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Research report

Loss of mRNA levels, binding and activation of GTP-binding proteins for cannabinoid CB_1 receptors in the basal ganglia of a transgenic model of Huntington's disease

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Abstract

Data obtained from the basal ganglia of postmortem Huntington's disease (HD) brains have revealed that the level of cannabinoid CB_1 receptors in striatal efferent neurons decreases in parallel to the dysfunction and subsequent degeneration of these neurons. These findings and others from rat models of HD generated by lesions with mitochondrial toxins, suggest that the loss of CB_1 receptors may be involved in the pathogenesis of the disease. To explore further the changes in the endocannabinoid system, as well as the potential of endocannabinoid-related compounds, we examined the status of CB_1 receptors in the HD94 transgenic mouse model of HD. These mice express huntingtin exon 1 with a polyglutamine tract of 94 repeats in a tissue-specifi and conditional manner using the tet regulatable system. They develop many features of HD, such as striatal atrophy, intraneuronal aggregates and progressive dystonia. In these animals, we analyzed mRNA levels for the CB_1 receptor, in addition to the number of specifi binding sites and the activation of GTP-binding proteins by CB_1 receptor agonists. mRNA transcripts of the CB_1 receptor were significantl decreased in the caudate-putamen of HD transgenic mice compared to age-matched littermate controls. The decrease concurred with a marked reduction in receptor density in both the caudate-putamen and its projection areas such as the globus pallidus, entopeduncular nucleus and substantia nigra pars reticulata. Furthermore, the efficac of CB_1 receptor activation was reduced in the globus pallidus, as determined by agonist-induced [^{35}S]GTPγS binding, and tended towards a decrease in the substantia nigra. None of these changes was seen in the cerebral cortex and hippocampus, despite high levels of expression of the mutant protein in these regions. The decrease in CB_1 receptor levels was accompanied by a decrease in the proenkephalin-mRNA levels but not in substance P-mRNA levels. Taken together, these results suggest that the loss of CB_1 receptor might be preferential to the enkephalinergic CB_1 receptor-containing striatopallidal neurons, and further implicate the CB_1 receptor to the subsequent HD symptomatology and neuropathology.

Theme: Disorders of the nervous system

Topic: Degenerative disease: other

Keywords: Cannabinoids; CB_1 receptor; Huntington's disease; Transgenic mouse; Basal ganglia

1. Introduction

It is well known that the administration of plant-derived, synthetic or endogenous cannabinoids [4,7,26,27,36] inhibits movement in rats in a dose-dependent and time-dependent manner [26,31]. The cannabinoid-induced motor inhibition is seen in parallel with changes in the activity of several neurotransmitters including GABA, dopamine and glutamate [18,26–28,34]. These effects are likely to be mediated by cannabinoid CB_1 receptors, which are densely distributed in the basal ganglia [11,12,17,35] and located presynaptically on striatal GABAergic projection neurons

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that project to the substantia nigra, globus pallidus and entopeduncular nucleus [11]. Another source of CB_1 receptors in the basal ganglia is the subthalamonigral glutamatergic pathway [17]. Cannabinoid agonists seem to block activity of these excitatory neurons, which then leads to an inhibition of their recipient neurons in the substantia nigra [32] and a subsequent reduction in motor activity [20].

The prominent presence of CB_1 receptors in the basal ganglia [11,12,17,35] and the pronounced effect of cannabinoids on movement [4,7,26,27,31,36] suggest a possible therapeutic usefulness of cannabinoids to improve movement in hyperkinetic disorders such as Huntington's disease (HD) (for review, see Ref. [3]). HD is a genetic neurodegenerative disorder caused by an unstable expansion of a CAG repeat in exon 1 of the human huntingtin gene, IT15. Translation through the CAG span results in a polyglutamine tract near the N-terminus of the protein, which leads to toxicity predominantly of striatal projection neurons [6,10,23]. The symptoms of this disease are primarily characterized by motor disturbances, such as chorea and dystonia, and secondarily by personality changes and cognitive decline [6,10,23]. Studies have demonstrated that CB_1 receptor binding was markedly reduced in the basal ganglia of HD patients [8,9,25], possibly due to the presence of these receptors on the striato-efferent GABAergic neurons that are especially susceptible in HD [11]. Similar results have been observed in a rat HD model generated by local [14] or systemic [22] application of 3-nitropropionic acid, a mitochondrial toxin that inhibits succinate dehydrogenase, which leads to a pattern of cell loss similar to the human disease (for review, see Ref. [2]).

