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Loss of cannabinoid CB₁ receptor expression in the 6-hydroxydopamine-induced nigrostriatal terminal lesion model of Parkinson's disease in the rat

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Abstract

The endocannabinoid system is emerging as a potential alternative to the dopaminergic system for the treatment of Parkinson's disease. Like all emerging targets, validation of this system's potential for treating human Parkinsonism necessitates testing in animal models of the condition. However, if components of the endocannabinoid system are altered by the induction of a Parkinsonian state in animal models, this could have an impact on the interpretation of such preclinical experiments. This study sought to determine if expression of the CB₁ subtype of cannabinoid receptor is altered in the two most commonly used rat models of Parkinson's disease. Parkinsonian lesions were induced by stereotaxic injection of 6-hydroxydopamine into the axons (medial forebrain bundle) or terminals (striatum) of the nigrostriatal pathway. On days 1, 3, 7, 14 and 28 post-lesion, rats were sacrificed and brains were processed for tyrosine hydroxylase and CB₁ receptor immunohistochemistry. The CB₁ receptor was expressed strongly in the substantia nigra *pars reticulata*, minimally overlapping with tyrosine hydroxylase immunoreactivity in the *pars compacta*. Interestingly, while there was little change in CB₁ receptor expression following axonal lesion, expression of the receptor was significantly reduced following terminal lesion. Loss of CB₁ receptor expression in the *pars reticulata* correlated significantly with the loss of striatal and nigral volume after terminal lesion indicating this may have been due to 6-hydroxydopamine-induced non-specific damage of striatonigral neurons which are known to express CB₁ receptors. Thus, this result has implications for the choice of model and interpretation of studies used to investigate potential cannabinoid-based therapies for Parkinson's disease as well as striatonigral diseases such as Huntington's disease and Multiple Systems Atrophy.

Keywords

Parkinson's disease; Endocannabinoid system; CB₁ receptor; 6-Hydroxydopamine; Substantia nigra

1. Introduction

The discovery in 1960 of a severe depletion of the neurotransmitter dopamine from the caudate and putamen of post-mortem Parkinsonian brains [17] revealed the first pharmacological target for this hitherto untreatable motor disorder. In 1961, it was reported that intravenous administration of the dopamine precursor, levodopa, to Parkinsonian patients was capable of reversing the akinesia associated with the disease [3]. Cotzias et al. [7] subsequently confirmed the anti-Parkinsonian effects of orally administered levodopa, but they also reported that long-term administration of levodopa caused the development of abnormal involuntary movements termed dyskinesias. Since then, the pharmacological treatment of Parkinson's disease has remained centred on the dopaminergic system despite the limitations and side effects associated with this approach.

The endocannabinoid system has recently emerged as a potential alternative target for the treatment of Parkinson's disease [5]. This proposition is based on a number of converging lines of evidence: firstly, brain regions involved in the control of movement such as the basal ganglia (especially the substantia nigra) possess the highest density of the CB₁ subtype of cannabinoid receptor [10,36] and the highest concentration of the endocannabinoids, anandamide and 2-arachidonylglycerol in the brain [4,20]; secondly, plant-derived, synthetic and endogenous cannabinoids exert inhibitory effects on motor activity in both humans [51] and experimental animals [8,45]. It has also been reported that endocannabinoid levels [41] and CB₁ receptor expression are altered in Parkinson's disease patients [27,31].

For Parkinson's disease, the most commonly used rat models are the unilateral 6-hydroxydopamine lesion models, where the catecholaminergic neurotoxin is administered directly into the nigrostriatal system—most commonly to the axons as they ascend along the medial forebrain bundle, or to the nigrostriatal terminals where they innervate the striatum. These models are suitable for testing the hypothesis that targeting the endocannabinoid system could be a valid therapeutic target for the treatment of the Parkinsonian motor disorder because they are associated with a well-characterised behavioural syndrome reminiscent of the human condition [48]. However, since there is some evidence that experimental Parkinsonian lesions induce alterations in the endocannabinoid system [23,44], this could have an impact on the interpretation of studies that seek to determine the anti-Parkinsonian potential of cannabinoid drugs in these models, particularly if the alterations do not model the changes which are known to occur in the human condition [27,31]. Therefore, it is vital to determine the effect of 6-hydroxydopamine-induced hemi-Parkinsonism on cannabinoid receptor expression if these models are to be used for validating the endocannabinoid system as a therapeutic target for Parkinson's disease.

