

1 Ken Sobel, Esq. [Bar No. 06551]  
2 5346 Soledad Rancho Court  
3 San Diego, CA 92109  
4 Phone: 619.208.2439  
5 Email: kennysocal711@gmail.com

6 *ATTORNEYS FOR APPELLANT,*  
7 *THE ARIZONA CANNABIS NURSES ASSOCIATION*

8 **SUPERIOR COURT OF THE STATE OF ARIZONA**

9 **COUNTY OF MARICOPA**

10 The ARIZONA CANNABIS NURSES ASSOCIATION ("AZCNA"), an Arizona  
11 Non-Profit Corporation,

12 Appellant,

13 v.

14 ARIZONA DEPARTMENT OF HEALTH SERVICES ("AZDHS"), an Arizona  
15 administrative agency; WILLIAM HUMBLE,  
16 Director of the AZDHS in his official  
17 capacity,

18 Appellees.

Case No.

19 **NOTICE OF APPEAL**

20 **ADHS/Clerk of the Department**  
21 **Administrative Counsel**

22 INTRODUCTION

23 The Arizona Cannabis Nurses Association ("AZCNA") submitted a timely Petition  
24 seeking to add Parkinson's Disease ("PD") as a "debilitating" condition under Arizona's  
25 voter-approved Medical Marijuana Act (Proposition 203), on July 9, 2014, and within the  
26 time period allowed by the Department.

A true and correct copy of the Petition and the Exhibits attached thereto is attached  
herewith as Exhibit "1", and incorporated by reference as though fully set forth herein.

MICHAEL A. JEANES, CLERK  
BY

RECEIVED AS LOBBY  
DOCUMENT  
OFFICE OF THE CLERK

14 SEP - 8 PM 5:03

**COPY**

SEP - 8 2014



MICHAEL A. JEANES, CLERK  
G. HOFFMANN, DEPUTY CLERK

**FILED**

SEP 18 2014

LC 2014-000421-001

1 The process and standards for the consideration and decision is set forth in the  
2 Arizona Administrative Code, Rule 9-17-106. Appellant met each and every element. In  
3 fact, it followed precisely – both in form and content – the specific requirements of the  
4 Rule.

5 Nevertheless, 26 days later, on August 5, 2014, the Arizona Department of Health  
6 Services (“AZDHS”), denied the Petition claiming that Petitioner (Appellant) “failed to  
7 satisfy the following elements as required in AAC R9-17-106(A)(4-7),” without providing  
8 “the specific reason for the determination” nor advising of “the process for requesting  
9 judicial review of the Department’s determination pursuant to A.R.S. Title 12, Chapter 7,  
10 Article 6;...”. AAC R9-17-106(B) (3) (b). A true and correct copy of the AZDHS denial  
11 letter is attached hereto as Exhibit “2” and incorporated by reference as though fully set  
12 forth herein.

13 There are now 23 states plus the District of Columbia that provide a medical  
14 marijuana program, 2 states are adult legal (Colorado, Washington), and 11 more states this  
15 year adopted “Charlotte’s Web” laws providing safe access for parents and their epileptic  
16 seizure-prone children allowing the use of cannabis oils. A total of 34 states plus the  
17 District of Columbia.

18 Several of the more recent medical states have added Parkinson’s Disease to the list  
19 of debilitating conditions including Massachusetts, Connecticut, Illinois and New Mexico.  
20 California has always allowed medical marijuana for Parkinson’s Disease as a result of it’s  
21 “catch-all” provision that allows the individual physician to recommend cannabis for any  
22 condition he/she believes will bring relief to the patient. All of these decisions have been  
23 based on the prevailing medical and scientific research, the same research supplied by  
24 Petitioner in this case in support of its Petition.

25 Because of the federal government’s vice grip on cannabis research – sometimes  
26 referred to as the NIDA blockade – it is nearly impossible to conduct high level, double-

1 blind human studies that investigate the positive medical benefits from the use of cannabis.  
2 Only two such studies have been approved in the past 10 years, and one of those, the PTSD  
3 study proposed by Dr. Sue Sisily in Arizona is bogged down by funding issues and her  
4 recent termination as a U of A Professor and Clinician.

5 AZDHS refused 19 Petitions to add debilitating conditions, until the 20<sup>th</sup> – a  
6 Petition to Add Post-Traumatic Stress Disorder – was approved by order of Administrative  
7 Law Judge Shedden with the State of Arizona’s Office of Administrative Hearings,  
8 *Arizona Cannabis Nurses Association v. Arizona Department of Health Services* (June 4,  
9 2014), Case No. 2014A-MMR-0254-DHS.

10 In this case, AZDHS rejected the Petition without allowing even a public hearing on  
11 the matter. This is a closed-universe case. All of the facts relevant to this Court’s  
12 determination can be found in the Appellant’s Petition (Exhibit 1) and the AZDHS’ letter  
13 response (Exhibit 2). The applicable law is found in ARS Section 36-2801.01 “Addition of  
14 Debilitating Medical Conditions”, and AAC R9-17-106 “Adding A Debilitating  
15 Condition”.

16 ARS Section 36-2801.01 specifically provides that “denial of a petition is a final  
17 decision of the department subject to judicial review pursuant to title 12, chapter 7, article  
18 6. Jurisdiction and venue are vested in the superior court.”

19 Again, that section also required ADHS to provide Petitioner with written notice to  
20 the requestor (Appellant) of “[t]he process for requesting judicial review of the  
21 Department’s decision pursuant to ARS Title 12, Chapter 7, Article 6”. ACC R9-17-  
22 106(B) (5) (ii). ADHS failed to do so. See Exhibit “2”.

23 Accordingly, based upon the fact that the Appellant complied with rules and met all  
24 of the essential requirements in its Petition, and the fact that AZDHS failed to follow its  
25 own rules in denying the Petition, the Appellant respectfully requests that the Superior  
26 Court grant the Petition, and approve Parkinson’s Disease as a new debilitating condition.

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26

JURISDICTION/VENUE/PARTIES

1. At all times relevant herein, Appellant AZCNA is a non-profit organization domiciled in Arizona, and the entity that filed a Petition to add Parkinson’s Disease as a Debilitating Condition pursuant to ARS Section 36-2801.01 and AAC R9-17-106.

2. Defendant Arizona Department of Health Services (“ADHS”) is an Arizona administrative agency with its principal place of business in Maricopa County responsible for implementing and administering the Arizona Medical Marijuana Act (“AMMA”).

3. Defendant William Humble is the Director of AZDHS and is believed to be a resident of Maricopa County. In his capacity as Director of AZDHS, Defendant Humble is responsible for implementing and administering the AMMA. He is sued in his official capacity.

4. Jurisdiction is proper pursuant to Arizona Revised Statutes, Section 36-2801.01, Sections 12-901 and 12-905.

5. Venue for the action in Maricopa County is proper pursuant to Arizona Revised Statutes, Section 36-2801.01 and Section 12-905, as the Defendants are located there and all or substantially all of the transactions related to the appeal occurred in Maricopa County, Arizona.

BACKGROUND

6. At all times relevant herein, Appellant AZCNA is a non-profit organization domiciled in Arizona, and the entity that filed a Petition to Add Parkinson’s Disease (“PD”) as a Debilitating Condition pursuant to ARS Section 36-2801.01 and AAC R9-17-106.

- 1 7. On July 9, 2014, the Arizona Cannabis Nurses Association (“AZCNA”) filed a  
2 9 page Petition plus 3 exhibits consisting of three articles published in peer  
3 reviewed scientific journals that support the use of medical marijuana for the  
4 condition or symptoms associated with Parkinson’s Disease. See Exhibit “1”  
5 8. However, AZDHS denied the Petition on August 5, 2014, solely on the basis  
6 that AZCNA failed to provide information supporting the requirements of R9-  
7 17-106(A) (4-7). ~~Such allegations are false. In fact, the AZCNA’s PD Petition~~  
8 specifically tracked each and every requirement set forth in sub-sections 4 – 7,  
9 and provided all of the relevant information to satisfy each such element.

10 9. R9-17-106(A)(4-7) provides as follows:

11 **“R9-17-106. Adding a Debilitating Medical Condition**

- 12 A. An entity may request the addition of a medical condition to the list of debilitating medical conditions in R9-17-201 by  
13 submitting to the Department, at the times specified in subsection (C), the following in writing:  
14 \*\*\*  
15 4. A description of the symptoms and other physiological effects experienced by an individual suffering from the medical  
16 condition or a treatment of the medical condition that may impair the ability of the individual to accomplish activities of  
17 daily living;  
18 5. The availability of conventional medical treatments to provide therapeutic or palliative benefit for the medical condition or a  
19 treatment of the medical condition;  
20 6. A summary of the evidence that the use of marijuana will provide therapeutic or palliative benefit for the medical condition  
21 or a treatment of the medical condition; and  
22 7. Articles, published in peer-reviewed scientific journals, reporting the results of research on the effects of marijuana on the  
23 medical condition or a treatment of the medical condition supporting why the medical condition should be added.”

24 In its Petition, for example, the AZCNA provided the following:

- 25 I. “A description of the symptoms and other physiological effects experienced by an individual suffering from  
26 the medical condition or a treatment of the medical condition that may impair the ability of the individual to  
accomplish activities of daily living

A. Symptoms:

According to the Mayo Clinic:

“Parkinson's signs and symptoms may include:

- **Tremor.** Your tremor, or shaking, usually begins in a limb, often your hand or fingers. You may notice a back-and-forth rubbing of your thumb and forefinger known as a pill-rolling tremor. One characteristic of Parkinson's disease is a tremor of your hand when it is relaxed (at rest).

- 1 • **Slowed movement (bradykinesia).** Over time, Parkinson's disease may reduce your ability to move and slow your movement, making simple tasks difficult and time-consuming. Your steps may become shorter when you walk, or you may find it difficult to get out of a chair. Also, you may drag your feet as you try to walk, making it difficult to move.
- 2
- 3 • **Rigid muscles.** Muscle stiffness may occur in any part of your body. The stiff muscles can limit your range of motion and cause you pain.
- 4
- 5 • **Impaired posture and balance.** Your posture may become stooped, or you may have balance problems as a result of Parkinson's disease.
- 6 • **Loss of automatic movements.** In Parkinson's disease, you may have a decreased ability to perform unconscious movements, including blinking, smiling or swinging your arms when you walk. You may no longer gesture when talking.
- 7
- 8 • **Speech changes.** You may have speech problems as a result of Parkinson's disease. You may speak softly, quickly, slur or hesitate before talking. Your speech may be more of a monotone rather than with the usual inflections. A speech-language pathologist may help improve your speech problems.
- 9
- 10 • **Writing changes.** Writing may appear small and become difficult.”

11 <http://www.mayoclinic.org/diseases-conditions/parkinsons-disease/basics/symptoms/con-20028488>”

12 Not only was the Petitioner’s response relevant and responsive to subsection (4), it  
13 even provided a cite to the Mayo Clinic, a well-recognized healthcare and research  
14 institution that has hospitals and facilities in several states, including Arizona.

15  
16 The same procedure was followed by Appellant with respect to each and every element  
17 required by the Rule. Please refer to Exhibit “1”, attached hereto and incorporated  
18 herein by reference as though fully set forth at length.

19  
20 APPEAL

21  
22 10. Proposition 203, commonly known as the Arizona Medical Marijuana Act  
23 (“AMMA”) was adopted by the voters in November 2010, and signed by the Governor  
24 in December, 2010. It was codified as Title 36, Chapters 28.1, et. seq. The purpose of  
25 the Act “*is to protect patients with debilitating medical conditions, as well as their*  
26

1 *physicians and providers, from arrest and prosecution, criminal and other penalties*  
2 *and property forfeiture if such patients engage in the medical use of marijuana.”*

3 11. ARS Section 36-2801.1 provides as follows:

4 36-2801.01. Addition of debilitating medical conditions

(Caution: 1998 Prop. 105 applies)

5 “The public may petition the department to add debilitating medical conditions or treatments to the list of  
6 debilitating medical conditions set forth in section 36-2801, paragraph 3. The department shall consider  
7 petitions in the manner required by department rule, including public notice and hearing. The department  
8 shall approve or deny a petition within one-hundred-eighty days of its submission. The approval or denial of  
9 a petition is a final decision of the department subject to judicial review pursuant to title 12, chapter 7,  
10 article 6. Jurisdiction and venue are vested in the superior court.”(Emphasis Added).

11 12. Similarly, the relevant Arizona Administrative Code, AAC Rule R9-17-106 provides in pertinent part  
12 as follows:

13 “Within 180 calendar days after receiving the request: a. Add the medical condition to the list of  
14 debilitating medical conditions, or b. Provide written notice to the requester of the Department’s decision  
15 to deny the request that includes:

16 i. The specific reasons for the Department’s decision; and

17 ii. The process for requesting judicial review of the Department’s decision pursuant to A.R.S.  
18 Title 12, Chapter 7, Article 6.” (Emphasis Added).

19 13. The Arizona Voter Protection Act, Proposition 105, adopted in 1998 by voter  
20 initiative amended the Arizona Constitution relating to initiative and referendum measures  
21 and protecting those measures from future interference by the executive or legislative  
22 branches of government. For example, it prohibits a governor's veto of the initiative;  
23 prohibits legislative repeal; requires a supermajority of three-fourths vote to amend or to  
24 supersede the measure, and allows amendment only if such “furthers the purpose of the  
25 measure.” It surely was intended to prevent a sub-division within the executive  
26 branch like an administrative agency or director from deviating from the specific  
requirements of the statute and rules as it relates to adding new debilitating medical  
conditions.

14. ARS Section 12-910 specifically provides this Court with the authority to “reverse” the  
agency action, ARS 12-910(E), if it concludes that “the action is not supported by  
substantial evidence [or] is contrary to law [or] is arbitrary and capricious [or] is an

1 abuse of discretion.” Appellant contends that ADHS’s denial meets that standard as  
2 does the Department’s failure or refusal to state the “specific reasons” for its denial or  
3 provide Petitioner with the “process for requesting judicial review.”

4 15. ARS Section 12-348 provides for an award of fees and expenses against a state agency  
5 if the AZCNA is the prevailing party. ARS 12-348(C) (3). The issues, as framed  
6 below, are essentially a matter of law. The only documents needed to resolve the legal  
7 issue include the Petition to Add Parkinson’s Disease (Exhibit “1”) and the Decision of  
8 the ADHS to summarily deny the Petition (Exhibit “2”). However, Appellant is  
9 prepared to provide expert medical opinion that PD is a serious, debilitating condition  
10 and that, from a medical and scientific view, Appellant satisfied all of the elements  
11 required by the Rule.

12 16. Pursuant to ARS Section 12-910(A), “[a]n action to review a final administrative  
13 decision shall be heard and determined with convenient speed.” There are at least  
14 60,000 Arizonans who suffer from PD. There is no cure for the disease. The medical  
15 and scientific evidence shows that these patients will receive a therapeutic or  
16 palliative benefit with safe access to medical marijuana, and they should be allowed  
17 to do so by adding PD to the list of debilitating medical conditions.

18 STATEMENT OF THE ISSUES PRESENTED FOR REVIEW

19 17. Pursuant to ARS Section 12-904(A), the following is the Appellant’s Statement of  
20 Issues Presented For Review:

- 21 1. Whether Petitioner provided sufficient information concerning PD  
22 as required pursuant to AAC R9-17-106, such that the ADHS’  
23 denial of same “is not supported by substantial evidence [or] is  
24 contrary to law [or] is arbitrary and capricious [or] is an abuse of  
25 discretion.”  
26

- 1                   2. Whether ADHS' failure to provide "specific reasons" for its  
2                   denial as required by AAC R9-17-106(B) (3) (b), such that the  
3                   ADHS' denial of same "is not supported by substantial evidence  
4                   [or] is contrary to law [or] is arbitrary and capricious [or] is an  
5                   abuse of discretion."
- 6                   3. Whether ADHS' failure to provide "the process for requesting  
7                   judicial review of the Department's determination" of its denial as  
8                   required by AAC R9-17-106(B)(3)(b), such that the ADHS' denial  
9                   of same "is not supported by substantial evidence [or] is contrary  
10                  to law [or] is arbitrary and capricious [or] is an abuse of  
11                  discretion."
- 12                 4. Whether the Court should order the addition of PD as a debilitating  
13                  condition pursuant to AMMA;
- 14                 5. Whether the Court should remand the case to ADHS with  
15                  instructions to proceed with the public hearing as Appellant met  
16                  the requirements of R9-17-106(B)(3)(a).

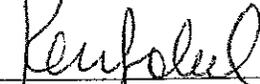
17  
18       WHEREFORE, Appellant respectfully requests relief, as follows:

- 19                 1. For An Order or Judgment adding Parkinson's Disease as a Debilitating Medical  
20                  Condition pursuant to ARS Section 36-2801.01.;
- 21                 2. For An Order or Judgment that Appellant met the preliminary requirements of  
22                  AAC R9-17-106(B)(3)(a) and remanding the case for further proceedings  
23                  consistent with that Rule;
- 24                 3. For An Evidentiary Hearing to be scheduled within 25 days of the filing of this  
25                  Notice of Appeal;

- 1 4. That at said Evidentiary Hearing, the ADHS be Ordered to Show Cause Why the  
2 Relief Should Not Be Granted, i.e. requiring ADHS to show legal justification for  
3 denying the Petition, for legal justification in not providing the "specific reason"  
4 for the denial, and the legal justification for failing to notify Appellant/Petitioner  
5 for the "process of requesting judicial review" of its decision;  
6 5. For Attorney's Fees and Costs, subject to proof;  
7 ~~6. Such other or further relief as this Honorable Court deems just and proper~~

8  
9 DATED this 8<sup>th</sup> day of September, 2014

10 LAW OFFICES OF KEN SOBEL

11 By: 

12 Ken Sobel, Esq. (Bar No. 06551)  
13 Attorney for Appellant/Plaintiff

14  
15 Original Filed with the Maricopa Superior  
16 Court on September 8<sup>th</sup>, 2014

17 Copy of the Foregoing served Via Certified  
18 Mail on September 6<sup>th</sup>, 2014, to:

19 Clerk of the Arizona Department of Health  
20 Services, 1740 West Adams, Room 203  
21 Phoenix, Arizona 85007

22 William Humble, Director  
23 Arizona Department of Health Services  
24 150 North 18<sup>th</sup> Avenue  
25 Phoenix, Arizona 85007

26 Office of the Attorney General  
State of Arizona  
1275 West Washington Street  
Phoenix, AZ 85007

AZCNA v. ADHS

Appeal – Parkinson’s Disease

EXHIBITS

---

**EXHIBIT “1”**



Arizona Cannabis Nurses Association

Heather Manus, RN, President

5505 E Paseo Cimarron

Tucson, AZ 85750

(505)716-6016

cannabisnurseheather@gmail.com

July 8, 2014

Hon. Will Humble, Director  
Arizona Department of Health Services

State of Arizona

PO Box 19000

Phoenix, AZ 85005

Re: Petition to Add Parkinson's Disease As A Debilitating  
Condition Under the Arizona Medical Marijuana Act

Dear Director Humble:

On behalf of the Arizona Cannabis Nurses Association ("AZCNA"), we respectfully submit the following Petition to Add Parkinson's Disease ("PD") to the list of debilitating conditions for which cannabis medicine may be used.

As requested, the following information is provided in support of this Petition:

- A description of the symptoms and other physiological effects experienced by an individual suffering from the medical condition or a treatment of the medical condition that may impair the ability of the individual to accomplish activities of daily living;
- The availability of conventional medical treatments to provide therapeutic or palliative benefit for the medical condition or a treatment of the medical condition;
- A summary of the evidence that the use of marijuana will provide therapeutic or palliative benefit for the medical condition or a treatment of the medical condition; and

- Articles, published in peer-reviewed, scientific journals, reporting the results of research on the effects of marijuana on the medical condition or a treatment of the medical condition supporting why the medical condition should be added.

I. **A description of the symptoms and other physiological effects experienced by an individual suffering from the medical condition or a treatment of the medical condition that may impair the ability of the individual to accomplish activities of daily living**

A. **Symptoms:**

According to the Mayo Clinic:

“Parkinson's signs and symptoms may include:

- **Tremor.** Your tremor, or shaking, usually begins in a limb, often your hand or fingers. You may notice a back-and-forth rubbing of your thumb and forefinger known as a pill-rolling tremor. One characteristic of Parkinson's disease is a tremor of your hand when it is relaxed (at rest).
- **Slowed movement (bradykinesia).** Over time, Parkinson's disease may reduce your ability to move and slow your movement, making simple tasks difficult and time-consuming. Your steps may become shorter when you walk, or you may find it difficult to get out of a chair. Also, you may drag your feet as you try to walk, making it difficult to move.
- **Rigid muscles.** Muscle stiffness may occur in any part of your body. The stiff muscles can limit your range of motion and cause you pain.
- **Impaired posture and balance.** Your posture may become stooped, or you may have balance problems as a result of Parkinson's disease.
- **Loss of automatic movements.** In Parkinson's disease, you may have a decreased ability to perform unconscious movements, including blinking, smiling or swinging your arms when you walk. You may no longer gesture when talking.
- **Speech changes.** You may have speech problems as a result of Parkinson's disease. You may speak softly, quickly, slur or hesitate before talking. Your speech may be more of a monotone rather than with the usual inflections. A speech-language pathologist may help improve your speech problems.
- **Writing changes.** Writing may appear small and become difficult.”

<http://www.mayoclinic.org/diseases-conditions/parkinsons-disease/basics/symptoms/con-20028488>

## **B. Parkinson's Disease Is a Serious and Debilitating Condition That Impairs the Ability of the Sufferer to Accomplish Activities of Daily Living.**

### **(i) Motor Symptoms:**

People are usually more familiar with the motor symptoms of PD, as these are the signs of the disease that are noticeable from the outside. These symptoms, known as the "cardinal" symptoms of PD, include:

- Bradykinesia (slowness of movement) – slowing down and loss of spontaneous and voluntary movement
- Rigidity – unusual stiffness in a limb or other body part
- Resting tremor – an uncontrollable movement that affects a limb when it is at rest and stops for the duration of a voluntary movement

Other motor symptoms also appear in PD:

- Postural instability – problems with standing or walking, or impaired balance and coordination
- Other physical symptoms, such as gait problems and reduced facial expression, may also occur due to the same disruption of movement that causes the better-known tremor and slowness

### **(ii) Non-Motor Symptoms:**

Doctors are increasingly recognizing the presence and effects of other symptoms of PD that are sometimes called "non-motor symptoms" or "dopamine-non-responsive." These symptoms are common and can have a major impact on Parkinson's patients. They can include:

- Cognitive impairment – decline in ability to multi-task and/or concentrate and potentially decline in intellectual functioning
- Mood disorders – depression and anxiety
- Problems sleeping – REM Sleep Disorder, where individuals act out their dreams
- Hyposmia – loss of sense of smell
- Constipation
- Speech and swallowing problems
- Unexplained pains, drooling and low blood pressure when standing

<https://www.michaeljfox.org/understanding-parkinsons/living-with-pd/topic.php?symptoms>

## **II. The availability of conventional medical treatments to provide therapeutic or palliative benefit for the medical condition or a treatment of the medical condition.**

According to UCSF Health:

“Medications can provide dramatic relief from Parkinson's symptoms, but no drug can stop the progression of the disease. In some cases, surgery is an option. Some doctors recommend physical therapy or muscle strengthening. For many patients, a combination of these approaches works best.

### Medication

Levodopa, combined with another drug called carbidopa or Sinemet, is the mainstay of Parkinson's therapy. Levodopa is rapidly converted into dopamine by the enzyme dopa decarboxylase (DDC), which is present in the central and peripheral nervous systems. Much of levodopa is metabolized before it reaches the brain.

Because these drugs are known to cause side effects and can become ineffective after prolonged periods of use, other drugs, such as dopamine agonists, amantadine, COMT inhibitors and anticholinergic medications also are used to treat Parkinson's disease.

### Surgery

When medication is ineffective, surgery may be an option to control symptoms and improve quality of life. However, not everyone is a good candidate for surgery. For example, if a patient never responded or no longer responds to levodopa and carbidopa, surgery is unlikely to help. Only about 10 percent of Parkinson's patients are candidates for surgery.

Three surgical procedures are performed to treat Parkinson's disease — ablative or destructive surgery, stimulation surgery or deep brain stimulation (DBS), and transplantation or restorative surgery.

- **Ablative Surgery** — This procedure locates, targets and then ablates or destroys a targeted area of the brain affected by Parkinson's. The object is to destroy tissue that produces abnormal chemical or electrical impulses that cause tremors and other symptoms of Parkinson's. A lesion of the globus pallidus, called pallidotomy, is the most common ablative surgery for Parkinson's disease.
- **Deep Brain Stimulation (DBS)**
  - Like ablative surgery, deep brain stimulation surgery treats the tremors and slowness associated with Parkinson's disease. The procedure involves inserting a deep brain stimulator into certain areas of the brain. Instead of destroying the overactive cells that cause symptoms of Parkinson's, DBS temporarily disables them by sending pulses of electricity. This therapy has been shown to provide greater relief of symptoms with fewer side effects than other treatments.
- **Transplantation** — In transplantation or restorative surgery, dopamine-producing cells are implanted into a certain part of the brain. The cells used for transplant may come from one of several sources — the patient's body, human embryos or pig embryos. Using stem cells for this procedure is currently being researched.

## Complementary Treatments

Complementary or integrative care combines the best of alternative therapies — such as acupuncture, massage, meditation, herbs and nutrition — and conventional medicine. A number of treatments such as simply physical activity, physical therapy, massage and support groups can help relieve symptoms and improve quality of life. Discuss with your doctor the possible treatments he or she recommends to treat your symptoms.”

### III. **A summary of the evidence that the use of marijuana will provide therapeutic or palliative benefit for the medical condition or a treatment of the medical condition.**

#### A. The Israeli Human Study: According to a MedPage Today report of the Movement Disorder Society...

“Smoking cannabis appeared to reduce tremor and pain and improve sleep among Parkinson's disease patients, researchers from Israel reported here.

Overall, patients' scores on the standard Unified Parkinson's Disease Rating Scale (UPDRS) averaged 33 before they smoked cannabis in the laboratory and averaged 24 after 30 minutes ( $P<0.01$ ), Ruth Djaldetti, MD, of Tel Aviv University Israel, reported at her poster presentation at the International Congress on Parkinson's Disease and Movement Disorders.

“We not only saw improvement in tremor in these patients, but also in rigidity and in bradykinesia,” Djaldetti told *MedPage Today*. “I would recommend use of marijuana to my patients as a last resort if nothing else was working for them or if they had pain.”