In a recent study, Denovan-Wright and Robertson [5] demonstrated that a decrease of CB_1 receptor-mRNA levels is also observed in the caudate-putamen of one of the HD transgenic mouse models, the Bates R6 lines [16]. However, they neither examined other parameters indicating the status of CB_1 receptors in the basal ganglia, nor the relationship of the receptor loss with other phenotypical markers of striatal projection neurons affected in HD. In the present study, we have used the conditional transgenic mouse model of HD (HD94) developed by Yamamoto et al. [37] to carry out a complete analysis of changes in CB_1 receptors in the basal ganglia of these animals. HD94 mice conditionally express, selectively in the forebrain, exon 1 of the human huntingtin gene with a polyglutamine expansion of 94 repeats using the tet-regulatable system. HD94 mice demonstrate many features of HD, such as striatal atrophy and reactive astrocytosis, intraneuronal aggregates and a progressive dystonia [37]. The neurological phenotype of these mice develops without neuronal loss, making these mice a useful model to dissociate changes related to cell dysfunction from changes related to cell death [19]. Although not examined here, this model also provides the possibility to abolish transgene expression [37], which allows for the study of reversion of different aspects of the disease at different stages.

In the basal ganglia of symptomatic transgenic mice, we have analyzed: (i) CB_1 receptor mRNA levels, by using in situ hybridization, (ii) CB_1 receptor binding capacity, by using [^{3}H]CP55,940 autoradiography, and (iii) the efficac of CB_1 receptor activation, using WIN55,212-2-stimulated [^{35}S]GTPγS binding. In addition, we have examined mRNA levels for: (i) neuronal-specifi enolase (NSE), a neuronal marker that may be indicative of any striatal neuronal cell loss; (ii) proenkephalin (PENK) and substance P (SP), which are phenotypical markers of CB_1 receptor-containing striatopallidal and striatonigral GABAergic neurons, respectively; and (iii) tyrosine hydroxylase (TH), the key enzyme of dopamine synthesis in nigrostriatal neurons, which are under the direct or indirect influenc of CB_1 receptor-containing striato-efferent neurons.

2. Materials and methods

2.1. Animals and sampling

All experiments were performed on CBA×C57BL/6 inbred mice. Wild-type, BiTetO, CamKIIα-tTA, and HD94 [37] mice were bred at the Centro de Biologí a Molecular 'Severo Ochoa' animal facility. As previously reported, all genotypes result from BiTetO×CamKIIα-tTA crosses, in which pregnant mothers received doxycycline 2 mg/ml in 5% sucrose solution instead of the normal drinking water for 5 days just prior to giving birth. This avoids the perinatal lethality observed in these mice [37]. The genotype of the mice was determined by PCR analysis of tail DNA biopsies. Mice were housed four per cage with food and water available ad libitum and maintained in a temperature-controlled environment on a 12/12 h light–dark cycle with light onset at 07:00 h. When animals were 4–5 months old, they were sacrifice by rapid decapitation and their brains were quickly and carefully removed and rapidly frozen by immersion in 2-methyl-butane cold in dry ice. All samples were stored at −80 °C until processed for the different analyses.

2.2. In situ hybridization and autoradiographic techniques

2.2.1. Brain slicing

Coronal sections 20-μm-thick were cut in a cryostat, according to the Lehmann atlas [15]. Sections, corresponding to different basal ganglia levels, were thaw-mounted onto RNAse-free gelatin/chrome alum-coated slides and dried briefl at 30 °C and stored at −80 °C until used. For the identificatio of the different brain nuclei, adjacent sections to those used for autoradiographic analy-

sis were stained with cresyl-violet and analyzed according to the Lehmann atlas [15].

2.2.2. LacZ staining

This was routinely carried out to verify the genotype of HD94 mice. Fresh frozen sections were post-fixe for 10 min in 4% paraformaldehyde in Sorensen's buffer. Slides were then incubated for 1 h at 30 °C in lacZ staining solution (1 mg/ml X-gal (4-chloro-5-bromo-3-indolyl-β-galactosidase), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide and 2 mM $MgCl_2$ in PBS). After staining, sections were rinsed and dry-mounted.