Therefore, the aim of this study was to determine if there are any changes in CB₁ receptor expression in the substantia nigra in the two most commonly used rat models of Parkinson's disease—those in which the nigrostriatal pathway is lesioned by unilateral axonal or terminal injection of 6-hydroxydopamine.

2. Materials and methods

2.1. Animals

Male Lister hooded rats ($n = 57$, Charles River, UK) were used in this study, weighing 225–250 g at the start of the experiment. They were housed under a 12 h light:dark cycle in a room maintained at 21 ± 2 °C and had access to food and water *ad libitum*. All procedures were carried out under license from the Irish Department of Health and Children, were approved by the Animal Ethics Committee of the National University of Ireland, Galway, and were in compliance with the European Communities Council directive 86/609/EEC.

2.2. Experimental design

The nigrostriatal pathway was lesioned on one side only by the injection of 6-hydroxydopamine into either the medial forebrain bundle (axonal lesion, $n = 41$) or the striatum (terminal lesion, $n = 16$). Rats were anaesthetised and sacrificed by transcardial perfusion with paraformaldehyde on days 1, 3, 7, 14 and 28 post-lesion at random. For the axonal lesion, 7–10 rats were sacrificed per time-point, whereas for the terminal lesion, 3–4 rats were sacrificed per time-point. Post-mortem, tyrosine hydroxylase immunohistochemistry was used to track the progress of the nigrostriatal lesions over time, and quantitative CB₁ receptor immunohistochemistry was used to determine whether there were any changes in CB₁ receptor expression in the two lesion models. Finally, quantitative DARPP-32 immunohistochemistry (as a marker of the striatonigral projection neurons) was carried out to determine if there was any correlation between CB₁ receptor expression and striatal or nigral volume.

2.3. 6-Hydroxydopamine lesions

Lesion surgery was conducted under isoflurane anaesthesia (5% in oxygen for induction; 2% in oxygen for maintenance) in a stereotaxic frame with the nose bar set at -2.3 mm. 6-Hydroxydopamine hydrobromide (Sigma, UK) was dissolved in 0.01% ascorbate saline. The axonal lesion was induced by injection of $12\text{ }\mu\text{g}$ (free-base) of 6-hydroxydopamine ($3\text{ }\mu\text{l}$ of $4\text{ }\mu\text{g }\mu\text{l}^{-1}$ solution at $1\text{ }\mu\text{l min}^{-1}$ with 2 min for diffusion) at stereotaxic coordinates AP -4.4 , ML ± 1.0 (from bregma) and DV -7.8 below dura. The terminal lesion was induced by injection of $28\text{ }\mu\text{g}$ (freebase) of 6-hydroxydopamine ($4 \times 3\text{ }\mu\text{l}$ injections of $2.33\text{ }\mu\text{g }\mu\text{l}^{-1}$ solution at $1\text{ }\mu\text{l min}^{-1}$ with 2 min for diffusion) at stereotaxic coordinates AP $+1.3$, ML ± 2.7 ; AP $+0.4$, ML ± 3.1 ; AP -0.4 , ML ± 4.3 ; AP -1.3 , ML ± 4.7 (from bregma) and DV -5.0 below dura.

