Fatty Acid Amide Hydrolase (FAAH) Inhibition Reduces L-3,4-Dihydroxyphenylalanine-Induced Hyperactivity in the 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine-Lesioned Non-Human Primate Model of Parkinson’s Disease

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ABSTRACT

Dopaminergic therapies remain the most efficacious symptomatic treatments for Parkinson’s disease (PD) but are associated with motor complications, including dyskinesia, and nonmotor complications, such as psychosis, impulse control disorders (ICD), and dopamine dysregulation syndrome (DDS). Nondopaminergic neurotransmitter systems, including the endocannabinoid system, are probably critical to the development of these complications. The role of fatty acid amide hydrolase (FAAH) in mediating L-3,4-dihydroxyphenylalanine (L-DOPA)-induced behaviors was explored in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-lesioned marmoset model of PD. Pharmacodynamic and locomotor effects of the selective FAAH inhibitor [3-(3-carbamoylphenyl)phenyl] N-cyclohexylcarbamate (URB597) were assessed via bioanalytical (liquid chromatography-tandem mass spectrometry) and behavioral observation approaches. URB597 (3, 10, 30, or 60 mg/kg p.o.) increased plasma levels of the FAAH substrates N-arachidonoyl ethanolamide (anandamide), N-oleoyl ethanolamide, and N-palmitoyl ethanolamide by 10.3 ± 0.3-, 7.8 ± 0.2-, and 1.8 ± 0.1-fold (mean of URB597 groups ± S.E.M.), respectively, compared with vehicle (all p < 0.001) 4 h after administration. Treatment with L-DOPA (20 mg/kg s.c.) alleviated parkinsonism but elicited dyskinesia, psychosis-like-behaviors and hyperactivity, a potential correlate of ICD and DDS. During the 2 to 4 h after L-DOPA, corresponding to 4 to 6 h after URB597 administration, URB597 reduced total L-DOPA-induced activity and the magnitude of hyperactivity by 32 and 52%, respectively, to levels equivalent to those seen in normal animals. Treatment with URB597 (10 mg/kg p.o.) did not modify the antiparkinsonian actions of L-DOPA or L-DOPA-induced dyskinesia and psychosis. URB597 did not alter plasma L-DOPA levels and was without behavioral effects when administered alone. Inhibition of FAAH may represent a novel approach to reducing L-DOPA-induced side effects, such as ICD and DDS, while maintaining the antiparkinsonian benefits of L-DOPA treatment.

Introduction

Dopamine replacement therapy remains the most efficacious symptomatic treatment for Parkinson’s disease (PD). However, this strategy is associated with motor complications such as dyskinesia, wearing off, and on-off fluctuations (Fox and Lang, 2008). Increasingly, nonmotor-related complications of treatment are recognized as undesirable consequences of aberrant, treatment-related dopamine receptor stimulation. Thus, treatment with the dopamine precursor, L-DOPA, or with dopamine agonists can result in excessively heightened mood and psychomotor activity even to the extent of psychosis (Racette et al., 2002). In addition, some patients experience hyperactivity with compulsive, repetitive, non-goal-directed motor activity (e.g., “punding,” stereotypies, “hobbyism,” and “walkabouts”). This activity is a part of the dopamine dysregulation syndrome (DDS), which is related

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to, but probably distinct from, impulse control disorders (ICD) such as pathological gambling, excessive eating or shopping, and hypersexuality (Evans and Lees, 2004). The usual treatment for such disorders is a reduction of dopaminergic treatments. However, this often results in worsening of motor function (O’Sullivan et al., 2009). Thus, novel treatment options are required to specifically target these problematic side effects.

The mechanisms underlying these motor and nonmotor complications are not completely elucidated, but it seems clear that, in addition to abnormal dopamine receptor stimulation, nondopaminergic transmitter systems are involved (Cenci, 2007; Fox et al., 2008). For instance, the basal ganglia are enriched with endogenous fatty acid amides (FAAs) such as the endocannabinoid N-arachidonoyl-ethanolamide (AEA; anandamide) (Bisogno et al., 1999), the enzyme responsible for fatty acid amide (FAA) catabolism, FAA hydrolase (FAAH) (Cravatt et al., 1996; McKinney and Cravatt, 2005), as well as cannabinoid receptor type 1 (Herkenham et al., 1991). It has been demonstrated in both rodent and primate models of PD that there are abnormalities in FAA levels, within the basal ganglia, in both parkinsonian per se, and after the emergence of complications of long-term dopaminergic therapy (Di Marzo et al., 2000; van der Stelt et al., 2005), leading to the suggestion that modulation of FAA levels via FAAH inhibition may have therapeutic benefit in patients with PD (Sieradzan et al., 2001).

In the present study, we investigated the potential role of FAAH in modulating the motor and nonmotor response to L-DOPA in MPTP-lesioned primates by using the prototypical FAAH inhibitor [3-(3-carbamoylphenyl)phenyl] N-cyclohexylcarbamate (URB597) (Piomelli et al., 2006). Systemic administration of URB597 and other selective FAAH inhibitors has previously been shown to inhibit brain FAAH enzyme activity in rodents and primates and to elevate AEA, N-oleoyl ethanolamide (OEA), and N-palmityl ethanolamide (PEA) in brain tissue (Kathuria et al., 2003; Lichtman et al., 2004; Justinova et al., 2008; Ahn et al., 2009).