Medical marijuana is legal in Israel for the treatment of Parkinson's disease, Djaldetti explained. “All of these 20 patients were cannabis users before we studied them. They were tested before they smoked cannabis in the clinic and then they were tested 30 minutes after smoking.” The patients were about 66 years of age and had been diagnosed with Parkinson's disease for a mean of 7.5 years.

Prior to smoking, the 20 patients had an average score of 7.5 in the tremor domain of the UPDRS, but after 30 minutes following smoking the average tremor score declined to 3.5 ( $P<0.001$ ), she said. The rigidity score declined from 7.4 to 6.4 ( $P=0.007$ ). The bradykinesia score declined from an average of 13.2 to an average of 8.6 ( $P<0.001$ ).

“There had been reported marked reductions in the Israeli media about the ability of marijuana to reduce tremor,” Djaldetti said. “We saw a reduction in tremor but it was less dramatic in our clinic than on television. The patients told us that the beneficial effect of cannabis smoking lasts for about 2 to 3 hours.”

Since 1996, when Californians approved medical marijuana, 17 other states and the District of Columbia have also approved its use. It is still illegal under federal law, although the Obama Administration has signaled that enforcement of the federal ban in states that have legalized it is not a government priority. It is legal for medical use in Canada and in many other countries.

Karin Gmitterova, MD, assistant professor of neurology at the University of Bratislava in Slovakia, told *MedPage Today*, “There is a community of patients that shares their experiences in using

alternative forms of medication ... They are more experienced with cannabis smoking in the Czech Republic and patients report that it can be helpful."

"It won't replace levodopa, of course," continued Gmitterova, who did not participate in the study. However, "The reduction in the UPDRS score that we see here is not only statistically significant but this is clinically important as well."

"When doctors can't help patients, they will find other methods of treatment through word of mouth or the Internet or from family members or friends," he added.

Djaldetti said that the researchers "were more taken with the improvements in rigidity and bradykinesia. On cannabis they were able to improve their fine motor skills. We did not see an improvement in gait and posture. I doubt that increasing the size of this study would result in a significant finding for gait or posture."

In addition, all of the patients in the study were already on medications for pain relief but those therapies were not providing the relief required, she noted. "We saw a dramatic reduction in pain in our patients and in their ability to sleep. When their pain was reduced, they slept better."

- B. Cannabis is a safe alternative to pharmaceuticals, and though it is difficult for scientists to conduct studies on cannabis, preliminary evidence indicates that it is a powerful treatment for PD and other neurological disorders.

Published in July of 2011 this British study reports:

Given its antioxidant properties and its ability to activate CB<sub>2</sub> but to block CB<sub>1</sub> receptors, Δ<sup>9</sup>-THCV [a principle compound in cannabis] has a promising pharmacological profile for delaying disease progression in PD and also for ameliorating parkinsonian symptoms.

Diseases such as Parkinson's Disease where the neurological system of the body is disrupted are counteracted by the neuroprotective properties of cannabis.

Cannabidiol (CBD) and Tetrahydrocannabinol (THC) are the principal cannabinoids found in cannabis. When ingested they have a synergistic effect, reducing inflammation, controlling spasms, and preventing neurological damage. Cannabis is an ideal candidate in the treatment of PD, but its legal status makes it difficult for those with PD to obtain medicine, or to consider using it, as often doctors do not mention the option.

- C. Michael J. Fox and others describe the palliative benefit for PD:

<https://www.youtube.com/watch?v=En5HJBxstVk>

- D. Observational studies show cannabis relieves motor and non-motor symptoms of Parkinson's disease.

"Studies focused on cannabinoid-based treatments for Parkinson's disease have been conducted since the seventies, due to the important number of patients suffering from the illness reporting an improvement of their symptoms following the consumption of cannabis.

Motor symptoms have been at the centre of these studies, as they constitute the most visible and most debilitating disorders in relation with Parkinson's disease. As early as the eighties, cannabinoids have been shown to alleviate dystonia, dyskinesia, and akinesia, all of them motor symptoms which have been known to impact certain Parkinson's patients. For instance, controlled doses of synthetic cannabinoids have been able to reduce dyskinesia in patients suffering from Parkinson's disease or Parkinsonism for years by 30%. Incidentally, medicinal cannabis as well as cannabis extracts-based medications are already being used to treat general spasticity in the context of other conditions such as multiple sclerosis or Gilles de la Tourette's syndrome.

---

Research focusing on the degeneration of brain cells and its resulting impact on cognitive capacities – which is the origin of the aforementioned motor symptoms – has also been relatively active, with a few breakthrough studies published during the previous decade. The neuroprotective properties of cannabinoids had already been proven on many occasions, and the impact it could have on Parkinson's disease was confirmed as well. In 2004, a research laboratory injected rats with an agonist of Tetrahydrocannabinol (THC), one of the major psychoactive substances of cannabis, followed by the injection of a toxin triggering an animal version of Parkinson's. Upon testing of these rats in parallel with a control group of healthy subjects, researchers observed that their brains were virtually indistinguishable. In a second series of tests, other rats were this time first injected with the toxin, then with the THC agonist, with positive results as well, especially when THC was combined with Cannabidiol (CBD), a non-psychoactive cannabinoid known for its medicinal properties. In human terms, the second test suggested that cannabinoids intake could slow down the progression of the disease for several years.

Despite numerous studies underlining the multiple benefits of cannabis on Parkinson's, no palliative or curative treatment has been developed, partly due to the lack of availability of medicinal cannabis dedicated to research. This is why the aforementioned studies have so far been considered with relative caution, especially since it was observed that an excessive dose of cannabis could reverse its own effects and temporarily worsen some of the motor symptoms. These results were however partly linked to the relative inefficacy of clinical tests conducted with synthetic cannabinoids, as well as the lack of familiarity of the subjects tested with medicinal marijuana or cannabis extracts-based medication.

In order to bypass the legal restriction of cannabis being illegal in most countries these last two years, many observational studies were published, focusing on patients already self-medicating with cannabis. The tests measured their reaction to a "dose" of medicinal cannabis, once again with positive outcomes in regards to motor symptoms as well as non-motor symptoms. Patients participating to the study reported that a "dose" of cannabis could relieve them for a period of 2 to 3 hours.

Finally, in March 2014, researchers from Tel-Aviv managed to show results for 22 patients suffering from Parkinson's Disease, whose symptoms, both motor and non-motor, were relieved following the use of cannabis. The medical team registered important fluctuations in pain, sleep, and several motor symptoms, namely tremor, rigidity and bradykinesia. In addition to these results being the first study showing cannabis relieving motor and non-

motor symptoms alike, no adverse effects were observed following the intake of cannabis. It is likely that these recent advances will trigger a newfound enthusiasm from the medical community to pursue research in this direction, especially since large amounts of medicinal cannabis have been unlocked for research in the United States.” <http://www.collective-evolution.com/2014/05/27/cannabis-relieves-parkinsons-disease/>

See Exhibit “1”

**IV. Articles, published in peer-reviewed, scientific journals, reporting the results of research on the effects of marijuana on the medical condition or a treatment of the medical condition supporting why the medical condition should be added.**

The following articles, published in peer-reviewed, scientific journals, are attached as exhibits hereto, as follows:

- A. **EXHIBIT ONE:** Lotan, I., Treves, T., Roditi, Y., Djaldetti, R.; *Medical Marijuana (cannabis) treatment for motor and non-motor symptoms in Parkinson’s Disease. An open-label observational study.* Movement Disorders 2013; 28 Suppl 1: 448.
- B. **EXHIBIT TWO:** Lastres-Becker, Isabel, et. al.; *Cannabinoids provide neuroprotection against 6-hydroxydopamine toxicity in vivo and in vitro: Relevance to Parkinson’s Disease,* Neurobiology of Disease 19 (2005) 96-107.
- C. **EXHIBIT THREE:** C.B. Carroll, M.-L. Zeissler, C. O. Hanemann and J. P. Zajicek, *D<sup>9</sup>-tetrahydrocannabinol (D<sup>9</sup>-THC) exerts a direct neuroprotective effect in a human cell culture model of Parkinson’s disease,* Neuropathology and Applied Neurobiology (2012) 38 535 – 547.

**V. Conclusion:**

Based on the foregoing, the Arizona Cannabis Nurses Association submits that it has met the standards required by Arizona Administrative Code, Rule 9-17-106, and respectfully requests that this Petition be set for a public hearing which will prove that

- The specified medical condition or treatment of the medical condition impairs the ability of the individual to accomplish activities of daily living; and
- Marijuana usage provides a therapeutic or palliative benefit to an individual suffering from the medical condition or treatment of the medical condition.

And, that Parkinson’s Disease be added to the list of debilitating conditions allowing patients to use medical cannabis for therapeutic and/or palliative benefits.



---

# EXHIBIT ONE

# Cannabis (Medical Marijuana) Treatment for Motor and Non-Motor Symptoms of Parkinson Disease: An Open-Label Observational Study

Itay Lotan, MD, Therese A. Treves, MD, Yaniv Roditi, MD, and Ruth Djaldetti, MD

**Objective:** The use of cannabis as a therapeutic agent for various medical conditions has been well documented. However, clinical trials in patients with Parkinson disease (PD) have yielded conflicting results. The aim of the present open-label observational study was to assess the clinical effect of cannabis on motor and non-motor symptoms of PD.

**Methods:** Twenty-two patients with PD attending the motor disorder clinic of a tertiary medical center in 2011 to 2012 were evaluated at baseline and 30 minutes after smoking cannabis using the following battery: Unified Parkinson Disease Rating Scale, visual analog scale, present pain intensity scale, Short-Form McGill Pain Questionnaire, as well as Medical Cannabis Survey National Drug and Alcohol Research Center Questionnaire.

**Results:** Mean (SD) total score on the motor Unified Parkinson Disease Rating Scale score improved significantly from 33.1 (13.8) at baseline to 23.2 (10.5) after cannabis consumption ( $t = 5.9$ ;  $P < 0.001$ ). Analysis of specific motor symptoms revealed significant improvement after treatment in tremor ( $P < 0.001$ ), rigidity ( $P = 0.004$ ), and bradykinesia ( $P < 0.001$ ).

**Conclusions:** There was also significant improvement of sleep and pain scores. No significant adverse effects of the drug were observed. The study suggests that cannabis might have a place in the therapeutic armamentarium of PD. Larger, controlled studies are needed to verify the results.

**Key Words:** cannabis,  $\Delta^9$ -THC, Parkinson disease, pain

(*Clin Neuropharm* 2014;37: 41–44)

Cannabis, also known as marijuana (from the Mexican Spanish, *marihuana*), is prepared from the *Cannabis sativa* plant. Its principal psychoactive constituent,  $\Delta^9$ -tetrahydrocannabinol (THC), was isolated in 1964 by Gaoni and Mechoulam.<sup>1</sup> Other cannabinoids among the 400 compounds contained in the plant are cannabidiol, cannabivarin, and tetrahydrocannabivarin. The potential use of cannabis in the pharmacotherapy of pain and various diseases (medical marijuana), including cancer, glaucoma, and multiple sclerosis, has been well documented.<sup>2,6</sup>

In Israel, marijuana was legalized for medical use in various conditions in the 1990s. The authorization of cannabis treatment for Parkinson disease (PD) was prompted by Israeli media reports of dramatic improvement of tremor in 1 patient and amelioration of symptoms in others. However, the findings have

not been corroborated in controlled studies. Overall, there are currently very few double-blind, controlled studies on the effect of cannabis on motor deficits in PD, and the results are often conflicting. Most of the existing literature focuses mainly on the effect of cannabinoid agonists on dyskinesias.

The aim of the present study was to evaluate the efficacy of cannabis treatment in alleviating the motor and non-motor symptoms of PD in a clinical setting.

## PATIENTS AND METHODS

Twenty-eight patients with PD attending the movement disorder clinic at Rabin Medical Center, a tertiary, university-affiliated hospital, had received permission to smoke cannabis from the Israel Ministry of Health as an add-on therapy because their anti-Parkinson medications had proved insufficient or to combat severe PD-related pain and tremor from June 2011 to April 2012. Patients were eligible for the study if they were treated with cannabis on a daily basis for at least 2 months and tolerated the drug with no major adverse effects. Six patients could not tolerate the drug and discontinued treatment after a short period because of severe adverse effects (inability to smoke, vomiting, dizziness, and psychosis). Twenty-two patients were included in the study. Seven patients had response fluctuations. The study protocol was approved by the local research ethics committee, and all patients gave written informed consent.

On the day of the study, eligible patients were instructed to arrive at the clinic without taking their regular medications so that their baseline motor status could be assessed. Patients with motor fluctuations were to be examined during the “off” period: Those who could not delay their morning dose were asked to wait at the clinic for onset of the off period before smoking cannabis; if they were unable to wait, they were examined during the “on” period.

At baseline, disease staging was performed using the Hoehn and Yahr rating scale. Additional data on motor symptoms and signs were collected with the motor part of the Unified PD Rating Scale (UPDRS); those on non-motor symptoms, with a visual analog scale and present pain intensity scale, the Short-Form McGill Pain Questionnaire, as well as the Medical Cannabis Survey National Drug and Alcohol Research Center Questionnaire. The latter questionnaire was adopted from a previous survey conducted in Australia on mode of use, subjective efficacy, and adverse effects of cannabis.<sup>7</sup> Thereafter, the patients were asked to smoke their regular dose of cannabis (amount inhaled, 0.5 g). Thirty minutes later, the motor and non-motor battery was repeated.

The effect of cannabis consumption on motor symptoms was evaluated by 2 raters (I.L. and R.D.) to avoid diversions and assure the credibility of the results. Interrater variability was analyzed with the Pearson correlation. Paired sample *t* test was used to compare values of the various parameters before

Department of Neurology, Rabin Medical Center, Beilinson Hospital, Petach Tikva; and Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel. Conflicts of Interest and Source of Funding: The authors have no conflicts of interest to declare.

Address correspondence and reprint requests to Itay Lotan, MD, Department of Neurology, Rabin Medical Center, Beilinson Campus, Petach Tikva 49100, Israel; E-mail: itayl@clalit.org.il

Copyright © 2014 by Lippincott Williams & Wilkins  
DOI: 10.1097/WNF.000000000000016

TABLE 1. Clinical Characteristics of Patients With PD Treated With Cannabis

Patient No.	Age, y/Sex	Disease Duration, y	Response Fluctuations	Past Pain	Current Medications
1	62/M	3	Yes	Yes	Levodopa
2	63/M	5	No	Yes	Levodopa, amantadine
3	70/F	11	Yes	No	Rasagiline, ach, selegiline
4	57/M	8	No	Yes	Levodopa, amantadine
5	54/M	9	Yes	Yes	Levodopa, rasagiline
6	77/M	6	No	No	Levodopa, ach, selegiline
7	58/M	18	No	Yes	Levodopa
8	64/F	7	Yes	Yes	Levodopa, pramipexole, rasagiline
9	60/F	14	No	No	Levodopa
10	42/M	3	No	Yes	Stalevo, amantadine, pramipexole, selegiline, ach
11	73/F	3	No	No	Levodopa, ach, selegiline
12	74/M	5	No	Yes	Levodopa, pramipexole, amantadine
13	52/F	5	No	Yes	Levodopa, rasagiline
14	73/M	11	Yes	Yes	Levodopa, ropinirole, amantadine
15	65/F	16	No	No	Levodopa, rasagiline, ach, selegiline
16	63/F	2	No	Yes	Levodopa, rasagiline
17	80/M	4	No	No	Rasagiline, ach, selegiline
18	70/M	2	No	Yes	Levodopa, selegiline
19	71/M	5	No	Yes	Levodopa
20	79/F	14	Yes	Yes	Levodopa
21	75/F	7	Yes	Yes	Levodopa, pramipexole, amantadine
22	48/M	2	No	Yes	Levodopa

Clinical data and current medical treatment of all patients included in the study.

Ach, anticholinergics; F, female; M, male.

and after treatment. All statistical analyses were done with Statistical Package for the Social Sciences software, version 19.

## RESULTS

### Patient Characteristics

The study group consisted of 13 men and 9 women with a mean (SD) age of 65 (10.2) years. The clinical characteristics and regular medications of the patients are described in Table 1. Mean (SD) disease duration was 7.3 (4.8) years. The median score on the Hoehn and Yahr scale was 1.5 (range, 1 to 3). Seven patients (3 men, 4 women) had motor fluctuations. The patients who had fluctuations were younger and had a shorter disease duration than those who did not, but the differences were not significant (mean [SD] age, 63.7 [12.7] years vs 65.6 [9.4] years; mean [SD] disease duration, 7.7 [4] years vs 7 [5.2] years). Three were assessed during the off period; 4, during the on period.

### Effect of Cannabis on Motor Symptoms

Analysis of the interrater variability yielded a high concordance in motor scores between the 2 examining physicians both before treatment (Pearson correlation, 8.4) and after (Pearson correlation, 8.8). Therefore, for convenience, we present only the results of one of the raters (L.L.).

The mean (SD) total motor UPDRS score improved significantly from 33.1 (13.8) at baseline to 23.2 (10.5) after cannabis consumption ( $t = 5.9, P < 0.001$ ). The change in motor UPDRS score was significant in both patients with and without response fluctuations (Fig. 1). Among the patients with response fluctuations, the off UPDRS score improved by 55% in 2 patients,

with no change in 1 patient; the on UPDRS score improved by 50% in 2 patients, with no change in 2. Analysis by specific motor symptoms revealed a significant improvement in tremor, rigidity, and bradykinesia after cannabis consumption. There was no effect on posture (Table 2).

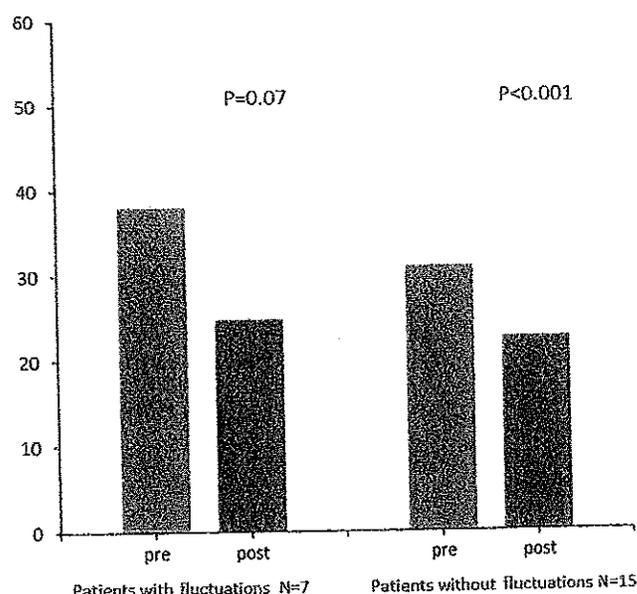


FIGURE 1. The effect of cannabis on the motor UPDRS score in the patients with and without response fluctuations.

TABLE 2. Effect of Cannabis on Motor UPDRS Score

	UPDRS		P
	Before Smoking Cannabis	After Smoking Cannabis	
Tremor (items 20–21)	7.55 (4.79)	3.64 (2.8)	0.000
Rigidity (item 22)	7.55 (3.79)	6.48 (3.56)	0.004
Bradykinesia (items 23–27, 30–31)	13.12 (6.88)	8.62 (5.5)	0.000
Posture (items 28–29)	1.90 (1.58)	1.55 (1.1)	0.056

The effect of Cannabis on different categories of the UPDRS.

### Effect of Cannabis on Non-Motor Symptoms

The visual analog scale score decreased significantly, from 5.4 (3.7) at baseline to 1.7 (2.6), after cannabis smoking ( $t = 5.3$ ;  $P < 0.001$ ). Corresponding scores on the present pain intensity scale were 2.7 (1.7) and 0.8 (1.1) ( $t = 5.9$ ,  $P < 0.001$ ). Twelve patients reported greatly improved quality of sleep during cannabis treatment, and 8 had mild relief.

No significant adverse effects were observed during the study. One patient had hypoglycemia that resolved after oral glucose intake, and 1 patient complained of dizziness. The main adverse effects of long-term smoking reported by the patients were somnolence, drowsiness, palpitations, and bad taste.

### DISCUSSION

The use of the *C. sativa* plant as a medicinal preparation dates back to ancient Asian pharmacopeia. Among its well-documented medical benefits are amelioration of nausea and vomiting, stimulation of hunger in patients receiving chemotherapy or with AIDS, lowered intraocular eye pressure, as well as general analgesic effects.<sup>2–6</sup> Research on the neuroprotective and therapeutic effects of cannabis in neurodegenerative diseases was spurred by the discovery of the endogenous cannabinoid system.<sup>8</sup> The cannabinoid signaling system in the brain interacts with G-protein-coupled cannabinoid receptors. Endocannabinoids, synthesized on demand, activate the cannabinoid receptors, thereby depressing the release of neurotransmitters, mainly glutamate. Tetrahydrocannabinol, the main psychoactive component of cannabis, exerts its most prominent effects via its actions on 2 types of cannabinoid receptors: the CB1 receptor, found primarily in the brain as well as in some peripheral tissues, and the CB2 receptor, found primarily in peripheral tissues but also expressed in neuroglial cells.<sup>9,10</sup>

Studies of the potential therapeutic effect of cannabinoids on PD have produced conflicting results. Among those conducted in the MPTP and 6-OHDA primate models, some found that cannabinoid improved motor activity,<sup>11–13</sup> whereas others reported that it did not.<sup>14,15</sup> Given that the mechanism of action of cannabinoids is mediated by glutamate, several clinical trials focused on the effect of cannabis on dyskinesias in PD. Again, the results were inconclusive. One randomized, double-blind, placebo-controlled crossover trial in 7 patients found a significant reduction in dyskinesias in response to treatment with the cannabinoid receptor agonist, nabilone.<sup>16</sup> However, a larger double-blind crossover study in 19 patients yielded no beneficial effect with Cannador (an extract of *C. sativa* containing  $\Delta^9$ -THC and cannabidiol) on either dyskinesias or UPDRS scores.<sup>17</sup> An observational study of 5 patients found no effect on tremor,<sup>18</sup> but an anonymous questionnaire survey reported that bradykinesia seemed to be the symptom most commonly improved by cannabinoids, followed by muscle rigidity and tremor.<sup>19</sup>

The present study suggests that smoking cannabis has a beneficial effect on tremor and rigidity, a lesser effect on bradykinesia, and only a trend for improvement of posture. The findings were consistent in patients with and without response fluctuations. In patients with fluctuations, both the off and on motor UPDRS scores improved. One patient with young-onset PD examined in the off period responded dramatically to inhaled cannabis, to the extent of a clear “on” gained by levodopa. Cannabis also had a positive impact on non-motor symptoms. Scores on pain scales dropped significantly, and the patients reported better quality of sleep. The latter finding might be attributable partly to nocturnal pain relief and partly to the tranquilizing and somnolent effect of the drug. The psychotropic effects of cannabis and the perception of well-being often associated with its use may also be responsible for the favorable response here and in other studies. Although cannabinoids have high lipid solubility and THC is still detected weeks after drug intake,<sup>20</sup> most of our patients reported that the benefits of a single dose were short-lasting (2–3 hours). During the study, cannabis was generally well tolerated.

The open-label design of this study has inherent limitations of a placebo effect and rater bias. We tried to overcome the latter problem by using 2 raters, and the low interrater variability partly ensures the reliability of the results. Nevertheless, bias and placebo effect can explain the discrepancy between the favorable results of the present study and the negative results of other clinical, double-blind studies. In the setting of the present study, it was difficult to perform a placebo-controlled trial because of the conspicuous and characteristic smell of the cannabis cigarette. Another drawback of the study is that the patients were assessed at 1 time point only. Longer assessment of the clinical response is warranted to clearly establish a beneficial effect of cannabis on the motor symptoms of PD.

In conclusion, this observational study is the first to report an amelioration of both motor and non-motor symptoms in patients with PD treated with cannabis (medical marijuana). The study opens new venues for treatment strategies in PD especially in patients refractory to current medications. It may promote legalization of cannabis in other countries and should encourage pharmaceutical companies to conduct controlled studies with a more purified substance. Although promising, our results should be interpreted with caution and confirmed in larger double-blind, placebo-controlled studies conducted over a longer term, with special attention to the possible addictive potential of the drug.

### REFERENCES

1. Gaoni Y, Mechoulam R. Isolation, structure and partial synthesis of an active constituent of hashish. *J Am Chem Soc* 1964;86:1646–1647.
2. Walker J, Huang S. Cannabinoid analgesia. *Pharmacol Ther* 2002;95:127–135.

3. Kumar RN, Chambers WA, Pertwee RG. Pharmacological actions and therapeutic uses of cannabis and cannabinoids. *Anaesthesia* 2001;56:1059–1068.
4. Baker D, Pryce G, Croxford JL, et al. Cannabinoids control spasticity and tremor in a multiple sclerosis model. *Nature* 2002;404:84–87.
5. Martin RS, Luong LA, Welsh NJ, et al. Effects of cannabinoid receptor agonists on neurally-evoked contractions of urinary bladder tissues isolated from rat, mouse, pig, dog, monkey and human. *Br J Pharmacol* 2000;129:1707–1715.
6. Jarvinen T, Pate D, Laine K. Cannabinoids in the treatment of glaucoma. *Pharmacol Ther* 2002;95:203–220.
7. Swift W, Gates P, Dillon P. Survey of Australians using cannabis for medical purposes. *Harm Reduct J* 2005;2:18.
8. Scotter EL, Abood ME, Glass M. The endocannabinoid system as a target for the treatment of neurodegenerative disease. *Br J Pharmacol* 2010;160:480–498.
9. Pertwee RG, Ross RA. Cannabinoid receptors and their ligands. *Prostaglandins Leukot Essent Fatty Acids* 2002;66:101–121.
10. Pertwee RG. Pharmacology of cannabinoid CB1 and CB2 receptors. *Pharmacol Ther* 1997;74:129–180.
11. van der Stelt M, Di Marzo V. Cannabinoid receptors and their role in neuroprotection. *Neuromolecular Med* 2005;7:37–50.
12. Kelsey JE, Harris O, Cassin J. The CB(1) antagonist rimonabant is adjunctively therapeutic as well as monotherapeutic in an animal model of Parkinson's disease. *Behav Brain Res* 2009;203:304–307.
13. Martinez A, Macheda T, Morgese MG, et al. The cannabinoid agonist WIN55212-2 decreases L-DOPA-induced PKA activation and dyskinetic behavior in 6-OHDA-treated rats. *Neurosci Res* 2012;72:236–242.
14. Meschler JP, Howlett AC. Signal transduction interactions between CB1 cannabinoid and dopamine receptors in the rat and monkey striatum. *Neuropharmacology* 2001;40:918–926.
15. Cao X, Liang L, Hadcock JR, et al. Blockade of cannabinoid type 1 receptors augments the antiparkinsonian action of levodopa without affecting dyskinesias in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated rhesus monkeys. *J Pharmacol Exp Ther* 2007;323:318–326.
16. Sieradzan KA, Fox SH, Hill M, et al. Cannabinoids reduce levodopa-induced dyskinesia in Parkinson's disease: a pilot study. *Neurology* 2001;57:2108–2111.
17. Carroll CB, Bain PG, Teare L, et al. Cannabis for dyskinesia in Parkinson disease: a randomized double-blind crossover study. *Neurology* 2004;63:1245–1250.
18. Frankel JP, Hughes A, Lees AJ, et al. Marijuana for parkinsonian tremor. *J Neurol Neurosurg Psychiatry* 1990;53:436.
19. Venderová K, Růžicka E, Vorisek V, et al. Survey on cannabis use in Parkinson's disease: subjective improvement of motor symptoms. *Mov Disord* 2004;19:1102–1106.
20. Dewey WL. Cannabinoid pharmacology. *Pharmacol Rev* 1986;38:151–178.

