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Short communication

Identification of CB_2 receptors in human nigral neurons that degenerate in Parkinson's disease

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HIGHLIGHTS

- CB_2 receptors were found in nigrostriatal neurons in the human substantia nigra.
- Their levels were lower in the substantia nigra of Parkinson's disease patients.
- The levels of this receptor could serve as a biomarker for this disorder.

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ABSTRACT

It is well-demonstrated that cannabinoid CB_2 receptors located in glial cells are up-regulated in neurodegenerative disorders serving as a target to control glial influences to neurons. Recent evidence indicates that CB_2 receptors may be also located in certain neuronal subpopulations and serve as a marker of neuronal losses. We investigated this possibility in the post-mortem substantia nigra of Parkinson's disease (PD) patients and controls. Immunostaining for the CB_2 receptor was found in tyrosine hydroxylase-positive neurons in the substantia nigra, a fact confirmed with double-staining analyses. The signal was found in controls but also in PD patients, in which CB_2 receptor labelling was significantly lower, in parallel to the losses of these neurons experienced in the disease. These data show for the first time that CB_2 receptors are located in tyrosine hydroxylase-containing neurons in the substantia nigra at levels significantly lower in PD patients compared to controls.

1. Introduction

The cannabinoid type-2 (CB_2) receptor was identified first in the immune system [1] but not in the central nervous system (CNS) [2], being proposed as the peripheral receptor for the endocannabinoid system [3]. However, further studies re-examined their presence in the healthy brain and identified CB_2 receptors in astrocytes [4], oligodendrocytes [5], quiescent [6] and perivascular microglia [7], neural progenitors [8], and even in a few subpopulations of neurons [6], although with a much more restricted distribution in the CNS compared to the CB_1 receptor. Additional studies demonstrated that the levels of CB_2 receptors experience a dramatic increase in numerous pathological conditions affecting the CNS, particularly those that are produced by inflammatory/degenerative stimuli, and in which these up-regulatory responses have been identified predominantly in reactive glial cells [9–11]. This includes Alzheimer's disease, amyotrophic lateral sclerosis, multiple sclerosis, Huntington's chorea, Parkinson's disease (PD) and others [9–11]. These observations provided arguments supporting the idea that this receptor may serve: (i) as a pathological biomarker, for example, the extent of CB_2 receptor expression has been related to malignancy in glial tumours [12]; and (ii) as a pharmacological target to control glial activation and their effects on neuronal homeostasis in neurodegenerative disorders [9,10].

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As mentioned above, CB_2 receptors are also present in neurons in the healthy brain, but only in a few subpopulations predominantly located in the brainstem [13], cerebellum, e.g. Purkinje [4] and granular [14] neurons, and basal ganglia, e.g. pallidothalamic neurons [15]. Some studies extended this presence to numerous cortical and subcortical structures [16,17], but the problems of specificity of the anti-CB_2 receptor antibodies used and the lack of the necessary methodological controls [6,18] have generated some controversy about these data. We recently found CB_2 receptor immunostaining in different neuronal subpopulations in the human cerebellum, e.g. Purkinje cells, using the necessary negative and positive controls to ensure the specificity of the immunolabelling found [4]. We also demonstrated how this immunostaining is affected in autosomal-dominant hereditary ataxias [4], for which it appears to serve as a marker of neuronal injury. We have now extended our interest to another neuronal subpopulation, neuromelanin-containing neurons in the substantia nigra that are dramatically affected in PD. In the present study, we used immunohistochemical procedures to label CB_2 receptors in the human post-mortem substantia nigra from PD patients and control subjects, and confirmed their presence in tyrosine hydroxylase-containing neurons by double-staining methods.

