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Ecstasy (3,4-Methylenedioxymethamphetamine) limits murine gammaherpesvirus-68 induced monokine expression

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Abstract

While Ecstasy (3, 4-methylenedioxymethamphetamine, MDMA) has been shown to modulate immune responses, no studies have addressed drug-induced alterations to viral infection. In this study, bone marrow-derived macrophages were exposed to MDMA, then infected with murine gammaherpesvirus-68, and the expression of monokines assessed. MDMA-induced reductions in virus-stimulated monokine mRNA expression were observed in a dose-dependent manner. In particular, IL-6 mRNA expression and secretion was significantly decreased in gammaherpesvirus-infected macrophages exposed to MDMA. Concentrations of MDMA capable of reducing monokine production did not induce significant cell death and allowed normal viral gene expression. These studies represent the first to demonstrate the ability of this drug of abuse to alter a viral-induced macrophage response.

Keywords

macrophage; gammaherpesvirus; Ecstasy; 3,4-methylenedioxymethamphetamine

1. Introduction

Ecstasy, (+/-)-3,4-methylenedioxymethamphetamine (MDMA), is a ring-substituted amphetamine derivative that is also structurally related to the hallucinogenic compound mescaline (Green et al., 2003). As a potent releaser of the neurotransmitters, serotonin and noradrenaline, and to a lesser extent, dopamine, MDMA creates a relaxed, euphoric state, including emotional openness, increased empathy, and a decrease in inhibitions (Green et al., 1995; Green et al., 2003; Kalant, 2001). The drug produces an array of physiological responses including hyperthermia, acute sympathomimetic effects (e.g. increased heart rate and blood pressure), transient increases in anxiety, and increased activation of the hypothalamic-pituitary-adrenal axis. Ecstasy is also associated with a number of serious side effects including cardiac arrhythmias, renal failure, seizures, and intracranial hemorrhage.

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Ingestion of the drug may result in long-term neurotoxic effects on central serotonergic neurons, a potential predisposing factor to psychological disturbances or psychiatric disease.

Although Ecstasy is one of the more popular club drugs used by teenagers and young adults (Landry, 2002), very little consideration has been given to the consequences of MDMA abuse on the immune response against infectious diseases (Connor, 2004; Pacifici et al., 2000). In general, drug abusers have long been known to be more susceptible to infectious diseases (Friedman et al., 2003), however, the exact mechanisms for increased susceptibility from drug abuse are not always clear. *In vivo*, MDMA decreases neutrophil phagocytosis (Connor, 2004), suppresses the generation of LPS-induced pro-inflammatory cytokines TNF- α and IL-1 β (Connor et al., 2001b; Connor et al., 2000b) and increases the LPS-induced production of the anti-inflammatory cytokine IL-10 (Connor et al., 2005). The increased production of IL-10 suppresses IFN- γ secretion and signaling (Boyle and Connor, 2007). The number of circulating natural killer cells increases (Pacifici et al., 2000; Pacifici et al., 2001a; Pacifici et al., 1999; Pacifici et al., 2002; Pacifici et al., 2001b), and as with LPS treatment, MDMA boosts the production of IL-10 and TGF- β 1 in PHA stimulated animals (Pacifici et al., 2001b). MDMA decreases the number of CD4⁺ T-helper cells (Pacifici et al., 2004) and reduces PHA stimulated T-cell proliferation (Pacifici et al., 1999). In rats, the drug alters the ability to switch from IgM to IgG_{2a}, possibly by reducing the secretion of INF- γ (Connor et al., 2001a).

In vitro, LPS-stimulated murine peritoneal macrophages exposed to MDMA at up to 100 μ M exhibited a generalized trend toward lower TNF- α production with increased MDMA concentration, although results were not statistically significant (House et al., 1995). In CD4⁺ T cells, exposure to MDMA resulted in a biphasic effect on IL-2 secretion, with pronounced stimulation of IL-2 production at very low MDMA concentrations and suppression at 100 μ M MDMA ((House et al., 1995). In a recent study, however, exposure of diluted whole blood to MDMA failed to alter LPS-induced IL-10 production (Connor, 2004).

Despite these previous investigations, no studies have addressed the effects that exposure to MDMA might have on the immunity to viral infections. In the present study, we have assessed whether the mouse macrophage response to murine gammaherpesvirus-68 (γ HV-68) is altered by the presence of Ecstasy. The virus is a naturally occurring, nonfatal mouse pathogen that provides an excellent model for the investigation of immune responses to gammaherpesvirus infections in humans (Gasper-Smith and Bost, 2004; Rajcani and Kudelova, 2005). γ HV-68 shares sequence homology and pathological similarities with Epstein Barr Virus (EBV) and human herpes virus-8 (HHV-8) (Virgin et al., 1997). Although B cells are considered the major reservoir for latent virus, latency also occurs in splenic macrophages after intranasal infection (Flano et al., 2000), and in peritoneal macrophages following intraperitoneal infection (Weck et al., 1999). To begin to address the effects of Ecstasy on viral-induced immune responses, bone marrow-derived macrophages were infected with γ HV-68 and exposed to MDMA. Significant reductions in virus-induced expression of monokines were observed in cultures treated with this drug of abuse.