2.4. Perfusion and immunohistochemistry

Rats were deeply anaesthetised with pentobarbital and transcardially perfused with 100 ml of ice-cold heparinised saline followed by 150 ml of 4% paraformaldehyde (pH 7.4). Their brains were removed and placed into 4% paraformaldehyde for 4 h post-fixation prior to transfer to 25% sucrose. After 48 h equilibration in sucrose solution, $40\text{ }\mu\text{m}$ serial sections of the fixed brains were cut using a freezing sledge microtome and immunohistochemical staining was performed as previously described [22]. Briefly, following quenching of endogenous peroxidase activity (using a solution of 3% hydrogen peroxide/10% methanol in distilled water) and blocking of non-specific secondary antibody binding (using 3% normal serum in tris-buffered saline containing 0.2% Triton-X, pH 7.4), sections were incubated at room temperature either overnight (tyrosine hydroxylase: mouse monoclonal from Millipore, UK (MAB318) used at 1:1000; DARPP-32: rabbit polyclonal from Millipore (AB1656) used at 1:1000) or for 48 h (anti-CB₁: rabbit polyclonal from Prof. Ken Mackie, Indiana University, Bloomington, IN, USA, used at 1:50) in the appropriate primary antibody (in 1% normal serum and tris-buffered saline containing 0.2% Triton-X, pH 7.4). The sections were then incubated with the appropriate biotinylated secondary antibody (in 1% normal serum and tris-buffered saline, pH 7.4), followed by a streptavidin–biotin–horseradish peroxidase solution (Vector). Immunolabelling was revealed by incubating the sections in a 0.5% solution of diaminobenzidine tetrahydrochloride in tris-buffered saline containing 30% hydrogen peroxide. Sections were mounted on gelatine-coated microscope slides, dehydrated in an ascending series of alcohols, cleared in xylene, and coverslipped using DPX mountant.

2.5. Image analysis

For rats subjected to both terminal and axonal lesions the density of CB₁ receptor expression in three coronal photomicrographs (taken using a Nikon WD70 microscope) through the substantia nigra (AP distance from bregma (mm): -6.0, -6.4, -6.8) was analysed by an experimenter blind to the lesion. For rats subjected to both terminal and axonal lesions, striatal volume was determined from three DARPP-32 immunostained coronal sections through the striatum (AP distance from bregma (mm): +0.7, -0.3, -0.7). For rats with terminal lesions only, the volume of DARPP-32 staining in three coronal sections through the substantia nigra (AP distance from bregma (mm): -6.0, -6.4, -6.8) was also analysed. All image analysis was carried out using Image J software and in all cases the values obtained for the lesioned side were expressed as a percentage of the intact side.

2.6. Statistical analyses

Statistical analyses were carried out using Graphpad Prism 5.0 software. Changes in CB₁ receptor expression and striatal volume at the different post-lesion time-points were analysed using one-way ANOVA followed by a *post hoc* Newman-Keuls multiple comparisons test ($P < 0.05$ considered statistically significant). Linear regression analysis was used to determine if there was any correlation between CB₁ receptor expression and striatal or nigral volume. All data are expressed as mean $\pm$ s.e.m.

3. Results

3.1. CB₁ receptor expression

In accordance with previous literature [16,40,49], the CB₁ subtype of cannabinoid receptor was strongly expressed in the *pars reticulata* region of the substantia nigra and there was minimal overlap with the dopaminergic (tyrosine hydroxylase immunoreactive) neurons in the *pars compacta* (Fig. 1A).

3.2. Effect of axonal nigrostriatal lesion on CB₁ receptor expression

As expected, injection of 6-hydroxydopamine into the nigrostriatal neurons at the axonal level in the medial forebrain bundle caused near-complete loss of tyrosine hydroxylase immunopositive cells from both the substantia nigra *pars compacta* and the ventral tegmental area by 28 days after lesioning (Fig. 1A). In addition, this lesion caused a small increase in expression of the CB₁ receptor in the substantia nigra *pars reticulata* (Fig. 1A and B) which was sustained for 2 weeks after the lesion.

3.3. Effect of terminal nigrostriatal lesion on CB₁ receptor expression

As expected, injection of 6-hydroxydopamine into the nigrostriatal neurons at the terminal level in the striatum caused a pronounced lesion of the substantia nigra *pars compacta* by 28 days after lesioning whilst sparing the ventral tegmental area (Fig. 1A). In addition, this lesion caused a significant reduction in the density of CB₁ receptor expression in the substantia nigra *pars reticulata* (Fig. 1A and C). Thus, as the dopaminergic lesion progressed in the *pars compacta* region, there was also a progressive decline in CB₁ receptor expression in the *pars reticulata*. Quantification of the density of CB₁ receptor expression over the different time-points confirmed that the density of CB₁ receptor expression was significantly decreased by Day 14 and Day 28 post-lesion ($F_{(4,11)} = 4.28$, $P < 0.05$ followed by *post hoc* Newman-Keuls test).