In rodent models of parkinsonism, URB597, administered as a monotherapy, has been reported to have no significant effect on motor activity, although a significant increase was observed when URB597 was combined with a subtherapeutic dose of the dopamine agonist quinpirole (Kreitzer and Malenka, 2007). Rodent studies have also suggested that FAAH inhibition could be a viable approach to reducing L-DOPA-induced dyskinesia, although this might require concomitant blockade of the vanilloid receptor type 1 (TRPV1) (Lee et al., 2006; Morgese et al., 2007). However, this hypothesis has never been tested in primate models of PD. Moreover, until recently, nonmotor complications of PD, particularly those of a psychiatric nature, such as ICD, DDS, and psychosis, were subject to scant consideration in large part because of the limited availability of animal models to study these phenomena (Visanji et al., 2006; Barraud et al., 2009). With regard to L-DOPA-induced psychosis in PD, a model has been developed and characterized, using L-DOPA-treated, MPTP-lesioned primates (Visanji et al., 2006) in which abnormal psychotomimetic behaviors, which are distinct from dyskinesia and parkinsonism, are observed. More recently, it has been shown that hyperactive components of the motor activity response of parkinsonian primates to L-DOPA are quantitatively distinct from the antiparkinsonian or dyskinetic actions of dopamine replacement (Visanji et al., 2009). It is hypothesized that this hyperactivity (i.e., activity that is above that seen in the normal, nonparkinsonian state) might represent a behavioral correlate of DDS and ICD (Visanji et al., 2009). It is thought that both of these treatment-related conditions may involve pathophysiological changes in striatal dopamine release, especially in the ventral striatum (Evans et al., 2006). Given the central role of endocannabinoids in modulating striatal neurotransmitter release (Maccarrone et al., 2003), it is possible that any action of FAAH inhibitors to modulate motor aspects of PD might also affect nonmotor complications.

Here, we assessed the effects of oral treatment with URB597 in parkinsonian MPTP-lesioned marmosets. We conducted an initial pharmacodynamic study to define a dose of orally administered URB597 that would enhance AEA, OEA, and PEA levels. With an optimal dosing regimen defined, we then examined the effect of URB597 alone or in combination with L-DOPA on parkinsonian deficits, L-DOPA-induced dyskinesia, psychosis, and hyperactivity.

Materials and Methods

Preparation of Animals. Six common marmosets (Callitrichus jacchus; female; weight, 350 to 500 g; Harlan Teklad, Madison, WI) were kept under conditions of controlled temperature (25 ± 2°C) and a 12-h light/dark cycle (lights on at 6:30 AM). Animals were group-housed and cared for in accordance with an approved Animal Care Committee protocol (AUP 1022.10) from the Institutional Animal Care and Use Committee of the University Health Network, Toronto, ON, Canada. Animals had access to food, fresh fruit supplements, and water ad libitum, and their home cage environment was enriched with primate toys, perches, and auditory stimuli. Before the start of the studies, animals were acclimatized to handling, blood collection, and administration of treatments (oral syringe feeding and subcutaneous injection), as well as transfer to observation cages for assessment of behavior. Activity of normal animals was quantified before any test article treatment or MPTP intoxication as described below (see Behavioral Assessment). A parkinsonian syndrome was induced by once-daily subcutaneous administration of MPTP hydrochloride (Sigma-Aldrich, Oakville, Canada; 2 mg/kg base concentration in 0.9% saline) for 5 consecutive days. After this, animals were allowed to recover for 12 weeks to allow parkinsonian symptoms to develop and stabilize. MPTP intoxication resulted in a syndrome characterized by bradykinesia, hunched posture, and a reduced range of movement. Treatment-related complications including dyskinesia and psychosis-like behaviors were evoked by twice-daily treatment with oral L-DOPA (as Prolopa; Roche Canada, Ontario, Canada; equivalent to 15 mg/kg L-DOPA and 3.5 mg/kg benzerazide) for a minimum of 30 days. This treatment regimen has been demonstrated previously to produce a stable model of L-DOPA-induced complications (Visanji et al., 2006). After this time, the animals were used in several studies to evaluate potential antiparkinsonian and antidyskinetic agents. A minimum period of 2 weeks was allowed between the completion of any prior study and the commencement of this study, a period in excess of 10 half-lives since administration of any previously used experimental drug. Throughout the course of the study, on days not requiring plasma sampling or behavioral observations, animals were treated once daily with oral L-DOPA therapy at 10:00 AM to maintain stable levels of treatment-related complications.

Assessment of Plasma FAAH Substrate and L-DOPA Levels in Response to Treatment with URB597. The effect of oral treatment of MPTP-lesioned marmosets with vehicle or URB597 (0, 10, 30, or 60 mg/kg) on plasma levels of endogenous FAAH substrates, AEA, OEA, and PEA were measured by liquid chromatography-tandem mass spectrometry assays (see Supplemental Materials). For
animal welfare reasons, only a single blood sample (1 ml) was taken from each animal on each day of treatment.

Behavioral Effect of URB597 Alone or in Combination with L-DOPA in MPTP-Lesioned Primates. The effects of URB597 (10 mg/kg p.o.) alone or in combination with L-DOPA (20 mg/kg s.c.) on motor activity, parkinsonian disability, dyskinesia, and psychosis were assessed in a group of MPTP-lesioned marmosets (n = 6) with stable L-DOPA-induced dyskinesia. Based on previous dose-finding studies in these animals (data not shown), a dose of L-DOPA (20 mg/kg/benserazide (5 mg/kg), was chosen such that it provided the best antiparkinsonian effect achievable while eliciting hyperactivity, dyskinesia, and psychosis that was stable and reproducible on successive L-DOPA administrations. For behavioral observations, L-DOPA was administered subcutaneously at a dose volume of 1 ml/kg, as L-DOPA methyl ester (Sigma-Aldrich) in combination with benserazide (Sigma-Aldrich). Based on its ability to maximally elevate plasma levels of AEA, OEA, and PEA in MPTP-lesioned marmosets, a dose of 10 mg/kg URB597 was used for all behavioral observations and was administered orally at a dose volume of 5 ml/kg.

