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Title Page:

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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Running Title Page: Effects of URB597 in parkinsonian marmosets

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Abbreviations:

PD, *Parkinson's disease*; ICD, *impulse control disorders*; DDS, *dopamine dysregulation syndrome*; L-DOPA, *L-3,4-dihydroxyphenylalanine*; MPTP, *1-methyl-4-phenyl, 1,2,3,6-tetrahydropyridine;* AEA, *N-arachidonoyl-ethanolamide*; OEA, *N-oleoyl-ethanolamide*; PEA, *N-palmitoyl-ethanolamide*; LID, *L-DOPA-induced-dyskinesia*; URB597, *[3-(3-carbamoylphenyl)phenyl] N-cyclohexylcarbamate*; ANOVA, *Analysis of variance;* LC-MS/MS, *Liquid Chromatography-Tandem Mass Spectrometry; cf., Compare [Lat.]*

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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 non-motor complications, such as psychosis, impulse control disorders (ICD) and dopamine dysregulation syndrome (DDS). Non-dopaminergic neurotransmitter systems, including the endocannabinoid system, are likely critical to the development of these complications. The role of fatty acid amide hydrolase (FAAH) in mediating L-DOPA-induced behaviours was explored in the MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine)-lesioned marmoset model of PD. Pharmacodynamic and locomotor effects of the selective FAAH inhibitor URB597 were assessed via bioanalytical (LC-MS/MS) and behavioural observation approaches. URB597 (3, 10, 30, or 60 mg/kg, *p.o.*) increased plasma levels of the FAAH substrates N-arachidonoyl-ethanolamide (AEA, anandamide), N-oleoyl-ethanolamide (OEA), and N-palmitoyl-ethanolamide (PEA) by 10.3±0.3, 7.8±0.2 and 1.8±0.1-fold (mean of URB597 groups ± SEM), respectively, *cf.* vehicle (all $p<0.001$) four hours after administration. Treatment with L-DOPA (20 mg/kg, *s.c.*) alleviated parkinsonism but elicited dyskinesia, psychosis-like-behaviours and hyperactivity, a potential correlate of ICD and DDS. During the period 2-4 h post-L-DOPA, corresponding to 4-6 hours after URB597 administration, URB597 reduced total L-DOPA-induced activity, as well as 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 anti-parkinsonian actions of L-DOPA, or L-DOPA-induced dyskinesia and psychosis. URB597 did not alter plasma L-DOPA levels and was without behavioural 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 anti-parkinsonian benefits of L-DOPA treatment.

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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, non-motor-related complications of treatment are recognised 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 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 non-motor complications are not completely elucidated but it seems clear that, in addition to abnormal dopamine receptor stimulation, non-dopaminergic 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 FAA catabolism, fatty acid amide hydrolase (FAAH) (Cravatt et al., 1996; McKinney and Cravatt, 2005), as well the cannabinoid receptor type 1 (CB1) (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 parkinsonism *per se* and following 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 PD patients (Sieradzan et al., 2001).

In the present study, we investigated the potential role of FAAH in modulating the motor and non-motor response to L-DOPA in MPTP-lesioned primates 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-palmitoyl 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 sub-therapeutic 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, though this might require concomitant blockade of the vanilloid receptor TRPV1 (Lee et al., 2006; Morgese et al., 2007). However, this hypothesis has never been tested in primate models of PD. Moreover, until recently, non-motor complications of PD, particularly those of a psychiatric nature, such as impulse control (ICD), dopamine dysregulation syndrome (DDS) and psychosis, were subject to scant consideration in large part due to 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 characterised, using L-DOPA-treated, MPTP-lesioned primates (Visanji et al., 2006) in which abnormal psychotomimetic behaviours, 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 quantifiably distinct from the anti-parkinsonian or pro-dyskinetic actions of dopamine replacement (Visanji et al., 2009). It is hypothesised that this hyperactivity (*i.e.*, activity that is above that seen in the normal, non-parkinsonian state) might represent a behavioural 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 impact non-motor 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.

