Fatty acid amide hydrolase inhibition for the symptomatic relief of Parkinson's disease

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Abbreviations

2-AG: 2-arachidonoyl glycerol

AEA: anandamide

CB₁: cannabinoid receptor type 1

CB₂: cannabinoid receptor type 2

CNS: central nervous system

DOPAC: 3,4-Dihydroxyphenylacetic acid

DA: dopamine

D₂: dopamine receptor subtype 2

ECS: endocannabinoid system

eCBs: endocannabinoids

FAAH: fatty acid amide hydrolase

GFAP: glial fibrillary acidic protein

GPe: external segment of the globus pallidus

HVA: homovanillic acid

Iba-1: ionized calcium-binding adapter molecule 1

LTD: long-term depression

MAGL: monoacylglycerol lipase

MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

MPTPp: MPTP plus probenecid

NAEs: N-acylethanolamines

OEA: oleoylethanolamine

PEA: palmitoylethanolamine

PD: Parkinson's disease

SNpc: *substantia nigra pars compacta*

TH: tyrosine hydroxylase

TRPV1: transient receptor potential vanilloid-type 1

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ABSTRACT

Elements of the endocannabinoid system are strongly expressed in the basal ganglia where they suffer profound rearrangements after dopamine depletion. Modulation of the levels of the endocannabinoid 2-arachidonoyl glycerol by inhibiting monoacylglycerol lipase alters glial phenotypes and provides neuroprotection in a mouse model of Parkinson's disease. In this study, we assessed whether inhibiting fatty acid amide hydrolase could also provide beneficial effects on the time course of this disease. The fatty acid amide hydrolase inhibitor, URB597, was administered chronically to mice treated with probenecid and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTPp) over 5 weeks. URB597 (1 mg/kg) prevented MPTPp induced motor impairment but it did not preserve the dopamine levels in the nigrostriatal pathway or regulate glial cell activation. The symptomatic relief of URB597 was confirmed in haloperidol-induced catalepsy assays, where its anti-cataleptic effects were both blocked by antagonists of the two cannabinoid receptors (CB₁ and CB₂), and abolished in animals deficient in these receptors. Other fatty acid amide hydrolase inhibitors, JNJ1661010 and TCF2, also had anti-cataleptic properties. Together, these results demonstrate an effect of fatty acid amide hydrolase inhibition on the motor symptoms of Parkinson's disease in two distinct experimental models that is mediated by cannabinoid receptors.

HIGHLIGHTS

- FAAH inhibition reverses the motor symptoms in Parkinson's disease.
- The anti-parkinsonian effects of URB597 involve CB₁ and CB₂ receptors in the indirect pathway.
- AEA and 2-AG interact with the dopaminergic circuit by different mechanisms of action.

KEYWORDS

Parkinson's disease, endocannabinoid system, FAAH, URB597, CB₁ receptor, CB₂ receptor.

1. INTRODUCTION

Different elements of the endocannabinoid system (ECS) are expressed strongly in the basal ganglia, where they are involved in motor control (Fernandez-Ruiz, 2009; Fride and Mechoulam, 1993; Herkenham et al., 1991). Profound rearrangements of the ECS have been described after dopamine (DA) depletion (Pisani et al., 2011). Indeed, in experimental models of Parkinson's disease (PD) there is more mRNA encoding the cannabinoid receptor type 1 (CB₁), the CB₁ receptor-mediated signaling is enhanced (Lastres-Becker et al., 2001; Romero et al., 2000), the levels of anandamide (N-arachidonoyl-ethanolamine, AEA) increase in the external segment of the globus pallidus (GPe) and in the striatum (Di Marzo et al., 2000; Gubellini et al., 2002; van der Stelt et al., 2005), and fatty acid amide hydrolase (FAAH) activity is reduced (Gubellini et al., 2002). Significantly, DA-replacement therapy returns the elevated AEA levels to control levels in PD patients (Pisani et al., 2010). Regardless of whether these changes contribute to movement suppression or are compensatory mechanisms to counterbalance DA depletion, it is clear that the ECS is affected in parkinsonian conditions and its modulation is a promising means to improve PD motor symptoms and/or PD-related neurodegeneration.

Administration of cannabinoids induces a wide range of effects in both, PD animal models and patients (Kluger et al., 2015). Although CB₁ receptor agonists potentiate bradykinesia in the MPTP primate model of PD (Meschler et al., 2001), studies in the 6-OHDA rat model and in the marmoset model have shown that such agonists improve motor impairment (Sanudo-Pena et al., 1998; van Vliet et al., 2008). On the other hand, CB₁ receptor antagonists have shown to consistently improve amphetamine-induced turning behavior, hypokinesia and bradykinesia in the 6-OHDA rat model (Cao et al., 2007; Fernandez-Espejo et al., 2005; Gonzalez et al., 2006; Kelsey et al., 2009) and to enhance the anti-parkinsonian action of levodopa (L-DOPA) in the MPTP monkey model (Cao et al., 2007; Fernandez-Espejo et al., 2005; Gonzalez et al., 2006; Kelsey et al., 2009). In humans, there is evidence that cannabinoids may improve motor and

non-motor symptoms of (Chagas et al., 2014a; Lotan et al., 2014; Zuardi et al., 2009), although benefits for motor symptoms are not always seen and there have been mixed results regarding L-DOPA-induced dyskinesia (Carroll et al., 2004; Chagas et al., 2014b; Mesnage et al., 2004; Sieradzan et al., 2001). Both agonists and antagonists of CB₁ receptors modulate the effects of L-DOPA, the main drug used in replacement therapy, and they may therefore be useful to treat dyskinesia (Martinez et al., 2012; Segovia et al., 2003).

Cannabinoids are considered to be neuroprotective agents, and they appear to exert their neuroprotective effects in animal models of PD through cannabinoid receptor-dependent and -independent mechanisms (Garcia et al., 2011; Lastres-Becker et al., 2005; Price et al., 2009). Hence, to identify ECS-modulating agents capable of providing therapeutic or neuroprotective benefits in patients with movement disorders, it is necessary to better understand cannabinoids mode of action, pharmacology and pharmacokinetics.

AEA and 2-arachidonoyl-glycerol (2-AG) are endogenous ligands of cannabinoid CB₁ and CB₂ receptors (Devane et al., 1992; Di Marzo et al., 2002; Mechoulam et al., 1995). The level of these endocannabinoids (eCB) in the central nervous system (CNS) is influenced by two catabolic enzymes, FAAH and MAGL (Cravatt et al., 1996; Nomura et al., 2008). Selective and efficacious inhibitors of FAAH and MAGL have been developed, and they produce a specific increase in eCB concentrations *in vivo* (Fegley et al., 2005; Long et al., 2009). Thus, inhibiting these enzymes constitutes an indirect strategy to modulate the ECS and this approach may have better outcomes than the use of cannabinoid receptor ligands (Chhatwal et al., 2005; Gaetani et al., 2003; Marsicano et al., 2003; Van Sickle et al., 2005). Specific inhibition of MAGL with JZL184 has a neuroprotective effect in animal models of PD due to the activation of a neuroprotective glial phenotype and a decrease in prostaglandin synthesis (Fernandez-Suarez et al., 2014; Nomura et al., 2011). Inhibition of FAAH has been investigated to characterize the modulation of DA receptor-mediated motor responses by eCBs (Kreitzer and Malenka, 2007).