2.2.3. Autoradiography of cannabinoid receptor binding

The protocol used is the method described by Herkenham et al. [12], with small modification [29]. 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 [^{3}H]CP-55,940 (Du Pont NEN) prepared in the same buffer, in the absence or the presence of 10 μM non-labelled CP-55,940 (Tocris) to determine the total and the non-specifi binding, respectively. Following incubation, slides were washed in 50 mM Tris buffer with 1% bovine serum albumin (fatty acid-free), pH 7.4, for 4 h (2×2 h) at 0 °C, dipped in ice-cold distilled water and then dried under a stream of cool dried air. Autoradiograms were generated by exposing the labelled sections and autoradiographic standards ([^{3}H] micro-scales, Amersham Ibérica), to tritium-sensitive fil ([^{3}H]-Hyperfilm Amersham Ibérica) for a period of 10 days, and developed (D-19, Kodak) for 4 min at 20 °C. Developed film were analyzed and quantifie in a computer-assisted videodensitometer using the standard curve generated from [^{3}H]-standards.

2.2.4. Analysis of mRNA levels for CB_1 receptor, NSE, TH, PENK and SP by in situ hybridization

The analysis of CB_1 receptor mRNA levels was carried out according to Rubino et al. [30], with some modification [29]. Briefly sections were fixe 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_1 receptor cDNA (Du Pont; the specificit of the probes used was assessed by Northern Blot analysis) was 3′-end labelled with [^{35}S]dATP using terminal deoxynucleotidyl-transferase. Sections were, then, hybridized with [^{35}S]-labelled oligonucleotide probes (7.5×10^5 dpm per section), washed and exposed to X-ray fil (βmax, Amersham Ibérica) for 10 days, 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 film 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 specificit of the signal. Several measures were taken to avoid that results might be influence by methodological variations: (i) the analysis for each brain section level was always carried out in a unique batch; (ii) if the use of more than one fil was required, they contained always the same proportion of wild and transgenic samples; and (iii) the measurements were taken by researchers blinded to the experimental conditions. Similar procedures were used for the analysis of mRNA levels of NSE, TH, PENK and SP. We used commercial probes (Amersham Ibérica) for PENK [38] and TH [29], and synthetic 45-base probes, selected from their previously-published sequences, for SP (5′-CCGTTTGCCCATCAATCCAAAGAACTGCTGAGGC-TTGGGTCTCCG-3′; [21]) and NSE (5′-TCTGGGTGAC-TTGGGGCTCAAGGTATCAAGGTAACTATGGCGGG-T-3′; [13]). As all these probes had been developed in rats, we firs confirme that they also recognized PENK and TH in mice (data not shown).

2.2.5. Autoradiography for WIN55,212-2-stimulated [^{35}S]GTPγS binding

We used the protocol described by Sim et al. [33]. Briefly slide-mounted brain sections were firs rinsed in assay buffer (50 mM Tris, 3 mM $MgCl_2$, 0.2 mM EGTA, 100 mM NaCl and 0.5% bovine serum albumin (fatty acid-free), pH 7.4) at 25 °C for 10 min, then pretreated for 15 min with an excess concentration (2 mM) of GDP (Boehringer Mannheim) in assay buffer. Afterwards, sections were incubated at 25 °C for 2 h in assay buffer containing 0.04 nM [^{35}S]GTPγS (Amersham Ibérica), 2 mM GDP and 1 μM WIN-55,212-2 (RBI, Natick, MA, USA). Basal activity was assessed in the absence of agonist, whereas non-specifi binding was measured in the presence of 10 μM unlabeled GTPγS (Boehringer Mannheim). In pilot experiments, additional brain sections were incubated in the presence of the CB_1 receptor antagonist SR141716 (0.3 μM; kindly supplied by Sanof Recherche, Montpellier, France) in addition to 0.04 nM [^{35}S]GTPγS, 2 mM GDP and 1 μM WIN-55,212-2. SR141716 signifi cantly antagonized the increase in WIN-55,212-2-stimulated [^{35}S]GTPγS binding, thus supporting the idea that this increase was caused through activation of cannabinoid receptors (data not shown; see details in Ref. [29]). Slices were rinsed twice in 50 mM Tris buffer, pH 7.4, and once in deionized water, then dried under a stream of cool dry air. Autoradiograms were generated by apposing the labelled tissues to fil (Hyperfilm βmax, Amersham Ibérica) for a period of 2 days, and developed (D-19, Kodak) for 4 min at 20 °C. Developed film were analyzed and quantifie by a computer-assisted video-

densitometer (Image Quant 3.3, Molecular Dynamics). As for the in situ hybridization method, several measures were also taken in this autoradiographic method to avoid that results might be influence by methodological variations.

