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Prior stimulation of the endocannabinoid system prevents methamphetamine-induced dopaminergic neurotoxicity in the striatum through activation of CB₂ receptors

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Abstract

Methamphetamine toxicity is associated with cell death and loss of dopamine neuron terminals in the striatum similar to what is found in some neurodegenerative diseases. Conversely, the endocannabinoid system (ECS) has been suggested to be neuroprotective in the brain, and new pharmacological tools have been developed to increase their endogenous tone. In this study, we evaluated whether ECS stimulation could reduce the neurotoxicity of high doses of methamphetamine on the dopamine system. We found that methamphetamine alters the levels of the major endocannabinoids, anandamide (AEA) and 2-arachidonoyl glycerol (2-AG) in the striatum, suggesting that the ECS participates in the brain responses to methamphetamine. ⁹-tetrahydrocannabinol (THC), a cannabis-derived agonist of both CB₁ and CB₂ cannabinoid receptors, or inhibitors of the main enzymes responsible for the degradation of AEA and 2-AG

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.neuropharm.2014.03.014>.

(URB597 and JZL184, respectively), blunted the decrease in striatal protein levels of tyrosine hydroxylase induced by methamphetamine. In addition, antagonists of CB₂, but not of CB₁, blocked the preventive effects of URB597 and JZL184, suggesting that only the former receptor subtype is engaged in neuroprotection exerted by ECS stimulation. Finally, we found that methamphetamine increases striatal levels of the cytokine tumor necrosis factor alpha, an effect that was blocked by ECS stimulation. Altogether, our results indicate that stimulation of ECS prior to the administration of an overdose of meth-amphetamine considerably reduces the neurotoxicity of the drug through CB₂ receptor activation and highlight a protective function for the ECS against the toxicity induced by drugs and other external insults to the brain.

This article is part of the Special Issue entitled ‘CNS Stimulants’.

Keywords

Endocannabinoids; Methamphetamine; Neurotoxicity; Neuroprotection; THC; Neuroinflammation

1. Introduction

The endocannabinoid system (ECS) is an endogenous neuromodulatory system comprised of: i) two main endogenous ligands of type-1 (CB₁) and type-2 (CB₂) cannabinoid receptors (Howlett et al., 2010), namely *N*-arachidonylethanolamine (anandamide, AEA) (Devane et al., 1992) and 2-arachidonoyl glycerol (2-AG) (Mechoulam et al., 1995); ii) a putative membrane transport system (Fowler, 2013); iii) intracellular storage organelles (Oddi et al., 2009) and cytosolic carriers (Maccarrone et al., 2010); and iv) enzymes responsible for the synthesis and degradation of these compounds. Fatty acid amide hydrolase (McKinney and Cravatt, 2005; Fezza et al., 2008), and monoacylglycerol lipase (Dinh et al., 2002) are the most important degradative enzymes for AEA and 2-AG, respectively. The ECS has been shown to participate in a broad range of functions including anxiety, depression, neurogenesis, reward, cognition, learning, and memory (Mechoulam and Parker, 2013). Converging evidence suggests that endogenous cannabinoids also play a neuroprotective role in pathological situations (van der Stelt and Di Marzo, 2005). Endocannabinoids bind mainly (but not exclusively) to CB₁ (Matsuda et al., 1990) and CB₂ (Munro et al., 1993) receptors that are G-protein coupled receptors. CB₁ receptor appears to be largely expressed in the brain, and particularly in the striatum, the hippocampus, the amygdaloid nucleus and the substantia nigra, and it is believed to play a major role in the ECS activity on movement control, learning, memory, reward and emotions (Freund et al., 2003; Solinas et al., 2008; Viscomi et al., 2009). For more than 10 years, it was believed that CB₂ receptors were absent from the brain, and that they were involved only in peripheral effects of endocannabinoids (Raitio et al., 2005). However, later on the presence of these receptors was clearly demonstrated also in the brain, and since then their functional role in pathological conditions has been suggested by several studies (Van Sickle et al., 2005; Viscomi et al., 2009; Onaivi et al., 2012). Recently, these receptors were also proposed to play a role in addiction and neuropsychiatric diseases (Morales and Bonci, 2012; Onaivi et al., 2012).

Methamphetamine is one of the most abused psychostimulants around the world, due to its inexpensive production and long lasting action (Krasnova and Cadet, 2009). A plethora of

