3,4-Methylenedioxymethamphetamine (MDMA, ecstasy) disrupts blood-brain barrier integrity through a mechanism involving P2X7 receptors

Ana Rubio-Araiz*, Mercedes Perez-Hernandez*, Andres Urrutia, Francesca Porcu, Erika Borcel, Maria Dolores Gutierrez-Lopez, Esther O'Shea and Maria Isabel Colado

Departamento de Farmacologia, Facultad de Medicina, Universidad Complutense, Instituto de Investigación Sanitaria del Hospital Clínico San Carlos (IdISSC), Red de Trastornos Adictivos del Instituto de Salud Carlos III, Madrid, Spain

Abstract

The recreational drug 3,4-methylenedioxymethamphetamine (MDMA; ‘ecstasy’) produces a neuro-inflammatory response in rats characterized by an increase in microglial activation and IL-1β levels. The integrity of the blood-brain barrier (BBB) is important in preserving the homeostasis of the brain and has been shown to be affected by neuro-inflammatory processes. We aimed to study the effect of a single dose of MDMA on the activity of metalloproteinases (MMPs), expression of extracellular matrix proteins, BBB leakage and the role of the ionotropic purinergic receptor P2X7 (P2X7R) in the changes induced by the drug. Adult male Dark Agouti rats were treated with MDMA (10 mg/kg, i.p.) and killed at several time-points in order to evaluate MMP-9 and MMP-3 activity in the hippocampus and laminin and collagen-IV expression and IgG extravasation in the dentate gyrus. Microglial activation, P2X7R expression and localization were also determined in the dentate gyrus. Separate groups were treated with MDMA and the P2X7R antagonists Brilliant Blue G (BBG; 50 mg/kg, i.p.) or A-438079 (30 mg/kg, i.p.). MDMA increased MMP-3 and MMP-9 activity, reduced laminin and collagen-IV expression and increased IgG immunoreactivity. In addition, MDMA increased microglial activation and P2X7R immunoreactivity in these cells. BBG suppressed the increase in MMP-9 and MMP-3 activity, prevented basal lamina degradation and IgG extravasation into the brain parenchyma. A-438079 also prevented the MDMA-induced reduction in laminin and collagen-IV immunoreactivity. These results indicate that MDMA alters BBB permeability through an early P2X7R-mediated event, which in turn leads to enhancement of MMP-9 and MMP-3 activity and degradation of extracellular matrix.

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Introduction

3,4-Methylenedioxymethamphetamine (MDMA) results in both acute and long-term neurotoxic effects in the brain of rodents. The major acute changes observed are an intense cellular stress, reflected by an increase in hydroxyl radical formation and in lipid peroxidation (Colado et al., 1997a, b) and signs of neuro-inflammation consisting of microglial activation (Orío et al., 2004) and several changes in interleukin-1 signal modulators in discrete brain areas (Torres et al., 2010, 2011). In addition, shortly after administration of MDMA there is microglial overexpression of the cannabinoid CB2 receptor, which is thought to be aimed at controlling the production of pro-inflammatory cytokines. In fact, repeated administration of the CB2 agonist JWH-015 prevents the MDMA-induced reduction in IL-1 receptor type I (IL-1RI) expression and the increase in microglial activation and IL-1β and IL-1 receptor antagonist (IL-1ra) release (Torres et al., 2011) produced by the drug.

There is evidence showing that blood-brain barrier (BBB) integrity is altered by a number of factors including increased levels of inflammatory cytokines (Shaftel et al., 2007; Tian and Kyriakides, 2009) and free radicals (Gasche et al., 2001; Katsu et al., 2010), factors/mediators, which are increased following administration of MDMA. In turn, both reactive oxygen species and pro-inflammatory cytokines, particularly IL-1β, are involved in the activation and induction of metalloproteinases (MMPs) in vivo as well as in vitro (Vecil et al., 2000; Liu and Rosenberg, 2005; Katsu et al., 2010). MMPs are zinc- and calcium-dependent endopeptidases that degrade components of the extracellular matrix and tight-junction proteins in endothelial cells (Rosenberg, 2009). While these functions are essential for remodelling,
MMPs have also been implicated in the disruption of the BBB, neuro-inflammatory response and neuronal cell death in many neurological diseases (Lindberg et al., 2001; Montaner et al., 2001; Zhou et al., 2011). To our knowledge the effect of MDMA on MMP expression and its consequences on basal lamina degradation have not been studied.