Treatments. The effect of URB597 alone and in combination with L-DOPA was assessed. On days before behavioral assessment, animals were fed normally and received a maintenance oral L-DOPA dose at 9:00 AM. At 4:00 PM animals were administered either vehicle (per os) or URB597 (10 mg/kg p.o.). On days of behavioral assessment, animals were fed their normal diet between 7:00 and 7:30 AM, after which time all food was removed from their cages. Water was available ad libitum. At approximately 9:00 AM, each animal received either vehicle (per os) or URB597 (per os). Two hours after this, at approximately 11:00 AM, animals received either vehicle or L-DOPA (subcutaneously). Behavioral assessment, as described below, commenced directly after this second treatment. To prevent any confounding effects of prior treatment with oral URB597 on the assessment of response to vehicle treatment, the order of these treatments was randomized in each animal. A minimum of 48 h was left between behavioral observations in the same animal.

Behavioral Assessment of Marmoset Activity. A quantitative assessment of marmoset activity was made using computer-operated passive infrared sensors as described previously (Visanji et al., 2009). A single sensor containing a hemispherical lens (Guardall, Mississauga, ON, Canada) was mounted 1.5 m above the top of each observation cage. The sensor was positioned so that motion was detected throughout the entirety of the cage below. The signal was fed via an RS-232 input to a computer. Proprietary Motion Detector software (Research Electronics, Toronto Western Hospital, Toronto, ON, Canada) was used that displayed activity counts within various activity ranges (IQR) in the text description. In all cases, a paired, two-tailed test was used. All marmoset activity time course and cumulated activity (total, high, and low activity counts) data are presented as the group mean and S.E.M. For measures of parkinsonian disability, dyskinesia, and psychosis, data were analyzed via Friedman’s test with Dunn’s multiple comparison post hoc test was used. To assess the effect of treatment on plasma L-DOPA level, a paired, two-tailed t test was used.

All marmoset activity time course and cumulated activity (total, high, and low activity counts) data are presented as the group mean and S.E.M. For measures of parkinsonian disability, dyskinesia, and psychosis, data were analyzed via Friedman’s test with Dunn’s multiple comparison post hoc test. Disability data are displayed as the median, with individual values on graphs and with interquartile range (IQR) in the text description. In all cases, p < 0.05 was taken to represent a significant difference.

Drugs and Formulation. MPTP hydrochloride (Sigma-Aldrich) was dissolved in 0.9% NaCl sterile solution to a concentration of 0.2 mg/ml (freebase) and administered at 1 ml/kg. Maintenance L-DOPA, administered as Prolopa capsules (Roche, Mississauga, ON, Canada) containing 50 mg of L-DOPA and 12.5 mg of benserazide (freebase) was dissolved in Gatorade sports beverage (The Gatorade Company, Chicago, IL) before oral administration by syringe at a volume of 10 ml/kg. On days of behavioral assessment, L-DOPA (20 mg/kg, free base) was prepared from the methyl ester form (Sigma-Aldrich) in combination with benserazide hydrochloride (5 mg/kg, freebase; Sigma-Aldrich) dissolved in 0.9% sterile saline containing 0.1% ascorbate (Sigma-Aldrich) and 0.05% absolute ethanol. URB597

analyzed post hoc by a movement disorder neurologist blinded to the treatment. Methods for assessment of behavior were essentially as described previously (Visanji et al., 2006). Parkinsonian disability scores were assessed every 10 min for the duration of assessment. In brief, range of movements were rated on a scale of 0 to 9: 0 = running, jumping between roof, walls, and perch, use of limbs through a wide range of activity; 9 = no movement. Bradykinesia was rated from 0 to 3: 0 = normal initiation and speed of movement; 1 = slight slowing of movement; 2 = moderate slowing of movement, marked freezing, difficulty initiating and maintaining movement; 3 = prolonged freezing, akinetic, inability to move. Postural abnormalities were rated 0 or 1: 0 = normal balance, upright posture, head held up; 1 = impaired balance, bended posture, head down. Attention/alertness was rated 0 or 1: 0 = normal head checking movements, movement of neck in variable directions, smooth, small movements; 1 = reduced or absent head checking, head in one position for more than 50% of observation period. A global parkinsonian disability score was rated as a combination of the behaviors mentioned above according to the following formula: disability score = (range of movement × 1) + (bradykinesia × 3) + (posture × 9) + (alertness × 9). The maximum score of the global parkinsonian disability was thus 36 per 10-min period. These scores were cumulated for each 2-h period. L-DOPA-induced dyskinesia and psychosis were also assessed. For each 10-min epoch, dyskinesia and psychosis were rated from 0 to 4: 0 = absent; 1 = mild, fleeting, rare, present less than 30% of the observation period; 2 = moderate, not interfering with normal activity, present more than 30% of the observation period; 3 = marked, at times interfering with normal activity, present less than 70% of the observation period; 4 = severe, continuous, replacing normal activity, present more than 70% of the observation period. For dyskinesia, chorea and dystonia were graded separately and scored to represent the most disabling dyskinesia observed, whether chorea or dystonia, in any 10-min period of assessment. For psychosis, hyperkinesia, response to nonapparent stimuli (hallucinatory behavior), repetitive grooming, and stereotypies were graded separately (Fox et al., 2006; Visanji et al., 2006). For this measure, the score given represented the most disabling of any of the four subscore levels observed in any 10-min period of assessment. These scores were cumulated for each 2-h period.

Data Presentation and Statistical Analysis. To assess the effect of treatment on the 6-h time course profile of total activity counts, a two-way repeated measures analysis of variance (RM-ANOVA) with a Bonferroni post hoc analysis was used. To assess the effect of treatment on cumulated levels of total activity, high and low activity and plasma FAAH substrate levels a parametric one-way ANOVA followed by Newman Keul’s multiple comparison post hoc test was used. To assess the effect of treatment on plasma L-DOPA level, a paired, two-tailed t test was used.
Cayman Chemical, Ann Arbor, MI) was formulated in a vehicle containing 50% corn oil (Professional Compounding Centers of America, Houston, TX) and 50% nutritional drink Ensure (Abbott Nutrition, Chicago, IL) to make the suspension palatable for marmoset oral dosing. Requisite amounts of URB597 were weighed into sterile 50-ml polypropylene tubes (for the 3, 10, 30, and 60 mg/kg doses, 12, 40, 120 and 240 mg were weighed, respectively). Ten milliliters of corn oil were then added and mixed until homogenous. Ten milliliters of Ensure were then added, and the mixture was mixed gently at first, vortexed, and finally sonicated (Ultrasonic Dismembrator, Fisher Scientific model 100, 1/8”) on a medium setting for 30 s to ensure homogeneity of the suspension. URB597 or vehicle was dosed orally at a volume of 5 ml/kg body weight.

