2, for 20 h at 4 °C), then washed three times with wash buffer (0.5 M NaCl, 2.5 mM NaH₂PO₄, 7.5 mM Na₂HPO₄, 0.1% Tween 20, pH 7.2). A 1% (w/v) ovalbumin (Sigma Chemical Co., Poole, Dorset, UK) solution (100 μ l) in bicarbonate coating buffer was added to each well and incubated at 37 °C for 1 h. Following three washes, 100 μ l of samples and standards were added and plates were incubated at 4 °C for 20 h. After three washes, 100 μ l of the biotinylated anti-rat IL-6 antibody (1:2000 dilution in wash buffer containing 1% sheep serum, Sigma Chemical Co., Poole, Dorset, UK) was added to each well. A further incubation was carried out for 1 h at room temperature. After three washes, 100 μ l avidin-HRP (Dako Ltd, UK) (1:5000 dilution in wash buffer) was added per well and plates were incubated at room temperature for 15 min. Following three washes, 100 μ l of TMB substrate solution (Dako Ltd.) was added per well and the plates were incubated for 20–30 min at room temperature. At the end of the incubation period 100 μ l of 1 M H₂SO₄ was added per well to stop the reaction and to facilitate colour development. Absorbance was read at 450 nm on a microtitre plate reader. Results were expressed as pg IL-6/ml.

2.7. Statistical analysis of data

The immunoglobulin data were analysed using a two-way repeated measures analysis of variance with the first and second factors being treatment and serum dilution respectively. The cytokine data were analysed using a two-way analysis of variance with the first and second factors being in vitro KLH stimulation and treatment respectively. If any significant change was found, post hoc comparisons were performed using Fishers LSD. Data was deemed significant when $P < 0.05$ and data are expressed as mean $\pm$ SEM.

3. Results

3.1. KLH-specific IgM and IgG

MDMA administration did not significantly alter KLH-specific IgM at any of the doses employed in the present study (Fig. 1a). In contrast, KLH-specific IgG was dose-dependently suppressed by MDMA administration [ANOVA treatment effect: $F(3,60) = 3.67$, $P < 0.05$]. Post hoc analysis demonstrated that MDMA (5 and 10 mg/kg) significantly ($P < 0.01$) suppressed KLH-specific IgG at all three serum dilutions employed in the present study (Fig. 1b).

3.2. KLH-specific IgG₁ and IgG_{2a}

Upon analysis of the IgG subtypes IgG₁ and IgG_{2a} it was demonstrated that KLH-specific IgG₁ was not significantly altered by MDMA administration (Fig. 2a). In contrast, KLH-specific IgG_{2a} was dose-dependently suppressed by MDMA administration [ANOVA treatment effect: $F(3,60) = 4.06$, $P = 0.01$]. Post hoc analysis demonstrated that MDMA (5 and 10 mg/kg) significantly suppressed ($P < 0.01$) KLH-specific IgG_{2a} at all three serum dilutions employed in the present study (Fig. 2b).

3.3. KLH-specific IFN- γ production

3.3.1. Day 7

There was a significant effect of in vitro KLH stimulation [$F(1,63) = 48.44$, $P < 0.001$] and drug treatment [$F(1,63) = 3.20$, $P < 0.05$] on splenocyte IFN- γ production 7 days following KLH/MDMA administration. However there was no significant in vitro KLH stimula-