The ionotropic purinergic receptor P2X-R is an ATP-gated cation channel whose activation by ATP allows influx of Ca²⁺ and Na⁺ and concomitant efflux of K⁺ (Burnstock, 2007). It is highly expressed in macrophage-like cells of the brain (Sim et al., 2004) and is implicated in the microglia response to inflammation (Collo et al., 1997), microglial proliferation (Bianco et al., 2005; Monif et al., 2009), and maturation and release of the pro-inflammatory cytokine IL-1β (Solle et al., 2001; Chakfe et al., 2002; Clark et al., 2010). To our knowledge there are no data on the role of P2X-R linking inflammatory responses with neuronal damage in the MDMA-induced model of neuro-inflammation.

The present study was undertaken to determine: (1) the effect of MDMA on the activity of MMP-9 and MMP-3, expression of laminin and type IV collagen (collagen-IV) and extravasation of IgG in rat hippocampus, (2) the ability of P2X-R antagonists to prevent the changes induced by MDMA in MMP activities, basal lamina and BBB disruption in the hippocampus, and (3) time-course expression and localization of P2X-R using double immunostaining for microglial cells.

Materials and method

Animals and drug administration

Male Dark Agouti rats (175–200 g, Harlan Laboratories Models, Spain) were used. Rats were housed in groups of six in conditions of constant temperature (21 ± 2°C) and a 12 h light/dark cycle (lights on: 08:00) and given free access to food and water. MDMA was given at the dose of 10 mg/kg (i.p.), animals being sacrificed 1, 3, 6 or 24 h later. Room temperature at the time of MDMA administration was 21–22°C.

Brilliant Blue G (BBG, 50 mg/kg, i.p. Sigma-Aldrich, Madrid, Spain) was given 10 min before MDMA administration (Peng et al., 2009). This compound was chosen because of its low toxicity (Remy et al., 2008) and high selectivity (Jiang et al., 2000) for blocking the potential adverse effects of P2X-R activation. In addition, as recent studies have reported the ability of this compound to inhibit voltage gated sodium channels (Jo and Bean, 2011) and to discard that this property might have relevance in the ex vivo effects, some of the studies were replicated by administering A-438079 (30 mg/kg, i.p. Tocris Bioscience, UK), a more selective P2X-R antagonist (Nelson et al., 2006; Marcellino et al., 2010). This compound was injected 60 and 30 min before MDMA. MDMA (Servicio de Estupefacientes y Psicotropos, MSPSI, Spain) and BBG were dissolved in saline (0.9% NaCl) while A-438079 was dissolved in saline containing ethanol (5%) and Tween 80 (10%). All compounds were given in a volume of 1 ml/kg. Doses of MDMA, BBG and A-438079 are reported in terms of the base.

All experimental procedures were performed in accordance with the guidelines of the Animal Welfare Committee of the Universidad Complutense de Madrid (following European Council Directives 86/609/CEE and 2003/65/CE).

Zymography of matrix metalloproteinases (MMP-9 and MMP-3)

Hippocampi were homogenized and lysed in NP-40 lysis buffer (150 mM NaCl, 50 mM Tris-HCl, 1% NP-40, pH 8.0) supplemented with 5% protease inhibitor cocktail (Sigma-Aldrich). Protein concentrations were measured using a DC Protein Assay kit (Bio-Rad, Spain). The activity of MMP-9 was assessed by gelatin zymography and that of MMP-3 by casein zymography. Similar protein quantities were mixed with non reducing Laemmli buffer for 15 min at 37°C and, for MMP-9 activity, subjected to SDS-PAGE using 9% acrylamide gel containing 0.1% gelatin (Sigma-Aldrich). For MMP-3 activity, gels were stained in Coomasie Brilliant Blue R-250 (Bio-Rad) until blue, followed by immersion in destaining solution (40% methanol, 10% acetic acid, 50% water) until bands were clear and the reaction was stopped by placing gels in 10% acetic acid. Gels were dried and digitized. A total of three independent experiments were run, which were densitometrically analysed.