However, the specific effect of FAAH inhibition on the motor symptoms of PD remains to be defined. Thus, this study was set out to determine whether enhancing AEA levels by inhibiting FAAH has beneficial effects on PD symptoms and/or prevents PD-related neurodegeneration. In the chronic MPTP mouse model, a five-week treatment with a specific FAAH inhibitor, URB597, significantly improved the motor symptoms of these animals without any sign of nigral neurorestoration. The symptomatic effect was further characterized in haloperidol-induced cataleptic animals treated with cannabinoid and vanilloid receptor antagonists, and it was shown that CB₁ and CB₂ but not the transient receptor potential vanilloid-type 1 (TRPV1) receptors were involved in the reversal of catalepsy provoked by different FAAH inhibitors. These results point to FAAH inhibition as a promising non-dopaminergic therapeutic strategy for the symptomatic treatment of PD.

2. MATERIALS AND METHODS

2.1. Animals

Adult (3 month old) male C57BL/6J mice (25-30 g) age were obtained from Charles River (Barcelona, Spain) and 5 mice per cage were housed at 21°C in a humidity-controlled environment, on a 12/12 h light/dark cycle (lights on at 8 am), with *ad libitum* access to food and water. All the procedures involving animals were carried out in accordance with the EU Directive 2010/63/EU governing the care and use of laboratory animals. The experimental design was approved by the Ethical Committee for Animal Testing of the University of Navarra. Male, 7 month old CB₁ receptor knockout (KO) mice (CB₁^{-/-}) and their wild type (CB₁^{+/+}) littermates (24-26 g), and male CB₂ receptor knock-out mice (CB₂^{-/-}) and their wild type (CB₂^{+/+}) littermates (30-35 g) of the same age were also used in these experiments. The mice lacking CB₁ cannabinoid receptor were generated on a C57BL/6N congenic background, as described previously (Marsicano et al., 2002). The CB₁ KO mouse on a C57BL/6N genetic background was kindly donated by Giovanni Marsicano (Marsicano et al., 2002). These animals were backcrossed to C57BL/6NCrl for 8 generations and generated by heterozygous breeding to avoid possible genetic differences in maternal care. The CB₂ KO mice on a C57BL/6J genetic background were kindly provided by Nancy E. Buckley (Cal. State Polytechnic University, Pomona, CA) (Buckley et al., 2000). These animals were backcrossed to C57BL/6J for 3 generations and share 93.75% of genetic background with their congenic strain.

2.2. Chronic MPTP mouse model

To induce a bilateral and progressive but partial dopaminergic lesion, mice received 10 intraperitoneal (i.p.) injections of MPTP hydrochloride (20 mg/kg in saline: Sigma-Aldrich, St. Louis, MO, USA) plus probenecid (250 mg/kg in saline: Invitrogen, Paisley, UK), administered twice a week over 5 weeks (Fig. 1A). Probenecid and MPTP (MPTPp) were administered in two consecutive injections, while control animals received probenecid and saline (Braun, Barcelona, Spain) on an identical administration regime. To be able to compare the effect of

FAAH inhibition with MAGL inhibition in MPTPp treated mice the administration regime of the inhibitor was the same used previously (Fernandez-Suarez et al., 2014). This regime (Fig. 1A) was selected after observing in a pilot experiment that animals receiving MPTPp treatment for 5 weeks and daily injections of the inhibitor got ulcers in the area of administration. Therefore, one-half of the MPTPp-treated animals and one-half of the control animals were i.p. injected with 1 mg/kg URB597 (Merck Millipore, Darmstadt, Germany) 5 days per week along the 5 weeks of MPTPp treatment (Fig.1A). The second half of animals was treated with the vehicle of URB597, i.e. with saline containing 15% dimethylsulfoxide (DMSO, Sigma-Aldrich), 5% PEG (Sigma-Aldrich) and 5% Tween-80 (Guinama, Valencia, Spain). Mice receiving 5 doses of the inhibitor per week did not show any obvious alteration in the administration areas. Mice were sacrificed 48 h after the last MPTPp administration (40 h after the last URB597/vehicle administration).

2.3. Haloperidol-induced catalepsy mouse model

Catalepsy was induced in naïve animals with an i.p. injection of haloperidol (Hal, 1 mg/kg; Sigma-Aldrich) dissolved in 1.3% DMSO and 1.3% Tween-80. Two hours later, distinct drugs were administrated (i.p.): SCH442416 (1 mg/kg, Tocris, Bristol, UK), an adenosine receptor subtype 2 (A_{2A}) antagonist used as anti-cataleptic positive control; URB597 (1 mg/kg); JZL184 (8 mg/kg, Cayman Chemical, Ann Arbor, MI, USA); Rimonabant (3 mg/kg, Cayman Chemical); AM630 (3 mg/kg, Tocris); capsazepine (10 mg/kg, Tocris); WIN55,212-2 (3 mg/kg, Tocris); JNJ1661010 (1 mg/kg, Sigma); and TCF2 (40 mg/kg, Tocris). The cataleptic status was evaluated in the bar test 60 min later (180 min after haloperidol injection).

2.4. Behavioral tests

All the behavioral tests using the MPTPp mice were performed under low light conditions 24 h after the last MPTP and probenecid injection, and 16 h after the last URB597 (or vehicle) administration. In the rotarod assay (LE8200, Panlab, Barcelona, Spain), the animals were placed on the rod and the rotarod was programmed to rotate at a speed increasing linearly

from 4 to 40 r.p.m. over 5 min. The time each mouse remained on the rod was recorded for a maximum of 5 min. All animals were pre-trained before MPTPp intoxication until they were able to remain on the rod for at least 60 s. The average permanence was calculated from 3 consecutive trials with a resting time of 30 min between each of them. For the pole test, the animals were placed with their head facing upwards on the top of a 50 cm high and 1 cm diameter vertical wooden pole covered with a bandage. All the animals were pre-trained before MPTPp intoxication until they were able to reverse their position and to descend the pole head first in less than 5 s. The animal's movement was recorded and all videos were analyzed blindly. The average time taken to turn their head and descend the entire pole was measured in 3 trials with a resting time of 30 min between each of them. Cataleptic status was evaluated using the bar test 60 min after the last WIN55,212-2 or URB597 administration. Animals were placed with their forepaws on a cylindrical metal bar oriented parallel to the ground at a height of 4 cm. The time elapsed before animals moved from this position was the parameter measured, with a maximum permitted time of 1 min on the bar per trial. The rectal temperature of the mice was measured and analgesia was evaluated using the tail-flick test, in which mice were placed in an animal restrainer while the tail was immersed into pre-heated water (52°C), measuring the time that passed before the animal flicked its tail. Locomotor activity was evaluated in the open field, placing individual mice into a plastic box divided into four equal quadrants. The movement of each mouse was recorded over 5 min using an infrared camera connected to the SMART software (Panlab), allowing the total distance moved by each animal to be calculated.