2. Materials and methods

2.1. Subjects

We used post-mortem human substantia nigra from control subjects and patients with diagnosis of PD that were obtained from two biobanks: "Fundación CIEN", Madrid, Spain, and "Banc de Teixits Neurològics, Universitat de Barcelona-Hospital Clínic", Barcelona, Spain. All material has been collected from donors for and from whom a written informed consent for a brain autopsy and the use of material and clinical information for research purposes had been obtained from both biobanks. Our study was approved by the Committee for Clinical Research of our institution. Samples (one section/patient) were received once fixed, paraffinized, sliced (3–5 μm) and mounted on glass slides. Table 1 summarizes the major characteristics of all individuals included in our analyses. They were selected to have an adequate matching by age, gender and post-mortem delay, as well as to avoid the presence of potential influencing factors, e.g. additional diseases in the case of PD patients, interfering causes of death in the case of controls. The neuropathological data provided by the biobanks confirmed that all patients exhibited a marked atrophy of the substantia nigra with evidence of neuronal loss. We also considered the pharmacological information (medication and intoxication history) of PD patients provided by the biobanks, which included the classic dopaminergic replacement therapy, e.g. levodopa, dopamine agonists. Medication and intoxication history of control subjects were also available and revised in the selection of cases to avoid any possible influence in the parameters controlled in this study.

2.2. Immunohistochemistry

The protocol used was previously described [19]. Briefly, tissue sections were deparaffinized, rehydrated in grade alcohol and washed extensively in 50 mM, pH 7.4, potassium phosphate-buffered saline (KPBS). To obtain more efficient immunostaining, tissue sections were irradiated in microwaves in a solution containing antigen retrieval solution pH 6 (Dako Cytomation, Glostrup, Denmark) [20]. After irradiated, samples were removed and washed extensively in KPBS. Then, endogenous peroxidase was blocked by 30 min incubation at room temperature in peroxidase blocking solution (Dako Cytomation). After several washes with KPBS, tissues were incubated with the monoclonal anti-CB_2 receptor antibody (R and D systems, Minneapolis, MN, USA, diluted 1:100 in KPBS containing 5% horse serum albumin (Sigma Chem., Madrid, Spain) and 0.1% Triton X-100 (Sigma Chem.), overnight at room temperature. After incubation, sections were washed in KPBS, followed by incubation with biotinylated horse anti-mouse antibody (1:200) for 1 h at room temperature. Avidin–biotin complex (Vector Laboratories, Burlingame, CA, USA) and a 3,3′-diaminobenzidine substrate–chromogen system (Dako Cytomation) were used to obtain a visible reaction product. Negative controls were used in each case by omitting primary antibody as well as by using an immunogenic blocking peptide following a previously-published procedure [4]. Sections were dehydrated, sealed, and coverslipped. A Leica DMRB microscope with DFC300FX camera (Leica, Wetzlar, Germany) were used for the observations and photography of the slides, respectively. Quantification of CB_2 immunostaining was carried out on high-resolution digital microphotographs that were taken with the 5× objective and under the same conditions of light and brightness/contrast. They were used to measure the mean density of labelling in the selected area, using the analysis software ImageJ (NIH, USA), which allows to be calibrated in a way that minimizes the influence of different backgrounds. Five images were taken for each case. All data were expressed in arbitrary units.

2.3. Immunofluorescence

To identify the specific cell population in which CB_2 receptor labelling is located, we performed double-staining analysis combining the anti-CB_2 antibody (diluted 1/50) with a monoclonal antibody against tyrosine hydroxylase (Chemicon-Millipore, Temecula, CA, USA; diluted 1:500). After the antigen retrieval procedure, tissue sections were washed with pH 7.4 PBS before overnight incubation at room temperature with the anti-CB_2 receptor antibody, followed by incubation with an anti-mouse secondary antibody conjugated with Alexa 546 (Invitrogen, Carlsbad, CA, USA) at 37 °C for 2 h. Afterwards, the anti-tyrosine hydroxylase anti-

Table 1
Major characteristics of patients and control subjects whose postmortem samples were used in this study (they were obtained from the biobanks of the "Fundación CIEN", Madrid, Spain, and "Banc de Teixits Neurològics, Universitat de Barcelona-Hospital Clínic", Barcelona, Spain).

Subject #	Age	Gender	Diagnosis	Postmortem delay (min)	Biobank
1	57	Female	Control	60	FCIEN
2	81	Male	Control	540	FCIEN
3	56	Male	Control	<480	FCIEN
4	66	Male	Control	420	HClinic
5	74	Female	Control	220	HClinic
6	82	Female	Parkinson's disease	<480	FCIEN
7	75	Male	Parkinson's disease	480	FCIEN
8	75	Male	Parkinson's disease	<480	FCIEN
9	81	Male	Parkinson's disease	330	HClinic
10	79	Female	Parkinson's disease	210	HClinic