---

# EXHIBIT TWO

## Cannabinoids provide neuroprotection against 6-hydroxydopamine toxicity in vivo and in vitro: Relevance to Parkinson's disease

Isabel Lastres-Becker,<sup>a,1</sup> Francisco Molina-Holgado,<sup>b</sup> José A. Ramos,<sup>a</sup>  
Raphael Mechoulam,<sup>c</sup> and Javier Fernández-Ruiz<sup>a,\*</sup>

<sup>a</sup>Departamento de Bioquímica y Biología Molecular III, Facultad de Medicina, Universidad Complutense, 28040-Madrid, Spain

<sup>b</sup>Wolfson Centre for Age-Related Diseases, Division of Biomolecular Science, GKT School of Biomedical Sciences, Hodgkin Building, Guy's Campus, London SE1 1UL, UK

<sup>c</sup>Department of Medicinal Chemistry and Natural Products, Medical Faculty, Hebrew University, Jerusalem 91120, Israel

Received 6 May 2004; revised 19 November 2004; accepted 22 November 2004  
Available online 16 February 2005

Cannabinoids have been reported to provide neuroprotection in acute and chronic neurodegeneration. In this study, we examined whether they are also effective against the toxicity caused by 6-hydroxydopamine, both in vivo and in vitro, which may be relevant to Parkinson's disease (PD). First, we evaluated whether the administration of cannabinoids in vivo reduces the neurodegeneration produced by a unilateral injection of 6-hydroxydopamine into the medial forebrain bundle. As expected, 2 weeks after the application of this toxin, a significant depletion of dopamine contents and a reduction of tyrosine hydroxylase activity in the lesioned striatum were noted, and were accompanied by a reduction in tyrosine hydroxylase-mRNA levels in the substantia nigra. None of these events occurred in the contralateral structures. Daily administration of  $\Delta^9$ -tetrahydrocannabinol ( $\Delta^9$ -THC) during these 2 weeks produced a significant waning in the magnitude of these reductions, whereas it failed to affect dopaminergic parameters in the contralateral structures. This effect of  $\Delta^9$ -THC appeared to be irreversible since interruption of the daily administration of this cannabinoid after the 2-week period did not lead to the re-initiation of the 6-hydroxydopamine-induced neurodegeneration. In addition, the fact that the same neuroprotective effect was also produced by cannabidiol (CBD), another plant-derived cannabinoid with negligible affinity for cannabinoid CB<sub>1</sub> receptors, suggests that the antioxidant properties of both compounds, which are cannabinoid receptor-independent, might be involved in these in vivo effects, although an alternative might be that the neuroprotection exerted by both compounds might be due to their anti-inflammatory potential. As a second objective, we examined whether cannabinoids also provide neuroprotection against the in vitro toxicity of 6-hydroxydopamine. We found that the non-selective cannabinoid agonist HU-210 increased cell survival in cultures of mouse cerebellar granule cells exposed to this toxin. However, this effect was significantly lesser when the cannabi-

noid was directly added to neuronal cultures than when these cultures were exposed to conditioned medium obtained from mixed glial cell cultures treated with HU-210, suggesting that the cannabinoid exerted its major protective effect by regulating glial influence to neurons. In summary, our results support the view of a potential neuroprotective action of cannabinoids against the in vivo and in vitro toxicity of 6-hydroxydopamine, which might be relevant for PD. Our data indicated that these neuroprotective effects might be due, among others, to the antioxidant properties of certain plant-derived cannabinoids, or exerted through the capability of cannabinoid agonists to modulate glial function, or produced by a combination of both mechanisms.  
© 2004 Elsevier Inc. All rights reserved.

**Keywords:** Cannabinoids; Parkinson's disease; 6-Hydroxydopamine; Basal ganglia; Neurodegeneration; Neuroprotection; Glial cells; Antioxidant properties; Anti-inflammatory effects

### Introduction

In addition to brain functions, such as the control of nociception, motor activity, emesis, body temperature, appetite, and memory and learning, the endogenous cannabinoid signaling system has been recently implicated in the control of the cell survival/death decision in the CNS and also in the periphery (for a review, see Guzmán et al., 2001). This finding is based, among others, on the observation that cannabinoids protect neurons from toxic insults such as glutamatergic excitotoxicity (Shen and Thayer, 1998), ischemic stroke (Nagayama et al., 1999), hypoxia (Sinor et al., 2000), trauma (Panikashvili et al., 2001), oxidative stress (Hampson et al., 1998; Marsicano et al., 2002), ouabain-induced secondary excitotoxicity (van der Stelt et al., 2001a,b), and others (see recent reviews in Grundy, 2002; Grundy et al., 2001; Mechoulam et al., 2002a,b). Most of these protectant effects appear to be mediated by the activation of the cannabinoid CB<sub>1</sub> receptor subtype (Pannentier-Bateur et al., 2002), although the contribution

\* Corresponding author. Fax: +34 91 3941691.

E-mail address: jifr@med.ucm.es (J. Fernández-Ruiz).

<sup>1</sup> Present address: J.W. Goethe Universität, Section Molecular Neurogenetics, Building 26, Room 509, Theodor Stern Kai 7, 60590 Frankfurt am Main, Germany.

Available online on ScienceDirect (www.sciencedirect.com).

of other different mechanisms (i.e., antioxidant and/or anti-inflammatory properties of cannabinoids) cannot be ruled out (see Grundy, 2002; Grundy et al., 2001; Mechoulam et al., 2002a,b).

Cannabinoids may be also neuroprotectant in Parkinson's disease (PD) (for a review, see Romero et al., 2002), a motor neurodegenerative disorder characterized by progressive death of nigrostriatal dopaminergic neurons that mainly results in bradykinesia (slowness of movement), rigidity, and tremor as major motor abnormalities (Sethi, 2002). The motor symptoms of this disorder may be significantly reduced with therapy of dopaminergic replacement, at least in the first and middle phases of the disease (Carlsson, 2002), but this does not delay/arrest the progress of neuronal injury. A possible delay/arrest has been tried with a variety of compounds that are potentially useful in acute or chronic neurodegeneration (for a review, see Vajda, 2002), such as: (i) chemical antioxidants (for a review, see Moosmann and Behl, 2002), (ii) NMDA receptor antagonists (for a review, see Alexi et al., 2000), (iii)  $Ca^{++}$  channel blockers (for a review, see Rodnitsky, 1999), and (iv) anti-inflammatory substances (for a review, see McGeer et al., 2001). However, the results obtained so far are not as promising as expected (Tintner and Jankovic, 2002). As cannabinoids share many of the above potentially neuroprotective properties (for a review, see Grundy, 2002; Grundy et al., 2001; Mechoulam et al., 2002a,b), they could be promising molecules to investigate for delaying/arresting the neuronal injury in PD, as recently reported for other motor neurodegenerative disorders, such as Huntington's disease (Lastres-Becker et al., 2004) or amyotrophic lateral sclerosis (Raman et al., 2004). In order to evaluate whether cannabinoids might provide neuroprotection also in PD, we have conducted two series of differentiated experiments addressed to demonstrate that cannabinoids were effective against the *in vivo* and *in vitro* toxicity of 6-hydroxydopamine, a toxin currently used to generate parkinsonism in laboratory animals (for a review, see Blum et al., 2001).

In the first series of experiments, we examined the ability of  $\Delta^9$ -THC, or another related plant-derived cannabinoid, cannabidiol (CBD), which shares with  $\Delta^9$ -THC some properties (i.e., antioxidant capability) but differs in its absence of psychotropic effects and its negligible affinity for the cannabinoid  $CB_1$  receptor (Pertwee, 1997), to alter *in vivo* the progress of neurodegeneration in rats subjected to unilateral injections into the medial forebrain bundle of 6-hydroxydopamine. Thus,  $\Delta^9$ -THC or CBD was daily administered to 6-hydroxydopamine-lesioned rats as of the first day post-lesion (to ensure an action of the cannabinoid against the appearance of first signs of toxicity) and the animals were tested for the progress of neurodegeneration after 2 weeks of daily cannabinoid administration. This was evaluated by analyzing the depletion of dopamine (DA) in the striatum, as well as by analyzing mRNA levels (in the substantia nigra) and activity (caudate-putamen) of tyrosine hydroxylase (TH), the rate-limiting enzyme for DA synthesis in these neurons. These measures were done in ipsilateral structures of lesioned animals and their sham-operated controls, but also in their corresponding contralateral structures (used as an internal control to test the effects of  $\Delta^9$ -THC or CBD in the absence of lesion), which allow (i) to differentiate the potential neuroprotective effects of cannabinoids (observed only in ipsilateral structures) from mere up-regulatory effects that, if occurring, would be also observed in contralateral structures, and (ii) to control the occurrence of compensatory mechanisms. Other accompanying analyses consisted of determinations of the mRNA

levels of proenkephalin and substance P in the caudate-putamen, since these two peptides are selective markers for striatal-efferent neurons which (i) serve to control the specificity of the lesion (striatal-efferent neurons do not degenerate in this model), and (ii) are under the influence of nigrostriatal dopaminergic neurons (Gerfen, 1992), so they might exhibit dysfunctional effects. In addition, we also conducted a further experiment to evaluate whether termination of  $\Delta^9$ -THC administration to 6-hydroxydopamine-lesioned rats after 2 weeks would result in a re-initiation of the process of neuronal injury during two subsequent weeks. This experiment also serves to control whether the potential effects of  $\Delta^9$ -THC against *in vivo* toxicity of 6-hydroxydopamine are mainly neuroprotective (they do not disappear after discontinuation of cannabinoid treatment) or due to up-regulatory responses (they would disappear after discontinuation of cannabinoid treatment). In a parallel study, we also tested whether the lesions caused by 6-hydroxydopamine were accompanied by changes in the effectiveness of  $CB_1$  receptors in the caudate-putamen and the substantia nigra 2 weeks after the application of the toxin. Previous studies have shown that overactivity of these receptors developed after longer periods of time (>4 weeks) following 6-hydroxydopamine application (Mailleux and Vanderhaeghen, 1993; Romero et al., 2000) as seen in other models of PD (Lastres-Becker et al., 2001). However, there are no indications that this also happens after shorter periods of time such as those used here and whether it may influence the potential neuroprotective action of cannabinoid agonists. In this additional experiment, we also analyzed the changes in mRNA levels for the vanilloid VR1 receptor subtype, which has been recently reported to be located onto nigrostriatal dopaminergic neurons (Mezey et al., 2000) that degenerate in this PD model.

As mentioned above, in addition to their antioxidant properties, cannabinoids might be neuroprotective also because of their anti-inflammatory properties, which are likely related to their ability to modulate glial influence to neurons (for a review, see Walter and Stella, 2004). This might also be important in PD since nigral cell death is accompanied by astrocyte proliferation and reactive microgliosis at the sites of neurodegeneration (McGeer et al., 2001). Even, since the cause of dopaminergic cell death in PD is still unknown, it has been postulated that alterations in glial cell function (i.e., microglial activation) may play an important role in the initiation and/or early progression of the neurodegenerative process (Chao et al., 1996; Gao et al., 2002; Hirsch et al., 1998), especially in a region like the substantia nigra which is particularly enriched in microglia and other glial cells (Kim et al., 2000). In this sense, it is well demonstrated that activated microglia produce a wide array of cytotoxic factors, including tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ), eicosanoids, nitric oxide, and reactive oxygen species, that impact on neurons to induce neurodegeneration (Hirsch, 2000; Minghetti and Levi, 1998), and some of them have been reported to be increased in the substantia nigra and the caudate-putamen of PD patients (Mogi et al., 1994; Nagatsu et al., 2000). Based on the above findings and on the fact that cannabinoids have been reported to possess anti-inflammatory properties (Jaggari et al., 1998; Richardson et al., 1998) which may be relevant in terms of neuroprotection—i.e., cannabinoid agonists down-regulated inflammatory cytokines (TNF- $\alpha$  and IL-12) and up-regulated anti-inflammatory ones (IL-10) from glial cells (Smith et al., 2000)—we conducted a second series of experiments addressed to test whether the protective effects of cannabinoids against the *in vivo* toxicity of 6-hydroxydopamine might also be

observed *in vitro* and exerted by regulating glial trophic support to neurons (i.e., by increasing pro-survival factors, and/or by reducing cytotoxic ones). In these experiments, we used cerebellar granule cell cultures exposed to 6-hydroxydopamine, a model of neuronal apoptosis that some authors have used as an *in vitro* model to study 6-hydroxydopamine neurotoxicity relevant to PD (Daily et al., 1999; Dodel et al., 1999). Neurons were exposed to the cannabinoid agonist HU-210 either directly, by adding the cannabinoid in their culture medium, or indirectly by exposing the neuronal cultures to conditioned medium obtained from mouse mixed glial cell cultures that had been exposed to the cannabinoid agonist.

## Materials and methods

### *Experimental design I: In vivo effects of $\Delta^9$ -THC or CBD in the progress of neurodegeneration in rats unilaterally lesioned with 6-hydroxydopamine*

#### *Animals, surgical procedures, treatments, and sampling*

**Animals.** Male Sprague–Dawley rats (>8 weeks; approximately 250 g weight) were housed in a room with controlled photoperiod (08:00–20:00 light) and temperature ( $23 \pm 1^\circ\text{C}$ ). They had free access to standard food and water. All experiments were conducted according to European rules (directive 86/609/EEC).

**Unilateral injection of 6-hydroxydopamine.** After pretreatment (30 min before) with desipramine (25 mg/kg, ip), and under equithesin anesthesia (3 mg/kg, ip), rats were injected stereotaxically [coordinates:  $-2.5$  mm in reference to bregma,  $-1.8$  mm from the midline,  $-8.9$  mm ventral from the dura mater, according to Paxinos atlas (Paxinos and Watson, 1986)] into the medial forebrain bundle with 6-hydroxydopamine free base (8  $\mu\text{g}$  in a volume of 2  $\mu\text{l}$  of saline containing 0.05% ascorbate to avoid oxidation). The correct location of this stereotaxic injection was routinely checked in a few additional animals subjected to injections of black ink and further inspection of their brains (see details in Romero et al., 2000). This was also checked at the time that rat brains were sliced for *in situ* hybridization analyses. Those animals showing an incorrect location of the lesion were discarded. To control the damage produced by the stereotaxic surgery itself, control rats were subjected to sham-operation (without injecting the toxin) in the ipsilateral side, whereas, in all groups, contralateral structures were always intact, allowing to measure the effects of the administered substances in the absence of lesion (contralateral structures), or after lesion or sham-operation (ipsilateral structures).

**Treatment with  $\Delta^9$ -THC and CBD.**  $\Delta^9$ -THC was kindly provided by GW Pharmaceuticals Ltd (Salisbury, UK) and CBD was purified from hashish in the Hebrew University laboratory as previously described (Gaoni and Mechoulam, 1971). They were prepared in Tween 80–saline solution (1:16 v/v) for ip administration. The doses used for each experiment were selected from previous studies reporting protective effects of these compounds in equivalent injury models (see Grundy, 2002; Grundy et al., 2001; Mechoulam et al., 2002a,b). In separate experiments, 6-hydroxydopamine-injected animals were ip administered with either  $\Delta^9$ -THC (3 mg/kg weight) or CBD (3 mg/kg weight), and with their corresponding vehicles,

16 h after the local injection of 6-hydroxydopamine. The injections were repeated daily for a period of 2 weeks post-lesion, when the animals were killed 2 h after the last injection. Their brains were rapidly removed and frozen in 2-methylbutane cooled in dry ice, and stored at  $-80^\circ\text{C}$  for neurochemical evaluation indicative of the degree of 6-hydroxydopamine-induced neuronal injury. In an additional experiment, 6-hydroxydopamine-injected rats were daily injected, starting at 16 h post-lesion, with  $\Delta^9$ -THC (3 mg/kg weight) or vehicle during a period of 2 weeks. Then, the treatment was interrupted for an additional period of 2 weeks at the end of which, the animals were killed and their brains removed and processed as described for the above experiments.

#### *Neurochemical evaluation of neuronal injury*

**Dissection procedure.** Coronal slices (around 500  $\mu\text{m}$  thick) were manually obtained at the caudate-putamen level (Palkovits and Brownstein, 1988). Subsequently, this structure was dissected and homogenized in 40 vol of cold 150 mM potassium phosphate buffer, pH 6.8. Each homogenate was distributed for the analysis of DA and DOPAC contents, and of TH activity described below.

**Analysis of DA and DOPAC contents.** The contents of DA and its major intraneuronal metabolite, DOPAC, were analyzed using HPLC with electrochemical detection according to our previously published method (González et al., 1999; Romero et al., 1995). Briefly, homogenates were diluted (1/2) in ice-cold 0.4 N perchloric acid containing 0.4 mM sodium disulfite and 0.90 mM EDTA. Dihydroxybenzylamine was added as an internal standard. The diluted homogenates were then centrifuged and the supernatants injected into the HPLC system, which consisted of a Spectra-Physics 8810 isocratic pump. The column was a RP-18 (Spherisorb ODS-2; 125 mm, 4.6 mm, 5  $\mu\text{m}$  particle size; Waters, Massachusetts, USA). The mobile phase consisted of 100 mM citric acid, 100 mM sodium acetate, 1.2 mM heptane sulphonate, 1 mM EDTA, and 7% methanol (pH 3.9), and the flow rate was 0.8 ml/min. The effluent was monitored with a coulometric detector (Coulchem II, ESA) using a procedure of oxidation/reduction (conditioning cell: +360 mV; analytical cell #1: +50 mV; analytical cell #2:  $-340$  mV). The signal was recorded from analytical cell #2, with a sensitivity of 50 nA (10 pg per sample), on a Spectra-Physics 4290 integrator, and the results were given as area under the peaks. Values were expressed as ng/area.

**Assay of TH activity.** The activity of this enzyme was measured according to Nagatsu et al. (1979). Homogenates were incubated at  $37^\circ\text{C}$  in the presence of 0.1 M sodium acetate, 1 mM 6-methyl-5,6,7,8-tetrahydropterine (prepared in 1 M mercapto-ethanol solution), 0.1 mg/ml catalase, and 0.2 mM L-tyrosine. For the blank incubation, L-tyrosine was replaced by D-tyrosine. Blank tubes containing 1  $\mu\text{M}$  L-3,4-dihydroxyphenylalanine (L-dopa) were also used as an internal standard for each tissue. After 30 min of incubation, the reaction was stopped by the addition of 0.2 N perchloric acid containing 0.2 mM sodium disulfite and 0.45 mM EDTA. Dihydroxybenzylamine was also added as an internal standard for HPLC determination. The amounts of L-dopa formed were evaluated by HPLC following the same procedure as for the direct analysis of DA and DOPAC contents, with the only difference of a previous extraction with alumina. Values were expressed as ng of L-dopa formed/area h.

*Autoradiography and in situ hybridization techniques*

**Brain slicing.** Coronal sections, 20- $\mu$ m-thick, were cut in a cryostat, according to the Paxinos and Watson atlas (1986). Sections were thaw-mounted onto RNase-free gelatin/chrome alum-coated slides and dried briefly at 30°C and stored at –80°C until used.

**Autoradiography of cannabinoid receptor binding.** The protocol used is basically the method described by Herkenham et al. (1991). Briefly, slide-mounted brain sections were incubated for 2.5 h, at 37°C, in a buffer containing 50 mM TRIS with 5% bovine serum albumin (fatty acid-free), pH 7.4, and 10 nM [<sup>3</sup>H]-CP-55,940 (Du Pont NEN) prepared in the same buffer, in the absence or the presence of 10  $\mu$ M non-labeled CP-55,940 (kindly supplied by Pfizer) to determine the total and the non-specific binding, respectively. Following this incubation, slides were washed in 50 mM TRIS buffer with 1% bovine serum albumin (fatty acid-free), pH 7.4, for 4 h (2  $\times$  2 h) at 0°C, dipped in ice-cold distilled water, and then dried under a stream of cool dried air. Autoradiograms were generated by apposing the labeled tissues, together with autoradiographic standards ([<sup>3</sup>H] micro-scales, Amersham), to tritium-sensitive film ([<sup>3</sup>H]-Hyperfilm MP, Amersham) for a period of 2 weeks. An intensifying screen (Biomax Transcreen LE, Kodak) was also used. Autoradiograms were developed (D-19, Kodak) for 4 min at 20°C, and the films were analyzed and quantitated in a computer-assisted videodensitometer using the standard curve generated from [<sup>3</sup>H]-standards.

**Analysis of mRNA levels for CB<sub>1</sub> receptor, VR1 receptor, TH, proenkephalin, and substance P by in situ hybridization.** The analysis of CB<sub>1</sub> receptor mRNA levels was carried out according to Rubino et al. (1994). Briefly, sections were fixed in 4% paraformaldehyde for 5 min and, after rinsing twice in phosphate buffer saline, were acetylated by incubation in 0.25% acetic anhydride, prepared in 0.1 M triethanolamine/0.15 M sodium chloride (pH 8.0), for 10 min. Sections were rinsed in 0.3 M sodium chloride/0.03 M sodium citrate, pH 7.0, dehydrated, and delipidated by ethanol/chloroform series. A mixture (1:1:1) of the three 48-mer oligonucleotide probes complementary to bases 4–51, 349–396, and 952–999 of the rat CB<sub>1</sub> receptor cDNA (Du Pont; the specificity of the probes used was assessed by Northern Blot analysis) was 3'-end labeled with [<sup>35</sup>S]-dATP using terminal deoxynucleotidyl-transferase. Sections were, then, hybridized with [<sup>35</sup>S]-labeled oligonucleotide probes (7.5  $\times$  10<sup>5</sup> dpm per section), washed and exposed to X-ray film ( $\beta$ max, Amersham) for 1 week, and developed (D-19, Kodak) for 6 min at 20°C. The intensity of the hybridization signal was assessed by measuring the grey levels in the autoradiographic films with a computer-assisted videodensitometer. Adjacent brain sections were co-hybridized with a 100-fold excess of cold probe or with RNase to assert the specificity of the signal (data not shown). Similar procedures were used for the analysis of mRNA levels of proenkephalin, substance P, vanilloid VR1 receptor, and TH. We used commercial probes (NEN-Du Pont, Itisa, Madrid, Spain) for TH (Garcia-Gil et al., 1998) and proenkephalin (Young et al., 1986), a synthetic 45-base probe, selected from the previously-published sequence, for substance P (5'-CGTTTGCCCAT-CAATCCAAAGAACTGCTGAGGCTTGGGTCTCCG-3'; Nawa et al., 1984), and a cDNA kindly provided by Dr. David Julius (University of California, San Francisco, CA, USA) for VR1

receptor (Mezey et al., 2000). Details on these procedures have been already published (Lastres-Becker et al., 2002).

**Experimental design II: Effects of HU-210 on neuronal death induced by 6-hydroxydopamine in cultured cerebellar granule neurons**

*Cell culture, treatments, and sampling*

**Animals.** One-day-old C57BL/6 mice were obtained from Charles River (UK) and were used for experimental purposes in accordance with the guidelines set by the European Council directives (86/609/EEC) and the Home Office, Animals Scientific Procedures Act (1986, UK).

**Primary mixed glial cultures.** Primary mixed glial cultures were prepared from the whole brains of 1-day-old mice following well-established protocols (McCarthy and de Vellis, 1980; Molina-Holgado et al., 1995), and grown in T150 flasks for at least 14 days in Dulbecco's modified Eagle's medium (DMEM) and 10% heat-inactivated fetal bovine serum (FBS), 20 mM glutamine, and antibiotics (0.1 IU/ml penicillin, 0.1  $\mu$ g/ml streptomycin solution). The medium was changed twice per week. On reaching the confluence (usually at 2 weeks), the cells were trypsinized. The media were replaced and the cells (5  $\times$  10<sup>5</sup> cells/well) were allowed to recover for 2–3 days before the experiments. To visualize glial fibrillary acidic protein (GFAP) and CD11b (MAC-1 a<sup>M</sup> chain), the cells were washed three times with phosphate-buffered saline (PBS) at room temperature. Monoclonal antibodies to GFAP (1:500; Sigma-Aldrich Co., UK) and MAC-1 (1:100; Serotec Ltd, UK) were diluted in DMEM containing 5% FBS, 0.02% sodium azide, 0.2% bovine serum albumin (BSA), 5% goat serum, and 0.2% Triton X-100, and applied for 15 min at room temperature. Afterwards, the cells were washed three times with PBS at room temperature. The second antibodies Texas-red, conjugated donkey anti-mouse, and FITC goat conjugated anti-rat (Jackson ImmunoResearch, USA) were diluted (1:100) and applied under the same conditions. Cells were then fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. Cell nuclei were labeled with DAPI (present in the mounting medium) (Vectashield, Vector, Burlingame, CA). The resulting cultures consisted of 70% astrocytes as determined by staining with GFAP and 30% of cells were positive for the microglia marker, MAC-1 (not shown).

**Cerebellar granular neuronal cultures.** Primary cultures of cerebellar granule neurons were prepared from the cerebella of 7-day-old mice according to well-established protocols (Cambray-Deakin, 1995). In brief, cerebella were removed and cultured in basal Eagle's medium (BME), supplemented with 10% heat-inactivated fetal calf serum (FCS), 30 mM glucose, 2 mM glutamine, and antibiotics (0.1 IU/ml penicillin, 0.1  $\mu$ g/ml streptomycin solution) and 25 mM KCl. Cells were plated onto poly-lysine-coated Petri dishes, multiwells, or glass coverslips according to experimental requirements at a density of 2.5  $\times$  10<sup>5</sup> cells/cm<sup>2</sup>. To prevent glial cell proliferation, 20 h after plating, cultures were treated with cytosine- $\beta$ -D-arabinofuranoside at a final concentration of 10  $\mu$ M. These cultures were used at 7 days after plating, when the cell population comprises 95% granule neurons and 5% of other cell types including astrocytes (not shown).