Western blot analysis

Rats were killed and decapitated 1, 3, 6 and 24 h after MDMA administration, the brains rapidly removed and the hippocampus dissected out on ice. Samples were homogenized and lysed in NP-40 lysis buffer (150 mM NaCl, 50 mM Tris-HCl, 1% NP-40, pH 8.0) supplemented with 5% protease inhibitor cocktail (Sigma-Aldrich). Protein concentrations were measured using a DC Protein Assay kit (Bio-Rad). The samples were boiled in Laemmli buffer and proteins were resolved by 7% SDS-PAGE and transferred to PVDF membranes. Non-specific binding was blocked by incubation for 1 h in TBS buffer containing 0.1% Tween 20 and 5% skimmed milk. Membranes were incubated overnight at 4°C with the following primary antibodies: polyclonal anti-laminin...
Rats were anesthetized with sodium pentobarbital and IgG leakage from serum into the brain was assessed in IgG extravasation. Images were acquired with a Zeiss Axio Imager A1 microscope taking 8 fields of 40× magnifications per animal and condition. All images were converted to grey scale and blood vessels were outlined to provide an integrated grey scale value for image analysis with ImageJ Software (version 1.43; NIH, USA).

IgG extravasation
IgG leakage from serum into the brain was assessed in the dentate gyrus as a marker of vasculature damage. Rats were anesthetized with sodium pentobarbital and perfused transcardially through the left ventricle with 200 ml of 0.1 M PBS (pH 7.4) followed by 200 ml of 4% paraformaldehyde-PBS. Brains were removed, post-fixed in the same solution for 4 h and cryoprotected by immersion in 30% sucrose-PBS at 4 °C. The brains were sliced at 20 μm in the coronal plane through the hippocampus and stored at −80 °C. The brains were sliced at 20 μm in the coronal plane through the hippocampus and stored in cryoprotectant solution at −20 °C. For double-labelling studies, cerebral free-floating sections containing dentate gyrus were blocked by incubation with 0.5% BSA, 10% normal horse serum and 0.1% Triton X-100 for 1 h, incubated at 4 °C overnight with the antibody Alexa Fluor® 594 donkey anti-rat IgG (1:1000) and covered with ProLong® Gold (Life Technologies). Images were acquired with a Zeiss Axio Imager A1 microscope taking 8 fields of 40× magnifications per animal and condition. All images were converted to grey scale and blood vessels were outlined to provide an integrated grey scale value for image analysis with ImageJ Software (version 1.43; NIH, USA).

Immunohistochemistry
Rats were anesthetized with sodium pentobarbital and perfused transcardially through the left ventricle with 200 ml of 0.1 M PBS (pH 7.4) followed by 200 ml of 4% paraformaldehyde-PBS. Brains were removed, post-fixed in the same solution for 4 h and cryoprotected by immersion in 30% sucrose-PBS at 4 °C. The brains were sliced at 40 μm in the coronal plane through the hippocampus and stored in cryoprotectant solution at −20 °C. For double-labelling studies, cerebral free-floating sections containing dentate gyrus were blocked by incubation with 0.5% BSA, 10% normal horse serum and 0.1% Triton X-100 for 1 h and incubated at 4 °C with the appropriate primary antibodies (OX-42, Serotec, Spain; 1:500; P2X-R, Alomone, Israel, 1:250; laminin, Sigma-Aldrich, 1:1000; Collagen-IV, Abcam, 1:250) followed by the Alexa Fluor® 488 donkey anti-rabbit IgG (1:1000), Alexa Fluor® 594 donkey anti-mouse IgG (1:1000) and mounted in ProLong® Gold with the nuclear marker DAPI (Life Technologies). Images were acquired sequentially on a Leica TCS-SP2AOBS confocal microscope (Leica Microsystems, Germany) for each fluorophore to avoid any cross signal between them. The specificity of the antibodies used was confirmed in the literature and verified by carrying out the corresponding secondary antibody controls to rule out the possibility of reaction between them and images were taken using the same settings for each antibody staining. In the case of the anti-P2X-R antibody (Melani et al., 2006) we incubated tissue sections with the corresponding primary and secondary antibodies in the presence of the antigen specific control for the primary antibody, P2X-R (576–595) Peptide (Alomone, Israel). No signal was detected in the presence of this blocking peptide thus confirming the specificity of the primary antibody for the P2X-R.

Measurement of rectal temperature
Immediately before and up to 6 h after MDMA injection, temperature was measured by use of a digital readout thermocouple (BAT12 thermometer, Physitemp, USA) with a resolution of 0.1 °C and accuracy of ±0.1 °C attached to a RET-2 Rodent Sensor which was inserted 2.5 cm into the rectum of the rat, the animal being lightly restrained by holding it in the hand. A steady readout was obtained within 10 s of probe insertion. Temperature readings were taken every 30 min immediately before and after MDMA injection and hourly thereafter.