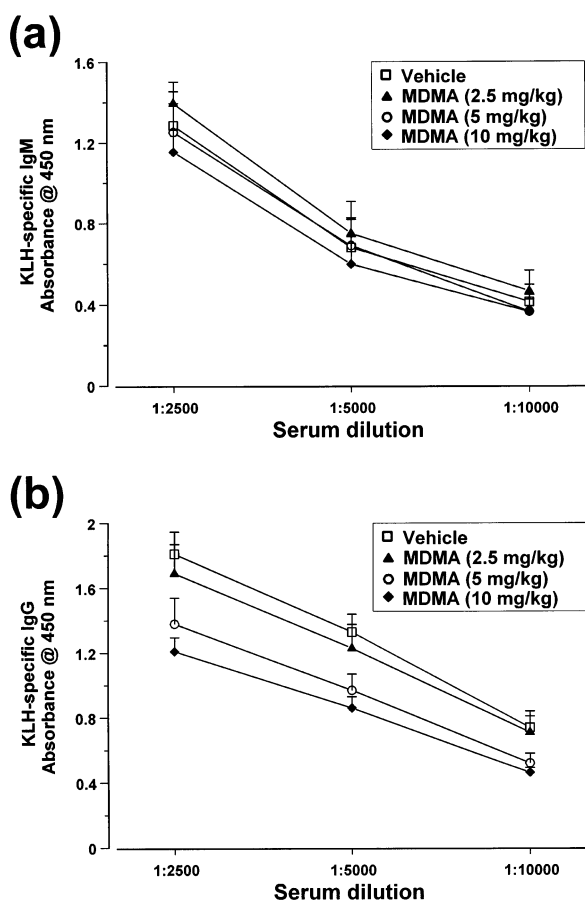


Fig. 1. Effect of acute MDMA administration on KLH specific (a) IgM and (b) IgG concentrations 7 and 14 days post KLH challenge respectively. Data expressed as means $\pm$ SEM ($n = 8-9$). MDMA (5 and 10 mg/kg) significantly suppressed ($P < 0.01$) KLH-specific IgG in comparison to vehicle treated counterparts (Fishers LSD).

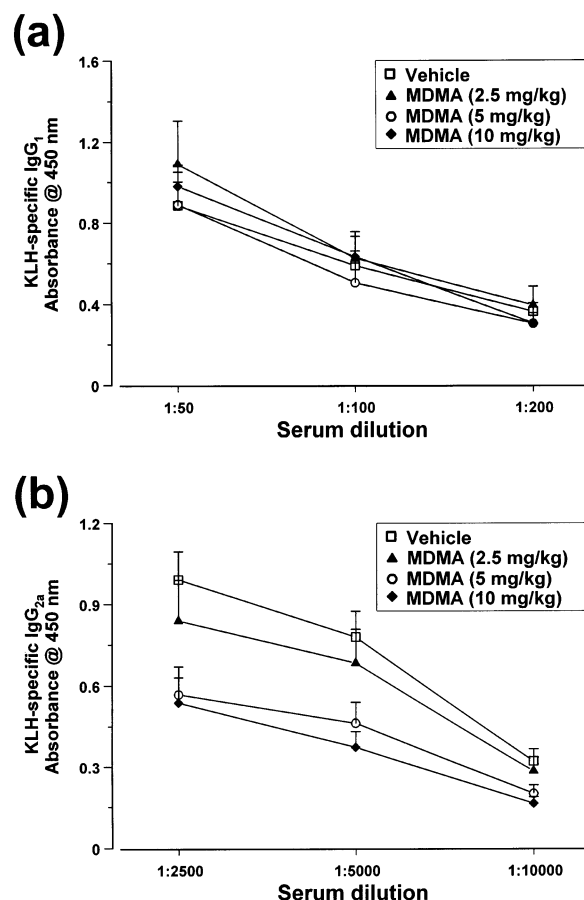


Fig. 2. Effect of acute MDMA administration on KLH specific (a) IgG₁ and (b) IgG_{2a} concentrations 14 days post KLH challenge. Data expressed as means $\pm$ SEM ($n = 8-9$). MDMA (5 and 10 mg/kg) significantly suppressed ($P < 0.01$) KLH-specific IgG_{2a} in comparison to vehicle treated counterparts (Fishers LSD).

tion $\times$ treatment interaction. Post hoc analysis demonstrated that stimulation of splenocytes with KLH (50 μ g/ml) significantly ($P < 0.01$) increased IFN- γ production compared to that produced by unstimulated splenocytes. However this was not altered by drug treatment (Fig. 3a).