**Results**

**URB597 Elevates Plasma Levels of AEA, OEA, and PEA in MPTP-Lesioned Marmosets.** Four hours after URB597 administration, plasma levels of three FAAH substrates (AEA, OEA, and PEA) were elevated (AEA, $F_{4,20} = 47.8$; OEA, $F_{4,20} = 32.7$; and PEA, $F_{4,20} = 15.2$; RM-ANOVA). Newman-Keuls analysis revealed a significant increase in plasma levels of all three FAAH substrates assessed compared with placebo treatment (all $p < 0.001$, see Supplemental Fig. S1, A–C, respectively). Mean plasma levels of AEA, OEA, and PEA were 0.49 ± 0.09, 0.98 ± 0.10, and 4.34 ± 0.21 ng/ml, respectively, in vehicle-treated monkeys and 5.0 ± 0.45, 7.7 ± 0.78, and 7.0 ± 0.27 ng/ml, respectively, in URB597 (10 mg/kg)-treated monkeys (Supplemental Fig. S1, A-C). All four doses of URB597 administered (3, 10, 30, and 60 mg/kg) resulted in similar fatty acid amide elevations (i.e., no dose response) and were not significantly different from each other (Newman-Keuls post hoc analysis; all $p > 0.05$), suggesting complete inhibition of FAAH at all dose levels studied. The observed pharmacodynamic effects of URB597 treatment at the four dose levels studied, expressed as mean fold change ($\pm$ S.E.M.) in plasma levels compared with vehicle treatment AEA, OEA, and PEA, were 10.3 ± 0.3, 7.8 ± 0.2, and 1.8 ± 0.1 for AEA, OEA, and PEA, respectively. The 10 mg/kg dose of URB597 was chosen for use in behavioral studies and was not associated with any change in L-DOPA levels 2 h after administration of L-DOPA, 4 h after administration of URB597 ($p > 0.05$, paired t test) (see Supplemental Table S1).

**FAAH Inhibitor Treatment Reduces L-DOPA-Induced Hyperactivity in MPTP-Lesioned Marmosets to the Level of Normal, Unlesioned Animals.** L-DOPA elicited a marked increase in total motor activity in MPTP-lesioned marmosets (Fig. 1). Examination of the time course of activity across the whole 6-h period of observation revealed a significant effect of time, and the interaction of the two on total activity counts ($F_{11,220} = 11.96; F_{3220} = 18.65, p < 0.001$; two-way RM-ANOVA; Fig. 1). Post hoc analysis demonstrated that L-DOPA-induced total activity was significantly higher than that of vehicle (subcutaneous)-treated, MPTP-lesioned animals for the first 3.5 h after drug administration (all $p < 0.05$). In comparison, URB597 (10 mg/kg p.o.) reduced L-DOPA-induced activity such that after approximately 2.5 h activity was no longer significantly different from that of vehicle (subcutaneous)-treated, MPTP-lesioned animals (Fig. 1). Thus, during the period 0 to 2 h, which included the time of peak L-DOPA effect (approximately 1–2 h) and the period 4 to 6 h, when animals were off, there were no differences in the magnitude of L-DOPA-evoked activity between animals coadministered L-DOPA and URB597 compared with those seen in L-DOPA-and vehicle-treated animals [both $p < 0.05$ compared with vehicle (subcutaneous)-treated animals]. As such, in the data presented below, further analysis of marmoset activity data from Fig. 1 was conducted for the period 2 to 4 h after L-DOPA administration. The start of this time period (2 h after L-DOPA and 4 h after URB597 administration) corresponds to the point at which AEA, OEA, and PEA levels in plasma were measured and found to be significantly elevated (Supplemental Fig. S1).

In the 2- to 4-h period after L-DOPA, there was a significant effect of treatment on total activity ($F_{4,25} = 8.98; p < 0.001$; one-way ANOVA; Fig. 2). Post hoc analysis demonstrated that total L-DOPA-induced activity was significantly increased (by 379%) compared with activity in vehicle (subcutaneous)-treated, MPTP-lesioned animals (2782 ± 682 compared with 581 ± 74 counts; $p < 0.001$). Furthermore, L-DOPA induced a level of total activity in MPTP-lesioned marmosets that was significantly increased, by 77%, compared with activity observed in nontreated, non-MPTP-lesioned (normal) animals (1572 ± 235 counts; $p < 0.05$; Fig. 2). This hyperactivity may represent a nonhuman primate correlate of dopamine dysregulation syndrome and impulse control disorder (Visanji et al., 2009). Treatment with
URB597 (10 mg/kg p.o.) attenuated L-DOPA-induced increases in total activity, in the 2- to 4-h period, to levels lower (by 32%) than that seen in the presence of L-DOPA alone (1879 ± 332 counts) and to a level that was not significantly different from that seen in normal, unlesioned animals (119% of normal; p > 0.05; Fig. 2).

Further analysis of the type of L-DOPA-evoked total activity during the 2- to 4-h period revealed a significant effect of URB597 treatment on the level of high activity counts, those that are above the average observed in a normal animal (F_{4,25} = 6.96; p < 0.001; one-way ANOVA; Fig. 2). L-DOPA significantly increased high activity counts (from 31 ± 17 to 2170 ± 778 counts) compared with vehicle treatment in MPTP-lesioned marmosets (p < 0.05). In addition, MPTP-lesioned marmosets treated with L-DOPA showed a significant increase in high activity counts (by 120%; p < 0.05) compared with normal, non-MPTP-lesioned animals (high activity counts; 985 ± 118; Fig. 2). It is noteworthy that there was no effect of any treatment on the level of low activity counts (those that are below the average observed in a normal animal) (F_{4,25} = 1.91; p > 0.05). As such, it seemed to be the hyperactive component, or high activity counts alone, that underlie the effect of L-DOPA on total activity in MPTP-lesioned marmosets. Indeed, in vehicle-treated MPTP-lesioned animals, high activity accounted for just 5% of total activity compared with 78% in the same animals treated with L-DOPA.