**Cell treatments.** In a first experiment, primary cultures of cerebellar granule neurons were incubated for 24 h with two doses of the synthetic and non-selective cannabinoid agonist HU-210 (1 or 10  $\mu$ M) (Mechoulam et al., 1990) or 6-hydroxydopamine (20  $\mu$ M). HU-210 is chemically related to classic cannabinoids, but it is much more potent than  $\Delta^9$ -THC or CBD at the two cannabinoid receptor subtypes, thus allowing to be used at lower concentrations in vitro and solving the solubility problems of  $\Delta^9$ -THC or CBD in aqueous solutions. In a second experiment, HU-210 (1 or 10  $\mu$ M) was first added to primary cultures of mixed glial cells, then incubated for 24 h, and their media removed and added to primary cultures of cerebellar granule neurons together with 6-hydroxydopamine (20  $\mu$ M), and incubated for another 24 h. The above concentrations and times of incubation were determined according to previously reported experiments in glial or neuronal cultures (Dodel et al., 1999; Galea et al., 1992; Simmons and Murphy, 1992; Molina-Holgado et al., 2003). The cells were checked for their viability and proliferation, using Trypan blue dye exclusion and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays (Carmichael et al., 1987). Glial cell treatments were performed at the same density ( $5 \times 10^5$  cells/well, 12-well dishes).

**Analysis of neuronal survival.** Hoechst 33342 (10  $\mu$ M) and propidium iodide (10  $\mu$ M) were used to stain viable and dead cells, respectively. Cells were counted by using a fluorescence microscope (Nikon EFD3). Five to ten microscopic fields were counted for each coverslip, and two to three coverslips per treatment were used for each experiment. Only neurons positive to Hoechst 33342, but negative to propidium iodide, were counted for the final analysis of cell survival.

#### Statistics

All data were assessed by the Student's *t* test or the one-way analysis of variance, followed by the Student–Newman–Keuls test, as required.

#### Results

##### *Experimental design I: In vivo effects of $\Delta^9$ -THC or CBD in the progress of neurodegeneration in rats unilaterally lesioned with 6-hydroxydopamine*

##### *Status of CB<sub>1</sub> receptors in the basal ganglia of 6-hydroxydopamine-injected rats*

Previous studies have revealed that 6-hydroxydopamine-induced lesions up-regulate CB<sub>1</sub> receptors in the basal ganglia (Mailleux and Vanderhaeghen, 1993; Romero et al., 2000), but this occurred after longer periods of time after the lesion than those used by us, namely when the dopaminergic injury is expected to be high. In the present study, however, we used a shorter period for the 6-hydroxydopamine action that likely causes a moderate lesion, which mimics that found in the first phases of PD in humans, possibly the most sensitive period during which the protective effects of cannabinoids may be more significant. Therefore, it was interesting to analyze the status of CB<sub>1</sub> receptors in the basal ganglia (and in other reference structures), before examining the neuroprotective effects of  $\Delta^9$ -THC and CBD in this rat model of PD. Our results indicated a complete lack of changes in both binding capacity and

mRNA levels for CB<sub>1</sub> receptors 2 weeks post-lesion in the caudate-putamen (medial and lateral parts) and also in the cerebral cortex (deep and superficial layers) (see Table 1). The same lack of changes for CB<sub>1</sub> receptor binding occurred in the substantia nigra (Table 1), although this structure showed a small but statistically significant reduction in mRNA levels for vanilloid VR1 receptors (Table 1) since this receptor subtype has been recently reported to be located on nigrostriatal dopaminergic neurons that degenerate by the application of 6-hydroxydopamine (Mezey et al., 2000). All the above data were seen by comparing both (i) the lesioned side versus the non-lesioned side in 6-hydroxydopamine-injected rats (data not shown), and (ii) the lesioned side in 6-hydroxydopamine-injected rats versus the equivalent side in control (sham-operated) rats (see values in Table 1).

##### *Effects of a chronic administration of $\Delta^9$ -THC to 6-hydroxydopamine-injected rats*

As expected, 6-hydroxydopamine injection produced, 2 weeks post-injection, a significant depletion of DA (−46.3%;  $F(2,29) = 4.323$ ,  $P < 0.05$ ) and DOPAC (−35.2%;  $F(2,29) = 3.70$ ,  $P < 0.05$ ) contents and a reduction of TH activity (−47.3%;  $F(2,29) = 9.473$ ,  $P < 0.005$ ) in the striatum of the lesioned side compared with the ipsilateral structure in sham-operated animals (see values in Table 2). There was also a reduction, to a lesser extent, in TH-mRNA levels in the substantia nigra (−19.9%;  $F(2,23) = 6.622$ ,  $P < 0.01$ ) (Table 2). None of these events occurred in the contralateral structures (all intact) for DA (controls:  $79.4 \pm 8.7$  ng/area; 6-hydroxydopamine:  $74.1 \pm 6.8$ ), DOPAC (controls:  $8.2 \pm 0.8$  ng/area; 6-hydroxydopamine:  $7.4 \pm 0.9$ ), TH activity (controls:  $242.3 \pm 24.0$  ng/area h;

Table 1

Cannabinoid CB<sub>1</sub> receptor binding (fmol/mg of protein) and mRNA levels (optical density), and vanilloid VR1 receptor mRNA levels (optical density), in the basal ganglia and some reference structures (cerebral cortex) of rats with unilateral lesions of the nigrostriatal dopaminergic neurons caused by local injection of 6-hydroxydopamine (2 weeks post-lesion) or controls (sham-operated)

Brain regions	Parameter	Control rats	6-Hydroxydopamine-lesioned rats
Lateral caudate-putamen	CB <sub>1</sub> receptor binding	71.5 $\pm$ 5.1	69.6 $\pm$ 3.9
	CB <sub>1</sub> receptor mRNA levels	0.238 $\pm$ 0.026	0.262 $\pm$ 0.019
Medial caudate-putamen	CB <sub>1</sub> receptor binding	55.8 $\pm$ 4.4	59.0 $\pm$ 3.3
	CB <sub>1</sub> receptor mRNA levels	0.143 $\pm$ 0.025	0.157 $\pm$ 0.015
Substantia nigra	CB <sub>1</sub> receptor binding	177.7 $\pm$ 10.5	178.5 $\pm$ 9.9
	VR1 receptor mRNA levels	0.62 $\pm$ 0.07	0.46 $\pm$ 0.06*
Cerebral cortex (deep layer)	CB <sub>1</sub> receptor binding	50.1 $\pm$ 3.0	52.7 $\pm$ 2.8
	CB <sub>1</sub> receptor mRNA levels	0.129 $\pm$ 0.027	0.144 $\pm$ 0.014
Cerebral cortex (superficial layer)	CB <sub>1</sub> receptor binding	40.0 $\pm$ 2.4	37.5 $\pm$ 3.2
	CB <sub>1</sub> receptor mRNA levels	0.112 $\pm$ 0.026	0.127 $\pm$ 0.012

Details in the text. Values are expressed as means  $\pm$  SEM of at least 7 determinations per group. Data were assessed by the Student's *t* test (\* $P < 0.05$ ).

Table 2

Effects of 2 weeks of daily administration of  $\Delta^9$ -THC (3 mg/kg) or CBD (3 mg/kg), or their corresponding vehicle, on dopamine and DOPAC contents, tyrosine hydroxylase (TH) activity, and mRNA levels for proenkephalin (PENK) and substance P (SP) in the caudate-putamen, and TH-mRNA levels in the substantia nigra of rats with unilateral lesions of the nigrostriatal dopaminergic neurons caused by local injection of 6-hydroxydopamine or controls (sham-operated)

Parameters	Controls	6-hydroxydopamine-lesioned rats	
		+vehicle	+ $\Delta^9$ -THC
<b>Caudate-putamen:</b>			
Dopamine contents (ng/area)	67.4 ± 10.5	36.2 ± 6.4*	48.2 ± 6.4
DOPAC contents (ng/area)	5.85 ± 1.16	3.79 ± 0.65*	5.32 ± 0.70
TH activity (ng/area.h)	237.6 ± 23.6	125.1 ± 15.3***	194.0 ± 19.2#
PENK-mRNA levels (optical density)	0.105 ± 0.023	0.112 ± 0.020	0.137 ± 0.012
SP-mRNA levels (optical density)	0.128 ± 0.004	0.121 ± 0.009	0.147 ± 0.011
<b>Substantia nigra:</b>			
TH-mRNA levels (optical density)	0.381 ± 0.009	0.305 ± 0.021**	0.401 ± 0.026#
Parameters	Controls	6-hydroxydopamine-lesioned rats	
		+vehicle	+CBD
<b>Caudate-putamen:</b>			
Dopamine contents (ng/area)	81.9 ± 7.8	52.2 ± 6.9**	70.0 ± 3.5#
DOPAC contents (ng/area)	7.50 ± 1.29	5.59 ± 0.60	8.02 ± 1.09
TH activity (ng/area.h)	222.6 ± 26.3	127.9 ± 13.8**	196.0 ± 13.4#
PENK-mRNA levels (optical density)	0.111 ± 0.007	0.113 ± 0.005	0.104 ± 0.009
SP-mRNA levels (optical density)	0.046 ± 0.005	0.043 ± 0.003	0.039 ± 0.003
<b>Substantia nigra:</b>			
TH-mRNA levels (optical density)	0.199 ± 0.023	0.117 ± 0.021*	0.149 ± 0.016

Data correspond to values measured in ipsilateral structures in three experimental groups, while the values in contralateral structures are included in the text. Values are expressed as means ± SEM of at least 7 determinations per group. Data were assessed by one-way analysis of variance followed by the Student–Newman–Keuls test (\* $P$  < 0.05, \*\* $P$  < 0.01, \*\*\* $P$  < 0.005 versus control rats; # $P$  < 0.05 versus vehicle-injected 6-hydroxydopamine-lesioned rats).

6-hydroxydopamine: 231.2 ± 17.5), and TH-mRNA levels (controls: 0.374 ± 0.009 units of OD; 6-hydroxydopamine: 0.401 ± 0.019). Daily administration of  $\Delta^9$ -THC (3 mg/kg) during 2 weeks after the lesion produced a significant waning in the magnitude of

the above reductions caused by the toxin in DA and DOPAC contents, and TH activity and mRNA levels, comparing the ipsilateral structures of the three experimental groups (see Table 2). No changes occurred in the contralateral non-lesioned structures by the exposure to  $\Delta^9$ -THC (DA: 78.9 ± 9.8 ng/area; DOPAC: 7.6 ± 1.0 ng/area; TH activity: 229.0 ± 27.3 ng/area h; TH-mRNA levels: 0.435 ± 0.014 units of OD). By contrast, the mRNA levels of proenkephalin and substance P in the caudate-putamen were not altered by either administration of 6-hydroxydopamine alone or when animals were also ip injected with  $\Delta^9$ -THC (Table 2).

#### Effects of chronic administration of CBD to 6-hydroxydopamine-injected rats

We next studied whether the above neuroprotective actions of  $\Delta^9$ -THC were also exerted by CBD, a cannabinoid also derived from *Cannabis sativa*, which shares with  $\Delta^9$ -THC some properties (i.e., antioxidant capability) but differs in its lack of affinity for the CB<sub>1</sub> receptors (for a review, see Bisogno et al., 2001; Pertwee, 1997). Also, in the animals of this experiment, 6-hydroxydopamine injection reduced, 2 weeks post-injection, DA (−36.3%;  $F(2,29) = 6.147$ ,  $P < 0.01$ ) contents and TH activity (−42.5%;  $F(2,29) = 7.766$ ,  $P < 0.005$ ) in the caudate-putamen, and TH-mRNA levels (−41.2%;  $F(2,29) = 4.767$ ,  $P < 0.05$ ) in the substantia nigra, whereas the reduction in DOPAC content in the caudate-putamen did not reach statistical significance in this experiment (see values in Table 2). As in the above experiment, these reductions were observed by comparing the ipsilateral structures of 6-hydroxydopamine-injected and sham-operated animals, whereas none of these events occurred in the contralateral structures for DA (controls: 102.0 ± 9.1 ng/area; 6-hydroxydopamine: 92.6 ± 7.1), DOPAC (controls: 10.4 ± 2.1 ng/area; 6-hydroxydopamine: 8.1 ± 0.8), TH activity (controls: 229.0 ± 19.9 ng/area h; 6-hydroxydopamine: 247.9 ± 19.3), and TH-mRNA levels (controls: 0.154 ± 0.028 units of OD; 6-hydroxydopamine: 0.146 ± 0.018). It is important to note that, in general, slightly different values were recorded for some of these parameters in this and the above experiment (see Table 2), differences that may be attributed to a normal interassay variation due to factors such as small differences in weight and age of animals or seasonal variations. Daily administration of CBD (3 mg/kg), during these 2 weeks post-lesion, also produced a significant waning in the magnitude of the above reductions caused by the toxin in DA and DOPAC contents and TH activity and mRNA levels, also causing a complete recovery of the control values in some cases (see Table 2). As occurred with  $\Delta^9$ -THC, the effects of CBD were observed comparing the ipsilateral structures of the three experimental groups, but they did not occur in the contralateral non-lesioned structures (DA: 107.6 ± 8.6 ng/area; DOPAC: 10.6 ± 0.8 ng/area; TH activity: 285.9 ± 23.5 ng/area h; TH-mRNA levels: 0.144 ± 0.016 units of OD). In addition, they were not accompanied by changes in mRNA levels of proenkephalin and substance P in the caudate-putamen in any of the three experimental groups analyzed (Table 2).

#### Effects of the interruption in the chronic administration of $\Delta^9$ -THC to 6-hydroxydopamine-injected rats

A further objective of our study was to examine whether 2 weeks after the end of the chronic  $\Delta^9$ -THC administration to 6-hydroxydopamine-lesioned rats, a re-initiation of the process of neuronal injury would take place. Our results indicated that the protective effect of  $\Delta^9$ -THC appeared to be irreversible since, 2

weeks after the interruption of this treatment, there were still statistical differences between the ipsilateral structures of  $\Delta^9$ -THC- and vehicle-treated 6-hydroxydopamine-injected rats as regards to DA ( $F(2,20) = 4.035$ ,  $P < 0.05$ ) and DOPAC ( $F(2,20) = 7.411$ ,  $P < 0.005$ ) contents and TH activity ( $F(2,20) = 4.49$ ,  $P < 0.05$ ) in the caudate-putamen, and to mRNA levels for TH ( $F(2,20) = 6.382$ ,  $P < 0.01$ ) in the substantia nigra (see Fig. 1). Again, no changes were noted in the contralateral non-lesioned structures for DA (+vehicle:  $50.2 \pm 7.3$  ng/area; + $\Delta^9$ -THC:  $51.5 \pm 5.2$ ), DOPAC (+vehicle:  $6.8 \pm 0.4$  ng/area; + $\Delta^9$ -THC:  $5.5 \pm 0.8$ ), TH activity (+vehicle:  $120.5 \pm 11.6$  ng/area h; + $\Delta^9$ -THC:  $132.6 \pm 28.4$ ), and TH-mRNA levels (+vehicle:  $0.098 \pm 0.004$  units of OD; + $\Delta^9$ -THC:  $0.102 \pm 0.007$ ), whereas mRNA levels for proenkephalin and substance P in the caudate-putamen were not altered after injection of  $\Delta^9$ -THC or vehicle to 6-hydroxydopamine-lesioned animals (Fig. 1).

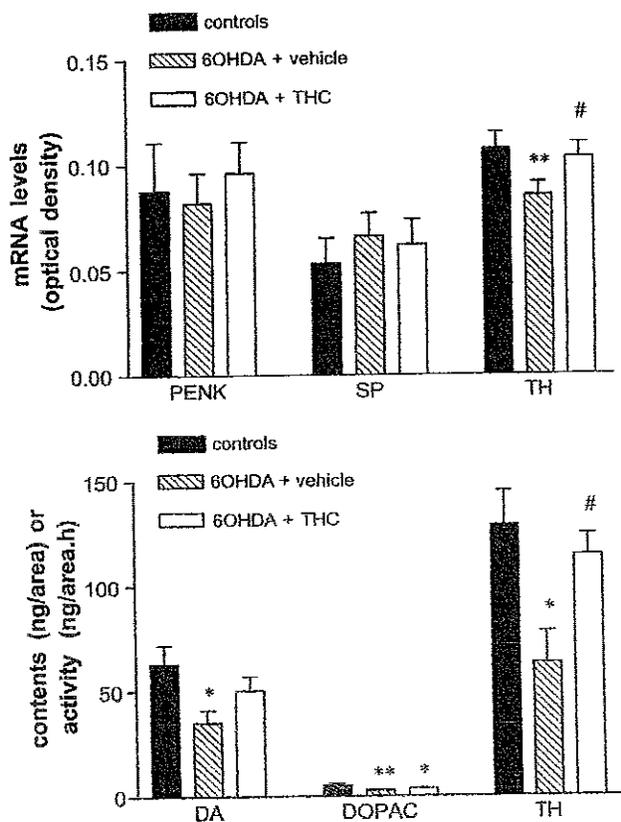


Fig. 1. Effects of 2 weeks of daily administration of  $\Delta^9$ -THC (3 mg/kg) or vehicle, followed by a period of another 2 weeks in which the treatment was interrupted, on dopamine and DOPAC contents, tyrosine hydroxylase (TH) activity, and mRNA levels for proenkephalin (PENK) and substance P (SP) in the caudate-putamen, and TH-mRNA levels in the substantia nigra of rats with unilateral lesions of the nigrostriatal dopaminergic neurons caused by local injection of 6-hydroxydopamine or controls (sham-operated). Data correspond to values measured in ipsilateral structures in the three experimental groups, while the values in contralateral structures are included in the text. Values are expressed as means  $\pm$  SEM of at least 8 determinations per group. Data were assessed by one-way analysis of variance followed by the Student–Newman–Keuls test (\* $P < 0.05$ , \*\* $P < 0.01$  versus control rats; # $P < 0.05$  versus vehicle-injected 6-hydroxydopamine-lesioned rats).

#### Experimental design II: Effects of HU-210 on neuronal death induced by 6-hydroxydopamine in cultured cerebellar granule neurons

To assess the neuroprotective effect of cannabinoid agonists on 6-hydroxydopamine-induced neuronal death in vitro, we used mouse cultures of cerebellar granule cells. These cells are quite sensitive to 6-hydroxydopamine, so that they have been used as an in vitro model to test the neurotoxicity of this toxin which may be relevant to PD (Daily et al., 1999; Dodel et al., 1999; Offen et al., 2000). We observed that the addition of 6-hydroxydopamine to differentiated cerebellar granule neurons during a period of 24 h caused a dramatic reduction in the number of surviving cells (Fig. 2), similar to that found by other authors (Kumar et al., 1995; Lotharius et al., 1999). Neuronal death developed rapidly, and the number of viable cells was reduced approximately to 35% of the total number of cerebellar granule neurons in culture (neuronal survival at control group was considered as 100%). Interestingly, the exposure of these neurons to the non-selective agonist HU-210, a cannabinoid much more better for in vitro studies than plant-derived cannabinoids, reduced 6-hydroxydopamine-induced cell death ( $F(5,35) = 41.59$ ,  $P < 0.0001$ ; Fig. 2), but this effect, compared with the effect of cannabinoids observed in the in vivo experiments, was small and did not exhibit dose-dependency (neuronal survival with HU-210 1  $\mu$ M: 55%, and with HU-210 10  $\mu$ M: 49%). It is possible that this might be related to the fact that some neuroprotective substances act in vivo by increasing prosurvival glial influence to neurons, which cannot be reproduced with this experimental approach. To solve this, we used the experimental design described by De Bernardo et al. (2003), who demonstrated that conditioned media obtained from glial cell cultures may increase neuronal survival in vitro. Thus, cerebellar granule neuronal cultures were treated with 6-hydroxydopamine and conditioned medium obtained by exposure of mixed glial cell cultures to HU-210 1 or 10  $\mu$ M, also for 24 h. We observed that, compared with the small effect when HU-210 is directly added to cultured neurons, the neuronal survival rate was quite increased when exposure to this cannabinoid was indirect (through generating glial conditioned media) (see Fig. 2). This suggests that the neuroprotective effect of HU-210 could be mainly exerted by increasing prosurvival glial influence to neurons. In addition, the effect showed a good dose-dependency (neuronal survival with HU-210 1  $\mu$ M: 44%, and 10  $\mu$ M: 88%, see Fig. 2), which might be indicative of the involvement of cannabinoid receptors, either CB<sub>1</sub> or CB<sub>2</sub>, or both, in these effects.

#### Discussion

The present study shows the first evidence for a neuroprotective action of cannabinoids in an animal model of PD, an adult-onset neurodegenerative disorder characterized by a preferential loss of the dopaminergic neurons of the substantia nigra pars compacta (for a review, see Sethi, 2002) triggered by three major pathogenic events: oxidative stress, mitochondrial dysfunction, and inflammatory stimuli (McGeer et al., 2001; Sherer et al., 2001). Previous studies relating cannabinoids to PD addressed questions as the changes in the endocannabinoid signaling system in postmortem basal ganglia of PD patients (Hurley et al., 2003; Lastres-Becker et al., 2001) or in animal models of this disease (Di Marzo et al., 2000; Gubellini et al., 2002; Lastres-Becker et al., 2001; Romero et al., 2000; Silverdale et al., 2001; Zeng et al.,

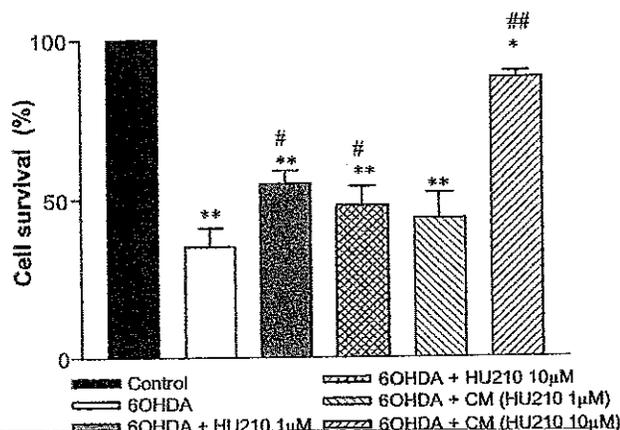


Fig. 2. Induction of cell death by 6-hydroxydopamine exposure of cultured mouse cerebellar granule neurons, and protective effects of HU-210 when added directly to neuronal cultures or through the generation of conditioned media (CM) from mixed glial cell cultures. Values are means  $\pm$  SEM of 4 to 6 independent experiments each carried out in triplicate. Data were assessed by one-way analysis of variance followed by the Student–Newman–Keuls test (\* $P < 0.05$ , \*\* $P < 0.005$  versus controls; # $P < 0.05$ , ## $P < 0.005$  versus 6-hydroxydopamine alone).

1999), studies that frequently, although not in all cases, revealed the occurrence of an overactivity of this system compatible with the hypokinesia characteristic of this disease (for a review, see Sethi, 2002). This overactivity, however, did not occur within 2 weeks after the lesion, as reported now. This period of time is significantly shorter than the periods used in previous studies reporting CB<sub>1</sub> receptor up-regulation (Lastres-Becker et al., 2001; Mailleux and Vanderhaeghen, 1993; Romero et al., 2000). We also found decreased mRNA levels for VR1 receptors 2 weeks after the lesion, a fact that was expected because of the location of this receptor subtype in nigrostriatal neurons (Mezey et al., 2000) that degenerate by 6-hydroxydopamine application. In our view, this shorter time period is more appropriate for the examination of the protective action of cannabinoids in this disease, since it mimics the first phases of PD in humans, probably the only one at which the neuroprotection by cannabinoids might be achieved. This lack of changes in CB<sub>1</sub> receptors at 2 weeks post-lesion indicates that the up-regulation only occurs when dopaminergic injury is strong and when there are less possibilities for a protectant therapy.

Previous studies have addressed the hypothetical efficacy of cannabinoid agonists or antagonists by reducing motor symptoms in PD (Di Marzo et al., 2000; Gilgun-Sherki et al., 2003; Maneuf et al., 1997; Meschler et al., 2001; Sañudo-Peña et al., 1998) or by alleviating the dyskinesia that develops after chronic dopaminergic replacement therapy (Brochie, 1998, 2000; Ferrer et al., 2003; Fox et al., 2002; Sieradzan et al., 2001). However, no evidence exists, to our knowledge, of a potential usefulness of cannabinoids to delay/arrest the progress of neurodegeneration in this disease, despite their well-demonstrated neuroprotectant efficacy in other models of acute or chronic degeneration (see references in Introduction). Here, we present the first evidence that  $\Delta^9$ -THC also acted as a neuroprotective substance in rats with hemiparkinsonism. Thus, the chronic administration of this cannabinoid to rats, starting 16 h (to avoid potential chemical interferences between the cannabinoid and the toxin) after they were subjected to unilateral lesions of the nigrostriatal dopaminergic neurons with 6-hydroxydopamine, produced a significant recovery in the impair-

ment of dopaminergic transmission caused by the toxin, likely indicating a reduction of dopaminergic cell death. This recovery modified neurochemical levels that become now, in most cases, similar or close to those observed in the ipsilateral structures of sham-operated animals. As we did not observe any changes of these neurochemical parameters in contralateral structures (all intact) by cannabinoid treatment, we assume that the changes observed in the lesioned structures are indicative of neuroprotection rather than of the occurrence of up-regulatory effects in surviving neurons (if this were the case, the effects would be recorded in both ipsilateral and contralateral structures). Interestingly, this recovery seemed to be persistent and irreversible since the interruption of chronic  $\Delta^9$ -THC treatment after 2 weeks did not result in a relapse of the dopaminergic injury. This last observation is also another data in support that the effect of cannabinoids in 6-hydroxydopamine-lesioned rats is produced by prevention of cell death and/or rescue of affected neurons, and does not indicate the occurrence of an upregulatory response of surviving neurons. If this were the case, the interruption of  $\Delta^9$ -THC treatment should have resulted in a loss of these effects and, then, dopaminergic parameters should have diminished again. Also supporting the view that the effect of  $\Delta^9$ -THC was produced by the arrest of cell death and/or the rescue of affected neurons is the fact that this cannabinoid has been shown already capable to increase the number of TH-containing neurons in studies with cultured fetal mesencephalic neurons (Hernández et al., 2000). On the other hand, it is less probable, but we cannot completely rule out, in absence of additional studies, that these data might also reflect an axonal sprouting response in surviving cell bodies, as has been previously reported that specific cannabinoids may produce in other pathological conditions (Zalish and Lavie, 2003).