Internet websites exist and are easily accessible to explain how to synthesize methamphetamine. Therefore, methamphetamine is often homemade clandestinely resulting in important variations in the concentration of different batches, which increase the risks of methamphetamine overdosing. Chronic use of methamphetamine is often associated with cognitive impairments such as deficits in attention, working memory and decision-making (Simon et al., 2000; Volkow et al., 2001). Post-mortem and brain imaging studies have shown that long-term use of methamphetamine induces specific damages for the dopaminergic neurotransmitter system, such as a reduction of dopamine levels, as well as a decreased level of dopamine transporter and tyrosine hydroxylase, the key player in dopamine synthesis (Wilson et al., 1996; Volkow et al., 2001). In animals, several protocols of administration have been developed to investigate neurotoxic effects of methamphetamine. These protocols mostly consist in repeated methamphetamine injections (4×10 mg/kg at 2 h intervals) or to single injections of 30 or 40 mg/kg of methamphetamine (Davidson et al., 2001; Zhu et al., 2005, 2006) which can be considered models of methamphetamine overdose. Importantly, these protocols produce similar neurotoxicity for the dopamine neurotransmission (Davidson et al., 2001; Zhu et al., 2005, 2006). As a matter of fact, these treatments reproduce the reductions in tyrosine hydroxylase activity and level, DAT activity and vesicular monoamine transporter-2 protein level in striatum and cortex observed in humans, and, thus these effects are often considered as hallmarks of the dopamine terminal loss induced by the drug (Wilson et al., 1996; Cadet et al., 2003; Krasnova and Cadet, 2009). In addition to the loss of monoaminergic terminals, methamphetamine treatment causes death of neuronal cell bodies in the striatum (Cadet et al., 2003; Thiriet et al., 2005). All these damages have been linked to the massive dopamine release induced by high doses of methamphetamine through an increased reverse transport of this monoamine through the DAT (Sulzer et al., 1995) and to the subsequent production of free radicals triggered by dopamine oxidation (Yamamoto and Zhu, 1998). The understanding of the mechanisms involved in methamphetamine neurotoxicity could lead to the discovery of new strategies to prevent or counter neurotoxic and neurodegenerative processes.

The aim of this study was to determine whether stimulation of the ECS could reduce methamphetamine neurotoxicity for dopamine terminals. We first evaluated whether a toxic regimen of methamphetamine modulates levels of endocannabinoids in the striatum. We then investigated whether THC, the main psychoactive ingredient of cannabis extracts, or synthetic compounds like URB597, a selective inhibitor of the fatty acid amide hydrolase that increases the *in vivo* levels of AEA (Kathuria et al., 2003), and JZL184, a selective inhibitor of the monoacylglycerol lipase that increases the *in vivo* levels of 2-AG (Long et al., 2009), could reduce the toxicity of methamphetamine on dopamine terminals. In addition, we sought to ascertain which cannabinoid receptor sub-type was involved in these effects of AEA and 2-AG. Finally, because the effects of endocannabinoids appeared to depend on CB₂ rather than CB₁ receptors, we investigated whether their effects were associated with neuroinflammatory mechanisms.

2. Materials and methods

2.1. Animals

Adult male mice C57Bl/6J were housed in a temperature-controlled environment on a 12 h light / 12 h dark cycle (light from 7 am till 7 pm). They were bred on-site and randomly housed in groups of four, directly after weaning (3 weeks of age). Mice were given free access to food and water. All experiments were conducted during the light period. Experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) for the care of laboratory animals.

2.2. Drugs and treatment

Adult male mice (about 4 months of age) received a single intraperitoneal (i.p.) injection of physiological saline or of a high dose of methamphetamine (Research Triangle Institute) (30 mg/kg), which aims to mimic an overdose of the drug. The fatty acid amide hydrolase inhibitor URB597 (synthesized at the University of Urbino Carlo Bo as previously reported) (Mor et al., 2004), the CB1 antagonist rimonabant (donated by the Research Triangle Institute, USA) and the CB2 antagonist AM630 (synthesized at Northeastern University) (1 mg/kg) were dissolved in 5% DMSO (Sigma, France), 5% Tween-80 (Sigma, France) and 90% sterile saline. The monoacylglycerol lipase inhibitor JZL184 (Interchim, France) (16 mg/kg) was dissolved in 20% DMSO (Sigma, France), 5% Tween-80 and 75% sterile saline.

9-Tetrahydrocannabinol (THC) (3 mg/kg) was dissolved in a solution of 5% ethanol, 5% Tween-80 and 90% physiological saline. Doses of each compound were chosen based on previously published papers (respectively: for URB597 (Kathuria et al., 2003; Moreira et al., 2008); JZL184 (Kinsey et al., 2011; Sumislowski et al., 2011); for AM630 and THC (Tourino et al., 2010)). Whereas some papers have used doses of rimonabant as high as 3 mg/kg in mice, this dose produces behavioral effects even in CB1 knock-out mice (Haller et al., 2002, 2004), suggesting that at such doses rimonabant produces non-specific effects likely related to its reported inverse agonist activity (Bergman et al., 2008). Therefore, we decided to use a dose of 1 mg/kg that is high enough to be effective in blocking the effects of exogenous cannabinoid (Solinas et al., 2003) while limiting the non-specific effects (Haller et al., 2002, 2004).

2.3. Measurement of endocannabinoids levels

For the detection of endocannabinoids, mice were treated with methamphetamine (30 mg/kg, i.p.) or physiological saline, and decapitated 1 h, 6 h, 12 h or 24 h after the treatment. Brains were removed and the striata were dissected on ice and frozen on dry ice, then tissues were subjected to lipid extraction with chloroform/ methanol (2:1, v/v), in the presence of d₈-AEA and d₈-2-AG as internal standards (Pucci et al., 2012). The organic phase was dried and then analyzed by liquid chromatography-electrospray ionization mass spectrometry (LCeESI-MS), using a single quadrupole API-150EX mass spectrometer (Applied Biosystem, CA, USA) in conjunction with a PerkinElmer LC system (PerkinElmer, MA, USA). Quantitative analysis was performed by selected ion recording over the respective sodiated molecular ions, as reported (Pucci et al., 2012).