In MPTP-lesioned marmosets, URB597 (10 mg/kg p.o.) significantly reduced high activity counts evoked by L-DOPA during the period of 2 to 4 h (by 52% to 1047 ± 222 counts) (p < 0.05) such that high activity counts of URB597/L-DOPA-treated MPTP-lesioned animals were no longer significantly different from those seen in normal, unlesioned animals (both p > 0.05) and the proportion of total activity that was high was reduced (to 56%) in the presence of URB597 (Fig. 2).

**URB597 Does Not Interfere with the Antiparkinsonian Actions of L-DOPA in MPTP-Lesioned Marmosets.** During the 2- to 4-h period, there was no change in the antiparkinsonian actions of L-DOPA (Fig. 3A). The MPTP-lesioned animals used in the current study displayed a moderate to marked level of parkinsonian disability (median, 277; IQ, 163–312). During this same period (2–4 h after L-DOPA administration), L-DOPA significantly attenuated the level of parkinsonian disability in MPTP-lesioned marmosets to a mild to absent level [median, 80; IQ, 57–111, Friedman statistic (FS) = 15.2; p < 0.05 with Dunn’s post hoc test]. L-DOPA in combination with URB597 (10 mg/kg p.o.) produced a similar significant alleviation of parkinsonism to mild to absent levels (median, 78; IQ, 65–114; p < 0.05) that was not significantly different from that exhibited by animals treated with L-DOPA alone (p > 0.05; Dunn’s multiple comparison test).

In the periods 0 to 2 and 4 to 6 h, there were no effects of URB597 on L-DOPA-induced antiparkinsonian benefits (data not shown).

**URB597 Does Not Modify L-DOPA-Induced Dyskinesia or Psychosis in MPTP-Lesioned Marmosets.** URB597 did not modify L-DOPA-induced dyskinesia during the 2- to 4-h period (Fig. 3B). The MPTP-lesioned marmosets used in the present study exhibited a mild to moderate level of L-DOPA-induced dyskinesia that was a combination of chorea and dystonia (median, 20; IQ, 10–29; FS = 15.9; p < 0.05 with Dunn’s post hoc test). The level of dyskinesia in MPTP-lesioned animals administered L-DOPA in combination with URB597 (10 mg/kg p.o.) was also mild to moderate (median, 18; IQ, 13–29) and, although significantly different from that seen with vehicle alone (median, 0; IQ, 0–0; p < 0.05), was not significantly different from that seen when treated with L-DOPA alone (p > 0.05; Dunn’s multiple comparison test).

URB597 did not modify L-DOPA-induced psychosis (Fig. 3C) during the 2- to 4-h period. The parkinsonian animals used in the present study exhibited a mild to moderate level of L-DOPA-induced psychosis after treatment with L-DOPA during the period in which URB597 exerted an effect on L-DOPA-induced activity (2–4 h after L-DOPA administration) (median, 0; IQ, 0–0; FS = 11.3; p < 0.05). The level of psychosis in MPTP-lesioned animals administered with L-DOPA in conjunction with URB597 (10 mg/kg p.o.) was also
Discussion

The FAAH inhibitor URB597, given orally at 10 mg/kg, a dose shown to significantly elevate systemic fatty acid amide levels, attenuated L-DOPA-induced hyperactivity in MPTP-lesioned marmosets without compromising the antiparkinsonian actions of L-DOPA or interfering with its metabolism. These data suggest that FAAH inhibitors may have therapeutic potential in ICD and DDS associated with Parkinson’s disease. The data do not support the use of FAAH inhibitors to reduce L-DOPA-induced dyskinesia or enhance antiparkinsonian actions of L-DOPA, nor for antiparkinsonian action of FAAH inhibitors as monotherapy.

The study provides the first demonstration in nonhuman primates that oral administration of a FAAH inhibitor can elevate systemic levels of three FAAH substrates (N-acyl ethanolamides; AEA, OEA, and PEA). Although we did not measure brain N-acyl ethanolamide levels or FAAH enzyme activity in these animals, studies by Fegley et al. (2005) suggest that complete FAAH inhibition is required for maximal AEA elevation in the brains of URB597-treated rats. Ahn et al. (2009) also observed a correlation among 1) elevated central AEA levels (brain), 2) elevated systemic AEA levels (plasma), 3) central FAAH inhibition, and 4) systemic FAAH inhibition after treatment of rats with the selective FAAH inhibitor PF-3845 compared with vehicle. It has been shown in squirrel monkeys that systemic administration of URB597 (intravenous route) elevated the brain levels of AEA and OEA and inhibited FAAH activity throughout the brain (Justinova et al., 2008). In this study in marmosets, orally administered URB597 elevated systemic levels of AEA, OEA, and PEA and established the basis for assessing the behavioral effects of FAAH inhibitor treatment in the nonhuman primate model of PD. Although a complete pharmacodynamic assessment of the effects of URB597 on L-DOPA levels would have been desirable, because of the small blood volume of the marmoset and consequent animal welfare reasons, we were unable to conduct repeat same-day sampling. We thus opted to remove a single sample of blood at a time point chosen to correspond to the beginning of the period of observed effect of URB597 on L-DOPA-induced activity (2 h after administration of L-DOPA, 4 h after oral administration of URB597 or vehicle). At this time point, 10 mg/kg URB597 was shown to mild to moderate and, although significantly greater than that seen with vehicle alone (p < 0.05), was not significantly different from those animals treated with L-DOPA alone (p > 0.05; Dunn’s multiple comparison test).