Statistics
Data are presented as mean±S.E.M. Data from MMP activity and expression, IgG extravasation, laminin, collagen-IV and P2X-R expression by Western blot and immunofluorescence and microglial activation were analysed using one-way ANOVA followed by Newman-Keuls test (GraphPad Prism 5.0, GraphPad Software Inc., USA). Rectal temperature measurements were analysed using two-way ANOVA for repeated measures (BMDP, Statistical Solutions, Ireland). Treatment was used as the between-subjects factor and time as the repeated measure. Differences were considered significant at p<0.05.

Results
MDMA increases the expression and activity of MMP-9 and MMP-3
Figure 1a, b illustrate a representative Western blot of the pro-active and active forms of MMP-9 and zymogram in the hippocampus of saline- and MDMA-treated rats. Gelatin zymography reveals a band at approximately 82 kDa corresponding to the active form of MMP-9 (Fig. 1b). Quantitative image analysis by one-way ANOVA revealed a significant effect of MDMA (10 mg/kg, i.p.) on MMP-9 activity (Fig. 1b; F4,28=8.87, p<0.0001). Post-hoc analysis indicated that MDMA produced a substantial increase in MMP-9 activity 1 h (75%) after administration. This effect was not evident at 6 or 24 h although MMP-9 activity remained slightly elevated at 3 h.

To study the possibility that augmented MMP-9 activity was due to increased enzyme expression, Western blot analysis was performed (Fig. 1a). Pro-active and
active MMP-9 bands were detected at 92 and 82 kDa, respectively (Fig. 1a). Quantitative image analysis of each of the two bands by one-way ANOVA revealed a significant effect of treatment on the expression of pro-active MMP-9 (Fig. 1b; $F_{4,28}=3.09$, $p=0.033$) and active MMP-9 (Fig. 1a; $F_{4,27}=4.68$, $p=0.005$). Post-hoc analysis indicated that MDMA significantly increased the expression of pro-active (36%) and active MMP-9 (35%) 1 h after drug administration. This effect was not evident 3, 6 or 24 h later.

Figure 1d, e show a representative Western blot of the pro-active and active forms of MMP-3 and zymogram in the hippocampus of saline- and MDMA-treated rats. Casein zymography shows a band at approximately 45 kDa corresponding to active MMP-3 (Fig. 1c).

Quantitative image analysis by one-way ANOVA revealed a significant effect of MDMA (10 mg/kg, i.p.) on MMP-3 activity (Fig. 1c; $F_{4,25}=4.24$, $p=0.009$). Post-hoc analysis indicated that MDMA produced a substantial increase in MMP-3 activity 3 h after administration (55%).

Pro-active and active MMP-3 bands were detected by Western blot at 53 and 45 kDa, respectively (Fig. 1d). Quantitative image analysis of each of the two bands by one-way ANOVA revealed no effect of treatment on the expression of pro-active MMP-3 (Fig. 1d; $F_{4,23}=2.31$, $p=0.089$) and a significant effect of treatment on the
expression of active MMP-3 (Fig. 1d; F4,23=3.39, p=0.026). Post-hoc analysis indicated that the expression of active MMP-3 6 h after MDMA injection was different from that observed at 3 h.

**MDMA reduces laminin and collagen-IV expression and increases IgG extravasation**

An increased activity of MMP-9 and MMP-3 is associated with laminin and collagen-IV degradation. Therefore, by means of immunohistochemical and Western blot studies we examined the effect of MDMA on laminin (Fig. 2, Supplementary Figure S1) and collagen-IV (Fig. 3) immunoreactivity.

**Figure 2** (upper panel) shows fluorescence images (40×) of representative laminin immunostained sections of dentate gyrus. DAPI staining shows nuclear staining in the dentate gyrus. Scale bar=100 μm. (a) Quantitative analysis of intensity of immunostaining at different times after MDMA administration. Rats received MDMA (10 mg/kg, i.p.) and were killed 1, 3, 6 or 24 h later. (b) Quantitative analysis of intensity of immunostaining showing effect of BBG on MDMA-induced changes. BBG (50 mg/kg, i.p.) was given 30 min before MDMA (10 mg/kg, i.p.) and rats killed 6 h after MDMA. Data expressed as per cent of control. Results shown as mean±S.E.M (n=4–9). Different from saline: *p<0.01. Different from MDMA-treated group at 1 h: †p<0.01. Different from MDMA-treated group at 3 h: ‡p<0.05. Different from MDMA-treated group at 6 h: §p<0.05, §§§p<0.001.