Methods

Preparation of animals

Six common marmosets (*Callithrix jacchus*; female; weight, 350-500 g (Harlan, Madison, WI, USA) were kept under conditions of controlled temperature (25 ± 2°C) and a 12-hour light/dark

cycle (lights on at 6.30 a.m.). Animals were group housed and cared for in accordance with an approved Animal Care Committee protocol (AUP 1022.10) from the local IACUC (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. Prior to 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 to transfer to observation cages for assessment of behaviour. Activity of normal animals was quantified prior to any test article treatment or MPTP intoxication as described below (in *Behavioural assessment* section). 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. Following this, animals were allowed to recover for 12 weeks to allow parkinsonian symptoms to develop and stabilise. MPTP intoxication resulted in a syndrome characterised by bradykinesia, hunched posture, and a reduced range of movement. Treatment-related complications including dyskinesia and psychosis-like behaviours were evoked by twice-daily treatment with oral L-DOPA (as Prolopa®, Roche Canada, Ontario, equivalent to 15 mg/kg L-DOPA and 3.5 mg/kg benserazide) for a minimum of 30 days. This treatment regimen has been previously demonstrated 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 anti-parkinsonian and anti-dyskinetic agents. A minimum period of two weeks was allowed between the completion of any prior study and the commencement of this study, a period in excess of ten half-lives since administration of any previously-employed experimental drug. Throughout the course of the study, on days not requiring plasma sampling or behavioural observations, animals were treated once daily with oral L-DOPA therapy at 10:00 a.m. 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 (3, 10, 30 or 60 mg/kg) on plasma levels of endogenous FAAH substrates, AEA, OEA and PEA were measured by LC-MS/MS 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.

Behavioural 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 anti-parkinsonian effect achievable while eliciting hyperactivity, dyskinesia and psychosis that was stable and reproducible on successive L-DOPA administrations. For behavioural observations, L-DOPA was administered *s.c.* at a dose volume of 1 ml/kg, as L-DOPA methyl ester (Sigma-Aldrich, Oakville, Canada) in combination with benserazide (Sigma-Aldrich, Oakville, Canada). 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 employed for all behavioural observations and was administered orally at a dose volume of 5 ml/kg.

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Treatments

The effect of URB597 alone and in combination with L-DOPA was assessed. On days prior to behavioural assessment, animals were fed normally and received a maintenance oral L-DOPA dose at 9:00 a.m. At 4:00 p.m. animals were administered either vehicle (*p.o.*) or URB597 (10 mg/kg, *p.o.*). On days of behavioural assessment, 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.*). Behavioural assessment, as described below commenced directly following 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 randomised in each animal. A minimum of 48 h was left between behavioural observations in the same animal.

Behavioural assessment of marmoset activity

A quantitative assessment of marmoset activity was made using computer-operated passive infra-red 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 utilised that displayed activity counts within Microsoft Excel (Microsoft, Redmond, WA). Total activity counts were logged in 30-min epochs across the entire 6 h duration of the

experiment. These activity data were analysed to define any period(s) during which URB597 produced a significant effect on L-DOPA induced activity. In the current study, we demonstrated significant effects of URB597 in the period 2-4 h following L-DOPA administration. Total activity data, cumulated into time bins of 2 h duration, were further analyzed to define activity that was above or below average, *i.e.*, hyper- or hypoactive behaviour. Firstly, the average normal activity, in counts per minute, was obtained from a separate group of animals prior to administration of MPTP (*i.e.*, in the normal state). In the experimental situation, the activity data from MPTP-lesioned marmosets were analyzed and the amount of high and low activity counts above and below the per-minute average of that seen in normal animals was calculated.