2. Materials and methods

2.1. Chemicals

Tyramine, β -phenylethylamine and octopamine were purchased from Sigma-Aldrich (St. Louis, MO). (+/-)-3, 4-Methylenedioxymethamphetamine HCl (MDMA; Ecstasy) was provided by the National Institute of Drug Abuse (NIDA). 100 mM stock solutions of these compounds were prepared in sterile RPMI tissue culture medium and aliquots stored at -80°C.

2.2. Animals

Six to eight week old female C57BL/6 mice (18-22 g) were purchased from Jackson Laboratories (Bar Harbor, ME) and housed in the vivarium in filter top cages containing sterile bedding. After arrival, mice were quarantined for at least five days, and fed chow and water *ad libitum*.

2.3. Cells

2.3.1. Isolation of bone marrow cells—Bone marrow-derived macrophages were matured in LADMAC-supplemented medium (Sklar et al., 1985) as previously described (Bowman and Bost, 2004; Elswa and Bost, 2004). Typically, a mouse was anesthetized with isoflurane and sacrificed by cervical dislocation. The hindquarters were sterilized with 95% ethanol, the femurs removed, and the exposed bone marrow flushed with a 3 ml syringe and 22 gauge needle into pre-warmed LADMAC (M-CSF containing) supplemented medium. The LADMAC supplemented medium contained RPMI 1640 tissue culture medium supplemented with 10% heat-inactivated and filtered fetal bovine serum (FBS), 25 mM HEPES, 4.5 g/L glucose, 1 mM Na pyruvate, 2 g/l NaHCO₃, 50 μ M 2-mercaptoethanol, 25 μ g/ml gentamicin, supplemented with 20% LADMAC cell (ATCC CRL-2420) conditioned medium. The flushed matrix material was pipetted up and down to release bone marrow cells, and the insoluble material allowed to settle to the bottom of the tube. Typically, 10 mls of medium containing bone marrow cells were then added to each of eight to twelve T-25 T-flasks, and the flasks incubated at 37°C in a CO₂ tissue culture incubator.

2.3.2 Generation of bone marrow-derived macrophages—Bone marrow cells were incubated for 2 days in LADMAC supplemented-medium, and then fed with an equal volume of pre-warmed medium. On day 4, the medium was replaced with 10 ml fresh pre-warmed LADMAC supplemented-medium. Virtually all cells were CD11b⁺ (Nelson et al., 2004) and ready for experiments after culturing for 5-6 days.

2.3.3. Pre-treatment with MDMA and activation with γ HV-68—Typically, macrophages were pre-treated with 500 μ M Ecstasy for 24 hours, followed by activation with γ HV-68 using a 1:1, virion to macrophage ratio. Macrophages attached to the T-flask were harvested in Trizol for the preparation of total RNA and stored at -80°C.

2.3.4. Cell viability and apoptosis assays—Macrophages were grown in 96-well plates and pretreated with varying concentrations of MDMA for 24 hours. This was followed by a further 24 hours in the presence of various ratios of γ HV-68 to macrophages.

Cell viability and apoptosis were then assayed using the Promega CellTiter-Glo Luminescent Cell Viability Assay and Caspase-Glo 3/7 Assay, respectively (Promega, Madison, WI).

2.3.5. Light microscopy—Macrophages were grown on tissue culture-treated cover slips in 24 well plates, and pretreated with MDMA for 24 hr. γ HV-68 was then added to appropriate wells for a further 24 hr to assess potential alterations in morphology. Cells were washed once with PBS, fixed for 10 min with 100% methanol at 4° C and washed 2X with PBS. Macrophages were stained with Wright's Accustain for 30 seconds and the wells washed 3X with deionized water. Cells were then washed 1X with 50% ethanol for 30-60 seconds followed by 100% ethanol for another 30-60 seconds. Coverslips were air dried and mounted on slides with Permount. Macrophages were photographed at 640X magnification using an Olympus IX71 light microscope with an attached Olympus DP40 digital camera.