2.5. Tissue processing

Animals were sacrificed by cervical dislocation for biochemical detections or by transcardial perfusion for histological analysis. For biochemical techniques, the brains were rapidly removed, frozen on dry ice and stored at -80°C. For histological techniques, mice were anesthetized with 10% chloral hydrate (Sigma-Aldrich) and perfused transcardially with 4%

paraformaldehyde (Merck, Darmstadt, Germany) in 0.125 M phosphate buffered saline (PBS, pH 7.4). The brain was then removed and cryoprotected for 48 h in 20% glycerol and 2% DMSO in PBS.

2.6. Dopamine quantification

Striatal concentrations of DA, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were determined by high performance liquid chromatography (HPLC) with electrochemical detection, as described previously (Goni-Allo et al., 2006).

Briefly, samples were injected using an automatic sample injector (Waters 717 plus, Waters Cromatografía, S.A., Barcelona, Spain) onto a Spherisorb ODS-2 reverse phase C¹⁸ column (5 m, 150 x 4.6 mm; Teknokroma, Barcelona, Spain) connected to a DECADE amperometric detector (Antec Leyden, Zoeterwoude, the Netherlands), with a glassy carbon electrode maintained at 0.7 V with respect to a Ag/AgCl reference electrode. The mobile phase consisted of 0.1 M citric acid, 0.1 M Na₂HPO₄, 0.74 mM octanesulphonic acid, 1 mM EDTA and 16% methanol (pH 3.4), pumped at a flow rate of 1 mL/min.

2.7. Mass spectrometry

Lipid extraction, and N-acylethanolamines (NAEs) and 2-AG determination, were performed as described previously, except that electrospray ionization was used instead of atmospheric pressure chemical ionization (Wang et al., 2012).

2.8. Histological techniques

Immunohistochemistry was performed on free floating sections (40 µm thick) and in a given experiment, all the sections were processed at the same time with each primary antibody. Sections were washed with PBS and endogenous peroxidase activity was inactivated by incubating them for 30 min with 0.03% H₂O₂ (Sigma-Aldrich) in methanol (Panreac, Barcelona, Spain). After washing 3 times with PBS, the tissue was incubated for 40 min with blocking solution [4% normal goat serum, 0.05% Triton X-100 (Sigma-Aldrich) and 4% BSA (Merck, Darmstadt, Germany) in PBS], and exposed overnight to the primary antibodies diluted in

blocking solution at room temperature (RT). The primary antibodies used were: rabbit anti-tyrosine hydroxylase (TH, 1:1000; Millipore, Temecula, CA, USA), rabbit anti-glial fibrillary acidic protein (GFAP, 1:500; Dako, Glostrup, Denmark) and rabbit anti-ionized calcium-binding adapter molecule 1 (Iba1, 1:500; Wako, Osaka, Japan). Antibody binding was detected by incubating the sections for 2 h at RT with a biotinylated goat anti-rabbit secondary antibody (1:1000; Jackson ImmunoResearch Laboratories, West Gore, PA, USA) diluted in blocking solution. The biotinylated antibodies were detected with peroxidase-conjugated avidin (1:5000; Sigma-Aldrich) for 90 min at RT followed by incubation with 0.05% diaminobenzidine (Sigma-Aldrich) in 0.03% H₂O₂/Trizma-HCl buffer [pH 7.6]. Sections were mounted on glass slides in a 0.2% solution of gelatin in 0.05 M Tris-HCl buffer [pH 7.6] (Sigma-Aldrich), dried and dehydrated in toluene (Panreac) for 12 min before coverslipping with DPX (BDH Chemicals, Poole, UK).

2.9. Image analysis

For a given experiment, immunostaining of the different samples was performed simultaneously and the microscope images were captured using the same parameters. Colorimetric images were digitized in a grey scale under constant light conditions using a digital camera (DXM1200F) coupled to an Eclipse microscope (Nikon, Barcelona, Spain). Sections were captured at 20X magnification and in each section, two portions (580 x 424 μ m) of the left and the right striatum, 0.38 mm from Bregma, according to the Mouse Brain Atlas (Paxinos and Franklin, 2001) were analyzed blindly using Metamorph image analysis software (Molecular Devices, Sunnyvale, CA). A fixed threshold was applied to all the images and the proportion of the pixels detected was calculated automatically for each image.

2.10. Stereological analysis

The number of TH-positive neurons present in the substantia nigra pars compacta SNpc was determined by unbiased design-based stereology. Stereological counting was performed on 6 coronal SNpc sections (40 μ m thick) taken at uniform intervals (120 μ m) that covered the

entire rostrocaudal extent of the nucleus between -2.92 mm and -3.64 mm relative to Bregma (Paxinos and Franklin, 2001). The reference volume of the SNpc was calculated from the images obtained with the 2X objective using a point count array according to Cavalieri principles (Gundersen and Jensen, 1987). The cross-sectional area of the nucleus was measured and the reference volume (V_r) for the entire SNpc was estimated using the following equation:

$$V_r = T \frac{a}{p} \sum P_i$$

where T is the section thickness, a/p is the area of each point and P_i is the number of points falling within the SNpc. All stereological counting was performed with newCAST Visiopharm software (Hoersholm) using a Bx61 microscope (Olympus, Hicksville, NY, USA) equipped with a DP71 camera (Olympus) and a stage connected to a xyz stepper (H101BX, PRIOR). The SNpc was outlined at low magnification (4X) to estimate the area, and the number of labeled neurons was calculated at 100X magnification under oil immersion using randomized meander sampling and the optical dissector methods. The optical dissector height was 11 μm to count 100-150 cells per animal using a sampling frame of 5,294 μm^2 and sampling steps of 145 μm x 145 μm (dx,dy). Unbiased counting was performed blindly and the total number of TH-positive neurons (N) was calculated using the following formula:

$$N = \sum Q^- \frac{t}{h} \frac{1}{asf} \frac{1}{ssf}$$

where $\sum Q^-$ is the total number of particles counted, t is the mean section thickness, h is the height of the optical dissector, asf is the area sampling fraction, and ssf is the section sampling fraction. Neuronal density (D) was determined using the formula: $D = N/V_r$.