As mentioned above, the present observation that chronic  $\Delta^9$ -THC treatment reduced the magnitude of dopaminergic injury in rats with hemiparkinsonism, is concordant with previous data showing that plant-derived, synthetic, or endogenous cannabinoids were neuroprotectant in a variety of in vivo and in vitro models of neuronal injury. However, it has been demonstrated that the mechanisms involved in these effects might be diverse, from events not mediated by cannabinoid receptors (NMDA antagonism, antioxidant properties; see Grundy et al., 2001, and Mechoulam et al., 2002a,b for review) up to CB<sub>1</sub> receptor-mediated phenomena (inhibition of glutamate release, stimulation of GABA action, reduction of Ca<sup>++</sup> influx, hypothermia, vascular effects, and others; see also Grundy et al., 2001, and Mechoulam et al., 2002a,b). The protective effects observed for  $\Delta^9$ -THC in the present study might be the result of an action independent of CB<sub>1</sub> receptors. This can be concluded from the fact that the two plant-derived cannabinoids,  $\Delta^9$ -THC and CBD, tested here were equally effective in attenuating the dopaminergic impairment following to the lesion with 6-hydroxydopamine, despite their differences in the affinity for CB<sub>1</sub> receptors (CBD has negligible activity at this receptor subtype; see Bisogno et al., 2001; Pertwee, 1997). A similar observation was made by Hampson et al. (1998) who examined the neuroprotective effects of  $\Delta^9$ -THC and CBD in rat cortical neuron cultures exposed to toxic levels of glutamate. These authors found that the ability of both cannabinoids to provide neuroprotection is CB<sub>1</sub> receptor-independent and based on the antioxidant properties of both compounds which are relatively equivalent (Hampson et al., 1998) and comparable, or even superior, to those reported for classic antioxidants such as ascorbate or  $\alpha$ -tocopherol (Hampson et al., 2000). Further studies by Chen and Buck (2000) and Marsicano

et al. (2002) also reported that cannabinoids protect cells from oxidative stress basically through a CB<sub>1</sub> receptor-independent mechanism. Therefore, our data, collectively, are concordant with the notion that these two plant-derived cannabinoids may function as neuroprotectant in PD based on their capability to reduce oxidative stress which represents a major hallmark in the pathogenesis of this disease (Blum et al., 2001). However, cannabinoids may also be effective in PD through mechanisms other than their antioxidant properties. For instance, the activation of non-CB<sub>1</sub>/non-CB<sub>2</sub> receptors may be of importance and, in view of the potent anti-inflammatory action of both cannabinoids, in particular CBD (Malfait et al., 2000), the blocking of the production of various factors associated with inflammation (nitric oxide, TNF $\alpha$ , and others) by these cannabinoids may be also relevant (see below). Even, it would be conceivable that the protective effect exerted by CBD might be produced through its recently reported ability to block anandamide breakdown and its uptake thus elevating anandamide levels (Bisogno et al., 2001) or, even, by its modest affinity for the CB<sub>2</sub> receptor subtype (Pertwee, 1997). In this sense, we have preliminary evidence that the blockade of the endocannabinoid inactivation with UCM707, a selective inhibitor of the endocannabinoid transport system (López-Rodríguez et al., 2003) that does not possess any antioxidant properties, did not reduce dopaminergic impairment caused by the application of 6-hydroxydopamine (data not shown). This discards that CBD might also act through blocking the endocannabinoid inactivation. As regards to a potential involvement of CB<sub>2</sub> receptors, it is important to remark that recent data have demonstrated that this receptor subtype, although relatively absent of the brain parenchyma in healthy conditions, is markedly expressed as a consequence of reactive astrocytosis and/or microglial cell activation that are produced by a degenerative insult (Benito et al., 2003). Other data have related CB<sub>2</sub> receptor to events involved in the progression or arrest of neurodegeneration, for instance, by influencing microglial cell migration at neuroinflammatory lesion sites (Walter et al., 2003). Therefore, further studies will have to explore whether other types of cannabinoids might provide neuroprotection by mechanisms distinct of those initially offered by  $\Delta^9$ -THC or CBD, and, in particular to examine the role of the CB<sub>2</sub> receptor subtype. The data obtained in the second group of experiments of this study support this possibility. These experiments were aimed at exploring whether the protective effects of cannabinoids against the *in vivo* toxicity of 6-hydroxydopamine might be also observed *in vitro* and exerted by regulating glial trophic support to neurons (i.e., by increasing pro-survival factors, and/or by reducing cytotoxic ones). Our results strongly support both hypotheses. First, HU-210 was able to reduce 6-hydroxydopamine induced cell death when added directly to cultured cerebellar neurons although these effects were small. We have recently described the same neuroprotective effect exerted by HU-210 in cultured cortical neurons subjected to excitotoxic stimulus and found that this effect is mediated by phosphatidylinositol 3-kinase/Akt signaling pathway (Molina-Holgado et al., *in press*). The interest of this last observation is that this signaling pathway has been strongly implicated in survival signaling in many cell types including neurons and glial cells (Brunet et al., 2001). Second, we have also found that glial cells are important in mediating part of the neuroprotective effects of cannabinoids against the *in vitro* toxicity of 6-hydroxydopamine. This can be concluded from the fact that conditioned media, generated by exposure of mixed glial cells to HU-210, produced a greater reduction of the rate of neuronal cell death induced by 6-

hydroxydopamine when they were added to neuronal cultures than in the case of direct exposure of these neuronal cultures to HU-210. In addition, in this last case, the effect of HU-210 was not dose-dependent thus indicating possible overlapping of different mechanisms activated by this cannabinoid. By contrast, there was a clear dose-dependent response when the cannabinoid was administered to mixed glial cell cultures, possibly indicating that it could be receptor-mediated, either CB<sub>1</sub> or CB<sub>2</sub> because of the lack of selectivity of HU-210 and because of the presence of both cannabinoid receptor subtypes in glial cells. It is well known that conditioned media generated by cultured glial cells are *per se* able to protect neurons from spontaneous and toxin-induced cell death (De Bernardo et al., 2003). This is likely related to the presence of pro-survival mediators (i.e., anti-inflammatory molecules) or the lack of death-induced factors (i.e., nitric oxide, TNF $\alpha$ , pro-inflammatory cytokines). It is possible that, in our study, the activation of CB<sub>1</sub> and/or CB<sub>2</sub> receptors by HU-210 in mixed glial cell cultures dose-dependently increased the presence of these pro-survival mediators and/or reduced that of death-induced factors, thus producing a greater neuronal survival. In support of this idea, it has been reported that cannabinoids inhibit the production of nitric oxide and pro-inflammatory cytokines (for a review, see Guzmán et al., 2001; Smith et al., 2000; Waksman et al., 1999). For instance, we have recently demonstrated that interleukin-1 receptor antagonist, an important anti-inflammatory cytokine that protects against experimentally-induced ischemic, excitotoxic, and traumatic brain insults, is produced in response to cannabinoid receptor activation in primary cultured glial cells (Molina-Holgado et al., 2003). Interestingly, cannabinoid receptor activation failed to do this in knockout mice for this anti-inflammatory cytokine (Molina-Holgado et al., 2003). In the same line of reasoning, we have also observed that 6-hydroxydopamine is also able to produce neuronal death through glial cell-mediated effects since neuronal cultures incubated with conditioned media obtained after adding this toxin to cultured mixed glial cells, showed similar rates of cell death than when the toxin was directly added to neuronal cultures. It is possible that interleukin-1 $\beta$  might be one of these critical factors since the above neurotoxic effects of 6-hydroxydopamine were significantly reduced when cultures were obtained from interleukin-1 $\beta$ -deficient mice (unpublished results). On the other hand, some studies reported that cannabinoids are also protective in glial cells and that this effect is mediated by activation of phosphatidylinositol 3-kinase/Akt signaling pathway (Gomez del Pulgar et al., 2002). As mentioned above, we have recently demonstrated that this mechanism, which has been strongly related to survival signaling (Brunet et al., 2001), is also mediating the protective effects of cannabinoids in neurons (Molina-Holgado et al., *in press*). It is possible that the greater neuroprotective effects observed for HU-210 when used to generate conditioned media than when added directly to neuronal cultures may be indicative of a more efficient activation of that signaling pathway by cannabinoids in glial cells than in neurons.

In summary, our results are compatible with a potential neuroprotective action of  $\Delta^9$ -THC against the progressive degeneration of nigrostriatal dopaminergic neurons occurring in PD, a neurodegenerative disorder with a useful symptomatic therapy but, as other neurodegenerative diseases, lacking an efficient neuroprotectant therapy. However, the fact that the same neuroprotective effects were elicited by CBD, a plant-derived cannabinoid with negligible affinity for the cannabinoid receptors, suggests a major involvement of CB<sub>1</sub> receptor-independent mechanisms, possibly

based on the antioxidative properties of both compounds and/or the effects associated with their well known anti-inflammatory activity, such as lowering the production of TNF $\alpha$ , nitric oxide, and other biologically active molecules. It is important to remark that the fact that CBD was equivalent to  $\Delta^9$ -THC in reducing dopaminergic injury in PD supports the assumption that CBD would be more advantageous for a potential neuroprotectant therapy in this disease, since it can be used at higher doses and for longer times than those possible with  $\Delta^9$ -THC, due to its lack of psychoactivity. An additional advantage for CBD is that its use in prolonged treatments does not induce tolerance (Malfait et al., 2000), a phenomenon often observed with  $\Delta^9$ -THC (Adams and Martin, 1996). In addition, the evidence provided by in vitro studies also indicates the occurrence of additional mechanisms of neuroprotection by cannabinoids that would include a modulation of glial function that would be effective in reducing inflammatory responses that usually accompany neurodegenerative insults.

### Acknowledgments

This work has been supported by grants from "Red CIEN" (C03/06), CAM-PRI (08.5/0063/2001), and MCYT (SAF2003-08269) to I.L.B., J.A.R., and J.F.R., and the Israel Science Foundation to R.M.  $\Delta^9$ -THC was kindly provided by GW Pharmaceuticals Ltd (Salisbury, UK).

### References

- Adams, I.B., Martin, B.R., 1996. Cannabis: pharmacology and toxicology in animals and humans. *Addiction* 91, 1585–1614.
- Alexi, T., Borlongan, C.V., Faull, R.L., Williams, C.E., Clark, R.G., Gluckman, P.D., Hughes, P.E., 2000. Neuroprotective strategies for basal ganglia degeneration: Parkinson's and Huntington's disease. *Prog. Neurobiol.* 60, 409–470.
- Benito, C., Nuñez, E., Tolon, R.M., Carrier, E.J., Rabano, A., Hillard, C.J., Romero, J., 2003. Cannabinoid CB2 receptors and fatty acid amide hydrolase are selectively overexpressed in neuritic plaque-associated glia in Alzheimer's disease brains. *J. Neurosci.* 23, 11136–11141.
- Bisogno, T., Hanus, L., De Petrocellis, L., Tchilibon, S., Ponde, D.E., Brandi, I., Moriello, A.S., Davis, J.B., Mechoulam, R., Di Marzo, V., 2001. Molecular targets for cannabidiol and its synthetic analogues: effects on vanilloid VRI receptors and on the cellular uptake and enzymatic hydrolysis of anandamide. *Br. J. Pharmacol.* 134, 845–852.
- Blum, D., Torch, S., Lamberg, N., Nisson, M.-F., Benabid, A.-L., Sadou, L.R., Verna, J.-M., 2001. Molecular pathways involved in the neurotoxicity of 6-OHDA, dopamine and MPTP: contribution to the apoptotic theory in Parkinson's disease. *Prog. Neurobiol.* 65, 135–172.
- Brotchie, J.M., 1998. Adjuncts to dopamine replacement: a pragmatic approach to reducing the problem of dyskinesia in Parkinson's disease. *Mov. Disord.* 13, 871–876.
- Brotchie, J.M., 2000. The neural mechanisms underlying levodopa-induced dyskinesia in Parkinson's disease. *Ann. Neurol.* 47, S105–S114.
- Brunet, A., Datta, S.R., Greenberg, M.E., 2001. Transcription-dependent and -independent control of neuronal survival by the PI3K-Akt signaling pathway. *Curr. Opin. Neurobiol.* 11, 297–305.
- Cambray-Deakin, M.A., 1995. Cerebellar granule cells. In: Cohen, J., Wilkin, G. (Eds.), *Neural Cell Culture: A Practical Approach*. IRL Press, Oxford, UK.
- Carlsson, A., 2002. Treatment of Parkinson's with L-DOPA. The early discovery phase, and a comment on current problems. *J. Neural Transm.* 109, 777–787.
- Carmichael, J., De Graff, W.G., Gazdar, A.F., Minna, J.D., Mitchell, J.B., 1987. Evaluation of the tetrazolium based semiautomated colorimetric assay: assessment of chemosensitivity testing. *Cancer Res.* 47, 936–942.
- Chao, C.C., Hu, S., Peterson, P.K., 1996. Glia: the not so innocent bystanders. *J. NeuroViro.* 2, 234–239.
- Chen, Y., Buck, J., 2000. Cannabinoids protect cells from oxidative cell death: a receptor-independent mechanism. *J. Pharmacol. Exp. Ther.* 293, 807–812.
- Daily, D., Barzilai, A., Offen, D., Kamsler, A., Melamed, E., Ziv, I., 1999. The involvement of p53 in dopamine-induced apoptosis of cerebellar granule neurons and leukemic cells overexpressing p53. *Cell. Mol. Neurobiol.* 19, 261–276.
- De Bernardo, S., Canals, S., Casarejos, M.J., Rodríguez-Martin, E., Mena, M.A., 2003. Glia-conditioned medium induces de novo synthesis of tyrosine hydroxylase and increases dopamine cell survival by differential signaling pathways. *J. Neurosci. Res.* 73, 818–830.
- Di Marzo, V., Hill, M.P., Bisogno, T., Crossman, A.R., Brotchie, J.M., 2000. Enhanced levels of endocannabinoids in the globus pallidus are associated with a reduction of movement in an animal model of Parkinson's disease. *FASEB J.* 14, 1432–1438.
- Dodel, R.C., Du, Y., Bales, K.R., Ling, Z., Carvey, P.M., Paul, S.M., 1999. Caspase-3-like proteases and 6-hydroxydopamine induced neuronal cell death. *Mol. Brain Res.* 64, 141–148.
- Ferrer, B., Asbrock, N., Kathuria, S., Piomelli, D., Giuffrida, A., 2003. Effects of levodopa on endocannabinoid levels in rat basal ganglia: implications for the treatment of levodopa-induced dyskinesias. *Eur. J. Neurosci.* 18, 1114–1607.
- Fox, S.H., Henry, B., Hill, M.P., Crossman, A.R., Brotchie, J.M., 2002. Stimulation of cannabinoid receptors reduces levodopa-induced dyskinesia in the MPTP-lesioned nonhuman primate model of Parkinson's disease. *Mov. Disord.* 17, 1180–1187.
- Galea, E., Feinstein, D.L., Reis, D.J., 1992. Induction of calcium-independent nitric oxide synthase activity in primary rat glial cultures. *Proc. Natl. Acad. Sci. U. S. A.* 89, 10945–10949.
- Gao, H.M., Jiang, J., Wilson, B., Zhang, W., Hong, J.S., Liu, B., 2002. Microglial activation-mediated delayed and progressive degeneration of rat nigral dopaminergic neurons: relevance to Parkinson's disease. *J. Neurochem.* 81, 1285–1297.
- Gaoni, Y., Mechoulam, R., 1971. The isolation and structure of  $\Delta^1$ -tetrahydrocannabinol and other neutral cannabinoids from hashish. *J. Am. Chem. Soc.* 93, 217–224.
- García-Gil, L., Ramos, J.A., Rubino, T., Parolaro, D., Fernández-Ruiz, J.J., 1998. Perinatal  $\Delta^9$ -tetrahydrocannabinol exposure did not alter dopamine transporter and tyrosine hydroxylase mRNA levels in midbrain dopaminergic neurons of adult male and female rats. *Neurotoxicol. Teratol.* 20, 549–553.
- Gerfen, C.R., 1992. The neostriatal mosaic: multiple levels of compartmental organization. *Trends Neurosci.* 15, 133–139.
- Gilgun-Sherki, Y., Melamed, E., Mechoulam, R., Offen, D., 2003. The CB1 cannabinoid receptor agonist, HU-210, reduces levodopa-induced rotations in 6-hydroxydopamine-lesioned rats. *Pharmacol. Toxicol.* 93, 66–70.
- Gomez Del Pulgar, T., De Ceballos, M.L., Guzman, M., Velasco, G., 2002. Cannabinoids protect astrocytes from ceramide-induced apoptosis through the phosphatidylinositol 3-kinase/protein kinase B pathway. *J. Biol. Chem.* 277, 36527–36533.
- González, S., Romero, J., de Miguel, R., Lastres-Becker, I., Villanua, M.A., Makriyannis, A., Ramos, J.A., Fernández-Ruiz, J.J., 1999. Extrapyramidal and neuroendocrine effects of AM404, an inhibitor of the carrier-mediated transport of anandamide. *Life Sci.* 65, 327–336.
- Grundy, R.I., 2002. The therapeutic potential of the cannabinoids in neuroprotection. *Expert Opin. Invest. Drugs* 11, 1–10.
- Grundy, R.I., Rabuffetti, M., Beltramo, M., 2001. Cannabinoids and neuroprotection. *Mol. Neurobiol.* 24, 29–52.
- Gubellini, P., Picconi, B., Bari, M., Battista, N., Calabresi, P., Centonze, D., Bernardi, G., Finazzi-Agrò, A., Maccarrone, M., 2002. Experimental

- parkinsonism alters endocannabinoid degradation: implications for striatal glutamatergic transmission. *J. Neurosci.* 22, 6900–6907.
- Guzmán, M., Sánchez, C., Galve-Roperh, I., 2001. Control of the cell survival/death decision by cannabinoids. *J. Mol. Med.* 78, 613–625.
- Hampson, A.J., Grimaldi, M., Axelrod, J., Wink, D., 1998. Cannabidiol and (-)  $\Delta^9$ -tetrahydrocannabinol are neuroprotective antioxidants. *Proc. Natl. Acad. Sci. U. S. A.* 95, 8268–8273.
- Hampson, A.J., Grimaldi, M., Lolic, M., Wink, D., Rosenthal, R., Axelrod, J., 2000. Neuroprotective antioxidants from marijuana. *Ann. N. Y. Acad. Sci.* 899, 274–282.
- Herkenham, M., Lynn, A.B., Little, M.D., Melvin, L.S., Johnson, M.R., de Costa, D.R., Rice, K.C., 1991. Characterization and localization of cannabinoid receptors in rat brain: a quantitative in vitro autoradiographic study. *J. Neurosci.* 11, 563–583.
- Hernández, M.L., Berrendero, F., Suarez, I., Garcia-Gil, L., Cebeira, M., Maekie, K., Ramos, J.A., Fernández-Ruiz, J.J., 2000. Cannabinoid CB1 receptors colocalize with tyrosine hydroxylase in cultured fetal mesencephalic neurons and their activation increases the levels of this enzyme. *Brain Res.* 857, 56–65.
- Hirsch, E.C., 2000. Glial cells and Parkinson's disease. *J. Neurol.* 247, 1158–1162.
- Hirsch, E.C., Hunot, S., Damier, P., Faucheux, B., 1998. Glial cells and inflammation in Parkinson's disease: a role in neurodegeneration? In: Olanow, W.C., Jenner, P. (Eds.), *Beyond the Decade of the Brain, Neuroprotection in Parkinson's Disease*, vol. 3. Wells Medical Ltd., Royal Tunbridge Wells, UK, pp. 227–237.
- Hurley, M.J., Mash, D.C., Jenner, P., 2003. Expression of cannabinoid CB1 receptor mRNA in basal ganglia of normal and parkinsonian human brain. *J. Neural Transm.* 110, 1279–1288.
- Jaggar, S.I., Hasnie, F.S., Sellaturay, S., Rice, A.S., 1998. The anti-hyperalgesic actions of the cannabinoid anandamide and the putative CB2 receptor agonist palmitoylethanolamide in visceral and somatic inflammatory pain. *Pain* 76, 189–199.
- Kim, W.G., Mohny, R.P., Wilson, B., Jeohn, G.H., Liu, B., Hong, J.S., 2000. Regional difference in susceptibility to lipopolysaccharide-induced neurotoxicity in the rat brain: role of microglia. *J. Neurosci.* 20, 6309–6316.
- Kumar, R., Agarwal, A.K., Seth, P.K., 1995. Free radical-generated neurotoxicity of 6-hydroxydopamine. *J. Neurochem.* 64, 1703–1707.
- Lastres-Becker, I., Cebeira, M., de Ceballos, M.L., Zeng, B.Y., Jenner, P., Ramos, J.A., Fernández-Ruiz, J.J., 2001. Increased cannabinoid CB1 receptor binding and activation of GTP-binding proteins in the basal ganglia of patients with Parkinson's syndrome and of MPTP-treated marmosets. *Eur. J. Neurosci.* 14, 1827–1832.
- Lastres-Becker, I., Hansen, H.H., Berrendero, F., De Miguel, R., Perez-Rosado, A., Manzanares, J., Ramos, J.A., Fernández-Ruiz, J.J., 2002. Alleviation of motor hyperactivity and neurochemical deficits by endocannabinoid uptake inhibition in a rat model of Huntington's disease. *Synapse* 44, 23–35.
- Lastres-Becker, I., Bizat, N., Boyer, F., Hantraye, P., Fernández-Ruiz, J.J., Brouillet, E., 2004. Potential involvement of cannabinoid receptors in 3-nitropropionic acid toxicity in vivo. *NeuroReport* 15, 2375–2379.
- López-Rodríguez, M.L., Viso, A., Ortega-Gutiérrez, S., Fowler, C.J., Tiger, G., de Lago, E., Fernández-Ruiz, J., Ramos, J.A., 2003. Design, synthesis, and biological evaluation of new inhibitors of the endocannabinoid uptake: comparison with effects on fatty acid amidohydrolase. *J. Med. Chem.* 46, 1512–1522.
- Lotharius, J., Dugan, L.L., O'Malley, K.L., 1999. Distinct mechanisms underlie neurotoxin-mediated cell death in cultured dopaminergic neurons. *J. Neurosci.* 19, 1284–1293.
- Mailleux, P., Vanderhaeghen, J.J., 1993. Dopaminergic regulation of cannabinoid receptor mRNA levels in the rat caudate-putamen: an in situ hybridization study. *J. Neurochem.* 61, 1705–1712.
- Malfait, A.M., Gallily, R., Sumariwalla, P.F., Malik, A.S., Andreaskos, E., Mechoulam, R., Feldmann, M., 2000. The nonpsychoactive cannabis constituent cannabidiol is an oral anti-arthritis therapeutic in murine collagen-induced arthritis. *Proc. Natl. Acad. Sci.* 97, 9561–9566.
- Maneuf, Y.P., Crossman, A.R., Brochie, J.M., 1997. The cannabinoid receptor agonist WIN55,212-2 reduces D2, but not D1, dopamine receptor-mediated alleviation of akinesia in the reserpine-treated rat model of Parkinson's disease. *Exp. Neurol.* 148, 265–270.
- Marsicano, G., Moosmann, B., Hermann, H., Lutz, B., Behl, C., 2002. Neuroprotective properties of cannabinoids against oxidative stress: role of the cannabinoid receptor CB1. *J. Neurochem.* 80, 448–456.
- McCarthy, K.D., de Vellis, J., 1980. Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue. *J. Cell Biol.* 85, 890–902.
- McGeer, P.L., Yasojima, K., McGeer, E.G., 2001. Inflammation in Parkinson's disease. *Adv. Neurol.* 86, 83–89.
- Mechoulam, R., Lauder, N., Breuer, A., Zahalka, J., 1990. Synthesis of the individual, pharmacologically distinct, enantiomers of a tetrahydrocannabinol derivative. *Tetrahedron: Asymmetry* 1, 315–319.
- Mechoulam, R., Panikashvili, A., Shohami, E., 2002a. Cannabinoids and brain injury: therapeutic implications. *Trends Mol. Med.* 8, 58–61.
- Mechoulam, R., Spatz, M., Shohami, E., 2002b. Endocannabinoids and neuroprotection. *Sci. STKE* (doi:10.1126/stke.1292002re5).
- Meschler, J.P., Howlett, A.C., Madras, B.K., 2001. Cannabinoid receptor agonist and antagonist effects on motor function in normal and 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP)-treated non-human primates. *Psychopharmacology* 156, 79–85.
- Mezey, E., Toth, Z.E., Cotright, D.N., Arzubi, M.K., Krause, J.E., Elde, R., Guo, A., Bhumberg, P.M., Szallasi, A., 2000. Distribution of mRNA for vanilloid receptor subtype 1 (VR1), and VR1-like immunoreactivity, in the central nervous system of the rat and human. *Proc. Natl. Acad. Sci. U. S. A.* 97, 3655–3660.
- Minghetti, L., Levi, G., 1998. Microglia as effector cells in brain damage and repair: focus on prostanoids and nitric oxide. *Prog. Neurobiol.* 54, 99–125.
- Mogi, M., Harada, M., Riederer, P., Narabayashi, H., Fujita, K., Nagatsu, T., 1994. Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) increases both in the brain and in the cerebrospinal fluid from parkinsonian patients. *Neurosci. Lett.* 165, 208–210.
- Molina-Holgado, F., Lledo, A., Guaza, C., 1995. Evidence for cyclooxygenase activation by nitric oxide in astrocytes. *Glia* 15, 167–172.
- Molina-Holgado, F., Pinteaux, E., Moore, J.D., Molina-Holgado, E., Guaza, C., Gibson, R.M., Rothwell, N.J., 2003. Endogenous interleukin-1 receptor antagonist mediates anti-inflammatory and neuroprotective actions of cannabinoids in neurons and glia. *J. Neurosci.* 23, 6470–6474.
- Molina-Holgado, F., Pinteaux, E., Hecnan, L., Moore, J.D., Rothwell, N.J., Gibson, R.M., 2004. Neuroprotective effects of the synthetic cannabinoid HU-210 in cortical neurons are mediated by phosphatidylinositol/Akt signaling. *Mol. Cell. Neurosci.* (in press).
- Moosmann, B., Behl, C., 2002. Antioxidants as treatment for neurodegenerative disorders. *Expert Opin. Invest. Drugs* 11, 1407–1435.
- Nagatsu, T., Oka, K., Kato, T., 1979. Highly sensitive assay for tyrosine-hydroxylase activity by high-performance liquid chromatography. *J. Chromatogr.* 163, 247–252.
- Nagatsu, T., Mogi, M., Ichinose, H., Togari, A., 2000. Changes in cytokines and neurotrophins in Parkinson's disease. *J. Neural Transm., Suppl.* 60, 277–290.
- Nagayama, T., Sinor, A.D., Simon, R.P., Chen, J., Graham, S.H., Jin, K.L., Greenberg, D.A., 1999. Cannabinoids and neuroprotection in global and focal cerebral ischemia and in neuronal cultures. *J. Neurosci.* 19, 2987–2995.
- Nawa, H., Kotani, H., Nakanishi, S., 1984. Tissue-specific generation of two preprotachykinin mRNAs from one gene by alternative RNA splicing. *Nature* 312, 729–734.
- Offen, D., Sherki, Y., Melamed, E., Fridkin, M., Breneman, D.E., Gozes, L., 2000. Vasoactive intestinal peptide (VIP) prevents neurotoxicity in neuronal cultures: relevance to neuroprotection in Parkinson's disease. *Brain Res.* 854, 257–262.
- Palkovits, M., Brownstein, J., 1988. *Maps and Guide to Microdissection of the Rat Brain*. Elsevier.
- Panikashvili, D., Simeonidou, C., Ben-Shabat, S., Hanus, L., Breuer, A.,