Behavioural assessment of parkinsonian disability, dyskinesia and psychosis

After administration of final treatment (L-DOPA, *s.c.*), animals were placed immediately into observation cages (0.8 × 0.8 × 0.7 m) containing food, water and a wooden perch and left undisturbed for the 6 h duration of the experiment. Behaviour was monitored via digital video disk (DVD) recording and analysed *post hoc* by a movement disorder neurologist blinded to the treatment. Methods for assessment of behaviour were essentially as described previously (Visanji et al., 2006). Parkinsonian disability scores were assessed every 10 min for the duration of assessment. Briefly, 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, crouched posture, head down. Attention/alertness was rated 0 or 1; 0 = normal head

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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 behaviours 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-minute period of assessment. For psychosis, hyperkinesia, response to non-apparent stimuli (hallucinatory behaviour), 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 sub-score 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 ANOVA with a Bonferroni *post hoc* analysis was used. To assess the effect of

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treatment on cumulated levels of total activity, high and low activity and plasma FAAH substrate levels a parametric 1-way analysis of variance (ANOVA) followed by Newman Keul's Multiple Comparison *post hoc* test was employed. 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 SEM. For measures of parkinsonian disability, dyskinesia and psychosis, data were analysed 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, Oakville, Canada) 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 L-DOPA and 12.5 mg benserazide (free base) was dissolved in Gatorade sports beverage (The Gatorade Company, Chicago, Illinois) prior to oral administration by syringe at a volume of 10 ml/kg. On days of behavioural assessment, L-DOPA (20 mg/kg, free base) was prepared from the methyl ester form (Sigma-Aldrich, Oakville, Canada) in combination with benserazide hydrochloride (5 mg/kg, freebase, Sigma-Aldrich, Oakville, Canada) dissolved in 0.9% sterile saline containing 0.1% ascorbate (Sigma-Aldrich, Oakville, Canada) and 0.05% absolute ethanol. URB597 (Cayman Chemical, Ann Arbor, Michigan) was formulated in a vehicle containing 50% corn oil (Professional Compounding Centers of America (PCCA), Houston, Texas) and 50%

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nutritional drink Ensure™ (Abbott Nutrition, Chicago, Illinois) 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). 10 ml corn oil was then added and mixed until homogenous. 10 ml Ensure™ was then added and the mixture mixed gently at first, vortexed and finally sonicated (Ultrasonic Dismembrator, Fisher Scientific model 100, 1/8") on a medium setting for 30 sec 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 to placebo treatment (all $p<0.001$, see Figures S1A-C, respectively, in Supplementary Materials). Mean plasma levels of AEA, OEA and PEA were 0.49 ± 0.09, 0.98 ± 0.10 and 4.34 ± 0.21 ng/ml in vehicle-treated monkeys and were 5.0 ± 0.45, 7.7 ± 0.78 and 7.0 ± 0.27 ng/ml, respectively, in URB597 (10 mg/kg)-treated monkeys (Figures S1A-C in Supplementary Materials). All four doses of URB597 administered (3, 10, 30, 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. This observed pharmacodynamic effects of URB597 treatment at the 4 dose levels studied, expressed as mean fold change (± SEM) in

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plasma levels compared to 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 behavioural studies and was not associated with any change in L-DOPA levels 2 h post administration of L-DOPA, 4 h post administration of URB597 ($p > 0.05$, paired t-test) (see Table S1 in Supplementary Materials).

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 (Figure 1). Examination of the time-course of activity across the whole 6 h period of observation revealed a significant effect of time, treatment and the interaction of the two on total activity counts ($F_{time\ 11,220} = 11.96$, $F_{treatment\ 3,220} = 18.65$ and $F_{interaction\ 33,220} = 5.18$, all $p<0.001$, 2-way RM-ANOVA, Figure 1). *Post-hoc* analysis demonstrated that L-DOPA-induced total activity was significantly higher than that of vehicle (*s.c.*)-treated, MPTP-lesioned animals for the first 3.5 h following 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 (*s.c.*)-treated, MPTP-lesioned animals (Figure 1). Thus, during the period 0-2 h, which included the time of peak L-DOPA effect (approximately 1-2 h) and the period 4-6 h, when animals were OFF, there were no differences in the magnitude of L-DOPA-evoked activity between animals co-administered L-DOPA and URB597 compared to those seen in L-DOPA and vehicle treated animals (both $p<0.05$ *cf.* vehicle (*s.c.*)- treated animals). As such, in the data presented below, further analysis of marmoset activity data from Figure 1 was conducted on the period 2-4 h post L-DOPA administration. The start of this time period (2 h after L-DOPA and 4

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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 (Fig. S1).