2.4. Western blot analysis

To evaluate dopamine terminal loss, we measured tyrosine hydroxylase levels in the striatum 7 days after the drug treatment by Western Blot. Mice were treated with selective antagonists of CB₁ or CB₂ receptors (or their vehicle as a control) 1 h prior to methamphetamine injection. Inhibitors of monoacylglycerol lipase and fatty acid amide hydrolase (or their vehicle as a control) were administered 40 min prior to methamphetamine injection. THC (or its vehicle as a control) was administered 30 min prior to methamphetamine injection. Animals were sacrificed 7 days after drug treatment by decapitation. Brains were removed and striatum was dissected on ice, frozen on dry ice and stored at 80 °C until Western blotting was performed (Thiriet et al., 2011). Tissues were homogenized (350 ml/25 mg) by ultra-sonication in a 0.2 M TrisHCl (pH 6.9) buffer solution containing 8% SDS and 35% glycerol. Samples were then heated to 90 °C for 3 min and centrifuged at 4 °C for 10 min (13000 g). Supernatants were collected and analyzed using a DC protein assay kit (BioRad, France). Fifty mg of proteins per sample were loaded on an SDS-page gel, and then gels were transferred onto nitrocellulose membranes that were blocked for 1 h at room temperature in Tris-Buffered Saline (TBS) solution containing 3% Bovine Serum Albumin (BSA) and 0.1% Tween-20. Membranes were incubated with primary antibodies: mouse anti-tyrosine hydroxylase antibody (1:5000) (Immunostar, France) and rabbit anti-Actin antibody (1:10,000) (Sigma, France), diluted in the same buffer for 2 h at room temperature. Several rinses were made (3 × 10 min) with TBS containing 0.1% Tween-20 before incubation for 1 h with secondary antibodies: goat anti-rabbit coupled to Alexa Fluor 488, and goat anti-mouse coupled to Alexa Fluor 568 (1:1000, Invitrogen, France), diluted in the same buffer. Membranes were rinsed as described before and analyzed using Typhoon Imaging system (GE Healthcare). Image J software was used for quantification. Actin signal was used to normalize tyrosine hydroxylase signal, in order to avoid loading artifacts.

2.5. Enzyme-linked immunosorbent assay (ELISA)

For the ELISA experiments, mice were killed 24 h after methamphetamine injections. Striata were dissected and homogenized in 25 mM Tris buffer (pH 7.4), containing 150 mM NaCl and 1 mM EDTA. Commercially available ELISA kits were used in order to assess TNF alpha levels (sensitivity: 4 pg/mL), according to the manufacturer's instructions (BioLegend, Ozyme, St Quentin Yvelines, France). The range of analysis was between 4 and 500 pg/ml. Lysates were diluted (1:2) with the assay diluents and all steps were performed at room temperature, except for plate coating and samples incubation (4 °C). The enzymatic reaction was performed by incubating for 15 min with the tetramethylbenzidine substrate, and then was stopped by adding 2N H₂SO₄. The optical density (OD) was read at 450 nm within 30 min, using the Mithras LB940™ (Berthold) multimode microplate reader. The TNF alpha levels were then calculated by plotting OD values of each sample against the standard curve. For convenience all results were expressed as pg/mg protein.

2.6. Statistical analysis

All results are presented as group means (±SEM). Differences between groups were assessed by ANOVA. For the effects of methamphetamine on endogenous cannabinoid levels a one-way ANOVA was used with time after methamphetamine as a factor. For the

effects of THC on tyrosine hydroxylase protein levels a two-way ANOVA with pre-treatment (Veh or THC) and treatment (Sal or METH) as factors. For the effects of URB597 or JZL184 on methamphetamine-induced decreases in TH levels and increases in TNF-alpha levels, we used a one-way ANOVA with treatment as a factor. Results showing significant differences were subjected to Fischer LSD *post-hoc* test except for the data of the endocannabinoids levels that were subjected to a Dunnet's post-hoc test. The level of significance was always set at $p < 0.05$.

3. Results

3.1. Methamphetamine treatment modulates endocannabinoids levels in the striatum

In order to evaluate the impact of methamphetamine treatment on the levels of endocannabinoids in the striatum of mice, we measured AEA and 2-AG levels at different time points after an acute toxic methamphetamine treatment (30 mg/kg). We found that striatal AEA levels show two peaks one at 1 h and one at 12 h after methamphetamine injection ($p < 0.05$) (Fig. 1A), two time points that appear critical for the toxic effects of methamphetamine (see Discussion). Then, AEA levels returned to basal levels after 24 h. Instead, 2-AG levels slowly decreased after methamphetamine administration, and this decrease reached significance 12 h after drug injection ($p < 0.05$), returning to basal levels after 24 h (Fig.1B).

3.2. THC prevents methamphetamine-induced reduction of tyrosine hydroxylase levels in the striatum

To evaluate the protective role of endocannabinoids, mice were treated with the natural agonist of CB₁/CB₂ receptors THC (3 mg/kg), 30 min prior to methamphetamine (30 mg/kg) injection. After seven days, tyrosine hydroxylase levels were measured in the striatum by Western blotting in order to evaluate the long-term impact of methamphetamine treatment on dopamine neurotransmission (Fig. 2). Tyrosine hydroxylase levels of methamphetamine-treated mice were significantly reduced (by 40%) when compared to vehicle-treated animals ($p < 0.001$), a result that extends the known toxicity of methamphetamine for dopamine neurotransmission within the striatum (Bowyer et al., 2008). When mice were treated with THC prior to methamphetamine injection, their striatal tyrosine hydroxylase levels remained similar to those of control mice, and were significantly higher than those of mice that received methamphetamine alone ($p < 0.01$). Administration of THC alone had no effect on striatal tyrosine hydroxylase levels. These results indicate that THC pretreatment prevents methamphetamine-induced toxicity in the striatum.