- Mechoulam, R., Shohami, E., 2001. An endogenous cannabinoid (2-AG) is neuroprotective after brain injury. *Nature* 413, 527–531.
- Parmentier-Batteur, S., Jin, K., Mao, X.O., Xie, L., Greenberg, D.A., 2002. Increased severity of stroke in CB1 cannabinoid receptor knock-out mice. *J. Neurosci.* 22, 9771–9775.
- Paxinos, G., Watson, C., 1986. *The Rat Brain in Stereotaxic Coordinates*. Academic Press, London.
- Pertwee, R.G., 1997. Pharmacology of cannabinoid CB<sub>1</sub> and CB<sub>2</sub> receptors. *Pharmacol. Ther.* 74, 129–180.
- Raman, C., McAllister, S.D., Rizvi, G., Patel, S.G., Moore, D.H., Abood, M.E., 2004. Amyotrophic lateral sclerosis: delayed disease progression in mice by treatment with a cannabinoid. *Amyotrophic Lateral Scler., Other Mot. Neuron Disord.* 5, 33–39.
- Richardson, J.D., Kilo, S., Hargreaves, K.M., 1998. Cannabinoids reduce hyperalgesia and inflammation via interaction with peripheral CB1 receptors. *Pain* 75, 111–119.
- Rodnitsky, R.L., 1999. Can calcium antagonists provide a neuroprotective effect in Parkinson's disease? *Drugs* 57, 845–849.
- Romero, J., de Miguel, R., Garcia-Palomero, E., Fernández-Ruiz, J.J., Ramos, J.A., 1995. Time-course of the effects of anandamide, the putative endogenous cannabinoid receptor ligand, on extrapyramidal function. *Brain Res.* 694, 223–232.
- Romero, J., Berrendero, F., Pérez-Rosado, A., Manzanera, J., Rojo, A., Fernández-Ruiz, J.J., de Yébenes, J.G., Ramos, J.A., 2000. Unilateral 6-hydroxydopamine lesions of nigrostriatal dopaminergic neurons increased CB1 receptor mRNA levels in the caudate-putamen. *Life Sci.* 66, 485–494.
- Romero, J., Lastres-Becker, I., de Miguel, R., Berrendero, F., Ramos, J., Fernández-Ruiz, J., 2002. The endogenous cannabinoid system and the basal ganglia. Biochemical, pharmacological, and therapeutic aspects. *Pharmacol. Ther.* 95, 137–152.
- Rubino, T., Massi, P., Patrini, G., Venier, I., Giagnoni, G., Parolaro, D., 1994. Chronic CP-55,940 alters cannabinoid receptor mRNA in the rat brain: an in situ hybridization study. *NeuroReport* 5, 2493–2496.
- Sañudo-Peña, M.C., Patrick, S.L., Khen, S., Patrick, R.L., Tsou, K., Walker, J.M., 1998. Cannabinoid effects in basal ganglia in a rat model of Parkinson's disease. *Neurosci. Lett.* 248, 171–174.
- Sethi, K.D., 2002. Clinical aspects of Parkinson disease. *Curr. Opin. Neurol.* 15, 457–460.
- Shen, M., Thayer, S.A., 1998. Cannabinoid receptor agonists protect cultured rat hippocampal neurons from excitotoxicity. *Mol. Pharmacol.* 54, 459–462.
- Sherer, T.B., Betarbet, R., Greenamyre, J.T., 2001. Pathogenesis of Parkinson's disease. *Curr. Opin. Invest. Drugs* 2, 657–662.
- Sieradzian, K.A., Fox, S.H., Hill, M., Dick, J.P., Crossman, A.R., Brotchie, J.M., 2001. Cannabinoids reduce levodopa-induced dyskinesia in Parkinson's disease: a pilot study. *Neurology* 57, 2108–2111.
- Silverdale, M.A., McGuire, S., McInnes, A., Crossman, A.R., Brotchie, J.M., 2001. Striatal cannabinoid CB1 receptor mRNA expression is decreased in the reserpine-treated rat model of Parkinson's disease. *Exp. Neurol.* 169, 400–406.
- Simmons, M.L., Murphy, S., 1992. Induction of nitric oxide synthase in glial cells. *J. Neurochem.* 59, 897–905.
- Sinor, A.D., Irvin, S.M., Greenberg, D.A., 2000. Endocannabinoids protect cerebral cortical neurons from in vitro ischemia in rats. *Neurosci. Lett.* 278, 157–160.
- Smith, S.R., Terminelli, C., Denhardt, G., 2000. Effects of cannabinoid receptor agonist and antagonist ligands on production of inflammatory cytokines and anti-inflammatory interleukin-10 in endotoxemic mice. *J. Pharmacol. Exp. Ther.* 293, 136–150.
- Tintner, R., Jankovic, J., 2002. Treatment options for Parkinson's disease. *Curr. Opin. Neurol.* 15, 467–476.
- Vajda, F.J., 2002. Neuroprotection and neurodegenerative disease. *J. Clin. Neurosci.* 9, 4–8.
- van der Stelt, M., Veldhuis, W.B., Bar, P.R., Veldink, G.A., Vliegthart, J.F., Nicolay, K., 2001a. Neuroprotection by  $\Delta^9$ -tetrahydrocannabinol, the main active compound in marijuana, against ouabain-induced in vivo excitotoxicity. *J. Neurosci.* 21, 6475–6579.
- van der Stelt, M., Veldhuis, W.B., van Haften, G.W., Fezza, F., Bisogno, T., Bär, P.R., Veldink, G.A., Vliegthart, J.F., Di Marzo, V., Nicolay, K., 2001b. Exogenous anandamide protects rat brain against acute neuronal injury in vivo. *J. Neurosci.* 21, 8765–8771.
- Waksman, Y., Olson, J.M., Carlisle, S.J., Cabral, G.A., 1999. The central cannabinoid receptor (CB1) mediates inhibition of nitric oxide production by rat microglial cells. *J. Pharmacol. Exp. Ther.* 288, 1357–1366.
- Walter, L., Stella, N., 2004. Cannabinoids and neuroinflammation. *Br. J. Pharmacol.* 141, 775–785.
- Walter, L., Franklin, A., Witting, A., Wade, C., Xie, Y., Kunos, G., Mackie, K., Stella, N., 2003. Nonpsychotropic cannabinoid receptors regulate microglial cell migration. *J. Neurosci.* 23, 1398–1405.
- Young, W.S., Bonner, T.I., Brann, M.R., 1986. Mesencephalic dopamine neurons regulate the expression of neuropeptide mRNAs in the rat forebrain. *Proc. Natl. Acad. Sci. U. S. A.* 83, 9827–9831.
- Zalish, M., Lavie, V., 2003. Dexanabinol (HU-211) has a beneficial effect on axonal sprouting and survival after rat optic nerve crush injury. *Vision Res.* 43, 237–242.
- Zeng, B.-Y., Dass, B., Owen, A., Rose, S., Cannizzaro, C., Tel, B.C., Jenner, P., 1999. Chronic L-DOPA treatment increases striatal cannabinoid CB1 receptor mRNA expression in 6-hydroxydopamine-lesioned rats. *Neurosci. Lett.* 276, 71–74.

---

# EXHIBIT THREE

# D<sup>9</sup>-tetrahydrocannabinol (D<sup>9</sup>-THC) exerts a direct neuroprotective effect in a human cell culture model of Parkinson's disease

C. B. Carroll, M.-L. Zeissler, C. O. Hanemann and J. P. Zajicek

Department of Clinical Neurobiology, Peninsula College of Medicine and Dentistry, University of Plymouth, Plymouth, UK

C. B. Carroll, M.-L. Zeissler, C. O. Hanemann and J. P. Zajicek (2012) *Neuropathology and Applied Neurobiology* 38, 535–547

## D<sup>9</sup>-tetrahydrocannabinol (D<sup>9</sup>-THC) exerts a direct neuroprotective effect in a human cell culture model of Parkinson's disease

**Aims:** D<sup>9</sup>-tetrahydrocannabinol (D<sup>9</sup>-THC) is neuroprotective in models of Parkinson's disease (PD). Although CB1 receptors are increased within the basal ganglia of PD patients and animal models, current evidence suggests a role for CB1 receptor-independent mechanisms. Here, we utilized a human neuronal cell culture PD model to further investigate the protective properties of D<sup>9</sup>-THC. **Methods:** Differentiated SH-SY5Y neuroblastoma cells were exposed to PD-relevant toxins: 1-methyl-4-phenylpyridinium (MPP<sup>+</sup>), lactacystin and paraquat. Changes in CB1 receptor level were determined by quantitative polymerase chain reaction and Western blotting. Cannabinoids and modulatory compounds were co-administered with toxins for 48 h and the effects on cell death, viability, apoptosis and oxidative stress assessed. **Results:** We found CB1 receptor up-regulation in response to MPP<sup>+</sup>, lactacystin and paraquat and a protective effect of D<sup>9</sup>-THC against all three toxins. This neuroprotective

effect was not reproduced by the CB1 receptor agonist WIN55,212-2 or blocked by the CB1 antagonist AM251. Furthermore, the antioxidants  $\alpha$ -tocopherol and butylhydroxytoluene as well as the antioxidant cannabinoids, nabilone and cannabidiol were unable to elicit the same neuroprotection as D<sup>9</sup>-THC. However, the peroxisome proliferator-activated receptor- $\gamma$  (PPAR $\gamma$ ) antagonist T0070907 dose-dependently blocked the neuroprotective, antioxidant and anti-apoptotic effects of D<sup>9</sup>-THC, while the PPAR $\gamma$  agonist pioglitazone resulted in protection from MPP<sup>+</sup>-induced neurotoxicity. Furthermore, D<sup>9</sup>-THC increased PPAR $\gamma$  expression in MPP<sup>+</sup>-treated SH-SY5Y cells, another indicator of PPAR $\gamma$  activation. **Conclusions:** We have demonstrated up-regulation of the CB1 receptor in direct response to neuronal injury in a human PD cell culture model, and a direct neuronal protective effect of D<sup>9</sup>-THC that may be mediated through PPAR $\gamma$  activation.

**Keywords:** D<sup>9</sup>-THC, cannabinoid, neuroprotection, Parkinson, PPAR $\gamma$ , SH-SY5Y

## Introduction

Cannabinoids are a group of compounds present in cannabis (*Cannabis sativa*) and include D<sup>9</sup>-

Science Park, Research Way, Plymouth PL6 8BU, UK. Tel: +44 1752 437420; Fax: +44 1752 517846; E-mail: camille.carroll@pms.ac.uk

© 2011 The Authors

Correspondence: Camille Carroll, Department of Clinical Neurobiology, Peninsula Medical School, John Bull Building, Tamar

Neuropathology and Applied Neurobiology © 2011 British Neuropathological Society

tetrahydrocannabinol (D<sup>9</sup>-THC) and cannabidiol [1,2]. There are two main cannabinoid receptor subtypes: CB1 (which is found primarily in the brain, particularly in the basal ganglia and in the limbic system) [3] and CB2 (which is primarily localized to cells of the immune system) [4]. Cannabinoid receptors are G protein-coupled receptors which inhibit adenylate cyclase [5].

Parkinson's disease (PD) is a neurodegenerative condition characterized by loss of dopaminergic neurones in

the substantia nigra pars compacta with resulting neurochemical imbalance throughout the basal ganglia [6]. Cannabinoids modulate neurotransmitter release within the basal ganglia [7,8] and have been demonstrated to result in symptomatic benefit in animal models [9], most likely mediated by the CB1 receptor. An increase in CB1 receptor level and efficacy of activation has been demonstrated in the striatum of PD patients and MPTP (1-methyl-4-phenylpyridinium iodide)-treated marmosets [10], most likely reflecting loss of dopaminergic inhibitory influences [7,10–13] and neurochemical compensatory mechanisms within the basal ganglia. Although these changes in humans may have been due to dopaminergic therapy, an *in vivo* positron emission tomography study of CB1 receptor binding in PD patients demonstrated increased binding in the putamen even in untreated patients [13]. It is, however, not known to what extent the up-regulation of the CB1 receptor seen in PD may reflect a direct response to neuronal damage.

There is increasing evidence that, in addition to symptomatic effects, cannabinoids may have neuroprotective properties which could be exploited for the treatment of neurodegenerative conditions including PD [14–16]. Although these protective effects may be receptor-independent [9,17], studies in a range of *in vivo* and *in vitro* excitotoxicity models have suggested that the neuro-protective effect of cannabinoids may be mediated via the CB1 receptor [18–24]. A protective effect mediated via the CB1 receptor and increased sensitivity to insult in CB1 receptor-deficient mice have been demonstrated in models of multiple sclerosis and closed head injury [18,19]. A similar CB1 receptor-mediated protective effect has also been demonstrated in rat models of cerebral ischaemia [22]. One mechanism by which this protective effect could be exerted is by the presynaptic inhibition of glutamate release and suppression of excitotoxic mechanisms [20,23,25]. However, a study of excitotoxicity in mouse spinal cord suggested that CB1 receptor agonists may have an effect by acting directly on postsynaptic neuronal CB1 receptors [21]. Importantly, the spinal cord cultures used in these experiments also contained glial cells, and later studies have provided evidence of CB1-mediated modulation of glial activity as being neuroprotective [15,26]. Nevertheless, a direct neuronal CB1-mediated protective effect has been demonstrated in a model of diabetic neuropathy [27].

A neuroprotective effect of cannabinoids has been demonstrated in both *in vivo* and *in vitro* PD models, which has

been attributed to their antioxidant properties [16], CB2 receptor activation [15,28] or glial CB1 receptor activation [26]. Additionally, a key role for the CB1 receptor has been demonstrated in a mouse model of PD [29]. CB1-mediated neuroprotective effects may therefore be direct neuronal effects, the result of modulation of neurotransmitter release or modulation of microglial activation.

We utilized a human cell culture model of PD that enabled us to determine direct neuronal effects, namely SH-SY5Y neuroblastoma cells, differentiated with retinoic acid and exposed to toxins that mimic the various abnormalities that have been implicated in the pathogenesis of PD: inhibition of mitochondrial function [1-methyl-4-phenylpyridinium (MPP<sup>+</sup>) iodide] [30,31], free radical generation (paraquat) [32–34] and inhibition of the ubiquitin proteasome system (UPS) (lactacystin) [35]. The use of differentiated SH-SY5Y cells is well established as a cell culture model of PD [36]. In this paper, we investigated whether up-regulation of the CB1 receptor can be induced as a direct response to neuronal injury and investigated the neuroprotective mechanisms of D<sup>9</sup>-THC.

Medium was changed every 48 h.

## Materials and methods

### Materials

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich Chemicals (Dorset, UK). WIN55,212-2, AM251 and cannabidiol were purchased from Tocris (Bristol, UK). Nabilone was a kind gift from MEDA Pharmaceuticals UK Ltd (Bishop's Stortford, UK).

### Culture of neuroblastoma cells

Human neuroblastoma cells (SH-SY5Y) were obtained from European Collection of Cell Cultures (ECACC), transferred to 75-cm<sup>2</sup> filter vent flasks (VWR, Leicestershire, UK), grown in Dulbecco's modified Eagle's medium (Invitrogen, Paisley, UK) containing 10% (v/v) foetal bovine serum (FBS) (PAA, Yeovil, UK), glutamine, 4.5 g/l glucose, 1 ml uridine (25 mg/ml), 5 ml pyruvate, 25 units/ml penicillin and 25 mg/ml streptomycin, and incubated at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air. For experiments, cells were seeded in six-well dishes (VWR) (200 000 cells/well) or 96-well plates (VWR) (10 000 cells/well) and treated with 10 mM retinoic acid for 5 days

© 2011 The Authors  
 Journal compilation © 2011 British Neuropathological Society

## Cell treatments

After differentiation, toxins [MPP<sup>+</sup>, lactacystin (Merck, Nottingham, UK) and paraquat] were added to retinoic acid-supplemented cell culture medium (total 100 ml). Concentrations of toxin were chosen that resulted in about two to threefold cell death at 48 h compared with vehicle: 5–7 mM MPP<sup>+</sup>, 20 mM lactacystin and 500 mM paraquat. For quantitative polymerase chain reaction (QPCR) and protein determination experiments, cells were harvested at 6, 12, 24 and 48 h. To investigate their effects, cannabinoids (D<sup>9</sup>-THC, WIN55,212-2, cannabidiol and nabilone) and modulators (AM251, T0070907) were co-administered with toxins in retinoic acid-supplemented medium. To determine the neuroprotective potential of antioxidant compounds, differentiated SH-SY5Y cells were treated with a range of concentrations of the antioxidants butylated hydroxytoluene (BHT) and  $\alpha$ -tocopherol in retinoic acid-supplemented medium in combination with MPP<sup>+</sup>. For pretreatment, BHT was applied for 1 h prior to co-application of MPP<sup>+</sup> and BHT. All plates contained positive (toxin or agent alone) and vehicle controls. Cell death was assessed after 48 h.

## Lactate dehydrogenase (LDH)-release assay

To assess the cytotoxicity of the toxins under our experimental conditions, LDH release of cells grown and treated in 96-well plates was measured. Cell culture medium (50 ml) was used to analyse the LDH activity by measuring the oxidation of nicotinamide adenine dinucleotide hydride (NADH) at 450 nm as described in the manufacturer's protocol (Promega, Southampton, UK). The remaining cells were lysed and LDH activity similarly measured to allow the percentage of cell death relative to the total number of cells to be calculated.

## MTT assay

Cells were treated in 96-well plates as described above. Ten microlitre of 5 mg/ml sterile-filtered 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Invitrogen, Paisley, UK) solution in serum-free medium was added to each well and incubated for 4 h. The medium was removed and 100 ml of dimethyl

sulphoxide (DMSO) per well added to dissolve the formazan precipitate. Plates were incubated on a shaker for 10 min and the

absorbance read at 562 nm using 650 nm as reference wavelength.

significant difference (HSD) *post hoc* test.

### Measurement of reactive oxygen species

SH-SY5Y cells were seeded into 96-well plates and treated for 48 h as described above. The medium was removed and cells were incubated with 10 mM 2',7'- Dichlorodihydrofluorescein diacetate (DCFDA) in serum-free medium for 30 min. Cells were then washed three times with phosphate-buffered saline (PBS) after which the fluorescence was measured at Ex: 485 nm and Em: 535 nm. To determine cell number, cells were lysed at -80°C and cell number estimated using the LDH assay. Experiments were carried out four times in triplicate.

### Total RNA extraction and PCR

Total RNA was extracted using GenElute (Sigma-Aldrich) and treated with DNA-free (Ambion, Huntingdon, UK) according to the manufacturer's instructions and reverse transcribed in a 50-ml reaction using cDNA Archive Kit (Ambion). To determine whether differentiated SH-SY5Y cells expressed cannabinoid receptors, we used the following PCR primers: CB1 - forward 5'-aggggatgcaagggatt-3', reverse 5'-agtggatggtgcggaag-3' giving an amplified fragment of 131 bp; CB2 - forward 5'-tcaaccctgtcatctatgctc-3', reverse 5'-agtcagtccaaca ctatc-3' giving an amplified fragment of 353 bp. Thermocycling was carried out as follows: 95°C for 10 min, 40 cycles of 95°C for 30 s, 54°C for 30 s, 72°C for 1 min, followed by 72°C for 5 min. QPCR was employed to detect changes of CB1 receptor mRNA. [FAM]-labelled CB1 receptor primers were purchased from Applied Biosystems (Warrington, Cheshire, UK) (Hs 01038522). Reactions were carried out on a Bio-Rad ICycler (Bio-Rad, Hemel Hempstead, UK). Each reaction was run in triplicate with 1-ml sample in a total volume of 20 ml. Amplification and detection were performed with the following conditions: an initial hold at 95°C for 10 s followed by 50 cycles at 95°C for 15 s and 60°C for 60 s. Gene expression was normalized to 18S expression run in triplicate concurrently. All samples were analysed in triplicate on three samples from four separate experiments. Statistical significance was determined using one-way analysis of variance (anova) in SPSS with Tukey honestly

## Protein extraction and Western blot analysis

Cells were lysed for protein extraction at 6, 12, 24 and 48 h following exposure to the toxins. Cells were washed with ice-cold PBS and protein extracted with NET-Triton buffer [150 mM NaCl, 5 mM EDTA (ethylenediaminetetraacetic acid), 10 mM Tris (tris(hydroxymethyl)aminomethane), pH 7.4, 1% Triton X-100] supplemented with protease inhibitor cocktail (Sigma-Aldrich) according to the manufacturer's instructions.

The proteins were resolved by SDS/PAGE (10% gels) and blotted onto polyvinylidene fluoride (PVDF) membranes. Membranes were washed with tris-buffered saline (140 mM NaCl, 50 mM tris/HCl, pH 7.2) containing 0.1%

Tween 20, 5% skimmed milk and 2% bovine serum albumin to block nonspecific protein binding. Membranes were incubated with primary antibody against CB1 receptor (1:200 CB1 1A, Alpha Diagnostic International, San Antonio, TX, USA) (approx. 60 kDa), CB2 receptor (1:200, #101550, Cayman Chemical, Ann Arbor, MI, US) (45 kDa) and cleaved caspase 3 (1:1000 #9664S, New England Biolabs) in tris-buffered saline (140 mM NaCl, 50 mM tris/HCl, pH 7.2) containing 0.1% Tween 20, 5% skimmed milk and 2% bovine serum albumin overnight at 4°C, washed three times and incubated with horseradish peroxidase-conjugated secondary antibody (Bio-Rad) for 1 h at room temperature. The protein bands were detected using the enhanced chemiluminescence method (Amersham Biosciences, Buckinghamshire, UK). Membranes were probed with b-actin (1:5000; Abcam, Cambridge, UK) to control for loading. Samples were analysed from at least four separate experiments. Statistical significance was determined using one-way anova in SPSS with Tukey HSD *post hoc* test.

## Results

### MPP<sup>+</sup>

MPP<sup>+</sup> at 5 mM resulted in a greater than twofold increase in mRNA and protein levels for the CB1 receptor at 24-h and 48-h exposure ( $P < 0.05$ ) (Figure 1a). A protective effect of 10 mM D<sup>9</sup>-THC against MPP<sup>+</sup> was demonstrated with the LDH release assay ( $P < 0.001$ ) and confirmed using the MTT assay ( $P < 0.005$ ) (Figure

2b,c) and consequently 10 mM D<sup>9</sup>-THC was used for all future experiments. The LDH assay was chosen for the following experiments as calibration curves indicated a stronger

linear relationship between optical density readings and cell number (Figure S1). D<sup>9</sup>-THC protected against the toxic effects of MPP<sup>+</sup> ( $P < 0.01$ ) (Figure 2d), an effect that was not blocked by co-administration of the selective CB1 receptor antagonist AM251 (1 mM). Additionally, the CB1 agonist, WIN55,212-2 (0.1–10 mM; data for 1 mM shown in Figure 2d) did not have the same protective properties. These results suggest that the protective effects of D<sup>9</sup>-THC against MPP<sup>+</sup> are not mediated by the CB1 receptor. D<sup>9</sup>-THC is also an agonist at the CB2 cannabinoid receptor. However, we were unable to detect expression of CB2 in our differentiated SH-SY5Y cells, either by reverse transcription PCR (RT-PCR) or Western blotting (Figure 3a,b).

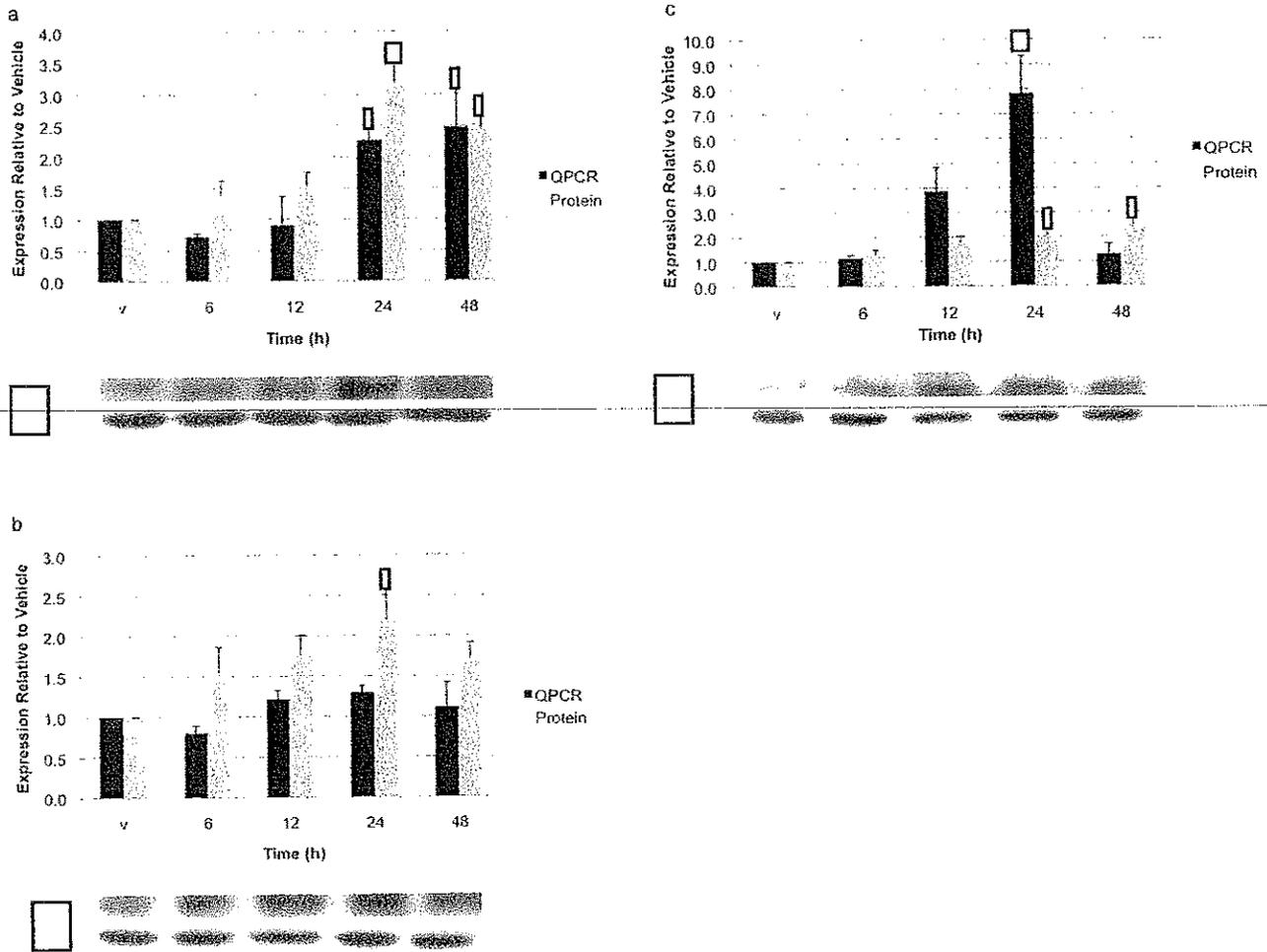
the toxicity of MPP<sup>+</sup>. Cannabidiol, another antioxidant phytocannabinoid, was similarly ineffective (Figure 4c). In addition,

### Paraquat and lactacystin

Although MPP<sup>+</sup> is widely employed to model PD both *in vivo* and *in vitro*, other cellular pathways are also implicated in the pathogenesis of the disease, including free radical damage and dysfunction of the UPS. We were interested to see whether cellular models involving these pathways also resulted in up-regulation of the CB1 receptor, and whether D<sup>9</sup>-THC would be protective.