3.4. Correlation between loss of CB₁ receptor expression and striatonigral degeneration

Since CB₁ receptors have been shown to be expressed on presynaptic terminals of GABAergic medium spiny neurons projecting from the striatum to the substantia nigra *pars*

reticulata [24,33,37] and since 6-hydroxydopamine has been shown to cause low levels of non-specific toxicity [28], we hypothesised that the loss of CB₁ receptor expression following injection of 6-hydroxydopamine into the striatum may have been due to a non-specific effect of this neurotoxin on the striatonigral projection neurons. To test this hypothesis, we used DARPP-32 immunohistochemical staining to determine if the terminal lesion led to loss of either striatal or nigral volume and whether this loss correlated with the decrease in CB₁ receptor expression.

Injection of 6-hydroxydopamine at the nigrostriatal terminals in the striatum resulted in decreased striatal volume over the 28-day period post-lesion (Fig. 2). Representative images of DARPP-32 immunostaining in terminal lesion sections at each time-point show an initial slight increase in striatal volume (probably reflecting an inflammatory response to injection of the toxin [6,9]), followed by a loss of DARPP-32 immunopositivity, a gradual decrease in striatal volume, and enlarging of the lateral ventricle on the lesioned side (Fig. 2A). Quantification of striatal volume, expressed as a percentage of the intact side, showed a significant decrease in striatal volume at Day 28 post-lesion in the terminal lesion model (Fig. 2Bi; $F_{(4,11)} = 4.07$, $P < 0.05$ followed by *post hoc* Newman-Keuls test) and this decrease correlated with the loss of CB₁ receptor expression in the substantia nigra *pars reticulata* (Fig. 2Bii; $r = 0.60$, $P < 0.05$). In addition to a loss of these DARPP-32 immunopositive cell bodies from the striatum, we also found that injection of 6-hydroxydopamine into the striatum also caused a loss of DARPP-32 immunopositive terminals from the substantia nigra *pars reticulata* (Fig. 2Ci; $F_{(4,11)} = 11.24$, $P < 0.001$ followed by *post hoc* Newman-Keuls test) and this also correlated significantly with the loss of CB₁ receptor expression from the same region (Fig. 2Cii $r = 0.67$, $P < 0.005$). Injection of the toxin at the axonal level did not affect the volume of DARPP-32 immunostaining in either the striatum or substantia nigra (data not shown).

4. Discussion

This study sought to determine if there were any changes in CB₁ receptor expression in the substantia nigra in the two most commonly used rat models of Parkinson's disease. Hemiparkinsonism was induced in male rats by either axonal or terminal injection of the catecholamine neurotoxin 6-hydroxydopamine and the density of CB₁ receptor immunoreactivity in the substantia nigra was analysed over 28 days of post-lesion. Although CB₁ receptor expression was transiently increased in the axonal lesion model of Parkinson's disease, it was significantly reduced when Parkinsonism was induced by terminal injection of 6-hydroxydopamine. Because reductions in striatal and nigral volumes after terminal lesion correlated significantly with loss of CB₁ receptor expression in the substantia nigra *pars reticulata*, it appears that the loss of CB₁ receptor expression seen in the terminal lesion model may be due to non-specific toxic effects of 6-hydroxydopamine.

The distribution of the CB₁ receptor within the brain has been well characterised by *in situ* hybridisation [33,35], receptor autoradiography [25] and immunohistochemistry [16,37,39,40,49]. In agreement with previous immunohistochemical studies, we have observed a clearly defined expression of the CB₁ receptor within the two zones of the substantia nigra, with strong immunoreactivity in a classic 'inverted teardrop' staining pattern in the *pars reticulata* and minimal staining in the *pars compacta*. The specific cellular localisation of the receptor was first revealed by Herkenham et al. [24], when they reported that excitotoxic lesions of the striatum resulted in loss of CB₁ receptor binding in the substantia nigra. This work indicated that the CB₁ receptor was expressed on the presynaptic terminals of striatal GABAergic medium spiny neurons projecting to the substantia nigra and this was later confirmed in a number of reports [21,26,33,50]. It is therefore thought that

CB₁ receptors are involved in modulating neurotransmission of the striatonigral (direct) pathway of motor control in the basal ganglia [11,29,46,47].