In the 2-4 h period post-L-DOPA, there was a significant effect of treatment on total activity ($F_{4,25}$ = 8.98, p<0.001, 1-way ANOVA, Figure 2). *Post-hoc* analysis demonstrated that total L-DOPA-induced activity was significantly increased (by 379%), compared to activity in vehicle (*s.c.*)-treated, MPTP-lesioned animals (2782 ± 682 *cf.* 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 to activity observed in non-treated, non-MPTP-lesioned (normal) animals (1572 ± 235 counts, p<0.05, Figure 2). This hyperactivity may represent a non-human 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 2-4 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, Figure 2).

Further analysis of the type of L-DOPA-evoked total activity during the 2-4 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, 1-way ANOVA, black bars, Figure 2). L-DOPA significantly increased high activity counts (from 31 ± 17 to 2170 ± 778 counts) compared to vehicle treatment in MPTP-lesioned marmosets (p<0.01). In addition, MPTP-lesioned marmosets treated with L-DOPA showed a significant increase in high activity counts (by 120%, p<0.05) as compared to normal, non-MPTP-lesioned animals (high activity counts; 985 ± 118 Figure 2). Notably, 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 appeared 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 to 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 2-4 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, un-lesioned animals (both p>0.05) and the proportion of total activity that was high was reduced (to 56%) in the presence of URB597 (Figure 2).

URB597 does not interfere with the anti-parkinsonian actions of L-DOPA in MPTP-lesioned marmosets

During the 2-4 h period, there was no change in the anti-parkinsonian actions of L-DOPA (Figure 3A). The MPTP-lesioned animals used in the current study displayed a moderate to marked level of parkinsonian disability (median, 277; IQR, 163-312). During this same period (2-4 h post L-DOPA administration), L-DOPA significantly attenuated the level of parkinsonian disability in MPTP-lesioned marmosets to a mild to absent level (median, 80; IQR, 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; IQR, 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, 0-2 h and 4-6 h, there were no effects of URB597 on L-DOPA-induced anti-parkinsonian 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 2-4 h period (Figure 3B). The MPTP-lesioned marmosets employed 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; IQR, 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; IQR, 13-29) and, while significantly different to that seen with vehicle alone (median, 0; IQR, 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 (Figure 3C) during the 2-4 h period. The parkinsonian animals used in the present study exhibited a mild to moderate level of L-DOPA-induced psychosis following treatment with L-DOPA during the period in which URB597 exerted an effect on L-DOPA-induced activity (2-4 h post L-DOPA administration) (median, 0; IQR, 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 mild to moderate and, while 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, 0-2 h and 4-6 h, there were no effects of URB597 on L-DOPA-induced dyskinesia or psychosis (data not shown).

URB597 monotherapy has no effect on behaviour in the MPTP-lesioned marmoset

URB597 given alone did not modify total activity, high or low activity count, parkinsonian disability or elicit dyskinesia or psychosis (Newman-Keuls or Dunn's *post-hoc* analysis, all $p>0.05$ *cf.* vehicle) (Figures 1, 2 and 3).

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 anti-parkinsonian actions of L-DOPA or interfering with its metabolism. These data suggest that FAAH inhibitors may have therapeutic potential in impulse control disorder (ICD) and dopamine dysregulation syndrome (DDS) associated with Parkinson's disease. The data do not support the use of FAAH inhibitors to reduce L-DOPA-induced dyskinesia or enhance anti-parkinsonian actions of L-DOPA, nor for anti-parkinsonian action of FAAH inhibitors as monotherapy.