The free radical-generating toxin, paraquat (500 mM), resulted in a modest 1.3-fold increase in CB1 receptor mRNA level and a significant twofold increase in protein level at 24 h (Figure 1b). Lactacystin (20 mM), which is a potent and specific inhibitor of the UPS, resulted in an almost eightfold increase in CB1 receptor mRNA level ( $P < 0.001$ ) within 24 h, but this had returned to baseline by 48 h (Figure 1c). This was associated with a significant increase in CB1 protein level. D<sup>9</sup>-THC was protective against both toxins, and similar to the effect against MPP<sup>+</sup>; this protective effect was not blocked by AM251 or reproduced by WIN55,212-2 (Figure 2d).

It is possible that the protective effects of D<sup>9</sup>-THC are mediated by its antioxidant properties conferred by the presence of a phenolic ring. We therefore investigated the effect of the synthetic cannabinoid, nabilone, whose structure also contains a phenolic ring and which is known to have antioxidant properties [37], on the toxic effects of MPP<sup>+</sup>. Concentrations of 20 mM and higher were toxic to the cells (Figure 4a). However, lower concentrations failed to demonstrate a protective effect (Figure 4b) against MPP<sup>+</sup>, while 10 mM potentiated



**Figure 1.** Cannabinoid CB1 receptor expression is increased in response to toxin exposure. Effect of exposure of differentiated SH-SY5Y cells to 5 mM 1-methyl-4-phenylpyridinium (MPP<sup>+</sup>) (a), 500 mM paraquat (b) and 20 mM lactacystin (c) on CB1 receptor mRNA and protein levels displayed graphically as mean ± SEM (\**P* < 0.05, \*\**P* < 0.001), with images of representative Western blots below (CB1 above and actin as loading control below). Protein data are normalized to vehicle-treated samples and corrected to the loading control actin. QPCR, quantitative polymerase chain reaction.

co-application of the antioxidants BHT or α-tocopherol failed to reproduce the protective effect of D<sup>9</sup>-THC (Figure 5). In contrast, pretreatment with BHT was protective.

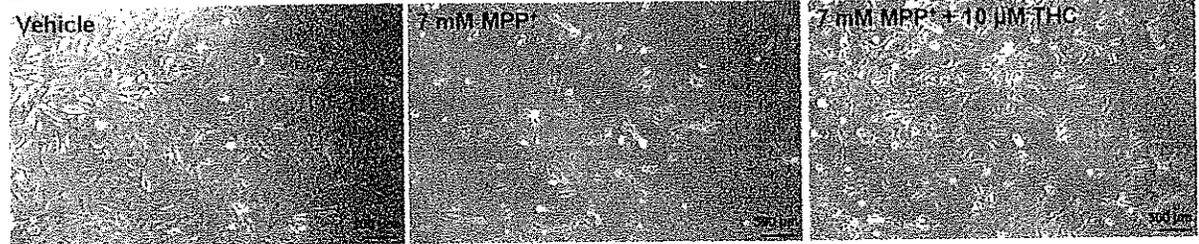
It is known that cannabinoids including D<sup>9</sup>-THC are able to bind to receptors other than CB1 and CB2. To investigate whether D<sup>9</sup>-THC mediates its neuroprotective effect through peroxisome proliferator-activated receptor-γ (PPARγ) activation, we examined the effects of the specific PPARγ antagonist, T0070907. T0070907 was not toxic to SH-SY5Y cells at concentrations between 1 mM and 10 mM (Figure 6a); this range of concentrations also had no significant

effect on MPP<sup>+</sup>-induced neurotoxicity (Figure 6b). We therefore selected this concentration

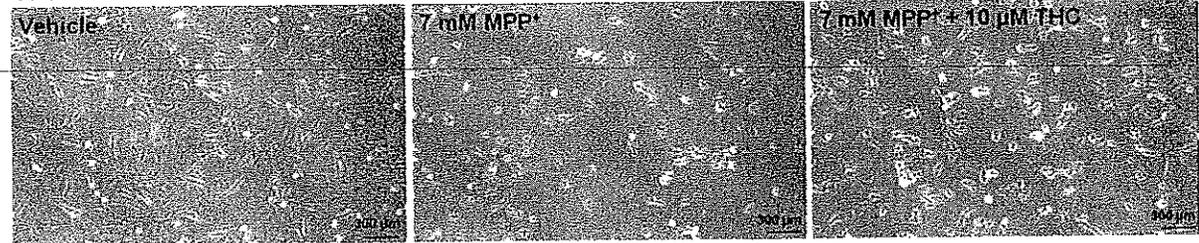
range for further investigation and found that T0070907 was able to dose-dependently inhibit the protective effect of D<sup>9</sup>-THC (Figure 6c). Furthermore, the specific PPAR $\gamma$  agonist pioglitazone resulted in significant neuroprotection against MPP<sup>+</sup> toxicity (Figure 7a) which was completely reversed by 5 mM T0070907 (Figure 7b). A protective effect of D<sup>9</sup>-THC blocked by T0070907 was further confirmed by Western blotting of activated caspase 3 cleaved at Asp 175, a marker of apoptosis. Addition of 10 mM D<sup>9</sup>-THC or 5 mM pioglitazone significantly reduced cleaved caspase 3 levels compared to MPP<sup>+</sup> ( $P < 0.005$ ), and this reduction was inhibited by 10 mM and 5 mM of the PPAR $\gamma$  antagonist, T0070907 for D<sup>9</sup>-THC and pioglitazone respectively (Figure 8). We also found

a

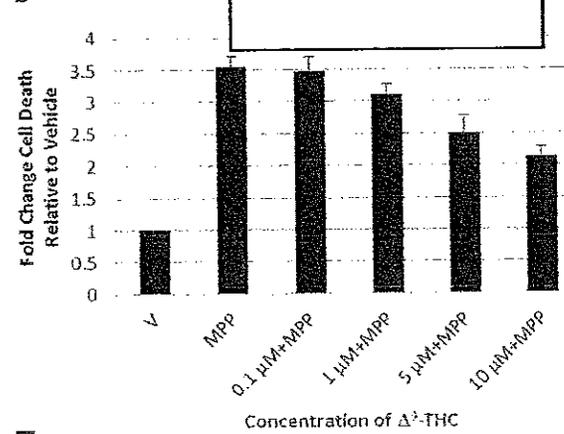
24 h



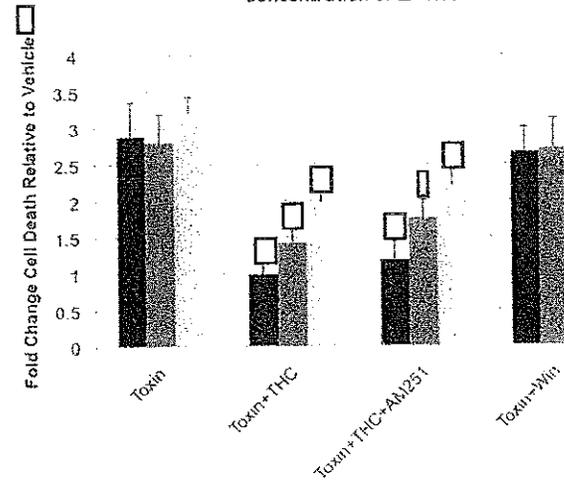
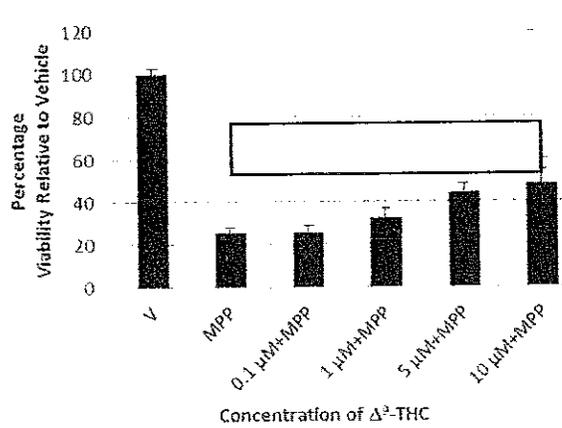
48 h



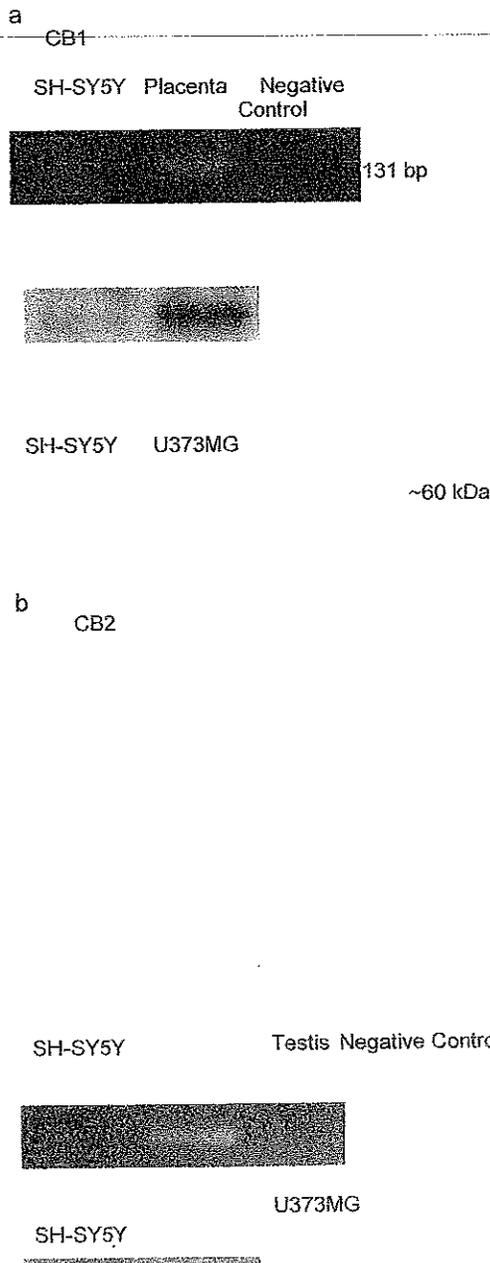
b



c



**Figure 2.** D<sup>9</sup>-THC exerts a protective effect against the toxins. (a) Microscopy image demonstrating protective effect of 10 mM D<sup>9</sup>-tetrahydrocannabinol (D<sup>9</sup>-THC) against 7 mM 1-methyl-4-phenylpyridinium (MPP<sup>+</sup>) in a representative field of view. (b) Lactate dehydrogenase (LDH) assay showing the effect of increasing concentrations of D<sup>9</sup>-THC on MPP<sup>+</sup> toxicity [one-way anova with Tukey honestly significant difference (HSD) *post hoc* test: \*\**P* < 0.001 vs. MPP<sup>+</sup>]. (c) MTT assay showing the effect of increasing concentrations of D<sup>9</sup>-THC on MPP<sup>+</sup> toxicity (one-way anova with Tukey HSD *post hoc* test: \**P* < 0.05, \*\**P* < 0.005 vs. MPP<sup>+</sup>). (d) Co-administration of 10 mM D<sup>9</sup>-THC to differentiated SH-SY5Y cells resulted in significantly reduced cell death in response to 5 mM MPP<sup>+</sup>, 500 mM paraquat (PQT) and 20 mM lactacystin (Lact). This protective effect could not be blocked by co-administration of 1 mM AM251, the specific CB1 antagonist, nor could it be reproduced by co-administration of the CB1 agonist 1 mM WIN55,212-2 (Win) with the toxins. Each bar represents the mean ± SEM of quadruplicate measurements from at least four independent experiments (one-way anova with Tukey HSD *post hoc* test compared with toxin alone: \**P* < 0.05, \*\**P* < 0.01).



## Discussion

We have demonstrated up-regulation of CB1 receptor mRNA in a human neuronal cell culture model of PD in direct response to an inhibitor of mitochondrial complex I (MPP<sup>+</sup>) and an inhibitor of the UPS (lactacystin), both mechanisms relevant to PD pathogenesis. There was a modest increase following 24-h exposure to paraquat, a free-radical generator, but this failed to reach significance. These changes were accompanied by significant increases in CB1 protein expression in response to all three toxins. There have been no previous reports of alteration in neuronal CB1 mRNA and protein level in direct response to toxin administration in cell culture

models of PD. Interestingly, similar changes in CB1 receptor level have not been consistently found in the substantia nigra of PD patients or animal models [10,12,13], although one study has demonstrated increased nigral CB1 receptor activity [10]. The failure to demonstrate an increase in CB1 mRNA in the nigra may reflect loss of dopaminergic neurones. Nevertheless, our finding suggests that up-regulation of the CB1 receptor may occur as a direct response to neuronal injury caused

**Figure 3.** Differentiated SH-SY5Y cells express CB1 but not CB2 receptors. Expression of CB1 (a) and CB2 (b) in differentiated SH-SY5Y cells. PCR on cDNA demonstrated CB1 expression (human placenta positive control) (a) but not CB2 (human testis positive control) (b). Western blot showed expression of CB1 (a) but not CB2 (b) with human glioma U373MG cell line as positive control.

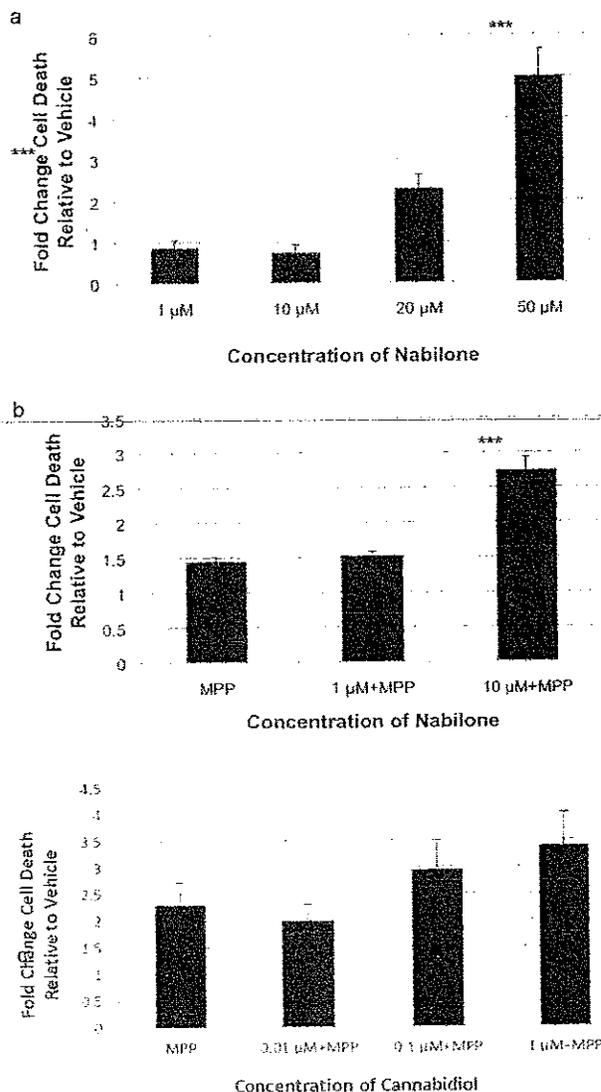
properties of D<sup>9</sup>-THC may be mediated via PPARγ through which it may exert its antioxidant effect.

that D<sup>9</sup>-THC treatment led to a significant reduction ( $P < 0.0001$ ) in reactive oxygen species (ROS) production, an effect that was blocked by 10 mM T0070907 (Figure 9). In addition, we found that D<sup>9</sup>-THC treatment induced up-regulation in PPARγ expression at the protein level (Figure 10). Thus we show that the neuroprotective

by the disease process. The mechanisms underlying this increased transcription remain unclear. It is known that sequences flanking exon 1 of the CB1 gene have marked promoter activity [38,39], and it is possible that transcription factors produced in response to toxin exposure result in increased gene transcription.

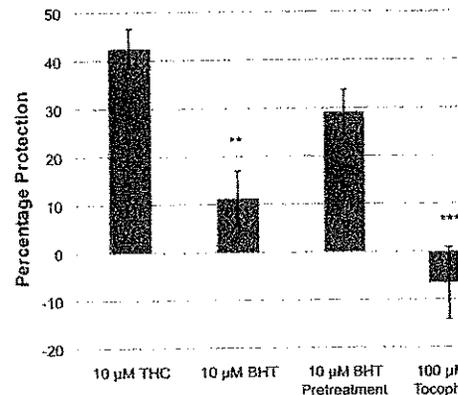
Given the up-regulation of the CB1 receptor in our cell culture model of PD, we hypothesized that agonists at the CB1 receptor would have neuroprotective effects. We found that 10 mM D<sup>9</sup>-THC was protective against all three toxins tested – MPP<sup>+</sup>, lactacystin and paraquat – showing a direct neuroprotective effect of D<sup>9</sup>-THC in a human cell culture PD model. However, this protective effect was not blocked by the CB1 receptor antagonist AM251 and was not reproduced by the CB1 receptor agonist

---



**Figure 4.** The antioxidant cannabinoids, nabilone and cannabidiol, fail to exert a protective effect. Effect of nabilone on differentiated SH-SY5Y cells (a). Co-application of nabilone failed to protect against 7 mM 1-methyl-4-phenylpyridinium (MPP<sup>+</sup>) toxicity (b). Cannabidiol did not protect against 7 mM MPP<sup>+</sup> (c). Each bar represents the mean ± SEM of quadruplicate measurements from four independent experiments (one-way anova with Tukey honestly significant difference *post hoc* test: \*\*\**P* < 0.001).

WIN55,212-2, suggesting that the protective effect of D<sup>9</sup>-THC is unlikely to be mediated by the CB1 receptor.



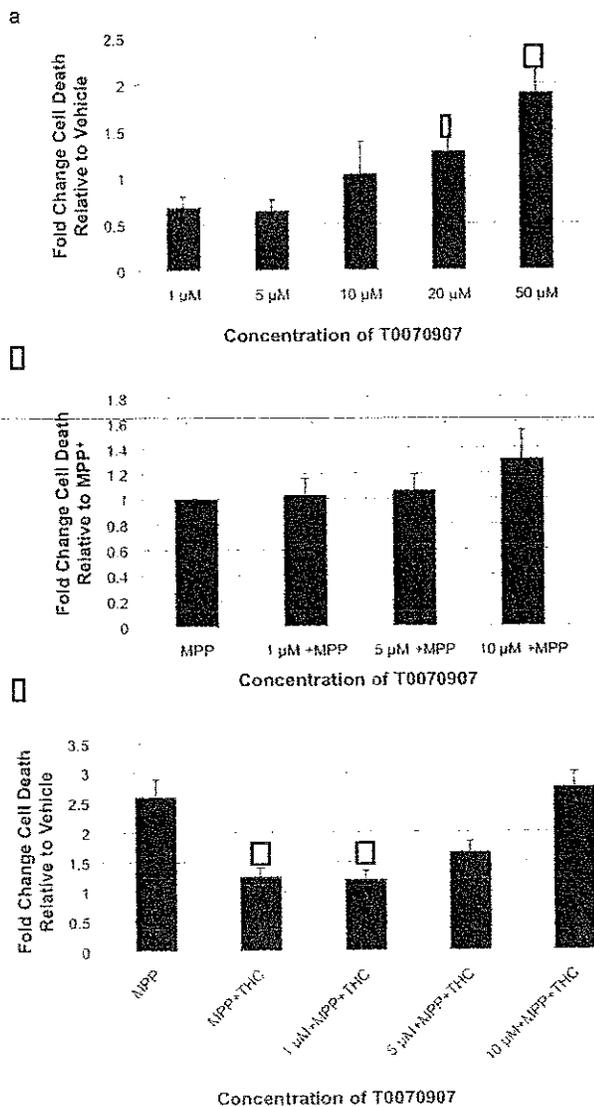
**Figure 5.** Percentage protection afforded by antioxidants against 7 mM 1-methyl-4-phenylpyridinium (MPP<sup>+</sup>) in differentiated SH-SY5Y cells compared with that of D<sup>9</sup>-tetrahydrocannabinol (D<sup>9</sup>-THC). Each bar represents the mean ± SEM of quadruplicate measurements from at least six independent experiments (one-way anova with Tukey honestly significant difference *post hoc* test, comparison with protective effect of D<sup>9</sup>-THC: \*\**P* < 0.01, \*\*\**P* < 0.001).

It has previously been shown that undifferentiated SH-SY5Y cells express the CB2 cannabinoid receptor [40] which may be an alternative site of action of D<sup>9</sup>-THC. However, in our differentiated cell culture model, we were unable to detect the presence of CB2 receptors either by Western blotting or RT-PCR. The lack of effect of

WIN55,212-2, which is also a CB2 receptor agonist, makes it further unlikely that the CB2 receptor is the site of action mediating the protective effect, although future work may include specifically investigating the effects of CB2 receptor antagonists. We therefore investigated the PPAR $\gamma$  as a potential site of action of D<sup>9</sup>-THC in our model. PPAR $\gamma$  is a nuclear receptor which, upon ligand binding, heterodimerizes with the retinoic X receptor to initiate transcription of genes featuring a specific PPAR response element within their promoter region [41]. There are three PPAR $\gamma$  variants all of which can be found in the brain and peripheral nervous system [42,43]. Studies in vascular endothelial cells showed that D<sup>9</sup>-THC is able to initiate PPAR $\gamma$  transcriptional activity, making the receptor a potential target for D<sup>9</sup>-THC [44–47]. Whether D<sup>9</sup>-THC is able to directly bind the receptor or modulate the concentrations of endogenous ligands through indirect pathways is to date unclear [44].

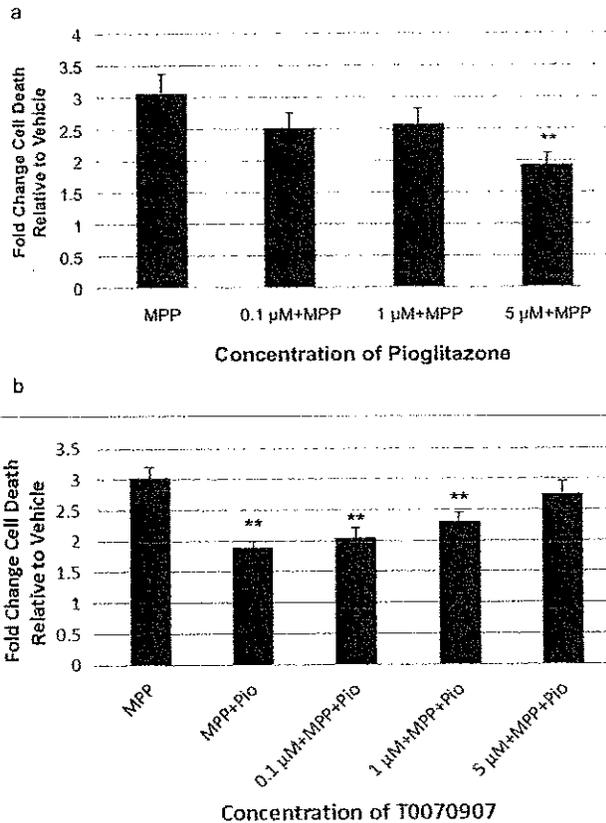
Interestingly, activation of PPAR $\gamma$  by rosiglitazone and pioglitazone has been found protective in both animal and cell culture models of PD [48–51]. Most importantly, however, a protective effect of the PPAR $\gamma$  agonist rosiglitazone against MPP<sup>+</sup>-induced neurotoxicity has been demonstrated in SH-SY5Y cells [49]. However, reasons for this neuroprotective effect are not known. Here, we present the first evidence of a PPAR $\gamma$ -mediated protective effect of D<sup>9</sup>-THC in MPP<sup>+</sup>-treated SHSY-5Y cells, as the protective effects of D<sup>9</sup>-THC on cell death, apoptosis and ROS

significant up-regulation of PPAR $\gamma$  expression in response to D<sup>9</sup>-THC



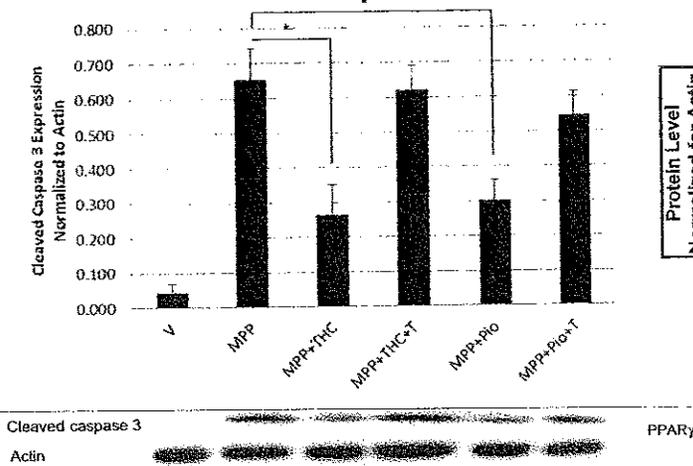
**Figure 6.** The effect of the peroxisome proliferator-activated receptor-gamma (PPAR $\gamma$ ) antagonist, T0070907. T0070907 was toxic at concentrations higher than 20 mM (a) [one-way anova with Tukey honestly significant difference (HSD) *post hoc* test: \* $P$  < 0.05, \*\* $P$  < 0.001 vs. vehicle]. T0070907 had no effect on 7 mM 1-methyl-4-phenylpyridinium (MPP<sup>+</sup>) toxicity (b) (one-way anova with Tukey HSD *post hoc* test: non-significant vs. MPP<sup>+</sup>). T0070907 dose-dependently inhibited D<sup>9</sup>-tetrahydrocannabinol (D<sup>9</sup>-THC)-mediated neuroprotection against 7 mM MPP<sup>+</sup> (c) (one-way anova with Tukey HSD *post hoc* test: \* $P$  < 0.05, \*\* $P$  < 0.001 vs. MPP<sup>+</sup>). Each bar represents the mean  $\pm$  SEM of quadruplicate measurements from at least three independent experiments.

production could be blocked by the specific PPAR $\gamma$  antagonist, T0070907, and reproduced by the PPAR $\gamma$  antagonist, pioglitazone. Furthermore, we detected

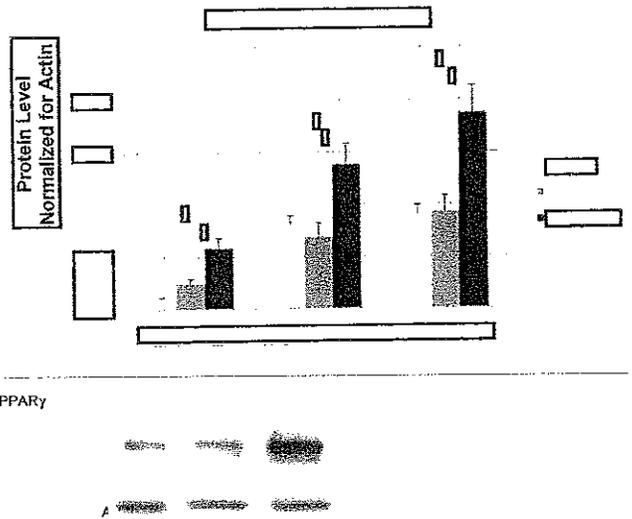


**Figure 7.** Pioglitazone was protective against MPP<sup>+</sup>. The peroxisome proliferator-activated receptor-gamma (PPAR $\gamma$ ) agonist pioglitazone (Pio) was significantly protective against 7 mM 1-methyl-4-phenylpyridinium (MPP<sup>+</sup>)-induced neurotoxicity at a concentration of 5 μM (a) [one-way anova with Tukey honestly significant difference (HSD) *post hoc* test: \*\**P* < 0.001 vs. MPP<sup>+</sup>]. This protective effect could be blocked dose-dependently by T0070907 (b) (one-way anova with Tukey HSD *post hoc* test: \*\**P* < 0.001 vs. MPP<sup>+</sup>). Each bar represents the mean ± SEM of quadruplicate measurements from at least three independent experiments.