2.4. Nucleic acid isolation and analysis

2.4.1. Total RNA isolation and synthesis of cDNA—RNA was isolated using the Trizol method (Invitrogen; Carlsbad, CA), as described by the manufacturer. For γ HV-68 expression (ORF50 and ORF65), RNA samples were incubated with RNase-free pancreatic DNase (RQ1 DNase, Promega, Madison, WI) as per the manufacturer's instructions, the RNA precipitated with EtOH and resuspended in 50 μ l nuclease-free H₂O. RNA concentrations were determined with a Gene Spec III spectrophotometer (Naka Instruments, Japan) using a 10 μ l cuvette. For cDNA synthesis, one μ g of RNA was reverse-transcribed in the presence of random hexamers (50 ng/ μ l), 10 mM dNTPs, 2.5 mM MgCl₂ using ImProm-II reverse transcriptase (Promega) in the buffer supplied by the manufacturer. cDNA was precipitated with one-tenth volume of 3 μ M sodium acetate (pH 5.2) and 3 volumes of EtOH, and resuspended in 50 μ l of nuclease-free H₂O.

2.4.2. Semiquantitative PCR and DNA product analysis—mRNA transcript levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), cytokines and chemokines, and viral open reading frames (ORFs) were examined by PCR using the synthesized cDNA. Typically, 100 ng (10%) of the cDNA was combined with 2.5 U of Taq polymerase (Promega), 0.2 mM each dNTP, 25 pmol of each primer and PCR buffer containing 2.5 mM MgCl₂ as provided by the manufacturer. Samples were cycled using 95° denaturation for 35 seconds, 60°C annealing for 75 seconds and 72°C extension for 90 seconds, with the first three cycles using extended denaturation, annealing and extension times. PCR was for 30 cycles except for the housekeeping gene, GAPDH (25 cycles). The extension time of the last cycle was for 5 min at 72°C. Forty percent of each amplified PCR product was electrophoresed on an ethidium bromide-stained 2% agarose gel and photographed under UV illumination.

PCR primer sets were designed by either using Oligo 6 primer analysis software (Molecular Biology Insights, Cascade, CO) or SciTools from IDT (Integrated DNA Technologies, Coralville, IA). Primer sets used for amplification are as follows:

GAPDH (accession no. NM_001001303; 346 bp - spans exons 5 to 7):

Forward: 5' - CCA TCA CCA TCT TCC AGG AGC GAG - 3'

Reverse: 5' - ATC ACT GCC ACC CAG AAG ACT GTG - 3'

TNF- α (accession no. NM_013693; 325 bp – spans exons 2 to 4):

Forward: 5' – GGC CTC CCT CTC ATC AGT TCT ATG – 3'

Reverse: 5' – GCT CTT GAC GGC AGA GAG GAG G – 3'

IL-6 (accession no. NM_031168; 268 bp – spans exons 3 to 5):

Forward: 5' - GAT GCT ACC AAA CTG GAT ATA ATC - 3'

Reverse: 5' - GGT CCT TAG CCA CTC CTT CTG TG - 3'

RANTES (Ccl5; accession no. NM_013653; 207 bp – spans exons 2 to 3):

Forward: 5' - TCG TGC CCA CGT CAA GGA GTA TTT – 3'

Reverse: 5' - ACT AGA GCA AGC GAT GAC AGG GAA – 3'

IL-10 (accession no. NM_010548; 278 bp - spans exons 2 to 5):

Forward: 5' – GGA CAA CAT ACT GCT AAC CGA CT – 3'

Reverse: 5' – ACA CCT TGG TCT TGG AGC TTA TTA – 3'

IL-12p40 (accession no. NM_008352; 330 bp – spans exons 4 to 6):

Forward: 5' – GCA CCA AAT TAC TCC GGA CGG TTC – 3'

Reverse: 5' – GCA AGT TCT TGG GCG GGT CTG – 3'

γ HV-68 ORF50 (murid herpesvirus 4; accession no. NC 001826; 355 bp):

Forward: 5' – ATG GCA CAT TTG CTG CAG AAC – 3'

Reverse: 5' – ACG GCG CCT GTG TAC TCA A – 3'

γ HV-68 ORF65 (murid herpesvirus 4; accession no. NC 001826; 221 bp):

Forward: 5' – ATG CTC CAG AAG AGG AAG GGA CAC – 3'

Reverse: 5' – TTG GCA AAG ACC CAG AAG AAG CC – 3'

2.4.3. Real Time Polymerase Chain Reaction (RT-PCR) to quantify mRNA

expression—Cytokine and chemokine mRNA expression was quantified relative to GAPDH expression by real time PCR using a Roche LightCycler for amplification and SYBR Green I for double-stranded DNA detection. Amplifications were performed in a total volume of 20 μ l containing QuantiTect SYBR Green PCR Master Mix (Qiagen), primer pairs (0.5 μ M), template cDNA (40 ng) and nuclease-free water. Samples were cycled 45 times, starting with an initial activation step of 15 min at 95°C, followed by denaturation for 15 sec at 95°C, annealing for 30 sec at 60°C and extension for 30 sec at 72°C. Data were typically acquired at 80°C for 2 sec after extension.