The study provides the first demonstration, in non-human 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 brains of URB597 treated rats. Ahn *et al.* (2009) also observed a correlation among (a) elevated central AEA levels (brain) (b) elevated systemic AEA levels (plasma), (c) central FAAH inhibition and (d) systemic FAAH inhibition following treatment of rats with the selective FAAH inhibitor PF-3845 compared to vehicle. It has been shown in squirrel monkeys that systemic administration of URB597 (*i.v.* route) elevated 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 behavioural effects of FAAH inhibitor treatment in the non-human primate model of PD. While a complete pharmacodynamic assessment of the effects of URB597 on L-DOPA levels would have been desirable, due to 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 (two hours post administration of L-DOPA, four hours post oral administration of URB597 or vehicle). At this time-point, 10 mg/kg URB597 was shown to 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 Table in Supplemental info) and thus, the behavioural effects of URB597 are unlikely due to an alteration in L-DOPA availability.

The increased levels of 3 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 anti-parkinsonian 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). While 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 due to any generalised sedation as neither the anti-parkinsonian 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 shown to exert little influence upon spontaneous motor activity in those species (reviewed in (Piomelli et al., 2006)).

The differential effects of URB597 on hyperactivity, parkinsonism, dyskinesia and psychosis-like behaviours exhibited in the present study are worthy of further consideration and highlight the importance of assessing multiple aspects of behaviour 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 2-4 h period following L-DOPA administration. Notably, 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 anti-parkinsonian benefit, dyskinesia nor psychosis-like behaviours. 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 previously been described with the alpha-adrenergic antagonist prazosin in the MPTP-lesioned primate (Visanji 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 (Visanji et al., 2009). In this respect, the hyperactivity exhibited following L-DOPA treatment in MPTP-lesioned marmosets may represent a correlate of the hedonistic, reward-driven compulsive behaviours characteristic of dopamine-dysregulation syndrome exhibited by some PD patients. In addition, it would appear that these effects are disease-specific and closely related to the extent of striatal dopamine denervation since the dose of L-DOPA employed 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, non-goal 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 (ADHD) in dopamine transporter knockout mice (Tzavara et al., 2006). FAAH inhibitors may thus have therapeutic potential across a range of psychiatric disorders involving obsessions and compulsive behaviours.

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, while we demonstrate that URB597 can elevate AEA, OEA and PEA, given the actions of these molecules, the effects observed here could be due to interactions with one or more of cannabinoid CB1 receptors (Di Marzo, 2009), TRPV1 receptors (van der Stelt and Di Marzo, 2004; Morgese et al., 2007), PPARα (Mascia et al., 2010), GPR119 (Chu et al., 2010), GPR55 (Whyte et al., 2009) or with an, as yet, uncharacterized FAA receptor.

The findings of a lack of effect of URB597 on anti-parkinsonian action of L-DOPA were surprising in the light of previous rodent data. FAAH inhibition has been reported to enhance anti-parkinsonian actions of dopamine replacement therapy in rodent models of PD (Kreitzer and Malenka, 2007). However, these 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 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 utilizing 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 only seen when FAAH inhibition was combined with an antagonist of the TRPV1 receptor (Morgese et al., 2007). As discussed above, FAAH inhibition has potential to indirectly stimulate multiple

receptor types. However, the ability to reduce dyskinesia appears to be suppressed by an elevation of fatty acid amides, likely 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 maximise the benefit of dopamine replacement therapy in PD.

In conclusion, we observe positive effects of FAAH inhibition on L-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 $\geq$ 1000 mg/kg for several weeks (Piomelli et al., 2006).

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Author Contributions

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

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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.