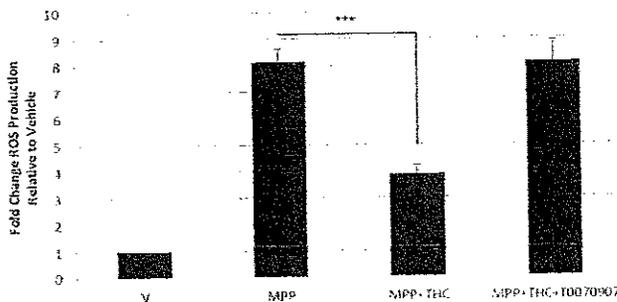
treatment, a finding indicative of PPAR $\gamma$  activation as the receptor has previously been shown to induce its own expression upon activation in a positive feedback response [52]. These data support PPAR $\gamma$  as a potential site of action of D<sup>9</sup>-THC. The synthetic CB1/CB2 receptor agonist WIN55,212-2 has also been reported to induce PPAR $\gamma$  expression in hepatoma HepG2 cells leading to induction of apoptosis [53]. However, in that study the increase in PPAR $\gamma$  expression could partly be blocked by the CB2 antagonist AM630 and the cholesterol depletor methyl-



**Figure 8.** 1-Methyl-4-phenylpyridinium (MPP<sup>+</sup>) (7 mM)-induced apoptosis was inhibited by both D<sup>9</sup>-tetrahydrocannabinol (D<sup>9</sup>-THC) and pioglitazone (Pio), an effect that was reversed by T0070907 (T) (10 mM and 5 mM respectively) as indicated by protein levels of cleaved caspase 3 (Asp175) (5A1E). Protein data are corrected to the loading control. Each bar represents the mean ± SEM of triplicate measurements from at least four independent experiments (one-way anova with Tukey honestly significant difference *post hoc* test: \**P* < 0.005 vs. MPP<sup>+</sup>).



**Figure 10.** Expression of peroxisome proliferator-activated receptor-gamma (PPARγ) in differentiated SH-SY5Y cells. Cells were treated with 7 mM 1-methyl-4-phenylpyridinium (MPP<sup>+</sup>) and 7 mM MPP<sup>+</sup> + 10 mM tetrahydrocannabinol (THC) for 48 h after differentiation (one-way anova with Tukey honestly significant difference *post hoc* test: \**P* < 0.05). Each bar represents the mean ± SEM of three independent experiments.



**Figure 9.** D<sup>9</sup>-tetrahydrocannabinol (D<sup>9</sup>-THC) significantly reduced reactive oxygen species (ROS) production following 7 mM 1-methyl-4-phenylpyridinium (MPP<sup>+</sup>) administration; this effect was inhibited by the peroxisome proliferator-activated receptor-gamma (PPARγ) inhibitor, T0070907 (10 mM) (one-way anova with Tukey honestly significant difference *post hoc*: \*\*\**P* < 0.0001 vs. MPP<sup>+</sup>). Each bar represents the mean ± SEM of four independent experiments in triplicate.

Previous evidence supports antioxidant properties underlying the neuroprotective effects of D<sup>9</sup>-THC and other cannabinoids, possibly conferred by the presence of phenolic ring [15,16,37]. Although this hypothesis would be supported by our finding of a lack of protective

adipocytes [54], indicating that this response may be highly cell type-specific, which may explain why WIN55,212-2 was not protective in our model.

effect of WIN55,212-2, which does not contain a phenolic ring, the phytocannabinoid cannabidiol and the synthetic cannabinoid and D<sup>9</sup>-THC analogue, nabilone, which do contain a phenol ring, were not protective, despite previous reports of their antioxidant properties [37]. In addition, we were unable to reproduce the same protective effect with co-application of the antioxidants  $\alpha$ -tocopherol or BHT. We therefore measured ROS production within our cells and confirmed the antioxidant capacity of D<sup>9</sup>-THC. However, rather than being mediated through its structural properties, we provide evidence that the antioxidant effects of D<sup>9</sup>-THC are mediated by its interaction with PPAR $\gamma$ . Possible PPAR $\gamma$ -mediated antioxidant effects include down-regulation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase expression [55] and enhanced activity of superoxide dismutase (SOD) and catalase [49] as well as increased expression of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1- $\alpha$ ) [56,57] which up-regulates oxidative stress response pathways such as nuclear respiratory factor 1 (NRF-1) and nuclear factor erythroid 2-related factor 2 (NRF-2) and has been implicated in mitochondrial biogenesis [58]. This is the subject of ongoing investigation.

## Conclusions

In conclusion, we have demonstrated up-regulation of the CB1 receptor in a human cell culture model of PD, as well as a direct neuroprotective effect of the phytocannabinoid, D<sup>9</sup>-THC, not mediated by the CB2 receptor. Although a CB1 receptor-mediated effect cannot totally be excluded, we propose that activation of PPAR $\gamma$  leading to antioxidant effects is highly relevant in mediating the neuroprotection afforded by D<sup>9</sup>-THC in our model.

- 10 Lastres-Becker I, Cebreira M, de Ceballos ML, Zeng BY, Jenner P, Ramos JA, Fernandez-Ruiz JJ. Increased cannabinoid CB1 receptor binding and activation of GTP-binding proteins in the basal ganglia of patients with

## Acknowledgements

Dr Carroll was an MRC post-doctoral fellow at the time of undertaking this work.

## Conflict of interest

The authors have no conflict of interest.

## References

- 1 ElSohly MA, Slade D. Chemical constituents of marijuana: the complex mixture of natural cannabinoids. *Life Sci* 2005; **78**: 539–48
- 2 Zeissler M-L, Zajicek J, Carroll C. CBD & D9-THC – the two faces of cannabis. *Cell Sci Rev* 2010; **7**: 63–83
- 3 Matsuda LA, Lolait SJ, Brownstein MJ, Young AC, Bonner TI. Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature* 1990; **346**: 561–4
- 4 Munro S, Thomas KL, Abushaar M. Molecular characterization of a peripheral receptor for cannabinoids. *Nature* 1993; **365**: 61–5
- 5 Pertwee RG. Pharmacology of cannabinoid CB1 and CB2 receptors. *Pharmacol Ther* 1997; **74**: 129–80
- 6 Brotchie J, Fitzer-Attas C. Mechanisms compensating for dopamine loss in early Parkinson disease. *Neurology* 2009; **72**: S32–8
- 7 Brotchie JM. CB1 cannabinoid receptor signalling in Parkinson's disease. *Curr Opin Pharmacol* 2003; **3**: 54–61
- 8 Benarroch E. Endocannabinoids in basal ganglia circuits: implications for Parkinson disease. *Neurology* 2007; **69**: 306–9
- 9 Fernandez-Ruiz J. The endocannabinoid system as a target for the treatment of motor dysfunction. *Br J Pharmacol* 2009; **156**: 1029–40

- Parkinson's syndrome and of MPTP-treated marmosets. *Eur J Neurosci* 2001; **14**: 1827-32
- 11 Papa SM. The cannabinoid system in Parkinson's disease: multiple targets to motor effects. *Exp Neurol* 2008; **211**: 334-8
  - 12 Lastres-Becker I, Romero J, Berrendero F, Perez-Rosado A, Manzanares J, Rojo A, Fernandez-Ruiz J, de Yebenes JG, Ramos JA. Unilateral 6-hydroxydopamine lesions of nigrostriatal dopaminergic neurons increased cannabinoid CB1 receptor mRNA levels in the rat striatum: possible therapeutic implications. In *2nd Parkinsons Disease Symposium on Neurotoxic Factors in Parkinsons Disease and Related Disorders*. Eds A Storch, MA Collins. Ulm: Kluwer Academic/Plenum Publ, 1999; 301-5
  - 13 Van Laere K, Casteels C, Lunsken S, Goffin K, Grachev ID, Bormans G, Vandenberghe W. Regional changes in type 1 cannabinoid receptor availability in Parkinson's disease in vivo. *Neurobiol Aging* (in press). DOI:10.1016/j.neurobiolaging.2011.02.009
  - 14 Grant I, Cahn B. Cannabis and endocannabinoid modulators: therapeutic promises and challenges. *Clin Neurosci Res* 2005; **5**: 185-99
  - 15 Lastres-Becker I, Molina-Holgado F, Ramos JA, Mechoulam R, Fernandez-Ruiz J. Cannabinoids provide neuroprotection against 6-hydroxydopamine toxicity in vivo and in vitro: relevance to Parkinson's disease. *Neurobiol Dis* 2005; **19**: 96-107
  - 16 Garcia-Arencibia M, Gonzalez S, de Lago E, Ramos JA, Mechoulam R, Fernandez-Ruiz J. Evaluation of the neuroprotective effect of cannabinoids in a rat model of Parkinson's disease: importance of antioxidant and cannabinoid receptor-independent properties. *Brain Res* 2007; **1134**: 162-70
  - 17 Sagredo O, Garcia-Arencibia M, de Lago E, Finetti S, Decio A, Fernandez-Ruiz J. Cannabinoids and neuroprotection in basal ganglia disorders. *Mol Neurobiol* 2007; **36**: 82-91
  - 18 Panikashvili D, Simeonidou C, Ben-Shabat S, Hanus L, Breuer A, Mechoulam R, Shohami E. An endogenous cannabinoid (2-AG) is neuroprotective after brain injury. *Nature* 2001; **413**: 527-31
  - 19 Pryce G, Ahmed Z, Hankey DJR, Jackson SJ, Croxford JL, Pocock JM, Ledent C, Petzold A, Thompson AJ, Giovannoni G, Cuzner ML, Baker D. Cannabinoids inhibit neurodegeneration in models of multiple sclerosis. *Brain* 2003; **126**: 2191-202
  - 20 Gilbert GL, Kim HJ, Waataja JJ, Thayer SA. [Delta]9-Tetrahydrocannabinol protects hippocampal neurons from excitotoxicity. *Brain Res* 2007; **1128**: 61-9
  - 21 Abood ME, Rizvi G, Sallapudi N, McAllister SD. Activation of the CB1 cannabinoid receptor protects cultured mouse spinal neurons against excitotoxicity. *Neurosci Lett* 2001; **309**: 197-201
  - 22 Nagayama T, Sinor AD, Simon RP, Chen J, Graham SH, Jin KL, Greenberg DA. Cannabinoids and neuroprotection in global and focal cerebral ischemia and in neuronal cultures. *J Neurosci* 1999; **19**: 2987-

- 23 Shen MX, Thayer SA. Cannabinoid receptor agonists protect cultured rat hippocampal neurons from excitotoxicity. *Mol Pharmacol* 1998; **54**: 459–62
- 24 van der Stelt M, Veldhuis WB, Bar PR, Veldink GA, Vliegthart JFG, Nicolay K. Neuroprotection by Delta(9)-tetrahydrocannabinol, the main active compound in marijuana, against ouabain-induced in vivo excitotoxicity. *J Neurosci* 2001; **21**: 6475–9
- 25 Twitchell W, Brown S, Mackie K. Cannabinoids inhibit N- and P/Q-type calcium channels in cultured rat hippocampal neurons. *J Neurophysiol* 1997; **78**: 43–50
- 26 Iuvone T, Esposito G, De Filippis D, Bisogno T, Petrosino S, Scuderi C, Di Marzo V, Steardo L. Cannabinoid CB1 receptor stimulation affords neuroprotection in MPTP-induced neurotoxicity by attenuating S100B up-regulation in vitro. *J Mol Med* 2007; **85**: 1379–92
- 27 Zhang F, Challapalli SC, Smith PJW. Cannabinoid CB1 receptor activation stimulates neurite outgrowth and inhibits capsaicin-induced Ca<sup>2+</sup> influx in an in vitro model of diabetic neuropathy. *Neuropharmacology* 2009; **57**: 88–96
- 28 Price DA, Martinez AA, Seillier A, Koek W, Acosta Y, Fernandez E, Strong R, Lutz B, Marsicano G, Roberts JL, Giuffrida A. WIN55,212-2, a cannabinoid receptor agonist, protects against nigrostriatal cell loss in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson's disease. *Eur J Neurosci* 2009; **29**: 2177–86
- 29 Pérez-Rial S, García-Gutiérrez MS, Molina JA, Pérez-Nievas BG, Ledent C, Leiva C, Leza JC, Manzanares J. Increased vulnerability to 6-hydroxydopamine lesion and reduced development of dyskinesias in mice lacking CB1 cannabinoid receptors. *Neurobiol Aging* 2011; **32**: 631–45.
- 30 Kalivendi SV, Kotamraju S, Cunningham S, Shang T, Hillard CJ, Kalyanaraman B. 1-Methyl-4-phenylpyridinium (MPP<sup>+</sup>)-induced apoptosis and mitochondrial oxidant generation: role of transferrin-receptor-dependent iron and hydrogen peroxide. *Biochem J* 2003; **371**: 151–64
- 31 Kalivendi SV, Cunningham S, Kotamraju S, Joseph J, Hillard CJ, Kalyanaraman B. alpha-synuclein up-regulation and aggregation during MPP<sup>+</sup>-induced apoptosis in neuroblastoma cells – intermediacy of transferrin receptor iron and hydrogen peroxide. *J Biol Chem* 2004; **279**: 15240–7
- 32 Dinis-Oliveira RJ, Remiao F, Carmo H, Duarte JA, Navarro AS, Bastos ML, Carvalho F. Paraquat exposure as an etiological factor of Parkinson's disease. *Neurotoxicology* 2006; **27**: 1110–22
- 33 Yang W, Tiffany-Castiglioni E. Paraquat-induced apoptosis in human neuroblastoma SH-SY5Y cells: involvement of p53 and mitochondria. *J Toxicol Environ Health A* 2008; **71**: 289–99
- 34 Yang WS, Tiffany-Castiglioni E. The bipyridyl herbicide paraquat produces oxidative stress-mediated toxicity in human neuroblastoma SH-SY5Y cells: relevance to the dopaminergic pathogenesis. *J Toxicol Environ Health A* 2005; **68**: 1939–61
- 35 Zhang X, Xie WJ, Qu S, Pan TH, Wang XT, Le WD. Neuroprotection by iron chelator against proteasome inhibitor-induced nigral degeneration. *Biochem Biophys Res Commun* 2005; **333**: 544–9
- 36 Lopes FM, Schröder R, da Frota ML Jr, Zanotto-Filho A, Müller CB, Pires AS, Meurer RT, Colpo GD, Gelain DP, Kapczinski F, Moreira JCF, Fernandes Mda C, Klamt F. Comparison between proliferative and neuron-like SH-SY5Y cells as an in vitro model for Parkinson disease studies. *Brain Res* 2010; **1337**: 85–94
- 37 Hampson AJ, Grimaldi M, Axelrod J, Wink D. Cannabidiol and (-)-Delta(9)-tetrahydrocannabinol are neuroprotective antioxidants. *Proc Natl Acad Sci U S A* 1998; **95**: 8268–73
- 38 Zhang PW, Ishiguro H, Ohtsuki T, Hess J, Carillo F, Walther D, Onaivi ES, Arinami T, Uhl GR. Human cannabinoid receptor 1: 5' exons, candidate regulatory regions, polymorphisms, haplotypes and association with polysubstance abuse. *Mol Psychiatry* 2004; **9**: 916–31
- 39 Borner C, Bedini A, Holtt V, Kraus J. Analysis of promoter regions regulating basal and interleukin-4-inducible expression of the human CB1 receptor gene in T lymphocytes. *Mol Pharmacol* 2008; **73**: 1013–19
- 40 Pasquariello N, Catanzaro G, Marzano V, Amadio D, Barcaroli D, Oddi S, Federici G, Urbani A, Agro AF, Maccarrone M. Characterization of the endocannabinoid system in human neuronal cells and proteomic analysis of anandamide-induced apoptosis. *J Biol Chem* 2009; **284**: 29413–26
- 41 Kota BP, Huang THW, Roufogalis BD. An overview on biological mechanisms of PPARs. *Pharmacol Res* 2005; **51**: 85–94
- 42 Moreno S, Farioli-Vecchioli S, Ceru MP. Immunolocalization of peroxisome proliferator-activated receptors and retinoid X receptors in the adult rat CNS. *Neuroscience* 2004; **123**: 131–45
- 43 Cullingford TE, Bhakoo K, Peuchen S, Dolphin CT, Patel R, Clark JB. Distribution of mRNAs encoding the peroxisome proliferator-activated receptor alpha, beta and gamma and the retinoid X receptor alpha, beta and gamma rat central nervous system. *J Neurochem* 1998; **70**: 1366–75
- 44 O'Sullivan SE. Cannabinoids go nuclear: evidence for activation of peroxisome proliferator-activated receptors. *Br J Pharmacol* 2007; **152**: 576–82
- 45 O'Sullivan SE, Tarling EJ, Bennett AJ, Kendall DA, Randall MD. Novel time-dependent vascular actions of Delta(9)-tetrahydrocannabinol mediated by peroxisome proliferator-activated receptor gamma. *Biochem Biophys Res Commun* 2005; **337**: 824–31

- 46 O'Sullivan SE, Kendall DA, Randall MD. Further characterization of the time-dependent vascular effects of Delta(9)-tetrahydrocannabinol. *J Pharmacol Exp Ther* 2006; **317**: 428–38
- 47 O'Sullivan SE, Kendall DA. Cannabinoid activation of peroxisome proliferator-activated receptors: potential for modulation of inflammatory disease. *Immunobiology* 2010; **215**: 611–16
- 48 Schintu N, Frau L, Ibba M, Caboni P, Garau A, Carboni E, Carta AR. PPAR-gamma-mediated neuroprotection in a chronic mouse model of Parkinson's disease. *Eur J Neurosci* 2009; **29**: 954–63
- 49 Jung TW, Lee JY, Shim WS, Kang ES, Kim SK, Ahn CW, Lee HC, Cha BS. Rosiglitazone protects human neuroblastoma SH-SY5Y cells against MPP<sup>+</sup> induced cytotoxicity via inhibition of mitochondrial dysfunction and ROS production. *J Neurol Sci* 2007; **253**: 53–60
- 50 Dehmer T, Heneka MT, Sastre M, Dichgans J, Schulz JB. Protection by pioglitazone in the MPTP model of Parkinson's disease correlates with I kappa B alpha induction and block of NF kappa B and iNOS activation. *J Neurochem* 2004; **88**: 494–501
- 51 Breidert T, Callebert J, Heneka MT, Landreth G, Launay JM, Hirsch EC. Protective action of the peroxisome proliferator-activated receptor-gamma agonist pioglitazone in a mouse model of Parkinson's disease. *J Neurochem* 2002; **82**: 615–24
- 52 Davies GF, Khandelwal RL, Roesler WJ. Troglitazone induces expression of PPAR[gamma] in liver. *Mol Cell Biol Res Commun* 1999; **2**: 202–8
- 53 Giuliano M, Pellerito O, Portanova P, Calvaruso G, Santulli A, De Blasio A, Vento R, Tesoriere G. Apoptosis induced in HepG2 cells by the synthetic cannabinoid WIN: involvement of the transcription factor PPAR $\gamma$ . *Biochimie* 2009; **91**: 457–65
- 54 Pagano C, Pilon C, Calcagno A, Urbanet R, Rossato M, Milan G, Bianchi K, Rizzuto R, Bernante P, Federspil G, Vettor R. The endogenous cannabinoid system stimulates glucose uptake in human fat cells via phosphatidylinositol 3-kinase and calcium-dependent mechanisms. *J Clin Endocrinol Metab* 2007; **92**: 4810–19
- 55 Calnek DS, Mazzella L, Hart CM. Peroxisome proliferator-activated receptor gamma ligands alter endothelial NADPH oxidase activity and expression. *J Investig Med* 2003; **51**: S258
- 56 Hondares E, Mora O, Yubero P, de la Concepcion MR, Iglesias R, Giralt M, Villarroya F. Thiazolidinediones and rexinoids induce peroxisome proliferator-activated receptor-coactivator (PGC)-1 alpha gene transcription: an autoregulatory loop controls PGC-1 alpha expression in adipocytes via peroxisome proliferator-activated receptor-gamma coactivation. *Endocrinology* 2006; **147**: 2829–38
- 57 Miglio G, Rosa AC, Rattazzi L, Collino M, Lombardi G, Fantozzi R. PPAR gamma stimulation promotes mitochondrial biogenesis and prevents glucose deprivation-induced neuronal cell loss. *Neurochem Int* 2009; **55**: 496–504
- 58 Wareski P, Vaarmann A, Choubey V, Safiulina D, Liiv J, Kuum M, Kaasik A. PGC-1 alpha and PGC-1 beta regulate mitochondrial density in neurons. *J Biol Chem* 2009; **284**: 21379–85

### Supporting information

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Calibration curve for MTT and LDH assays. Using the linear regression model, the regression coefficients  $R^2$  were calculated in SPSS, indicating a stronger linear relationship between optical density readings and cell number for the LDH assay. Each data point represents the mean  $\pm$  SEM of three independent experiments.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

Received 29 June 2011

Accepted after revision 20 December

2011 Published online Article Accepted on 26

December 2011

Copyright of *Neuropathology & Applied Neurobiology* is the property of Wiley-Blackwell and its content may not be copied or emailed to multiple sites or posted to a listserv without the copyright holder's express written permission. However, users may print, download, or email articles for individual use.



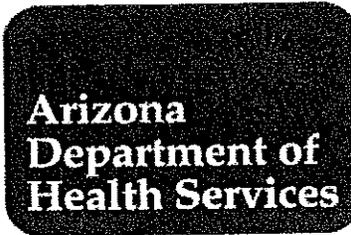
AZCNA v. ADHS

Appeal – Parkinson’s Disease

EXHIBITS

---

**EXHIBIT “2”**



***Division of Public Health Services***

*Medical Marijuana Program*

P.O. Box 19000  
Phoenix, Arizona 85005

JANICE K. BREWER, GOVERNOR  
WILL HUMBLE, DIRECTOR

August 5, 2014

Arizona Cannabis Nurses Association  
Attn: Heather Manus, RN  
5505 E. Paseo Cimarron  
Tucson, AZ 85750

---

Dear Ms. Manus:

Thank you for your petition to add Parkinson's Disease to the list of debilitating medical conditions set forth in Arizona Revised Statutes (A.R.S.) § 36-2801(3) and Arizona Administrative Code (A.A.C.) R9-17-201.

Pursuant to A.R.S. §§ 36-2801.01 and 36-2803, the Arizona Department of Health Services (ADHS) adopted rules governing the manner in which ADHS shall consider petitions from the public to add debilitating medical conditions. *See* A.A.C. R9-17-106. According to A.A.C. R9-17-106(A), in order for the Department to consider a petition for review, the requestor is required to submit *each* of the elements listed.

ADHS determined that your petition does not meet the requirements in A.A.C. R9-17-106(A). Specifically, your petition failed to satisfy the following elements as required in A.A.C. R9-17-106(A)(4-7):

4. A description of the symptoms and other physiological effects experienced by an individual suffering from the medical condition or treatment of the medical condition that may impair the ability of the individual to accomplish activities of daily living;
5. The availability of conventional medical treatments to provide therapeutic or palliative benefit for the medical condition or treatment of the medical condition;
6. A summary of the evidence that the use of marijuana will provide therapeutic or palliative benefit for the medical condition or a treatment of the medical condition; and
7. Articles, published in peer-reviewed scientific journals, reporting the results of research on the effects of marijuana on the medical condition or a treatment of the medical condition supporting why the medical condition should be added.

Due to its failure to satisfy the requirements set forth in A.A.C. R9-17-106(A), your petition is incomplete and as a result, ADHS will not consider your petition for review.

ADHS will be accepting petitions to add to the list of debilitating medical conditions again in

Ms. Manus

August 5, 2014

January 2016. Please visit our website at <http://www.azdhs.gov/medicalmarijuana/debilitating/> for the most current information and updates about this process.

Sincerely,

The Arizona Medical Marijuana Program

---