3.3.2. Day 14

There was a significant effect of in vitro KLH stimulation [$F(1,62) = 16.77$, $P = 0.001$], of MDMA treatment [$F(3,62) = 6.80$, $P < 0.001$] on splenocyte IFN- γ production 14 days following KLH/MDMA administration. Post hoc analysis demonstrated that stimulation of splenocytes with KLH (50 μ g/ml) significantly ($P < 0.01$) increased IFN- γ production compared to that produced by unstimulated splenocytes. MDMA treatment (2.5–10 mg/kg) produced a significant ($P < 0.01$) suppression of KLH-induced IFN- γ production (Fig. 3b).

3.4. KLH-specific IL-6 production

3.4.1. Day 7

There was a significant effect of in vitro KLH stimulation [$F(1,62) = 88.66$, $P < 0.001$] on splenocyte IL-6 production 7 days following KLH/MDMA administration. However there was no significant treatment effect or in vitro KLH stimulation $\times$ treatment interaction. Post hoc analysis demonstrated that stimulation of splenocytes with KLH (50 $\mu\text{g/ml}$) significantly ($P < 0.01$) increased IL-6 production compared to that produced by unstimulated splenocytes. However this was not altered by drug treatment (Fig. 4a).

3.4.2. Day 14

There was a significant effect of in vitro KLH stimulation [$F(1,62) = 85.66$, $P < 0.001$] on splenocyte IL-6 production 14 days following KLH/MDMA administration. However there was no significant treatment effect or of in vitro KLH stimulation $\times$ treatment interaction. Post hoc analysis demonstrated that stimu-

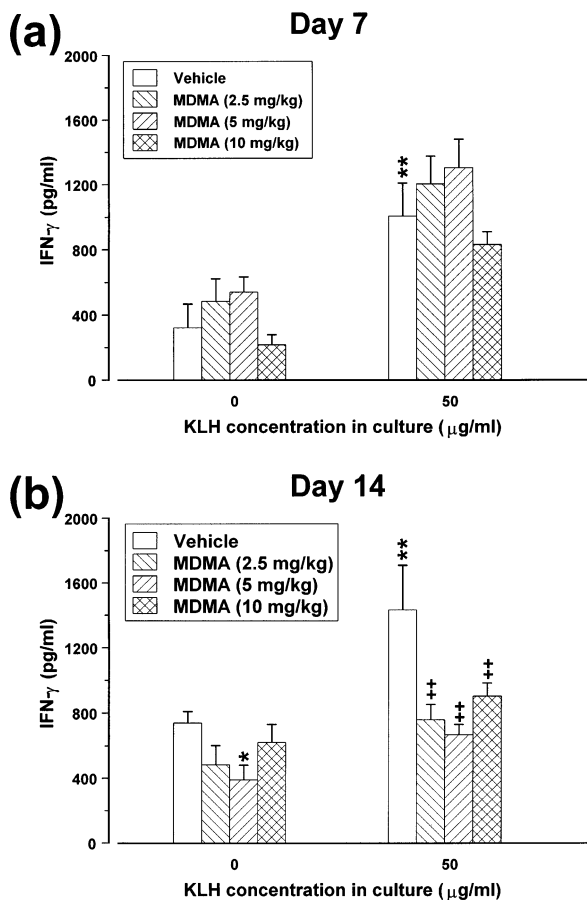


Fig. 3. Effect of acute MDMA administration on KLH specific IFN γ production in cultured splenocytes (a) 7 days and (b) 14 days post KLH challenge. Data expressed as means $\pm$ SEM ($n = 8-9$). ** $P < 0.01$ versus Unstimulated counterparts, + + $P < 0.01$ versus KLH stimulated vehicle (Fishers LSD).