In the periods 0 to 2 and 4 to 6 h, there were no effects of URB597 on L-DOPA-induced dyskinesia or psychosis (data not shown).

**URB597 Monotherapy Has No Effect on Behavior in the MPTP-Lesioned Marmoset.** URB597 given alone did not modify total activity, high or low activity count, or parkinsonian disability or elicit dyskinesia or psychosis (Newman-Keuls or Dunn’s post hoc analysis; all p > 0.05 compared with vehicle) (Figs. 1–3).
significantly elevate plasma fatty acid amide levels in all animals. Higher or lower doses of URB597 showed similar elevation of plasma fatty acid amide levels in these animals, suggesting maximal inhibition of FAAH. At this same time point, plasma L-DOPA levels were not altered by treatment with URB597 (see Supplemental Table 1) and thus the behavioral effects of URB597 are unlikely because of an alteration in L-DOPA availability.

The increased levels of three biomarkers of FAAH inhibition (AEA, OEA, and PEA) in marmosets treated with URB597 found herein are similar to the pharmacodynamic effect observed in studies with URB597 and other selective FAAH inhibitors in squirrel monkeys (Justinova et al., 2008) and in rodents (Fegley et al., 2005; Kinsey et al., 2009). The doses of URB597 found to be effective here also correspond closely to those that modulate motor responses to L-DOPA and other antiparkinsonian agents in rodent models of PD (Lee et al., 2006; Kreitzer and Malenka, 2007). URB597 treatment effects have been demonstrated in animal models of other neurological diseases including neuropathic pain (Kinsey et al., 2009), depression (Gobbi et al., 2005), and anxiety (Naidu et al., 2007). Although there have been suggestions that URB597 can cause sedation in a ferret model of morphine-6-glucuronide-induced emesis (Sharkey et al., 2007), the effects we observe are unlikely to be caused by any generalized sedation as neither the antiparkinsonian effects of L-DOPA nor L-DOPA-induced dyskinesia were affected by URB597. Moreover, a range of oral doses of URB597 have been tested in single or repeat daily administration tolerability studies in rodents (rats and mice) or primates (cynomolgus macaques) and have been shown to exert little influence on spontaneous motor activity in those species (reviewed in Piomelli et al., 2006).

The differential effects of URB597 on hyperactivity, parkinsonism, dyskinesia, and psychosis-like behaviors exhibited in the present study are worthy of further consideration and highlight the importance of assessing multiple aspects of behavior in MPTP-lesioned primates. Thus, in L-DOPA-treated, MPTP-lesioned animals, URB597 reduces both total activity and high activity to levels equivalent to those seen in normal animals. Specifically, the action of URB597 to ameliorate L-DOPA-induced hyperactivity occurred in the 2- to 4-h period after L-DOPA administration. It is noteworthy that there was no effect of URB597 during the earlier period of peak L-DOPA action. During the period of significant effect of URB597 on activity, there was neither a reduction in antiparkinsonian benefit, dyskinesia, nor psychosis-like behaviors. The selective reduction in high activity by URB597 thus reflects an attenuation of the quantity, but not quality, of motor activity. Similar effects to reduce L-DOPA-induced hyperactivity have been described previously with the α-adrenergic antagonist prazosin in the MPTP-lesioned primate (Visani et al., 2009). It is suggested that a change in amount and quality of movement may reflect an alteration in motivational aspects of motor control that relate to the degree to which the animals are driven or compelled to move (Visani et al., 2009). In this respect, the hyperactivity exhibited after L-DOPA treatment in MPTP-lesioned marmosets may represent a correlate of the hedonistic, reward-driven compulsive behaviors characteristic of dopamine dysregulation syndrome exhibited by some patients with PD. In addition, it would seem that these effects are disease-specific and closely related to the extent of striatal dopamine denervation because the dose of L-DOPA used in this study (20 mg/kg p.o.) has been shown to be devoid of effect on motor activity when administered to normal, non-MPTP-treated primates (Boyce et al., 1990). We hypothesize that such problems may comprise compulsive, repetitive, nogoal directed motor activity, e.g., “punding,” stereotypies, “hobbyism,” and “walkabouts” (as part of DDS) or ICDS such as pathological gambling, excessive eating or shopping, and hypersexuality (Evans and Lees, 2004; O’Sullivan et al., 2009; Weintraub et al., 2010). These actions are especially interesting when considered in light of data suggesting that FAAH inhibition might reduce hyperdopaminergic symptoms that may relate to psychosis and attention-deficit hyperactivity disorder in dopamine transporter knockout mice (Tzavara et al., 2006). FAAH inhibitors thus may have therapeutic potential across a range of psychiatric disorders involving obsessions and compulsive behaviors.

From a mechanistic point of view, we show that the effects of URB597 are consistent with inhibition of FAAH and elevation of fatty acid amides. However, we have not defined which fatty acid amides elevated by FAAH inhibition are responsible for the effects observed, nor which receptor might in turn be stimulated by such increases. Thus, although we demonstrate that URB597 can elevate AEA, OEA, and PEA, given the actions of these molecules, the effects observed here could be caused by interactions with one or more of the cannabinoid type 1 receptors (Di Marzo, 2009), TRPV1 receptors (van der Stelt and Di Marzo, 2004; Morgese et al., 2007), peroxisome proliferator-activated receptor α (Mascia et al., 2010), G protein-coupled receptor 119 (Chu et al., 2010), G protein-coupled receptor 55 (Whyte et al., 2009), or with an as yet uncharacterized FAA receptor.

The findings of a lack of effect of URB597 on antiparkinsonian action of L-DOPA were surprising in light of previous rodent data. FAAH inhibition has been reported to enhance antiparkinsonian actions of dopamine replacement therapy in rodent models of PD (Kreitzer and Malenka, 2007). However, those findings were seen in acute models of PD, and it is quite possible that the endocannabinoid system is altered in chronic disease states. Indeed it has been suggested that down-regulation of FAAH in the striatum might represent a compensatory mechanism for the loss of dopamine (Gubellini et al., 2002).