3.3. Blockade of endocannabinoids degradation prevents methamphetamine-induced reduction of tyrosine hydroxylase levels in the striatum

To activate the ECS, we then used two different synthetic compounds: URB597, an inhibitor of the fatty acid amide hydrolase activity that increases brain levels of AEA (Kathuria et al., 2003), and JZL184, an inhibitor of the monoacylglycerol lipase activity that increases brain levels of 2-AG (Long et al., 2009). Again, striatal tyrosine hydroxylase levels were significantly reduced by methamphetamine injection, compared to controls (Figs. 3 and 4). Pretreatment with URB597 at the dose of 1 mg/kg completely prevented methamphetamine-

induced decreases in tyrosine hydroxylase striatal levels, suggesting that fatty acid amide hydrolase inhibition blocked methamphetamine-induced striatal dopaminergic terminal loss ($p < 0.001$) (Fig. 3). Importantly, URB597 produced similar protective effects on methamphetamine-induced reduction in striatal dopamine levels in the striatum (Supplementary Fig. 1) and these effects were independent from effects on methamphetamine-induced hyperthermia (Supplementary Fig. 2). Administration of JZL184 at a dose of 16 mg/kg 40 min prior to methamphetamine treatment also prevented methamphetamine-induced decrease in striatal tyrosine hydroxylase levels ($p < 0.001$) (Fig. 4). Incidentally, URB597 or JZL184 did not modify striatal tyrosine hydroxylase levels when used alone (Figs. 3 and 4, respectively).

3.4. CB₂, but not CB₁, receptors mediate the protective effects of URB597 and JZL184 against methamphetamine-induced toxicity

To further dissect the mechanisms by which endocannabinoids can provide neuroprotection against methamphetamine-induced dopamine terminal loss, we used selective antagonists of CB₁ and CB₂ receptors, namely rimonabant and AM630, which were injected 1 h prior to methamphetamine injection, and 20 min prior to URB597 or JZL184 injection. Blockade of CB₁ receptors by rimonabant (1 mg/kg) did not modify the protection against methamphetamine-induced decreases in tyrosine hydroxylase levels exerted by URB597 (Fig. 3) or JZL184 (Fig. 4) pre-administration, suggesting that this receptor subtype was not engaged in the neuroprotective effects of endocannabinoids. Conversely, when the CB₂ antagonist AM630 (1 mg/kg) was pre-administered, the beneficial effects of both URB597 ($p < 0.05$) (Fig. 3) and JZL184 ($p < 0.01$) (Fig. 4) on methamphetamine-induced decreases in striatal tyrosine hydroxylase levels were blunted, suggesting that the neuroprotective actions of endocannabinoids engaged mainly the CB₂ receptor subtype. Neither CB₁ nor CB₂ receptor antagonists affected methamphetamine-induced decreases in striatal tyrosine hydroxylase levels, when used alone (data not shown).

3.5. The protective effects of endocannabinoids are associated with a reduced striatal accumulation of TNF alpha after methamphetamine administration

Because CB₂ receptors are known to play a role in regulating neuroinflammatory reactions (Cabral et al., 2008), which are involved in methamphetamine-induced neurotoxicity (Kita et al., 2009; Goncalves et al., 2010), we measured striatal levels of the cytokine TNF alpha as a marker of neuroinflammation 24 h after methamphetamine injection and we evaluated whether ECS stimulation could modulate these levels (Fig. 5). As previously reported (Kita et al., 2009), we found that methamphetamine administration increased the level of TNF alpha (+100%, $p < 0.001$) in the striatum. This increase was blunted by pre-administration of THC, URB597 or JZL184 ($p < 0.01$), though TNF alpha levels remained slightly higher than those of controls ($p < 0.01$).

4. Discussion

In this study, we found that stimulation of the ECS prior to methamphetamine administration has protective effects against methamphetamine-induced neurotoxicity to dopamine neurons. Indeed, administration of a toxic dose of methamphetamine alters striatal levels of

endocannabinoids, the endogenous ligands of CB₁ and CB₂ receptors, and inhibition of the hydrolysis of these compounds prevents methamphetamine-induced reduction of tyrosine hydroxylase levels, a hallmark of dopamine terminal loss in the striatum (Cadet et al., 2003). In particular, it appears that the beneficial effects of endocannabinoids are mediated by stimulation of CB₂, rather than CB₁ receptors, and involve a reduction of neuroinflammatory processes triggered by methamphetamine.