Because of the high density of CB₁ receptors in the substantia nigra and the central role that this structure plays in the pathogenesis of Parkinson's disease, a number of studies have investigated the effect of experimental Parkinsonian lesions on expression of this receptor at the mRNA and protein levels. When, as in the present study, the Parkinsonian neurotoxin 6-hydroxydopamine, has been injected into the nigrostriatal axons at the level of the medial forebrain bundle, previous studies have reported either an increase [34,44] or no change [53] in CB₁ receptor mRNA in the striatum. Interestingly, the elevation in CB₁ receptor mRNA following axonal injection of 6-hydroxydopamine was shown to be temporary (albeit over a longer time-course [44]) which reflects the transient increase seen in the present study. Thus, our finding supports that of the previous literature and indicates that the changes observed in CB₁ receptor expression in the substantia nigra *pars reticulata* to axonal injection of a catecholaminergic toxin are not sustained. Interestingly, we have been unable to find any reports of the effect of terminal injection of 6-hydroxydopamine on expression of the CB₁ receptor. Thus, the present finding that CB₁ receptor expression in the substantia nigra is decreased when the toxin is injected into the nigrostriatal terminals at the level of the striatum is particularly novel. The effect of Parkinsonian lesions on the other subtype of cannabinoid receptor, the CB₂ receptor, was not investigated in the present study. However, given that neuroinflammation has been implicated in the neuropathology of Parkinson's disease [18,38], the CB₂ receptor is up-regulated on activated microglia [2,43], and cannabinoid receptor agonists are neuroprotective in an animal model of Parkinson's disease [19,42], it would be interesting to determine if expression of this subtype is altered in the experimental Parkinsonian state.

It is well known that the axonal and terminal lesion protocols used in the present experiment lead to different magnitudes of nigrostriatal degeneration [12,13,28]. However this is unlikely to account for the differences in CB₁ receptor expression observed because the axonal lesion (which causes near-complete nigrostriatal degeneration [13,14]) did not affect CB₁ receptor expression, whereas the terminal lesion (which leaves ~25% of the nigrostriatal neurons intact [13,14]) did result in altered CB₁ receptor expression. This indicates that the difference between the lesion models in terms of the magnitude of dopamine cell loss cannot account for the changes seen. Rather, because we found that injection of 6-hydroxydopamine into the striatum caused a significant decrease in both striatal and nigral volume over time, this suggests that the "catecholaminergic" neurotoxin caused degeneration of, not only the nigrostriatal terminals, but also a subpopulation of the indigenous striatal cells themselves. It is reasonable to suggest that the loss of CB₁ receptor expression we observed in the substantia nigra *pars reticulata* was a consequence of a non-specific toxic effect of 6-hydroxydopamine on the GABAergic medium spiny striatonigral projection neurons that are known to express the receptor [24,33].

Validation of any novel target for the treatment of a human condition necessitates testing in animal models of that condition. The 6-hydroxydopamine-induced axonal and terminal models used in the present study are the most commonly used rat models of Parkinson's disease and are frequently used in the preclinical validation of novel anti-Parkinsonian therapies. However, the terminal lesion model has a number of advantages which makes it a more valid model of Parkinson's disease. Injection of 6-hydroxydopamine into the nigrostriatal terminals results in a relatively progressive degeneration in which the nigrostriatal axons gradually die-back from the lesioned striatum, and it also selectively destroys the nigrostriatal pathway whilst leaving the mesolimbocortical pathway intact [28]. Therefore, due to its relatively progressive time-course and nigrostriatal selectivity the nigrostriatal terminal lesion model is considered to be a more relevant model of Parkinson's