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Footnotes

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Legends for Figures

Figure 1. The effect of treatment with URB597 on time course of total activity in MPTP-lesioned marmosets. On days prior to behavioural observation, six female marmosets with L-DOPA-induced motor complications, including dyskinesia, were pre-treated at 4:00 p.m. with either vehicle or URB597 (10 mg/kg, *p.o.*). In addition, at 9:00 a.m. animals received oral L-DOPA (Prolopa™ 20 mg/kg) in order to maintain motor complications. At 9:00 a.m. on days of behavioural observation, animals were treated acutely with oral vehicle or URB597 (10 mg/kg, *p.o.*). Two hours later (11:00 a.m.), animals also received vehicle or L-DOPA / benserazide (20/ 5 mg/kg, *s.c.*) after which animals were transferred to observation cages and total activity counts assessed over the entire 6 h period of observation. $N = 6$ for all groups. Data are mean values with SEM. * represents $p < 0.05$ *cf.* vehicle (*p.o.*) / vehicle (*s.c.*) treated, MPTP-lesioned animals. 2-way, RM ANOVA with Bonferroni *post hoc* test. Dotted vertical lines (at 2 and 4 h) indicate the period of effect of URB597 on L-DOPA-induced activity selected for detailed analysis.

Figure 2. The effect of treatment with URB597 on total motor activity counts and proportion comprising either high and low activity in MPTP-lesioned marmosets. On days prior to behavioural observation, six female marmosets with L-DOPA-induced motor complications, including dyskinesia, were pre-treated at 4:00 p.m. with either oral vehicle or URB597 (10 mg/kg, *p.o.*). In addition, at 9:00 a.m. animals received oral L-DOPA (Prolopa™ 20 mg/kg) in order to maintain motor complications. At 9:00 a.m. on days of behavioural observation animals were treated acutely with oral vehicle or URB597 (10 mg/kg, *p.o.*). Two hours later (11:00 a.m.) animals also received vehicle (*s.c.*) or L-DOPA / benserazide (20/ 5 mg/kg, *s.c.*) after which animals were transferred to observation cages and activity assessed via computerised activity monitoring. Total activity scores were obtained every min during the

period 2-4 h after administration of L-DOPA and also further compared to that same time period obtained pre-MPTP (normal). High (black bar) and low (white bar) activity counts (that were respectively above or below the average of animals in the normal state) were cumulated. *n*=6 for all groups. Data are mean values with SEM. ** represents $p < 0.01$ total and high activity counts *cf.* vehicle (*p.o.*) / vehicle (*s.c.*) treated, MPTP-lesioned animals. # represents $p < 0.05$ high activity counts *cf.* vehicle (*p.o.*) / L-DOPA (*s.c.*) treated, MPTP-lesioned animals. † represents $p < 0.05$ total and high activity counts *cf.* normal (untreated pre-MPTP) animals. ∧ represents $p < 0.05$ total activity counts *cf.* vehicle (*p.o.*) / vehicle (*s.c.*) treated, MPTP-lesioned animals. Multiple 1-way ANOVA with Newman Keul's Multiple Comparison Test.

Figure 3. The effect of treatment with URB597 on parkinsonian disability, dyskinesia and psychosis in MPTP-lesioned marmosets. On days prior to behavioural observation, six female marmosets with L-DOPA-induced motor complications, including dyskinesia, were pre-treated at 4:00 p.m. with either vehicle or URB597 (10 mg/kg, *p.o.*). In addition, at 9:00 a.m. animals received oral L-DOPA (Prolopa™ 20 mg/kg) in order to maintain motor complications. At 9:00 a.m. on days of behavioural observation, animals were treated acutely with oral vehicle or URB597 (10 mg/kg, *p.o.*). Two hours later (11:00 a.m.), animals also received vehicle or L-DOPA / benserazide (20/ 5 mg/kg, *s.c.*) after which animals were transferred to observation cages and assessed via *post-hoc* analysis of DVD recordings by a neurologist blinded to treatment. Scores for parkinsonian disability (**A**), dyskinesia (**B**) and psychosis (**C**) were obtained every 10 min and summed over the period 2-4 h after L-DOPA administration. *n*=6 for all groups. Data are expressed as medians with individual values. * represents $p<0.05$ *cf.* vehicle (*p.o.*) / vehicle (*s.c.*) treated animals, Friedman's test with Dunn's multiple comparison's test.

Figure 1.