4.1. Methamphetamine alters the ECS

In this study, we found that administration of a toxic dose (30 mg/kg) of methamphetamine altered striatal levels of endogenous cannabinoids in a time-dependent manner. First of all, we observed two peak increases in AEA striatal levels at 1 and 12 h after injection of methamphetamine, and these levels returned to basal values after 24 h. As discussed below, these time points have already been demonstrated to be crucial for methamphetamine-induced toxicity (Pereira et al., 2002; Bowyer et al., 2008; Krasnova and Cadet, 2009). Indeed, within the synapse, methamphetamine stimulates a massive release of dopamine as soon as 30 min after its injection, and this release is maximal after about 90 min (Pereira et al., 2002; Lan et al., 2009). Since endocannabinoids have been described to play a neuromodulatory role within the brain (Marsicano and Lutz, 2006), it could be speculated that the methamphetamine-induced dopamine release leads to the synthesis and release of AEA (Giuffrida et al., 1999), which would act retrogradely (Wilson and Nicoll, 2001) to produce a negative feedback on dopamine release (Melis et al., 2004). As a result, AEA would limit the damage triggered by dopamine oxidation, as well as by generation of reactive oxygen species (Cadet et al., 2003). Twelve hours after injection, methamphetamine toxicity reaches a peak in terms of striatal tyrosine hydroxylase immunoreactivity, and glial reactions become apparent (Bowyer et al., 2008); therefore, increases in AEA levels at this later time point may represent a second wave of reactions to help the system to recover from the toxic effects of methamphetamine.

Interestingly, at variance with what we found with AEA, the administration of methamphetamine did not produce an increase but rather a decrease in striatal 2-AG levels that became significant 12 h after methamphetamine administration. Because 2-AG has been suggested to play a house-keeping role that is necessary for basal functioning and survival of the cell (Piomelli, 2003), it could be speculated that the decreases in 2-AG levels represent a sign of cell damage that takes place 12 h after the injection of methamphetamine (Bowyer et al., 2008; Krasnova and Cadet, 2009). Therefore, the brain would respond to toxic insults induced by methamphetamine by releasing AEA and, if this reaction is not sufficient to avoid brain damage, subsequent toxicity and temporary malfunctioning would be associated with low levels of 2-AG.

4.2. Endocannabinoids provide neuroprotection against methamphetamine-induced dopamine terminal loss

To evaluate the potential protective effect of endocannabinoids against methamphetamine-induced degeneration of dopamine terminals, we stimulated the ECS by using a direct agonist of CB₁ and CB₂ receptors, or inhibitors of the degradation of their endogenous ligands AEA and 2-AG, previously used to increase endocannabinoids levels in the brain

(Kathuria et al., 2003; Long et al., 2009) and we measured striatal levels of tyrosine hydroxylase. In fact, in models of methamphetamine toxicity, tyrosine hydroxylase levels are often used as an index of loss of dopamine terminals and their reduction closely parallels depletion in dopa-mine levels measured by HPLC (Hayashi et al., 2001; Thiriet et al., 2005, and Supplementary Fig. 1). Administration of the CB₁/CB₂ receptors agonist THC significantly reduced methamphetamine-induced decreases in tyrosine hydroxylase levels. This finding is in agreement with previous cell culture studies that showed that THC could reduce the neurotoxicity induced by the neurotoxin 1-methyl-4-phenyl pyridinium (MPP⁺), which shares mechanisms of action with methamphetamine (Moldzio et al., 2012). In line with this, in an *in vivo* mouse model of Parkinson's disease, dopamine neurons are also protected against MPP⁺ neurotoxicity by the injection of WIN55,212-2, another non-specific CB₁/CB₂ receptor agonist (Price et al., 2009). Similarly, the administration of exogenous plant-derived cannabinoids (i.e., THC or cannabidiol) also reduces the dopaminergic toxicity induced by 6-OHDA lesion in rats, another model of Parkinson's disease (Lastres-Becker et al., 2005). Altogether, our results and literature data concur to suggest that THC injection could have neuroprotective properties, thus preventing damage to dopamine neurons.

The medical use of THC or other direct CB₁/CB₂ receptor agonists is complicated by their reinforcing effects and their well-known abuse liability. The discovery of the ECS has led to the development of new pharmacological tools that allow indirect stimulation of CB₁/CB₂ receptors by blocking metabolic degradation of AEA and 2-AG (Kathuria et al., 2003; Piomelli, 2003; Di Marzo et al., 2004; Long et al., 2009; Bisogno and Maccarrone, 2013). Although these compounds have not yet been tested in humans, several studies in animals suggest their lack of abuse liability (Gobbi et al., 2005; Solinas et al., 2006; Justinova et al., 2008). Interestingly, the administration of the fatty acid amide hydrolase inhibitor URB597 or of the monoacylglycerol lipase inhibitor JZL184 blocked the effects of the neurotoxic regimen of methamphetamine on striatal tyrosine hydroxylase levels. For URB597, this protection was also confirmed for methamphetamine-induced reductions in striatal dopamine levels (Supplementary Fig. 1). In addition, URB97 did not alter methamphetamine-induced hyperthermia suggesting that the effects of stimulation of the endogenous cannabinoid system are not simply secondary to non-specific effects on temperature (Supplementary Fig. 2). The protection by the endocannabinoids is consistent with previous studies showing that N-acylethanolamines, to which AEA belongs (Degn et al., 2007), and 2-AG (Melis et al., 2006) can protect neurons against oxidative stress due to ischemia, both *in vitro* and *in vivo*. These substances have also beneficial effects against excitotoxicity (Marsicano et al., 2003), a process that contributes with oxidative stress to the neurotoxicity of methamphetamine (Cadet et al., 2003; Krasnova and Cadet, 2009). Therefore, drugs that enhance the levels of endogenous CB₁/CB₂ receptor agonists may be more useful than THC for therapeutic use in humans, because they are devoid of risks of abuse (Gobbi et al., 2005; Solinas et al., 2006; Justinova et al., 2008).