The inability of FAAH inhibition to reduce L-DOPA-induced dyskinesia is consistent with some, but not all, rodent data. On the one hand, in a simple rodent assay using acute challenge with L-DOPA in reserpinised rats to assess compounds with potential to reduce L-DOPA-induced dyskinesia, it was suggested that FAAH inhibition could be beneficial (Lee et al., 2006). However, in what is arguably a better validated model, repeated L-DOPA-treatment in the 6-hydroxydopamine-lesioned rat, a reduction in abnormal involuntary movements was seen only when FAAH inhibition was combined with an antagonist of the TRPV1 receptor (Morgese et al., 2007). As discussed above, FAAH inhibition has the potential to indirectly stimulate multiple receptor types. However, the ability to reduce dyskinesia seems to be suppressed by an elevation of fatty acid amides, probably AEA, which stimulates the TRPV1 receptor (van der Stelt and Di Marzo, 2004). These findings highlight the differences in pharmacology between the diverse complications of L-DOPA therapy and demonstrate the need for multiple therapeutic approaches to maximize the benefit of dopamine replacement therapy in PD.
In conclusion, we observe positive effects of FAAH inhibition on \( \alpha \)-DOPA-induced hyperactivity, which may be relevant to dopamine dysregulation syndrome and impulse control disorders that have emerged as increasingly appreciated problems in PD. As the potential of FAAH inhibition in PD is investigated, further studies will be required to define whether benefits would be maintained with long-term treatment and whether long-term therapy with FAAH inhibitors might be associated with adverse effects unrelated to impulse control disorders, for instance, obesity and insulin resistance (Tourino et al., 2010). Thus far, no untoward side effects or health issues have been reported in studies of oral daily administration of URB597 to normal rats or cynomolgus macaques at toxicological doses of \( \leq 1000 \text{ mg/kg} \) for several weeks (Piomelli et al., 2006).

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Authorship Contributions

Participated in research design: Johnston, Fox, Milne, Pearson, and Brotchie.

Conducted experiments: Johnston, Sykes, Wakefield, and Bartolini.

Contributed new reagents or analytic tools: Wakefield and Sykes.

Performed data analysis: Johnston, Fox, Huot, Wakefield, Sykes, Bartolini, and Pearson.

Wrote or contributed to the writing of the manuscript: Johnston, Fox, Milne, Pearson, and Brotchie.

References


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We thank Ironwood scientists Sylvie Bernier, Galen Carey, Peter Germano, Elaine Liong, Wayne Schairer, Alex Bryant, Ada Silos-Santiago, Mark Currie, Albert Profy, Gerhard Hanning, and Rob Busby for helpful discussions and assistance.
Supplemental materials:

Fatty acid amide hydrolase (FAAH) inhibition reduces L-DOPA-induced hyperactivity in the MPTP-lesioned non-human primate model of Parkinson’s disease

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Methods

Assessment of plasma FAAH substrate levels in response to treatment with URB597

Treatments

The effect of oral treatment of MPTP-marmosets with either vehicle or URB597 (3, 10, 30 or 60 mg/kg) on plasma levels of specific endogenous FAAH substrates was assessed. Levels of the endocannabinoid AEA, in addition to the non-cannabinoid fatty acid amides OEA and PEA, were measured by LC-MS/MS assays (described below). At 4:00 p.m. on days prior to plasma sampling, animals were orally administered vehicle (described below) or URB597 (3, 10, 30 or 60 mg/kg). Doses were given in a non-randomised fashion, being administered in ascending order of dose. At 7:00 a.m. the following day, all food was removed from the animals’ cages and, at 10:00 a.m. on the day of sampling, animals were again treated with the same dose of URB597 or vehicle. Four hours later, at 2:00 p.m., under light isoflurane anaesthetic (Forane®, Baxter Healthcare, Canada), 1 ml of blood was drawn from the great saphenous vein using a 27G butterfly needle and collected into 2 x 0.5 ml K₂-ethylenediaminetetraacetic acid- (EDTA)-containing vials (BD microtainer®, BD, Mississauga, Canada). Plasma was prepared within 10 min of collection by centrifugation of blood at 13,000 x g for 10 min at room temperature. Plasma was then transferred to sterile 1.5 ml tubes prior to immediate freezing on dry ice and subsequent storage at -80°C.

Bioanalytical assessment of AEA, OEA and PEA in MPTP-marmoset plasma

The concentrations of endogenous AEA, OEA and PEA in the above plasma samples collected from MPTP-lesioned marmosets were determined by LC-MS/MS as follows. Standard curves were generated using d8-AEA, d4-OEA, and d4-PEA (Cayman Chemical, Ann Arbor, MI) as stable isotope-labelled surrogate calibrators which contained 8 or 4 deuterium atoms per fatty
acid amide, respectively. These standards were prepared by diluting each in marmoset plasma (Bioreclamation Inc, Rochester, NY). An internal standard, d4-AEA (AEA labelled with 4 deuterium atoms, Cayman Chemicals, Ann Arbor, MI), was added to all samples, including those from MPTP-lesioned marmosets, and the standards. The fatty acid amides were extracted from 200-µl plasma samples or plasma standards by protein precipitation with three volumes of chilled chloroform:methanol (1:2, v:v), followed by liquid-liquid extraction with chloroform. After evaporation under nitrogen, the extracts were reconstituted in 60 µl of acetonitrile/isopropanol/water (20:5:75, v:v). The samples were injected (20 µl) on a Cliqueus C8 HPLC column (2.1 x 30 mm; 5 µm particle size; Higgins Analytical, Mountain View, CA) and chromatographed under reverse phase conditions using a gradient system with 0.1% formic acid in water and 0.1% formic acid in acetonitrile/isopropanol/water (85:10:5, v:v). The compounds were detected and quantified by tandem mass spectrometry in positive ion mode on an API 4000 instrument (Applied Biosystems; Framingham, MA). The limit of quantitation for all three analytes was 0.3 ng/ml.