2.11. Statistical analysis

The data are presented as the mean $\pm$ SEM. Normality was analyzed with the Kolmogorov–Smirnov test and the data following a normal distribution were analyzed by means of one-way

ANOVA followed by Tukey's multiple comparisons test, two-way ANOVA followed by Bonferroni's post-hoc test, or with a t-test. The data that do not follow a normal distribution were evaluated using the Mann-Whitney test. Data were analyzed using GraphPad Prism 5 (La Jolla, CA, USA) and in all instances, p values < 0.05 were considered statistically significant.

3. RESULTS

3.1. Effect of chronic URB597 treatment on MPTPp mice

URB597 is a specific inhibitor of FAAH (Fegley et al., 2005), the predominant catabolic enzyme of AEA in the CNS (Cravatt et al., 2001). It has been reported that this inhibitor provides a rapid onset (<15 min) and persistent (>12 h) FAAH inhibition (Piomelli et al., 2006). The effect of FAAH inhibition with URB597 was evaluated in the chronic MPTPp mouse model of PD. In this model, a characteristic partial, bilateral and progressive dopaminergic lesion is induced by 5 weeks of combined treatment with MPTP and probenecid (Petroske et al., 2001). Probenecid reduces the renal clearance of MPTP and its metabolites, provoking a sustained toxic response (Lau et al., 1990). Starting on the same day as the first MPTPp injection and with a delay of 8 h, URB597 (1 mg/kg) was administered to MPTPp animals for 5 days a week over 5 weeks. MPTPp and either URB597 or vehicle were administered (i.p.) to animals in the MPTPp group, while animals in the control groups were injected (i.p.) with probenecid/saline and either URB597 or vehicle (see Material and Methods and Fig. 1A). Motor behavior was evaluated 24 h after the last MPTPp administration (16 h after the last injection of URB597 or vehicle) using the pole test (Fig. 1B). Analysis of the data using two-way ANOVA demonstrated a significant interaction between the two factors, MPTPp lesion and URB597 treatment ($F_{1,46} = 13.3$, $p = 0.0007$). Indeed, MPTPp mice took significantly longer to descend the 50 cm pole than the controls and, while URB597 administration did not affect the performance of control mice, URB597-treated MPTPp mice performed significantly better than MPTPp mice in this test (Fig. 1B). A two-way ANOVA analysis of data from the rotarod test also revealed a significant interaction between the MPTPp lesion and the URB597 treatment ($F_{1,46} = 9.8$, $p = 0.003$). As

such, while MPTPp administration significantly reduced the time mice could remain on the rotarod compared to the controls, URB597 administration significantly increased the time the MPTPp mice stayed on the rod, although it had no effect on the performance of the control animals (Fig. 1C). These results indicate that the inhibition of FAAH through chronic URB597 administration significantly improved the motor behavior of MPTPp mice.

A neuroprotective effect of URB597 could be responsible for the motor benefits observed in MPTPp mice. To test this possibility, the preservation of the nigrostriatal pathway was evaluated by TH immunostaining (Figs. 2A and 2B). At the striatal level (Fig. 2A), the density of TH-positive terminals was analyzed (Fig. 2C) and while a two-way ANOVA revealed a significant effect of MPTPp administration ($F_{1,26} = 105.8$, $p < 0.0001$), there were neither interactions nor differences between the groups receiving URB597 (Fig. 2C). In fact, MPTPp administration significantly reduced the number of striatal TH-positive terminals and this effect was not reversed by chronic URB597 treatment. The number of TH-positive neurons in the SNpc was quantified by unbiased stereology and a two-way ANOVA for stereological counting demonstrated a significant effect of MPTPp injection ($F_{1,15} = 20$, $p < 0.0001$) but neither differences between the groups treated with the FAAH inhibitor, nor interaction effect were observed (Fig. 2D). Injection of MPTPp induced a significant decrease in the number of TH-positive neurons in the SNpc, which was unaffected by the chronic administration of URB597. These results indicate that FAAH inhibition does not provide neuroprotection to dopaminergic neurons.

We addressed the possibility that URB597 could upregulate DA levels in the remaining striatal dopaminergic terminals by assessing the striatal levels of DA and its metabolites in samples obtained 48 h after the last MPTPp injection (40 h after the last injection of URB597 or vehicle). One-way ANOVA showed a significant decrease of DA (Fig. 3A; $F_{2,22} = 242.9$, $p < 0.0001$), DOPAC (Fig. 3B; $F_{2,22} = 61.8$, $p < 0.0001$) and HVA (Fig. 3C; $F_{2,22} = 19.3$, $p < 0.0001$) in MPTPp mice, while URB597 administration did not augment the striatal DA levels in lesioned

animals (Fig. 3A, 3B and 3C). These data indicate that URB597 administration to MPTPp animals does not result in increased striatal DA levels.

As any MPTP lesion provokes glial cell recruitment and activation, the status of glial cells was analyzed after the distinct treatments. Immunohistochemistry for GFAP revealed a small number of reactive astrocytes in the striatum of control animals and a marked increase of the staining in those that received MPTPp and MPTPp plus URB597 (Suppl. Fig. 1A). Two-way ANOVA of the densitometry data revealed a significant effect on the area of GFAP immunoreactivity in the striatum when animals received MPTPp ($F_{1,26} = 154.3$, $p < 0.001$) that was not affected by administration of URB597 (Suppl. Fig. 1B). Microglia was analyzed by immunohistochemistry using an antibody against Iba1, a specific marker of microglia (Suppl. Fig. 2A). Subsequent stereological analysis showed differences between control animals and mice receiving MPTPp. A significant effect of MPTPp administration was detected in microglial cell density (Suppl. Fig. 2B; $F_{1,18} = 6$, $p = 0.02$) and in the length of the microglial ramifications (Suppl. Fig. 2D; $F_{1,16} = 5.2$, $p < 0.03$). URB597 treatment did not revert the cell density and ramification changes induced by MPTP. In contrast, two-way ANOVA of data on cell body area revealed a significant interaction between the MPTPp lesion and URB597 administration (Suppl. Fig. 2C; $F_{1,18} = 10.1$, $p = 0.005$). In fact, the post hoc test showed that the increase in cell body area in MPTPp animals was significantly reduced when MPTPp mice were treated with URB597.

FAAH regulates the concentration of NAEs, including the endocannabinoid AEA and the non-cannabinoid NAEs: N-palmitoylethanolamine (PEA) and N-oleoylethanolamine (OEA). NAEs concentrations were determined in the midbrain and the cerebellum. Midbrain AEA (Fig. 4A) and OEA (Fig. 4B) levels were unaffected in the control mice and those that received MPTPp, whereas PEA levels decreased significantly in MPTPp mice (Fig. 4C). This decrease in PEA concentration was not observed in the cerebellum of MPTPp-treated animals. The MPTPp treatment led to a decrease of 2-AG levels in the cerebellum but not in the midbrain (Figs. 4D

and 4H). The effect of MPTP is qualitatively different in the two brain areas analyzed. The effect of URB597 administration was also tested in midbrain and cerebellum from animals sacrificed 48 h after the last administration of MPTPp (40 h after the last injection of URB597 or vehicle). URB597 induced a significant increase in the concentration of AEA in the midbrain from naïve animals. A greater increase in the levels of AEA, OEA and PEA was observed in the midbrain from MPTPp-lesioned mice (Fig. 4A-C). These changes were qualitatively similar but of lower magnitude in the cerebellum from MPTPp-treated animals (Fig. 4E-G). The levels of 2-AG were not affected by URB597 in any of the two regions analyzed (Figs. 4D and 4H). Taken together, these data highlight a specific increase in midbrain and cerebellar AEA, PEA and OEA levels in response to FAAH inhibition and suggest a differential metabolism of NAEs in healthy vs. MPTPp animals.