4.3. Role of CB₂ receptors in the effects of endocannabinoids

In this study, the protective effects of the ECS against methamphetamine-induced toxicity appear to be mediated by CB₂ receptors. THC, AEA and 2-AG all activate both CB₁ and

CB₂ receptors, although AEA and 2-AG have a slightly higher affinity for CB₁ receptors (Pertwee et al., 2010). Our results are in agreement with previous literature data that document a neuroprotective function for CB₂ receptor within the brain (Fernandez-Ruiz et al., 2008; Viscomi et al., 2009; Onaivi et al., 2012). For example, a recent study has shown that protection provided by WIN55,212-2 against MPP⁺ toxicity is mediated by the stimulation of CB₂ receptors, since ablation of these receptors exacerbated the effects of the neurotoxin (Price et al., 2009). In addition, other studies have shown that CB₂ receptors stimulation reduce the neurotoxicity of MDMA (Torres et al., 2010; Tourino et al., 2010; Torres et al., 2011), a substituted amphetamine that produces toxicity in the brain with mechanisms that are largely overlapping on those induced by methamphetamine (Quinton and Yamamoto, 2006). These results suggest that CB₂ agonists may be beneficial in preventing the toxic consequences of overdose induced by methamphetamine or other amphetamine derivatives.

4.4. Neuroinflammation, endocannabinoids and methamphetamine

The prominent role of CB₂ receptors in the neuroprotective effects of endocannabinoids against methamphetamine toxicity suggests that the effects of endocannabinoids may depend on the reduction of methamphetamine-induced neuroinflammation. Indeed, endocannabinoids modulate inflammatory responses by regulating microglia function via receptor dependent and independent mechanisms, and consequently they control the generation of cytotoxic factors such as TNF alpha (Walter and Stella, 2004). In this study, we found that pretreatment with a CB₁/CB₂ receptor agonist, or with inhibitors of the degradation of their endogenous ligands (AEA and 2-AG), both reduce the accumulation of TNF alpha in the striatum 24 h after methamphetamine injection. In agreement with this finding, several studies have demonstrated the involvement of CB₂ receptors in the progression/arrest of brain damage, by influencing events such as microglial cell proliferation, differentiation and migration at neuroinflammatory lesion sites (Walter et al., 2003; Fernandez-Ruiz et al., 2008, 2011), for example in models of neurodegenerative diseases like Parkinson's and Huntington's diseases (Price et al., 2009; Fernandez-Ruiz et al., 2011). THC also reduces MDMA-induced microglial activation by acting on CB₂ receptors (Torres et al., 2010; Tourino et al., 2010). It is also noteworthy that CB₂ receptor expression increases in micro-glial cells in case of noxious conditions associated with inflammatory events (Fernandez-Ruiz et al., 2007). Interestingly, such inflammatory processes appear to be part of methamphetamine-induced toxicity, because this drug stimulates microglial activation in dopaminergic regions such as the striatum (Sekine et al., 2008), and increases TNF alpha levels (Nakajima et al., 2004; Kita et al., 2009). Altogether, these observations suggest that activation of CB₂ receptors may modulate neuroinflammatory responses induced by methamphetamine, may involved a reduction of TNF alpha production triggered by methamphetamine (Fernandez-Ruiz et al., 2008) and, consequently, limiting methamphetamine-induced neurotoxicity.

5. Conclusions

Altogether, the present results demonstrate that stimulation of the ECS prior to the administration of a neurotoxic dose of methamphetamine considerably reduced the toxicity

of this drug for dopamine terminals in the striatum, mainly by stimulating CB₂ receptors. These results highlight the importance of endocannabinoids as neuroprotective modulators against neurotoxic processes observed within the brain during some neurodegenerative diseases. Further investigation will be needed to determine whether similar neuroprotective effects could be found if the ECS is stimulated after the administration of methamphetamine.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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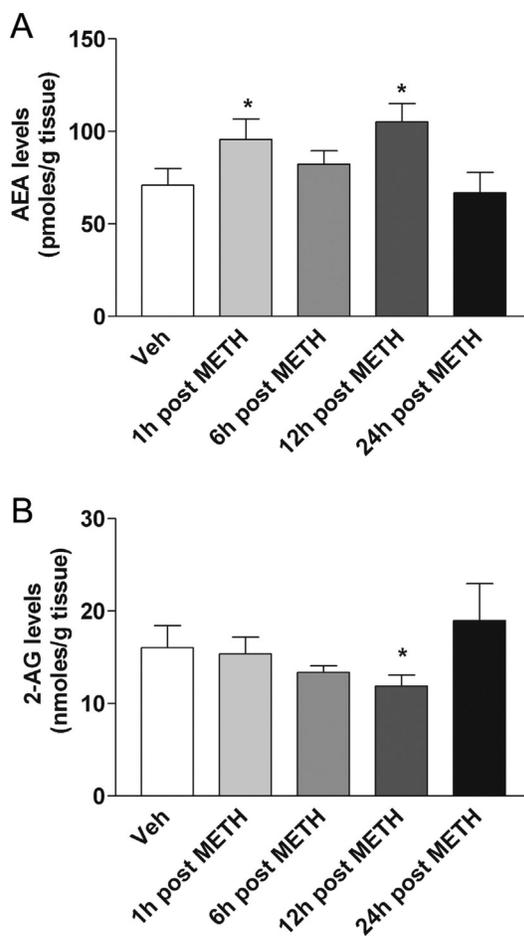