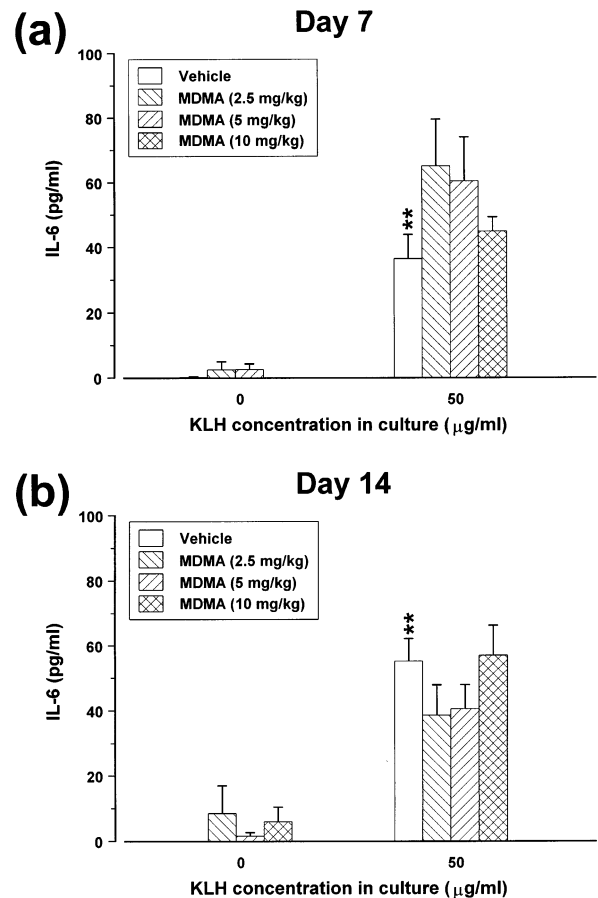


Fig. 4. Effect of acute MDMA administration on KLH specific IL-6 production in cultured splenocytes (a) 7 days and (b) 14 days post KLH challenge. Data expressed as means $\pm$ SEM ($n = 7-9$). ** $P < 0.01$ versus Unstimulated counterparts (Fishers LSD).

lation of splenocytes with KLH (50 $\mu\text{g/ml}$) significantly ($P < 0.01$) increased IL-6 production compared to that produced by unstimulated splenocytes. However this was not altered by drug treatment (Fig. 4b).

4. Discussion

In the present study acute treatment with MDMA did not alter the KLH-specific IgM response. In contrast, MDMA (5 and 10 mg/kg) significantly suppressed KLH-specific IgG production. Thus MDMA administration did not alter the initial generation of the antibody response, but rather inhibited antibody class switching from IgM to IgG. It is of interest that the effect of MDMA on the antigen-specific antibody response is confined to IgG, and that IgM production is not altered. This is similar to what has been previously reported following acute morphine administration in rats [9].

IgM antibodies have low affinity for antigen, therefore antibody class switching is required to produce