3.2. URB597 relieves PD symptoms via CB₁ and CB₂ receptors.

Since the improved motor behavior of MPTPp mice treated chronically with URB597 was not mediated by a neuroprotective effect on dopaminergic neurons or by a higher DA tone, the possible involvement of cannabinoid receptors in this symptomatic relief was investigated using the haloperidol-induced catalepsy assay. This assay involves antagonizing D₂ receptors in medium striatal spiny neurons of the indirect pathway and it has been used to assess the potential symptomatic efficacy of novel non-dopaminergic agents in PD, including mGlu₄-positive allosteric modulators, adenosine A_{2A}/A₁ antagonists and mGlu₇ agonists (Greco et al., 2010; Neustadt et al., 2009; Niswender et al., 2008; Shook et al., 2010). The blockade of D₂ receptors by haloperidol (1 mg/kg) results in rigidity and catalepsy in rodents that mimics a key symptom of PD, namely the difficulty of patients to initiate movement.

Initially, the effect of URB597 (1 mg/kg) administration alone was assessed in the bar test performed 3 h after drug administration (Fig. 5A). The cataleptic effect of haloperidol was evident while URB597 lacked this effect (Fig. 5D). When haloperidol was co-injected with drugs (Fig. 5B), haloperidol-induced catalepsy was reversed by the co-administration of SCH442416

(1 mg/kg), a selective adenosine A_{2A} receptor, which served as a positive control for the restoration of motor control (Fig. 5B and 5E). Similarly, co-administration of URB597 significantly reduced the cataleptic effect of haloperidol (Fig. 5B and 5E), although JZL184, a MAGL inhibitor that restores motor symptoms in the MPTPp model by a neuroprotective mechanism (Fernandez-Suarez et al., 2014), failed to show anti-cataleptic properties (Fig. 5B and 5E). When URB597 was administered prior to haloperidol it was evident that its anti-cataleptic effect was independent of the order in which the compounds were administered (Fig. 5C and 5F). The level of NAEs was determined in the striatum of mice treated with haloperidol plus/minus URB597 (as in Fig. 5B). Administration of URB597 to haloperidol-treated mice increased significantly the levels of AEA, OEA, and PEA (Suppl. Fig. 3). No effect was detected on 2-AG concentrations. These results indicate that the anti-cataleptic effect of FAAH inhibition correlated with the increase in NAEs levels.

The effect of URB597 on catalepsy was then tested in animals treated with cannabinoid receptor antagonists. The mice were injected with haloperidol and 2 h later, each group received either: i) URB597, now used as a positive control; ii) a cannabinoid receptor antagonist; iii) URB597 plus the antagonist; or iv) the vehicles of the drugs. Cataleptic status was evaluated 1 h later in the bar test. The cannabinoid receptor antagonists, rimonabant (3 mg/kg) for CB₁ receptor (Fig. 6A), AM630 (3 mg/kg) for CB₂ receptor (Fig. 6B) and capsazepine (10 mg/kg) for the TRPV1 (Fig. 6C), did not have anti-cataleptic properties in the mice. However, the effect afforded by URB597 against haloperidol-induced catalepsy was reversed by the administration of rimonabant (Fig. 6A) and AM630 (Fig. 6B). By contrast, the anti-cataleptic effect of URB597 was not affected by the administration of the TRPV1 receptor antagonist, capsazepine (Fig. 6C). To further explore the involvement of cannabinoid receptors in the mechanism of action of URB597, catalepsy assays were performed in animals deficient for CB₁ or CB₂ receptors. A low dose of haloperidol (0.1 mg/kg) was used in CB₁^{-/-} mice and in their age-matched littermates due to their high sensitivity to haloperidol. Although the mouse

strain used in this study was C57BL, the CB₁ KO mice were on a CB57BL/6N background as opposed to the C57BL/6J background of the rest of the mice, this genetic variation could account for the different susceptibility to haloperidol (Mekada et al., 2009). Remarkably haloperidol-induced catalepsy was not reverted by URB597 in either the CB₁ (Fig. 6D) or CB₂ (Fig. 6E) receptor KO mice.

Finally, to demonstrate that the anti-cataleptic effect of URB597 is specific to FAAH inhibition, two different FAAH inhibitors were tested. The irreversible FAAH inhibitor JNJ1661010 (1 mg/kg) and TCF2 (40 mg/kg), a reversible inhibitor, were administered to the mice 120 min after they received haloperidol (Figs. 7A and 7B, respectively). When the cataleptic status of these animals was assessed in the bar test, both inhibitors had a significant anti-cataleptic effect. Together, these results indicate that CB₁ and CB₂ receptors are involved in the anti-parkinsonian effect of URB597, which acts as a specific FAAH inhibitor.

3.3. Chronic URB597 administration does not induce the cannabinoid tetrad

Cannabinoids acting via CB₁ and/or CB₂ receptors have long been associated with a set of four well-characterized responses: hypothermia, antinociception, hypolocomotion and catalepsy (Little et al., 1988; Wiley et al., 2014). This cannabinoid tetrad was reproduced in mice receiving WIN55,212-2, a non-selective CB₁ receptor agonist, and thus, it was investigated in mice receiving URB597. Drugs were administered for 7 days and the tetrad was assessed one hour after the last administration. The administration of WIN55,212-2 significantly reduced the body temperature (Fig. 8A), it had an analgesic effect in the tail-flick test (Fig. 8B) and it induced catalepsy (Fig. 8C), as well as significantly dampening the locomotor activity of the mice in the open field test (Fig. 8D). By contrast, URB597 did not affect any of these parameters (Figs. 8A-8D), indicating that the elevation of AEA is not equivalent to administering cannabinoid receptor CB₁ agonists.

4. DISCUSSION

In this study, the anti-parkinsonian effect of chronic FAAH inhibition in the MPTPp mouse model is described. The significant improvement of the parkinsonian motor deficits in these mice is due to a symptomatic rather than a neuroprotective effect of FAAH inhibition, which was further characterized using haloperidol-induced catalepsy. URB597 administration has anti-cataleptic properties that involve CB₁ and CB₂ but not TRPV1 receptors. Moreover, FAAH inhibitors other than URB597 also showed a similar anti-cataleptic effect.