Fig. 1. AEA (A) and 2-AG (B) levels in the striatum at 1 h, 6 h, 12 h and 24 h after methamphetamine treatment (30 mg/kg). Columns represent the means, and vertical lines the standard error of the mean (SEM) ($n = 8-9$ mice per group). Administration of methamphetamine induced an increase in AEA levels 1 h and 12 h after drug injection (A), and a decrease in 2-AG levels 12 h after drug injection (B). One-way ANOVA followed by post-hoc Dunnett's test. * $p < 0.05$ compared values from the methamphetamine-treated mice to vehicle-treated mice.

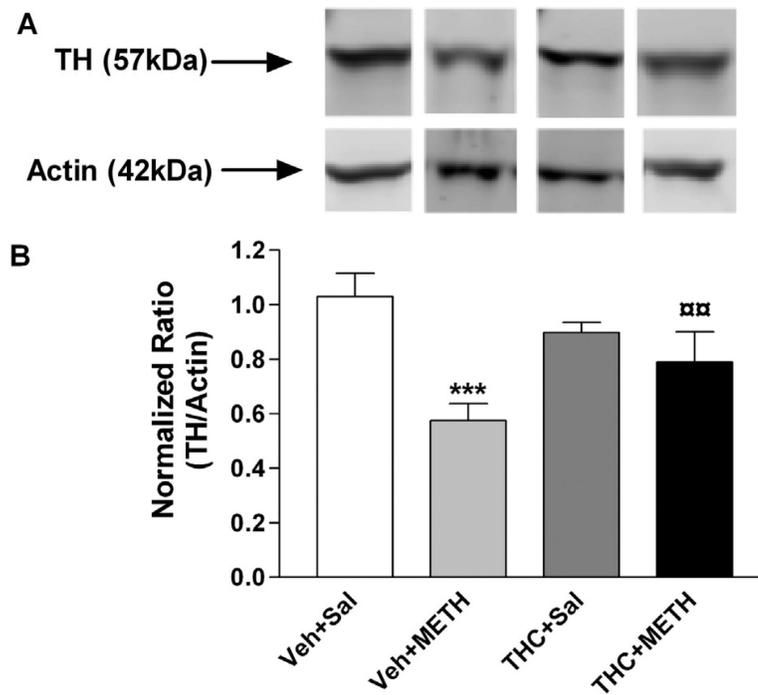


Fig. 2. Effect of THC pre-treatment on the striatal levels of tyrosine hydroxylase protein in mice treated with methamphetamine. (A) Representative immunoblots of levels of tyrosine hydroxylase (TH) in the striatum of mice treated with vehicle (NaCl 0.9%), THC (3 mg/kg), methamphetamine-HCl (METH; 30 mg/kg), or with THC (3 mg/kg) 30 min prior to methamphetamine injection, and killed 7 days later. (B) Graphical representation of tyrosine hydroxylase levels (data are expressed as means + SEM, and $n = 6-7$ mice per group). Administration of methamphetamine induced a decrease in the striatal tyrosine hydroxylase expression of about 40%, and THC pre-administration blunted this decrease. Two-way ANOVA followed by *post-hoc* Fischer LSD's test. *** $p < 0.001$ compared with vehicle-treated group. ** $p < 0.01$ compared with methamphetamine-treated group.

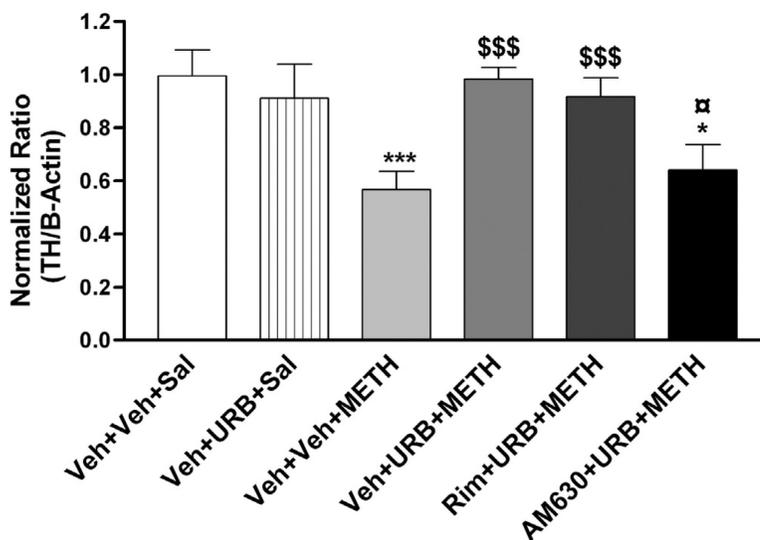


Fig. 3.