Fig. 2. Time-course of MDMA-induced changes in dentate gyrus laminin immunoreactivity and effect of Brilliant Blue G (BBG). Upper panel shows fluorescence images (40×) of representative laminin immunostained sections of dentate gyrus. DAPI staining shows nuclear staining in the dentate gyrus. Scale bar=100 μm. (a) Quantitative analysis of intensity of immunostaining at different times after MDMA administration. Rats received MDMA (10 mg/kg, i.p.) and were killed 1, 3, 6 or 24 h later. (b) Quantitative analysis of intensity of immunostaining showing effect of BBG on MDMA-induced changes. BBG (50 mg/kg, i.p.) was given 30 min before MDMA (10 mg/kg, i.p.) and rats killed 6 h after MDMA. Data expressed as per cent of control. Results shown as mean±S.E.M (n=4–9). Different from saline: *p<0.01. Different from MDMA-treated group at 1 h: †p<0.01. Different from MDMA-treated group at 3 h: ‡p<0.05. Different from MDMA-treated group at 6 h: §p<0.05, §§§p<0.001.

reduced laminin immunostaining at 1 h (37%), 3 h (35%) and 6 h (31%).

To further confirm data on laminin, Western blot analyses were carried out. A major band for laminin was detected at 220 kDa (Supplementary Figure S1A). One-way ANOVA revealed a significant effect of treatment (F4,24=10.05; p<0.0001). Post-hoc analysis indicated that MDMA produced a substantial decrease in laminin expression 1 h (48%), 3 h (47%) and 6 h (27%) after dosing.

**Figure 3** (upper panel) shows fluorescence images (40×) of representative collagen-IV immunostained sections of dentate gyrus at different times after MDMA administration. Quantitative image analysis by one-way ANOVA revealed a significant effect of treatment on the immunoreactivity of collagen-IV (Fig. 3a; F4,21=6.28, p=0.0017). Post-hoc analysis indicated that MDMA significantly reduced collagen-IV immunostaining at 1 h (60%), 3 h (54%), 6 h (60%) and 24 h (29%).

BBB permeability was visualized and quantified by means of IgG immunostaining (Fig. 4). IgG expression in the brain parenchyma is indicative of disruption of
the BBB. Figure 4 (upper panel) shows fluorescence images (40×) of representative IgG immunostained sections of dentate gyrus at different times after MDMA administration. One-way ANOVA revealed a significant effect of treatment (Fig. 4a; F_{4,22}=2.96, p=0.043). Post-hoc analysis indicated that MDMA substantially increased dentate gyrus IgG leakage 3 h post-injection (38%).

**Brilliant Blue G (BBG) prevents the MDMA-induced changes in basal lamina proteins and IgG extravasation**

To investigate the role of P2X7R on the changes induced by MDMA in MMP activity, the ability of BBG to prevent these changes was examined. Figure 1c, f show representative zymograms of MMP-9 and MMP-3. Quantitative analysis of MMP-9 and MMP-3 activity by one-way ANOVA revealed a significant effect of treatment (for MMP-9: Fig. 1c; F_{3,16}=10.35, p=0.0005 and for MMP-3: Fig. 1f; F_{3,30}=7.25, p=0.0008). Post-hoc analysis indicated that BBG prevented the MDMA-induced increases in MMP-9 and MMP-3 activities.

BBG was able to prevent degradation of basal lamina proteins (Fig. 2, Supplementary Figure S1B) and IgG extravasation (Fig. 4).

Fig. 3. Time-course of MDMA-induced changes in dentate gyrus collagen-IV immunoreactivity and effect of Brilliant Blue G (BBG). Upper panel shows fluorescence images (40×) of representative collagen-IV immunostained sections of dentate gyrus. DAPI staining shows nuclear staining in the dentate gyrus. Scale bar=100 μm. (a) Quantitative analysis of intensity of immunostaining at different times after MDMA administration. Rats received MDMA (10 mg/kg, i.p.) and were killed 1, 3, 6 or 24 h later. (b) Quantitative analysis of intensity of immunostaining showing effect of BBG on MDMA-induced changes. BBG (50 mg/kg, i.p.) was given 30 min before MDMA (10 mg/kg, i.p.) and rats killed 6 h after MDMA. Data expressed as per cent of control. Results shown as mean±S.E.M (n=4–8). Different from saline: *p<0.05, **p<0.01. Different from MDMA-treated group: ̲̅̃ p<0.05.
ANOVA revealed a significant effect of treatment (Fig. 3b; $F_{3,13}=4.92, p=0.017$). Post-hoc analysis indicated that BBG attenuated the MDMA-induced reduction in collagen-IV immunoreactivity.