2.3. Statistics

Data were assessed by unpaired Student's *t*-test.

3. Results

Results demonstrate a significan decrease of CB_1 receptor-mRNA transcripts in the caudate-putamen, mainly in the medial portion (−19.2%), of HD transgenic mice compared to wild-type mice (Table 1; see representative autoradiograms in Fig. 1). As expected, this reduction does not appear to be due to neuronal loss, as revealed by the similar mRNA levels of NSE in both genotypes (transgenic mice: 0.34±0.08 units of OD, $n=5$; wild-type mice: 0.29±0.06 units of OD, $n=8$). However, the reduction could be due to a preferential decrease of striatal neurons projecting to the globus pallidus, since mRNA levels for PENK were reduced by ~43.5% (wild-type mice: 0.23±0.04 units of OD, $n=9$; transgenic mice: 0.13±0.02 units of OD, $n=5$; $P<0.05$; see representative autoradiograms in Fig. 2). In contrast, there were not changes in the expression of SP, which is expressed in striatal neurons projecting to the substantia nigra (transgenic mice: 0.25±0.06 units of OD, $n=4$; wild-type mice: 0.24±0.04 units of OD, $n=7$; see representative autoradiograms in Fig. 2). TH mRNA levels were also similar between transgenic (0.27±0.02 units of OD, $n=4$) and wild-type (0.27±0.03 units of OD, $n=10$) mice.

Autoradiographic binding studies revealed a marked reduction in the density of CB_1 receptors in the caudate-putamen (lateral: −35.7%; medial: −32.2%) and in the striatal projection areas, the globus pallidus (−32.6%), entopeduncular nucleus (−27.5%) and substantia nigra pars reticulata (−20.8%) (Table 2; see representative autoradiograms in Fig. 1). This loss of receptor density was also reflecte in a loss of the efficac of receptor activation, since the magnitude of the stimulation of

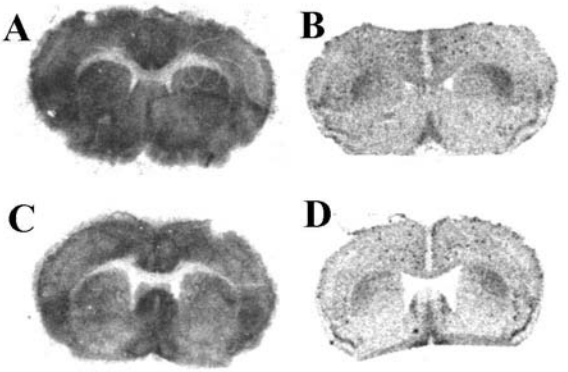

Fig. 1. Representative autoradiograms corresponding to the analysis of binding capacity (panels A and C) and mRNA levels (panels B and D) for CB_1 receptors in the caudate-putamen of adult (4–5 month-old) transgenic HD (panels C and D) or wild-type (panels A and B) mice. See details in the text.

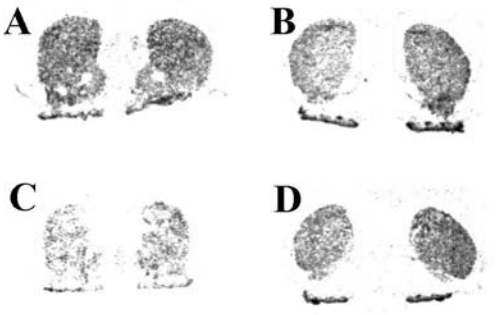

Fig. 2. Representative autoradiograms corresponding to the analysis of mRNA levels for proenkephalin (panels A and C) or substance P (panels B and D) in the caudate-putamen of adult (4–5 month-old) transgenic HD (panels C and D) or wild-type (panels A and B) mice. See details in the text.