Primer sets used for real time amplification are as follows:

GAPDH (133 bp – spans exons 5 to 6):

Forward: 5' - CAA TGT GTC CGT CGT GGA TCT GAC – 3'

Reverse: 5' - AGA CAA CCT GGT CCT CAG TGT AGC – 3'

TNF- α (101 bp – spans exons 1 to 2):

Forward: 5' - GCC TCT TCT CAT TCC TGC TTG TGG – 3'

Reverse: 5' - GGC CAT TTG GGA ACT TCT CAT CCC – 3'

IL-6 (126 bp – spans exons 1 to 2):

Forward: 5' - TCC TCT CTG CAA GAG ACT TCC ATC C – 3'

Reverse: 5' - ACA GGT CTG TTG GGA GTG GTA TCC – 3'

IL-12p40 (127 bp – spans exons 3 to 4):

Forward: 5' - AGA CTC TGA GCC ACT CAC ATC TGC – 3'

Reverse: 5' - GAA CCG TCC GGA GTA ATT TGG TGC – 3'

Other primer sets for real time PCR were the same as used for semiquantitative PCR.

2.5. Enzyme Linked Immunosorbent Assay (ELISA) to quantify protein secretion

After incubation of macrophages in T-flasks, medium was removed and enzyme-linked immunosorbent assays (ELISAs) performed to quantify mouse IL-6 secretion as described previously (Gasper et al., 2002). Capture and biotinylated detection antibodies were purchased from BD Pharmingen (San Diego, CA). The presence of bound antibody was detected with streptavidin-horseradish peroxidase and tetramethylbenzidine (BioRx Laboratories, Owings Mills, Md.). Colorimetric reactions were subsequently stopped by the addition of H₂SO₄ (diluted 1:3 with deionized H₂O) and the *Abs*_{450 nm} measured (model 550 microplate reader; Bio-Rad, Hercules, Calif.). Standard curves were generated by determining absorbance values using limiting dilutions of recombinant murine IL-6 (BD Pharmingen). Results are presented as means $\pm$ standard error of the mean, based on triplicate determinations. ELISA sensitivity was 10 pg/ml.

2.6 Statistics

Where applicable, data are presented as mean values $\pm$ the standard error of the mean (SEM). The number of times an experiment was repeated (N values) are provided in the captions to the figures. Statistical analysis consisted of ANOVA followed by post hoc comparisons. Tukey's multiple comparison was used following one-way ANOVA and Bonferroni posttests following two-way ANOVA. F values are presented where applicable. Relevant statistically significant differences are marked by asterisks in the figures and p values are provided in the text and figure legends.

3. Results

3.1. MDMA-induced reduction of IL-6 mRNA expression by virus-infected macrophages

To ascertain whether Ecstasy might alter virus-induced monokine expression, we examined IL-6 mRNA levels of cultured macrophages infected with γ HV-68. We have previously successfully used IL-6 mRNA expression and protein production after acute γ HV-68 infection of antigen-presenting cells to monitor cellular activation (Gasper-Smith and Bost, 2004; Gasper-Smith et al., 2006). As shown in Figure 1A, the induction of IL-6 mRNA following viral infection was greatly reduced in the presence of 500 μ M MDMA. To assure that the decreases observed were not due to significant differences in input RNA or efficiencies of reverse transcription between samples, amplification of the housekeeping gene, GAPDH, was performed on the same cDNA samples for comparison.

A dose response to MDMA treatment showed that concentrations of the drug as low as 100 μ M could reduce IL-6 mRNA expression (Fig. 1B). Doses of MDMA at or above 3000 μ M also affected GAPDH expression, suggesting that cellular toxicity or death might be occurring at these high drug concentrations (Fig. 1B).

3.2. MDMA-induced morphological changes and cell death following γ HV-68 infection

To demonstrate that the reduction in IL-6 mRNA expression was not the result of MDMA-induced cell death, assays for viability and caspase activity were performed. Luminosity levels are directly related to cell ATP levels, which in turn are a measure of cell viability. Mouse macrophages continue to maintain ATP levels, and remain viable at MDMA concentrations up to 1.25 mM at all virus concentrations (Fig. 2A). At 2.5 mM MDMA, viability is statistically significantly reduced (as indicated by the asterisk for the 1:1 macrophage to viral ratio) at all viral levels shown. At 5 mM MDMA and above, cells are no longer viable, and visually have detached from the tissue culture wells, an indication of cell death. Note that 1 nM ATP (right side of the figure) yields a luminescence signal above that of the wells containing macrophages, indicating that luminosity from the cell-containing wells is well within the linear range.