Figure 4 (upper panel) shows fluorescence images ($40\times$) of representative IgG immunostained sections of dentate gyrus. Quantitative analysis of intensity of immunostaining at different times after MDMA administration revealed a significant effect of treatment ($F_{3,16}=5.61, p=0.008$). Post-hoc analysis indicated that BBG attenuated the MDMA-induced increase in IgG immunostaining.

A-438079 prevents the MDMA-induced changes in basal lamina proteins

Figure 5 shows fluorescence images ($40\times$; upper panel) of representative laminin (left panel) and collagen-IV (right panel) immunostained sections of dentate gyrus. Quantitative analysis by one-way ANOVA revealed a significant effect of treatment ($F_{3,14}=7.80, p=0.0024$; $F_{3,13}=5.05, p=0.0155$). Post-hoc analysis indicated that A-438079 prevented the MDMA-induced reduction in laminin and collagen-IV immunoreactivity.

MDMA increases P2X$_{7}$R expression and microglial activation

The effect of MDMA on P2X$_{7}$R expression in dentate gyrus was determined at different post-injection time-points (1, 3, 6 and 24 h) by immunohistochemical analyses (Fig. 6). Figure 6 (upper panel) shows fluorescence images ($40\times$) of representative sections of dentate gyrus stained for P2X$_{7}$R (green). Quantitative analysis of intensity of P2X$_{7}$R immunostaining revealed a significant effect of treatment ($F_{4,24}=9.28, p=0.0001$). Post-hoc analysis indicated that MDMA significantly increased P2X$_{7}$R expression 3 h (59%) and 6 h (57%) after dosing. Little modification of the expression of the purinergic receptor was found at 1 and 24 h.

Next, expression of P2X$_{7}$R in microglia was examined. First of all, the effect of MDMA on OX-42 immunostaining (red) in dentate gyrus at several post-injection time-points was evaluated (Fig. 6). Figure 6 (upper panel)
shows fluorescence images (40×) of representative sections of dentate gyrus stained for OX-42. Quantitative analysis of intensity of OX-42 immunostaining by one-way ANOVA revealed a significant effect of treatment (Fig. 6; $F_{4,24}=10.31$, $p<0.0001$). Post-hoc analysis indicated that there was increased OX-42 immunoreactivity 6 h (54%) after dosing. Merged images of double-immunofluorescence staining showed P2X7R expression to be localized in microglia at 6 h (Fig. 6, upper panel).

Because P2X7R could modulate the microglial activation (Monif et al., 2009), we investigated the effects of BBG on OX-42 immunostaining following MDMA (Fig. 6). Quantitative analysis of OX-42 immunostaining by one-way ANOVA revealed a significant effect of treatment (Fig. 6d; $F_{3,10}=28.32$, $p<0.0001$). Post-hoc analysis indicated that OX-42 immunostaining in rats treated with BBG and MDMA was similar to that observed in the saline group.

**BBG reduces the MDMA-induced changes on P2X7R expression and microglial activation**

Figure 6 (upper panel) shows fluorescence images (40×) of representative sections of dentate gyrus stained for P2X7R (green) and OX-42 (red). Quantitative analysis of P2X7R immunostaining by one-way ANOVA revealed a significant effect of treatment (Fig. 6c; $F_{3,12}=58.64$, $p<0.0001$). Post-hoc analysis indicated that MDMA increased P2X7R staining at 6 h and this was reduced by BBG pre-treatment such that P2X7R immunoreactivity in rats treated with BBG and MDMA was similar to that observed in the saline group.

**P2X7R antagonists do not modify MDMA-induced hyperthermia**

Given the importance of hyperthermia in the integrity of the BBB, we studied the effect of BBG and A-438079 on MDMA-induced hyperthermia (Supplementary Figure S2). Two-way ANOVA indicated that there was a significant effect of treatment (Figure S2A, $F_{3,14}=70.78$, $p<0.0001$; Figure S2B, $F_{3,16}=35.59$, $p<0.0001$). Post-hoc analysis revealed that MDMA increased rectal temperature peaking 30–60 min after injection (Figure S2A, $F_{1.7}=68.18$, $p<0.0001$; Figure S2B, $F_{1.8}=60.74$, $p=0.0001$). BBG (Figure S2A) or A-438079
Figure S2B did not modify this hyperthermic response ($F_{1,8}=0.62$, $p=0.45$; $F_{1,8}=0.25$, $p=0.63$, respectively) and did not modify the temperature of saline-treated rats ($F_{1,6}=0.74$, $p=0.42$; $F_{1,8}=4.17$, $p=0.08$, respectively).