The two main eCBs in the CNS, 2-AG and AEA, can activate both CB₁ and CB₂ receptors (Devane et al., 1992; Mechoulam et al., 1995; Pertwee, 1999). Modulating eCB levels by administering inhibitors of their catabolic enzymes shows that the two eCBs regulate antinociceptive and anxiolytic-like responses, and that AEA may fulfill a central role in the modulation of memory consolidation (Busquets-Garcia et al., 2011) without the side effects derived from the administration of CB₁ receptor agonists. It is likely that the pharmacological effect of URB597, which is sensitive to CB₁ and CB₂ receptor antagonists, is primarily due to the increase in AEA but not in 2-AG levels. As URB597 increases endogenous AEA levels, it does not mimic the spectrum of pharmacological responses produced by administration of exogenous AEA or CB₁ receptor agonists (Kathuria et al., 2003). FAAH inhibition also produces large elevations in the levels of two AEA analogs, OEA and PEA, whose biological effects are neither mediated by CB₁ receptors nor fully elucidated (Calignano et al., 1998; Rodriguez de Fonseca et al., 2001). Thus, the possibility that URB597 has additional properties mediated by these NAEs cannot be ruled out.

We previously reported a neuroprotective effect of JZL184, a MAGL inhibitor, in the nigrostriatal pathway (Aymerich et al., 2015; Fernandez-Suarez et al., 2014), which was accompanied by a marked improvement in motor behavior of MPTPp mice (Fernandez-Suarez et al., 2014). Here, we demonstrate that chronic administration of URB597 to MPTPp mice produces a robust improvement in motor behavior that is not due to the protection of neurons

in the SNpc, indicating that inhibition of FAAH has symptom-relieving properties. The effect of URB597 on behavior was not associated with an increase in dopamine levels in the striatum. A symptomatic effect of the cannabinoid Δ^9 -tetrahydrocannabivarin was described in the parkinsonian rat model (Garcia et al., 2011). The reduction of motor inhibition was dopamine-independent and could be associated with changes in glutamatergic transmission in the striatum and SNpc (Garcia et al., 2011). Naïve animals treated with URB597 presented an increase in AEA levels in was much lower than that obtained in MPTPp animals suggesting that the metabolism of NAEs is markedly altered in PD animals. The URB597 efficacy is not due to 2-AG since the levels of this compound did not change due to URB597 treatment. These results highlight the different roles of the two main eCBs and the distinct anti-parkinsonian effects induced by inhibiting the two main metabolizing enzymes of the ECB system, FAAH or MAGL. MPTPp toxicity induces a strong astroglial response that was not affected by chronic URB597 administration but was downregulated by MAGL inhibition, which seemingly contributes to nigral neuroprotection (Fernandez-Suarez et al., 2014). The most notable microglial phenotypic change after administering a MAGL inhibitor was a large-scale increase in branching (Fernandez-Suarez et al., 2014). In fact, the changes induced by JZL184 resemble those in alternatively activated microglia, M2, (Wirjatijasa et al., 2002) and they may account for the positive influence of MAGL inhibition on preventing dopaminergic denervation. In the same MPTPp-based PD model MAGL inhibition resulted in a glia-dependent neuroprotection ((Fernandez-Suarez et al., 2014), whereas FAAH inhibition affected glial phenotype but without a clear correlation with the anti-symptomatic actions of URB597. The most remarkably change induced by chronic URB597 administration in MPTPp mice was the decrease in the microglial cell soma size, which is compatible with an anti-inflammatory phenotype but it is not correlated with a neuroprotective activity. Actually, the efficacy of acute administration of URB597 in the haloperidol test suggests that the symptomatic relief is not due to glial involvement. Furthermore, the reported anti-inflammatory effect of URB597 (Murphy et al.,

2012; Tham et al., 2007) and the reduction in microglial cells that accompanies the protection of retinal ganglion cells by URB597 (Slusar et al., 2013) does not seem to underlie the beneficial effects on PD symptoms. Although FAAH and MAGL inhibition results in a marked improvement of motor behavior in the MPTP mice, the mechanism of action seems quite different. Depending on which is the endocannabinoid whose concentration is chronically elevated, motor improvement is achieved by nigral neuroprotection or by compensatory mechanisms that do not require neuronal survival.

Motor control is achieved via the direct and indirect basal ganglia pathways due to a flow of information between the input nucleus, the striatum and the output nuclei (Albin et al., 1989; DeLong, 1990). An imbalance between neural activity in the direct and indirect pathways has been proposed to underlie the profound motor deficits observed in PD (Albin et al., 1989; DeLong, 1990). Neural activity stimulates AEA but not 2-AG release in the dorsal striatum through the D_2 receptors present on the medium spiny neurons of the indirect pathway (Giuffrida et al., 1999). These neurons present selective eCB-mediated long-term depression (LTD) that is also dependent on D_2 receptor activation (Kreitzer and Malenka, 2007). In the 6-OHDA and the reserpine PD models, the loss of eCB-mediated LTD can be rescued by administering either quinpirole, a D_2 receptor agonist, or URB597, and the co-administration of these drugs reduces the parkinsonian motor deficits (Kreitzer and Malenka, 2007). Here, we show that URB597 administration is sufficient to reverse haloperidol-induced catalepsy and to reduce the motor symptoms elicited by MPTP administration without the addition of a D_2 receptor agonist. In the reserpine and 6-OHDA mouse models, DA depletion is almost complete at the time behavior was analyzed (18-24 h and 48-60 h, post injection). Hence, eCB-mediated LTD would require increases of AEA and enhanced dopaminergic tone induced by the synthetic D_2 receptor agonist. The use of a D_2 receptor agonist would further contribute to enhance the AEA levels and reinforce LTD, reducing the activity of the indirect pathway and restoring motor control (Kreitzer and Malenka, 2007). The chronic MPTP mouse model used

here has a partial dopaminergic lesion making administration of an exogenous DA receptor agonist dispensable. However, URB597 failed to alter the anti-parkinsonian action of L-DOPA in MPTP marmosets (Johnston et al., 2010). In Kreitzer and Malenka's study, the effect of URB597 was detected in an acute disease model, while the marmosets studied by Johnston suffered a chronic parkinsonian condition. Compensatory mechanisms affecting the eCB system in chronic parkinsonian conditions may account for these differences, with L-DOPA increasing the AEA levels in intact animals, whereas the levels of AEA fell in 6-OHDA rats but were unaffected by L-DOPA (Ferrer et al., 2003). In addition, our findings suggest that chronic inhibition of FAAH activity may be beneficial to treat PD, especially in early stages of the disease when there is still significant striatal DA tone.