An assay for macrophage apoptosis (caspase 3 and 7 activity) at various viral levels and MDMA concentrations demonstrated that at virus to macrophage levels of 1:1 or less, MDMA promoted apoptosis at 1.25, 2.5 and 5 mM concentrations (Fig. 2B). For example, at a 1:1 virus to macrophage ratio, asterisks indicate the statistically significant increase in luminosity at the 1.25 to 5 mM MDMA concentrations. Notably, at concentrations of MDMA less than 1.25 mM, in the presence or absence of virus, increased caspase 3 and 7 activity were not observed.

Phenotypic changes in macrophage morphology were noted using concentrations of MDMA that did not induce cell death. As shown in Figure 3, when mouse macrophages were exposed to 500 μ M MDMA with subsequent viral infection, changes in cell morphology could be observed. Typically, cells treated with drug and infected with virus remained attached, but had a reduction in pseudopodia length, becoming less elongated. This length reduction with the combination treatment of Ecstasy and virus was more pronounced (Fig. 3D) than that observed with virus (Fig 3B) or Ecstasy (Fig 3C) alone.

3.3. MDMA does not function via trace amine receptors to reduce monokine expression

A number of monoamines were tested for their effects on IL-6 induction with gammaherpesvirus. As illustrated in Fig. 4A, we first showed that trace amines such as tyramine (500 μ M), as well as β -phenylethylamine and octopamine (data not shown), did not by themselves induce IL-6 transcription. When these trace amines were compared to MDMA for suppression of induction of IL-6 transcription in macrophages after 6 hr in the presence of γ HV-68, only MDMA showed the suppressive effect. 500 μ M tyramine, β -phenylethylamine and octopamine did not significantly reduce γ HV-68 induction of IL-6 transcription (Fig. 4B), as did 500 μ M MDMA. These results support previous work demonstrating that trace amine receptors are absent on mouse macrophages (Nelson et al., 2007) and that these monoamines would be predicted to have little effect on cellular activation.

3.4. MDMA-induced reduction of monokine mRNA expression by virus-infected macrophages

To address whether MDMA might modify the expression of additional monokine mRNA expression following gammaherpesvirus infection, real time PCR analyses were performed. Macrophages were cultured in the presence or absence of 500 μ M MDMA and the levels of mRNAs encoding GAPDH, TNF- α , IL-6, RANTES, IL-10 and IL-12p40 were determined. As shown in Figure 5, expression of all monokines were significantly reduced. Approximately 50-fold reductions in mRNA expression for IL-6, TNF- α , IL-10 and RANTES were observed, with an approximate 5 fold decrease in IL-12p40 expression. The amount of GAPDH mRNA in each sample relative to the zero time control is shown in the figure to reinforce the fact that cells remain viable and nonapoptotic, and that the MDMA at this concentration did not alter GAPDH mRNA levels.

3.5. MDMA-induced reduction of IL-6 secretion by virus-infected macrophages

To demonstrate that a reduction in IL-6 mRNA expression by MDMA also translated into decreased monokine secretion, ELISA analyses were performed on culture supernatants. Figure 6A demonstrates a significant reduction in IL-6 secretion in cultures treated with MDMA and infected with virus. This suppression was not observed when the trace amine receptor agonist, tyramine, was used (Fig. 6B). Results in Figure 6B further demonstrate that the suppressive effects that MDMA has on IL-6 expression by virus infected macrophages is not via trace amine receptors.

3.6. MDMA effects on γ HV-68 viral gene expression

Early γ HV-68 genes are expressed during the acute phase of viral attack, and therefore their expression may be used as a proxy for the effects of MDMA on productive infection during the lytic cycle. If viral gene expression is unaffected by the presence of 500 μ M MDMA, this would suggest both that macrophages remain healthy and susceptible to infection, and would reinforce the idea that transcription *per se*, as found for the GAPDH gene, is unaffected by the presence of this concentration of Ecstasy. Two viral genes, ORF50 coding for the lytic cycle transcription activator, RTA, and ORF65 coding for the M9 capsid protein, were used to monitor the early, lytic phase of infection (Martinez-Guzman et al.,

2003; Stevenson and Efstathiou, 2005). ORF50 has been used previously by other researchers to monitor viral activity *in vitro* in dendritic cells (Flano et al., 2005) and macrophages (Weck et al., 1999).