**Discussion**

The current study shows for the first time that: (i) administration of a single dose of MDMA produces a marked reduction in laminin and collagen-IV expression associated with a rapid and transient increase in MMP-9 and MMP-3 activity and a short-lived increase in IgG extravasation, (ii) the P2X$_7$R antagonist BBG prevents the changes induced by MDMA in basal lamina and BBB. A-438079 also attenuates the MDMA-induced reduction in laminin and collagen-IV immunoreactivity, (iii) P2X$_7$Rs are up-regulated shortly after MDMA administration and colocalize with microglia and (iv) BBG reduces the microglial activation and increase in P2X$_7$R induced by MDMA. These results indicate that MDMA disrupts BBB integrity and strongly implicate P2X$_7$Rs in the regulation of MDMA-induced changes.

Immediately after MDMA administration there is an enhanced MMP-9 proteolytic activity in the hippocampus that is more pronounced and appears before the increased activity of MMP-3. Western blot analysis indicates that the increased MMP-9 proteolytic activity is probably due to increased total protein. The enhanced activity of these MMPs is associated with a greater degradation of the basement membrane proteins, collagen-IV and laminin and with a transient increased in BBB permeability. These observations are consistent with a recent study showing increased MMP-9 activity and expression in striatum following repeated methamphetamine administration (Urrutia et al., 2013). This effect is specifically involved in the disruption of BBB induced by...
methamphetamine since administration of a broad spectrum MMP inhibitor, BB-94, is able to prevent laminin degradation and reduce the BBB damage induced by the drug (Urrutia et al., 2013). The cellular source of MMP-9 has not been firmly established but in the striatum of methamphetamine-treated mice the increase in IgG immunoreactivity was seen in the regions where gelatinase activity was most prominent (Urrutia et al., 2013).

The effect of MDMA on MMP-3 activity and expression is a novel finding in this study. Stromelysin-1, known also as MMP-3, likewise contributes to BBB disruption by attacking the components of basal lamina during neuro-inflammation. MMP-3 degrades various collagens, elastin, fibronectin and laminin but also activates latent forms of other matrix metalloproteinases (Ramos-De Simone et al., 1999). MMP-3 knockout mice show less disruption of the BBB after intracerebral LPS injection than that observed in wild-type mice (Gurney et al., 2006). MMP-3 has been reported to be involved in the conversion of pro-MMP9 to the active form (Kim and Hwang, 2011). However, following MDMA the participation of MMP-3 in MMP-9 activation appears unlikely since MMP-3 activity is increased at a later time-point to MMP-9 activity.

To our knowledge there are no previous data on the effect of MDMA on MMP activation and basal lamina degradation. In addition, the effect of MDMA on BBB integrity has scarcely been investigated. There is only a study examining the effects of acute MDMA on BBB dysfunction, brain oedema, and cell injury in rats and mice (Sharma and Ali, 2008). This investigation shows leakage of Evans Blue dye, particularly in the cerebellum, hippocampus, cortex, thalamus and hypothalamus shortly after MDMA. This effect was more pronounced in mice than in rats. A marked increase in brain water along with Na⁺, K⁺, and Cl⁻ content was also seen in the aforementioned brain regions. The presence of distorted neuronal and glial cells in brain regions associated with leakage of Evans Blue was quite common in MDMA-treated animals. Furthermore, increased albumin immunoreactivity, indicating breakdown of the BBB, and up-regulation of glial fibrillary acidic protein (GFAP), suggesting activation of astrocytes, were seen in most brain regions showing oedematous changes. These observations constitute the only evidence that MDMA has the capacity to disrupt BBB permeability to proteins and to induce the formation of oedema, probably by inducing hyperthermia and cellular stress.