[^{35}S]GTPγS binding with the receptor agonist WIN55,212-2 was significantl lower in the globus pallidus (−27.3%) of transgenic mice, but only tended towards a decrease in the substantia nigra (−24.3%) (Table 3). Interestingly, the reductions in both CB_1 receptor binding capacity and stimulation of GTP-binding proteins were always more marked in the globus pallidus than the substantia nigra. This is in concordance with the increased vulnerability of PENK-positive striatopallidal neurons than the SP-positive

Table 1
CB_1 receptor-mRNA levels (arbitrary units of optical density) in the basal ganglia and other brain regions of adult (4–5 month-old) transgenic HD or wild-type mice

Brain structures	Wild-type mice	Transgenic HD mice
Basal ganglia		
Lateral caudate-putamen	0.385±0.025 (6)	0.318±0.030 (4)
Medial caudate-putamen	0.282±0.014 (6)	0.228±0.022 (4)*
Reference structures		
Cerebral cortex (II–III)	0.243±0.035 (6)	0.214±0.018 (4)
Cerebral cortex (V–VI)	0.221±0.010 (6)	0.225±0.026 (4)
Hippocampus (Ammon's horn)	0.276±0.014 (7)	0.300±0.010 (4)

Values are means±S.E.M. with the number of determinations per group in parentheses. Data were assessed by Student's *t*-test (*$P<0.05$).

Table 2
CB_1 receptor binding capacity (fmol/mg tissue) in the basal ganglia and other brain regions of adult (4–5 month-old) transgenic HD or wild-type mice

Brain structures	Wild-type mice	Transgenic HD mice
Basal ganglia		
Lateral caudate-putamen	153.9±7.2 (6)	98.9±9.2 (5)***
Medial caudate-putamen	143.6±7.2 (6)	97.3±8.8 (5)**
Globus pallidus	234.9±13.5 (7)	158.4±10.5 (5)***
Entopeduncular nucleus	265.9±8.7 (9)	192.8±12.8 (5)***
Substantia nigra	297.7±5.7 (9)	235.8±8.6 (5)***
Reference structures		
Cerebral cortex (I–II)	122.6±7.8 (6)	113.4±9.2 (5)
Cerebral cortex (V–VI)	132.1±2.8 (6)	123.5±13.6 (5)
Hippocampus (Ammon's horn)	187.6±11.3 (9)	199.9±19.2 (5)

Values are means±S.E.M. with the number of determinations per group in parentheses. Data were assessed by the Student's *t*-test (*$P<0.05$, **$P<0.01$, ***$P<0.005$).

striatonigral ones. Finally, no changes were seen for the CB_1 receptor parameters in the cerebral cortex and hippocampus (Tables 1–3), thus indicating a specificit to the changes in CB_1 receptors to regions controlling movement and those that are particularly vulnerable in HD.

4. Discussion

The present study strongly demonstrates that CB_1 receptors are significantl reduced in striatal projection neurons in a mouse model overexpressing a mutant form of huntingtin, as occurs in the brain of HD patients. These losses of CB_1 receptors seem to occur in a situation where neuronal loss did not take place, as reflecte by the lack of changes in NSE-mRNA levels in the caudate-putamen of HD transgenic mice. Stereological analysis of HD94 striata at an older age (40 weeks) also indicates that cell death does not occur [19]. Hence, we have to conclude that changes observed in the present study in HD94 mice must be attributed to neuronal plasticity rather than cell loss, and may implicate a role of CB_1 receptor loss in the early stages of HD. This has been recently claimed by some authors [5,8], considering: (i) that losses of CB_1 receptors in the basal ganglia of HD patients occurred in advance of other receptor losses and even before the appearance of major HD symptomatology [8], and (ii) that a decrease in CB_1 receptor-mRNA levels was evident in the caudate-putamen of another HD transgenic mouse model that also exhibited no neuronal death [5]. This contrasts, however, with the studies conducted in other rodent models for HD, such as rats injected with 3-nitropropionic acid, where a profound loss of striatal projection neurons is the major histopathological event that takes place (for review, see Ref. [2]). In these rats, there are also marked losses in the population of CB_1 receptors in the basal ganglia [14,22] that are possibly a secondary consequence to the neuronal loss.