As shown in Fig. 7, 500 μ M MDMA appeared to have little effect on the time course of expression of the viral genes, ORF50 and ORF65. As expected, viral mRNA was not detected prior to the addition of virus (0 hr lanes for ORF50 and ORF65), but increased in amount within hours after the addition of γ HV-68 to the macrophage cultures. Since these PCR assays are easily confounded by the presence of viral genomic DNA, this genomic DNA was removed by DNase digestion prior to cDNA synthesis. Mock cDNA synthesis (in the absence of reverse transcriptase), followed by PCR for ORF50 and ORF65 confirmed that the RNA used for this analysis was free of contaminating viral genomic DNA (data not shown). As controls, the same cDNA was used to assess macrophage IL-6 and GAPDH mRNA levels, with the expected results: IL-6 induction was reduced by the presence of 500 μ M MDMA, whereas the amount of GAPDH mRNA detected was unaffected.

The cDNA samples used for Fig. 7 were subjected to further, quantitative analysis by real time PCR. We were unable to detect a statistically significant difference in the time course of ORF50 expression (Fig. 8, top panel), however, expression of ORF65 suggested a slight time lag in expression. Adjusting for this time lag (Fig. 8, bottom panel insert) showed that the same levels of ORF65 expression are reached in the presence or absence of 500 μ M MDMA. Taken together, the effects of 500 μ M Ecstasy on viral gene expression appear to be relatively modest compared to the attenuated host cytokine/chemokine mRNA induction.

4. Discussion

To begin to investigate the possibility that exposure to Ecstasy might alter the host's response against viral infections, mouse bone marrow-derived macrophages were incubated with this drug and then monitored during activation by a viral pathogen. Macrophages infected with γ HV-68 during exposure to 500 μ M MDMA remained viable and nonapoptotic, although distinct reductions in cell length and changes in morphology were apparent. The induction of cytokine and chemokine mRNA, and the production of IL-6 protein, were greatly reduced in the presence of Ecstasy, an effect relatively specific when compared to actions of other monoamines. The presence of Ecstasy did not alter the acute expression of the viral γ HV-68 ORF50, and only modestly delayed the production of ORF65 mRNA. Taken together, these results suggest direct immunomodulatory effects of MDMA on macrophages that may compromise the host's anti-viral response.

Concentrations of MDMA below 1.25 mM did not reduce macrophage viability or induce apoptosis in the presence of virus. At or above this level, MDMA did induce macrophage death, consistent with literature reports that other cell types exposed to high micromolar, or low millimolar concentrations of MDMA, resulted in cell death *in vitro*. The drug induces apoptosis in primary, cortical neuronal cultures (Capela et al., 2007; Capela et al., 2006; Stumm et al., 1999), cerebellar granule cells (Jimenez et al., 2004), the neuronal cell line, PC12 (Milhazes et al., 2006), choriocarcinoma placental (JAR) cells (Simantov and Tauber, 1997), rat primary hepatocytes (Montiel-Duarte et al., 2004; Montiel-Duarte et al., 2002)

and hepatic stellate cells (Montiel-Duarte et al., 2004). The cytotoxicity of MDMA and its metabolites is reported to be by an oxidative mechanism (Milhazes et al., 2006).

However our results clearly show that at concentrations which do not promote cell death (e.g. 500 μ M), MDMA can reduce monokine expression following viral infection. These findings suggest a receptor-mediated mechanism for such *in vitro* effects, though it is clear that trace amine receptors are not responsible (Nelson et al., 2007). Which receptor mediates such effects is at present unclear, although numerous proteins including monoamine transporters (Battaglia et al., 1988; Han and Gu, 2006; Pacifici et al., 2004; Partilla et al., 2006; Rudnick and Wall, 1992; Verrico et al., 2007), monoamine receptors (Battaglia et al., 1988; Kramer et al., 1997; Nash et al., 1994) and cholinergic or histamine receptors (Battaglia et al., 1988) have been implicated. We have initiated a survey of the expression of monoamine transporters and receptors in mouse macrophages, and find, intriguingly, expression of the mRNA for serotonin receptor Htr2a (unpublished results). Binding of MDMA to this receptor has recently been coupled to apoptosis in cortical neurons (Capela et al., 2007; Capela et al., 2006), and may account for both macrophage immunomodulation and, at higher concentrations, cell death. Further studies will need to be performed to ascertain if this macrophage receptor mediates these effects following drug exposure.