Cannabinoid-based therapies have been proposed for the treatment of PD. Since CB₁ receptor stimulation promotes hypokinesia and catalepsy, CB₁ receptor antagonists would be preferable to counteract the consequences of ECS upregulation in advanced stages of the disease. However, studies evaluating the anti-parkinsonian effect of CB₁ receptor antagonism have produced conflicting findings (Fernandez-Espejo et al., 2005; Gonzalez et al., 2006; Kelsey et al., 2009). Indeed, no therapeutic effect was observed in a clinical trial with rimonabant (Mesnage et al., 2004). By contrast, treatment of MPTP mice with the non-selective CB₁ and CB₂ receptor agonist WIN55,212-2, reversed the motor deficits induced by the neurotoxin when analyzed 24 h post-injection (Price et al., 2009). URB597 decreases L-DOPA-induced dyskinesia when co-administered with the TRPV1 antagonist capsazepine (Morgese et al., 2007). Our results show that both, CB₁ and CB₂ but not TRPV1 receptors are involved in the anti-cataleptic effect of URB597 administration. Although AEA binds to and activates CB₁ and CB₂ receptors (Pertwee, 1999), increasing AEA levels through FAAH inhibition does not produce the hypokinesia and catalepsy observed after WIN55,212-2 administration. Furthermore, the anti-cataleptic effect of FAAH inhibition was completely abolished by specifically blocking CB₁ or CB₂ receptors or in animals deficient for these receptors. A partial

effect would have been expected for each of these KO mice, since both receptors are involved. However, it has been seen that these two cannabinoid receptors interact in the indirect pathway and in fact, CB₁ and CB₂ receptors form functional heteromers in the GPe (Callen et al., 2012). One specific characteristic of this heteromer is that *in vitro* co-stimulation of CB₁ and CB₂ receptors results in a negative cross-talk in intracellular signaling and neurite outgrowth (Callen et al., 2012). GPCR heteromers often display cross-antagonism, i.e. the antagonist of one receptor blocks the agonist-mediated response originated in the partner receptor (Franco et al., 2016). Thus, binding of AEA to functional heteromers in the GPe could explain the complete blockade of the anti-cataleptic effect of URB597 with either CB₁ or CB₂ antagonists or in KO animals. It would be interesting to explore the effect of FAAH inhibition using the MPTPp model in KO mice for CB₁ or CB₂ receptors, but it has been reported that administration of MPTP to CB₂ KO mice leads to death in 24-48 h indicating that genetic ablation of CB₂ receptors increases the sensitivity to MPTP-induced systemic toxicity (Price et al., 2009). Interestingly, we show for the first time that CB₂ receptors are involved in the anti-cataleptic effect due to increased endocannabinoid levels.

Together, our findings suggest that modulation of the ECS may be beneficial for the treatment of PD. Inhibition of FAAH activity has a symptomatic effect that is mediated by CB₁ and CB₂ receptors. Also, these and previous results emphasize the distinct role of the two main eCBs in the brain and their therapeutic potential, and suggest a differential regulation of ECB metabolism under parkinsonian conditions.

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8. FIGURE LEGENDS

Figure 1: Effect of URB597 on the motor performance of MPTPp mice. (A) Schedule of MPTPp and URB597 administration, and of the subsequent motor function tests. Control animals were treated with saline, probenecid (250 mg/kg) and URB597 (1 mg/kg) or the vehicle used to prepare the URB597. The MPTPp animals were injected with MPTP (20 mg/kg), probenecid and 8 h later with URB597 or the vehicle used to prepare it (all compounds were administered by i.p. injection). Parkinsonian motor symptoms were evaluated 24 h after the cessation of MPTPp (16 h after the last URB597 or vehicle) administration. Animals were sacrificed 48 h after the last injection of MPTPp (40 h after the last injection of URB597 or vehicle). (B) In the pole test, each animal was placed facing upwards on the top of a 50 cm pole and the time they took to turn their head around, face down and descend was recorded. (C) In the rotarod test, the animals were placed on the rotarod device and the latency until they fell off was recorded as the rotarod accelerated. Two-way ANOVA followed by a Bonferroni's multiple comparison

post-hoc test were used to determine statistical differences of the data. $n = 12-13$ animals per group, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Figure 2: Chronic administration of URB597 does not produce neuroprotection in the nigrostriatal pathway. The extent of neurodegeneration in the nigrostriatal pathway was analyzed to determine whether the motor improvement induced by URB597 administration to MPTPp mice was due to neuroprotection. (A) Representative photomicrographs showing TH-immunoreactivity in the striatum. (B) Representative photomicrographs showing TH-immunoreactivity in the SNpc. (C) Densitometry quantification of TH-immunostained fibers in the striatum. (D) Quantification of TH expressing neurons in the rostrocaudal extension of the SNpc by unbiased stereology. A two-way ANOVA followed by Bonferroni's multiple comparison post-hoc test was used to determine statistical differences: $n = 5-7$ animals per group, $***p < 0.001$. Scale bar: 1 mm

Figure 3: Effect of chronic URB597 administration on the levels of DA, DOPAC and HVA in the striatum. Striatal content of DA (A) and its metabolites, DOPAC (B) and (C) HVA, was determined by HPLC in control, MPTPp and MPTPp mice treated with URB597. One-way ANOVA followed by a Bonferroni's multiple comparison post-hoc test was used to determine statistical differences: $n = 7-8$ animals per group, $***p < 0.001$.

Figure 4: Effect of chronic URB597 administration on the midbrain and cerebellar NAEs and 2-AG content. The concentrations of AEA (A, E), OEA (B,F), PEA (C,G) and 2-AG (D, H) were determined by liquid chromatography-mass spectrometry in control (plus/minus URB597 treatment) and MPTPp (plus/minus URB597 treatment) animals. Open bars correspond to data from animals treated with vehicle, whereas solid bars correspond to data from animals treated with URB597. Two-way ANOVA followed by a Bonferroni's multiple comparison post-hoc test

was used to determine statistical differences: $n = 7-8$ animals per group $**p < 0.01$, $***p < 0.001$.

Figure 5: Effect of URB597 administration on the cataleptic behavior of mice. (A, D) The ability to induce catalepsy was evaluated in the bar test 180 min after the i.p. administration of haloperidol (Hal, 1 mg/kg) or URB597 (1 mg/kg). Only mice receiving haloperidol showed cataleptic behavior. (B,E) To study the anti-cataleptic properties of different drugs, catalepsy was induced in all the animals by administering haloperidol and, 120 min later all animals received either SCH442416 (1 mg/kg) as a positive control, URB597 (1 mg/kg) or JZL184 (8 mg/kg). Catalepsy was evaluated 180 min after haloperidol injection in the bar test. URB597 administration produced an anti-cataleptic effect similar to SCH442416. (C,F) The order of administration of haloperidol and URB597 was changed, URB597 was injected 60 min prior haloperidol. URB597 induced an evident reduction of catalepsy in the bar test performed 180 min after haloperidol injection. A Mann-Whitney test was used to determine the statistical differences in (D) and (F), and a Kruskal-Wallis test followed by Dunn's post-hoc test was used in (E): $n = 7$ animals per group, $*p < 0.05$, $**p < 0.01$.