Effect of pre-treatment with URB597 and cannabinoid receptors antagonists on the striatal levels of tyrosine hydroxylase protein of mice treated with methamphetamine. Graphical representation of the striatal tyrosine hydroxylase protein levels (data are expressed as means + SEM, and $n = 6-9$ mice per group). Administration of methamphetamine (METH; 30 mg/kg) induced a decrease of tyrosine hydroxylase expression, and pre-administration of URB597 (1 mg/kg) 40 min before blunted this decrease. The prior administration (1 h before methamphetamine) of the CB₂ antagonist AM630 (1 mg/kg), but not of the CB₁ antagonist rimonabant (1 mg/kg), abolished the beneficial effect of URB597. One-way ANOVA followed by *post-hoc* Fischer LSD's test. * $p < 0.05$ and *** $p < 0.001$ compared to vehicle-treated group. \$\$\$ $p < 0.001$ compared with methamphetamine-treated group. □ $p < 0.05$ compared to Vehicle-URB-methamphetamine-treated animals.

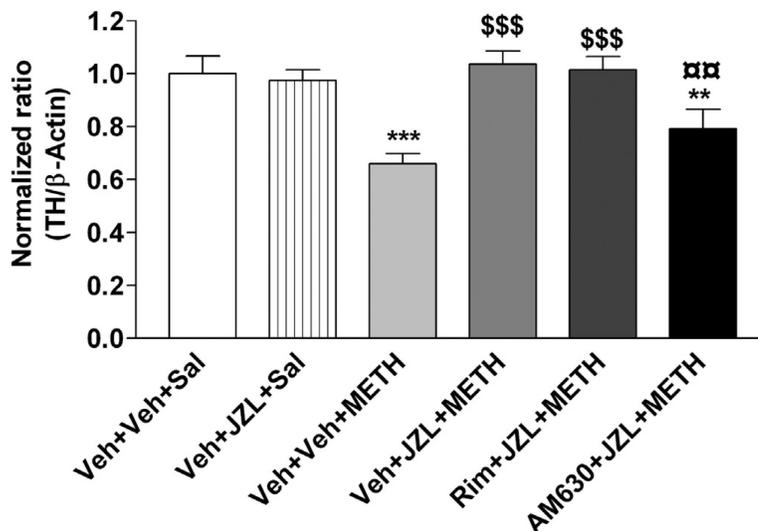


Fig. 4. Effect of pre-treatment with JZL184 and cannabinoid receptors antagonists on the striatal levels of tyrosine hydroxylase protein of mice treated with methamphetamine. Graphical representation of the striatal tyrosine hydroxylase protein levels (data are expressed as means + SEM, and $n = 6-7$ mice per group). Administration of methamphetamine (METH; 30 mg/kg) induced a decrease of tyrosine hydroxylase expression, and pre-administration of JZL184 (16 mg/kg) 40 min before blunted this decrease. The prior administration (1 h before methamphetamine) of the CB₂ antagonist AM630 (1 mg/kg), but not of the CB₁ antagonist rimonabant (1 mg/kg), abolished the beneficial effect of JZL184. One-way ANOVA followed by *post-hoc* Fischer LSD's test. ** $p < 0.01$ and *** $p < 0.001$ compared to vehicle-treated group. \$\$\$ $p < 0.001$ compared with methamphetamine-treated group. □□ $p < 0.01$ compared to Vehicle-JZL-methamphetamine-treated animals.

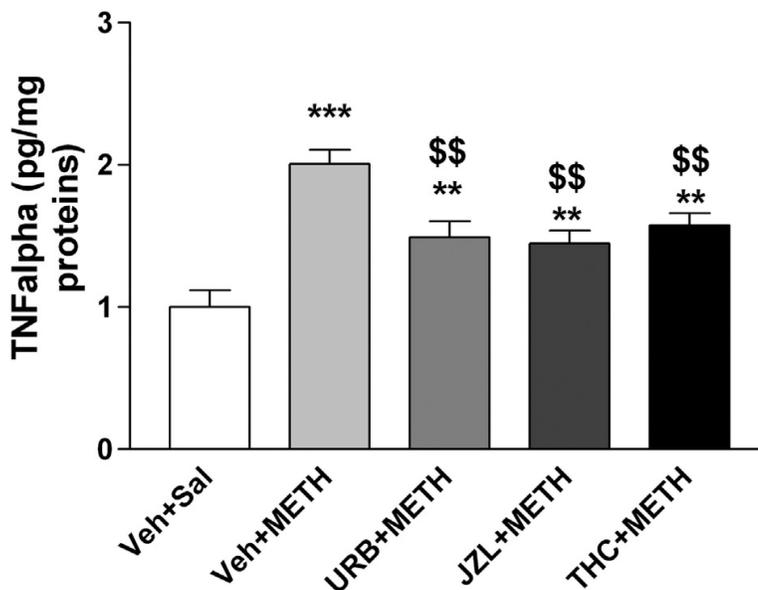


Fig. 5. Changes in TNF alpha striatal levels in response to methamphetamine, alone or in combination with cannabinoid receptors agonists

Graphical representation of the TNF alpha levels (data are expressed as means + SEM, and $n = 7-8$ mice per group). Administration of methamphetamine (METH; 30 mg/kg) increased TNF alpha tissue concentration and pre-administration of THC (3 mg/kg) 30 min before, and of URB597 (1 mg/kg) or JZL184 (16 mg/kg) 40 min before, reduced this increase. One-way ANOVA followed by post-hoc Fischer LSD's test. ** $p < 0.01$ and *** $p < 0.001$ compared to vehicle-treated group. \$\$ $p < 0.01$ compared to methamphetamine-treated group.