Ecstasy has been characterized as a “stressor” of the immune system (Connor, 2004; Pacifici et al., 2000), modulating, or suppressing immune function. In LPS-stimulated rats, serum levels of IL-1 β and TNF- α were reduced (Connor et al., 2005; Connor et al., 2000b) and IL-10 levels increased (Connor et al., 2005) with the addition of MDMA. Blood lymphocyte levels were in general decreased (Connor et al., 2000a), and serum IgG2a antibodies reduced in the presence of Ecstasy (Connor et al., 2001a). In humans, there was an early decrease in blood CD4⁺ T cell numbers (Pacifici et al., 2004; Pacifici et al., 2000; Pacifici et al., 2001a; Pacifici et al., 2001b), and increases (Pacifici et al., 2004; Pacifici et al., 2000; Pacifici et al., 2001a; Pacifici et al., 2001b), or decreases (Pacifici et al., 2002) in blood NK cell numbers dependent on acute or chronic MDMA administration. *In vitro*, the trend is toward reduced cytokine production in the presence of MDMA, although there was no affect on IL-10 production in whole blood (Connor, 2004), or a statistically significant decrease in TNF- α secretion from mouse peritoneal macrophages (House et al., 1995). Results presented here add to this body of work by demonstrating the effects of MDMA during infection with a gammaherpesvirus.

Many viral infections are spread by close contact and/or the exchange of oral or nasal secretions. Ecstasy abuse is coupled to its physiological effects which include euphoria and a loss of inhibitions. These effects have made Ecstasy a popular drug of abuse for young adults. Unfortunately, the social behaviors which accompany its abuse also promote close contact and an increased potential for the spread of infectious diseases. Results presented in this work also suggest that macrophage function can be altered following MDMA exposure when challenged with a viral infection. Ongoing studies are aimed at confirming such a possibility using animal models of gammaherpesvirus infection. If true, abuse of Ecstasy may facilitate behaviors which increase the transmission of infectious diseases, while simultaneously limiting the protective host response against these pathogens.

In summary, MDMA severely reduced the monokine response to acute infection with γ HV-68. Since macrophages play a role in the initiation of the immune response, the host's innate response against viral infection may suffer, suggesting the potential for increased susceptibility of drug abusers to infectious diseases, such as herpesviruses.

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Figure 1. MDMA time course and dose-response assessed by semiquantitative PCR of IL-6 mRNA induction in mouse macrophages after γ HV-68 infection

Panel A - Macrophages were untreated or pretreated with 500 μ M MDMA for 24 hrs followed by the addition of γ HV-68 for the times indicated in the figure. cDNA was then prepared from total RNA and PCR performed. Expression of the housekeeping gene, GAPDH, is shown as a positive control to indicate that similar amounts of cDNA are present in each sample. While GAPDH expression is unaffected, IL-6 mRNA induction is greatly reduced by the presence of MDMA. The results are presented as amplified products electrophoresed on ethidium bromide stained agarose gels. DNA sizes in base pairs are shown to the left of the DNA standard. **Panel B** - Mouse macrophages were incubated for 24 hr with various concentrations of MDMA and the incubation in Ecstasy continued for another 6 hr after the addition of γ HV-68 (1:1). Total RNA was isolated, cDNA prepared and PCR results shown for GAPDH and IL-6 expression.

Figure 2. Cell viability and caspase 3/7 activity in MDMA treated mouse macrophages
Mature macrophages were incubated for 24 hr with MDMA, and then γ HV-68 added for an additional 24 hr in the amounts shown in the figure. **(Panel A)** Macrophages were grown in 96-well plates and viability assayed by quantification of the amount of ATP present in each well, as described in the CellTiter-Glo Luminescent Cell Viability Assay. The number of viable macrophages in culture is directly proportional to the amount of ATP. Luminescence from a well with 1 nM ATP is included to demonstrate that light release from sample wells had not reached a plateau. The horizontal bar shows the mean luminescence from cells without MDMA. The Viability assay was performed four separate times (N = 4) and values are presented as means $\pm$ SEM. Two-way ANOVA using MDMA concentration and γ HV-68 titers as sources of variation indicate that MDMA has a major effect on cell viability (F = 124.2). For the virus to macrophage ratio of 1:1, wells with luminescence statistically significantly below the horizontal bar ($p < 0.001$) are marked by an (*), indicating reduced macrophage viability. **(Panel B)** Caspase 3 and caspase 7 activity, a proxy for apoptosis, in macrophages assayed by the Caspase-Glo 3/7 Assay. The amount of caspase activity, and therefore the amount of apoptosis, is proportional to the luminescence signal from each well. The horizontal bar shows the mean luminescence from cells without MDMA. The Caspase 3/7 assay was performed four separate times (N = 4) and values are presented as means $\pm$ SEM. Two-way ANOVA using MDMA concentration and γ HV-68

titers as sources of variation indicate that MDMA has a major effect on cell apoptosis ($F = 31.6$). For the virus to macrophage ratio of 1:1, wells with luminescence statistically significantly above the horizontal bar ($p < 0.05$) are marked by an (*), suggesting increased macrophage apoptosis.