disease than the axonal lesion model. In the context of the preclinical validation of novel Parkinson's disease therapies, our finding that CB₁ receptor expression is decreased in the terminal lesion model suggests that this model may have its limitations in terms of predicting the anti-Parkinsonian effects of experimental cannabinoid drugs. Thus, because the direct target of cannabinoid agonists and antagonists, as well as the indirect target of drugs affecting endocannabinoid synthesis, metabolism, and transport is decreased in the terminal lesion model, this suggests that even though it is considered to be a more relevant model of Parkinson's disease, it may not be the best model for testing the anti-Parkinsonian efficacy of drugs targeting the endocannabinoid system. Moreover, evidence from *post-mortem* studies in Parkinson's disease patients suggests that the CB₁ receptor [31] and CB₁ receptor mRNA [27,31] expression in the substantia nigra remains unchanged, whilst receptor coupling, as measured by WIN55,212-2 stimulated [³⁵S]GTPγS binding, increases [31]. Extending the observations of the present study beyond Parkinson's disease and its modelling, the effect of striatonigral loss on CB₁ receptor expression in the substantia nigra may also have implications for researchers studying the potential of cannabinoid pharmacotherapy in striatonigral diseases such as Huntington's disease and Multiple Systems Atrophy.

In summary, we have shown that expression of the CB₁ receptor is differentially altered in two of the most widely used rat models of Parkinson's disease: in the 6-hydroxydopamine-induced axonal lesion model, expression of the receptor is transiently up-regulated, whereas in the terminal lesion model, expression of the CB₁ receptor is significantly reduced in the substantia nigra *pars reticulata*. This study suggests that, although the terminal lesion model is considered a more relevant model of the human condition, the axonal lesion model may be more useful in testing the potential anti-Parkinsonian efficacy of drugs targeting the endocannabinoid system.

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Fig. 1.

CB₁ receptor expression in the 6-hydroxydopamine-induced axonal and terminal lesion models of Parkinson's disease. (A) Double immunohistochemistry for tyrosine hydroxylase (blue) and the CB₁ receptor (brown) revealed a clearly defined and regional pattern of expression within the substantia nigra with the dopaminergic marker being expressed in the *pars compacta* region and the CB₁ receptor being expressed in the *pars reticulata* region. Induction of hemi-Parkinsonism by either axonal or terminal injection of 6-hydroxydopamine caused a progressive loss of tyrosine hydroxylase immunoreactivity from the *pars compacta* on the lesioned side (left) of the midbrain. This was associated with a transient increase in CB₁ receptor expression in the *pars reticulata* following axonal lesion and a progressive decline in CB₁ receptor expression in the *pars reticulata* following terminal lesion. Scale bar represents 4 mm. (B and C) Quantification of the density of CB₁ receptor expression confirmed that expression of the receptor was slightly increased following axonal lesion but progressively declined after terminal lesion. * $P < 0.05$ vs. Day 1 by one-way ANOVA with *post hoc* Newman-Keuls. SN_{pc}, substantia nigra *pars compacta*; SN_{pr}, substantia nigra *pars reticulata*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Fig. 2.

Loss of CB₁ receptor expression in the substantia nigra correlates with loss of striatal and nigral volume in the 6-hydroxydopamine-induced terminal lesion model of Parkinson's disease. (A) Immunohistochemistry for striatal DARPP-32 (upper panels) revealed that the induction of hemi-Parkinsonism by terminal injection of 6-hydroxydopamine caused a progressive loss of DARPP-32 immunopositivity (insets), striatal volume, and enlargement of the ventricles on the lesioned side (left) of the forebrain. This corresponded with the loss of CB₁ receptor immunoreactivity in the substantia nigra (lower panels). Scale bar represents 6 mm in the upper panels and 4 mm in the lower panels. (B) Quantification of DARPP-32 immunoreactivity revealed that nigrostriatal terminal injection of 6-hydroxydopamine caused a significant decrease in striatal volume (Bi: * $P < 0.05$ vs. Day 1 by one-way ANOVA with *post hoc* Newman-Keuls) and this correlated significantly with the loss of CB₁ receptor expression in the substantia nigra *pars reticulata* (Bii). (C) Quantification of DARPP-32 immunoreactivity also revealed that the terminal lesion caused a significant loss of DARPP-32 immunopositive fibres from the substantia nigra *pars reticulata* (Ci: ** $P < 0.001$ vs. Day 1 by one-way ANOVA with *post hoc* Newman-Keuls) and this also correlated with the loss of CB₁ receptor expression from the same substructure (Cii).