high affinity IgG antibodies. Antibody switching from IgM to other subclasses is very important in maintaining immunocompetence. In the present study, two pathways for the genetic switch from IgM to IgG production were investigated. One pathway requires the Th₁ type cytokine IFN- γ to stimulate IgM-secreting cells to switch to IgG_{2a}-secreting cells [see [17]]. Another pathway requires the Th₂ type cytokines IL-4 and IL-6 to stimulate IgM-secreting cells to switch to IgG₁-secreting cells [see [17,18]]. IgG₁ and IgG_{2a} levels were measured to determine if these two pathways were differentially affected. KLH-specific IgG_{2a} was much more abundant than IgG₁, as indicated by the difference in the dilution range required for their analysis by ELISA (IgG₁: 1:50–1:200; IgG_{2a}: 1:2500–1:10,000). This shows that KLH provokes a Th₁ polarized IgG response. With regard to the effect of MDMA on KLH-specific IgG isotypes, only IgG_{2a} levels were decreased following MDMA administration. Furthermore, in the present study this decrease in IgG_{2a} was accompanied by decreased KLH-specific IFN- γ production 14 days, but not 7 days, following KLH administration. However it is likely that reduced IFN- γ production at a timepoint earlier than 14 days was responsible for the suppression of the IgM to IgG_{2a} antibody switch observed. In this regard we have previously demonstrated that acute MDMA administration suppresses concanavalin A-induced IFN- γ production within 3 h of administration [6]. The fact that MDMA did not alter KLH-specific IgG₁ production may indicate MDMA has less of an effect on Th₂ type cytokines such as IL-4 and IL-6 than on the Th₁ type cytokines such as IFN- γ . In this regard, KLH-specific IL-6 production was not altered by MDMA administration either 7 or 14 days following the KLH challenge. However, it is noteworthy that in a previous preliminary study, we observed that a higher dose of MDMA (20 mg/kg) in addition to suppressing KLH-specific IgG_{2a}, also produced a modest, but significant suppression of KLH-specific IgG₁ [19]. This indicates that at very high doses of MDMA its effects are not confined to the suppression of IgG_{2a} as observed in the present study. Nonetheless, the doses of MDMA used in the present study yield plasma drug levels that are in the same range as those reported by human MDMA abusers [see [7]], and are therefore of most relevance to the human situation.

The mechanism(s) by which MDMA suppresses KLH-specific IgG_{2a} production remains to be elucidated. However, it is unlikely that the suppressive effect of MDMA on KLH-specific IgG_{2a} production is mediated by a direct effect of the drug on IFN- γ production, as in vitro exposure to MDMA does not alter Concanavalin A-stimulated IFN- γ production (Connor, unpublished data). However, it is possible that the well documented ability of MDMA to activate the hypothalamic

pituitary adrenal (HPA) axis and the sympathetic nervous system (SNS) [4,5,20] may play a role in its suppressive effect on antigen-specific IgG_{2a} production. In this regard, the dose-dependent suppression of KLH specific IgG_{2a} production observed in this study mirrors the dose-dependent increase in circulating corticosterone concentrations that we have previously described following acute MDMA administration [5]. In addition, it has been previously demonstrated that the glucocorticoid receptor antagonist RU 486 blocks the suppressive effect of stress on KLH-specific IgG_{2a} [21]. In addition, the catecholamines noradrenaline and adrenaline suppress IFN- γ production from PHA-stimulated splenocytes [22], thus presenting themselves as potential mediators of the MDMA-induced suppression of IgG_{2a}. However, further studies are required in order to elucidate the exact mechanism(s) that mediate the suppression of KLH-specific IgG_{2a} observed as a result of acute MDMA administration.

IgG_{2a} is the dominant antibody responsible for complement-mediated lysis reactions and is therefore an important player in anti-viral immunity. In fact reports indicate that viral infections produce a ten to 100-fold excess in IgG_{2a} over IgG₁ [23,24]. IgG_{2a} activates both the classical and alternative complement pathways which are responsible for the control of inflammation aiding antigen clearance [25]. The complement cascade is a vital component of the immune system as it promotes opsonization, chemotaxis, lysis and increased blood flow to the infected area. The fact that IgG_{2a} is a critical antibody subtype in the antiviral response and an inducer of the complement cascade, may suggest that exposure to MDMA in the period close to viral infection could have an adverse effect on antiviral immunity.

In conclusion, these data indicate that MDMA administration at doses that yield circulating drug concentrations in the same range as those reported in human MDMA abusers (see [7]), suppresses the ability to switch from IgM to IgG_{2a} production, possibly by reducing IFN- γ production. Considering the important role of IgG_{2a} in antiviral immunity and host resistance in general, it is possible that MDMA abuse could reduce host resistance to disease. In this regard it was previously demonstrated that administration of d-amphetamine the parent compound of MDMA reduced host resistance to infection by influenza A virus and the bacteria *Listeria monocytogenes* [26,27]. In addition, there have been cases where MDMA abuse in humans closely preceded the development of meningococcal meningitis [28].

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