Figure 3. Mouse macrophage morphology in the presence of MDMA and γ HV-68
Macrophages were stained, fixed, mounted and photographed at 640X magnification, as described in the Materials and Methods. **Panel A** – Control macrophages. **Panel B** – Macrophages infected 1:1 with γ HV-68 (24 hr). **Panel C** – Macrophages incubated with 500 μ M MDMA (48 hr). **Panel D** – Macrophages incubated with 500 μ M MDMA (48 hr) and γ HV-68 (1:1; 24 hr). Cells incubated with MDMA and activated with γ HV-68 appeared reduced in length relative to macrophages with MDMA, or γ HV-68 alone.

Figure 4. Semiquantitative PCR analysis of IL-6 mRNA induction after γ HV-68 infection of mouse macrophages in the presence of 500 μ M MDMA or other monoamines

Panel A – A comparison of IL-6 expression in the presence of glycine and the monoamines tyramine and MDMA. Macrophages were pretreated with the various compounds for 24 hr and the cells further incubated for 6 hr in the presence and absence of γ HV-68. Relative to glycine or tyramine, IL-6 mRNA induction by γ HV-68 is reduced in the presence of 500 μ M MDMA. **Panel B** – Results similar to panel A, showing the specificity of the suppression of IL-6 induction by γ HV-68 in the presence of 500 μ M MDMA relative to other trace amines.

Figure 5. Relative quantification of mouse macrophage monokine mRNA induction by γ HV-68 in the presence or absence of 500 μ M MDMA using real time PCR

Expression of monokine mRNA was monitored by real time PCR during a time course of macrophage activation with γ HV-68 (1:1; cell: virus) in the presence or absence of MDMA. 500 μ M MDMA was added to appropriate cells 24 hr prior to the addition of virus. Data for each graph are from three separate experiments ($N = 3$), with the cycle threshold (C_T) value for each sample from each experiment determined in triplicate. The fold-change (fold induction) is relative to the 0 time control, and was determined for each sample using the equation, fold-change = $2^{C_T(\text{control}) - C_T(\text{sample})}$. Mean values of the fold-change $\pm$ SEM are then presented in each graph.

Figure 6. Effects of 500 μM MDMA and other monoamines on IL-6 secretion after γHV-68 infection of mouse macrophages

Panel A – Macrophages were incubated for 24 hr in the presence or absence of 500 μM

MDMA and then virus added for a further 12 or 24 hr. **Panel B** – Macrophages were incubated for 24 hr in the presence, or absence of 500 μM monoamines and the incubation continued with, or without virus, for a further 24 hr.

For both panels, the amount of IL-6 in the medium was measured by ELISA and the results presented as mean IL-6 concentrations $\pm$ SEM. Each experiment was performed 3 separate

times ($N = 3$), with each value obtained in duplicate. One-way ANOVA, followed by Tukey's Multiple Comparison Test indicate the two 12 hr time points +/-MDMA, and 24 hr time points +/- MDMA were statistically significantly different at the $p < 0.001$ level (panel A). Similarly, after viral activation, only MDMA significantly reduced IL-6 secretion relative to control macrophages at the $p < 0.001$ level (panel B).

Figure 7. Semiquantitative PCR analysis of γ HV-68 mRNA expression in mouse macrophages in the presence and absence of 500 μ M MDMA

Macrophages were untreated, or pretreated with MDMA for 24 hrs followed by the addition of γ HV-68 for the times indicated in the figure. PCR was then performed for two viral genes, ORF50 and ORF65, expressed early in the infection. Total RNA was treated with DNase prior to cDNA synthesis to remove viral genomic DNA. PCR using the same cDNA samples was also performed for GAPDH as a control for the presence of cDNA, and for IL-6 to illustrate the effect of MDMA on cytokine mRNA induction.

Figure 8. Relative quantification of γ HV-68 mRNA expression in mouse macrophages in the presence and absence of 500 μ M MDMA using real time PCR

The ORF50 and ORF65 viral gene expression shown in figure 7 was quantified by real time PCR. Cells were pretreated for 24 hr with 500 μ M MDMA, followed by the addition of virus for the times indicated. **Panel A** - Expression of ORF50 mRNA was unaffected by pre-incubation for 24 hr with 500 μ M MDMA. The experiment was performed three separate times ($N = 3$), with the value of each sample determined in duplicate. The fold-change for each time point was determined as described in the legend to figure 5 and normalized to the fold-change in mRNA expression for the housekeeping gene, GAPDH. Mean values $\pm$ SD.

SEM are presented. One-way ANOVA followed by Tukey's multiple comparison test suggests that for each time point, the presence of MDMA did not alter the relative expression of ORF50 mRNA ($p>0.05$). **Panel B** – ORF65 showed a lag time of expression when MDMA was present in the medium, as can be seen in the insert. Data was acquired as in panel A, and the mean values of the relative fold-change $\pm$ SEM are presented.