Figure 6: Study of the cannabinoid receptors involved in the anti-cataleptic effect of URB597. In all the experimental animal groups, catalepsy was induced by administering haloperidol (Hal, i.p.) at a dose of 1 mg/kg and the mice were evaluated in the bar test 180 min after the haloperidol injection. (A) The results of the bar test in the presence of a CB_1 receptor antagonist (Rimonabant, 3 mg/kg) alone or in combination with URB597. (B) The results of the bar test in the presence of a CB_2 receptor antagonist (AM630, 3 mg/kg) alone or in combination with URB597. (C) The results of the bar test in the presence of a TRPV1 antagonist (Capsazepine, 10 mg/kg) alone or in combination with URB597. (D) Haloperidol-induced catalepsy evident in the bar test of CB_1 receptor knockout mice and their corresponding wild

types, in the presence or absence of URB597. In this case, catalepsy was induced with 0.1 mg/kg of haloperidol. (E) Haloperidol-induced catalepsy (1 mg/kg) evident in the bar test in the CB₂ receptor knockout mice and their corresponding wild types, in the presence or absence of URB597. A Kruskal-Wallis test followed by Dunn's post-hoc test was used to determine statistical differences in (A), (B) and (C, n = 7 animals per group), and a Mann-Whitney test was used to determine statistical differences in (D) and (E, n = 5-6): $p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Figure 7: Anti-cataleptic effect of different FAAH inhibitors. Catalepsy was induced by administering haloperidol (Hal, 1 mg/kg) and it was evaluated in the bar test 180 min later. (A) JNJ1661010 (1 mg/kg), an irreversible FAAH inhibitor, and (B) TCF-2 (40 mg/kg), a reversible FAAH inhibitor, were administered 120 min after haloperidol. A Mann-Whitney test was used to determine statistical differences: $n = 6-7$, $*p < 0.05$, $**p < 0.01$.

Figure 8: Cannabinoid tetrad. The effect of URB597 (1 mg/kg) was compared to that of WIN55,212-2 (3 mg/kg), both administered (i.p.) to mice daily for 7 days. (A) Body temperature, (B) analgesia, (C) catalepsy (D) and spontaneous locomotor activity. A Student's T-test was used to determine the statistical differences in (A), (B) and (D), and the Mann-Whitney test was used to determine the statistical differences in (C, $n = 8-10$): $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

SUPPLEMENTARY INFORMATION

1. GFAP-immunolabeling of astroglia

Immunohistochemistry and image analysis were performed as described in the Materials and Methods section. The effect of chronic URB597 on GFAP immunolabeling was studied in MPTPp animals by immunohistochemistry (Suppl. Fig. 1A). The densitometry analysis (Suppl. Fig. 1B) revealed an increase in GFAP immunoreactivity in MPTPp animals that was not affected by the administration of URB597.

Supplementary figure 1: Changes in GFAP expression in the striatum of MPTPp animals treated with URB597. (A) Representative photomicrographs of GFAP-immunoreactivity in the striatum. (B) Densitometry quantification of the GFAP-immunopositive area relative to the total area. All samples were processed simultaneously and all the images were obtained under identical confocal parameters. A two-way ANOVA followed by Bonferroni's multiple comparison post-hoc test was used to determine statistical differences ($n = 5$ animals per group): *** $p < 0.001$. $n = 7-8$ animals, *** $p < 0.001$. Scale bar: 100 μm .

2. Stereological analysis of microglial cell parameters

Colorimetric immunohistochemistry was performed as described in the Materials and Methods section. The following parameters were estimated by unbiased stereology in striatal Iba1 immunopositive cells (Suppl. Fig. 2A): cell density (Suppl. Fig. 2B), soma area and length of microglial processes (Suppl. Fig. 2C and 2D). The unbiased stereological analysis was performed on 5 coronal striatal sections (40 μm thick) between +1.34 mm and -0.26 mm relative to Bregma (Paxinos and Franklin, 2001). Approximately 100-150 cells were counted per animal using a sampling frame of 3,781 μm^2 and sampling steps of 389 $\mu\text{m} \times 389 \mu\text{m}$ (dx,dy). The soma area was estimated with 2D nucleator (Visiopharm). The total length of the neurites was estimated by global spatial sampling, counting the intersections of fibers with isotropically oriented virtual planes within a virtual box in a thick section with an arbitrary

orientation. The total fiber length (L) was calculated according to Larsen et al. (Larsen et al., 1998):

$$L = \frac{1}{hsf} \frac{1}{asf} \frac{1}{ssf} d \times 2 \times \sum Q^{-}$$

where d is the plane separation distance and $\sum Q^{-}$ is the total number of fiber-plane intersections counted (hsf, asf and ssf). Counting was performed with a height of 10 μm , using a frame area of 454 μm^2 , sampling steps of 426 $\mu\text{m} \times 426 \mu\text{m}$ (dx,dy) and a plane separation distance (d) of 20 μm (Drojdahl et al., 2010).

The results in Suppl. Fig. 2 showed that MPTP-induced an increase in cell body area that was prevented by chronic URB597 treatment. However, cell density and ramification length of Iba1-immunopositive cells increased significantly in MPTP animals and URB597 did not prevent these changes.

Supplementary figure 2: Morphological analysis of Iba1-immunopositive cells from striatal sections. (A) Representative photomicrographs of Iba1-immunostained microglia in the striatum of animals treated with MPTP or with MPTP plus URB597. Stereological quantification of microglial cell density (B), cell body area (C) and microglial ramification length (D).

* $p < 0.05$, *** $p < 0.001$. (n=5 animals per group). Scale bar: 50 μm .

3. Determination of NAEs and 2-AG levels in haloperidol-treated mice

NAEs and 2-AG determination in the striatum of animals treated with haloperidol or with haloperidol plus URB597 was performed by HPLC-MS as described in the Materials and

Methods section. Except 2-AG all other assayed compounds were significantly increased upon URB597 treatment.

Supplementary figure 3: Effect of URB597 in the haloperidol-injected mice on the striatal NAEs and 2-AG content. The concentration of AEA (A), OEA (B), PEA (C) and 2-AG (D) was determined by liquid chromatography-mass spectrometry in haloperidol and haloperidol mice treated with URB597. Haloperidol (Hal) was i.p. injected at a dose of 1 mg/kg; URB597 was i.p. administered (1 mg/kg) 2 h later and animals were sacrificed 1 h after (3 h after haloperidol injection). Student's t test was used to determine statistical differences: n = 7 animals per group *p < 0.05, ***p < 0.001.

3. References

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Figure 1: Celorrio et al.

Figure 2: Celorrio et al.

Figure 3: Celorrio et al.

Figure 4: Celorrio et al.

Figure 5: Celorrio et al.

Figure 6: Celorrio et al.

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Figure 8: Celorrio et al.