In addition to the alterations in BBB permeability, MDMA induces signs of neuro-inflammation reflected as microglial activation. Microglial activation is regulated by various subtypes of nucleotide (P2X, P2Y) and adenosine (A1, A2A and A3) receptors, which control ionic conductances, membrane potential, gene transcription, the production of inflammatory mediators and cell survival. Among them, the role of P2X₇-R is especially well established. The P2X₇-R is found in various brain cell populations, and in particular in microglia, whose expression is increased in response to certain experimental cues (Grygorowicz et al., 2010; Engel et al., 2012; Kimbler et al., 2012). Increased microglial activation and up-regulation of P2X₇-R is seen in a wide variety of conditions including Alzheimer’s disease (Lee et al., 2011), Parkinson’s disease (Marcellino et al., 2010), multiple sclerosis and amyotrophic lateral sclerosis (Yiangou et al., 2006), Huntington’s disease (Díaz-Hernández et al., 2009), in the penumbra following cerebral ischemia in rodents (Melani et al., 2006) and following LPS application to rat striatum (Choi et al., 2007). Therefore, we examined the effect of MDMA on the microglial expression of P2X₇-R and the ability of a P2X₇-R antagonist to reduce the MDMA-induced changes in microglial activation and BBB disruption. The current study shows that MDMA causes overexpression of P2X₇-R which is evident 3 h post-injection although increased colocalization was not evident until 6 h, time at which microglial activation occurs. The fact that P2X₇-R up-regulation appears before microglial activation indicates that enhanced P2X₇-R expression may be driving microglial activation and proliferation (Monif et al., 2009) making their transition from resting to a reactive state.

Administration of the P2X₇-R antagonist BBG prevents the increased expression of P2X₇-R induced by MDMA in microglial cells and reduces microglial activation. To our knowledge there are no previous data on the effect of a P2X₇-R antagonist on the density of P2X₇-R in brain tissue and our results suggest that BBG inhibits microglial activation by a mechanism involving a reduction in P2X₇-R expression.

Numerous stimuli have been reported to be involved in the increase in MMP activity and the subsequent basal laminin degradation. According to the data in the literature, cytokine release could be involved in the increase in MMP-9 activity (Gottschall and Deb, 1996; Vecil et al., 2000). Previous studies have shown an early increase in IL-1β following MDMA (Orio et al., 2004), which may induce an increment in MMP expression through NFkB as is observed in other conditions (Wang et al., 2011; Lee et al., 2012). In line with this, MDMA induces an early increase in p65/p50 heterodimer DNA binding in areas such as the frontal cortex and hypothalamus (Orio et al., 2010). The current study does not allow us to establish an accurate time-course. However, the results suggest that MDMA induces an early increase in IL-1β release, which would in turn increment MMP activity leading to the degradation of laminin and collagen-IV with concomitant and subsequent IgG extravasation (3 h) and followed by microglia activation (6 h).

A relevant result from this study is that administration of the P2X₇-R antagonist, BBG, prevents the changes induced by MDMA in MMP-9 and MMP-3 activities and consequently reduces extracellular matrix degradation and IgG leakage. Similar results were obtained
by using the more selective P2X7R antagonist, A-438079. These data strongly indicate that early activation of P2X7R is an initiating factor in this cascade of events. In fact, P2X7R activation promotes neuro-inflammation by causing the release of pro-inflammatory cytokines, such as IL-1β and TNF-α (Lister et al., 2007; Tschopp and Schroder, 2010).

The main function of the BBB is to maintain homeostasis of the brain and protect the CNS from endogenous or exogenous toxins within the circulation (Abbott et al., 2010). Drugs inducing BBB disruption may increase the concentration of any CNS active drugs in the brain but also may favour the infiltration of pathological mediators from periphery making the CNS more vulnerable to damage. As mentioned earlier several studies indicate that administration of methamphetamine increases BBB permeability probably leading to the entry into the CNS parenchyma of several small viruses and neurotoxic products of viral metabolism that are retained in the periphery under normal conditions. Methamphetamine is also able to enhance HIV infection of human blood monocyte-derived macrophages, the primary target site for the virus (Liang et al., 2008) and could facilitate the transport of HIV-infected leukocytes into the brain. This effect could explain the high incidence of AIDS-related neurologic disease in methamphetamine users (Cisneros and Gharpade, 2012; Weber et al., 2013). The interaction between MDMA and HIV remains to be characterized but there is good evidence showing that ecstasy users may be at a heightened risk for the transmission of HIV and other blood-borne viral infections (BBVI) through risky sexual behaviours (Dunn et al., 2010; Klein et al., 2010).

In conclusion, the current study indicates that MDMA increases BBB permeability through an early P2X7R-mediated event, which enhances MMP-9 and MMP-3 activities and degrades laminin and type IV collagen.

**Supplementary material**

For supplementary material accompanying this paper, visit http://dx.doi.org/10.1017/S1461145714000145

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**Statement of Interest**

None.

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