The cannabinoid CB_1 receptors are located presynaptically on striatal GABAergic neurons that project to the substantia nigra, globus pallidus and entopeduncular nucleus [11]. One hallmark of HD is the selective loss or dysfunction of enkephalinergic projections to the globus pallidus [1,24]. We therefore hypothesized that the loss of CB_1 receptors might be preferentially observed in striatopallidal neurons. Although PENK-mRNA levels (striatopallidal), but not SP-mRNA levels (striatonigral), were reduced in the caudate putamen of HD transgenic mice, CB_1 receptor binding was reduced in all projection areas. Nevertheless, the extent of the decrease of CB_1 receptor binding in the substantia nigra is less than in the globus pallidus. Moreover, the WIN-55,212-stimulated

Table 3
Percent of stimulation by WIN55,212-2, a CB_1 receptor agonist, of [^{35}S]GTPγS binding in the basal ganglia and other brain regions of adult (4–5 month-old) transgenic HD or wild-type mice

Brain structures	Wild-type mice	Transgenic HD mice
Basal ganglia		
Globus pallidus	227.1±16.5 (6)	165.1±20.5 (5)*
Substantia nigra	275.3±43.6 (6)	208.5±14.3 (5)
Reference structures		
Cerebral cortex (I–II)	26.9±5.6 (7)	25.9±8.3 (4)
Cerebral cortex (V–VI)	26.0±3.8 (7)	23.9±8.6 (4)
Hippocampus (Ammon's horn)	87.5±7.7 (5)	95.1±4.8 (4)

Values are means±S.E.M. with the number of determinations per group in parentheses. Data were assessed by the Student's *t*-test (*$P<0.05$).

[^{35}S]GTPγS binding was significantl decreased in the globus pallidus, but did not reach significanc in the substantia nigra. Taken together, this indicates the existence of differences in the vulnerability of these two pathways to mutated huntingtin, as was suggested in a previous study that might occur in the human pathology [24], but that has been questioned recently in a rat model of HD where a marked cell loss takes place [14].

An important difference between this study and the report by Denovan-Wright and Robertson [5] is that they found a decrease in CB_1 receptor-mRNA levels within a subset of neurons in the cerebral cortex and the hippocampus, while we did not observe these changes in HD94 mice. This difference may be attributed to the difference in length of the expanded CAG repeat between our model (94) as opposed to the R6/2 transgenic model (>115) used by Denovan-Wright and Robertson [5,16]. Another key difference between models is the promoters used to drive transgene expression. However, this is unlikely since the expression levels of the mutant protein in the cortex and hippocampus are very robust in the HD94 mice. Another remarkable difference is that the decrease in CB_1 receptor-mRNA levels found in HD94 mice was mainly located in the medial portion of the caudate putamen, which is similar to the decrease in dopamine D1 receptors reported previously [37]. By contrast, Denovan-Wright and Robertson [5] reported that their decreases in CB_1 receptor-mRNA levels occurred exclusively in the lateral caudate putamen. The observed differences between the two models are difficul to elucidate. We can only surmise that since both models express the transgene relatively homogeneously throughout the striatum, and both models express similar protein fragment lengths, perhaps the differences in polyglutamine length or transgene expression levels could account for the difference.

In summary, our data further supports the finding that CB_1 receptors down-regulate rapidly in the basal ganglia because of plastic changes caused by expression of a mutant huntingtin fragment. Hypofunction of the endocannabinoid system may thus be a possible therapeutic target for the motor deterioration seen in HD. The similar pattern of loss of CB_1 receptors between this model and HD patients makes HD94 mice a valuable tool for the study of dysfunctional endocannabinoid transmission. Two interesting possibilities are offered for future studies. Firstly, as the HD94 mouse is a conditional model [37], it would be possible to further explore the involvement of CB_1 receptor changes in the pathogenesis of HD, by examining their behavior after blocking transgene expression with doxycycline. Secondly, this model can be used to test the efficac of substances that increase endocannabinoid transmission, and determine whether they are able to alleviate motor abnormalities observed in this disease, which lacks efficien symptomatic and/or neuroprotectant pharmacological therapy. In this respect, we have recently demonstrated that inhibitors of endocannabinoid uptake, such as AM404, were able to reduce hyperkinesia and recover from neurochemical deficit in a rat model of HD generated by bilateral intrastriatal injections of 3-nitropropionic acid [14].

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