The effect of URB597 on plasma L-DOPA levels in MPTP-lesioned marmosets

Treatments

The effect of oral treatment with either vehicle or URB597 (10 mg/kg) on plasma levels of L-DOPA was assessed at the beginning of the period of peak L-DOPA effect, i.e., 2 h after L-DOPA administration. At 4:00 p.m. on days prior to plasma sampling, animals were treated orally with either vehicle or URB597 (10 mg/kg). On days of plasma sampling, animals were fed their normal diet between 7:00 and 7:30 a.m., after which time all food was removed from their cages. Water was available ad libitum. At approximately 9:00 a.m., each animal received
either vehicle (p.o.) or URB597 (p.o.). Two hours after this, at approximately 11:00 a.m., animals received either vehicle or L-DOPA (s.c., 20 mg/kg) treatment. Two hours later, at 2:00 p.m., animals were lightly anaesthetised as described above and 1 ml of blood was removed from the great saphenous vein using a 27G butterfly needle and collected into 2 x 0.5 ml K2-EDTA-containing vials (BD microtainer®, BD, Mississauga, Canada). Plasma was prepared within 10 min of collection by centrifugation of blood at 13,000 x g for 10 min at room temperature. Plasma was then transferred to sterile 1.5 ml tubes prior to immediate freezing on dry ice and subsequent storage at -80°C.

Bioanalytical assessment of L-DOPA levels

The concentrations of L-DOPA in marmoset plasma samples were determined using LC-MS/MS by modification of a published procedure for quantification of L-DOPA in human plasma samples (Li et al., 2000). Plasma samples were transferred between laboratories on dry ice and then stored at -80°C until analysis. A standard curve of L-DOPA was prepared in marmoset plasma using a 0.5 mg/ml stock solution of L-DOPA (Sigma-Aldrich, St. Louis, MO) in water.

Plasma samples from MPTP-lesioned marmosets that had been treated orally with vehicle or URB597 and injected subcutaneously with L-DOPA and benserazide were thawed at room temperature and a 100-µl aliquot was taken from each sample. To each aliquot, 400 µl of ice cold acetonitrile containing 100 ng of deuterium-labelled L-DOPA [L-DOPA-d3 internal standard 3-(3,4-Dihydroxyphenyl-2,5,6-d3)-L-alanine obtained from Sigma-Aldrich, St. Louis, MO] was added. The individual plasma and acetonitrile samples were centrifuged at 15,000 x g for 10 min at room temperature. The supernatant was collected and dried under a stream of
nitrogen. The dried pellet was resuspended in 2% acetonitrile in water containing 0.1% formic acid.

Ten microliters of each sample were injected onto a Waters Atlantis T3 column (2 x 100 mm, 3 µm particle size) using a Waters Acquity HPLC system (Waters Corporation, Milford, MA). L-DOPA and L-DOPA-d3 were detected as they eluted from the column using a mobile phase gradient over 1.7 min, beginning with 2% acetonitrile in water and increasing to 20% acetonitrile in water. The aqueous mobile phase contained 0.1% formic acid. Detection of L-DOPA and L-DOPA-d3 was conducted using a Waters TQD mass spectrometer (Waters Corporation, Milford, MA) operating in MRM mode. The lower limit of quantification of L-DOPA by this LC-MS/MS method (or lowest usable standard for this assay) in plasma from MPTP-lesioned marmosets was 5 ng/ml.
Figure legends

Figure S1. The effect of treatment with URB597 on plasma FAAH substrate levels in MPTP-lesioned marmosets. On days prior to sampling, six female marmosets with L-DOPA-induced motor complications were pre-treated at 4:00 p.m. with either vehicle or URB597 (3, 10, 30 or 60 mg/kg, p.o.). At 9:00 a.m. the following day, animals were treated again with the same dose of vehicle or URB597. Four hours later (1:00 p.m.), animals were lightly anaesthetised and a blood sample removed. Plasma levels of AEA, OEA and PEA were assessed via LC-MS/MS. \( n = 6 \) for all groups. Data are mean values with SEM. *** represents \( p < 0.001 \) cf. vehicle treated animals (RM-1-way-ANOVA with Newman Keul’s Multiple Comparison Test).

Table S1. Effect of URB597 treatment on plasma L-DOPA levels in MPTP-lesioned marmosets. On days prior to sampling six female marmosets with L-DOPA-induced motor complications were pre-treated at 4:00 p.m. with either vehicle or URB597 (10 mg/kg, p.o.). At 9:00 a.m. the following day, animals were treated again with the same dose of vehicle or URB597. L-DOPA containing benserazide (5 mg/kg) was given at 11:00 a.m. on day of sample and four hours later (at 1:00 p.m.), animals were lightly anaesthetised and a blood sample removed. Plasma levels of L-DOPA were assessed via LC-MS/MS. \( n = 6 \) for all groups. Data are mean values with SEM. \(^a\) represents \( p < 0.05 \) cf. vehicle treated animals (paired t-test).
Table S1.

<table>
<thead>
<tr>
<th>Treatment 1</th>
<th>Treatment 2</th>
<th>Plasma L-DOPA concentration (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>vehicle (p.o.)</td>
<td>L-DOPA (20 mg/kg, s.c.)</td>
<td>$^{a}1618 \pm 135$</td>
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<tr>
<td>URB597 (10 mg/kg, p.o.)</td>
<td>L-DOPA (20 mg/kg, s.c.)</td>
<td>$1543 \pm 162$</td>
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</tbody>
</table>
References

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method for L-dopa and dopamine in rat plasma using electrospray LC/MS/MS. *J Pharm

Pharmacological characterization of psychosis-like behavior in the MPTP-lesioned
Figure S1.

A. AEA

B. OEA

C. PEA